Stem Cell Biology and Regenerative Medicine

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Editors

Stem Cells & Regenerative Medicine

From Molecular Embryology to Tissue Engineering

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Foreword by
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Preface

Embryology is a branch of biology that has an immediate bearing on the problem of "life." Life cannot be fully accounted for without an understanding of its dynamic nature, which expresses itself in the incessant production of new organisms in the process of ontogenetic development. Therefore, embryology is defined as the science of the development of an embryo from the fertilization of the ovum to the fetus stage. Teaching of embryology has long been an established feature at universities throughout the world, both for students in biology and students in medical sciences. During the twentieth century most of this science has been overshadowed by experimental-based genetics and cell biology, which have turned classical embryology into "developmental biology." Several universities are now teaching developmental biology instead of embryology as a course in biology programs. Significant contributions made in the twenty-first century in the fields of molecular biology, biochemistry, and genomics, integrated with embryology and developmental biology, provide an understanding of the molecular portrait of a "developmental cell." This integrated approach to development is incorporated in the present book as "stem cell biology," a new sister branch of embryology/developmental biology that emphasizes the study of self-renewal, differentiation, pluripotency, and nuclear programming, which are characteristics of stem cells. In a broad sense, *stem cell biology is nothing more than an understanding of embryology and development together at the molecular level using engineering, imaging, and cell culture principles*. With such a wide scope, this book can only be an introduction to stem cell biology.

Stem Cells and Regenerative Medicine: From Embryology to Tissue Engineering is mainly intended for readers in the biotechnology and molecular medicine fields. Although quite a number of books already exist covering stem cells, this book differs, in that is the first text completely devoted to the basic developmental, cellular, and molecular biologic aspects of stem cells and their clinical applications in tissue engineering and regenerative medicine. We took serious consideration in choosing the chapters and sections in this book to maintain the theme of *Molecular Embryology to Tissue Engineering*.

This book focuses on the basic biology of embryonic and cancer cells and their key involvement in self-renewal, muscle repair, epigenetic processes, and therapeutic applications. Significant contributions, such as nuclear reprogramming–induced pluripotency, and stem cell culture techniques using novel biomaterials, are also

covered. This text consists of 36 chapters, grouped into six parts. Most of the chapters are written by experts in the field from academia and industry. The goal is to have this book serve as a reference for graduate students, post-docs, and teachers and as an explanatory analysis for executives and scientists in biotech and pharmaceutical companies. Our hope is that this volume will provide a prologue to the field for both newcomers and those already active in the field.

The term "stem cell" appeared in the scientific literature as early as 1868 in the work of the eminent German biologist Ernst Haeckel. Haeckel, a supporter of Darwinian evolution, developed a number of phylogenetic trees to represent the evolution of organisms from common ancestors and called these trees *Stammbaume* ("stem trees"). In this context, he used the term *Stammzelle* ("stem cell") to describe the ancestor unicellular organism from which he presumed all multicellular organisms evolved. He referred to fertilized egg as the source that gives rise to all cells of the organism. Later, in 1887, Theodor Boveri and Valentin Hacker identified the earliest germ cells in animal embryos. In 1892, Valentin Hacker described stem cells as the cells that later in development produce oocytes in the gonads. Thus, in these early studies, the term stem cell referred to what we call the "germline lineage," "primordial germ cells," and "germline stem cells." In 1896, Edmund Wilson, an embryologist, reviewed the finding of these German scientists in his book *The Cell in Development and Inheritance*, which was published in English and became an inspirational work for a generation of embryologists and geneticists, especially in United States. Given his wide readership, Wilson is generally credited as having coined the term "stem cell."

Nuclear programming is the process that instructs specialized adult cells to form early pluripotent stem cells. Pluripotent stem cells posses the capacity to become any type of mature cell in the body and therefore have great potential for experimental and therapeutic purposes. Using the concept of "cellular reprogramming," Briggs and King in 1952 produced normal tadpoles by transplanting nuclei from blastula cells to enucleated eggs in the frog *Rana pipens*. However, transplanting nuclei from differentiated cells was achieved by John Gurdon in 1962 in the African clawed toad, *Xenopus laevis*, which is now known as the classic nuclear transfer experiment. It took more than another decade (1975) for Gurdon to succeed in producing healthy and sexually mature fertile frogs with functional muscle, beating hearts, well-differentiated eyes, and all of the other organs. This experiment provided the first clear evidence that cell specialization does not involve irreversible inactivation in the genes required for development of an animal. This conceptual framework led to the start of the field of nuclear reprogramming, and Gurdon became known as the "father" of nuclear reprogramming (cloning). It took almost another 10 years to clone an adult sheep, Dolly (in 1996), by Kevin Campbell and Ian Wilmut of the Roslin Institute in Edinburgh, Scotland. This experiment dramatically extended Gurdon's concept from frogs to mammals. The Dolly-related work of somatic cell nuclear transfer was further extended to produce monkeys, cows, dogs, mice, and other animals. These remarkable contributions stimulated other researchers to think about using nuclear transfer to generate pluripotent human embryonic stem cells for cell replacement therapy.

The road to embryonic stem cells and beyond began in the 1960s with the work of Leroy Stevens from the Jackson Labs, Bar Harbor, Maine, who discovered embryonal carcinoma cells while studying testicular carcinomas. Later Stevens and colleagues demonstrated that these embryonal carcinoma cells are indeed pluripotent stem cells. In the mid-1970s, Gail Martin's postdoctoral work with Martin Evans at the University of Cambridge led her to develop *new in vitro* clonal culture methods of embryoid cells. In the early 1980s, Martin, then at the University of California at San Francisco, and Martin Evans and Matthew Kaufman of the University of Cambridge independently isolated stem cells from mouse embryos and coined the term "embryonic stem cells." It took almost 10 years for Jamie Thompson of the University of Wisconsin to culture monkey embryonic stem cells and subsequently human embryonic stem cells in 1999. Thompson's work propelled the activity of stem cell research and cell propagation technologies in general.

There are two routes to producing a living animal: (1) injection of a somatic cell nucleus into an enucleated egg (nuclear reprogramming) and (2) use of an embryo to produce embryonic stem cells. In a quite astonishing discovery, Kazutoshi Takahashi and Shinya Yamanaka of Kyoto University in Japan in 2006 for the first time turned adult mouse skin fibroblast cells into pluripotent cells. This breakthrough of inducing fribroblasts was achieved by stable transfection of only four transcription factors (Oct4, Sox2, Klf4, and c-Myc), and these are now referred to as induced pluripotent stem (iPS) cells. The discovery of iPS cells turned the field of nuclear reprogramming upside down. This work was extended and further confirmed by several groups that generated iPS cells from individuals with various neurodegenerative diseases, raising the hope of cell replacement therapy and making personalized medicine a reality. A section of this book with six chapters details the concepts behind nuclear reprogramming and induced pluripotent stem cells.

In 1868, Ernst Neumann suggested that hematopoiesis occurs in bone marrow. He used the term "stem cell" to refer to the common precursor of the blood system in 1912. The debate about the existence of a common hematopoietic stem cell continued for several decades until definitive evidence was provided in 1961 by two Canadian scientists, James Till and Ernest McCulloch. Blood and the system that forms it, known as the hematopoietic system, consists of many cell types with specialized functions (some of these include red blood cells, platelets, granulocytes, macrophages, B and T lymphocytes, etc). Generally, the hematopoietic system is destroyed by radiation and chemotherapeutic agents that kill dividing cancer cells. In order to quantitatively assess the radiation sensitivity of normal bone marrow cells, a colony-forming unit assay was developed by Till and McCulloch, who coined the term "pluripotent hematopoietic stem cells" (HSCs). Today, we know that the best locations for HSCs are bone marrow, umbilical cord blood, and embryonic stem cells. In 1959, for the first time, Edward Donnall Thomas of the University of Washington used HSCs for treating leukemia and lymphomas through bone marrow transplantation. The efficient expansion of HSCs in culture remains one of the major research themes of stem cell biology. Combined applications of genomics, proteomics, and gene therapy approaches will further help to widen the

horizon for clinical applications. According to Irving Weissman of Stanford University Medical School, the progeny produced from hematopoietic stem cells exhibits properties that include self-renewal, differentiation, migration, and apoptosis. A few chapters in the third part of this book highlight of the use of HSCs for bone marrow cell therapy, heart transplantation, and cell replacement therapy for neurologic disorders.

The term "tissue engineering" was first used by Eugene Bell of MIT in 1984, and later was also used extensively by Wolter and Meyer in 1984. Tissue engineering combines cells, engineering, and materials methods with suitable biochemical and physiochemical factors to improve or replace biologic functions. In other words, it deals with the repair or replacement of portions of or whole tissues such as bone, blood vessels, bladder, skin, and artificial organs. According to Robert Langer and Joseph Vacanti, it "applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ." Powerful developments in the multidisciplinary field of tissue engineering have yielded a novel set of tissue replacement parts and implementation strategies. Scientific advances in biomaterials, stem cells, growth and differentiation factors, and biomimetic environments have created unique opportunities to fabricate tissues in the laboratory from combinations of engineered extracellular matrices ("scaffolds"), cells, and biologically active molecules. A section of this book with five chapters highlights recent developments in biomaterials, three-dimensional culture systems, lab-on-a-chip concepts, and microtechnologies used in attempts to understand stem cell biology.

Regenerative medicine is a new branch of medicine that attempts to change the course of chronic disease, in many instances regenerating failing organ systems lost due to age, disease, damage, or congenital defects. The term "regenerative medicine" was first referred to in 1992 by Leland Kaiser and then popularly used by William Haselstine of Human Genome Sciences. The term regenerative medicine is often used synonymously with tissue engineering, although those involved in regenerative medicine place more emphasis on the use of stem cells to treat diseases using cell therapies or transplantation methods. This field holds the promise of regenerating damaged tissues and organs in the body by stimulating previously irreparable organs to heal themselves. Regenerative medicine also empowers scientists to grow tissues and organs in the laboratory and safely implant them when the body cannot heal itself. A section in this book is entirely devoted to describing the use of stem cells in muscle repair and treating cardiac and urologic diseases.

Gurdon has spent much of his career deciphering the molecules and mechanisms that an egg uses to "rejuvenate" nuclei. We know a lot about nuclear transfer, but the question remains of how to regulate and control the most efficient way to reprogram the nucleus. Although both Gurdon's (nuclear reprogramming) and Yamanaka's (iPS) technologies can generate living animals, we do not know the molecular mechanisms underlying these two strategies. The potential of iPS cell technology in biology and medicine is enormous; however, it is still in its infancy, and there are many challenges to overcome before various applications can be used successfully. We still need to understand the components of oocytes or eggs used to depress

somatic gene expression and discover the direct cell-type switches by over-expressed transcription factors. It is also important to identify the basis for the stability of the differentiated state of cells, which will help us to understand how egg-reprogramming factors operate. Finally, mapping of the "embryome" is a necessity, and is looks as though it will become available soon, which will help us to understand the intricacies and epigenetic imprints of embryos.

Many people have contributed to making our involvement in this project possible. We thank our teachers for their excellent teaching, guidance, and mentorship, which helped us to bring this educational enterprise. We are extremely thankful to all of the contributors to this book, without whose commitment this book would not have been possible. Many people have had a hand in the preparation of this book. Each chapter has been passed back and forth between the authors for criticism and revision; hence each chapter represents a joint composition. We thank our readers, who have made our hours putting together this volume worth it. We are indebted to the staff of Springer Science + Business Media (Humana Press), and in particular Mindy Okura-Marszycki and Vindra Dass for their generosity in giving time and effort throughout the editing of this book. This book is dedicated to memory of my late friend, Prof. Xiangzhong (Jerry) Yang of the University of Connecticut, Storrs, who was the first to clone a cow (Amy, the calf) and a strong proponent of stem cell research here in US and China. This book is also dedicated to memory of my late friend, Prof. C.M. Habibullah of the Deccan College of Medical Sciences, India, who was a strong supporter of stem cell research in India. We especially thank Prof. John Gurdon, a researcher of great distinction, for his kindness and support in writing the Foreword to this book. Last, but not least, we thank Shyamala Appasani for her understanding and cooperation during the development of this book.

This book is the first joint project of father and son. A portion of the royalties will be contributed to the Dr. Appasani Foundation, a nonprofit organization devoted to bringing social change through the education of youth in developing nations.

Waltham, MA Krishnarao Appasani
Boston, MA Raghu K. Appasani

Foreword

I am very grateful to Krishnarao and Raghu Appasani for preparing this volume on the massively expanding fields of stem cells and regenerative medicine and for inviting me to offer a few introductory comments.

The prospect of being able to rejuvenate cell types of almost any kind from easily accessible cells of an adult makes it realistic to envisage cell replacement. Most important, this possibility would provide a patient with new cells of their own genetic type, thereby avoiding the necessity of immunosuppression, as would be required for any cells derived from any other individual, except an identical twin. The great interest in this field has been enormously stimulated by the recent discovery of induced pluripotency stem cells but has depended on several much earlier discoveries, most notably on that of embryonic stem cells.

There has been something of a tidal wave of interest in stem cells and regenerative medicine as researchers all over the world become active in it. Almost every day there are new papers published on various aspects of pluripotency, and it would be hard, even for those intimately involved in experimental work of this kind, to keep up to date with every advance. It is therefore very valuable to have a volume of 36 contributions summarizing the current status of progress in the various fields that contribute to regenerative medicine. Krishnarao and Raghu Appasani have assembled the contributing chapters into six main areas, ranging from stem cell biology through tissue engineering and therapeutic possibilities. The component chapters will be valuable not only to those who are experimentally active in an aspect of regenerative medicine, but also to those concerned with potential therapeutic applications. This volume also contains a valuable historical perspective by the Appasanis explaining key events in the development of this field over the last 150 years.

Although there is great enthusiasm for the eventual therapeutic value of work in this field for human health, scientists are very cautious about the time scale of human benefit. Bone marrow cells have been of great clinical value for a number of years. However, there is a long way to go before the brain and heart, to take two examples, can benefit from laboratory-created stem cells. It is indeed remarkable that beating heart cells or dopamine-secreting brain cells can now be derived from human skin and can be proliferated in the laboratory. However, substantial advances will be needed for it to be possible to integrate these laboratory-grown cells into

organs or tissues of living individuals and to arrange for these new cells to continue their newly acquired activity once transplanted into a patient. It is unlikely that a complex organ, often consisting of many different cell types, will soon be able to be constructed in the laboratory. The number of cells required for human therapy is also of concern, since a human heart or brain consists of more than 1 million million (10^{12}) cells. On the other hand, some cells make their contribution by secreting products or by providing critical neural connections, and even 10,000 cells of one kind could be valuable, as, for example, in the retina of the eye. I believe there is a cautious optimism in this field. It is generally true that once scientists find out how to achieve a desired result to a small extent, it is only a question of time before this advance is made to work enormously more efficiently.

My last comment concerns the reliability and safety of stem cells in regenerative medicine. There is understandable concern that any stem cells used for therapeutic purposes should be completely free of potential cancer cells or potentially harmful viruses. However, I submit that a situation might be reached where, even though one patient may suffer, more than 99.9% of other patients may derive enormous benefit. I hope that the fear of an occasional harmful replacement cell will not discourage continuing attempts to derive replacement cells that could be of enormous therapeutic value for a great number of other patients.

Cambridge, UK John. B. Gurdon

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Part VI Regenerative Medicine

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Part I
Stem Cell Biology

Introduction to Stem Cells and Regenerative Medicine

Krishnarao Appasani and Raghu K. Appasani

> The development of cell lines that may produce almost every tissue of the human body is an unprecedented scientific breakthrough. It is not too unrealistic to say that stem cell research has the potential to revolutionize the practice of medicine and improve the quality and length of life.

–Harold Varmus, Former Director, National Institutes of Health,
and 1989 Nobel Laureate in Physiology or Medicine,
testimony before the U.S. Senate Appropriations Subcommittee on Labor,
Health and Human Services, Education and Related Agencies, December 2, 1998

Embryology is a branch of biology that has an immediate bearing on the problem of life. Life cannot be fully accounted for without an understanding of its dynamic nature, which expresses itself in the incessant production of new organisms in the process of ontogenetic development. Therefore, embryology is defined as the science of the development of an embryo from the fertilization of the ovum to the fetus stage. Teaching of embryology has long been an established feature at universities throughout the world for students of medical and biological sciences. During the twentieth century most of this science has been overshadowed by experimentally based genetics and cell biology, which have in turn transformed classical embryology into developmental biology. Several universities are now teaching developmental biology instead of embryology as a course in their biology programs. Significant contributions made in the twenty-first century in the fields of molecular biology, biochemistry, and genomics integrated with embryology and developmental biology provide an understanding of the molecular portrait of a developmental cell. This integrated approach to development is incorporated in the present book as stem cell biology, a new sister branch of embryology/developmental biology that emphasizes the study of self-renewal, differentiation, pluripotency, and nuclear programming, all of which are characteristics of stem cells.

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The common hallmarks of stem cells include their unlimited self-renewal capacity and the high multilineage differentiation potential, which make them fundamental during embryo/fetal development and throughout the adult life. According to their developmental potential, stem cells can be divided into different categories: totipotent, pluripotent, multipotent, and unipotent. Totipotent stem cells can be found in the early stages of embryo development, after the very first cell divisions; these cells are able to differentiate into all types of embryo tissues in the trophoblast. After the segregation phase, the embryo, now called blastocyst, contains a cluster of cells, the inner cell mass, from which the embryo develops and embryonic stem cells (ESCs) are isolated; ESCs are defined as pluripotent because they can differentiate into cells of the three germinal layers (ectoderm, mesoderm, endoderm) but not the trophectoderm lineage. Lower down in the hierarchical tree there are the multipotent stem cells, isolated from specific niches in many adult tissue and organs that can produce a limited range of differentiated cell lineages. Finally, there are the unipotent stem cells, defined also as committed progenitors, as they can generate only one specific cell type and have a much more limited proliferative potential.

In a broad sense, stem cell biology is nothing more than an understanding of the integration of embryology and development at the molecular level using engineering, imaging, and cell culture principles. With such a wide scope, this book can only be an introduction to stem cell biology. We carefully chose the chapters and sections to maintain the theme expressed in the subtitle of this book: *Molecular Embryology to Tissue Engineering*. Many of the aforementioned applications are described within the various themes of the book, which are summarized as follows.

1 Section 1: Stem Cell Biology

The term "stem cell" appeared in the scientific literature as early as 1868 in the work of the eminent German biologist Ernst Haeckel. In demonstrating the evidence for Darwinian evolution, Haeckel developed phylogenetic trees and used the term "*Stammzelle*" (stem cell) to describe the ancestor unicellular organism from which he presumed all multicellular organisms evolved. In 1892 Valentin Hacker described "stem cells as the cells that later in development produce oocytes in the gonads." In 1896 the embryologist Edmund Wilson reviewed these German scientists' historical findings in his book *The Cell in Development and Inheritance*, which was published in English and was inspirational to a generation of turn-of-the-century embryologists and geneticists, especially in the United States. The "road to embryonic stem cells and beyond" began in the 1960s with the work of Leroy Stevens from the Jackson Laboratory, Bar Harbor, Maine, who discovered embryonal carcinoma cells while studying testicular carcinomas. Later Stevens and colleagues demonstrated that these embryonal carcinoma cells were indeed pluripotent stem cells. In the early 1980s Gail Martins of the University of California at San Francisco in the United States and Martin Evans and Matthew Kaufman of the University of Cambridge in England independently isolated stem cells from mouse

embryos and coined the term "embryonic stem cells." The greatest achievement of the ESC field during the mid 1980s is considered to be the advent of gene targeting and germline modification, for which Mario Capecchi, Martin Evans, and Oliver Smithies shared the 2007 Nobel Prize in Physiology or Medicine. In 1995, Jamie Thompson of the University of Wisconsin cultured monkey ESCs for the first time and subsequently human embryonic stem cells in 1999. Thompson's work propelled the activity of stem cell research and cell propagation technologies in general. The first seven chapters of this book detail the historical aspects of ESCs, self-renewal, cancer stem cells, the use of stem cells in tissue repair, and the regeneration of sensory cells.

The historical aspects of ESC discovery are detailed in Chapter 2 by Theodorou and Snyder. In a mammalian organism homeostasis is the central mechanism maintaining and preserving organ and tissue integrity. Stem cells are the main players of homeostatic balance through self-renewal and multilineage differentiation. *Bmi1* is one of many genes that play a crucial role in mitochondrial function, leading to the hypothesis that this gene is one of the master regulators of tissue maintenance. In Chapter 3, Sangiorgi and Capecchi detail the role of *Bmi1* in stem cell self-renewal and mitochondrial function using knockout mice technology. The existence of cancer stem cells (CSCs) continues to be an extensively debated topic among scientists, and with mounting evidence it is an area that needs further examination. Currently there is no single method for the isolation of CSCs; a combination of techniques such as surface markers, sphere formation, and chemotherapy resistance assay are used, which are only partially efficient. Recent efforts in this research have identified tumor-initiating cells in nearly all types of cancers, which exhibit self-renewal, unlimited propagation *in vitro*, and chemoresistance. In order to effectively treat cancer according to the CSC theory, du Potet et al. describe novel therapy methods in Chapter 4.

Treatments for muscular dystrophies constitute the goal of a very active field of research, and the characteristics of mesenchymal stem cells make them a promising alternative for cell-based therapy. In this context, adipose-derived stem cells (ADSCs) present very interesting intrinsic features in terms of abundance and easy access. ADSCs exhibit a myogenic differentiation potential lower than the adipogenic, osteogenic, or chondrogenic potential. The physiologic importance of this plasticity remains to be elucidated, but it is conceivable that better knowledge may potentially lead to their use in muscle dystrophy therapy. The myogenic potential of ADSCs has been investigated using *MyoD*, a master gene of embryonic myogenesis, which is detailed in Chapter 5 by Dechesne et al.

Hearing loss presents a significant social and economic burden. It is commonly associated with an irreversible loss of hair cells and their innervating spiral ganglion neurons. Attempts at integrating new supplementary cell sources into the damaged inner ear have been tested extensively. In Chapter 6, Wei and Yamoah summarize the history of available cell sources, their achievements and limitations, and future developments for hearing rehabilitation. Utilization of multiple classes of progenitor and stem cells as cell therapy holds the potential to effectively treat these patients. The main integral cellular parts of bone in this process are the bone-forming osteoblasts, which are surrounded by osteoclasts. Compromised osteoprogenitor function

can thus subsequently lead to osteodegenerative diseases, including osteoarthritis, osteoporosis, and many more. In Chapter 7, Baumgartner et al. describes the clinical use of stem cells, specifically adult mesenchymal and embryonic stem cells, as cellular sources for the treatment of osteodegenerative diseases.

2 Section 2: Epigenetic and MicroRNA Regulation in Stem Cells

Epigenetic changes are commonly accepted as important events in cancer development and progression. A series of intriguing results suggests that epigenetic deregulations may occur in stem/progenitor cells and precede genetic alterations. Epigenetic mechanisms are essential for maintaining the identity and regulation of stem cells. Therefore a dysfunction of these mechanisms could lead to the transformation of the normal stem cells into cancer stem cells. This section is devoted to summarizing the role of epigenetics and gene regulation that is mediated by microRNAs.

As described by Ouzounova et al. in the opening chapter in this section, Chapter 8, epigenetic alterations may be considered as one of the first events in stem/progenitor cells leading to cancer development. Epigenetic disruption of "stemness" genes in stem/progenitor cells may be one of the initial steps of carcinogenesis. The subsequent genetic and epigenetic deregulation of molecular pathways involved in survival and differentiation may result in the formation of CSCs. Better understanding of epigenetic mechanisms during cancer development and progression is essential for the development of novel therapeutic strategies. Epigenetic alterations could be principal targets for inhibitors of histone deacetylases or DNA methyltransferases. Therefore, establishing epigenetics-based therapeutic strategies at the CSC level would theoretically prevent regeneration of the tumor. More research on the study of epigenetic mechanisms could also simultaneously facilitate the development of novel and efficient strategies for cancer prevention and treatment.

For decades, researchers in the embryonic stem cell field have been trying to uncover the molecular mechanisms that govern stem cell fate. Recent results indicate that microRNAs (miRNAs), tiny molecules about 22 nucleotides long, which are small, noncoding RNAs, play important regulatory roles in animal stem cell fate. More than 600 miRNAs have been identified in humans. It has been proposed that more than one third of all human genes may be regulated by miRNAs, and one gene could also be repressed by multiple miRNAs.

Self-renewal refers to the unlimited proliferation potential of cultured ESCs *in vitro*. Pluripotency refers to the unique potential of ESCs to generate any differentiated cell type in the adult organism. A series of genetic studies elucidated the requirement for miRNAs in the maintenance of ESC self-renewal and pluripotency. Several reports revealed that a core regulatory circuitry of transcription factors controls the self-renewal and pluripotency properties of ESCs. *Oct4*, *Sox2*, and *Nanog* function as central regulators to the transcriptional control hierarchy that determines

the fates of ESCs. Moreover, miRNAs, including miR-134, miR-296, miR-470, and miR-145, have been demonstrated to modulate the pluripotency of ESCs by repressing the expression of *Oct4*, *Sox2*, and *Nanog*. Knowing that both protein-coding genes and noncoding genes are regulated by key transcription factors provides novel insights into the molecular mechanism of cell differentiation and reprogramming of ES cells. As described by Xu and Kosik in Chapter 9, the cell-type-specific expression signature of miRNAs in mouse and human ESCs has been used successfully to distinguish ESCs from differentiated cell types. The characterization of the miRNA pathways and their underlying molecular mechanisms is of great importance to the understanding of ESC self-renewal and pluripotency.

The liver is an extraordinary organ that sustains its regenerative power throughout life. The precise molecular mechanisms regulating liver regeneration have yet to be unveiled, but a number of cell types have been postulated to be involved in the process, such as resident liver stem cells, differentiated hepatocytes, and extrahepatic stem cells. Liver regeneration from extrahepatic stem cells, hepatic lineage plasticity of mesenchymal stem cells (MSCs), and the applications of MSCs for the treatment of liver diseases in preclinical and clinical studies are discussed by Kuo et al. in Chapter 10. They also describe the signaling pathways and mechanisms including miRNAs that are involved in the regulatory control of hepatic fate specification. Thus far, little has been reported on the role of miRNAs in MSCs, and miRNA expression and function in the differentiation of MSCs remain largely unknown. Use of microarrays and quantitative PCR analyses has shown the alteration of miRNAs during MSC differentiation and osteogenesis.

miRNA regulation in hepatocyte generation has been demonstrated to be important in controlling cell behavior and differential fates and contributes an additional layer of control in the regulatory network. In liver, miR-122 is the most abundant miRNA, and it is recognized as a liver-specific miRNA. A number of studies have revealed that miR-122 mediates cholesterol and lipid metabolism. In addition, the expression level of miR-122 is associated with liver diseases, such as hepatitis C virus infection, and hepatocellular carcinoma. Recently, the function of hepatocytes and the expression of miRNAs were examined in liver-specific Dicer-knockout mice, and the results suggest an essential role for miRNAs in sustaining normal hepatic functions throughout the lifetime of the organism.

3 Section 3: Stem Cells for Therapeutic Applications

Stem cell therapy holds enormous potential for treating a wide range of genetic and sporadic degenerative disorders. However, one of the major hurdles facing stem cell therapy is the ability to assess cell fate and outcome prior to transplantation. Before stem cell therapy can become a reality, new techniques will be needed to assess cell viability prior to transplantation. Section 3 of this book highlights various types of stem cells and their applications for cell therapy, notably for muscle, liver, cardiac, and neurodegenerative diseases.

Recent studies have shown that time-lapse microscopy can address these needs by observing dynamic events at the single-cell level such as differentiation, asymmetric versus symmetric divisions, and fate specification. Time-lapse microscopy can be performed in a high-throughput and noninvasive or minimally invasive manner and has the potential to become a powerful predictive tool when combined with an understanding of the underlying biologic processes. The idea of correlating imaging technology with gene expression profiling has also been investigated in computed tomography and magnetic resonance imaging. Chapter 11 by Loewke and Reijo Pera from Stanford University Medical School reviews the role of time-lapse microscopy in stem cell research and therapy. Stem cell therapies offer enormous hope for treating many tragic diseases and tissue defects. In particular, mesenchymal stem/multipotent stromal cells (MSCs) are capable of differentiating into multiple types of connective tissues (i.e., bone, cartilage, and even muscle and neuron) and have proangiogeneic and immunomodulatory effects. MSCs have potential utility for treating a variety of diseases and disorders, including graft versus host disease, organ transplantation, cardiovascular disease, brain and spinal cord injury, lung, liver and kidney diseases, and skeletal injuries. In Chapter 12, Zhao et al. summarize the current status of therapeutic applications of MSCs, mechanism of action, delivery routes, MSC homing, the current status of clinical trials, and potential challenges and safety issues. In addition, this chapter also includes novel chemical approaches developed in their laboratory at MIT and Harvard to promote homing and engraftment.

The gastrointestinal (GI) tract is a rich repository of stem cells. There is continuous and rapid renewal of the epithelial lining of the GI tract. Attempts have been made in mice to harvest neural stem cells (NSCs) from the GI tract. This has potential in the treatment of motility disorders like achalasia, congenital hypertrophic pyloric stenosis, and diabetic gastroparesis. Chapter 13 by Khan et al. discusses the isolation of gut and esophageal epithelial stem cells and demonstrate the differentiation pathway and tissue renewal. The lung epithelium is structurally and functionally a complex tissue composed of different cell types exposed to toxic agents and pathogens that can with time result in various lung diseases, including asthma, chronic obstructive pulmonary disease, pulmonary fibrosis, and lung cancer. The stem cell niche in the lung is poorly defined, but as in many other organs, stromal cells and extracellular matrix likely play a fundamental role in regulating stem cell activity. Basal cells and cells in the neck of the submucosal glands have been shown to contain stem cell characteristics in trachea and large bronchi. Chapter 14 by Magnusson et al. describes the lung development and methods to track stem cells *in vitro* and *in vivo*. In addition, this chapter also focuses on the cellular hierarchy in the lung epithelium, the potential location of endogenous lung stem cells, and current evidence indicating that stem cells are potential cancer-initiating cells in the lung.

Placental-derived stem cells are easily accessible and do not have many of the limitations of ESCs and their derivatives for their use in clinical trials. Graham Jenkin's group from Monash University in Australia have developed methods to isolate, characterize, and store human amnion epithelial cells (hAECs) for human

use in cell therapies. These cells have conserved long telomere lengths and, unlike ESCs, do not form teratomas *in vivo*; they are very safe for cell therapy and transplantation. Chapter 15 by Murphy et al. summarizes the characterization of hAECs from term gestational tissues and investigates their potential application in the treatment of adult and perinatal lung injury. In addition, this chapter shows that hAECs exhibit key features of pluripotent stem cells and do not form teratomas.

Cardiovascular disease remains the single most potent killer in all developed societies. A handful of pharmacologic strategies are available to treat coronary artery disease. However, no conventional treatment is available to reverse a failing and structurally deranged heart to transition back toward more efficient and healthful structure and cardiac pump function. Despite advances in reperfusion therapies for acute myocardial infarction (MI), many patients are left with large scars and significant adverse remodeling, underscoring the need for new approaches for myocardial preservation and regeneration. Ideally, cell therapy would provide readily available and well-characterized cell type(s) capable of homing to the infarct zone and altering the infarction healing process to limit or prevent ventricular remodeling. In this section, Chapter 16 by Angeli and Yeghiazarians, clinicians, and Chapter 17 by Michael Mann, a surgeon, from the University of California at San Francisco provide insight into the use of bone marrow cell therapy (BMCT) and stem cell transplantation for cardiac diseases, especially MI.

Angeli and Yeghiazarians review some of the clinical trials of BMCT in post-MI patients and address safety concerns. They point out that the cell retention and engraftment rate after cell delivery is very low. Potential alternatives using biomaterials and cell engineering techniques will increase cell retention and engraftment and are in development; however, future clinical studies will need to be conducted to evaluate their safety and efficacy. Ongoing research will continue to improve both our understanding of developmental cardiac myocyte and our ability to enhance the therapeutic properties of donor stem and progenitor cells. In a nutshell, we are in the early days of stem cell therapy to treat ischemic heart disease. Much more work remains to be performed to better understand the potential of cell therapy post-MI.

Over the last few years, there has been a tremendous surge of interest in NSC-based therapies for treating neurologic disorders. The cells for brain repair may be derived from endogenous NSC populations or exogenous sources, such as ESCs. For these therapies to be effective it is imperative to be able to recruit transplanted neural progenitors to the damaged sites. In this context, stem cell therapies targeted for the treatment of adult brain injuries must invoke the mechanisms that govern normal migration of neural progenitors during embryogenesis and adult neurogenesis. Significant progress has been made toward development of stem cell–based therapies to treat neurodegenerative diseases such as Huntington disease, epilepsy, and Alzheimer disease. In this section three chapters describe the latest highlights in this field.

Huntington disease (HD) is an autosomal dominant genetic neurodegenerative disorder. Progressive degeneration of (γ-amino butyric acid) GABAergic medium spiny projection neurons in the basal ganglia and loss of cognitive and psychiatric

symptoms are hallmarks of HD patients. With no efficient treatment available in the clinic to alleviate or compensate for the progressive neuronal cell loss in HD, novel treatment strategies such as endogenous cell replacement therapy need to be investigated. In Chapter 18, Connor reviews the highlights of the use of adult neural progenitor cell replacement therapy in the HD brain. In Chapter 19, Hartman et al. review current knowledge regarding neuronal and glial migration in development and adulthood, as well as the factors that promote or limit cell migration in degenerative disorders, including demyelinating diseases, stroke, and epilepsy. In addition, this chapter provides the current literature on the molecular and cellular mechanisms guiding neuronal and glial migration.

Alzheimer disease (AD) is an incurable, degenerative, and terminal disease that was first described by Alois Alzheimer in 1906. Patients with AD can be categorized as either having early-onset or late-onset AD. Early-onset AD is caused through mutations in the amyloid precursor protein and accounts for less than 5% of cases. Late-onset AD is most often diagnosed in people over 65 years of age. Typically these early symptoms include short-term memory loss and clumsiness. Progress in NSC research is offering greater understanding and better-tuned cellular resources for potential cellular therapeutics. Nevertheless, AD with its heterogeneity in presentation and diffuse pathology remains a challenging cellular therapeutic target. NSCs cultured *in vitro* as neurospheres or adherent cultures may offer potential cellular resources. In Chapter 20 Gorba et al. discuss various options of using NSC therapy in treating AD patients.

4 Section 4: Nuclear Reprogramming and Induced Pluripotent Stem Cells

Nuclear programming is the process that instructs specialized adult cells to form early pluripotent stem cells. Pluripotent stem cells posses the capacity to become any type of mature cell in the body and therefore have great potential for experimental and therapeutic purposes. Using the concept of cellular reprogramming, Briggs and King in 1952 produced normal tadpoles by transplanting nuclei from blastula cells to enucleated eggs in the frog *Rana pipens*. Transplantation of nuclei from differentiated cells was achieved by John Gurdon in 1962 in the African clawed toad, *Xenopus laevis*, which is now known as the classic nuclear transfer experiment. It took more than another decade (1975) for Gurdon to succeed in producing healthy and sexually mature fertile frogs with functional muscle, beating hearts, well-differentiated eyes, and all of the other organs. This experiment provided the first clear evidence that cell specialization does not involve irreversible inactivation in the genes required for development of an animal. This conceptual framework led to the start of the field of nuclear reprogramming, and Gurdon became known as the "father" of nuclear reprogramming (cloning). It took almost another 10 years to clone an adult sheep, Dolly (in 1996), by Kevin Campbell and Ian Wilmut of the Roslin Institute in Edinburgh, Scotland. This experiment dramatically

extended Gurdon's concept from frogs to mammals. The Dolly-related work of somatic cell nuclear transfer was further extended to produce monkeys, cows, dogs, mice, and other animals. These remarkable contributions stimulated other researchers to think about using nuclear transfer to generate pluripotent human embryonic stem cells for cell replacement therapy.

There are two routes to producing a living animal: (1) injection of a somatic cell nucleus into an enucleated egg (nuclear reprogramming) and (2) use of an embryo to produce ESCs. In a quite astonishing discovery, Kazutoshi Takahashi and Shinya Yamanaka of Kyoto University in Japan for the first time in 2006 turned adult mouse skin fibroblast cells into pluripotent cells. This breakthrough of inducing fibroblasts was achieved by stable transfection using only four transcription factors (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*), now referred to as induced pluripotent stem (iPS) cells. This work was extended and further confirmed by several groups to generate iPS cells from individuals with various neurodegenerative diseases, leading to the hope of cell replacement therapy and the dream that personalized medicine can become a reality. The crowning achievement of cDNA reprogramming has obviously been the creation of iPS cells, which will not only prove invaluable in the laboratory, but also represent a significant step forward in stem cell therapy without the ethical issues posed by ESCs. A section of this book with seven chapters is devoted to nuclear reprogramming and iPS cells.

Many human diseases, such as myocardial infarction, diabetes, retinal degeneration, and spinal cord injury, occur because of cell loss, degeneration, and injury. Theoretically, with the transplantation of specific cells created from autologous iPS cells, the cells that are lacking can be replenished and replaced by cells with the defects corrected, and thereby a patient's symptoms can be relieved. So far, the transplantation of iPS-derived cells has shown promising therapeutic effects in a few animal disease models, for example, in a rat model for Parkinson disease and a mouse model for acute myocardial infarction.

Wakayama et al. developed nuclear transfer ES (ntES) cells that were identical to the ES cells derived by fertilization in terms of their global gene expression and their differentiation potential. The results are discussed in detail in Chapter 21. In addition, by combining the cloning and the ntES cell techniques, this approach could be applied to fertility treatments using somatic cells instead of gametes. Generation of haploid human germ cells from pluripotent stem cells will be a remarkable achievement in the future. In human germ cell development, the primordial germ cell (PGC) is the most promising platform on which to build further differentiation processes. There is a great interest in the potential use of *in vitro*–derived germ cells in regenerative medicine to treat infertility. In Chapter 22 Lindgren et al. discuss the current state of the technology used for PGC differentiation *in vitro* and the future outlook for the use of regenerative medicine to treat infertility. Treatment of infertility will require the generation of a haploid gamete. No study to date has convincingly showed that human pluripotent stem cell–derived germ cells can effectively undergo meiosis and recombination.

Diabetes mellitus is a metabolic disease that is characterized by high blood glucose levels caused by the progressive failure of the insulin-secreting beta cells of

the islets of Langerhans in the pancreas. It can be subdivided into type I (insulin dependent) and type II disease. Current therapy still only gives limited protection against late complications of diabetes. It has therefore become increasingly apparent that new therapeutic options in the treatment of diabetes are desperately needed. The ideal therapy would allow effective cell replacement to restore the patient's normal physiologic insulin secretion without having to resort to repeated invasive monitoring and exogenous insulin administration by injection. Chapter 23 by Drummond et al. summarizes the significant contributions made toward the use of stem cell technologies to generate beta cells as a potential therapy for patients suffering from diabetes. Although this rapidly developing iPS technology offers a promising route to finding a cure for this destructive disease, there are still many hurdles to overcome before it can be safely used in clinics.

The generation of iPS cells has spawned unprecedented opportunities for investigating the molecular mechanisms that underlie cellular pluripotency and reprogramming, as well as for obtaining patient-specific cells for future clinical applications. However, both prospects are hampered by the low efficiency of the reprogramming process. Skin keratinocytes comprise one cell type that is easily obtainable and can be reprogrammed to pluripotency by retroviral transduction with Yamanaka factors (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*). As described by Aasen and Belmonte in Chapter 24, the keratinocyte-derived iPS (KiPS) cells appear indistinguishable from human embryonic stem cells in colony morphology, growth properties, expression of pluripotency-associated transcription factors, and surface markers, as well as in their capacity to differentiate both *in vitro* and *in vivo*. In addition, keratinocytes can be cultured from a single plucked hair from adult individuals, permitting generation of iPS cells without the need for invasive biopsies. The system of induced reprogramming of keratinocytes to pluripotency provides both a practical and advantageous alternative for the generation of patient- and disease-specific pluripotent stem cells.

The pig is an attractive species for creating pluripotent cell lines because, unlike the currently preferred mouse model, the pig resembles the human quite closely in size, anatomy, and physiology. Recently, elegant work on the generation of porcine induced pluripotent stem cells (piPSCs) was performed, and this is described in Chapter 25 by Roberts and his colleagues from the University of Missouri at Columbia. The derivation of piPSCs is a significant milestone in transplantation biology. The piPSC affords advantages in developing clinical models for human regenerative medicine and for testing the safety and efficacy of "personalized" transplants and may also have application in agriculture. piPSCs have an edge over ESC in transplantation studies because of the possibility of generating patient-specific stem cells that can be grafted back into the same animal from which they were derived. Human embryonic stem cells and induced pluripotent stem cells (iPSCs) provide great promise for development of clinical applications of cell-based therapies in regenerative medicine, as well as improvement of *in vitro* disease modeling and drug screening. In Chapter 26, Zeng and Zhou summarize the therapeutic potential of patient-specific iPS cells and the derivation of disease-corrected hematopoietic progenitors from Fanconi anemia–induced pluripotent stem cells.

As is seen in all of the foregoing chapters, Yamanaka's four transcription factors (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*), or at least three of them (*Oct4*, *Sox2*, and *Klf4*), are important for inducing pluripotency. However, recently Hans Scholer and his colleagues from the Max Planck Institute for Molecular Medicine, Munster, Germany, have demonstrated that only one factor, *Oct4*, is sufficient to directly reprogram adult mouse neural stem cells into iPS cells. In addition, this group has also shown that these human neuronal stem cell–derived 1-F iPS cells are indistinguishable at the molecular level from human embryonic stem cells and can differentiate into cells of all three germ lineages both *in vitro* and *in vivo*. Kim et al. describe some of the highlights of this work in Chapter 27.

Gurdon has spent much of his career deciphering the molecules and mechanisms that the egg uses to "rejuvenate" nuclei. We know a lot about nuclear transfer, but the question still remains of how to regulate and control the reprogramming of the nucleus most efficiently. Although both Gurdon's (nuclear reprogramming) and Yamanaka's (iPS) technologies can generate living animals, we do not know the molecular mechanisms underlying these two strategies. Are they the same or different? The potential of iPS cell technology in biology and medicine is enormous, although it is still in its infancy and there are many challenges to be overcome before various applications can be used successfully. We still need to understand the components of oocytes or eggs used to depress somatic gene expression and discover the direct cell-type switch by overexpressed transcription factors. It is also important to identify the basis of the stability of the differentiated state of cells, which will help to understand how egg-reprogramming factors operate. Finally, mapping of the "embryome" is a must and is likely to become available soon, which will help us to understand the intricacies and epigenetic imprints of embryos.

Using nuclear transfer embryonic stem technology, researchers can now generate live mice from even frozen dead bodies, suggesting that extinct animals, such as the mammoth, can be possibly resurrected by this technology if nondamaged nuclei are retrieved from the permafrost. One of the biggest challenges for using ESCs is immune rejection after transplantation into patients for clinical therapy. iPSCs thus provide a great alternative since they can avoid immune rejection/histocompatibility if derived from a patient's own somatic cells. However, the concern of teratoma or teratocarcinoma formation still applies in the recipient owing to the presence of residual undifferentiated pluripotent cells in transplants. Induced pluripotency technology is a new avenue for treating severe diseases and certain cancers. It has excited the scientific community. The technical race for iPS cell generation is well along, but the race for applying these cells to answer basic research questions and for therapeutic regenerative medicine is only in its infancy.

Reprogramming biology may still need to be ironed out, but on another playing field, chemical biologists are busy looking for a chemical route to iPSCs using small molecules. However, small molecules can also cause unwanted side effects. The idea and hope of the chemists is to reprogram any cell of the body into another by means of a simple molecular kit. Peter Schultz's group from the Scripps Institute, La Jolla, California, and Douglas Melton's group from the Harvard Stem

Cell Institute, Cambridge, Massachusetts, have shown independently that the small molecules lactam kenpaullone and RepSox induce pluripotency, respectively. In addition, stem cell biologist Judith Kimble from the University of Wisconsin at Madison has used butanedinitrile, just one chemical molecule, and converted sperm cells into oocytes in a worm, *Caenorhabditis elegans*. Very recently, Marius Wernig's group from the Stanford University Medical School, Stanford, California, has used a combination of only three factors, *Ascl1, Brn2* (also called *Pou3f2*), and *Myt1l*, to rapidly and efficiently convert mouse embryonic and postnatal fibroblasts into functional neurons *in vitro*. These induced neuronal (iN) cells express multiple neuron-specific proteins that generate action potentials and form functional synapses. Generation of iN cells from nonneural lineages could have important implications for studies of neural development, neurologic disease modeling, and regenerative medicine.

5 Section 5: Tissue Engineering

The term "tissue engineering" was first used by Eugene Bell of MIT in 1984, and later was also referred to extensively by Wolter and Meyer in 1984. Tissue engineering studies "a combination of cells, engineering, and materials methods, and suitable biochemical and physio-chemical factors to improve or replace biological functions." In other words, it intends "to repair or replace portions of or whole tissues such as bone, blood vessels, bladder, skin and artificial organs." According to Robert Langer and Joseph Vacanti, it "applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ." Powerful developments in the multidisciplinary field of tissue engineering have yielded a novel set of tissue replacement parts and implementation strategies. Scientific advances in biomaterials, stem cells, growth and differentiation factors, and biomimetic environments have created unique opportunities to fabricate tissues in the laboratory from combinations of engineered extracellular matrices ("scaffolds"), cells, and biologically active molecules. A section in this book has five chapters that highlight recent work relating to biomaterials, three-dimensional culture systems, "lab-on-a-chip" development, and microtechnologies aimed at understanding stem cell biology.

Although use of one body part for another or the exchange of parts from one person to another has been mentioned in literature for centuries, actual investigations into organ transplantation began in the early 1900s. The field of urology was the earliest to benefit from these new transplantation techniques. Synthetic materials/or devices such as tetrafluoroethylene (Teflon) and silicone have been introduced to replace or rebuild diseased tissues in the human body. However, all these devices can only provide a structural replacement; the functional components of the original tissue cannot be achieved using these materials.

Tissue engineering has emerged as an excellent approach for the repair/regeneration of damaged tissues, with the potential to circumvent the limitations of

autologous and allogeneic tissue repair. Nonetheless, significant efforts are still necessary to achieve a better understanding of stem cell biology, on one hand, and an optimal microenvironment capable of stimulating transplanted cells and host tissue regenerative "response," on the other hand, by developing functionalized scaffolds and choosing the appropriate set of cytokines to be slowly delivered locally. Indeed, although considerable progress has been made toward understanding ESCs, we cannot yet claim that they can be used safely in clinical applications. At the same time, a number of challenges remain in the design of materials that are nonimmunogenic, scalable, mechanically tunable, and bioactive in their presentation of key regulatory signals to cells.

Chapter 28 by Antonini et al. discusses the use of electrospun constructs and self-assembling peptides (both characterized by their nanoscale architecture) in the context of tissue engineering. Self-assembling peptides alone cannot provide directed spatial guidance to regenerating cells. On the other hand, electrospun scaffolds have been adopted in regenerative approaches targeting musculoskeletal and nervous system injuries due to the ability to spatially guide tissue regeneration. Innovative microscale technologies can contribute to quantitative understanding of how phenomena at the microscale can determine stem cell behavior, increasing the fidelity in controlling the culture conditions and the throughput of the data while reducing times and costs. While acknowledging the advantages of applying these technologies to stem cell culture, Serena et al. in Chapter 29 focus on the relevant issues related to the control and mimicking of the microenvironmental cues of the stem cell niche such as substrate properties, cell topology, the soluble environment, and electrophysiology. Techniques such as microcontact printing, microtransfer molding, and solvent-assisted micromolding can be applied. Alternatively, microscale features could be created on supporting materials via selective chemical vapor deposition via wet or dry etching processes.

With the introduction of microtechnology and microfluidic platforms for cell culture, the pace of stem cell research can be dramatically enhanced. Using microfluidic-based techniques, extracellular microenvironments can be controlled in a precise manner and their influence on various cellular behaviors can be studied. Microfluidic devices made of transparent materials allow real-time and high-throughput monitoring of cell functions and cell fate by utilizing fluorescence microscopy and other optical techniques. Chapter 30 by Gupta et al. discusses the considerable capability of microfluidic devices, which still remains an underutilized technology, for stem cell research. Microfluidics will provide insights into creating integrated, modular, and easy-to-use devices to perturb stem cells. Microfluidics are commonly referred to as the "lab-on-a-chip" approach. The advantages of microfluidics will be fully realized when this technology is used in a modular manner in tightly integrated fluidic systems where each module can be treated as a fully characterized, separate unit with defined input and output ports.

Small molecules have played a large part in the research associated with neuronal differentiation of stem cells. Natural compounds incorporated into culture medium and specific neural supplements enhance the neuronal cell output. However, there are certain limitations to these approaches when using either natural

small molecule supplementation or the widely used commercial neural supplements. Synthetic small molecules can be designed with a specific biologic outcome in mind and can exhibit very tightly regulated biologic responses. Through the collaborative interactions between biologists and chemists, there is enormous potential to develop innovative approaches to control cell differentiation responses for the benefit of basic research and potential therapeutic applications. In Chapter 31, Christie et al. review research using small molecules to control the differentiation of stem cells into neural phenotypes, mentioning in particular work on pluripotent stem cells and adult neuroprogenitor cells.

Neural stem cells hold great promise in regenerative therapy. Within a few years cells derived from NSCs are expected to be used for cell replacement therapy in neurodegenerative diseases like Parkinson and Huntington diseases, where defined populations of dopaminergic and GABAergic neurons are lost. The number of neurons that can be derived from NSCs in culture depends particularly on the age of the donor, region of origin, mitogens used for propagation, and longevity in culture. Chapter 32 by Andersen et al. focuses on three-dimensional culture systems for long-term propagation of fetal NSCs and mitogens commonly used for maintenance of their proliferative capacity and neurogenic potential. Increased knowledge about the microenvironment and stem cell niches is, moreover, mandatory for the development of therapies targeting the patient's endogenous NSCs and stem cell niches for the purpose of improving their reparative responsiveness to ongoing neurodegenerative diseases.

6 Section 6: Regenerative Medicine

Regenerative medicine is a new branch of medicine "that attempts to change the course of chronic disease and in many instances will regenerate tired and failing organ systems lost due to age, disease, damage, or congenital defects." The term "regenerative medicine" was first used in 1992 by Leland Kaiser and then popularized by William Haseltine of Human Genome Sciences, Rockville, Maryland. The term regenerative medicine is often used synonymously with tissue engineering, although those involved in regenerative medicine place more emphasis on the use of stem cells to treat diseases using cell therapies or transplantation methods. This field holds the promise of regenerating damaged tissues and organs in the body by stimulating previously irreparable organs to heal themselves. In support of regenerative medicine, scientists grow tissues and organs in the laboratory and safely implant them when the body cannot heal itself. Section 6 of this book is devoted to the use of stem cells for muscle repair and treatment of cardiac and urologic diseases. In addition, a chapter on the collection and use of cord blood stem cells for several clinical applications is also included.

Organ transplantation remains a mainstay of treatment for patients with severely compromised organ function. In the last two decades, as a response to the requirements of these tissues, scientists have attempted to grow native stem cells, engineer

tissues, and design treatment modalities using tissue engineering and regenerative medicine techniques for virtually every tissue of the human body. In Chapter 33, Atala reviews some of his team's elegant work on tissue engineering techniques and the progress that has been achieved in the field of urologic regenerative medicine. The kidney was the first entire organ to be replaced in a human. This was done in 1955 using identical twins, and thus the immunologic barrier to allotransplantation was not addressed. Then, in the early 1960s, Joseph E. Murray of the Harvard Medical School (who later received a Nobel Prize in 1990 for his work), performed a nonrelated kidney transplantation from a non–genetically identical patient into another. This transplant marked a new era in medicine, and transplantation became a viable therapy for failures in various organ systems. However, immune suppression and tissue rejections are still major drawbacks, which has opened the door for other alternatives.

In Chapter 34, Gharaibeh et al. demonstrate the use of muscle-derived stem cells (MDSCs) as a model for stem cell therapy in regenerative medicine. The MDSCs' ability to proliferate *in vivo* for an extended period of time, combined with their strong capacity for self-renewal, resistance to stress, ability to undergo multilineage differentiation, ability to induce neovascularization, and paracrine effects, partially explain the high regenerative capacity of these cells *in vivo*. In addition, Gharaibeh et al. review current knowledge on the utility of the MDSCs to improve the healing of various musculoskeletal tissues and injured cardiac muscle and list several clinical applications. Stem cell transplantation is an innovative therapy for tissue regeneration and repair after an injury. MDSCs are an excellent choice for cell therapy, as they exhibit stem cell characteristics, are easy to isolate, and can be used for autologous therapy. In order to improve cell transplantation for all types of repair in clinical translation, it is imperative to understand inherent differences between stem cell populations.

Ischemic heart disease (IHD) is characterized by reduced blood flow to the heart muscle, or myocardium. Established treatments for IHD include addressing risk factors that contribute to the disease, such as high cholesterol, hypertension, tobacco use, and diabetes, and improving impaired heart function directly by mechanical measures, such as biventricular pacemaker implantation, valve repair, bypass surgery, and heart transplant. Of these treatments, only transplant of a new heart resolves the underlying pathology of IHD, which is death of the heart muscle cells, or cardiomyocytes. Cardiac transplant is the major exception, but its efficacy as a general treatment is limited by the small number of available donor organs. The goal of cardiac regenerative medicine is to create robust, renewable sources of heart cells, particularly cardiomyocytes that can be used to restore lost heart function.

Recent findings in stem cell and developmental biology have suggested the possibility of generating new heart muscle using cells derived from a variety of sources. These include adult autologous stem cells found in bone marrow or skeletal muscle, autologous cardiac progenitor cells, embryonic stem cells, and induced pluripotent stem cells or other types of reprogrammed cells. In Chapter 35, Huang et al. focus on cell-based methods for regenerating cardiomyocytes that are lost due to IHD or other injury, with the end goal of restoring compromised heart function.

Results from early clinical trials of injected skeletal myoblasts and bone marrow cells show that minor improvement in heart function can be obtained without cardiomyocyte regeneration; however, these results are generally not regarded as successful examples of true regenerative medicine. The most promising of these from a clinical and commercial prospective is the directed differentiation of ESCs. IPS-based approaches, direct reprogramming, and expansion of native cardiac stem cells are also highly promising methods, as they are patient-specific therapies that may represent viable alternatives to the use of ESCs.

ESC therapies have been most often touted as the optimal stem cell source for regenerative medicine applications due to their ability to become any tissue in the body that might need therapy. Unfortunately, ESC applications are currently limited by ethical, political, biologic, and regulatory hurdles. However, cord blood stem cells provide a better alternative for regenerative medicine applications. In Chapter 36, Harris summarizes the latest developments in cord blood collection, processing, and banking, as well as the recent use of cord blood stem cells in transplant and regenerative medicine. As Harris discusses in this chapter, cord blood stem cells are the best source of these stem cells, as they can be used to derive tissues from all three (mesodermal, endodermal, and ectodermal) germ lineages.

Overall, regenerative medicine is a multidisciplinary field that requires expertise in a wide variety of scientific disciplines, including cell and molecular biology, physiology, pharmacology, chemical engineering, biomaterials, nanotechnology, and clinical sciences. Although modest clinical success has been achieved in specific areas, the field is still in its infancy. Long-term studies are still essential to assure safety and efficacy before these technologies can have widespread clinical application.

Embryonic Stem Cells: Discovery, Development, and Current Trends

Elias Theodorou and Michael Snyder

Abstract Murine embryonic stem cells were first derived almost 30 years ago from cultured blastocysts and have been primarily used as a tool to better understand development through targeted gene deletions. Only recently has the focus has shifted toward embryonic stem cells themselves and the molecular mechanisms by which they choose a specific cell fate. Through rapid advances in cell culture and genomic modification techniques researchers are beginning to regularly utilize embryonic stem cells for in vitro gene function assays. More important, the mechanisms critical for establishing the pluripotent sate of embryonic stem cells have been elucidated to the point that clinically beneficial stem cell–like counterparts can now be generated from nonembryonic sources.

Keywords Embryonic • Stem • Differentiation • Genomic • iPS

1 Embryonic Stem Cells

1.1 History

The events that lead to the research resulting in the isolation of the first mouse and human embryonic stem cells began in 1953 at the Jackson Laboratory in Bar Harbor, Maine. This initial research was performed by Leroy Stevens, who was funded by a tobacco company determined to prove that the harmfulness of cigarettes stemmed from the wrapping paper and not the tobacco. Stevens observed that male mice of the 129Sv strain would consistently develop teratomas of the testis [63]. Over the course

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of almost two decades, Stevens' work showed that cells giving rise to teratomas were even present as early as the blastocyst stage, within the inner cell mass [128]. From that observation, the term "embryonic stem cell" was coined. Teratomas were subsequently used to derive cell lines with the ability to differentiate into several cell types [80]. However, because these cell lines had abnormal properties (e.g., abnormal ploidy) and retained the ability to generate teratomas, they were named "embryonic carcinoma" cells. Steven's groundbreaking discovery that pluripotent stem cells existed in early embryos resulted in multiple laboratories determining the proper culture conditions that would allow the propagation of mouse [33, 74] embryonic stem (ES) cells.

From the onset of culturing murine ES cells, it became apparent that having the technology to introduce heritable genetic changes in the germline would be a powerful asset to studying development and disease in the mouse. Because of that fact, the focus of embryonic stem cell research in the 1980s was not the basic biology of stem cells; instead, the greatest achievement of the ES cell field at that time was considered to be the advent of gene targeting and germline modification [26, 142]. Research conducted along these lines by Mario Capecchi, Martin Evans, and Oliver Smithies led to their joint award of the 2007 Nobel Prize in Physiology or Medicine.

A massive resurgence of interest in the properties of ES cells took place in the late 1990s after the efforts of John Hearn's laboratory resulted first in the isolation of ES cells from rhesus monkey and marmoset ([144, 145], Table 1) and subsequently to the derivation of human ES (hES) cell lines [143].

Surprisingly, even though mouse ES cells were first isolated in 1981, rat ES proved to be much more difficult to derive. Rat ES cells were highly desirable because rats are easier than mice to manipulate for physiologic studies, yet no efficient method to precisely modify the germline existed. Rat ES cells eluded researchers until 2008, when two independent research groups succeeded in their derivation [16, 65].

Table 1 Embryonic stem cells derived to date

Mouse [33]
Hamster [27]
Sheep [96]
Rabbit [86]
Mink [131]
Pig [137]
Cow [37]
Rhesus monkey [144]
Zebrafish [132]
Chicken [102]
Marmoset [145]
Human [143]
Horse [114]
Rat [16]

1.2 Properties of Mouse Embryonic Stem Cells

Embryonic stem cells have several distinctive features that cumulatively set them apart from all known cell types. First, they are immortal and, like cancer cells, exhibit the ability to undergo limitless self-renewal. It has been shown in mouse ES (mES) cells through targeted deletion of the telomerase RNA component [93] that proliferative lifespan is directly dependent on telomerase activity. Second, ES cells possess the ability to form teratomas when transferred subcutaneously to syngeneic [117] or immunocompromised [35] mice. The final and most important characteristic of embryonic stem cells is their ability to contribute to all three major germ layers (ectoderm, mesoderm, and endoderm) either in vitro and, ideally, in vivo [25]. Testing differentiation capabilities of mES cells in vivo is has typically been done by injecting mES cells into blastocysts and then examining the tissue contribution of those mES cells in the resulting chimeras [51, 159]. In recent years the gold standard test for the "stemness" of mES or ES-like cells has been the use of tetraploid blastocyst [40, 81, 154]. Embryos resulting from the outcome of such injections must be wholly derived from the 2N stem cells injected into 4N tetraploid blastocysts, making it the most stringent test of pluripotency available.

1.3 Self-Renewal of Embryonic Stem Cells

An important goal in the field of embryonic stem cells is determining what mechanism(s) allow ES cells to endlessly maintain their self-renewal potential. The exact mechanisms controlling stemness have not been determined, but research has revealed key extracellular and intracellular players. When mES cells were first derived they could only be maintained in their proliferative and pluripotent state by growing them on layer of permanently growth arrested mouse embryonic fibroblasts (MEFs). Almost a decade later it was found that leukemia inhibitory factor (LIF) was the soluble factor being produced by MEFs [108]. Part of the interleukin-6 (IL-6) superfamily of cytokines, LIF functions by binding to a heterodimeric receptor composed of gp130 and LIF receptor-beta (LIFR-beta). LIF and LIFR-beta are expressed in a complementary pattern in the developing blastocysts. As the LIF receptor is expressed in the inner cell mass of the blastocyst, LIF cytokine itself is expressed in the differentiated trophoblast layer [92]. Once LIF has bound the LIF/gp130 complex, the JAK-STAT pathway is activated, resulting in homodimers of Stat3 translocating to the nucleus and transcribing genes critical for self-renewal [78, 94]. IL-6 can substitute for LIF when present at high concentrations and complexed with soluble IL-6 receptor (sIL-6R) [166]. Although LIF is critical in culture, blastocysts are only altered in their ability to undergo delayed implantation during times of stress or when women conceive while still nursing, a phenomenon known as diapause [91]. The effects exerted by LIF on mES cells in culture can only occur in the presence of fetal bovine serum. Although serum has a complex mixture of

soluble proteins, the presence of serum is only required to make available BMP4 to mES cells. BMP4 cytokine binds to the BMP receptor, BMPR1, which activates SMAD and induces the expression of the helix-loop-helix transcription factors known as inhibitors of differentiation (Id). As their name implies, Id proteins cooperate with Stat3 in maintaining self-renewal by repressing genes involved in neural differentiation [165]. In addition to Stat3 and Id factors, the proto-oncogene c-Myc has been shown to be a central player in maintaining the undifferentiated state. The levels of c-Myc are regulated through several mechanisms. Upon activation by the LIFR/gp130 complex of the JAK-STAT pathway, JAK phosphorylates and activates PI3 kinase (PI3K), which then phosphorylates the c-Myc inhibitor glycogen synthase kinase 3 (GSK3). The importance of PI3K's indirect maintenance of c-Myc's activity via GSK3 regulation is evident in cells that have been forced to differentiate in the presence of LIF by the addition of the PI3K inhibitor LY294002 [103]. Moreover, GSK3 inhibition of c-Myc can potentially be blocked by direct signaling through the Wnt pathway. The GSK3 inhibitor 6-bromoindirubin-3′-oxime (BIO) allows mES cells to be propagated without the presence of LIF in the media [80, 116]. The importance of c-Myc in maintaining the self-renewal of mES cells is at least partially tied to c-Myc's upregulation of the catalytic subunit of telomerase [152], although telomerase alone is not sufficient to explain c-Myc's role in self-renewal.

Along with Stat3 and c-Myc, two other transcription factors, Oct3/4 and Nanog, have been shown to be important in maintaining ES cell self-renewal. The POU domain transcription factor Oct3/4 was initially cloned from mES cells and shown to be uniquely regulated, in that its expression would be turned off during mES cell differentiation [99, 112]. mES cells have been shown to be very sensitive to changes in levels of Oct3/4 protein, with reduction in levels resulting in dedifferentiation into trophoectoderm and upregulation driving differentiation into primitive endoderm and mesoderm [95]. While it has been known that absence of Sox2 protein influences the pluripotency of mES cells, it was only recently reported that Sox2 regulates the expression of several transcription factors that in turn are important for ensuring the appropriate levels of Oct3/4 [75]. Of the four transcription factors associated with the self-renewal of mES cells, the latest addition has been Nanog. Originally identified from a functional expression screen, Nanog has been shown to be highly expressed in mES cells and can maintain the self-renewal of mES cells without the addition of LIF or BMP to the media [17, 84]. Not surprisingly, when overexpressed, Nanog is not enough in and of itself to reprogram differentiated cells into ES-like cells. However, it is the only factor to date that can increase the efficiency of mES cell derivation. Likewise, ectopic expression of Nanog was able to boost the efficiency of fusions between mES cells and neural stem cells by over 100-fold in order to generate ES-like hybrid cell lines [123]. Boyer et al. [8] used chromatin immunoprecipitation (ChIP)-on-chip technology to conclude that Oct3/4, Nanog, and Sox2 share many overlapping transcriptional targets, including each other's promoters. Aside from the obvious implication of cross-regulation, the significance of the majority of the targets shared by the self-renewal trio remains to be discovered.

1.4 Differentiation of Mouse Embryonic Stem Cells

The first report describing the multilineage capability of mES cells in vitro with some detail was by Gail Martin, whose report shortly followed the landmark work by Evans and Kaufman [33]. By using embryoid bodies as had been done with mouse embryonic carcinoma cells, Martin was able to differentiate mES cells [74], although conditions were not defined for each observed lineage. A few years later a stand-alone landmark publication did describe the differentiation of embryoid bodies (EBs) in more detail, including the appearance of endothelial cells [28]. Surprisingly, more than 7 years after the initial isolation of mES cells passed before routine studies surfaced that attempted to use mES cells for modeling in vivo development events. Following the lead of work carried out by Evans and Martin, early studies relied on the use of EBs as well as later staged cystic embryoid bodies (CEBs), which resemble the 6- to 8-day cylinder stage of mouse development. Unlike early-stage mouse embryos, embryoid bodies could be generated much more easily and thus were utilized to mirror events observed in vasculogenesis and angiogenesis [110, 151] and globin switching [66]. Hematopoietic assays involving secondary reagents, such as methylcellulose [117], or the addition of cell lines, such as the coculture of a bone marrow stromal layer [41], expanded the repertoire of lineages obtainable in vitro.

The differentiation of mES cells into highly sought after cell types such as neurons [74] and myocardium [28] was observed when untreated embryoid bodies were allowed to adhere and differentiate. However, protocols that involved the use of specific reagents such as dimethylsulfoxide (for muscle) and retinoic acid (for neurons) were not employed for years, even though such reagents had proven successful in the directed differentiation of P19 EC cells [31, 32]. Instead, early methods used to generate cardiomyocytes and neurons, like those used for blood cell differentiation, focused on low-level manipulation of EBs. Emphasis was placed on how well the timing of EB differentiation paralleled embryonic development [82, 171]. As protocols became more refined, there was a shift to alter variables such as EB cell number and better define media conditions (e.g., lower serum content, insulin supplementation, etc.).

Early neuronal differentiation studies had demonstrated that EC cells could be differentiated into cells that displayed morphologic characteristics of neurons [49, 121]. The transition to studying neuronal differentiation of mES cells took off in the mid-1990s with more detailed characterization of the “neuron-like” cells that were produced from EB differentiation protocols. In research published by David Gottlieb’s laboratory it was shown by several tests, namely immunostaining and voltage-clamp readings in response to excitatory and inhibitory agonists, that neuron-like cells possessed key characteristics of true neurons [1]. Furthermore, they were the first group to quantitate the effectiveness of the embryoid body differentiation protocol and to implement the now widely used 4–/4+ retinoic acid differentiation protocol. Almost in parallel, Fraichard et al. [38] demonstrated that they too could differentiate mES cells into neurons that passed several stringent criteria.

Differentiated neurons derived through an EB protocol involving much longer incubation postplating were found to be immunopositive for neuronal cytoskeletal marker beta III tubulin. Neuron-like cells also displayed current induction when treated with glycine, *N*-methyl-D-aspartate (NMDA), and kainite and furthermore were shown to express glutamic acid decarboxylase (GAD) and acetylcholinesterase (AchE), two enzymes involved in neurotransmitter metabolism. Previous work had demonstrated that cells expressing the marker Nestin shared characteristics to neuroepithelial precursor cells that give rise to both neurons and glial cells [63]. Although the percentage of glial cells derived from embryoid bodies was not high, there was a subpopulation that expressed either the astrocyte marker GFAP or oligodendrocyte marker O4. Research using EB formation to differentiate mES cells into neurons continued; meanwhile, generating cells representing different developmental stages gained momentum. In 1996 it was found that the Nestin-positive neural precursor cells present in differentiating mES cells culture could be expanded with the strong mitogen bFGF so that "cleaner" populations of either glial cell or neurons could be obtained [98].

Following preliminary studies involving the derivation of postmitotic neurons, there was a steady progression in determining ways to differentiate mES cells into different types of neurons and supportive cells, as well as identifying basic mechanisms of mES differentiation. After gaining experience in differentiating mES cells into postmitotic neurons. some groups were able to enhance or direct the differentiation mES cells into specific neuronal subtypes. Renoncourt et al. first demonstrated the potential to generate cranial and somatic motorneurons as interneurons. The main limitation of the study was that the evidence for differentiation was based on cells in culture expressing homeodomain proteins associated with certain cell types such as HB9 or Ilset-1 for motorneurons [109]. It was later demonstrated that functional motorneurons could be derived from mES cells based on the ability of the motorneurons to incorporate into the spinal cords of chick embryos and form synapses with muscle [157]. The authors were able to make specific neurons by using a combination of a HB9-GFP transgenic mES reporter line (derived from transgenic mice) and retinoic acid as well as sonic hedgehog (Shh) differentiation of EB-derived neuronal precursors. The systematic derivation of dopaminergic midbrain neurons and serotonergic hindbrain neurons was also achieved through the use of a multistep protocol involving EBs [61]. To eliminate the large variation seen in most mES cell neuronal differentiation protocols, neuronal differentiation methods have been devised that center around the use of stromal cells [2]. Stromal cell–induced neuronal differentiation of mES cells using defined media results in the reliable production of beta III tubulin–positive neurons and, after lengthy culture, tyrosine hydroxylase positive dopaminergic neurons ([54]; E. Theodorou, unpublished observations). The work done by Barberi et al. demonstrated that by using defined media along with a bone marrow stromal cell feeder layer, mES cells could be induced to differentiate into key neuronal lineages of interest: neural stem cells (NSCs), dopaminergic, serotonergic, GABAergic, and spinal motor neurons. Significant progress has also very recently been made in differentiation of mES cells using the tried and proven 4–/4+ retinoic acid differentiation protocol.

When embryoid bodies taken through a 4–/4+ differentiation protocol were dissociated and plated in well-defined neural precursor survival media followed by neuronal maintenance media, yields of neurons were brought to a very high level [6]. A surprising finding that has only begun to be explored is that, if grown in minimal media, mES cell will by default differentiate into neuroectodermal precursors. These findings were first described in work detailing how autocrine bFGF signaling was necessary for mES cells to go down this default pathway, without the use of cellular aggregation or specialized stromal cell layer [164]. The results of Ying et al. were later used as the basis for forming neural stem cells without the aid of neurospheres, but by proliferation of neuroectodermal precursors treated with EGF and FGF-2 [21]. Protocols involving exploitation of this default differentiation pathway have only recently entered the mainstream in the mES cell field but will very likely increase the options available to study differentiation once detailed protocols have become available.

Just as highly sought as the neurons themselves, supportive glial cells were found to have been present in EBs that had been subjected to the 4–/4+ differentiation protocol. Although the cell populations were mixed, the oligodendrocytes that were present had the ability to myelinate neuronal axons in culture (as judged by scanning electron microscopy). The oligodendrocyte precursors could be further enriched with the proper supportive media and to a limited degree could differentiate into myelinating oligodendrocytes in a rat model [68]. In the same year this work was surpassed by two independent groups able to derive not only oligodendrocytes, but also astrocytes [15, 87]. From a technical point of view, what separated these latter publications was that they did not simply age EBs in order to allow production of oligodendrocytes. Instead, mES cells were differentiated into glial-restricted precursors (GRPs) that were self-renewing and could be maintained in culture. Although glial cell differentiation of mES cells has received far less attention than differentiating neurons, significant headway has been made toward producing impressive numbers of oligodendrocytes through the use of aggregate protocols [52].

Muscle cells of the heart, called cardiomyocytes, are another highly desirable cell type because of their immediate relevance to disease, with the ability to incorporate into postinfarcted hearts in rats and canines [67, 83]. The presence of cardiomyocytes was noted for many years in teratocarcinoma cells [130] well before mES cells were first derived. It was even determined that varying the levels of retinoic acid, typically used to signal differentiation of neurons, could induce cardiomyocyte differentiation [31]. The first indications of EBs differentiating into cardiac muscle were the presence of Z-bands in histologic sections. Along with reverse transcription-polymerase chain reaction (RT-PCR) for cardiac myosin, Western blots for alpha and beta cardiac proteins indicated that cardiac-like muscle in EBs was reminiscent of some aspects of heart development at the E9–E10 stage [111]. Later it was confirmed by immunostaining, in situ hybridization, and RT-PCR of markers of differentiation that indeed cardiomyocytes were being produced in embryoid bodies [82]. Unlike the differentiation of neurons from EBs, the size of the EBs used to produce cardiomyocytes had a bearing on the efficiency of differentiation. Generally, EBs that were formed through the use of the hanging drop method (in which the

specific number of cells per EB can be controlled) and comprised approximately 500–1000 cells gave the highest percentage of cells staining positive for alpha-cardiac myosin heavy chain [72]. Dissociation of the same EBs through enzymatic treatment allowed the authors to identify the presence of sinusnodal, atrial, and ventricular cell types. Evidence that mES cell–derived cardiomyocytes did indeed resemble their in vivo counterparts was independently demonstrated by researchers who used EB dissociation combined with cellular electrophysiology [29]. More recently, the use of EBs has allowed certain questions to be asked about cardiogenesis without the difficulty of having to culture live embryos. In addition, recent studies suggest the existence of a cardiovascular progenitor in differentiating EBs [53,160]. With the ability to isolate a cardiovascular progenitor, the cardiomyocyte lineage exemplifies how mES differentiation has allowed for a better understanding of the basic biology of a cell type with intrinsic medical value. The lineages covered in this review represent only a sampling of the cell types derived to date. A more extensive overview is presented by Keller [55].

2 High-Throughput Functional Assays

2.1 Large-Scale Differentiation Studies

Both ethical/social impact and experimental practicality must be taken into consideration when choosing a stem cell line as a model system. Murine ES cells naturally lend themselves to high-throughput functional screening for several reasons: (1) mES cells can easily be grown to large quantities and differentiated in defined media; (2) mES cells have been shown to differentiate into all tissues of the adult mouse [88, 101]; (3) unlike other mammalian ES cell lines, mES cells have been genetically manipulated for many years [9]; and (4) to prove in vivo efficacy of differentiated cells, they can be transplanted into mouse models of disease [15, 56]. Other embryonic and adult stem cell lines have potential benefits that could be taken advantage of once studies with an established system such as mouse ES cells have taken place. To date no other system has surpassed murine ES cells in overall utility.

In 2003 the power of cDNA functional screening in mES cells was brought into the mainstream with work done by Chambers et al. [17] that culminated in the cloning of the mES cell self-renewal factor Nanog. Oddly enough, the screen yielded one of the most critical and aggressively studied stem cell self-renewal factors, yet Nanog was the only hit from the screen. While the expression cloning of Nanog was an important step forward, it also served to highlight some of the limitations of relying on cDNA libraries, such as the absence of positives due to clones that are low abundance or that are not represented as in-frame, full-length cDNAs.

More recently, several groups have taken strides to bring functional ES cell screening up to speed with genomics technology. In an effort to logically go from a model of differentiation to systematic identification of genes responsible for the observed phenotype, Ihor Lemischka's laboratory combined a unique selection

method with microarray technology [106]. The method involved the transfection of an unbiased episomal cDNA library into mES cells that were transgenic for the large T antigen. The episomal library was based on the same vector as the library used in Chambers et al. and replicated in the presence of large T antigen. Once the mES cells were placed through a functional screen, the episomal library was extracted and labeled for microarray analysis. cDNAs that were selected for or against (depending on the functional screen being performed) were then used in one-gene-per-well assays for confirmation. Using microarrays to take a focused approach to screening paid off with a dramatic increase in positives. However, the use of cDNA libraries for focused screening brought about problems previously, seen such as false positives resulting from the expression of incomplete cDNAs. One more obstacle that arose was an increase of false positives as a result of incomplete alternate splice variants misidentified by microarray as full-length transcripts. A second large-scale cDNA functional screen in mES cells [34] was the first instance of (1) a screen involving full-length ORFs and (2) a screen directed at the differentiation of mES cells into a specific cell lineage—neurons. While the assay relied on a sensitive and rapid method for hit detection, it did not yield many positives. In addition, the type of assay conducted by Falk et al. reflected those previously published [18, 105] in which an enormous number of cDNAs were transfected in small pools or individually. Thus, the cost feasibility of such a screen would be outside the reach of many laboratories. What was lacking was a collection of stable mES cell lines, each expressing a defined factor, preferably in an inducible fashion. Theodorou et al. [141] delivered such a system when they generated more than 700 mES cell lines, each line transgenic for a unique transcription factor, which was expressed upon withdrawal of tetracycline from the culture media. While the collection of mES cells was generated though screening thousands of individual colonies, it represents the first such large-scale stable collection made available to the scientific community and which can be propagated long term. The collection pieced together by Theodorou et al. would not have been feasible without the foundation work involving targeted modification of mES cells.

2.2 Mouse Embryonic Stem Cell Modification and Expression Systems

Over the years there have been multiple attempts to genetically modify ES cells so that they could serve as a platform for large-scale gene expression studies. The difficulty has been in developing a methodology that could satisfy most of the requirements for an ideal expression system: (1) ease of inserting transgenes into a defined chromosomal locus, (2) inducible expression, (3) ubiquitous and constitutive expression, and (4) expression that is trackable. Inserting a transgene into a defined locus in a predictable and efficient manner has proven to be the most difficult requirement. Several traditional methods for making defined chromosomal modifications and insertions exist, but they all share a low success rate [12].

More advanced systems have taken advantage of unique restriction sites [20, 24] or improved electroporation and culture conditions [141]. The method that has proven most successful uses standard positive selection along with a counterselection cassette coding for either wild-type or attenuated diphtheria toxin [162, 163]. All of these methodologies rely on the delivery of large, linearized plasmids carrying fragments of modified genomic DNA via electroporation into high numbers of mES cells and therefore do not lend themselves to high throughput. As a way to circumvent repeated modification of large plasmids, large-scale DNA preparations, and electroporations, several groups have made advances using homologous recombination simply as a "priming" step. With this new method, a specific genomic locus is primed with recombination sites such as loxP or frt so that by standard transfection methods a plasmid coding for a corresponding recombination site could be introduced into cells [3, 76]. Such systems have been referred to as recombinase-mediated cassette exchange (RMCE). The locus of choice for RMCE priming via homologous recombination is ROSA26, located on chromosome 6 in mice. It has been shown the ROSA26 locus [169] allows for ubiquitous and constitutive expression of an inserted transgene. An added bonus to utilizing the ROSA26 locus is that it is readily targeted with high efficiency [127].

There have been multiple instances in the literature where mES cell lines were primed for RMCE in order to increase the efficiency of transgenic line derivation. Some systems relied on the endogenous ROSA26 locus directly driving the expression of an artificial transactivator for which a corresponding promoter and associated transgene were inserted elsewhere [59, 148, 161]. Invariably, this involves the picking of many colonies and screening (at best) with PCR and, in some cases, by Southern blot to check for the proper copy number of the transgene. A few reports described mES cells retrofitted with knock-in vectors dependent on unique loci other than the tried-and-proven ROSA26 locus [4, 70, 170]. In such proof-of-concept papers there is usually insufficient evidence provided to warrant the transfer of a large clone collection. One example involved the use of a ubiquitous Col1A collagen promoter to drive the expression of a small molecule–controlled transactivator, which could then transactivate a gene of interest [3]. However, even with a strong promoter, the system ultimately was ineffective at expressing the GFP transgene in the entire brain as well as skeletal muscle. This could have been a result of the Col1A promoter being inactivated in these tissues at the epigenetic level, although this was not determined. A more likely possibility is that transgene expression was lessened because of the incorporation of the bacterial elements of the inserted plasmid, which were previously shown to interfere with transgene expression [42, 147]. Yet one more promising system that has been shown to be effective in vitro as well as in vivo incorporated the use of Cre-lox recombination as well as doxycycline-inducible expression to control transgene timing and expression levels [73, 167]. While the tightly regulated expression from the ROSA26 locus is a desirable characteristic, it was outweighed by several key points: (1) Cell lines would have to be screened for positives by PCR, (2) expression induced by hydroxytamoxifen was mosaic and took 48 hours to achieve, and (3) monitoring of transgene expression was not incorporated into the system.

Building off of an earlier generation system, Masui et al. were able to take an inducible promoter system [95] that had been used for so-called "supertransfections" [38] and modify it so that it would satisfy many requirements that would make it amenable to high throughput ([76]; E. Theodorou, unpublished observations). Not only did their system incorporate the ROSA26 allele to allow for constitutive and ubiquitous expression, it also primed the ROSA26 allele with modified loxP recombination sites for RMCE. The loxPV (originally referred to as lox2272) site used for RMCE acted as a substrate for Cre recombinase but could not recombine with a wild-type loxP site. This allowed for recombination that was directional and would always insert the transgene in the proper configuration relative to the promoter element. An added set of modifications increased the ease and rapidity for which correctly targeted clones could be selected. Instead of employing the more typical strategies employed of screening for inserts by PCR and Southern blotting, Masui et al. [76] employed a rapid two-tiered approach. Clones were initially screened for resistance to puromycin antibiotic since a puromycin resistance cassette was shuttled into the modified ROSA26 allele. However, simply screening for puromycin resistance would identify the clones having undergone successful RMCE as well as clones containing the puromycin resistance cassette incorrectly inserted in the genome near an active promoter. Puromycin clones could be quickly tested for correct targeting by (1) growing cells and assaying for a renewed sensitivity to hygromycin or (2) inducing transgene expression and screening clones by fluorescent microscopy for the presence of Venus. This was a marked improvement over other available systems. The use of the IRES-Venus element added the benefit of not only making transgene-expressing clones easier to identify, but also to select clones with higher fluorescence, corresponding to higher expression of upstream transgene (E. Theodorou, unpublished observations). The ability to simultaneously detect correctly targeted clones and clones that have the highest levels of expression is another distinct advantage over more cumbersome methods such as RT-PCR or Western blots [170].

2.3 Large-Scale Gene Transfer

Traditional cloning of open reading frames (ORFs) into plasmid vectors typically requires one or more restriction enzymes, and success rate can vary greatly. A recently developed cloning system has alleviated the need for restriction enzyme cloning [150]. The Gateway system of recombinational cloning is based on site-specific recombination mediated by the λ-phage integrase family [44]. Recombination involves two different reactions and results in one relevant product after each reaction. As shown in Fig. 1c, the consensus att recombination sites are 25 nucleotides long (and thus create a short peptide linker whenever N- and/or C-terminal fusions are introduced). Step one of the cloning (Fig. 1a) involves incubating a PCR product amplified with attB primers with a donor vector (pDONR221) in the presence of λ-integrase (Int) and *Escherichia coli* integration host factor (IHF).

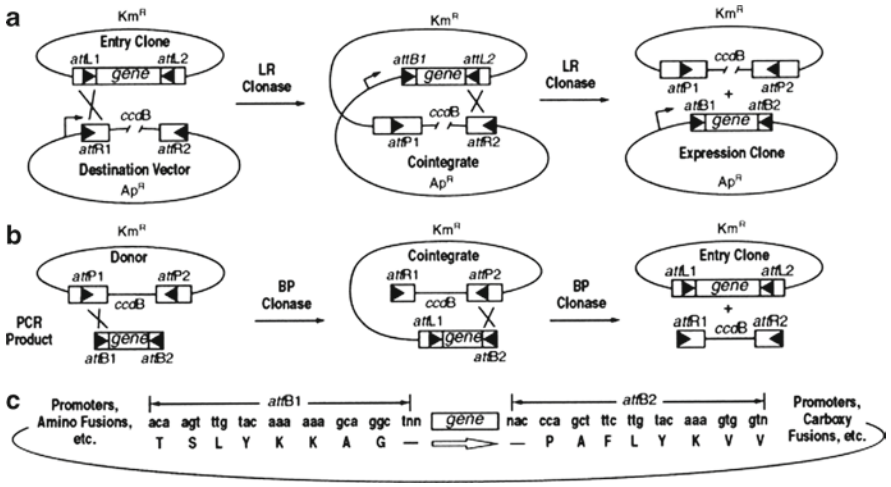

Fig. 1 Overview of the Gateway system. **a:** The entry clone allows transfer of the ORF into any destination vector with the appropriate recombination sites via an LR reaction. **b:** An entry clone containing a full-length open reading frame (ORF) is generated through the recombination of the donor vector with a polymerase chain reaction product with attB1/attB2 overhangs. **c:** Destination vectors may be designed to produce N- or C-terminal fusions proteins that link to the translated ORF through either an N- or C-terminal eight–amino acid linker peptide. (From Hartley, J.L., Temple, G.F., and Brasch, M.A. (2000) DNA cloning using in vitro site-specific recombination. Genome Res. 10, 1788–1795.)

Site-specific recombination occurs between the PCR product attB sites and donor vector attP sites [hence the name BP Clonase for the enzyme mix (Fig. 1a)]. The BP Clonase reaction is subsequently transformed into *E. coli* strain DH5α or any ccdB-sensitive strain and spread on antibiotic-containing agar plates. Of the colonies that grow, the majority will be correctly recombined entry clones because of the potency of the ccdB counterselection. The donor vector will not grow in DH5α because the ccdB gene blocks the growth of DH5α through the inhibition of topoisomerase. Individual plasmid minipreps of colonies can be screened for correct insert size by performing Taq polymerase PCR with primers identical to the attB sites produced from the LR Clonase reaction or by digestion with restriction endonuclease BsrG1 (New England Biolabs, Ipswich, MA), whose restriction site is present in all att recombination sequences.

Shortly after the Gateway system of cloning was first unveiled, several groups reported the beginnings of human ORF collections using Gateway [11,124]. As of yet, no complete Gateway clone collection exists, although some companies, such as OriGene Technologies (Rockville, MD) and Invitrogen Corporation (Carlsbad, CA), have made a large percentage of the estimated 25,000 human ORFs [19] available for purchase and modification. Although it is not cost-feasible for most laboratories to purchase large-scale ORF collections, the situation has improved thanks to the efforts of the National Institutes of Health (NIH) to clone every human ORF as part of the Mammalian Gene Collection (MGC) [129]. The availability of the MGC

collection has enabled academic laboratories to become involved in clone generation and to significantly reduce the cost per clone [39,60,113]. Furthermore, the NIH has recently taken notice of the utility of the Gateway system and as part of the newly established ORFeome Collaboration will attempt to transfer all human ORFs into the Gateway system (http://www.orfeomecollaboration.org/). Lastly, it will soon be possible for most laboratories to shuttle ORFs without the high cost associated with the Gateway system. This will be possible because of the MAGIC clone shuttling system, which relies on phage recombination occurring within bacteria rather than depending on the use of purified recombinant proteins [64].

3 Transcription Factor Studies

3.1 Transcription Factor Functional Determination in Murine Embryonic Stem Cells

Murine ES cells have been used for more than a decade to study transcription factor function. Two of the earliest targeted knockouts in mES cells were the noted transcription factors pRb [50] and p53 [30]. Although in many cases ES cells were used as a tool to generate mice with null mutations, in some cases the differentiation properties of the mES themselves were more informative than the knockout mice [10, 156]. For example, overexpression of transcription factors can, in some instances, effectively force the differentiation of ES cells. In a study conducted by Fujikura et al. [38] cDNAs for 10 transcription factors were delivered (by transfection or electroporation) into wild-type mES cells. Six factors produced no phenotypic changes, while 4 other transcription factors induced the formation of extraembryonic endoderm in some colonies. Thus, there is evidence to show that using mES cells as in vitro tools to model transcription factor lineage commitment is feasible; however, better methods of cDNA delivery to allow homogeneous expression throughout a cell culture, as well as combining such systems with reporter cell lines, would be desirable.

3.2 Forward Differentiation of Murine Embryonic Stem Cells by Ectopic Expression of Defined Factors

The power of exogenous gene delivery in ascertaining protein function and deriving a desired cell type has been realized for decades. First on the scene was MyoD [138], the canonical “forced differentiation” transcription factor that was able to transdifferentiate a variety of cell lines simply through ectopic expression [155]. PPARgamma also followed suit with its ability to convert mouse fibroblasts into adipocytes when ectopically expressed in the presence of the appropriate ligand [14, 146].

The use of transient transfections to establish stable cell lines for studying differentiation progression naturally first occurred with embryonic carcinoma (EC) cells because of their availability, prior characterization, and ease of culture. EC cells have been adopted for experiments involving transgene induction [127] and inhibition [86] of differentiation. In addition, the high efficiency of EC cell transfection made it possible in one instance to test a handful of bHLH transcription factors for induction of neuronal differentiation [36].

mES cells very quickly took the lead over EC cells for differentiation studies due to their normal karyotype and development in vivo, potentially making any discovery of greater relevance to basic biology. Most often the genes of choice in ectopic expression assays were transcription factors, although the overall gene choice and target cell type varied. The earliest attempt to test the ability of a factor to promote the differentiation of mES cells into a specific cell lineage began with the well-known differentiation-inducing factor MyoD. Ectopic expression of MyoD did promote muscle differentiation in mES cells partially differentiated via embryoid bodies [121]. Teratomas formed from transgenic MyoD mES cells, however, failed to show any added increase in muscle formation. Nonetheless, the work marked the first use of mES cells for examining the function of a specific protein, specifically a transcription factor. Most transcription factors, unlike MyoD, did not have successful track records of effectively differentiating multiple cells lines, and as a result likely candidates were often chosen from factors with very potent in vivo phenotypes. For instance, only mild effects were seen in the case of Pax4, a transcription factor essential for pancreatic beta cell development. The overexpression of Pax4 did lead to an increase in Nestin-positive pancreatic precursors as well as Pdx-1–positive cells but failed to form fully functional insulin-producing beta cells [7]. In another instance a protein critical for eye formation in mice, the Rx/rax transcription factor [78], had an effect on morphology of mES cells and resulted in their displaying characteristics indicative of retinal neurons [134]. The mES cell differentiation reports mentioned thus far involved the use of screening for stably expressing clones from pools of cells transfected with randomly integrating expression vectors. Although the method of individual clone testing is tedious, it has proven to be effective with transcription factors, possibly because of the potency of transcription factors even if expressed at low levels. In at least once instance transgenic mES cell stable clones were generated as pools with the transcription factor Osterix. Osterix had been shown to be critical for generation of osteoblasts in vivo [91], so it was believed that overexpression would lead to differentiation. Pools of clonal mES cell lines transfected with Osterix were tested, and it was found that 46% of cells expressed the transgene [136]. Although clones were pooled and varied in expression, Osterix managed to stimulate osteogenic differentiation in transgenic cells. By far the most impressive demonstration of single-factor differentiation guided by in vivo data is Sox9. Sox9 was the first factor shown to be essential for cartilage formation [5]. Ectopic expression of Sox9 as a stable mES transgenic cell line [58] or adenoviral delivery with related family members Sox5 and Sox6 [48] resulted in direct differentiation of mES cells into mature chondrocytes.

Single-factor induction of mES cell differentiation continues, but systems for expression have become progressively more sophisticated. Zebrafish with deficiencies in the caudal homeobox transcription factor cdx4 fail to specify blood progenitors [23]. By using the Ainv15 tet conditional expression system, Wang et al. [153] were able to conditionally increase mesoderm specification and hematopoietic progenitor formation. While the Ainv15 system was based on the use of an established knock-in cell line, some groups have achieved the same effect by using multiplasmid transfections. Recently, it was shown that the nuclear protein Chibby (Cby) could induce cardiogenesis in a tetracycline-inducible fashion [125]. Lastly, after performing two rounds of knock-in electroporations, Serafimidis et al. [119] generated a cell line that was capable of inducible Ngn3 expression. The use of an inducible system allowed the authors to carry out a lengthy and complex differentiation protocol with precise timing for Ngn3 expression. Not only was the expression system and protocol more sophisticated than many of the previous stable cell line experiments executed in mES cells, but it also allowed for the generation of difficult-to-obtain endocrine pancreas progenitors.

3.3 Reverse Differentiation of Murine Embryonic Stem Cells by Ectopic Expression of Defined Factors

What has turned out to be the highest-impact discovery in stem cell biology since the derivation of stem cell lines has been the use of a defined set of cDNAs for cellular dedifferentiation. Research done in the laboratory of Shinya Yamanaka proved that differentiated cells could be reprogrammed into ES-like cells called induced pluripotent stem (iPS) cells through overexpression of a small number of cDNAs. While seemingly isolated and completely without precedent, efforts to dedifferentiate fully differentiated or partially differentiated cells has a firm foundation in the literature. The earliest interest in the biology of cellular reprogramming (likely) originated from observations of animals such as newt [45] and starfish [118] that have the ability to regenerate severed limbs. Such observations fueled work done in the 1980s and 1990s that began to apply biochemistry and molecular biology to the study of regeneration [13, 58, 69]. The work carried out in newts specifically encouraged Mark Keating's group at Harvard Medical School to go beyond studying reprogramming in tissues and focus on the level of single cells. By ectopic expression of the transcription factor msx1, Keating's group managed to convert a small percentage of multinucleated myotubes into dividing, mononucleated cells [98]. Not only was this work pivotal in being the first to show that differentiation could be induced by a gene normally expressed in progenitor cells, but it also accomplished this feat in mammalian cells. Equally as impressive, the mononucleated cells had regained the ability of their parental cell line, C2C12, to differentiate into several lineages, including adipocytes, myocytes, and chondrocytes. Prior to Keating's work, the only success in reprogramming in mammalian cells was in the complex milieu of the oocyte during cloning [149]. Of course, embryonic stem cells were

known to have greater differentiation potential than the immortalized C2C12 cell line and represented the next logical step in dedifferentiation research. Although some progress was made demonstrating that mouse ES cells could reprogram adult cells to a degree [131], it was only recently shown that fibroblasts fused to human ES cells could result in cells with 4N DNA content yet all the major characteristics of hES cells [22]. Reprogramming of differentiated cells with hES cells was independently reproduced with the aid of hES cells expressing an OCT4-GFP reporter [167]. Once it was determined that fibroblasts could be essentially reprogrammed into fully functioning hES-like cells, the difficulty lay in finding a way to deconvolute which proteins were essential to the dedifferentiation process. Using a brute force approach, Takahashi et al. [136] first used microarray analysis to examine which transcription factors had the highest levels of expression in mES cells. They then performed a series of batch retroviral infections into Fbx15-Neo reporter tail tip fibroblasts until the fibroblasts were infected with all 24 transcription factors. Reprogrammed ES-like cells (referred to as iPS) were generated, and further analysis determined that the minimal combination of factors needed for dedifferentiation was Oct2/4, Myc, Sox2, and Klf4 [136]. Remarkably, mouse iPS cells were identically grown as mES cells and were successfully used to generate EBs, displayed markers of various lineages when differentiated, and produced teratomas, but when injected into blastocysts they could only produce embryonic mouse chimeras.

Rapid progress has continued to be made in the area of dedifferentiation via overexpression of defined transcription factors. As a direct follow=up to the original work in Yamanaka's laboratory, Okita et al. delivered the set of four transcription factors into fibroblasts to generate iPS cells. As previously mentioned, ectopic Nanog has shown the ability to increase the percentage of mES-neural stem hybrids [123]. Taking advantage of this, Okita et al. [100] took the published iPS protocol one step further by selecting out clones that had high levels of Nanog expression. The iPS clones high in Nanog expression were able to generate chimeras and, in one instance, transmit through the germline. A major drawback of the work done by Okita et al. was that the presence of the Myc proto-oncogene resulted in approximately 20% of the germline pups rapidly developing tumors. The problem of tumor formation was eventually circumvented by using a conditionally expressed Myc transgene [43] so that iPS-derived myeloid precursors were rendered safe before transplantation into a mouse model of sickle cell anemia. Another report from the same laboratory demonstrated that drug selection of retrovirus-generated iPS cells was not necessary to select reprogrammed clones, and that iPS clones generated through a gentler, morphology-based protocol could contribute to adult chimeras [81].

The most exciting results surrounding iPS cells have come from reprogramming experiments carried out with human cell lines. Defying expectations [107], several groups were able to apply similar techniques used to derive mouse iPS cells to derive human iPS or hES-like cells. Most fascinating is that each research group managed to contribute to at least one unique finding to the generation of human iPS cells. The first group to publish success at generating human iPS from fibroblasts proved that the human orthologs OCT4, SOX2, MYC, and KLF4 could work across

species, and the iPS cells they generated were able to differentiate and form EBs and teratomas [136]. In the same year Yu et al. [168] was able to accomplish the same goal but managed to do it without using transcription factors identical to those of Takahashi et al. Instead, a miniature screen involving 14 transcription factors highly expressed in human oocytes was carried out. The 14 transcription factors were narrowed down to OCT4, SOX2, NANOG (not essential, but improved efficiency), and LIN28. This demonstrated that reprogramming fibroblasts into iPS was not limited to the originally published four transcription factors. A recent report focused on the translational applications of iPS by using human orthologs of the original four factors to convert primary biopsied skin cells into iPS cells [104]. Finally, in an effort to eliminate any chance of Myc inducing tumors, Nakagawa et al. [89] were able to generate both mouse and human iPS without the use of Myc. The three-factor mouse iPS cells were the first and only reported instance of iPS cells being transmitted through the germline without inducing tumors in resulting offspring. One additional interesting find by Nakagawa et al. was that homologous transcription factors could substitute, to a lesser degree, for the three programming factors (e.g., Oct1 and Oct6 could substitute for Oct4).

Early work done by the Keating laboratory and very recently by the Yamanaka and other laboratories relied on the use of defined cDNAs to induce cellular reprogramming in a defined and controlled manner. Indeed, it has been the use of full-length cDNAs that has allowed researchers in the field of dedifferentiation to achieve what others previously could not through indirect methods involving cell fusion [22, 134] or whole-cell extracts [139]. The crowning achievement of cDNA reprogramming has obviously been the creation of iPS cells, which will not only prove invaluable in the laboratory, but also are a significant step forward in stem cell therapy without the ethical issues posed by embryonic stem cells. After iPS cell derivation was reproduced, the floodgates opened, and the race has been on to generate iPS cells solely through the use of non–viral-based reagents such as chemical compounds [46, 71, 122], excisable transgenes [158], and transducible proteins [171]. As of this writing, no single method has superseded all others for the generation of ES-like iPS cells. It is likely that in the exploding field of cellular reprogramming we will not have to wait too long.

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Bmi1 in Self-Renewal and Homeostasis of Pancreas

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Abstract In mammalian organisms homeostasis is the central mechanism maintaining and preserving organ and tissue integrity. Stem cells are the main players in homeostatic balance: through self-renewal and multilineage differentiation, stem cells provide an endless supply of fresh, new cells throughout the lifetime of an organism. *Bmi1* is one of the genes with a role in stem cell self-renewal and homeostasis in several organs and tissues. Its role in pancreas has been recently highlighted by two studies. In exocrine pancreas *Bmi1* labels a population of differentiated acinar cells able to self-renew for more than 1 year, while in the endocrine pancreas absence of *Bmi1* is associated with impaired beta cell regeneration upon damage. These experiments highlight *Bmi1*'s central position in maintaining organ homeostasis. The recent discovery that *Bmi1* plays a crucial role in mitochondrial function makes it possible to hypothesize that this gene is one of the master regulators of tissue maintenance controlling stem cell self-renewal and mitochondrial metabolism.

Keyword Self-renewal • Homeostasis • Polycomb group genes • *Bmi1*-knockout mice

1 Introduction

The word homeostasis comes from the Greek words *hómos* ("similar") and *histēmi* ("to stand"), indicating a process by which balance is achieved and maintained. Homeostasis is the central mechanism around which the life of a mammalian organism revolves. Every cell, tissue, organ, and body system has to maintain its

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functions in a constantly changing environment and modify its needs accordingly. A disease is usually the result of a failed homeostatic mechanism. For example, a mammalian organism has to maintain its blood sugar level within a narrow range. This balance is kept in an apparently simple way through insulin, making glucose readily available when needed or storing it as glycogen or fat. The multiple aspects of sugar absorption, storage, and use require thousands of cells, signaling pathways, and genes, each one finely tuned. When one or more homeostatic controls fail, diabetes ensues, with all of its consequences.

Homeostasis does not apply only to metabolism; it is required in a number of regulated functions, from blood pressure control to DNA repair and tissue proliferation/apoptosis. In particular, cell proliferation and apoptosis represent the essence of a mammalian organism. Mammals are not born with a fixed number of cells. After zygote formation an incredible number of cell divisions takes place, and in just 19 days for a mouse or 9 months for a human, a fully-grown organism is born and viable. All of the organs are continuously reshaped by a combination of cell proliferation and apoptosis; even after birth and throughout adulthood, cells are continuously added and replace old ones until the individual's death. This process provides an incredible resilience against damaging external factors.

The central players in proliferative homeostasis are adult stem cells. Most adult mammalian tissues are able to constantly regenerate; liver is just the most famous example. For instance, the epithelial layers of the gastrointestinal tract and of the respiratory tree, as well as muscle and hematopoietic cells, can undergo a compensatory proliferation upon damage, although, unlike liver, the extent of this regeneration is often limited; cells in many organs are able to maintain an incredible proliferative capacity during ordinary daily conditions. In particular, all tissues interacting directly with the external environment, which are hence strongly exposed to damage, have a strategy to preserve their integrity by using "preventive" replacement [1]. Hematopoietic, gastrointestinal, respiratory, and skin cells are constantly subject to damage due to their exposure to pathogens, drugs, toxic substances, and ultraviolet rays. Instead of repairing them, a better and safer method has been adopted that consists in replacing all cells that are lost. One of the most sensitive and delicate steps during cell proliferation is DNA replication. Stem cells were "created" to protect DNA integrity. In particular, adult stem cells must be able to self-renew for the entire lifespan of an organism in order to maintain tissue homeostasis. Stem cells represent a reservoir of slowly proliferating cells, relatively protected against replicative errors, generating undifferentiated proliferating progenitors and differentiated cells (Fig. 1). In this way most proliferating cells are readily discarded after a few days of work, preventing possible tissue failure or, worse, propagation of DNA damage. This model, proposed when stem cells were still uncharacterized, implies that in most tissues their role is to allow constant adaptation to the environment and to provide a constant supply of new cells, preventing disease and aging.

Self-renewal, a central feature of stem cells, is the result of the dynamic balance between proliferation and maintenance of the developmental potential [2]. Most human diseases can be considered the result of an imbalance of this process, ultimately

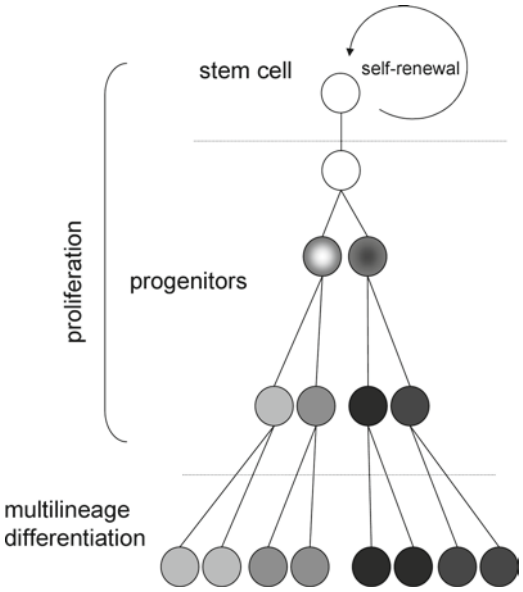

Fig. 1 Stem cell tree. Simplified representation of stem cells, progenitors, and differentiated cells. Stem cells proliferate and self-renew, while progenitors proliferate but do not self-renew. In most organs terminally differentiated cells are not able to proliferate

leading to tissue maintenance failure [3]. Cancer is the typical disease caused by an imbalance in stem cell homeostasis. Tumors arise in different tissues and from different cell types as a result of complex extrinsic (environment) and intrinsic (germline or somatic mutations) interactions. Colon cancer is a good example. In order to develop, it has to accumulate genetic mutations over time, making it progressively more aggressive [4]. Because most of the colonic epithelial cells are short lived, the only cells living long enough to accumulate mutations leading to cancer transformation are probably stem cells. Activation of known oncogenes in intestinal stem cells (ISCs) in the mouse, coupled with careful lineage tracing, showed that ISCs are indeed the cells of origin of intestinal adenomas [5–7]. There is increasing evidence in mice and in humans that stem cells are not only the cells of origin of intestinal cancer. Once a tumor is established, there is a cell population with stem cell features that is capable of supporting and maintaining cancer growth [8].

For many tissues (e.g., kidney, pancreas, adrenal glands, and thyroid), however, it is not known whether stem cells really exist. In those organs most of the terminally differentiated cells are able to proliferate in vivo, as demonstrated by proliferative markers or by incorporation of nucleotide analogs. These findings were complemented and extended recently by the use of genetic fate mapping of differentiated cells, unequivocally showing that differentiated cells are able to proliferate. Tissue maintenance is guaranteed by differentiated cells without the need for an undifferentiated stem cell population constantly providing the renewal capacity. These organs (e.g., kidney and pancreas) have a slow proliferation rate, and hence

it is conceivable that their proliferative homeostasis can be maintained by their slow renewal capacity and relatively long lived, well-differentiated cells.

2 Stem Cells in Pancreas

Pancreas is an organ known more for its diseases (diabetes and pancreatic adenocarcinoma) than for its normal physiologic functions. For this reason the proliferative capacity of the exocrine and endocrine pancreas is under intense scrutiny with the hope of finding new cell-based treatments for diabetes and new strategies to treat pancreatic cancer. The identification of the cell of origin of pancreatic cancer will eventually lead to new forms of treatment specific for those cells. Insulin-producing beta cells in endocrine pancreas are examples of adult differentiated cells that are well characterized with respect to their proliferative skills. Several papers [9–11] showed that beta cells maintain organ and glucose homeostasis in vivo under normal physiologic conditions through rounds of self-duplication.

As to exocrine acinar cells, it is well known that they exhibit proliferative behavior compatible with self-duplication [12, 13]. These conclusions were reached through a "reverse" approach, given that there were no identified markers for pancreatic stem cells. A tamoxifen-inducible Cre driver mouse line was generated that was expressed only in differentiated cells. In beta cells, Cre was driven by the insulin locus, while in acinar cells, it was driven from the elastase locus. Labeling differentiated cells (through the combination of the Cre driver with a conditional mouse reporter line) and following them over time generated the following predictions. If there are undifferentiated progenitors giving rise to differentiated acinar/beta cells, the originally labeled population would be progressively diluted, while if differentiated cells give rise to more differentiated cells the pool of cells originally labeled would stay undiluted. Using insulin::CreER [10] and two different elastase::CreER [12,14] drivers, it was demonstrated that all of the differentiated cell pools remained virtually undiluted, confirming that all cells were proliferating through rounds of self-duplication. These studies, however, labeled the whole differentiated acinar/insular compartment, or at least most of it, without leaving the possibility of analyzing whether among differentiated cells there is a small or large compartment of cells able to maintain proliferative and self-renewing capacity as "monopotent" stem cells.

Another paper [15], however, showed that beta cell progenitors exist and reside within or around the duct epithelium. These progenitor cells are identified by *Neurog3*, are activated following injury, and generate all of the endocrine cell types. Presence of *Neurog3*$^{+}$ cells giving rise to insulin-producing beta cells opens up the possibility for in vivo cell-based therapy for diabetic patients, but so far it so not clear whether *Neurog3*$^{+}$cells have a significant role in organ homeostasis under normal physiologic conditions [16].

A different approach to studying these cell populations has also been undertaken. Inducible Cre drivers expressed in other stem cell populations are already

available, and, if they are expressed in exocrine and endocrine pancreas, their analysis would help to identify their properties. As mentioned before, self-renewal is an exclusive feature of stem cells or of cells with stem cell properties. Genes and mechanisms underlying adult stem cell self-renewal are being characterized. A few genes are known to have a role in self-renewal of adult stem cells in vivo. One of them is *Bmi1*.

3 *Bmi1*'s Role in Stem Cells and Development

Bmi1 is part of the Polycomb group gene (PcG) group in flies and vertebrates involved in establishing, along with the Trithorax group proteins, expression boundaries of the *Hox* genes [17]. There are two major PcG complexes, PRC1 and PRC2. Bmi1, along with Ring1a and Ring1b, belongs to the PRC1 complex. PRC1 has a histone H2A-K119 ubiquitin E3 ligase activity, Ring1b is the catalytic subunit, and Bmi1 maintains the integrity of the complex and is essential for enzymatic activity [18]. To repress transcription, the PRC1 complex is dependent on the continuous presence of PRC2, which has Ezh2-mediated H3K27 methyltransferase activity [19].

Bmi1 has a fundamental role through this repressor activity in setting up the expression boundaries of the *Hox* genes. A skeletal analysis of the *Bmi1*-knockout (KO) mouse showed a series of defects along the anteroposterior axis reminiscent of the skeletal defects found in *Hox* mutants [20]. Its role is well established in vivo in several tissues and organs. *Bmi1*$^{-/-}$ mice die 3–5 weeks after birth with progressive ataxia, failure to thrive, and inability of hematopoietic stem cells (HSCs) to self-renew [20–23]. Because of the early death of the *Bmi1*$^{-/-}$ mice, this gene's role in tissue homeostasis and maintenance in adult organs has not been fully evaluated.

The investigation of the hematopoietic system failure and ataxic phenotype led to the discovery of its central function in self-renewal of HSCs, neural progenitors, and leukemic cells, firmly establishing its role in self-renewal. Multipotent hematopoietic progenitors in *Bmi1* KO mice are unable to self-renew and undergo apoptosis or early senescence upon activation of the *Ink4a/Arf* locus with subsequent p53-mediated apoptosis. The *Ink4a/Arf* locus expresses the p16^{Ink4a} and p19^{Arf} proteins and interacts at different levels with the *Bmi1* gene, with which it has established an inverse correlation. High levels of Bmi1 are associated with low level of p16^{Ink4a} and vice versa, suggesting an inhibiting role of Bmi1 on the *Ink4a/Arf* locus. The genetic interaction was tested in vivo, generating double KO *Bmi1*/*Ink4a/Arf* mice. These mutants show an improvement in some of the symptoms of the *Bmi1*$^{-/-}$ mice, in particular regarding HSC potential and in neuronal progenitor proliferative capacity, while shortened mouse lifespan and failure to thrive were largely unaffected [24]. Overexpression of *Ink4a/Arf* locus in vivo in different organs is responsible for early aging, indicating that most of the phenotypic effects in *Bmi1* KO mice are probably mediated through the activation of the

Ink4a/Arf locus [25, 26]. In vitro, it has been demonstrated that Bmi1 is able to bind the promoter region of the *Ink4a/Arf* locus after Ezh2-mediated H3k27 methylation. Upon Bmi1 binding, the histone H2A is ubiquitylated and the *Ink4a/Arf* locus silenced. In this way cells are maintained in an active proliferative status [19]. Upon Bmi1 removal, *Ink4a/Arf* is again actively transcribed, and cells stop dividing.

A *Bmi1*^{CreER} allele (a tamoxifen-inducible Cre recombinase expressed by the *Bmi1* locus through an internal ribose entry site) was generated in the mouse with the specific aim of analyzing the lineage of stem cell populations throughout the body [5]. Expression of *Bmi1* should presumably be active only in a very specific subset of self-renewing cells or when those cells undergo self-renewal. The *Bmi1*^{CreER} allele was generated with the goal of identifying and following stem cells in different tissues and organ. The temporal control provided by tamoxifen predicts the following outcomes (Fig. 2): if cells labeled by *Bmi1*$^{+}$ at time 0 are proliferating but not self-renewing, their progeny would be temporarily labeled, and they would disappear. Instead, labeling of self-renewing cells at time 0 will be associated with the presence of labeled cells even after 1 year. This system has the advantage of demonstrating the presence of *Bmi1*$^{+}$ cells, showing their behavior over time, and evaluating their "stemness." Moreover, the presence of a Cre enzyme allows

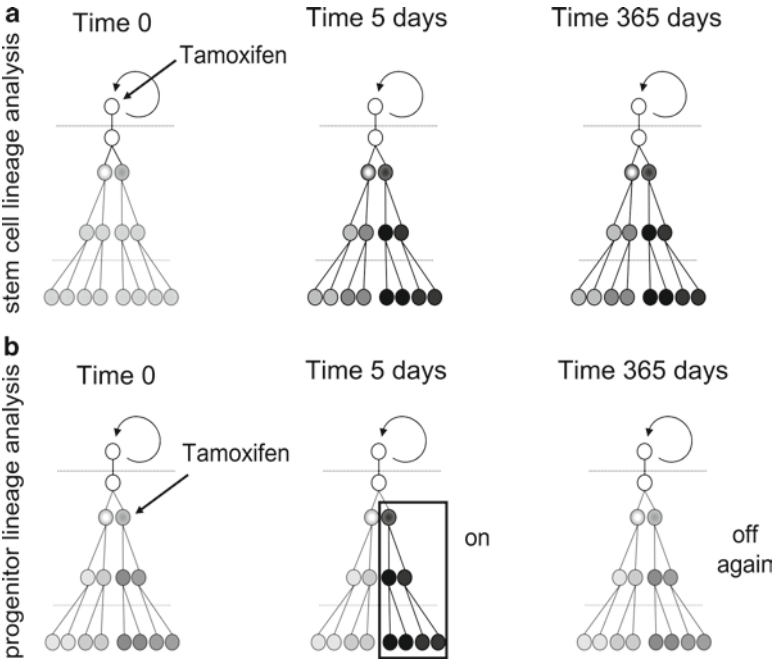

Fig. 2 Different outcomes of a lineage analysis in a stem cell and in a progenitor population. **a:** Upon tamoxifen injection, when the CreER driver is expressed in a stem cell population, progressively more cells will be labeled at 5 days. Because stem cells self-renew, labeling is still present after 1 year. **b:** When CreER is expressed in a progenitor, proliferating but not self-renewing, there are more cells labeled at 5 days, but then the label progressively disappears

efficient recombination not only of reporter genes, but also of many already available conditional alleles to test the effect of their inactivation inside those cells, as well as the complete ablation mediated by the diphtheria toxin of the cells expressing *Bmi1*.

The analysis of the *Bmi1*^{CreER} allele in the small intestine allowed the identification in the adult mouse of a population of ISCs [5]. *Bmi1* ISCs are located in the so-called position +4, are able to self-renew for more than 1 year, are undifferentiated, and are able to give rise to all differentiated lineages of the small intestine. Cre-mediated *Beta-catenin* stable expression in *Bmi1*$^{+}$ cells was able to generate intestinal adenomas [5]. These data were independently confirmed by two other groups using two different drivers (*Lgr5* and *CD133*) to label ISCs and to recapitulate adenomatogenesis starting from ISCs. The expression domains of *Bmi1*, *Lgr5*, and *CD133* partly overlapped, indicating that although the general properties were the same, each gene labeled a specific domain or a specific function within a group of cells located at the bottom of an intestinal crypt.

4 *Bmi1*'s Role in Exocrine Pancreas

The adult pancreas is organized in a three-dimensional network of acini (from the Latin *acinus*, meaning "berry"), which are the functional units producing the pancreatic enzymes. All the acini "drain" their juice of digestive enzymes into a progressively larger series of ducts conveying them into the duodenum. The epithelial layer of the ducts produces mucin. There is a particular cell type located at the junction between the smallest duct and the acinus: the centroacinar cell. This cell, because of its location and intermediate differentiation between ducts and acini, has been considered for a long time the pancreatic stem cell [27], but no lineage analysis has evaluated its properties. Each acinus does not have a clearly defined stem cell compartment, and there is no apparent migration inside each acinus. As mentioned before, acinar cells can proliferate through self-duplication. The acini and ducts represent the exocrine pancreas; interspersed among them are "islets" of cells representing the endocrine pancreas that produce hormones, the best-known being insulin and glucagon, produced by beta and alpha cells, respectively, and involved in glucose homeostasis.

Use of the *Bmi1*^{CreER} allele in the exocrine pancreas demonstrates that *Bmi1* is expressed in a small population of acinar cells (approximately 2.5% of all acinar cells), showing a completely differentiated phenotype [28]. In spite of their differentiated phenotype, cells derived from *Bmi1*-expressing cells (or their progeny), labeled by a single administration of tamoxifen, are still present 1 year later. This finding prompted a more careful evaluation of their proliferative behavior early on after tamoxifen. The initial population analyzed 5 days after tamoxifen was able to expand during the first month from 2.5% to 11% of the whole acinar population. To confirm the proliferative capacity of *Bmi1*$^{+}$ acinar cells, it was shown that the lineage is labeled by Ki-67 staining and bromodeoxyuridine (BrdU) incorporation

(two commonly used markers for cell proliferation). Because there is no apparent migration inside each acinus, every new cell will derive from an adjacent one, and the increase of the number of cells over the first month can be easily correlated with the proliferation of originally labeled cells. Cells were counted at 5, 15, and 30 days and scored as single clones or expanded clones. Single clones are defined as isolated labeled cells, without any of their cell membranes "touching" another labeled cell. Expanded clones were considered two or more adjacent cells having in common one of their membranes (the epithelial marker E-cadherin was used to identify cell boundaries). At 5 days most of the cells were scored as single clones, while at 15 and 30 days most of the cells were expanded clones, strongly suggesting that the original labeled cells gave rise in 1 month to two or more adjacent cells (Fig. 3).

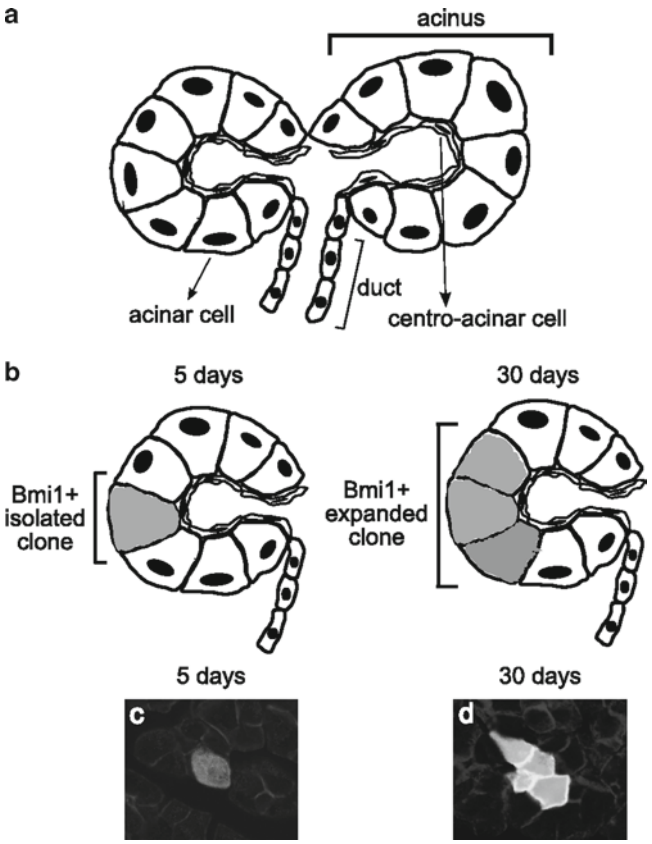

Fig. 3 *Bmi1* in exocrine pancreas. **a:** Schematic representation of a pancreatic acinus showing acinar cells, centroacinar cells, and duct cells. **b:** Dynamic expansion of the *Bmi1*$^+$ cells 5 and 30 days after tamoxifen. At 30 days this is referred to as an expanded clone to indicate that a cluster of cells is usually present derived from the few cells labeled by tamoxifen at the beginning of the experiment. **c, d:** Immunofluorescence experiment indicating a single *Bmi1*$^+$ cell at 5 days and a group of cells present at 30 days after tamoxifen

Upon diphtheria toxin cell ablation and caerulein-induced pancreatitis, *Bmi1*-differentiated acinar cells and their progeny undergo compensatory proliferation in order to maintain organ homeostasis. A final experiment with the *Bmi1* acinar lineage was done using long-term chase with BrdU. BrdU was administered at weaning along with tamoxifen (TM), and mice were sacrificed several months later. The BrdU long-chase experiments show that the BrdU lineage persists for at least 6 months, representing an indirect and independent confirmation of the persistence of the *Bmi1* lineage. Moreover, the newly generated *Bmi1*$^+$ lineage at 6 months is present in cells that were BrdU labeled 7 months earlier. The presence of strong BrdU label in the *Bmi1*-differentiated acinar cells after a 6-month chase suggests that some of these cells are set aside until needed during the life cycle of the mouse. An advantage of setting cells aside, whether "classic" stem cells or a subpopulation of differentiated cells used to maintain organ or tissue homeostasis, is protection of their genomes from errors generated during replication. It is interesting to compare the BrdU expression in other organs, like the small intestine, where the *Bmi1* lineage is present for 1 year [5]. In the small intestine, after 15 days the whole epithelium is completely labeled, followed by progressive washing out of the BrdU. The last cells to remain labeled are considered "putative stem cells," and the label-retaining ability is usually considered a property of stem cells [29]. The label-retaining ability can be interpreted either as a result of nonrandom segregation of the template strand [30] or of a slower cell cycle than the surrounding, rapidly dividing epithelium. The immortal-strand theory is still highly debated and controversial [31–33]. After 2–3 months, only rare BrdU$^+$ cells are left in the small intestine and probably represent stem cells because they are located in the +4 position [5]. The relatively slow BrdU disappearance in ISCs probably indicates the relative quiescent state of ISCs compared to the rest of the cells inside the crypt and could represent an indirect proof of their stemness. The BrdU retention in pancreas after 7 months probably reflects the even slower turnover of pancreatic cells compared to the ISCs. Presence of *Bmi1*-derived BrdU$^+$ cells is a strong suggestion that some of the acinar cells might have been in a relatively quiescent state reminiscent of ISCs.

The lifespan of acinar cells is not known. To rule out the possibility that the persistence of the *Bmi1* acinar lineage was related to the fact the acinar cells were long lived and not replicating, TM was given at weaning, followed 7 months later by BrdU administration. Then pancreas was then analyzed to check whether the *Bmi1* lineage was still proliferating after 8 months. This experiment confirmed that *Bmi1* progeny were proliferating, as indicated by the presence of cells of the BrdU$^+$*Bmi1*$^+$ lineage. This study highlights a new detail in the self-duplication model: presence of a "monopotent" acinar stem cell labeled by *Bmi1* expression. This model requires more confirmation and a more detailed analysis of a crucial, still unresolved, problem: it is not possible to identify discrete acini on sections.

Exocrine pancreas (as well endocrine pancreas or other organs such as liver and kidney) is a difficult organ to study because it is hard to establish the precise boundary of each acinus (the fundamental unit) and consequently to identify whether there is a stem cell–like population inside each acinus. While in the

small intestine it is clear that *Bmi1* is present only in a "subpopulation" of cells (mostly the so-called +4 cells), it is really difficult to prove in vivo in the whole pancreas whether, inside each acinus, *Bmi1* is expressed only in a "subpopulation" that has the potential to proliferate (the self-duplication hypothesis) or proliferate and self-renew (a stem cell–like population). If, for example, all of the cells inside an intestinal crypt were labeled with a genetic marker, after a 1-year chase it would immediately be evident that all of the cells inside a crypt are capable of self-duplication and one would missed the fact that there are a stem cell population, a population of progenitor cells, and a population of differentiated cells. Instead, using *Bmi1* (or *Lrg5* or *CD133* lineage tracing), it appears that there are discrete cells able to proliferate and self-renew (stem cells), giving rise to all of the other cells, which are proliferating but not self-renewing (progenitors).

Moreover, if all of the acinar cells can proliferate, it would be expected that the lineage would disappear in some of the analyzed mice because some of the cells would eventually die and disappear without replicating, especially considering the relatively low number of initially labeled cells (2.5%). Instead, over a 12-month period at 13 different time points, in a relatively small number of mice per each time point but collectively in 34 different mice the lineage never disappeared.

All of the previous reports labeled all of the acinar cells (or at least a large fraction of them) and were not able to discriminate whether all the cells or only a few within each acinus had the same proliferative/self-renewal potential. Saying that all the cells can proliferate (self-duplicate) does not mean (or rule out) that all the cells can self-renew (or just a small fraction of them). Inside an intestinal crypt only a few cells can self-renew, while all of them (except Paneth cells) proliferate, as shown by BrdU incorporation. Again, if pancreas was like intestine with discrete separate crypts/acini, this issue it would be immediately resolved.

Another open issue is the role of the centroacinar cells. Speculation about their role in exocrine pancreas homeostasis will be resolved when there is a genetic fate-mapping experiment clarifying the kind of progeny to which they are able to give rise. It is important to observe that the "immortal," differentiated *Bmi1*$^+$ acinar cells support the cell-of-origin studies that identified elastase$^+$ acinar cells as the source of mouse pancreatic adenocarcinoma and its preneoplastic lesions [27, 34–37]. Cancer is believed to derive from stem cells because these are virtually immortal and consequently are the only cells that live long enough to accumulate mutations to trigger the cancer phenotype. The *Bmi1* acinar lineage can survive up to 1 year or longer, and this explains why differentiated acinar cells can give rise to pancreatic adenocarcinomas.

This study characterizes *Bmi1*'s role in the exocrine pancreas and establishes a more general role for *Bmi1* not only in the self-renewal of "classic" ISCs, but also in the maintenance of the proliferative ability of differentiated cells. Self-renewal represents a more general property not only of classic stem cells, but also of differentiated acinar cells, retaining in this way their proliferative state in order to maintain pancreatic tissue homeostasis.

5 *Bmi1*'s Role in Endocrine Pancreas

Bmi1 lineage analysis in pancreas, starting at the weaning age, showed that besides acinar cells, *Bmi1* lineage is also present in alpha cells expressing glucagon and in endothelial cells, while it is mostly absent in other islet cells, duct cells, and centroacinar cells [28]. Another study [38], using immunofluorescence, showed that *Bmi1* was present in some beta cells at 2 and 4 weeks, then its expression progressively declined, and at 10 weeks only weak staining was present in some cells. When glucose homeostasis was analyzed in *Bmi1* KO mice, they showed an abnormal glucose homeostasis, and upon treatment with streptozotocin (a toxic drug that kills specifically beta cells) they showed incomplete regeneration, suggesting that beta cell proliferation is *Bmi1* dependent. Even though these data have to take into account that *Bmi1* KO mice are runted and smaller than their normal siblings, the molecular interaction in beta cells between *Bmi1* and the *Ink4a/Arf* locus is particularly interesting. As established before, there is an inverse correlation between *Bmi1* and *Ink4a/Arf* expression, with *Bmi1* decline correlated with increased $p16^{Ink4a}$ expression. *Bmi1* expression is coupled with histone H2A ubiquitylation and repression of the *Ink4a/Arf* locus.

Reduction in *Bmi1* expression leads to loss of H2A ubiquitylation and promotes mixed lineage leukemia–mediated histone H3K4 trimethylation and transcriptional activation of the *Ink4a/Arf* locus. This implies a specific and direct mechanism through which *Bmi1* levels are critical in determining proliferative potential of beta cells. Consequently a reduction in the level of *Bmi1* associated with aging translates into reduced proliferative potential and self-renewal of beta cells. As a confirmation of these findings, overexpression of *Ink4a/Arf* resulted in reduced proliferation index of beta cells, and after streptozotocin treatment, ablation of one or both copies of *Ink4a* allowed a quicker and better recovery of their weight and restoration of normoglycemia. Overexpression of *Ink4a/Arf* confirmed that this locus represents both a biologic marker and an effector of aging, mediating the decline of the replicative capacity of beta cells. Overall these studies highlight the molecular role of *Bmi1* in repressing the *Ink4a/Arf* locus through a regulatory mechanism involving both the PRC1 complex and the Trithorax group, competing for the silencing and activation of the *Ink4a/Arf* locus, one of the main regulator of beta cell renewal capacity.

6 Conclusions

A new and surprising role for *Bmi1* has been recently discovered, with *Bmi1* at the crossroads of stem cell self-renewal and mitochondrial function [39]. In *Bmi1* KO mice, reactive oxygen species (ROS) levels were found to increase, while treatment with *N*-acetylcysteine (NAC; a scavenger for ROS) greatly improves survival in the same mice. The negative effect of ROS is mediated through the activation of the DNA damage response, and one of the central mediators of this pathway is *Chk2*.

Double-KO mice for *Bmi1* and *Chk2* show a partial recovery of their failure-to-thrive phenotype, significantly extending their lifespan, and this effect is also increased by concomitant treatment with NAC. The antioxidant NAC and/or the concomitant KO of *Chk2* did not rescue the *Bmi1*$^{-/-}$ HSC phenotype in the long-term repopulation assay, indicating that *Bmi1* is at a pivotal position where the self-renewal and control of ROS levels meet. This is important, especially in light of the fact that cells expressing *Bmi1* must be able to keep both programs under strict control in order to prevent irreversible damage to stem cells responsible for organ homeostasis.

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Cancer Stem Cells in Solid Tumors

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Abstract The existence of cancer stem cells continues to be extensively debated among scientists, and given the mounting evidence, it is an area that needs further examination. The possible reason for resistance to current treatment modalities in cancer is that we have yet to discover a method to eradicate cancer stem cells. This chapter describes the theory of cancer stem cells, portrays the role of cancer stem cells in radiation and chemotherapy resistance, and describes methods for their isolation. Finally, it presents current knowledge on cancer stem cells in solid organ tumors and discusses the limitations of current isolation strategies.

Keywords Cancer stem cells • Tumor-initiating cells • Chemoresistance • Neurosphere assay

1 Introduction

Despite decades of effort and a diversity of treatments, cancer is still a disease with a poor prognosis. Cancer research has recently shifted to characterization and targeting of cancer stem cells (CSCs). This cutting-edge strategy aims at specifically eliminating the tumor-initiating and tumor-propagating cells that seem to be selectively spared by current treatments. The CSC model predicts that, as in any organ, cells in the tumor are hierarchically organized and derived from a rare population of CSCs that are able, like any somatic stem cells, to self-renew and differentiate [1]. Briefly, cancer is now considered as a stem cell disease affecting the proliferation, survival, and antiapoptotic pathways of normal stem cells [2,3].

The theory that cancer originates from "stem cells" was first postulated in the nineteenth century by observations of the histological similarities between fetal

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developing cells and teratocarcinoma [4]. During the twentieth century cancer research focused on the molecular biology of cancer. In 1971 a paper was published that described an in vitro study demonstrating that only a small percentage of myeloma cells were capable of forming tumor colonies [5]. This was followed in 1977 by a publication claiming the isolation of stem cell populations in different epithelial tumors [6]. The study hypothesized not only that tumors originated from a subset of cancer cell populations, but also that there were differences between CSCs of different tumors and between CSCs and normal granulocyte precursors. A seminal paper was published in 1997 proving in an in vivo experiment that acute myeloid leukemia originated from hematopoietic stem cells (HSCs) [1]. This initiated a flurry of research to establish CSCs and their markers in all cancers, but it took several years to establish their existence in solid organ tumors. Al-Hajj et al. [7] succeeded in identifying CSCs in breast cancer, followed by Singh et al., who isolated CSCs from brain tumors [8,9]. Later, CSCs were discovered in prostate [10], liver [11], skin [12], and colon cancer [13–15].

A CSC is a tumor cell capable of self-renewal and reconstitution of a morphologically identical tumor to the one from which it was isolated [16]. The similarities between CSCs and normal stem cells have led to the terminology of cancer "stem cells." The characteristic trait of both normal stem cells and CSCs is the ability to divide asymmetrically, producing one stem cell and one daughter cell dedicated to a specific line of differentiation [17]. The difference is that in a CSC population this trait is no longer under strict control [18], allowing extensive proliferation, unlike in normal tissue, when proliferation occurs only on demand. The ratio of CSCs to cancer (nonstem) cells is retained, but the overall cell numbers continually increase. Research has indicated that several signaling pathways, such as Wnt and Notch, are involved in the self-renewal capacity of stem cells. CSCs likely share many other properties of normal stem cells, including relative quiescence, resistance to drugs and toxins through the expression of several ABC transporters, resistance to apoptosis, and active DNA repair [19].

The origin of CSCs is under extensive investigation. A possible explanation for the origin of the CSC is that the cell is no longer under growth regulation and therefore remains a stem cell or a differentiated cell that has acquired the capabilities of self-renewal [20]. The biologic properties of CSCs are the focus of current research. By determining the origin of these cells, the pathways of self-renewal and differentiation, identification of their niches, and their surface markers, therapy can be appropriately targeted in the hope of a cancer cure [21]. This chapter considers the existence of CSCs and current research into CSCs in solid tumors and their clinical implications.

2 Chemoresistance and Radioresistance and Cancer Stem Cells

The link between CSCs and chemoresistance has not been established despite its importance with regard to clinical implications. In the conventional model of drug resistance, mutations occur randomly in one or several cells and confer a selective advantage after chemotherapy. The CSC hypothesis assumes that in most cases,

resistance is driven through a rare population, the CSCs, mainly as a consequence of their resting stem cell phenotype [22]. High levels of transporters (MDR1, ABCG1) [23–25] and of antiapoptotic proteins (Bcl-2 family members) [26], slow cell cycling [27], or enhanced DNA damage repair mechanisms [28] provide them with innate resistance. In contrast to CSCs, differentiated tumor cells lose their intrinsic resistance upon differentiation but can become resistant through acquired processes of gene amplification or chromosome rearrangement [29]. Sometimes chemotherapy does not even affect normal cancer cells. In these rare events, differentiated cancer cells seem to possess intrinsic resistance, and this weakens the theory that only the CSC population is chemoresistant. This rare phenomenon can be explained by the fact that the resistant CSC phenotype is abnormally persistent in the committed progenitors and consecutively to the rest of the differentiated cancer cells [19]. As evidence for CSC intrinsic chemoresistance, the transporter ABCB1 implied in drug resistance and expressed by most normal cancer cells is a major target in cancer therapy [30, 31]. However, several studies have shown that CSCs from breast and pancreas cancer express ABCG2, another transporter of the same family, instead of ABCB1 [25]. Such therapies would therefore miss CSCs, which would explain their low efficiency. Killing normal cancer cells can decrease the tumor size but cannot prevent the resistance to chemotherapy since only one of these tumor-initiating cells is able to grow a new tumor and cause tumor recurrence. To cure and eliminate cancer, CSCs need to be eradicated.

CSC resistance to radiotherapy was postulated in both breast and brain cancers and thought to be linked to an altered/damaged DNA checkpoint in the CSC cycle. It is thought that these checkpoints may initiate normal cells being transformed into cancer cells, and they also play a role in radioresistance [32]. The overexpression of the Wnt/β-catenin pathway was shown to increase radioresistance in murine mammary epithelial cells [33]. Reactive oxygen species (ROS) are involved in cellular apoptosis following radiation therapy, with high levels being associated with a greater amount of cell death due to increased amounts of free radical scavenging systems that increase DNA damage. An analysis of both murine breast and head and neck CSCs versus nontumor cells following coculture with irradiated feeder cells showed that not only did the CSC have low levels in ROS, but they were also radioresistant in comparison to the nontumor cells, implying that ROS was the mechanism by which cell death is caused by radiotherapy [34]. Examining these properties of cells will allow a focus on targeting these cells in future cell therapies.

3 Current Strategies for Isolating Cancer Stem Cells

3.1 Cell Markers

Only a few studies have reported the existence of CSCs, as shown in Table 1. Based on their similarities with normal stem cells, CSCs were first isolated with corresponding stem cell surface markers. CD133, initially referred to as AC133, was chosen as the most relevant candidate since it is an important hallmark of the “stemness” state

Table 1 The most common markers used for cancer stem cell isolation

Cancer Type	Cancer Stem Cell Marker	Reference
Leukemia	$CD34^+/CD38^-$	1
Breast	$ESA^+/CD44^+/CD24^{-/low}$	7
Brain	$CD133^+$	8, 66
	$CD44^+$	98
Prostate	$CD44^+/\alpha_2\beta_1^{hi}/CD133^+$	10
	$CD44^+/CD24^-$	99
Colorectal	$CD133^+$	14, 15
	$CD44^+/ESA^+/CD166^+$	13, 89
	$CD44^+$	88
Pancreatic	$CD133^+$	25, 91
	$ESA^+/CD44^+/CD24^+$	90
Head and neck	$CD44^+$	111
	$CD133^+$	11, 94, 95
Liver	$CD90^+$	40
	$EpCAM^+$	96
Retinoblastoma	$CD133^+$	112
Lung	$CD133^+$	104, 113
Kidney	$CD133^+$	114
Ovary	$CD133^+$	100
	$CD44^+/MyD88^+$	101
Melanoma	$CD90^+$	39
Larynx	$CD133^+$	115

in primitive cells from neural, epithelial, endothelial, and hematopoietic tissues [35–37]. It has also been reported to be present on CSC populations from different hematopoietic and solid tumors such as leukemia, brain [8], colon [14,15], liver [11], and prostate cancer [10]. The CD133 marker thus appears to be the most likely candidate for a universal "stemness" marker in cancer. Subsequently, other markers for the identification of CSCs were discovered, including the marker CD44 in breast cancer [7]. CD44 in combination with $CD24^-$ and ESA^+ allows the purification of breast tumor–initiating cells, with CD44 also present in pancreatic [38], breast [7], prostate [10], and colorectal cancer [13]. In colorectal cancer, the proposed combination of markers $CD44^+/CD166^+/ESA^{hi}$ is expressed by a highly tumorigenic population. Other markers, such as ABCB5 and CD90, are involved in melanoma [39] and liver [40] CSC isolation, respectively.

3.2 "Side-Population" Cells

Hoechst dye is the tool primarily used as a strategy to enrich human HSC subpopulations. The cells able to expel the dye form a distinct subset of cells, identifiable on flow cytometry, and have been called "side-population" (SP) cells. SP cells were identified in murine HSCs by Goodell et al. [41]. Their potential to repopulate

lethally irradiated mice was 1000-fold higher than whole bone marrow cells. Although a controversial matter, SP cells are considered to be enriched in stem cells and have subsequently been identified among gastrointestinal, liver, nasopharyngeal, and lung cancer cells [23,24,42,43].

3.3 Sphere Formation

The isolation of CSCs can be performed by a neurosphere assay set up by Reynolds and Weiss to isolate neural stem cells [44,45]. This assay relies on the unique ability of stem cells to form spheres, and growing cells in such conditions seems to favor proliferation rather than differentiation, thus enhancing the number of stem cells. The method has been adapted to purify putative CSC populations, especially in brain cancer [46–50], as well as in breast [51], colorectal [52], pancreatic [53], ovarian [54], and prostate cancer [55].

3.4 Aldehyde Dehydrogenase

Another strategy to isolate stem cells and CSCs is emerging based on the targeting of cells with high aldehyde dehydrogenase (ALDH) activity, especially ALDH1. This enzyme mediates the oxidation of intracellular aldehydes to carboxylic acids in the cytosol and is involved in retinoid metabolism [56]. It has been shown that in HSCs, retinoids are responsible for the terminal differentiation of late progenitors in mice and the self-renewal of early precursor cells [57,58]. ALDH1 activity is hence implicated in maintaining a stem cell phenotype and is believed to be present in elevated levels in HSC and neuronal stem cells. ALDH1 successfully allowed the isolation of HSCs in neural and breast stem cell populations [59–61]. Studies have isolated $ALDH^+$ populations in leukemia [62], breast [61,63], colorectal [13], liver [64], and lung [65] cancers.

4 Current Knowledge on Existence of Cancer Stem Cells in Solid Tumors

4.1 Brain Tumors

Brain tumorigenic stem cells (BTSCs) have been detected in primary brain tumors, including glioblastoma (GBM), medulloblastoma, ganglioglioma, and pilocytic astrocytoma [8,49,50,66–68]. They have also been identified in several brain cancer cell lines [46,69–71]. With use of the neurosphere assay, CSCs are grown at clonal

density on uncoated plastic in serum-free medium supplemented with endothelial growth factor (EGF) and basic fibroblast growth factor (bFGF) [44,45]. Cells responsive to these growth factors form floating spheres; they are considered CSCs if they can keep this sphere-forming property after several passages and give rise to a large number of differentiated progeny [72].

Neurospheres grown from primary brain tumors and brain cancer cell lines express CSC-like properties, including higher levels of stem cell markers CD133 and nestin, which is a cytoskeletal protein associated with neural stem cells [46,49,73]. Neurospheres originating from glioma have been shown to differentiate into neurons and astrocytes [74]. When derived from oligoastrocytoma, they have been shown to express glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP), which suggests their capacity for multilineage differentiation [50]. Neurospheres possess an enhanced sphere-forming potential in vitro and an increased cancer-initiating capability in vivo compared to the initial population, with only 1000–5000 cells from neurospheres necessary to reinitiate a tumor [46, 48–50, 73].

Besides neurosphere formation, brain CSCs have been targeted through the corresponding stem cell surface marker CD133. This marker is expressed at higher levels in neurospheres from brain tumors than in the primary tumor tissues [46,49,73]. Uchida et al. first showed that $CD133^+$ cells isolated from pediatric brain tumors had in vitro stem cell characteristics with high capacity for sphere formation and a potential for engraftment in neonatal rat brain [36]. Later, Singh et al. isolated a $CD133^+$ population from medulloblastomas and GBM. As few as 100 $CD133^+$ cells could reinitiate a tumor in NOD/SCID mice even after several passages, while 1000 times that many $CD133^-$ cells could not [8,9]. In addition, $CD133^+$ cells could generate clusters; they self-renewed and differentiated to recapitulate the original phenotype of the tumor from which they were derived.

A few studies confirmed the stem cell–like phenotype of $CD133^+$ glioma cells in vitro [75] and in vivo [28]. Bao et al. showed that 10,000 $CD133^+$ cells were able to reinitiate the tumor in immunocompromised mice brains, whereas 2×10^6 $CD133^-$ cells could not form tumors in three of five animals [28]. In vitro studies on $CD133^+$ cells also included resistance assays to chemotherapeutic agents. In cell lines derived from primary cultures of glioblastomas, $CD133^+$ cells were significantly more resistant than $CD133^-$ cells when exposed to a panel of four agents including paclitaxel, carboplatin, temozolomide, and etoposide, and they expressed higher levels of the drug resistance gene BCRP1 [26]. However, the use of CD133 as a universal BTSC surface marker has been compromised by studies reporting that $CD133^-$ cells were able to form neurospheres and were tumorigenic in nude rats [76,77]. Moreover, these cells were capable of generating $CD133^+$ cells after their reimplantation in rat brains [77]. It thus appears that CD133 may not be as essential in brain tumor initiation as first expected. Its role might be more critical for tumor progression rather than for initiation.

4.2 Breast Cancer

In breast primary tumors, the combination of the two markers $CD44^+/CD24^-$ enabled Al-Hajj et al. to isolate breast tumor–initiating cells. Among them, cells expressing the additional epithelial surface antigen (ESA) marker had a reinforced tumorigenic potential in NOD/SCID mice [7]. ESA was previously used to identify a breast stem cell population defined as MUC^-/ESA^+ [78]. As few as 200 cells presenting the $CD44^+/CD24^-/ESA^+$ phenotype generated a tumor in all of the mice tested when injected into the mammary fat pad of NOD/SCID mice. Furthermore, only the cells with $CD44^+/CD24^-$ expression progressed into the other phenotypes $CD44^+/CD24^+$, $CD44^-/CD24^+$, and $CD44^-/CD24^-$ after injection in the mice. This indicates that if the $CD44^+/CD24^-$ phenotype can differentiate and reconstitute all of the phenotypes present in the tumor bulk, it might be the most primitive phenotype among them. Similarly, in breast cancer cell lines SUM149 and SUM159, the cells with $CD44^+/CD24^-/ESA^+$ phenotype showed higher tumorigenicity, with as little as 100 cells forming tumors in NOD/SCID mice, confirming that $CD44^+/CD24^-/ESA^+$ might be the phenotype of breast CSCs in cell lines as well as in fresh tumor samples [79]. In addition, in two clinical studies in which patients received neoadjuvant chemotherapy, there was a marked increase in the levels of expression of $CD44^+/CD24^-$ compared to untreated patients, supporting the hypothesis that a resistant population exists among the cancer cells, and reinforcing the possibility that the combination $CD44^+/CD24^-$ is the breast CSC phenotype [80, 81].

Further work on breast cancer involved in vitro studies with assays primarily set up for the culture of breast stem cells as nonadherent "mammospheres" [82]. Similar to the neurosphere assay, mammary tissue can generate mammospheres with large populations of stem and progenitor cells able to differentiate in the three mammary epithelia [82]. Of interest, when such cultures were performed on breast tumors, mammospheres were obtained, and 95% of the cells showed the $CD44^+/CD24^-$ phenotype and were highly tumorigenic in vivo [51]. Moreover, $CD44^+/CD24^-$ cells in breast cancer cell lines were associated with invasive properties, as confirmed by their efficiency in invading Matrigel and by their gene expression profiling [83]. Resistances to radiation [84] and to chemotherapy [85] were suggested this population according to preliminary studies on breast cancer cell lines.

In connection with the $CD44^+/CD24^-/ESA^+$ phenotype, enzymatic assay allowing the targeting of cells with high ALDH activity has been confirmed as an alternative tool for extracting breast CSCs. In breast cancer primary cultures, a sorted $ALDH^+$ cell population representing 5% of the total population possessed the potential to form mammospheres, whereas $ALDH^-$ cells could not [61]. $ALDH^+$ cells were also highly tumorigenic in vivo and able to regenerate tumor diversity with only 500 cells injected. However, the overlap with the $CD44^+/CD24^-$ population was lower than 1.2%, which emphasizes the fact that isolating CSCs by specific cell surface markers may not comprise the whole stem cell population. In breast cancer cell lines, the combination of markers $ALDH^+$ and $CD44^+$ reinforced

the tumorigenic and metastatic potential of these cells [86]. Recently, the role of ALDH1 was elucidated in a study involving the analysis of 33 breast cancer cell lines. An $ALDH^+$ population with obvious stem cell properties was detected in 23 of these cell lines. These putative CSCs proved to be an important mediator of invasion in vitro and in vivo. $ALDH^+$ cells developed metastases in several distant sites, while $ALDH^-$ developed a metastasis only once, limited to the lymph nodes [63].

4.3 Colorectal Cancer

For colorectal cancer, CD133 was the first marker used to isolate a population with high tumorigenic potential [14, 15]. O'Brien et al. reported that on average 1 in 262 $CD133^+$ colorectal cancer cells was able to initiate cancer [14]. The $CD133^+$ population, as compared to the total tumor cell population, would represent a 200-fold enrichment in CSCs. Ricci-Vitiani et al. also confirmed the high tumorigenic potential of $CD133^+$ colorectal cancer cells as compared to $CD133^-$ cells [15]. The $CD133^+$ population isolated directly from primary colorectal specimens [15] possessed an exclusive sphere formation capability compared to the $CD133^-$ fraction and was able to retain the ability to initiate tumors for more than 1 year when cultivated under serum-free conditions.

Cells isolated directly from colorectal specimens have been propagated in serum-free conditions [52]. The resulting spheres expressed stem cell marker CD133 and were tumorigenic in NOD/SCID mice, whereas cells grown in the presence of serum were not. Moreover, upon deprivation of growth factors, the spheres differentiated and lost both CD133 expression and their tumorigenic potential.

Recent studies may undermine the use of CD133 as a marker to isolate colorectal CSC. Using a knock-in tumor mouse model, Shmelkov et al. reported on wide expression of CD133 in both cancer and noncancer epithelial cells in colon [87]. With the antibodies routinely used to isolate the CD133 population in colorectal cancer, it has not been possible to detect this wide expression of CD133 in normal colonic cells. In their study, Shmelkov et al. created a knock-in tumor mouse model in which CD133 expression could be detected by histochemistry using a LacZ reporter associated to the CD133 gene. The detection of the CD133 marker was therefore independent of the epitopes usually targeted by current anti-CD133 antibodies, which suggests that these antibodies might not detect all CD133 molecules.

Shmelkov et al. also reported that $CD133^-$ cells from metastatic colon were as tumorigenic as $CD133^+$, which was also recently confirmed by Chu et al. [88]. Like the two studies quoted earlier [14,15], they used available commercial antibodies to isolate CD133 cells. Their results showed that CD133 molecules may not be properly targeted by the available anti-CD133 antibodies and that CD133 is expressed by non-CSCs. Therefore, the targeting of CD133 with these antibodies and the use of CD133 as the main marker for CSC isolation needs to be questioned. As an alternative to CD133, the combination of markers $CD44^+/ESA^{hi}$ with $CD166^+$ has been proposed to identify a colorectal tumor-initiating population [13,89]. It was

shown recently that $CD44^+$ cells were tumorigenic in vivo [88]. In all the cases, these populations have been claimed to have an exclusive tumorigenicity property compared to their negative counterpart.

Among the populations used to isolate colorectal CSCs, it is unknown whether they differ in maturity. Little is known on the nature of CSCs and their progeny. In an effort to classify colorectal CSC, Todaro et al. recently proposed a hierarchical model in which $CD133^+$ cells are the stem cells and another important stem cell marker, Musashi-1 (msi1), is likely to be the marker of progenitor cells [52]. In this study, it was also shown that among a $CD133^+$/$msi1^+$-enriched population, $CD44^+$ cells are the ones that possess the highest metastatic potential.

In addition to cell surface markers, studies on the role of ALDH1 in colorectal CSCs have considered its role among the ESA^+CD44^+ CSC population. Results have been contradictory concerning the potential of the $ESA^+CD44^+ALDH^+$ cells to enhance tumorigenicity in vivo compared with their counterparts $ESA^+CD44^+ALDH^-$ [13, 88].

4.4 Other Organs

There are literature reports on the discovery of tumor-initiating cells among most organs, in particular liver, pancreas, prostate, and ovary. In primary pancreatic cancer cells, a population rich in tumor-initiating cells was found using the same approach as for breast CSC: ESA^+/$CD44^+$/$CD24^+$ [90]. This subpopulation comprised 0.2%–0.8% of the original tumor population. As few as 100 cells with this phenotype were able to regenerate the tumor with all cancer cell phenotypes. Some work has also been done in primary tumors and pancreatic cancer cell lines with use of the $CD133^+$ marker [25,91]. In fresh primary tumor samples, 1%–3% of the cells were $CD133^+$, and a population of 500 of them was able to reinitiate a tumor in NOD-SCID mice [91]. Only 14% of $CD133^+$ cells stained for the $CD44^+$/$CD24^+$/ESA^+ phenotype. This suggests, as in colorectal cancer, that different sets of markers can discriminate different populations with tumor-initiating potential.

Using the PANC-1 pancreatic cancer cell line, a colony-forming assay was established and allowed the detection of cells able to form adherent spheres over several generations in serum-free medium [53]. The cells isolated from the sphere-forming assay were capable of excluding Hoechst dye, and when cultured in medium with serum generated two types of cells—those with and those without the Hoechst dye exclusion capability [53]. A population of 5×10^5 PANC-1 cells dissociated from spheres and injected in nude mice formed a visible tumor nodule after 4 weeks, whereas it took 10^7 cells from the original population to form tumors. SP cells were also detected among this cell line [92, 93] and showed a high resistance to the antitumor agent gemcitabine [92]. The CSC nature of this population could not be definitely proven. SP and non-SP cells had the same sphere-forming efficiency [92]. and no in vivo work has been reported to confirm a high tumor-initiating efficiency of these SP cells.

In liver, early investigations used the marker CD133 to isolate putative CSCs. Highly in vivo tumorigenic $CD133^+$ cells were discovered in fresh primary tumor samples [94] and in several hepatocellular carcinoma (HCC) cancer cell lines [11, 94, 95]. In the PCL8024 HCC cell line, ALDH used in combination with CD133 [64] seemed to target an even more tumorigenic population, since significantly fewer $CD133^+/ALDH^+$ cells (500) were needed for tumor formation in comparison to $CD133^+/ALDH^-$ cells (10,000) and $CD133^-/ALDH^-$ cells (300,000). The marker CD90 (also described as Thy-1) has also been associated with tumorigenicity. $CD90^+$ cells were more tumorigenic than their counterpart in HCC cell lines, which was also validated in primary HCC [40]. In primary tumors, $CD90^+$ cells were 200 times more tumorigenic than the rest of the population. The same paper also reported that the CD44 marker is necessary for tumor engraftment and tumor metastasis, which has been confirmed in other cancers [52]. The marker EpCAM (=ESA) also distinguishes a highly tumorigenic and metastatic liver CSC population, with an interesting highlight on the role of the Wnt/β-catenin pathway in the survival of $EpCAM^+$ cells [96]. In another attempt to isolate liver CSCs, SP cells in the HCC cell line Huh7 promoted tumors in NOD/SCID xenograft experiments, with as few as 10^3 SP cells able to initiate a tumor, whereas 10^6 cells of the non-SP population were necessary to obtain the same result [23].

Prostate and ovary cancers also have been investigated. Mainly, the phenotypes $CD44^+$, $CD133^+$, $\alpha_2\beta_1^{hi}$ as well as androgen receptor $(AR)^-$ are expressed by cells presenting increased clonogenic, metastatic, and tumor-initiating potential [10, 97, 98]. The combination $CD44^+/CD24^-$ [99], as described previously in breast cancer, may also distinguish a prostate CSC population. In ovarian cancer, the main markers for isolating CSC-like populations were reported to be $CD133^+$ [100] and $CD44^+/MyD88^+$ [101]. In several prostate and ovarian cancer cell lines, sphere formation assays were established [54,55], and the resulting spheroids were able to expand exponentially after several passages and to establish tumors in mouse engraftment experiments.

5 Limitations of the Current Strategies to Isolate Cancer Stem Cells

Despite great effort, it has proven to be exceedingly difficult to find an ideal marker for the isolation of CSCs. A universal marker of CSCs would be a perfect tool for therapeutic purposes. Although some cell surface markers have been used to isolate CSCs, none of them is exclusively expressed by CSCs. For example, CD133 was considered the most likely candidate for a universal “stemness” marker in cancer, as it has been reported to be present on CSC populations from different hematologic and solid tumors [8, 10, 11, 14, 15]. However, it is present not only on putative CSCs, but also on many non stem cells in various tumors and normal tissues [8, 102]. In colorectal cancer, Shmelkov et al. reported that CD133 expression was largely extended to all sorts of differentiated colonic cells, and not only to a small percentage of stem cells as previously assumed [46].

The study by Shmelkov et al. also raises the issue of technical hurdles generated by the use of antibodies to detect cell surface markers [87]. Four monoclonal antibodies directed against one or another epitope of CD133 are commercially available. The locations of these epitopes have yet to be described but are known to be spatially distinct [35]. Both epitopes are glycosylated, and monoclonal antibodies recognize the glycosylated form of the CD133 molecule. The appearance of false-negative or false-positive binding of the antibodies is therefore highly probable [103].

A false-negative result might happen if the monoclonal antibodies are not able to recognize CD133 molecule when it is nonglycosylated. In addition, uneven glycosylation of the epitopes could generate discordant results according to the antibody used. Since little is known on the nature of the glycosylation of these two epitopes, it is then likely that other glycosylated extracellular molecules might be recognized, and this can provoke cross-reaction between antibodies. The glycosylation of the CD133 molecule is therefore a real concern and might be a plausible explanation of the contradictory results observed in brain and colorectal cancers. It is therefore necessary to use complementary techniques to isolate the CSC population.

It is important to consider the origin of cancer cells in order to define the best strategy to isolate them. The rationale behind finding a pan-CSC marker or an organ-specific CSC marker relies on the theory that CSCs originate from their respective organs. However, the stage at which the mutations leading to malignant transformations happens is not known yet, and it is conceivable that stem cells as well as early or even late progenitor cells can be the origin of CSCs, leading to a different panel of markers. The evidence that in the same organ two sets of CSC populations exist, such as $CD133^+$ and $CD44^+/CD166^+$ in colorectal cancer [13,15], and are similarly able to initiate tumors, proves that none of these markers is exclusively expressed by CSCs. Moreover, the proposed CSC markers are not expressed in 100% of the primary tumors examined, which seems to indicate that the search for a single marker or even combinations of markers might be a wrong strategy.

The growth of spheres is a possible way to isolate CSCs. In spite of the usefulness of the neurosphere assay, it is not free of limitations. In particular, the proliferating capacity is not restricted to stem cells. Progenitor cells are also able to proliferate, albeit on a shorter term. Reynolds et al. reported the existence of cells able to form spheres after two or three passages but not further [72]. It is therefore not possible to assume that all spheres derive from a stem cell, and it is of major importance to define the number of passages necessary to isolate true stem cells. Regardless of the fact that the neurosphere assay is so far one of the best in vitro tests to account for the existence of stem cells, it does not allow the isolation of a pure population of stem cells since spheres also comprise more differentiated populations. In brain cancer, the number of cells necessary to reproduce tumors in vivo was generally not lower than 5000 cells, which is still far from the theoretical value of 1 cell being able to reconstitute the whole tumor [46–50].

SP cell isolation has been the first attempt to purify cancer-initiating cells through a functional approach rather than by immunologic phenotype, and has led to other functional investigations. Isolated SP cells have stem cell–like properties,

such as increased invasive properties, high levels of telomerase [104], increased expression of drug resistance–associated genes, and evidence for self-renewal [24]. Although SP cells seem to be enriched in cancer-initiating cells [23], the SP content in CSCs remains controversial. For example, a recent study of SP cells from several gastrointestinal cancer cell lines could not establish any difference between the SP and non-SP cells [105]. Studies on tumorigenicity in vivo, on clonogenicity, on surface markers expression, and on drug resistance gene expression did not show any significant differences between the two populations, and the authors concluded that an SP cell could not be used as a CSC marker for intestinal cancers.

For the isolation and the purification of stem cells on the basis of a functional assay as an alternative to cell surface, measurement of ALDH activity appears to be an appropriate candidate. Its role has mainly been investigated in breast cancer, in which $ALDH1^+$ cells really display interesting stem cell–like properties [61, 63]. Current investigations in other solid tumors are in progress. ALDH1 is very promising as a CSC marker in liver, but a high ALDH activity does not seem improve the tumorigenicity of colorectal CSCs [13]. It is therefore too early to debate the universality of ALDH to isolate CSCs. In addition, this technique is confronted with the fact that there is no overlap with the tumor-initiating cells previously isolated with the cell surface markers [61].

Evidence regarding CSC chemoresistance is accumulating [19]. Several solid tumors have displayed a resistant phenotype with high expression of multidrug resistance genes [multidrug resistance gene 1 (MDR1), MDR related-protein 1 (MRP1)] [106] and apoptosis inhibition [107]. CSCs also showed higher resistance to chemotherapeutic drugs than somatic cancer cells [22,108]. Developing an assay in which putative CSCs could be isolated in relation to their property of resistance has been attempted in various studies. Of interest, long-term gemcitabine-resistant pancreatic cancer cell lines L3.6pl and AsPC-1 displayed more invasive and migratory properties than their nonresistant counterparts [109]. In these resistant cell lines, the usual CSC markers CD44, CD24, and ESA were also expressed in higher proportion (about 10%–15% more). Other studies have isolated tumor-initiating cells after short but lethal exposure to a chemotherapeutic drug, sparing only an “intrinsic” resistant population. Glioblastoma cells resistant to a lethal dose of 1,3-bis(2-chloroethyl)-1-nitrosourea showed cancer stem–like cell properties [110]. They were enriched in $CD133^+$ cells, were multipotent and able to generate neuron- and glial-like cells, and formed tumors when transplanted into brains of NOD/SCID mice.

6 Conclusion

The isolation of CSCs is required to establish their characteristics. Once the attributes of CSCs have been detailed, the most effective treatments may be discovered and instituted. Currently there is no single method for CSC isolation, and a combination the described isolation techniques should be used, that is, surface markers, sphere formation, ALDH assay, and chemotherapy resistance assay, as they are

partially efficient at isolation. The efforts invested in CSC isolation during these last few years have nevertheless strengthened the evidence for the existence of tumor-initiating cells in nearly all types of cancers. These tumor-initiating cells have shown various properties, mainly self-renewal and unlimited propagation in vitro, and in several cases chemoresistance and ability to metastasize.

At this premature stage in our understanding and knowledge of CSCs it would be unwise to embark on costly studies of oncologic treatments. In order to effectively treat cancer according to the CSC theory, we need to employ a therapy that would target the entire CSC population, and we should therefore persevere in our efforts to isolate and characterize CSCs.

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Adipose-Derived Stem Cells and Skeletal Muscle Repair

Claude A. Dechesne, Didier F. Pisani, Sébastien Goudenege, and Christian Dani

Abstract Treatments for muscular dystrophies constitute the goal of a very active field of research, and the characteristics of mesenchymal stem cells make them promising alternative candidates for cell-based therapy. In this context, adipose-derived stem cells (ADSCs) present very interesting intrinsic features in terms of abundance and easy access. Their myogenic potential has been investigated since the early 2000s. A synthesis of the findings is presented in this chapter. In fact, ADSCs harbor a limited autonomous myogenic differentiation potential. However, this capacity is increased when ADSCs are engrafted in a regenerating muscle. Very recently, it has been reported that an explanation for the relatively modest myogenic potential of ADSCs is that only a small perivascular cell subfraction carries an efficient myogenic potential. From a clinical perspective, significant results have been obtained by genetic modification of the whole ADSC population. Human ADSCs modified with MyoD, a master gene of embryonic myogenesis, have been shown in vitro and in vivo to be as myogenic as genuine myoblasts, and these cells are now being studied in animal models of dystrophic muscles.

Keywords Myogenesis • Muscle regeneration • Duchenne muscular dystrophy • Adipose-derived stem cells • Strand vascular fraction

1 Introduction

In 1995, the pioneering work of Arnold Caplan and colleagues reported that mesenchymal stem cells (MSCs) derived from bone marrow can be differentiated into the skeletal myogenic lineage [1, 2]. This extended the differentiation potential of

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MSCs and raised important new questions, including the physiologic relevance of the myogenic capacity of these cells. In addition, these results opened a new field of investigation—evaluation of MSC potential for muscle repair.

Skeletal muscle benefits from a strong power of regeneration, which allows recovery from rather severe injuries. Naturally, most if not all of the regenerated muscle fibers are generated by the satellite cells, which are the muscle progenitor cells. They lie along the fibers in a quiescent status and are recruited in case of injury. Upon different signals generated at the injury site, they proliferate to generate myoblasts that either fuse with preexisting fibers or fuse together into new multinucleated fibers. In the meantime the satellite cells replenish their original niche. A similar process occurs in muscle dystrophies to replace the necrotic fibers. This is the case, for instance, the Duchenne muscular dystrophy (DMD), a severe and common disease caused by the absence of dystrophin. After too many necrosis/regeneration cycles, the repair potential of satellite cells is exhausted, muscle fibers disappear, and the subsequent loss of function creates serious pathologic conditions. Although the origin of DMD and other muscle dystrophies has been described within the last 20 years, no treatment capable of stopping the deleterious evolution is available. Much effort has been devoted to that purpose, and various therapeutic strategies have been developed, including drug-, gene- and cell-based therapies. In this context, identification of any cell population that could efficiently regenerate muscle has special interest in assessing new cell-based therapies. MSCs are considered to be promising candidates because of their differentiation potential and their possibility of extensive multiplication in culture.

MSCs have now been found in several tissues, including adipose tissue. This tissue has special advantages from an eventual clinical perspective because it constitutes an abundant and easily accessible reservoir of MSCs. In addition, adipose tissue can provide much more stem cells than bone marrow, with a yield of 5000 cells per gram of tissue versus 100–1000 cells [3]. Although a relatively limited number of studies have been published on the myogenic potential of adipose-derived stem cells (ADSCs) and some data have to be confirmed by more laboratories, some significant conclusions can be drawn.

2 Adipose Tissue as a Source of Mesenchymal Stem Cells

Like bone marrow, adipose tissue is derived from the embryonic mesoderm and contains a heterogeneous population of stromal cells. The multilineage differentiation potential of adult adipose-derived cells was investigated in Marc Hedrick's laboratory in the early 2000s [4–6]. This group used human adipose tissue obtained by liposuction and extracted the stromal vascular fraction (SVF) using collagenase digestion and elimination of red blood cells. A routine yield of more than 10^8 cells per 300 cm^3 of liposuctioned tissue underlined the advantage of adipose tissue in directly providing a substantial amount of SVF cells. The adipose

SVF-derived adherent cells expanded easily in vitro and exhibited the same fibroblast-like phenotype as bone marrow MSCs. At passage 15, cultures had still a low level of senescence. Under the appropriate culture conditions, the cells were shown to differentiate toward the adipogenic, osteogenic, chondrogenic, and myogenic lineages on the basis of specific phenotype modifications, histologic staining, and marker expression. Of importance, self-replication and multiple-lineage differentiation were confirmed at the single-cell level. This indicated that SVF contains ADSCs and not only a mixture of different progenitor cells. All of these properties have been further confirmed with cells prepared from human [7, 8] and mouse [9–11] excised adipose tissues.

The initial studies reported by Zuk et al. [6] and further studies starting from liposuction material or excised adipose tissue fragments indicate that ADSCs express a CD surface marker profile very close, if not similar, to bone marrow MSCs. In humans, several studies found that these cells express CD29, CD44, CD90, and CD105 but are negative for CD13, CD31, CD34, CD45, and HLA-II [6–8, 12–14]. Together, these results are consistent with a MSC identity for ADSCs.

3 In Vitro Myogenic Potential of Adipose-Derived Stem Cells

3.1 Autonomous Myogenic Potential

ADSC autonomous myogenic differentiation has been reported by several labs, but the general point of view is that in culture, without any muscle-like environment, the myogenic differentiation is very limited. The full differentiation of myoblasts is obtained under appropriate culture conditions, including the absence (or low percentage) of serum when the cells are subconfluent. Then the myoblasts express myogenic determination factors (MyoD, Myf5) followed by other myogenic regulatory factors (myogenin, MRF4) and start to fuse into multinucleated myotubes that will express most of the muscle terminal markers. From the many reports studying the myogenic differentiation of ADSCs it is clear that, although ADSCs can express early and even late muscle markers under myogenic culture conditions, fusion into myotubes remains a rare event. This was observed for human ADSCs isolated from lipoaspirates [4–6], as well as from excised adipose tissues. Our laboratory has isolated ADSCs from adipose tissue samples from young donors as populations of human multipotent adipose-derived stem (hMADS) cells [7]. hMADS cell myotubes were found at a very low frequency (less than 0.1% of total plated cells) and not with all donor cells (Fig. 1). Similar observations were reported in mice with ADSCs prepared from subcutaneous or inguinal fat pads [10]. Thus, the yield of ADSC myogenic differentiation is obviously lower than that observed for adipogenic or osteogenic differentiation.

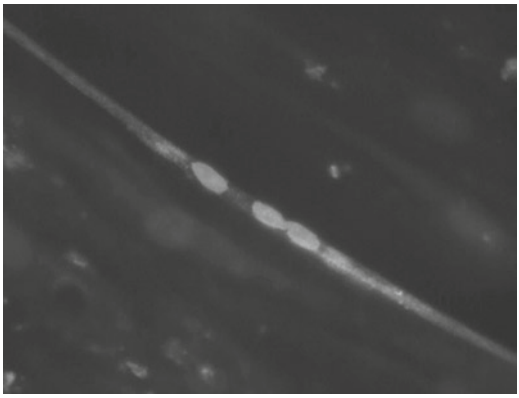

Fig. 1 Autonomous myogenic differentiation of human multipotent adipose-derived stem (hMADS) cells. A rare myotube is shown among hMADS cells placed under myogenic culture conditions for 3 weeks. It exhibits a typical elongated form and contains several nuclei. The nuclei were stained with 4–,6-diamidino-2-phenylindole and the sarcolemma with an antidystrophin antibody. Dystrophin is a terminal marker of myogenic differentiation

3.2 Myogenic Potential of Adipose-Derived Stem Cells Cultured with Myoblasts

The low autonomous myogenic potential of ADSCs raised the question of the importance of a muscle environment to achieve a more complete myogenic differentiation. In vitro this question has been addressed through the coculture with myoblasts. Human or mouse ADSCs traced with green fluorescent protein (GFP) were mixed with C2C12 murine myoblasts [8] or mouse primary myoblasts [10] and cultured under myogenic differentiation conditions. GFP-positive myotubes were found in both experiments, and the human GFP-positive myotubes expressed human nestin, which is known to be significantly expressed in muscle cells. This indicated that ADSCs can fuse with muscle cells and contribute to myotube formation. In addition, Di Rocco et al. found that soluble factors secreted by differentiating myogenic cells were involved in ADSC myogenic differentiation [10].

The coculture experiments showed that ADSCs share with muscle cells the capacity to fuse into myotubes and then to express muscle proteins. However, the yield remained very low, at best in a 1% range of ADSCs integrated into myotubes. Moreover, it is difficult to conclude that the coculture with muscle cells quantitatively increases the level of myogenic differentiation, considering the measurement accuracy of very low percentages and the occurrence of a possible fraction of ADSCs undergoing nonsignificant fusion with myoblasts cells. Indeed, it cannot be completely excluded that a few nonmyogenic cells, physically in contact with several muscle cells, may be enrolled in the massive wave of fusion of differentiating muscle cells.

4 In Vivo Myogenic Potential of Adipose-Derived Stem Cells

The most physiologic way to assess the myogenic potential of ADSCs is to examine their capacity to contribute to muscle regeneration in vivo. Several studies reported an engraftment of ADSCs in muscles of animal recipients. Bacou et al. were the first to show that rabbit autologous adipose-SVF cells, freshly harvested and traced with LacZ, contributed to up to 10% of fibers of regenerating muscles that had been injured before the cell transfer [15]. Thus, they established that adipose SVF contains cells with an in vivo myogenic potential. Similarly, mouse ADSCs injected into *mdx* mouse muscles or mouse adductor muscles after femoral artery removal were respectively found in up to 10% and 20% of the recipient's muscle fibers [10]. The *mdx* mouse is routinely used as a DMD murine model since it is deficient in dystrophin and consequently subjected to strong muscle regeneration. It is interesting to note that only a few muscle fibers derived from mouse donor's cells were found in *mdx* mice injected with ADSCs after in vitro expansion [11]. Finally, human ADSCs were also found incorporated in regenerated mouse muscle fibers. This was observed in our laboratory with the hMADS cells engrafted in muscles from *mdx* and preinjured, immunodeficient mice [7] (Fig. 2) and with ADSCs prepared from liposuction procedures engrafted in muscles of the SJL mouse, a murine model for limb girdle muscular dystrophy type 2B [16]. Therefore, in the regenerating muscle environment, transplanted ADSCs exhibit a significant potential to differentiate into skeletal muscle fibers.

5 Cellular Origin of Myogenic Adipose-Derived Stem Cells

Although ADSCs represent a purified cellular fraction from fat tissue, they constitute a heterogeneous cell population including at least mast cells, endothelial cells, pericytes, fibroblasts, and preadipocytes [4]. Only hMADS cells are a long-term expanded cell population, which are likely less heterogeneous than fresh ADSCs. Very recently, investigations have been carried out to identify which cell type(s) hold the myogenic potential of ADSCs. They target cells from the perivascular compartment.

A CD45-negative fraction representing less than 1% of total number of mouse adipose SVF cells was found in the "side population" (SP) of fresh adipose SVF [17]. SP cells actively pump out the Hoechst 33342 dye through multidrug resistance transporters, and it has been suggested that high activity of these transporters correlates with cell stemness [18]. Of importance, the SP CD45-negative fraction expresses endothelial lineage markers such as Angpt2, VE-cadherin, endoglin, Flk1, CD31, and Vcam1. Fresh SP CD45-negative cells participated to myotube formation when cocultured with C2C12 myoblasts and to regenerated myofibers of preinjured mouse muscles, in both cases with a much better efficiency than total SVF cells. In humans, Liu et al. used the VEGF receptor 2 (Flk1) as an endothelial progenitor cell marker to sort the Flk1-positive SVF fraction [12]. They showed that this fraction, expanded

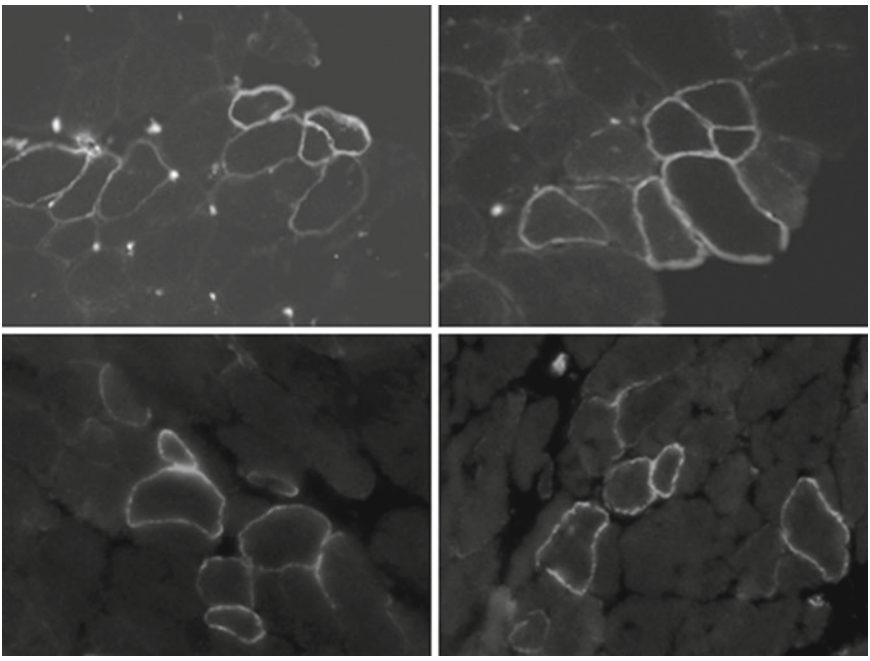

Fig. 2 Contribution of human multipotent adipose-derived stem (hMADS) cells to muscle regeneration. Approximately 500,000 hMADS cells were injected in the tibialis anterior muscles of *mdx* (*top*) and immunodeficient Rag2$^{-/-}\gamma C^{-/-}$ mice (*bottom*). Muscle regeneration is spontaneous in the *mdx* mouse and was triggered by cryoinjury in the Rag2$^{-/-}\gamma C^{-/-}$ mouse. Two weeks later, hMADS cell–derived regenerated muscle fibers were detected with a human-specific antidystrophin antibody in the *mdx* mouse. One month later, they were detected with a human-specific antispectrin antibody in the Rag2$^{-/-}\gamma C^{-/-}$ mouse. Like dystrophin, spectrin is expressed at the sarcolemma of muscle fibers

three passages in culture, is endowed with in vitro autonomous myogenic capacities as well as with the capacity to contribute to muscle regeneration in preinjured *mdx* or wild-type mouse muscles. Unfortunately, no quantitative comparison with total SVF cell population was reported in this study. Of interest, both the SP CD45$^-$ mouse cells and the Flk1$^+$ human cells were also recovered after transfer in blood vessel endothelium of recipient mice. It is also of interest that an endothelial origin for a subset of myogenic cells has also been reported within skeletal muscle [19].

Within the perivascular niche and besides the endothelial cells, it has also been demonstrated that the pericytes from human adipose as well as other tissues exhibit an active in vitro and in vivo myogenic differentiation potential [20]. Pericytes are mural cells found in both small and large vessels. They autonomously fuse into myotubes in vitro and contribute to muscle regeneration approximately four times more efficiently than myoblasts after injection in *mdx* or preinjured, regenerating mouse muscles from immunodeficient animals. Of interest, their expansion in culture retains their myogenic potential. These data are in accordance with the latest conclusions on

the pericyte origin of stem cells present in human adipose tissue [13,21]. Therefore, according to the most recent studies, ADSCs or MSCs in general could be, at least in part, vascular precursor stem cells at various stages of differentiation [22] or multipotent precursors derived from pericytes [23,24].

6 Genetic Modification of Adipose-Derived Stem Cells

From a potential clinical perspective, the identification of the most myogenic subpopulations of SVF cells opens the route for the improvement of muscle repair with ADSCs. However, the very small percentage of cells such as the perivascular precursors in the total SVF cell population raises practical difficulties, especially if these cells should be used without in vitro expansion as mentioned in some studies. A solution may be found in harvesting very large quantities of adipose tissue. We evaluated as another alternative the genetic modification of ADSCs to give them a higher myogenic potential.

We used hMADS cells and postulated that overexpression of a key myogenic gene such as MyoD, which is a master gene of embryonic myogenesis [25], may overcome their low myogenic potential. MyoD-hMADS cells were obtained by transduction with a recombinant HIV PGK-MyoD lentiviral vector encoding mouse MyoD [26]. MyoD-hMADS cells underwent spectacular modifications.

6.1 In Vitro Differentiation Potential of MyoD-Human Multipotent Adipose-Derived Stem Cells

Unlike wild-type- or LacZ-hMADS cells transduced with a PGK-nlsLacZ lentiviral vector and used as a negative control, MyoD-hMADS cells cultured under myogenic conditions formed multinuclear myotubes and expressed early and late differentiation myogenic markers to the same extent as genuine myoblasts (Fig. 3). Cocultured with DMD myoblasts, they fused into DMD-hMADS cell hybrid myotubes and restored the expression of dystrophin, the lacking protein in DMD. This capacity of fusion with myoblasts is a characteristic of muscle cells, and the expression of dystrophin from the hMADS cell genome indicates that the cellular fusion did not disturb the myogenic differentiation program of DMD myoblasts, which is a necessary condition for potential clinical use.

An important issue with multipotent cells is the risk of differentiation into undesirable lineages. Moreover, in many muscle dystrophies, muscles are infiltrated by fat cells, which aggravate muscle dysfunction and create an adipogenic environment. It was therefore crucial to investigate the adipogenic differentiation potential of MyoD-hMADS cells. In fact, the strong adipogenic differentiation potential of hMADS cells was inhibited by the forced expression of MyoD. Together, these results showed that MyoD-hMADS cells may have a promising in vivo myogenic potential.

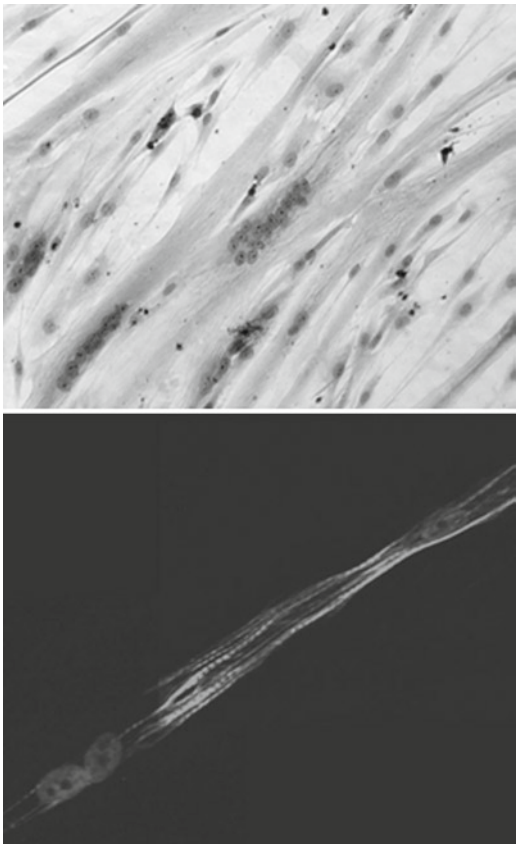

Fig. 3 In vitro myogenic differentiation of human multipotent adipose-derived stem (hMADS) cells forced by MyoD expression. Unlike wild-type hMADS cells, MyoD-hMADS cells fuse very efficiently into myotubes when cultured under myogenic conditions **(top)**. Many multinucleated myotubes are formed, and a field of a culture dish is visualized after eosin–hematoxylin staining. These myotubes expressed myogenic markers, as shown with an antimyosin antibody staining the striated myofibers **(bottom)**. Nuclei were detected with 4–,6-diamidino-2-phenylindole labeling in the lower panel

6.2 MyoD-Human Multipotent Adipose-Derived Stem Cells Contribute to Muscle Repair In Vivo

To assess the effect of MyoD expression in hMADS cells, the contributions of wild-type-, LacZ-, and MyoD-hMADS to muscle repair were compared after cell transfer in regenerating muscles of $Rag2^{-/-}\gamma C^{-/-}$ immunodeficient mice. One month later, hMADS cell–derived nuclei were examined with a human-specific anti–lamin A/C antibody. Positive nuclei were found either scattered within or among the muscle fibers or in clusters between fibers, showing that hMADS cells survived in the mouse muscles in different locations (Fig. 4a, b). Much more MyoD-hMADS cell–derived nuclei than wild-type- or LacZ-hMADS cell–derived nuclei were found integrated in muscle fibers. Serial sections showed that human nuclei were always detected among mouse nuclei within the same fiber, indicating that hMADS cells fused with mouse regenerating fibers, as could be expected from the *in vitro* results.

Then the presence of hMADS cell–derived fibers was studied by the expression of human-specific muscle markers. Human spectrin, which is expressed at

the sarcolemma of muscle fibers, was clearly detected at the membrane of many fibers, always located in regenerated regions displaying human lamin A/C–positive nuclei (Fig. 4b). The human spectrin labeling was used to quantify the in vivo effect of modification of hMADS cells by MyoD. The total number of human spectrin–positive fibers was about five times higher with MyoD-hMADS cells than with wild-type- or LacZ-hMADS cells (Fig. 4c). The expression of several other human muscle markers confirmed the presence of hMADS cell–derived muscle fibers.

Therefore MyoD expression in hMADS cells enhances their engraftment in regenerating muscles and their contribution to muscle repair. This property is now under investigation in animal models of dystrophic muscles to allow a comparison with the most efficient cells used in this field, namely the vessel-derived mesoangioblasts and the peripheral blood AC133^{+} cells [27, 28].

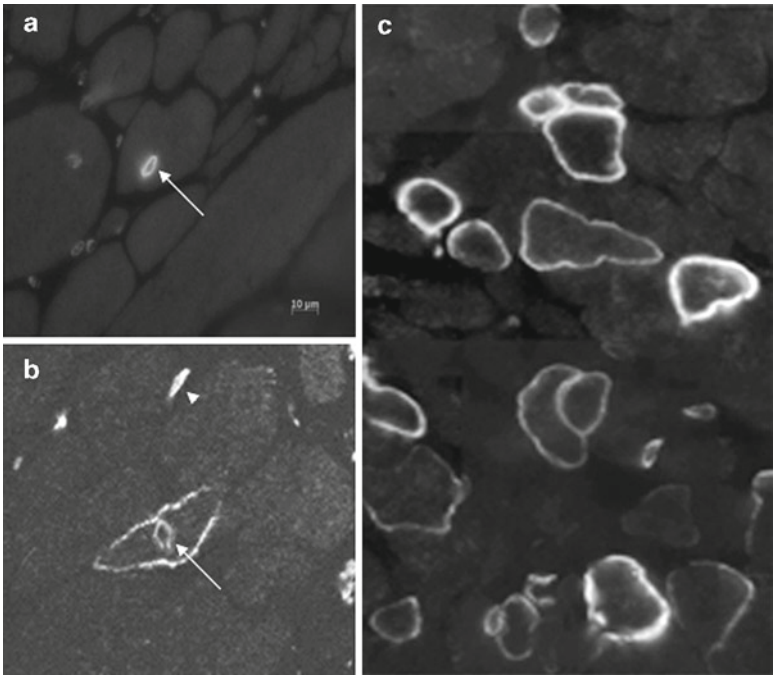

Fig. 4 In vivo muscle repair with MyoD-human multipotent adipose-derived stem (hMADS) cells. Approximately 500,000 MyoD-hMADS cells were injected in the cryoinjured tibialis anterior muscle of an immunodeficient Rag2$^{-/-}\gamma C^{-/-}$ mouse. The contribution of injected cells to muscle regeneration was studied 1 month later. hMADS cell–derived nuclei could be found either integrated in the mouse muscle fibers (**a**, **b**, *arrows*) or in the interstitial spaces between muscle fibers (**b**, *arrowhead*), as detected with a human-specific anti–lamin A/C antibody. In panel A, mouse nuclei are stained with 4–,6-diamidino-2-phenylindole only. hMADS cell–derived fibers were examined with a human-specific antispectrin antibody (**b**, **c**). Note in panel C the large extent of human spectrin–positive fibers found with MyoD-hMADS cells in comparison with wild-type hMADS cells (Fig. 2, bottom)

7 Conclusions

ADSCs are abundant adult stem cells that exhibit a myogenic differentiation potential lower than the adipogenic, osteogenic, or chondrogenic potential. The physiologic importance of this plasticity remains to be elucidated, but it is conceivable that better knowledge may potentially lead to their use in muscle dystrophy therapy. Since they are very efficiently transduced with lentiviral vectors, they can be engineered to express a corrected gene and therefore used in autologous cell-based treatments. The main limitation is the extent of their myogenic differentiation capacities. In this chapter we showed that two options have already been investigated. The first alternative is the identification of an ADSC fraction that would express a stronger potential than the whole population. To date, at least the relatively rare perivascular cells are very good candidates. The second alternative is the genetic modification of ADSCs with a key myogenic gene. MyoD has been used, but other genes, such as Pax3, should be tested [29]. At this point, the level of contribution to regeneration of muscle recipient remains to be assessed in dystrophic animal models.

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Regeneration of Sensory Cells of Adult Mammalian Inner Ear

Dongguang Wei and Ebenezer N. Yamoah

Abstract Irreversible loss of hair cells and their innervating spiral ganglion neurons is the major reason for hearing loss. Attempts at integrating new supplementary cell sources into the damaged inner ear have been tested extensively. This chapter reviews the history of available cell sources and their achievements, limitations, and future developments for hearing rehabilitation. It addressed issues regarding the "self-repair" of mammalian inner ear sensory epithelium, including (1) recruitment of the in situ proliferation and differentiation of endogenous cells at the damaged site and (2) autologous transplantation, which offer new optimism for helping hearing-impaired individuals.

Keywords Stem cells • Inner ear • Sensory epithelium • Rehabilitation

1 Introduction

Hearing loss presents a significant social and economic burden. More than three million people in the United States suffer from hearing loss, which is commonly associated with irreversible loss of hair cells (HCs) and their innervating spiral ganglion neurons (SGNs).

Hearing-impaired individuals find themselves cut off from social intercourse, leading to reductions in their quality of life. Attempts at integrating new supplementary cell sources into the damaged inner ear have been tested extensively. In this chapter, we introduce cell sources available for hearing rehabilitation. We describe the history of each cell source, along with its achievements, limitations, and future

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developments. The use of multiple classes of progenitor and stem cells is emphasized, which offer reasons for new optimism for hearing-impaired individuals.

2 Stratagems for Regenerating Sensory Cells of Adult Mammalian Inner Ear

Regeneration of mammalian inner ear sensory cells via cell-based therapy is becoming a widespread therapeutic modality. The goal of cell replacement therapy is to develop biologically competent substitutes that can restore and maintain lost or deficient functions. Regeneration of damaged inner ear HCs and/or SGNs presents a great challenge for hearing restoration because these two inner ear sensory cells do not regenerate after damage. The two major stratagems for cell replacement therapy are (1) transplantation therapy, using cells derived from autogenic, allogeneic, or xenogeneic sources, and (2) autorepair therapy, in which the remaining host cells and the endogenous stem or progenitor cells are mobilized to enhance repair. Transplantation therapy has been practiced for years. However, autorepair therapy of the inner ear is a new concept.

2.1 Advanced Therapy Depends on Better Understanding of the Fate of Inner Ear Sensory Cells

Although technical advances have considerably enriched our understanding of the general pathways that direct inner ear sensory cells toward the right fate, significant steps forward in hearing rehabilitation depend on advances in the ability to manipulate the fate of inner ear sensory cells, which in turn depends on a better understanding of development and regulation of the fate of inner ear sensory cells [1–3]. We thus highlight some recent work on this topic.

The retinoblastoma (Rb) pathway regulates the transition from the proliferating stage to the postmitotic stage of the mouse inner ear [4]. Hence, deletion of Rb leads to overproduction of hair cells [5]. Notch signaling is involved in prosensory path formation and the lateral inhibition–mediated differentiation of hair cells [6]. Loss of Notch signaling leads to an increase in the size of prosensory patches. Moreover, the basic helix-loop-helix transcription factor Atoh1 (Math1) was demonstrated to play a key role in hair cell differentiation during development [7, 8]. Of interest, expression of Math1 alone in postnatal and adult individuals enhances ectopic hair cell formation near the organ of Corti [9,10]. More recent research confirmed that putative hair cells induced by Atoh1 misexpression could generate functional hair cells, which highlights the possibility of gene therapy for ameliorating hearing loss [11]. Mammalian postmitotic supporting cells have recently been recognized as a potential target for therapeutic manipulation. These cells can reenter the cell cycle and transdifferentiate into new HCs in culture, and their

proliferative capacity depends, in part, on the activity of cyclin-dependent kinase inhibitor $p27^{kip1}$ [12]. Cross-regulation of Ngn1 and Math1 coordinates the production of neurons and sensory hair cells during inner ear development [13]. Therefore, manipulation of cell cycle regulatory genes and the key genes for HC commitment is another potential avenue for regenerating HCs within deaf auditory epithelium.

2.2 Transplantation Therapy

Transplantation therapies generally fall into two main categories: (1) allogeneic cells for short-term structural repair or restoration of physiologic function and (2) autologous cells for long-term structural repair and functional rehabilitation. Different cell sources have been tested to evaluate therapeutic approaches. The most commonly tested cellular paradigm uses various stem cells and fetal and adult cells of sensorineural origin. The use of these cells for hearing restoration raises a number of questions, including cell survival, differentiation, and biodistribution within the cochlea. For future effective transplantation therapy, it is necessary to understand the cellular mechanisms involved.

2.2.1 Allogeneic Cell Transplantation

Cell-based therapy using allogeneic and/or xenologous cells has long been tested in various applications. Implantation of allogeneic cell replacements has the potential to provide new essential HCs and to prevent the loss of SGNs before secondary degeneration of these neurons results in profound hearing loss. This stratagem has been extensively studied for more than 10 years in various animal models, including guinea pig, rat, mouse, and chinchilla, and some effective operating models has also been established [14]. However, future studies may be required before application in large-animal models and ultimately humans.

Embryonic Stem Cells

Embryonic stem cells (ESCs) are prepared from the inner cell mass of early embryos and are capable of indefinite in vitro self-renewal while maintaining their potential to develop into all cell lineages of the body. Ever since the first isolation of pluripotent stem cells from human embryos [15], stem cell research has been a topic of headlines in the United States. Although ESCs hold great promise in both regenerative medicine and basic biologic research, it, like other new human health care–related technologies, has raised many legal and ethical concerns, and it is subject to a number of policies and laws at the state and federal levels.

Unlike most adult stem cells (ASCs), ESCs are broadly pluripotent and can be expanded almost indefinitely to provide an enormous cell replacement supply.

This great degree of plasticity may turn into a major limitation in the use of ESCs unless pretransplantation education and/or adjustment of phenotypes are done. Appropriate pretransplantation education of ESCs will also provide a better match between the grafted elements and local functional circuits. A major challenge of this protocol is the efficient production of pure populations of HCs from ESCs. Recapitulation of the sequential differentiation steps during development is required to drive ESCs toward the HC lineage. However, given our limited understanding of this sequence and the fact that ESCs are still many steps away from mature HCs, the challenge remains to be resolved.

As opposed to earlier presumptions, differentiated ESCs express major histocompatibility complex class I antigens, and thus immune rejection is another major concern.

Evaluation of the possible contribution of ESCs to restore hearing is in its infancy. Small numbers of mouse ESCs were capable of survival in the deafened mammalian cochlea for up to 4 weeks without causing an inflammatory tissue response [16]. A stepwise in vitro exposure of murine ESCs to a set of regulatory factors could routinely create inner ear progenitors, which eventually differentiated into a specific cell type with defining molecular and structural characteristics of HCs from ESCs [17]. Future research needs to focus on developing more clinical paradigms and functional evaluations of newly differentiated cells in the cochlea.

Stem Cells from Amniotic Fluid and Placenta

Recent research has demonstrated the presence of stem cells with multiple potential in the amniotic fluid and placenta, and these they are now recognized as potential sources of stem cell therapy. Amniocentesis and chorionic villus sampling are widely accepted methods for prenatal diagnosis. Therefore, no ethical concerns are likely to impede progress if embryonic and fetal stem cells are taken from amniotic fluid and placenta before or at birth. Isolated human and rodent amniotic fluid–derived stem (AFS) cells express embryonic and ASC markers. Undifferentiated AFS cells expand extensively without feeders. Of interest, AFS cells are broadly multipotent. They can differentiate into cell types representing each germ layer, including neuronal, osteogenic, and hepatic phenotypes [18]. The demonstrated neuronal lineage indicates that this AFS could be used potentially to repopulate inner ear sensory cells. Moreover, banking these stem cells may provide a convenient source both for autologous therapy in later life and for the matching of histocompatible donor cells with recipients.

Fetal/Neonatal Cells and Fetal/Neonatal-Derived Stem Cells

There is an urgent need for a viable and functional cell source for many patients at the end stage of organ failure. In this regard, use of fetal and/or neonatal cells represents a possible approach to treating diseases that currently have no imminent

solutions. Although many individuals are willing to accept the use of cells from viable fetal tissues, opposing political and ethical views have stymied progress in this field. Despite the successful transplantation of primary fetal/neonatal cells and fetal/neonatal-derived stem cells into the cochlea, their widespread clinical application could be limited due to unresolved ethical and moral issues regarding the use of fetal tissues [19]. Thus, much effort has been devoted to other neuronal lineage sources.

Adult Stem Cells

Despite the promise of ESCs, controlled lineage commitment and elimination of residual ESCs are both time consuming and of low efficiency. ASCs have been identified in many tissues and have entered the public consciousness as a less controversial alternative to embryonic or neonatal cells. They are arrested in a preterminal differentiation phase of their developmental program within their tissues of residence, and most of them are hard to harvest and expand. However, they may provide a more direct route to clinical translation and could be used for bioengineered products. The most favorable ASCs tested for hearing repair are bone marrow–derived stem cells [20, 21].

Neural Stem Cells

Neurodegenerative diseases are prominent targets for stem cell therapy in general and for neural stem cells (NSCs) in particular. NSCs are capable of self-renewal and multidifferentiation and are believed to give rise to the vast array of various cell types of the nervous system. Transplantation of neural lineage–committed NSCs is a possible approach for ameliorating hearing deficits. They are a promising source of a virtually unlimited supply of sensorineural enriched cells. The NSCs could improve hearing through several different mechanisms: (1) their intrinsic propensity to supply neurotrophic/neuroprotective molecules and homeostasis-promoting agents that directly benefit host neuronal functioning [22]; (2) differentiation into SGN-like neurons [23, 24]; and (3) integration into Rosenthal's canal [23] and reestablishment of local interneuronal or neuron–hair cell connections [25]. Although it is generally believed that functional rewiring through grafted cells over a long distance is less likely, successfully integrated cells may use Rosenthal's canal as a regenerative highway to precisely guide the rewiring fibers to HCs.

Replacement of Auditory Neurons

Although the existence of progenitor cells in adult spiral ganglia has been demonstrated both in humans and in adult mice [26, 27], primary and secondary damage to auditory neurons does not readily elicit a corresponding proliferation of these

endogenous progenitor cells. On the other hand, advances in hair cell regeneration need progress in neuronal protection [28, 29] and replacement to connect new hair cells [10, 25]. Attempts to generate new auditory neurons from various exogenous cell sources have been reported with considerable success [30–32].

Recent reports have demonstrated innervation of new auditory neurons into HCs [25, 33, 34], and the synaptic coupling between HCs and their innervated new auditory neurons has suggested that these newly established innervations can eventually be established into functional synapses [25]. The upkeep of these new auditory neurons requires trophic inputs. Cografts of essential trophic factors may arrest the degenerative process. Since loss of HCs is the primary reason for peripheral hearing impairment, implantation of new auditory neurons alone will not be enough when HCs are not readily available. In this case, the combination of neural replacement therapy and cochlear prostheses appears as a reasonable alternative. The grafted cells will supplement the failing SGNs, and the coimplanted cochlear prostheses will bypass the absent HCs and directly stimulate the afferent neural branches projecting to the higher auditory perception center. Since it has been demonstrated that deafferented SGNs can establish synapse-like contacts with each other [26] and stem cell–derived auditory neurons can functionally rewire deafferented SGNs [25], the grafted auditory neurons may serve as a network for incorporating and retaining host SGNs. In addition, the use of cochlear prostheses and new auditory neurons as a working group may significantly enhance their efficiency.

To provide a better functional bridge between HCs and the brainstem nuclei, the efficacy of different graft routes has also been tested. The Scala tympani route is expected to enhance the function of a cografted cochlear prosthesis, which is implanted via the same route. This route has now been enriched through multiple alternatives; a new technique has been established to deliver cells to the internal auditory meatal portion of an auditory nerve without injuring the endolymphatic and perilymphatic chambers. Therefore, this technique could significantly reduce surgical trauma during cell transplantation [35]. It also provides a more controlled model for analysis of the potential for cell transplantation into the inner ear. Neural progenitor cells injected into the cochlear nerve trunk [30] sent out neurites into fasciculating bundles projecting through Rosenthal's canal, and ultimately into the organ of Corti, where they contacted hair cells. This technology revealed the potential of replacing auditory neurons lost to primary neuronal degeneration.

2.2.2 Autologous Transplantation

Autologous transplantation is a widely accepted and approved technique in regenerative medicine. It can be subdivided into (1) direct biopsy, taken from tissue that is the same as the engineered tissue type, and (2) indirect biopsy, taken from a similar tissue when a direct biopsy would cause considerable injury. Autologous transplantation has advantages that include lack of immune rejection and disease-free transmission from donor to recipient. The successful application of this stratagem to the inner ear needs the identification of new autologous cell sources. In addition to the traditional bone marrow–derived cells, ongoing efforts to evaluate

new cell sources for cell replacement therapy include ependymal cells, induced pluripotent stem (iPS) cells, and olfactory sheath cells. One of the major disadvantages of autologous transplantation is the limitation in the amount of available tissue.

Ependymal Cells

A recent study [25] reported an extensive analysis using structural, molecular, and functional criteria to demonstrate that adult brain germinal zone cells and ependymal cells, derived from the same neural ectodermal layer as the otic vesicle cells, have characteristic features of inner ear HCs. The ependymal layer is a polarized epithelium with actin-based stereocilia and microtubule-based kinocilia cells, similar to vestibular HCs. They express defining HC markers such as myosin VIIa, myosin VI, and CtBP2/RIBEYE. These cells have similar large conductance and nonselective ion channels, and they take up the styryl dye FM1-43 that permeates hair cell transduction channels. In addition, these cells integrated into the cochlear sensory epithelium and demonstrated the electrophysiologic characteristics of HCs, that is, they are electrically active, send synaptic input to SGNs, and release glutamate as their neurotransmitter. The in vivo and in vitro proliferative capacity suggests that ependymal cells can be used in autologous replacement of nonrenewable HCs. Further examination of the differences between the proliferative capacity of ependymal cells and the quiescence of hair cells may make ependymal cells a valuable comparative model for understanding some basic biologic questions. Clinical application of this cell type depends on further evaluation of their performance in animal models. Nonetheless, as a tool for understanding the basic science of cell replacement in the inner ear, this work represents an important step forward [36]. Understanding of the mechanisms of functional plasticity may open a new window for cell replacement therapy.

Olfactory Progenitors

The olfactory neuroepithelium in the mammalian nervous system is capable of generating new olfactory receptor neurons periodically throughout life. It also has the capacity to proliferate in response to acute injury. This unique proliferative feature results from the presence of multipotent stem cells in the olfactory neuroepithelium [37]. The in vitro differentiation of adult mouse olfactory precursor cells into hair cells has been reported, and thus these hair cell-like cells represent a potential autotransplantation therapy for hearing loss [38]. Precursor cells from mouse olfactory neuroepithelium were sphere forming and showed proliferative capacity. After coculturing with cochlear cells and/or cochlear supernatant, the olfactory progenitor cells expressed some hair cell markers, including myosin VIIa, calretinin, and espin. The results demonstrated for the first time that adult olfactory precursor cells could differentiate into hair cell–like cells. The olfactory neuroepithelium offers an abundant and easily accessible source of ASCs, which could be a

potential autotransplantation therapy for hearing loss. However, the functional potential of this source remains to be addressed.

Induced Pluripotent Stem Cells

One unavoidable issue accompanying the transplantation stratagem is immune rejection. A successful allograft is accompanied by the pitfalls of the use of lifetime immunosuppression. An attractive solution to both the shortage of reproducible cells and immune rejection is the use of autologous sources of stem cells, which are collected from the same patient. Several recent important reports confirmed that terminally differentiated somatic cells can acquire properties similar to those of ESCs after activation of a set of stem cell genes [39–41]. The reprogrammed cells are called iPS cells. The iPS cells demonstrate striking similarities with ESCs in morphology, proliferation, expression of marker genes, and multiple differentiations.

The unique features of iPS cells give them additional advantages for cell replacement therapy. First, since iPS cells can be generated from the patient, the issue of immune rejection is essentially overcome, and second, the application of somatic cell–derived iPS cells is free of ethical concerns regarding the use of human embryos. The potential of iPS cells is enormous. The iPS stratagem has been growing exponentially, and numerous iPS lines have been generated. Several iPS cell–derived somatic cell types are being evaluated for regenerative medicine and pharmaceutical application [ref]. The probability of teratoma formation calls for pregraft differentiation of iPS cells. Progress in iPS techniques and better understanding of the manipulation of HC differentiation may eventually enable us to perform a “space-time switch” to precisely reverse the retained HCs or supporting cells in the deafened ear to the unique embryonic stage so they can behave as proliferative progenitors with the committed lineage. The promise of silencing transgenes in the iPS cell genome is necessary to eliminate a greater risk of immature teratoma formation [42].

Bone Marrow–Derived Cells

Mesenchymal stem cells (MSCs) from bone marrow have been reported to differentiate into multiple lineages and have long been evaluated in clinical practice. An in vitro overexpression of the prosensory transcription factor Math1 in MSCs induces the MSCs into inner ear sensory cells that express myosin VIIa, espin, Brn3c, p27Kip, and jagged2. Some of the daughter cells demonstrate F-actin–positive protrusions, a structural analog of HC stereociliary bundles. Hair cell markers were also induced in a culture of mouse MSC-derived cells in contact with embryonic chick inner ear cells, and this induction was not due to a cell fusion event [20]. An in vivo evaluation demonstrated the homing capability of bone marrow-derived cells to the deafened cochlea, and these cells displayed mature hematopoietic properties. However, spontaneous transdifferentiation to any cochlea cell types after acoustic trauma was not observed [21]. These pilot studies suggest that education or predifferentiation of bone marrow–derived cells is required to achieve possible hearing rehabilitation.

2.3 *Self-Repair Therapy*

2.3.1 In Situ Proliferation of Endogenous Inner Ear Stem/Progenitor Cells

Unlike mammals, birds and amphibians are capable of regenerating inner ear HCs throughout life [43, 44]. In the mature mammalian inner ear, only the vestibular system can regenerate HCs upon damage [45, 46]. However, the mature mammalian cochlear system does not spontaneously regenerate HCs after damage. Grafting to repopulate the lost HCs will be challenged by the difficulty of in situ integration of grafted cells to fill vacant HCs. Functional restoration may be achieved by in situ proliferation and differentiation of endogenous stem cells. Tissue-specific stem and/or progenitor cells have been isolated and characterized from different tissues. The search for bona fide stem or progenitor cells within the mature mammalian inner ear was encourage by a recent ground-breaking study [47] in which pluripotent stem cells were identified from the adult mammalian vestibular sensory epithelium. The discovery of this true stem cell in the mature mouse inner ear justifies the use of in situ self-repair therapy. Selectively promoted in situ proliferation and proper differentiation of quiescent endogenous stem cells may offer a reconstruction of damaged structure and restoration of the failing inner ear. Characterization of this stem cell niche will help to establish a more specific and predictable program for native stem cell expansion. Identification of guiding molecules of each key differentiation stage will contribute to a better control of cell lineage commitment.

2.3.2 Conversion of Supporting Cells into Hair Cells Without Mitosis

Previous research established that adult cells could be epigenetically reprogrammed into another closely related phenotype without cell division. Although the molecular mechanisms underlying this phenotypic plasticity need further investigation, this phenotypic switch has been achieved by terminally differentiated supporting cells, a cell type that not only provides structural support for the HCs, but also can be induced to undergo a phenotypic switch to HCs. The transcription factor Math1, a key factor of hair cell differentiation, is essential for this phenotypic conversion. Not only is overexpression of Math1 sufficient to induce embryonic ectopic supporting cells into HCs, but also the terminal differentiated supporting cells within the mature organ of Corti can be transdifferentiated into HCs. This striking phenotypic switch has been confirmed by different research groups, and a pilot in vivo study even demonstrated a partially functional restoration [9, 10, 48, 49]. The common developmental lineage of supporting cells and hair cells may be the mechanism underlying this phenotypic switch. The major problem of this stratagem is that phenotypic conversion without mitosis leads to a reduced number of supporting cells, and the forced phenotypic conversion stratagem is only effective within a limited time window following ototoxic insult [50].

2.3.3 Generating New Hair Cells from Terminal Deafened Sensory Epithelium

To retrieve without destruction is the gold criterion of regenerative medicine. The goal in using cell replacement therapies in hearing loss is to repopulate damaged inner ear sensory cells in an appropriate organotypic manner. HCs die upon ototoxic insult. Eventually the remaining supporting cells also disappear, leaving only a layer of unfeasible flat epithelium. One potential concern associated with phenotypic conversion is the reduction in the number of supporting cells. Thus, a combined therapy of cell cycle reentry and phenotypic conversion in the deafened-ear epithelium may improve both structural and functional outcomes. To date, putative endogenous stem cells have only been identified in mature mice vestibular organs, and although stem cells have been found in neonatal auditory sensory epithelium [51, 52], similar endogenous stem cells in adult auditory epithelium have not been identified. Since the mature mammalian auditory sensory epithelium does not spontaneously turn on a regenerative response after damage, it may prove essential to temporally provide the additional regenerative and inductive signals to the damaged site. However, the supply of regenerative signals must be within a limited time window; otherwise, the regenerative attempt will end up with tumor formation.

The coxsackievirus and adenovirus receptor is widely expressed on many cell types, including the auditory epithelium. This makes the use of adenoviruses a powerful tool for delivering regenerative and inductive signals to the damaged site [53, 54]. Adenoviruses do not integrate into the host genome, and production of exogenous proliferative signals by the replication-deficient adenoviral vectors can only be maintained for several days. In addition, the cochlea is isolated from the surrounding tissues by the bony capsule, which limits the spread of the viral vectors into neighboring tissues. The molecular and anatomic features just mentioned should lead to a particularly effective approach in the proliferative replacement of HCs within deaf auditory sensory epithelium: sequential administration of adenoviral vectors carrying key cell cycle effectors (i.e., cyclin-dependent kinase) or inhibitors of cell cycle suppressor may recapitulate cell proliferation.

3 Perspectives on Future Research

Cell therapy holds the potential to effectively leverage nature's abilities into new hopes for patients with intractable diseases, although many issues remain to be addressed before novel cell therapy stratagems can become bedside protocols. Current rapid development of various hearing rehabilitation approaches have blossomed from advances in understanding in basic and clinical science. The integration of new ideas from molecular biologists, neural cell biologists, electro-bioengineers, and neurosurgeons will lead to future models of hearing restoration.

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Stem Cells and Their Use in Skeletal Tissue Repair

Laura Baumgartner, Vuk Savkovic, Susanne Trettner, Colette Martin, and Nicole I. zur Nieden

Abstract Bone has a limited capacity to repair injuries through remodeling. The main integral cellular parts of bone in this process are the bone-forming osteoblasts, which are surrounded by osteoclasts. The osteoclasts in turn remove the old bone matrix. Consequently, these two cell types essentially maintain the structural integrity and the metabolic capacity of the skeleton. Compromised osteoprogenitor function can thus subsequently lead to osteodegenerative diseases, including osteoarthritis, osteoporosis, and many more. This leads to a substantial problem in the public health sector. Several research groups have made remarkable efforts toward improving bone repair through the transfer of allogeneic or autologous tissue and through cell-based therapy, as both regenerate the area of bone loss with cells possessing osteogenic potential. This chapter concentrates on describing the clinical use of stem cells, specifically of adult mesenchymal and embryonic stem cells, as cellular sources for the treatment of osteodegenerative diseases.

Keywords Stem cells • Regenerative medicine • Osteodegenerative diseases • Bone development

1 Introduction

In the healthy body, a substantial part of the natural restoration of tissue structure and function within vertebrates is achieved by the activation of quiescent adult stem cells, which proliferate and differentiate to regenerate and repair the damaged area [1–3]. However, normal tissue function diminishes with increased age and can be

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further jeopardized by the onset of disease or injury. Progressive deterioration and loss of function of an organ or tissue in which this repair process is malfunctioning renders an irreversible loss of normal function and causes a diseased state, which is typically described with the adjective “degenerative.” The end consequences of the lack of restoration capacity are complete malfunction of the affected system and typically life-long disability, which can frequently lead to death. Unfortunately, effective cures for most degenerative diseases do not yet exist. Today, the treatment of degenerative diseases, among them the osteodegenerative diseases, focuses on symptomatic relief, which is mostly achieved through the administration of pharmaceuticals. Stem cells remain one of the truly promising solutions to fully heal such disorders in the future and truly cure the underlying cause of the malfunction. The types of stem cells with potential clinical use in the orthopedic field are discussed here, and the progression of these cells into possible clinical use is laid out.

2 Osteodegenerative Diseases

Mature osteoblasts, the bone-forming cells, secrete an extracellular matrix (ECM) that contributes to the characteristic mechanical properties of these tissues. The ECM of bone is inhabited by osteoblasts (which originate from either the mesoderm or the neural crest ectoderm), hematopoietic osteoclasts, and chondrocytes. Bone-lining cells periodically stimulate bone resorption by osteoclasts, and as a result, bone tissue as a whole is continually being modified through resorption/regeneration cycles [4, 5].

One substantial problem in the current public health sector is the number of patients affected by osteodegenerative diseases, including osteoarthritis and osteoporosis, in which the ratio in the function of osteoblasts and osteoclasts is disturbed. In osteoporosis, the mineral density of the bone is reduced and the osseous microarchitecture is altered. Furthermore, a change occurs with regard to the amount and diversity of noncollagenous proteins in bone, and therefore the risk of bone fractures increases due to bone fragility. While osteoporosis had primarily been considered to be a disease of the elderly [6], in recent years, awareness of osteoporosis in children and adolescents has increased [7]. The underlying condition can be undetectably present for decades without showing any symptoms, as there are no obvious symptoms for osteoporosis unless the bone fractures.

Osteoarthritis (OA), a noninflammatory joint disorder, is the most prevalent form of arthritis and affects approximately 27 million adults in the United States [8, 9]. The clinical picture primarily involves the degradation of joints, including articular cartilage, but also the subchondral bone next to it. The course of this disease progresses slowly, occurring over several years. The development of OA follows a multifarious pathway with gradually evolving changes in bone matrix composition, such as alteration in the water content, degradation of cartilage matrix components, collagen fibers, and glycosaminoglycans [9]. As the disease progresses, attack-like symptoms with joint pain, increasing restriction in joint mobility, and a reduced ability to walk occur more frequently.

Although osteoarthritis and osteoporosis are the best known of the osteodegenerative diseases, there are many more that are not as common but equally devastating, some of which will be discussed here in order to put current treatment modalities and the potential for stem cell therapy into context. Another osteodegenerative disorder involving joints and bones is Paget disease of bone (PDB), which is characterized by increased, highly distinctive, and permanent disorganized bone remodeling, causing hypertrophy and abnormal bone structure [10]. The typical clinical symptoms of this bone disease are bone pain and skeletal deformities and fractures, but they may also include hearing loss. Genetic factors play an important role in PDB. This is evident by the mutations or polymorphisms that have been identified in four genes associated with the RANK/NFκB signaling pathway that cause classic and related syndromes [11].

Osteogenesis imperfecta (OI), also referred to as "brittle-bone disease," is a genetic disorder of mesenchymal cells characterized by defective collagen type I, which is the major structural protein in bone and is vital for bone flexibility and strength. This heritable disorder affects around 1 in 12,000 people [12], although the clinical expression profile varies widely. In the mildest form of the disease, fractures occur occasionally before puberty, deformity is minimal, and stature is normal. In its most severe forms, the symptoms can be seen prenatally, as fractures of the ribcage often occur in utero, leading to pulmonary insufficiency and death [13].

Osteopetrosis, also known as marble bone disease or Albers-Schonberg disease, is possibly the rarest of the hereditary osteodegenerative diseases, with an incidence of 1 in 100,000–500,000 [14]. This disease is characterized by impaired osteoclast function that leads to an increase in bone density, in contrast to the more prevalent osteomalacia, in which the bones soften. Due to the scattered accumulation of bone tissue, the osseous microarchitecture is perturbed, followed by a decrease in mechanical stability [15].

3 Treatment Methods: State of the Art

For the majority of cases, current treatments for the described osteodegenerative diseases include physiotherapy, medication, and surgical procedures. In case of OI, for instance, surgeons widely use a procedure known as the rodding technique, which involves internal splinting of the long bones by inserting a metal rod into affected bones of the patients, mostly the femur or the tibia. However, the bones so treated typically remain fragile [12]. Another treatment modality is the use of bisphosphonates, a potent class of antiosteoclastic agents, which have been reported to have beneficial effects in patients with osteogenesis imperfecta as well as with Paget disease [16, 17]. In addition, a variety of agents, including anabolic steroids, sodium fluoride, magnesium oxide, and calcitonin, alone or in combination, have been used in attempts to increase bone mass and to reduce the risk of fracture. However, almost all of these treatments have shown limited effectiveness in randomized, controlled clinical trials [16].

For the treatment of all osteodegenerative diseases with an increased susceptibility to bone fractures, such as osteoporosis, the aim is to increase bone stability and to minimize the risk of bone fractures. In order to achieve this, drug treatments with appropriate calcium and vitamin D supplementation as well as osteoanabolic substances should be combined with antipain medication and rehabilitation [18].

Notably, none of the conventional treatments are long term or address the underlying causes. Given the limitations of current treatments, several innovative therapeutic approaches are being explored. For instance, allogeneic bone marrow transplantation was applied in OI patients to engraft and contribute to the formation of new dense bone [17]. In addition, stem cells from other origins may present a unique and promising source for such forms of regenerative therapy. For osteodegenerative bone diseases there has been a particular focus on the use of embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), mesenchymal progenitor cells, and marrow stromal cells, all of which might give rise to, among other cell types, bone cells.

4 Types of Stem Cells

Apart from their different morphologic appearance (Fig. 1), MSCs and ESCs differ in a variety of other features, although both are characterized by the two hallmark features of stem cells: the capacity to self-renew and to give rise to specialized daughter

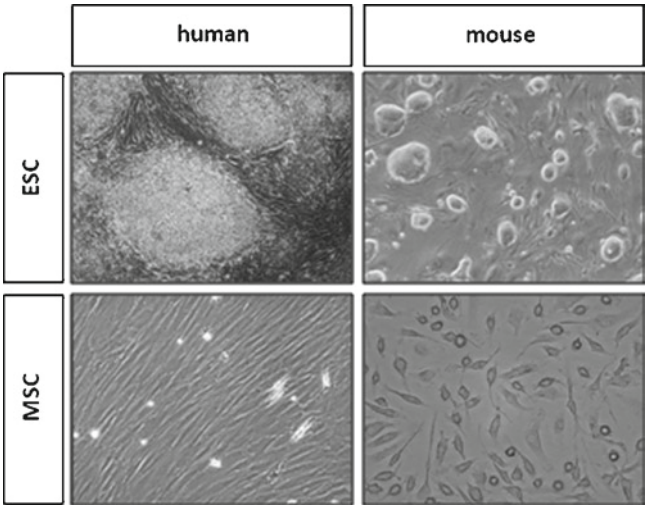

Fig. 1 Typical morphology found in undifferentiated human (CA-1) embryonic stem cells and murine embryonic stem cell (D3) colonies (ESCs), as well as human and murine primary bone marrow stem cells (MSCs)

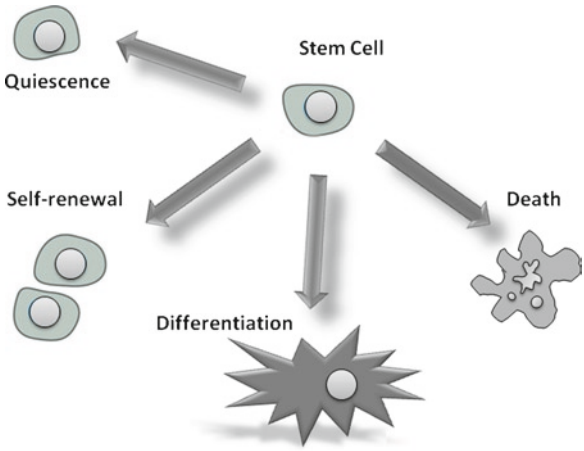

Fig. 2 The possible fates of stem cells

cells. Developmental signals such as cytokines, cell adhesion molecules, mechanical forces, or ECM molecules (the environment of the cell) determine whether they remain dormant, form scar tissue, or participate in regeneration (Fig. 2).

ESCs are derived from an early developmental stage, the blastocyst. Generally, the embryo is destroyed during the isolation procedure; however, recent studies have unexpectedly described the isolation of ESCs without altering the viability of the embryo [19].

In contrast, MSCs are unspecialized cells that reside in mature somatic tissues. At this time, the exact origin of MSCs is unclear; it may be that these cells are remnants from embryologic development. MSCs and ESCs further vary in the diversity of the array of specialized cells in which they are able to differentiate. As such, MSCs have been found to possess the capacity to generate cells of another tissue lineage [20].

4.1 Mesenchymal Stem Cells

More than 40 years ago, Friedenstein et al. described an ossific progenitor cell of stromal origin in rats, isolated from bone marrow, and later coined the term mesenchymal stem cells (MSCs) [21]. Friedenstein et al. proved the differentiation potential of those cells into fibroblasts and performed what we today consider pioneering work in the field of stem cell research [22, 23]. With their multilineage differentiation potential, MSCs have been successfully differentiated in vitro into an array of different cell types, for instance, osteocytes [23, 24] (see Fig. 3), chondrocytes [25, 26], and other cells of connective tissue [27, 28].

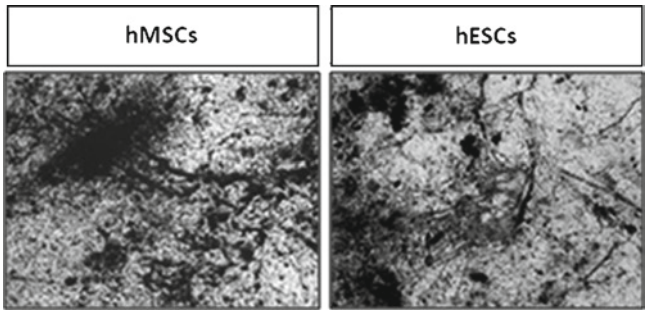

Fig. 3 Comparison of the morphology of mineralized osteoblasts between human mesenchymal stem cells (hMSCs) isolated from bone marrow and human embryonic stem cells (hESCs). Human MSCs were differentiated with dexamethasone (0.1 μM) for 21 days, and hESC cultures (CA-1) were time-dependently supplemented with LiCl (4 mM) and 1,25-OH_2 vitamin D_3 (5×10^{-8} M). The hESC picture was taken at differentiation day 28. Both cell types also received mineralizing factors, β-glycerophosphate (10 mM), and ascorbic acid (50 μg/mL). Note the typical nodule-like structure of mineralized areas

The characterization of MSCs is very complex. Initially, Friedenstein et al. isolated them by their tight adherence to plastic [22]. Today we know that these cells show a variable profile of surface marker expression [20, 29, 30], yet lack a specific exclusive marker. This fact makes it difficult to identify the MSCs with one universal marker. In contrast, all of the hematopoietic cells are characterized by at least one of the markers CD14 (monocytes and macrophages), CD34 (hematopoietic progenitor cells), or CD45 (leukocyte-common antigen), which are therefore considered negative markers for MSCs. The most commonly used markers for the detection and purification of MSCs are the SH2 and SH3 antibodies [31], which recognize CD105 (endoglin) and CD73 (ecto-5′-nucleotidase) [32]. Both markers are constitutively expressed by MSCs but are also expressed by endothelial cells [33, 34]. In view of those findings, it is recommended to use the combination of CD105 and CD106 (vascular cell adhesion molecule 1 [VCAM1]) to identify the MSCs, as CD106 is only expressed on the MSC surface but not on endothelial cells [32, 35].

In addition, newer studies suggest that by isolating MSCs based on their plastic adherence, a portion of MSCs is lost. Nonadherent bone marrow cells may be expanded in culture by “pouring off” culture supernatants. These nonadherent cells retain a potential to specialize into osteoblasts, chondrocytes, and adipocytes, the classic cell types that MSCs generate, but also fibroblastic and glial cell lineages [36].

One limiting factor of the adult stem cells for clinical applications as well as for some in vitro work is the severe aging of MSCs in culture [37]. Baxter et al. showed that in vitro culture for merely 7–10 population doublings reduced the length of the telomeres to what corresponds to the diminution of more than half of

their lifespan. MSCs derived from adult donors have already gone through some substantial shortening in their telomere length in vivo when compared to MSCs from young donors [38].

4.2 Embryonic Stem Cells

The second major type of stem cell is the ESC, made up of a population of cells defined by their origin. ESCs are derived from the inner cell mass (ICM) of the blastocyst, which is the embryonic structure that exists just prior to the implantation into the uterine wall. At this stage, the ICM is composed of approximately 50–60 uniform cells that, when isolated, can be cultured in vitro as ESCs [39]. These cells are pluripotent in nature, which implies that they have the potential to generate any cell type in the body. ESCs have been successfully differentiated in vitro into a variety of specialized cell types, including osteoblasts [40–42] (see Fig. 3) and chondrocytes [43, 44].

While adult stem cells can replicate only a defined number of population doublings in vitro, ESCs can be cultivated indefinitely in vitro without losing their characteristics [45]. In contrast to MSCs, ESCs do not senesce in culture due to significant telomerase activity.

Whereas the first vertebrate ESCs were isolated from the mouse blastocyst in 1981 [39, 46], the isolation of nonhuman primate and human embryonic stem cells (hESCs) took a good decade and a half longer to isolate and was largely made possible by advances in culture techniques for blastocysts [47–51].

ESCs from various species require different signals in vitro to maintain their undifferentiated state and prevent spontaneous differentiation. Initially, cultivation of murine embryonic stem cells (mESCs) on mitotically inactivated mouse embryonic fibroblasts was required to maintain their undifferentiated pluripotent state [52]. A similar result can be achieved through supplementation of ESC cultures with a cytokine called leukemia inhibitory factor (LIF) [53–56], and in this case, the need to use feeder layers is avoided. In contrast, LIF signaling has been found to be insufficient to maintain pluripotency in hESCs [49].

Recently, a number of groups found expression of Oct-3/4 and Rex-1, a pluripotency-associated transcription factor [57], in murine as well as human bone marrow MSCs [58–60], a finding that remains controversial [61, 62]. In the course of our studies, we were also able to demonstrate expression of Oct-3/4 in MSC cultures isolated from human and mouse bone marrow (Fig. 4), again challenging the clear distinction of the marker set between MSCs and ESCs. Despite the controversy around the overlapping expression of transcription factors between ESCs and MSCs, the ESC surface markers Tra-160, Tra-181, and SSEA-3/4 (SSEA-1 for mESCs) are unique, making pluripotent ESCs readily identifiable in a cell population [63].

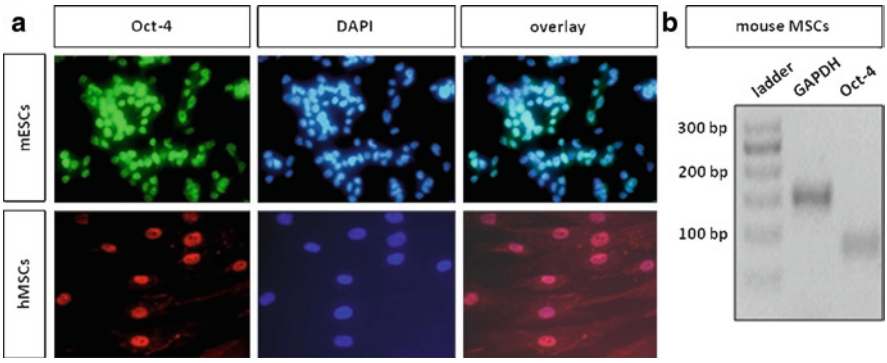

Fig. 4 Comparison of Oct-3/4 expression between embryonic stem cells (ESCs) and mesenchymal stem cells (MSCs). **a:** Murine ESCs and human MSCs stained with an Oct-3/4 antibody, DAPI nuclear counterstain. In our hands, both ESCs and MSCs stain positive for Oct-3/4. **b:** Reverse transcription-polymerase chain reaction for Oct-4 on primary mouse bone marrow MSCs

5 Stem Cells and Bone Differentiation: Features of Mesenchymal Stem Cells and Embryonic Stem Cells

5.1 Mesenchymal Stem Cells and Embryonic Stem Cells

During embryonic development, formation of bone begins with the proliferation of osteoprogenitor cells and is followed by ECM deposition, expression of alkaline phosphatase, and differentiation into osteoblasts through mineralization. Fully mature cells then express characteristic markers in the context of their tissue environment, in which they fulfill specialized functions. For some cell types, these characteristic functions are easier to assess than for others. As such, electrophysiologic determination of action potentials can characterize a cardiomyocyte or a neuron, although such functional features are more difficult to assess in osteoblasts in vitro. In order to evaluate whether a stem cell has successfully differentiated into an osteoblast in vitro, researchers therefore widely rely on the profiling of gene or protein expression patterns and the staging of the differentiation whenever possible. Minimally, the assessment of gene expression allows conclusions to be drawn as to the function of the cells.

The main products of osteoblasts are deposited extracellularly, the most exclusive feature of bone tissue clearly being the ECM calcification. Here, collagen is prevalently represented by collagen type I, a marker of preosteoblasts [64]. The expression of key proteins during osteogenesis, along with the collagens, is a tightly controlled set of events along a defined time scale. Additional proteins that flag the differentiation of osteoblasts are osteopontin [65, 66], osteonectin, alkaline phosphatase, collagen X [64], and core binding factor alpha 1 (Cbfa1), a bone-specific transcription factor and a member of the runt family of transcription factors, also named Runx2 [67, 68]. With increasing maturation, osteoblasts express bone

sialoprotein and osteocalcin, which are considered to be truly specific markers for fully functioning osteoblasts.

In vitro, MSCs respond to signals that are also present in the early embryo, and, if directed by such appropriate signals, they may give rise to specified osteoblasts. The differentiation-initiating factors are provided by neighboring cells in vivo. In culture, timed supplementation of the medium with growth factors and additives largely regulates cell fate, with main changes between the early and late stages of osteogenic differentiation. Usually MSCs are differentiated into the osteogenic lineage in the presence of ascorbic acid, β-glycerophosphate, and dexamethasone [24, 69]. Furthermore, there have been many trials to induce osteoblastic differentiation of MSCs by supplementation with growth factors such as bone morphogenic proteins (BMPs). BMPs are implicated in regulating osteoblast differentiation and bone formation together with Wnt signaling, with the most potent osteogenic inducers in vivo as well as in vitro being BMP-2, -4, -6, -7, and -9 [70, 71]. BMPs interact with serine/threonine kinase receptors, BMPR type I and BMPR type II, subsequently phosphorylating the transcription factors Smads1, 5, or 8. These form a heterodimeric complex with Smad4 in the nucleus, which then activates the expression of osteogenic target genes [71]. In addition, the administration in sequential pattern of growth factors like FGF-2 and BMP-2 were investigated to also reach a favorable effect in the osteogenic differentiation of MSCs [70]. During the 2- to 3-week period of monolayer cultures, the long, stretched, spindle-shaped MSCs differentiate into osteoblasts, exhibit mineralization, and time dependently express bone-specific markers, such as osteocalcin.

Similar to MSCs, ESCs can also be led to differentiate from pluripotency into mesoderm and mesenchyme, which could subsequently lead to cartilage, bone, or fatty tissue. Generally, the ESCs are thought to differentiate more efficiently if they are grown as embryoid bodies (EBs), with or without the cultivation in "hanging drops." Conditions within the hanging drops mimic that of an embryonic environment, and the EBs develop into a three-dimensional structure with appropriate conditions for a developing embryo. The EBs will then generate all three germ layers. As is true for MSCs, appropriate factors have to be supplied in culture in order for the cells to veer toward the osteogenic lineage. Inhibition of the canonical Wnt/CatnB pathway by dexamethasone or VD_3 is thought to be the key mechanism in fully differentiated osteoblasts by stimulating the production of osteoprotegerin, an inhibitor of osteoclast formation (reviewed in ref. 72).

Upon the generation of EBs, mESCs have been shown to generate osteoblasts within 3–4 weeks of differentiation when stimulated by mineralization factors such as ascorbic acid and β-glycerophosphate, as well as additional osteoinductive agents like vitamin D_3 and dexamethasone [41,73–75]. The resulting fully developed osteoblast phenotype is reached by the fifth week of differentiation and can be verified by timely coordinated peak expression patterns of bone-specific genes, for example, collagen type I. The first mineralized cells appear approximately by day 10 of differentiation [41]. Collagen expression is followed by timely activation of osteopontin, bone sialoprotein, and osteocalcin, transcription of which is in turn regulated by Cbfa1 [67, 68].

Differentiation methodology used for mESC differentiation into osteoblasts has been successfully translated into human and nonhuman primate ESCs [42,76–79]. The resemblance of differentiation between mESCs and hESCs has become even more obvious upon subcutaneous transplantation of such human cells into mice, where they were able to yield mineralized tissue in vivo, expressing human osteogenic differentiation markers [76].

Osteogenic differentiation within intact embryoid bodies has been reported in mouse by Chaudhry et al. [80] and by our group [41]. Nontrypsinized human EBs allowed to adhere to gelatin-coated plates differentiate and form osteogenic nodules that display an osteoblast phenotype within 12 days of culture with the osteogenesis-promoting medium [77]. It has been postulated that mesenchymal condensation is required as an initial step of osteogenic differentiation [81]. During condensation, the cells come into closer cellular contact and gap junctions mediate intercellular coupling, a process considered crucial for osteogenesis [82, 83]. The highly dense and compact mass of EBs is believed to be conducive to these processes and thereby is believed to endorse osteogenic differentiation. Since very few putative osteoprogenitor cells were found in the nodules, it has been hypothesized that the mesenchymal cells present at the nodule–dish interface are initially formed within the adherent EB by cellular condensation, migrate outward, and differentiate into the osteogenic lineage [77].

Recent studies have shown that a similarly efficient and spontaneous differentiation of hESCs and mESCs into osteoblasts takes place if the EB step is omitted, obtaining differentiated osteoblasts from a single cell suspension in 10–12 days [84, 85]. Moreover, differentiation of single cells into osteoblasts occurred spontaneously, simply requiring addition of β-glycerophosphate as a source of organic phosphate for mineralization. The same differentiation markers were found in the "quick" osteoblasts as in those obtained via EBs.

Despite these findings, spatial components of the microenvironment cannot be ignored as developmental factors. Garreta and colleagues [86] demonstrated that a three-dimensional structure appears to spontaneously enhance commitment to the osteogenic lineage, even without osteoinductive factors. Although levels of ALP, osteopontin, and collagen I expression, as well as mineralization content, were studied as differentiation markers, the levels of osteocalcin, which is considered to be an exclusive osteoblast marker, were not reported.

6 Signals That Steer Differentiation

In recent years several studies investigated the understanding of the molecular mechanisms that control bone development. One particular challenge researchers face when considering stem cells as sources for transplantable osteoblasts, as for any other cell, is the potential of the stem cells to become other cell types in addition to the desired one. Therefore, finding ways to purify progenitor cells, intentionally kill (purge) unwanted cells, and enhance the primary differentiation output

have been strategies that have been profoundly worked on in order to isolate transplantable cells. Knowledge of the molecules that can manipulate cell fate is imminent, especially in the context of steering differentiation appropriately. As a result of such studies, a multitude of different growth factors, morphogens, signaling molecules, and transcriptional regulators have been implicated in the regulation of the osteogenic differentiation of stem cells.

The Wnt/CatnB canonical signaling pathway, for instance, is critically involved in bone development and adult homeostasis with regard to proliferation, differentiation, migration, and determination in ESCs and MSCs [87, 88]. Upon activation of the canonical Wnt cascade, β-catenin (CatnB), a transcription factor at the end of the cascade, is being stabilized and translocated to the nucleus, where it complexes with T cell factor, LEF family transcription factors, and/or other cofactors to trigger downstream target gene transcription [89–91]. Noncanonical Wnt pathways have also been described to play a role in osteogenesis, among them Wnt5a signaling through activation of phosphatidylinositol and PKCdelta as well as Wnt7b, also acting via the PKCdelta-mediated pathway [92]. Noncanonical Wnt4 signaling through activation of p38MAPK has also been shown to enhance bone regeneration [93]. The inhibition of the Wnt pathway during osteogenic differentiation of adult MSCs is associated with a reduction in the expression profile of osteogenic transcription factors and an inhibition of c-Jun N-terminal and p38 mitogen-activated protein kinases [94]. Activation of canonical Wnt signal transduction by Wnt3a leads to improved cell proliferation in MSCs. In contrast, Wnt5a does not alter proliferation but instead enhances osteogenic differentiation of MSCs. According to Baksh et al. [95], Wnt3a is suppressed by Wnt5a, and Wnt3a in turn inhibits the Wnt5a-mediated improvement of osseous development, pointing to the fact that there seems to be an existing cross-talk between different Wnt signaling pathways that leads to functional antagonism during the osteogenic program.

In ESC osteogenesis, CatnB expression levels and nuclear activity are regulated in a fluctuating manner. Early differentiation of ESCs starts with a spontaneous increase in expression of T (brachyury), pinpointed as an early pan-mesodermal marker. Since ectodermal and endodermal markers are still expressed at this time, this state has been suggested to correspond to primitive streak formation in vivo. Brachyury-positive cells mark a population that has specified toward mesendoderm [96, 97] and retain the capability to differentiate into cells of the hematopoietic lineage as well as the mesodermal lineage [98]. It is intriguing that the Wnt/CatnB axis supports the expression of T (brachyury) [97, 99].

During subsequent development, CatnB is decreased during early osteogenic commitment in vitro [100] and increased in the late stages of osteogenesis, both in ESCs in vitro and in differentiating osteoblasts in vivo [100, 101].

In the past, CatnB has generally been perceived as necessary for the development of the osteoblast phenotype (reviewed in ref. 72), which could lead to a misinterpreted collision of its biphasic role and a necessity of its downregulation at the early osteogenic differentiation stages. CatnB is necessary for a completion of osteogenesis at its late stages; however, it needs to be downregulated at the early onset of osteogenic specification for osteogenesis to begin, as we expressed in a

recent review [102]. Ultimately, the therapeutic potential of MSCs and ESCs for skeletal tissue regeneration, particularly in bone, depends on their intrinsic features and integration/repair capability, which will be the focus of the next section.

7 Stem Cells and Transplantation Aspects

Stem cells, whether from the same organism (autologous), from another organism of the same species (allogeneic), or from a different species (xenogeneic), have the potential to be used in clinical therapies through the transplantation of the cells into the designated treatment site. To date, MSCs have been used in animal models of nonunion bone fractures, as well as a few early clinical trials [103], which have not provided any indication that would suggest that these cells would induce teratomas upon transplantation. In fact, adult stem cells have the ability to avoid rejection by the host [104]. MSCs synthesize the enzyme indoleamine 2,3-dioxygenase, which suppresses the T cell response of the immune system [105]. Therefore, MSCs seem to induce an advantageous local immune suppression, while the immune defense of the body persists overall. Due to this immune-modulating property, MSC coinjections or injections of MSC-conditioned medium have been shown to aid in the prevention of immune rejection of whole tissues [106,107].

Although MSC grafts provide successful tissue support for smaller defects, the amount of available autologous cells may be limited due to their in vitro senescence as described earlier in this chapter. Here, allogeneic tissues can provide adequate tissue volume; however, risk of disease transmittance and tissue rejection diminishes the efficacy of this treatment method.

In contrast, the clinical application of ESCs is largely hindered not only by the ethical issues that surround them, but also by their potential to induce teratomas upon transplantation, the insufficient control of their proliferation and differentiation once transplanted, and the fact that any cell type derived from an ESC will be allogeneic, not autologous, and therefore is likely to be rejected without immunosuppressants. The latter has recently been examined in several studies that have attempted to generate induced pluripotent stem cells (iPSCs), in which somatic cells of various origins have been reprogrammed to express pluripotency-associated transcription factors [108–110]. With this method, a patient's cell may be harvested and genetically modified in vitro to become a pluripotent cell, which in turn may be taken through a differentiation protocol. The end result would be a fully differentiated osteoblast that would possess very low host-graft rejection. Although iPSCs are a suggested solution to the immune rejection problem, their ultimate clinical use is still facing the other challenges that are associated with ESCs, such as karyotypic stability in culture, amenability to large-scale manufacturing, survival of transplanted cells, tumor formation potential, and lack of control of their differentiation once transplanted. In vivo rodent models and large preclinical animal studies have therefore been performed to find solutions to these issues (Table 1). It is significant that Brederlau and colleagues noted that the teratoma formation capacity of the ESCs decreases with progressing differentiation.

Table 1 Overview of transplantation studies using in vivo rodent and other preclinal animal models

Species	Lineage	Target organ	Purification based on	Recipient animal	Route of administration	Outcome measure	Time of assessment	Teratoma	References
									117
Hu	Cardiac	Heart	BMP-2, FGFR inhibitor predifferentiation	Immunosuppressed rats	Coronary artery ligation	Antihuman laminin staining, anti-MHC-beta	2 mo	No (n = 15)	118
					Intrascar injection	Cardiac-specific mRNA			
Hu	Myogenic	Muscle	Selective culture conditions, CD73+/NCAM+	SCID/Beige mice	Intramuscular injection into tibialis anterior muscle	Survival, expression of human myoblast-specific RNA and protein, expression of human laminin in 7% of the fiber in the grafted area	Up to 6 mo	No	119
Hu	Cardiac	Heart	Undifferentiated	Sprague-Dawley rat	Intracardial injection after myocardial infarction	Cardiac-specific mRNA	2 mo	No	120
Mo	Undiff	Heart	Undifferentiated	Nude mice SCID mice Allogeneic		Equal numbers of cardiomyocytes in cardiac tumors as in hind-limb tumors Equal numbers of cardiomyocytes in cardiac tumors as in hind-limb tumors Increased inflammation, upregulation of MHC I and II		100% 100% Rejected after several weeks	113

(continued)

Table 1 (continued)

Species	Lineage	Target organ	Purification based on	Recipient animal	Route of administration	Outcome measure	Time of assessment	Teratoma	References
Cym	Hemato	BM	Coculture with OP9, cytokine cocktail	Cynomolgus	In utero fetal liver	Donor-derived CFU in recipients at birth	3 mo	100% ($n = 3$) 100% in controls (undifferentiated ESCs, $n = 3$) None following SSEA-4 purging ($n = 6$)	121
Mo	Undiff	Heart	Undifferentiated	BALB/c, allogeneic	Intramyocardial injection after LAD ligation	Mild $CD3^+$ T lymphocyte infiltration, sparse $CD4^+$ T cell clusters, massive $CD4^+$ and $CD8^+$ T cells	1 wk	100% in allogeneic and syngeneic after 4 wk	112
				Sv129/SvJ, syngeneic		Limited numbers of infiltrating T lymphocytes	1 wk 4, 8 wk	0% in allogeneic after 8 wk, 100% in syngeneic	
Cym	Hemato	BM	OP9 coculture, cytokine cocktail	Sheep	In utero fetal liver	Donor-derived CFU in recipients at birth	Up to 17 mo	No	51
Mo	Undiff	Joint		Immunosuppressed rats	Intra-articular joint injection	Survival, cartilage production	8 wk	No	115
Hu	Osteo-genic	SC	Dexamethasone	Mice	PDLLA scaffold	Presence of mineralized cells	35 d	NA, $n = 5$	76

Ham	MSC	Muscle	Undifferentiated	Hamster	Direct delivery of \MSC into skeletal muscle bed	Improved ventricular function; increased circulating levels of HGF, LIF, M-CSF; mobilization of c-kit$^+$, CD31$^+$, and CD133$^+$ progenitor cells and subsequent increase in myocardial c-kit$^+$ cells; trophic effect of MSC further activated expression of HGF, IGF-2, and VEGF in the myocardium	1 mo	122
Hu	Osteogenic	SC	Dexamethasone	Balb/c nude mice	Umbilical MSC loaded with a biomimetic artificial bone scaffold; implantation subcutaneously	Capacity of osteogenic differentiation leading to osteogenesis with human origin in vivo	12 wk	123
Mo	Undiff	Heart	Undifferentiated, transduction with *Lentivirus*	C57BL/6J mice	Muscle patch placement	Functional improvement; MSCs exhibited the capacity to invade the scar and increased repopulation and thickness of the scar	14 d	124

BM, bone marrow; Cym, cynomolgus monkey; Ham, hamster; hemato, hematopoietic; HGF, hepatocyte growth factor; Hu, human; IGF-2, insulin-like growth factor-2; LIF, leukemia inhibitory factor; M-CSF, macrophage colony-stimulating factor; Mo, mouse; PDLLA, poly-DL-lactic acid; SC, subcutaneous; undiff, undifferentiated; VEGF, vascular endothelial growth factor.

Whereas hESCs that were predifferentiated in vitro for 16 days formed teratomas in 100% of the cases, the percentage dropped to 0% when the cells were predifferentiated for 23 days [111]. After transplantation of undifferentiated mESCs into infarcted hearts, no teratomas were observed in an allogeneic setting, as MHC class I and II antigens are upregulated with progressing differentiation [112, 113]. In a syngeneic setting, teratomas are formed in all cases. In vivo transplantations of ESCs into other target organs are not that well characterized. In the context of skeletal regeneration, Wakitani et al. [114] reported that undifferentiated mESCs, when injected into the knee joint of SCID mice, formed teratomas and irreparably destroyed the joint. However, in the xenogeneic setting (mESCs in immunosuppressed rats), and when the joint is encumbered by full-thickness osteochondral defects, the cells integrate and repair the defect within 8 weeks of transplantation [115]. Subsequently, the same group showed that the repair of the defect through undifferentiated ESCs does not occur in mice in which the joints were immobilized [116], suggesting a role for mechanical loading during in vivo repair.

Transplantations of undifferentiated ESCs or ESC-derived osteoblasts in vivo into the bone had not been performed until very recently. This recent data suggest that the teratoma formation capacity of mESCs decreases with prolonged directed in vitro differentiation into osteoblasts, underlining the findings just described. Moreover, hESCs that were steered into the osteogenic fate with dexamethasone in vitro and seeded onto poly-DL-lactic acid scaffolds showed no signs of teratoma formation when transplanted into the skin fold of SCID mice. Rather, new bone was formed around the scaffolds [76]. Finally, a recent paper by Jan de Boer's group showed that ESCs contribute to new bone formation in a critical size defect if they go through a cartilage stage, mimicking endochondral bone formation [117].

The first human trials using ESC-derived cells, not for the treatment of degenerative bone diseases, but for spinal cord injury, are finally underway, after the regulatory authorities had been approached with trial approval pleas for the last few years. We eagerly await the first results, hoping that these will pave the road for future widespread clinical use of stem cells for the treatment of degenerative diseases, including osteodegenerative diseases.

8 Conclusion

In summary, one has to conclude that various stem cell sources exist that are capable of generating transplantable osteoblasts or osteoprogenitors in vitro. Whereas the widespread clinical use of MSCs is possibly accompanied by the problem of generating enough transplantable cells, it is unclear whether the microenvironment, differentiation states, or an injured administration site can prevent teratoma formation and direct differentiation into functional mature specialized cells when either ESCs or iPSCs are the cell source for the transplantable material. In order to devise safe and effective clinical strategies however, collecting such data is imminent and justifies further in vivo studies in animals.

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Part II
Epigenetic and microRNA Regulation in Stem Cells

Epigenetic Identity in Cancer Stem Cells

Maria Ouzounova, Hector Hernandez-Vargas, and Zdenko Herceg

Abstract Growing evidence supports the existence of a subpopulation of cancer cells with stem cell characteristics within tumors. As occurs with normal embryogenesis, epigenetic changes define the balance between pluripotency and differentiation in cancer stem cells (CSCs). The basis and implications of this novel concept are discussed, together with the evidence supporting a role for epigenetic mechanisms in the induction of CSCs. We discuss the evidence favoring the plasticity of these mechanisms and its potential therapeutic implications.

Keywords Cancer stem cells • Histone modifications • DNA methylation • MicroRNA • Mammosphere model

1 Epigenetic Identity in Cancer Stem Cells

1.1 Epigenetic Control in Normal Tissue Development and Tumorigenesis

All cells in an organism are descendants of a single cell. Cells of the inner mass of the preimplantation blastocyst are able to divide and differentiate into all of the different cell types of the body, and when cultured in vitro these inner mass cells can be propagated for many cell generations as embryonic stem (ES) cells [1]. In a similar way, adult self-renewing tissues are characterized by the presence of a small subpopulation of somatic or adult stem cells (SCs). These cells have the ability to divide asymmetrically, producing the different types of cells within a given organ,

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and are characterized by their ability to self-renew and their multipotent differentiation, unique properties that have been defined as “stemness” [2].

Stem cells share an identical genome with differentiated cell types, making necessary the existence of mechanisms that will control the expression of differentiation programs and their heritability independent of the genetic code. Such mechanisms have been grouped by definition under the name of “epigenetics” and include chromatin organization, histone modifications, DNA methylation, and microRNAs. All of these processes appear to cooperate with and reinforce each other to either sustain pluripotency or produce cell specialization in response to specific stimuli [3]. In stem cells, gene expression patterns controlled by these epigenetic mechanisms confer “stemness” properties that are reverted to during the transition from a pluripotent to a committed state [4]. In addition, the plasticity of chromatin states in the maintenance of stem/progenitor cell pluripotency is illustrated by the fact that virtually all cell types can be converted into more immature cell stages with wider differentiation potentials [5].

In contrast to the idea of cancer as a genetic disease, it has become clear that the disorder of cell proliferation and cell death observed in cancer can better be explained by a combination of genetic and epigenetic events. Consistent with this idea, aberrant epigenetic regulation of a set of “stemness” genes may lie at the origin of tumor development and malignant phenotype. Indeed, recent studies indicate that aberrant epigenetic information plays an important role in virtually every type of human neoplasia [6].

Traditionally, tumorigenesis has been explained using a “clonal origin” model, whereby all cancer cells within the same tumor tissue are presumed to be endowed with the capacity to generate new tumors by simple proliferation of transformed cells. Recently, however, an alternative “hierarchical/stem cell” model has proposed that tumors are sustained in their pathologic growth by a minority subpopulation of tumor cells with “stemlike” properties in a way analogous to normal organogenesis. The “cancer stem cell” (CSC) is an operational term to functionally define this distinct subpopulation of tumor cells with unlimited renewal potential [7]. These stemlike tumor cells may have a deregulated potential for self-renewal, excessive proliferation, and aberrant differentiation into heterogeneous progeny of cancer cells, culminating in intratumor heterogeneity [7, 8]. Among the features shared between normal and cancer stem cells are the capacities to self-renew, give rise to a differentiated progeny, and migrate into surrounding tissue; however, the mechanisms that govern the establishment and maintenance of key characteristics of these cells remain poorly understood (Fig. 1). There is growing evidence that molecular pathways required for normal stem cell functions are deregulated in CSCs [9]. As occurs with normal organogenesis and cell differentiation, the selective activation and repression of these pathways may be mediated by epigenetic mechanisms [10]. However, there is an intense debate as to whether CSCs originate from adult stem cells or from mature, committed progenitors and/or even terminally differentiated cells that have abnormally acquired self-renewal capacity [11]. Regardless of these diverse origins, chromatin modification states and/or DNA methylation could represent the first step of tumorigenesis [6, 12].

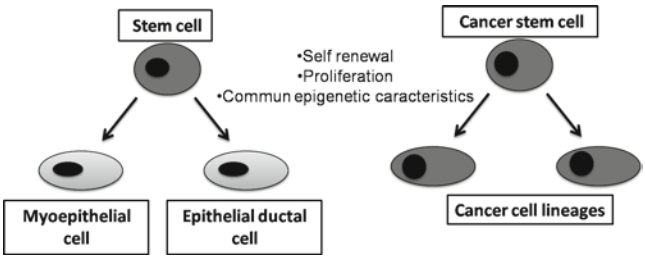

Fig. 1 The cancer stem cell hypothesis. Cancer stem cells share with normal stem cells the ability to self-renew and to proliferate, giving rise to different cell lineages. Moreover, normal and cancer stem cells may be epigenetically similar

There is cumulating evidence in favor of a CSC origin of several types of tumors, and the parallel between normal SCs and CSCs is becoming more and more apparent. However, there is not complete understanding of the epigenetic characteristics of CSCs, where the persistent activation of self-renewal and pluripotency pathways may lie at the core of the carcinogenesis process.

1.2 Epigenetic Mechanisms Involved in Pluripotency

The self-renewal potential of ES and SCs requires a long-term memory system for stable maintenance of transcriptional patterns. Epigenetic mechanisms contribute to the repression of inappropriate developmental programs in time and space while ensuring heritability of existing or newly acquired phenotypic states (Fig. 2). Therefore, the potential for multilineage differentiation requires plasticity of the genome, allowing multiple differentiation decisions. This apparent contradictory epigenetic behavior of stem cells is reflected by the presence of a specific chromatin configuration and a unique epigenetic signature, as has been shown in recent studies [13]. A similar epigenetic signature has begun to emerge for CSCs.

1.2.1 Chromatin Organization

Chromatin consists of nucleosomes, each containing 147 base pairs (bp) of DNA wrapped around an octamer of the core histone proteins H3, H4, H2A, and H2B. Major architectural chromatin proteins appear to be more dynamic than previously thought. Using the fluorescence recovery after photobleaching approach, Meshorer et al. [14] monitored the affinity and exchange rate of different chromatin-associated proteins in pluripotent mouse ES cells and differentiated cell types. They found that different histone variants are rapidly exchanged, ranging from several seconds to a few minutes. Moreover, a significantly larger fraction of total cellular pools of certain histone variants are loosely bound to chromatin in ES cells compared to differentiated cells. This hyperdynamic chromatin seems to be a hallmark of pluripotent cells, and may

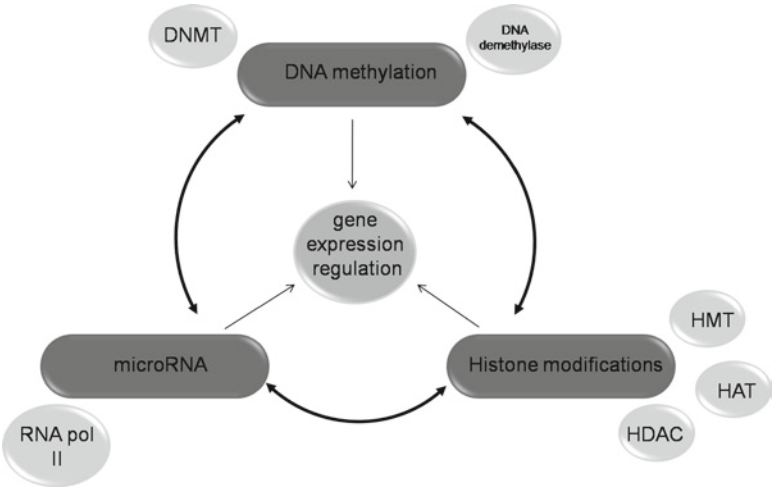

Fig. 2 Epigenetic mechanisms (DNA methylation, histone marks, siRNA). Gene expression regulation may be explained by epigenetic modifications. This regulation is a result of combined effects of DNA methylation (mediated by DNA methyltransferases [DNMTs]), histones modifications (mediated by chromatin-modifying complexes, including histone acetyltransferases [HATs], histone methyltransferases [HMTs], and histone deacetylases [HDACs], and microRNAs

play an important role in the maintenance of the pluripotency of ES cells. By maintaining loosely bound histone variants and other chromatin-associated proteins, pluripotent ES cells may preserve the potential to rapidly differentiate into multiple cell lineages according to changing needs of a tissue [6]. There are few reports in the literature on the chromatin dynamics of CSCs. We recently addressed this question by using the mammosphere model to study putative breast cancer stem cells (H. Hernandez-Vargas, M. Ouzounova, and Z. Herceg, unpublished observations). By using a similar strategy to that of Meshorer et al., we found an increased dynamicity of chromatin in putative breast cancer stem cells compared to the parental cells. Of interest, this dynamicity was modulated when cells were grown under attachment conditions, somehow reverting to their phenotype. Therefore, different cell populations within tumors may differ in their chromatin dynamics, and this may constitute an indirect mark of their ability to sustain pluripotency or differentiation. Further studies are required to understand how this correlates with the physiologic chromatin dynamics observed in ES cells.

1.2.2 DNA Methylation

DNA Methylation During Normal Development

The methylation of DNA is the covalent addition of a methyl group to the five-carbon (C5) position of cytosine bases in CpG dinucleotides. DNA methylation has long been considered a key mechanism of transcriptional regulation and has been implicated in a diverse range of cellular functions, including tissue-specific gene

expression, cell differentiation, cell fate determination, genomic imprinting, and X chromosome inactivation [15]. Indeed, the dynamic regulation of methylation patterns has been shown to be critical for embryonic development [16, 17], with genome-wide analyses of DNA methylation revealing tissue- and cell type–specific profiles [18, 19]. In mammalian development, the global DNA methylation profile of the genome is dynamically reprogrammed during gametogenesis and early embryogenesis [20] (Fig. 3). At both of these stages, the genome first undergoes global demethylation [21] followed by reestablishment of DNA methylation during implantation [22]. De novo methylation occurs postimplantation, thus reestablishing global methylation levels. Therefore, demethylation of the genome is generally correlated with permissive chromatin states and the acquisition of pluripotency, and, conversely, methylation is correlated with repressive states and loss of pluripotency [20]. The importance of DNA methylation in embryonic development and cell differentiation is reflected in the phenotype of mice deficient for the specific DNA methyltransferases that catalyze the de novo methylation of cytosine residues in gene promoters [23, 24]. In fact, mice deficient for Dnmt3a die at about 4 weeks after birth, whereas Dnmt3b–/– mice exhibit developmental defects including growth impairment and rostral neural tube defects [25].

Mechanistically, DNA methylation is considered as a means of shutting down genes whose activity is no longer required as cells differentiate. This methylation

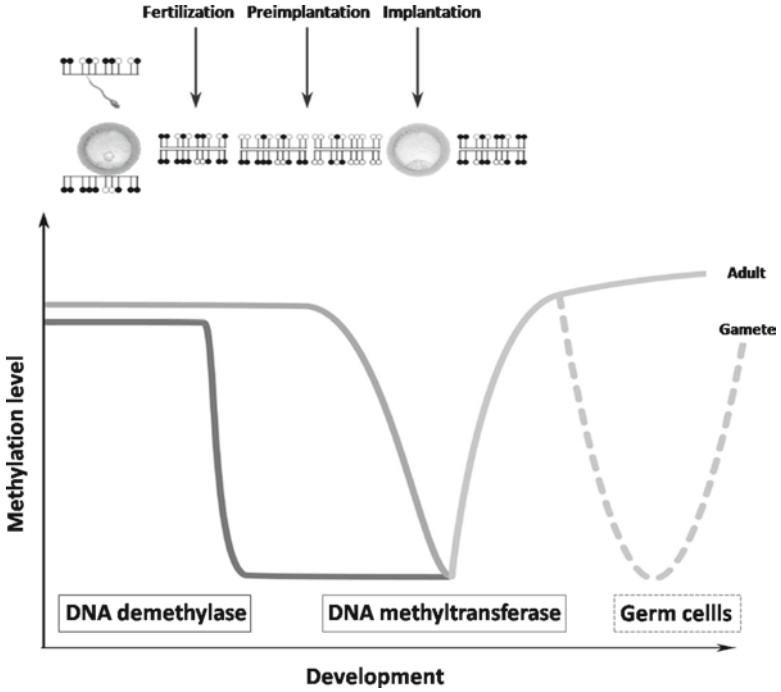

Fig. 3 DNA methylation evolution during early embryonic development

can prevent binding of certain transcription factors to CpG islands [26, 27] and can also induce the preferential association with deacetylated histones [28]. This association is mediated by methyl cytosine–binding proteins of the MBD and MeCP families that recruit transcription-repressing complexes containing histone deacetylases [29] and histone methylases [30, 31]. In stem cells this mechanism is responsible for reversibly repressing genes encoding transcription factors required for differentiation [32], while several genes important in pluripotency and self-renewal are silenced by methylation in differentiated cells [33]. In this sense, it has been shown that when ES cells differentiate into a particular cell lineage many genes are downregulated [34], while global methylation is increased [35, 36], favoring the idea that transcription accessibility is a major factor in determining the plasticity of differentiation in ES cells.

DNA Methylation and Carcinogenesis

It is now established that in cancer there is a global disruption of normal DNA methylation patterns. In general, most tumors display a global hypomethylation and a specific promoter hypermethylation that have been linked with genomic instability and inactivation of tumor suppressor genes (TSGs), respectively [37, 38]. Epigenetic gene silencing and associated promoter CpG island DNA hypermethylation are prevalent in all cancer types and are an alternative mechanism to mutations that inactivate tumor suppressor genes within a cancer cell [39]. These epigenetic changes may precede genetic changes in premalignant cells and foster the accumulation of additional genetic and epigenetic hits [40, 41], providing a selective advantage to neoplastic cells. An attractive idea is that disruption of DNA methylation patterns may reactivate a chromatin configuration common to pluripotent cells. Indeed, the global methylation pattern in CSCs and normal stem cells may be similar. In addition, it was recently shown that a stem cell–like chromatin pattern may predispose TSG to DNA hypermethylation and heritable silencing [42].

In a contrasting approach, several studies have described a cancer-related methylation profile in normal pluripotent and progenitor cells. It was found, for example, that epithelial progeny of estrogen-exposed breast progenitor cells display a cancer-like methylome [43]. This is in line with the presence of a subset of cancer-related genes frequently methylated in ES [44]. Moreover, analysis of ES cell–derived and primary cells revealed that “weak” CpG islands associated with a specific set of developmentally regulated genes undergo aberrant hypermethylation during extended proliferation in vitro [17], a pattern reminiscent of that reported in some primary tumors [45]. Therefore, it is tempting to speculate that the acquisition of promoter DNA methylation in a set of repressed genes could lock in stem cell phenotypes and initiate abnormal clonal expansion and thereby predispose to cancer, as recently proposed [46, 47].

DNA Methylation in Cancer Stem Cells

The notion of CSCs in breast tumors has been recently validated with the identification of a small cell population with tumor-initiating capacity and stemlike characteristics, the so-called breast cancer stem cells [48]. Recently, analysis of the DNA methylation profiles of four distinct cell populations from normal human breast tissue, including the putative $CD44^+CD24^-$ CSCs, demonstrated that epigenetically controlled transcription factors seem to help define progenitor and differentiated cell phenotypes [49]. Furthermore, genes encoding transcription factors with known stem cell function are similarly methylated in $CD44^+CD24^-$ cells from normal mammary epithelium and breast carcinomas. These findings imply conservation of epigenetic programs that define progenitor characteristics. The same study suggests that DNA methylation profiles may be used as markers of cellular differentiation states. However, in the mammary epithelium more differentiated cells maybe more methylated, whereas in ES cells differentiation may correlate with hypomethylation. It was previously demonstrated that a $CD44^+$ breast cancer cell gene expression signature correlates with shorter distant metastasis-free survival [50] and that distant metastases are enriched for hypermethylated $CD24^+$ breast cancer cells. This is consistent with the finding that $CD44^+$ cells are the most hypomethylated and highly express several transcription factors with known stem cell function, including HOXA10 and TCF3.

In a similar way we recently observed a significant reduction of the global DNA methylation in putative CSCs compared to parental differentiated cells (H. Hernandez-Vargas, M. Ouzounova, and Z. Herceg. submitted). Moreover, we found a distinct panel of genes differentially methylated in putative CSCs. Of interest, gene ontology analysis revealed that these cells were enriched in four main categories, including developmental processes, secreted proteins, extracellular matrix, and protein kinase activity. These findings show that putative CSCs exhibit a distinct methylation pattern, and the genes regulated by DNA methylation belong to processes that have been related to a specific stemlike signature [51] (Fig. 4).

In colon cancer and glioblastoma cancer stem cells, characterized by the expression of CD133 [52, 53], DNA methylation was found in the promoter CpG island of CD133 in cells with low or absent expression of the marker protein [54]. Of interest, this methylation pattern is specific for only primary tumors and is not found in normal brain and colon. Indeed, an abnormal DNA methylation profile associated with abnormal gene silencing seems to sharply distinguish $CD133^+$ from $CD133^-$ cells.

In summary, because of numerous studies showing its association with heterochromatin, DNA methylation has gained a reputation as a permanent, silencing mark. However, rather than a permanent mark, methylation has been shown to be dynamic, capable of temporally changing at gene promoters [55]. This makes it a key mechanism during normal differentiation and highlights a role of altered DNA methylation in cancer. In fact, cancer-specific DNA methylation predominantly involves the same sites that show normal DNA methylation variation among tissues, particularly at genes associated with development [17].

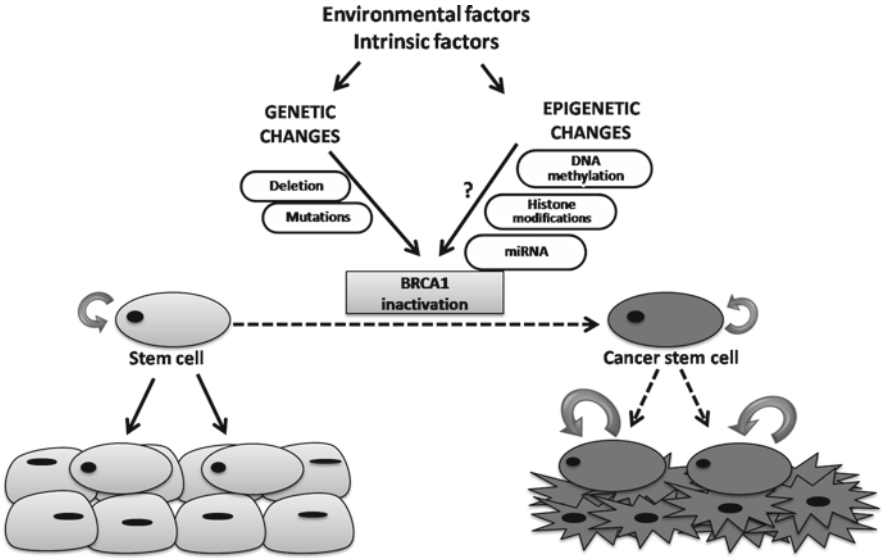

Fig. 4 Epigenetic deregulation in cancer stem cells. BRCA1 downregulation in breast cancer may be caused not only by genetic changes, but also by one or multiple epigenetic mechanisms, including DNA methylation, histone modifications, and microRNA (miRNA) regulation. BRCA1 is involved in the balance between self-renewal and differentiation of breast stem cells, and loss or silencing of BRCA1 may promote tumorigenesis at two levels. Inactivation of BRCA1 may induce transformation of stem cells into cancer stem cells and/or dedifferentiation of somatic cells into cancer cells with self-renewal and differentiation capacity

1.2.3 Histone Marks

The tertiary structure of chromatin and the activity of the associated genes are directly mediated by numerous modifications of the chromatin components. Chromatin histone residues undergo posttranslational modifications at the N-terminal tails that alter the ability for the DNA to interact with nuclear proteins. The described classes of histone modifications are acetylation, lysine methylation, arginine methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deamination, and proline isomerization, which function to regulate various processes such as transcription, repair, replication, and condensation [56].

With regard to transcriptional control, histone acetylation is primarily associated with activation, whereas lysine methylation correlates with both activation and repression, depending on the lysine residue that has been modified. For example, methylation of histone 3 lysine 4 (H3K4) is associated with active and open chromatin, whereas histone 3 lysine 9 (H3K9) and histone 3 lysine 27 (H3K27) methylations are associated with inactive and compacted chromatin [57]. The recent discovery of bivalent domains—those possessing both active (H3K4 methylation) and repressive (H3K27 methylation) modifications—complicates further the distinction between active and repressive marks [42]. Bivalent domains seem to be associated with the

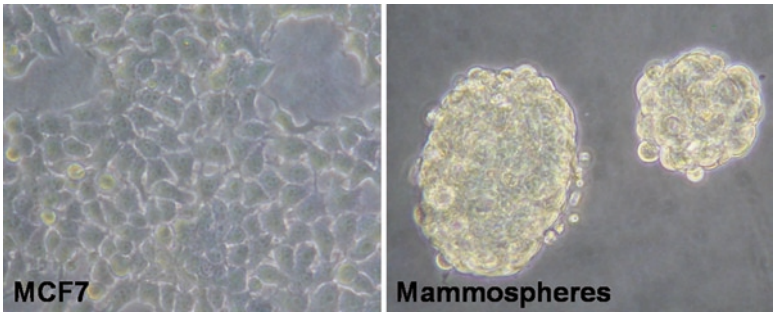

Fig. 5 Putative breast cancer stem cells (mammospheres)

regulation of genes involved in embryonic development and differentiation. These domains are expressed at low levels in ESCs and dissolve in differentiated cells where the genes are marked by either H3K27me3 or H3K4me3 [4].

Recently, in a model of breast cancer stem cells (Fig. 5) we observed a reduced acetylation and reduced H3K4me2/H3K27me3 ratio and we were able to link this epigenetic pattern to a flexible regulation a panel of developmental genes in CSCs compared to differentiated mammary epithelial cells (H. Hernandez-Vargas et al., submitted). In a recent report, Ohm et al. [42] suggested that similar mechanisms of epigenetic alterations occur in ES cells and in early carcinogenesis. The bivalent chromatin marks in ES cells are associated with two additional repressive histone marks, H3K9me2 and H3K9me3, leading to a hypermethylated chromatin. This pattern is similar to that in adult cancers, with the exception of the loss of the activating H3K4 mark [58]. These results lead to the hypothesis that CSCs also express the bivalent state plus the additional repressive marks and raise the possibility for a reversal of tumor cells to a bivalent state by epigenetic therapies.

1.2.4 MicroRNAs

MicroRNAs (miRNAs) are a class of approximately 22-nucleotide (nt)-long, non-coding RNAs found in eukaryotes. miRNA processing is mediated by the nuclear Drosha/Pasha complex with RNase III activity and further mediated by the RNase III enzyme Dicer to generate a 22-bp miRNA duplex. miRNA can inhibit gene expression by mRNA degradation or by translational inhibition of target genes. MiRNA genes constitute approximately 1%–5% of the predicted genes, with up to 1000 miRNA genes in the human genome [59]. They could act as tumor suppressors by inhibiting oncogenes or function as oncogenes by inhibiting tumor suppressors.

More than 50% of miRNA genes were localized in cancer-associated genomic regions or in fragile sites [60]. miRNAs are increasingly associated with cancer, either as tumor suppressors or oncogenes. An example is the let-7 miRNAs, which act as tumor suppressors in numerous cell types. Their important role in both developmental timing and cancer leads us to envisage how the disruption of the

temporal regulation of proliferation and differentiation could lead to the aberrant expansion of immature cells, especially in light of the recent validation of the CSC hypothesis [7, 61]. A recent study found that let-7 was reduced in breast tumor–initiating cells and increased with differentiation [62]. Moreover, low let-7 is involved in maintenance of the undifferentiated status and proliferating potential in tumor-initiating cells, and enforced let-7 expression inhibits tumor growth in NOD/SCID mice. Downregulation of let-7 in breast tumors compared to normal tissue was previously reported [63], and low levels of let-7 were correlated with poor prognosis in advanced ovarian cancer [64].

In a different way, miR-199b-5p was recently identified as a negative regulator of the proliferation rate and anchorage-independent growth of medulloblastoma cells [65]. Moreover, this miRNA was shown to inhibit the expression of several cancer stem cell genes and to decrease the stem cell–like ($CD133^+$) population of cells in medulloblastoma. The downregulation of miR-199b-5p in metastatic medulloblastoma could suggest a potential epigenetic silencing, confirmed by the fact that 5-aza-deoxycytidine treatment reduces miR-199b-5p expression in a panel of medulloblastoma cell lines.

miRNAs represent an intriguing link between early carcinogenesis and physiologic embryonic development. Furthermore, their role in cell differentiation and more specifically tumor-initiating cells may provide a precious diagnosis tool and hope for developing new therapeutic strategies.

1.3 Concluding Remarks

Epigenetic changes are commonly accepted as important events in cancer development and progression. Series of intriguing results suggest that epigenetic deregulations may occur in stem/progenitor cells and precede genetic alterations. Epigenetic mechanisms are essential for maintaining the identity and the regulation of stem cells; therefore, dysfunction of these mechanisms could lead to the transformation of the normal stem cells into CSCs. The detection of epigenetic modifications at early stages of tumorigenesis and in apparently healthy tissues surrounding the site of tumors argues in favor of the hypothesis that epigenetic alterations appear prior to genetic changes [66, 67]. A similar situation is likely present in stem cells, where the DNA methylation pattern is first erased and then reestablished, allowing the parental imprinting mechanism [25].

Epigenetic alterations may be considered as one of the first events in stem/progenitor cells leading to cancer development. A better understanding of epigenetic mechanisms during cancer development and progression is essential for the development of novel therapeutic strategies [68, 69]. Epigenetic alterations could be principal targets for inhibitors of histone deacetylases or DNA methyltransferases. Thus, establishing epigenetics-based therapeutic strategies at the CSC level would prevent regeneration of the tumor.

In summary, it is now well accepted that epigenetic changes play an important role in all stages of cancer and that these events occur not only in early stages of tumorigenesis, but also in CSCs. We can define a "stemness" phenotype as a flexible state associated with distinct epigenetic changes. Epigenetic disruption of stemness genes in stem/progenitor cells may be one of the initial steps in carcinogenesis. The subsequent genetic and epigenetic deregulation of molecular pathways involved in survival and differentiation may result in the formation of CSCs. Further studies are needed for a better understanding of the epigenetic mechanisms underlying neoplastic transformation and generation of CSCs. This information would facilitate the development of novel and efficient strategies for cancer prevention and treatment.

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Function of MicroRNA-145 in Human Embryonic Stem Cell Pluripotency

Na Xu and Kenneth S. Kosik

Abstract MicroRNAs are posttranscriptional regulators of gene expression, but their roles in controlling self-renewal and pluripotency of human embryonic stem cells (hESCs) remain unclear. Our recent study indicates that the level of microRNA-145 (miR-145) is low in hESCs but highly upregulated during differentiation. Endogenous miR-145 is sufficient to repress the 3′ untranslated regions of OCT4, SOX2, and KLF4. By downregulating OCT4 and SOX2, increased miR-145 inhibits hESC self-renewal, represses pluripotency, and induces lineage-restricted differentiation. Furthermore, the miR-145 promoter is bound and repressed by OCT4 in hESCs. Thus, miR-145 plays key roles in repressing hESC pluripotency and promoting differentiation. This work reveals a direct link between the core reprogramming factors and miR-145 and uncovers a double-negative feedback loop involving OCT4, SOX2, KLF4, and miR-145.

Keywords Embryonic stem cells • Self-renewal • Pluripotency • MicroRNA • miR-145

1 Human Embryonic Stem Cell

1.1 Self-Renewal and Pluripotency

Embryonic stem cells (ESCs or ES cells) are valuable resources for clinical therapies due to their two key properties: self-renewal and pluripotency. Self-renewal refers to the unlimited proliferation potential of cultured ESCs in vitro. Pluripotency refers to the unique potential of ESCs to generate any differentiated cell type in the adult organism. These properties distinguish ESCs from other lineage-restricted stem cells, which can only give rise to limited types of differentiated cells.

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Interesting yet puzzling is the coexistence of these two properties within every single ESC. An ESC has the program to initiate cellular decisions in completely opposite directions: maintenance in the pluripotent ESC state or differentiation into a somatic cell. For decades, researchers in this field have been trying to uncover the molecular mechanisms that govern stem cell fate.

1.2 Molecular Delineation of Key Regulators in Human Embryonic Stem Cells

Cell fate during development is tightly controlled by transcription factors that function as molecular switches of gene expression programs. Many ESC-specific transcription factors have been well characterized and reported [1, 2]. This chapter examines the functions and regulation of OCT4, SOX2, and KLF4 in ESCs.

OCT4, or OCT-3/4, is a POU family transcription factor expressed highly in embryos and germ cells [3, 4]. A mouse genetic knockout study established that Oct4-deficient embryos fail to develop the inner cell mass (ICM) from which ESCs originate [5]. The requirement of Oct4 for establishing and maintaining ESCs is further revealed in mouse and human ESCs. It is not surprising that homozygous Oct4-negative ESCs could not be obtained by homologous recombination, indicating loss of self-renewing undifferentiated ESCs [5]. Induced RNA interference (RNAi)–mediated OCT4 knockdown leads to endoderm and trophoblast differentiation that is similar in human and mouse ESCs [6, 7]. These studies indicate that transcription factor OCT4 is required for ICM formation and ESC self-renewal and pluripotency.

In addition to OCT4, two other factors, SOX2 and KLF4, are required for ESC self-renewal and pluripotency. Sox2, a high-mobility group DNA-binding domain transcription factor, is expressed at high levels in the ICM in mouse embryos and later germ cells. Sox2-homozygous mutant mouse embryos displayed peri-implantation lethality with defective ICM development and extraembryonic ectoderm formation [8]. In human ESCs, RNAi-mediated knockdown of SOX2 caused loss of stem cells and expression of trophectoderm markers [9].

In the search for Oct4 and Sox2 cofactors, Krüppel-like factor 4 (Klf4) was identified as a transcriptional coregulator that activates a subset of Oct4 target genes in ESCs [10]. In mouse ESCs, Klf4 alone is dispensable to the maintenance of self-renewal, yet depletion of Klf2, Klf4, and Klf5 together leads to ESC differentiation [11]. Furthermore, Klf factors regulate a common downstream target, Nanog, which indicates that the Klf circuitry controls ESC-specific gene expression [11].

It is well known that transcription factors do not act alone. Genome-wide mapping of transcriptional factor targets by chromatin immunoprecipitation (ChIP), combined with microarrays or sequencing methods, provides insights into the transcriptional network in ESCs [12–14]. Using genome-scale location studies, it was found that OCT4, SOX2, and NANOG co-occupy the promoters of a large number of developmentally important genes. These downstream genes can be classified into two categories: pro–self-renewal or prodifferentiation. The pro–self-renewal genes include chromatin-remodeling enzymes, histone modification enzymes,

signaling pathway components, and ESC transcription factors (e.g., REST, SKIL, HESX1, and STAT3). OCT4, SOX2, and NANOG activate these genes to promote and stabilize the ESC self-renewing fate. The prodifferentiation genes bound by OCT4, SOX2, and NANOG are inactive, and they encode transcription factors that may regulate development and lineage differentiation. Furthermore, OCT4, SOX2, and NANOG form regulatory circuitry with autoregulatory and feedforward loops. Recently, the core regulatory circuitry has been extended to include six other transcription factors—Klf4, c-Myc, Dax1, Rex1, Zpf281, and Nac1—in mouse ESCs [13]. The self-renewal and pluripotency properties are regulated by an integral network of transcription factors that may regulate hundreds to thousands of downstream genes and cooperatively specify cell fate.

1.3 Transcription Factors and Reprogramming

The importance of stem cell transcription factors was recently highlighted in the study of reprogramming, that is, the conversion of differentiated somatic cells back to the pluripotency state. Overexpression of OCT4, SOX2, and KLF4 along with other factors, such as NANOG, C-MYC or LIN28, can reprogram or dedifferentiate somatic cells into induced pluripotent stem (iPS) cells in both mouse [15–17] and human [18–20]. The success of iPS technology supports the idea that levels of key ESC transcription factors are crucial in achieving pluripotent stem cell fate. Recently, a group of noncoding microRNA (miRNA) genes have been linked to the transcriptional regulatory circuitry of ESCs [21]. However, relatively little is known about how miRNAs epigenetically modulate ESC self-renewal and pluripotency.

2 MicroRNAs

MicroRNAs are 21- to 23-nucleotide, noncoding RNAs that play important regulatory roles in animals and plants. miRNAs bind to partially complementary target sites in 3′ untranslated regions (UTRs) of mRNAs, which results in mRNA cleavage, or translational repression of the encoded proteins. miRNA functions in a wide range of cellular and developmental processes, such as control of cell proliferation, cell death, neuronal patterning in nematodes, leaf and flower development in plants, and lineage differentiation in mammals [22]. In many cases, miRNAs fine tune or restrict cellular identities by repressing important transcription factors or key pathways [23].

More than 600 miRNAs have been identified in humans. Because each miRNA recognizes its target through partial sequence complementarity, one miRNA could regulate hundreds of mRNAs. It was proposed that more than one third of all human genes may be regulated by miRNAs [24]. One gene could also be repressed by multiple miRNAs. Recognition among mRNAs and miRNAs may depend on the expression patterns of the genes and miRNAs in a specific cellular context or physiologic environment.

2.1 MicroRNA Expression in Embryonic Stem Cells

Many miRNAs have displayed intriguing expression patterns. The cell type–specific expression signature of miRNAs in mouse and human ESCs has been used successfully to distinguish ESCs from differentiated cell types [25]. Using various tools such as microarrays, sequencing, and real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis, several groups reported genome-wide miRNA expression profiles [26–29]. More than 100 miRNAs are differentially expressed in hESCs and differentiated embryoid bodies (EBs) [30]. The characterization of the miRNA pathways and their underlying molecular mechanisms is of great importance to the understanding of ESC self-renewal and pluripotency.

2.2 MicroRNA Processing

The requirement of miRNAs for the maintenance of ESC populations and pluripotency was initially demonstrated by genetic studies. Murine ESCs with genetic deletion of a key miRNA processing enzyme, Dicer [31, 32] or DGCR8 [33], lose their pluripotency and show defective differentiation. Dicer-deficient mutant ESCs can be partially rescued by the miR-290 cluster miRNAs that regulate Rbl2-dependent DNA methylation to downregulate Oct4 indirectly [34, 35]. However, miR-290 decreases during ESC differentiation when Oct4 needs to be switched off, which makes it less likely to be a dominant player in the differentiation process. On the other hand, mutant DGCR8 ESCs do not fully downregulate pluripotency markers such as Oct4, Rex1, Sox2, and Nanog in response to differentiation [33]. It remains unclear which miRNAs may be involved in direct repression of pluripotency markers. The study of the functions of miRNAs in ESCs is still in an early stage, and the direct roles of miRNAs in downregulating pluripotency genes are yet to be investigated.

3 MicroRNA-145: Regulator of Stem Cell Fate

3.1 Identification of miR-145 as a Temporally Regulated MicroRNA During Human Embryonic Stem Cell Differentiation

The goal of our study was to investigate the roles of miRNA in direct repression of pluripotency genes in hESCs. Based on previous research, we hypothesized that

some temporally regulated miRNAs participate in the downregulation of pluripotency genes upon differentiation of ESCs. Such miRNAs may likely present an expression profile in contrast to pluripotency genes. While OCT4, SOX2, and KLF4 are downregulated during differentiation, the negative miRNA regulators may be upregulated in their levels.

To identify human miRNAs that display increased expression upon differentiation, we compared the expression profiles of 466 miRNAs in undifferentiated hESC line H9 and differentiated EBs using the Taqman real-time multiplex RT-PCR method. Our analysis yielded results consistent with previous reports: two clusters of miRNAs, miR-302 and miR-371/372/373, were ESC specific [27, 28, 30]. From this analysis, we found that miR-145 was expressed at a relatively low level in undifferentiated hESCs but was significantly upregulated in EBs [36] (Fig. 1). In addition, miR-145 is predicted to target multiple hESC reprogramming factors: OCT4, SOX2, and KLF4 by TargetScan [37], miRBase [38], and Miranda [24]. On the basis of our preliminary evidence, miR-145 is a strong candidate repressor of pluripotency genes.

3.2 Defining Targets of miR-145: OCT4, SOX2, and KLF4

The critical question is which transcription factors are targets of miR-145. We employed luciferase reporter assays to evaluate the interaction of miR-145 and its predicted targets. We engineered luciferase reporters that have either the wild-type 3′ UTRs of OCT4, SOX2, and KLF4 or the mutant UTRs with a 6–base pair (bp) deletion in the target sites (Fig. 2a). Reporter constructs such as these are widely used to provide experimental evidence that miRNAs directly repress translation initiation. In both HeLa and 293 T cells, the pre-miR mimic of miR-145

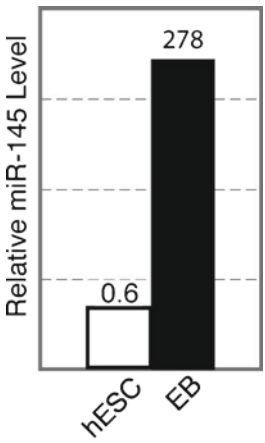

Fig. 1 The expression of miR-145 in human embryonic stem cells (hESCs). The relative level of miR-145 increased after hESC differentiation. In the Taqman real-time reverse transcription-polymerase chain reaction, the small nucleolar RNA h-NR003022 was used as an internal normalization control. EBs, embryoid bodies

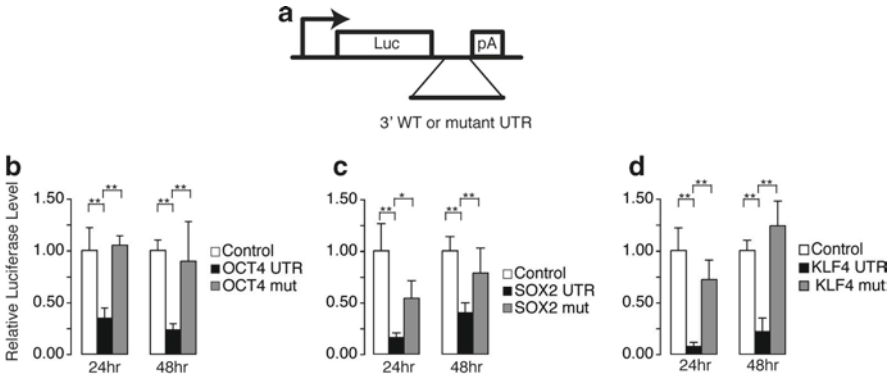

Fig. 2 Endogenous miR-145 represses the 3′ untranslated regions (UTRs) of OCT4, SOX2, and KLF4 in human embryonic stem cells (hESCs). **a:** The 3′ UTR reporters in target validation. Luc, firefly luciferase; pA, polyadenylation signal; WT, wild type. Mutant UTR has a 6–base pair (bp) deletion of the miR-145 target site. **b–d:** The relative luciferase level of 3′ UTR luciferase reporters of OCT4 **(b)**, SOX2 **(c)**, and KLF4 **(d)** in hESCs under self-renewal conditions at 24 or 48 hours posttransfection. Control, the basal luciferase reporter without UTR; mut, mutant UTR with a 6-bp deletion of the miR-145 target sites. $^{**}p < 0.01$. $^{*}p < 0.05$. The error bar is standard deviation from three independent experiments

("pre-miR-145") significantly reduced the luciferase activities of the wild-type OCT4, SOX2, and KLF4 reporters by 25%–33% compared to the negative control pre-scramble [36]. In contrast, mutant reporters were not repressed by pre-miR-145, which indicates that the target site directly mediates the repression. Taken together, our results show that miR-145 directly targets OCT4, SOX2, and KLF4 3′ UTRs.

3.3 Endogenous miR-145 Directly Targets OCT4, SOX2, and KLF4 3′ Untranslated Regions in Human Embryonic Stem Cells

Our next question concerns the extent to which endogenous miR-145 can repress the 3′ UTR reporters of OCT4, SOX2, and KLF4 in hESCs under self-renewal conditions. Since the endogenous miR-145 is expressed at relatively low levels, we were surprised to observe a significant repression of the wild-type 3′ UTR luciferase reporter activities of OCT4, SOX2, and KLF4 in comparison to the no-UTR control [36] (Fig. 2b–d). To validate the effect, the mutant reporters with a 6-bp deletion of the miR-145 target sites were tested. They were found to have significantly less repression than the wild-type reporters [36]. We conclude that the endogenous miR-145 in hESCs is sufficient to repress OCT4, SOX2, and KLF4 3′ UTR reporters directly.

Because the endogenous miR-145 level is increased upon hESC differentiation, we expected that the magnitude of repression of the OCT4, SOX2, and KLF4 UTRs by miR-145 also increases. Upon withdraw of bFGF in hESCs cultures, the magnitude of repression for the OCT4 and KLF4 UTRs indeed showed a significant increase as differentiation progressed to days 2, 4, and 8 [36]. These results indicate that miR-145 upregulation upon cellular differentiation further downregulates its direct targets OCT4, SOX2, and KLF4.

3.4 Effect of miR-145 on Endogenous OCT4, KLF4, and SOX2 in Human Embryonic Stem Cells

miRNAs can downregulate gene expression by either of two posttranscriptional mechanisms: mRNA cleavage and translational repression. Because the luciferase assay does not distinguish the two mechanisms well, we further examined how the levels of the endogenous OCT4, KLF4, and SOX2 mRNA and protein change in hESCs in response to miR-145 (Fig. 3a, b). In short, we found that the mRNA level of SOX2, but not those of OCT4 or KLF4, were decreased in the miR-145–overexpressing cells, while Western blot analysis showed that the protein levels of OCT4 and KLF4, but not that of SOX2, were reduced [36]. The reduction of the OCT4 and KLF4 protein and SOX2 mRNA levels indicates that these endogenous reprogramming factors are posttranscriptionally controlled by miR-145 in hESCs.

3.5 Induced miR-145 Regulates Human Embryonic Stem Cell Self-Renewal

Next, we studied the functional role of miR-145 in modulating hESC self-renewal by a gain-of-function approach. By increasing miR-145 expression from a lentiviral vector, we found a significant drop of the self-renewal marker SSEA4 and Tra-1-81 staining, an increase in apoptosis rate, and a reduction of S-phase population in virus-infected cells analyzed by flow cytometry [36] (Fig. 3c, d). These suggest that miR-145 upregulation disrupts the maintenance of the hESC self-renewal.

To assess the long-term effect of miR-145, we performed a colony formation assay. hESCs transduced with Lenti-miR-145 produced small and grossly differentiated colonies, and the negative control Lenti-scr yielded compact and undifferentiated colonies. Quantification of the phenotypes showed that the inhibition effect of miR-145 on hESC clonogenicity is greater than the effect from knocking down of OCT4 [36]. In summary, we found that upregulation of miR-145 alone in hESC is sufficient to prohibit the maintenance of self-renewal, and miR-145 has strong effects perhaps through its repression of all three pluripotency factors together.

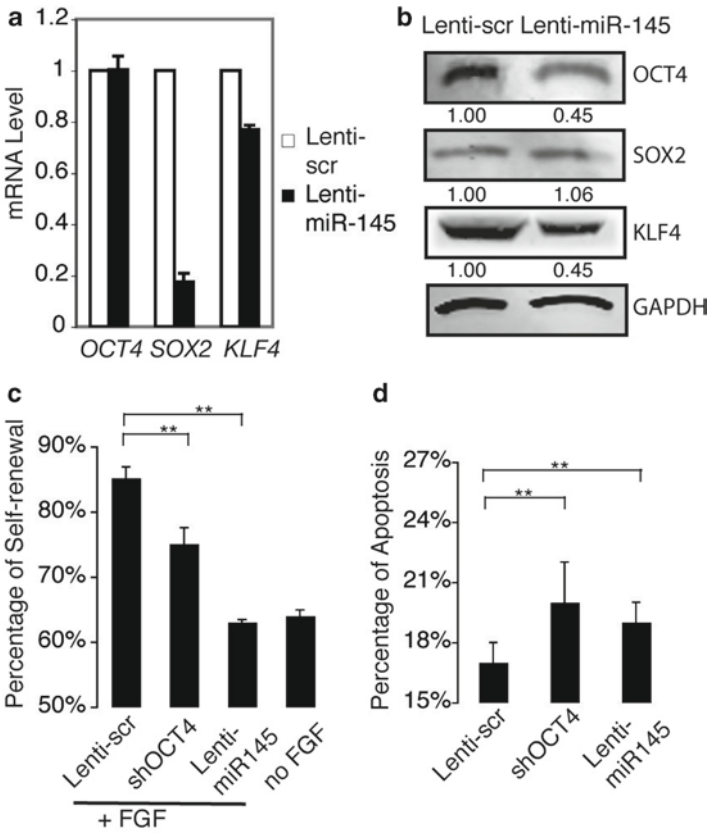

Fig. 3 Overexpression of miR-145 inhibits OCT4, KLF4, SOX2, and human embryonic stem cell (hESC) self-renewal. **a, b:** Relative mRNA **(a)** and protein **(b)** levels of OCT4, SOX2, and KLF4 in control Lenti-scr and Lenti-miR-145 GFP$^+$ cells. Isolation of the GFP$^+$ hESCs under self-renewal conditions expressing the control Lenti-scr or Lenti-miR-145 was performed by fluorescence-activated cell sorting. **c:** Inhibition of self-renewal during hESC maintenance by ectopic miR-145 in the Lenti-miR-145-expressing/GFP$^+$ cells. After regular mechanical splitting of the hESC colonies, the cells were transduced with lentiviruses and maintained under self-renewal conditions for 6 days. Self-renewal is assessed by flow cytometry analysis of the SSEA4 staining in lentiviral GFP-expressing cells (GFP$^+$) cells. Flow cytometry data collection was in two channels: GFP in the green channel and SSEA4 in the yellow channel. The self-renewal percentage was calculated as $N_{(\text{GFP+SSEA4+})}/(N_{(\text{GFP+ SSEA+})} + N_{(\text{GFP+ SSEA}-)})$, where N is the number of events. A t-test compared shOCT4 or Lenti-miR-145 to control Lenti-scr. $^{**}p < 0.01$. $N = 6$. **d:** Increased apoptosis in Lenti-miR-145 cells after 6 days, assessed by flow cytometry analysis of the annexin V staining in lentiviral GFP-expressing (GFP$^+$) cells. The percentage was calculated as $N_{(\text{GFP+annexin+})}/(N_{(\text{GFP+ annexin+})} + N_{(\text{GFP+ annexin}-)})$. $^{**}p < 0.01$. $N = 6$

3.6 *miR-145 Promotes Differentiation of Human Embryonic Stem Cells*

hESCs can differentiate into cells of extraembryonic trophectoderm and three different germ layers: ectoderm, endoderm, and mesoderm. To assess the effect of miR-145 in modulating differentiation, we examined the expression of lineage-specific markers and found a marked increase of the mesoderm-specific marker α-smooth muscle actin (SMA) and the ectoderm-specific marker β-III tubulin (TUJ1) by immunostaining in Lenti-miR-145 culture [36]. Consistently, mRNA expression analysis showed that miR-145 upregulation elevated the mesoderm marker *MIXL1, NODAL* and the ectoderm marker *VIMENTIN, OTX2* expression as well [36] (Fig. 4a). Our results demonstrated that increased miR-145 induced hESC differentiation into the mesoderm and ectoderm lineages.

To understand whether the miR-145–mediated differentiation is dependent on its targets OCT4 and SOX2, we designed a rescue experiment (Fig. 4b). When Lenti-miR-145 overexpression decreased the endogenous OCT4 and SOX2, we introduced ectopic OCT4 and SOX2 expression vectors lacking 3′ UTRs, resistant to the regulation by miR-145. Indeed, the cellular differentiation phenotype caused by miR-145 was compensated by the reciprocal rescuing of OCT4 and SOX2, indicating that miR-145 acts through repressing multiple pluripotency factors to promote differentiation [36].

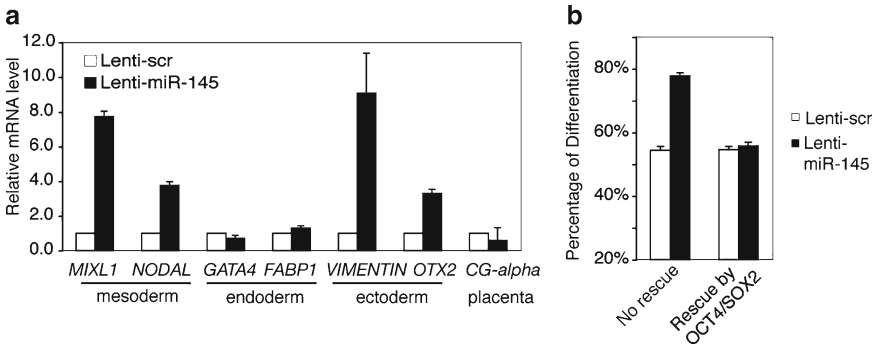

Fig. 4 miR-145 promotes differentiation of human embryonic stem cells (hESCs). **a:** Increased mesoderm and ectoderm differentiation in Lenti-miR-145–infected hESCs. Relative mRNA level of differentiation marker genes *MIXL1*, *NODAL*, *GATA4*, *FABP1*, *VIMENTIN*, *OTX2*, and *CG*-α. *GAPDH* was used as internal normalization in SYBR quantitative reverse transcription polymerase chain reaction. $^{**}p < 0.01$. $N = 6$. **b:** The effect of miR-145 on differentiation is mediated by OCT4 and SOX2. Expression of the OCT4/SOX2 rescue constructs completely inhibits the effect of ectopic miR-145 in promoting differentiation

3.7 Necessity of miR-145 During Human Embryonic Stem Cell Differentiation

Next, we studied the effect of miR-145 loss of function on differentiation progression by transfection of differentiating hESCs with the inhibitor LNA-miR-145. In LNA-miR-145 cells, the differentiation rate was significantly reduced compared to the LNA-scr control, and the S-phase population increased dramatically [36]. These results are further supported by the deregulation of a panel of differentiation markers and impairment of the downregulation of miR-145 targets [36]. Thus, miR-145 is not only sufficient, but it is also necessary in the downregulation of pluripotency genes during differentiation.

3.8 A Novel Feedback Loop of miR-145 and Transcription Factors

An important yet unclear question is what factors modulate the miR-145 upregulation during hESC differentiation. Using the luciferase reporter assay, we mapped an OCT4-binding motif ("Site 1") within a homologous region 1 kb upstream of the miR-145 transcription start site. This element, when linked to the 5′ UTR of the luciferase gene, showed OCT4-dependent inhibition of the reporter activity [36]. To confirm that the miR-145 promoter is bound by OCT4, the electrophoretic mobility shift assay and ChIP were employed to evaluate the in vitro and in vivo DNA–protein interactions. In summary, our experiments indicate that OCT4 acts as a transcriptional repressor on miR-145 in hESCs. This interesting result suggests that OCT4 and miR-145 form a reciprocal regulatory loop that helps to control the low level of miR-145 in hESC maintenance and switches to the high level of miR-145 upon differentiation.

3.9 Connection of miR-145 and Pluripotency Network

In brief, we investigated the roles of miR-145 as a temporally regulated miRNA during hESC differentiation and showed that it can target 3′ UTRs of OCT4, SOX2, and KLF4. In contrast to the decrease of the pluripotency factors during differentiation, the miR-145 level was relatively low in hESCs and increased during differentiation. By gain-of-function and loss-of-function approaches, we found that the endogenous levels of OCT4, SOX2, and KLF4 are controlled posttranscriptionally by miR-145 in hESCs. Ectopic miR-145 expression significantly disrupts the maintenance of hESCs in the self-renewal state, and miR-145 is necessary and sufficient to modulate the differentiation progression through the OCT4/SOX2 pathway. In addition, the promoter of miR-145 was repressed by OCT4 in hESCs. Therefore, miR-145 is connected to the pluripotency network in a double-negative feedback loop (Fig. 5). We uncovered a direct link between miR-145 and the core pluripotency factors OCT4/SOX2/KLF4 and demonstrated that miR-145 represses pluripotency and controls ESC differentiation.

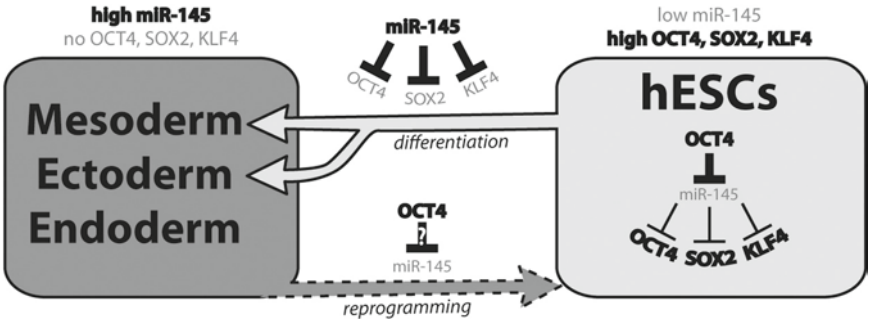

Fig. 5 Proposed model of the double-negative feedback loop by miR-145 and three factors. miR-145 inhibits self-renewal and pluripotency factors OCT4, SOX2, and KLF4 and controls differentiation. Gray indicates a low level of either miR-145 or pluripotency factors. Boldface indicates a high level of either miR-145 or pluripotency factors

4 Conclusions

The functions of miRNAs in mammalian development have attracted much research interest. Recent reports showed that differentiation of somatic stem cells [39] and mouse ESCs [40, 41] can be modulated by miRNAs through posttranscriptional attenuation of key ESC factors. Here, our study has demonstrated for the first time that in human ESCs a specific miRNA plays a key regulatory role by direct targeting of the pluripotency factors OCT4, SOX2, and KLF4.

Although OCT4 and SOX2 are well known for their high expression in ESCs, the consequence of abnormally elevated or repressed OCT4 and SOX2 doses on ESC self-renewal and pluripotency is detrimental. A less than twofold increase of Oct4 protein turns murine ESCs into primitive endoderm and mesoderm, and repression of Oct4 results in differentiation into trophectoderm [42]. A less than twofold increase in Sox2 protein triggers the ESC differentiation into neuroectoderm, mesoderm, and trophectoderm [43]. Both Oct4 and Sox2 are transcriptionally activated by themselves, as well as other factors such as Nanog and FoxD3 [44]. It poses a threat to the ESC identity when these strong positive feedback loops are not controlled properly and coordinately by negative signals. The presence of negative regulation by a miRNA such as miR-145 provides a solution to the dose control problem in hESCs.

miR-145 and OCT4 form a double-negative feedback loop that switches the hESCs between self-renewal and differentiation. The next unanswered question is how this pathway participates in dedifferentiation of somatic cells. Would turning off miR-145 expression allow the initiation of self-renewal? In future studies, uncovering the epigenetic regulation by miRNAs would be one of the next major steps in the delineation of reprogramming pathways. Discovery of new interactions between multiple transcriptional factors and miRNAs will add a new layer of complexity in the regulatory network that determines stem cell fates.

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Mesenchymal Stem Cells for Liver Regeneration

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Abstract The liver is an extraordinary organ that retains its regenerative power throughout life. The precise molecular mechanisms regulating liver regeneration are unknown, but a number of cell types have been postulated to be involved in the process, including (1) resident liver stem cells, (2) differentiated hepatocytes, and (3) extrahepatic stem cells. This chapter will discuss liver regeneration from the context of extrahepatic stem cells. Recent research findings have challenged the dogma of limited lineage commitment potency of somatic stem cells. This chapter reviews the hepatic lineage plasticity of mesenchymal stem cells in vitro and in. In addition, it discusses developments in the application of mesenchymal stem cells for the treatment of liver diseases in preclinical and clinical studies, as well as in possible signaling pathways and mechanisms, including microRNAs, which are involved in the regulatory control of hepatic fate specification.

Keywords Mesenchymal stem cells • Hepatogenesis • Liver disease • miRNA • Cell therapy

1 Introduction

The liver is the largest glandular organ, and is capable of expansion under functional deficiency and reduction under functional excess. The liver is responsible for a number of vital roles, including metabolism of carbohydrates, lipids, and proteins, secretion of metabolic wastes, synthesis of serum proteins, biotransformation and breakdown of xenobiotics, and storage of vital nutrients such as glycogen, vitamins, and minerals. The liver is made up of a number of cell types: hepatocytes, stellate

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cells, Kupffer cells, bile duct epithelial cells, and endothelial cells. Other cell types that constitute the liver include fibroblasts and hematopoietic cells. Hepatocytes constitute approximately 60% of all cells of the liver and are responsible for the majority of the biochemical functions of the organ. Stellate cells comprise 5%–10% of all cells in the liver and are responsible for the production of a number of growth factors and extracellular matrix proteins.

In the last few decades, somatic stem or progenitor cells of a variety of tissue lineages have been identified. However, liver stem or progenitor cells have remained elusive. Extensive research efforts have identified a population of small cells in the liver that in the presence of hepatic injury exhibit proliferative potential, as well as the capacity to differentiate into hepatocytes [1–4]. These cells are known as oval cells. However, oval cells have only been identified in rodents, and a definitive stem cell population in the human liver is yet to be identified.

2 Bone Marrow as a Source of Hepatic Progenitors

Approximately a decade ago, studies emerged reporting that transplanted bone marrow (BM) cells developed into hepatocytes in vivo [5–8]. Of interest, hematopoietic stem cells (HSCs) shared the expression of several markers genes in common with oval cells and liver progenitor cells, including CD34, Thy-1, c-kit, flt-3, sca-1, and CD45, which led to speculation that oval cells and HSCs may arise from a common cell population originating in the BM [5, 9–11]. This hypothesis was tested transplanting BM into recipient animals and assessing for donor-derived hepatocytes [5]. In female rats that were lethally irradiated and rescued with a BM transplant from a male animal, hepatocytes containing the Y chromosome were detected by day 13 posttransplantation. When host hepatocytes proliferation was inhibited by administration of 2-acetylaminofluorene (2-AAF), donor-derived hepatocytes accounted for approximately 0.14% of the reconstituted liver, and about 0.1% of the Thy-1$^+$ oval cells were positive for the Y chromosome. These findings were confirmed in an independent approach in which BM cells from dipeptidyl peptidase IV (DPPIV)–positive male rats were injected into lethally irradiated DPPIV-negative female rats in the presence of 2-AAF. Similar findings were shown in another study transplanting BM from male mice into lethally irradiated female recipients [6]. In addition, it was reported that transplantation of 200 male CD34$^+$lin$^-$ cells into female recipients gave rise to up to 2.2% of Y chromosome–positive hepatocytes. Such findings were corroborated in studies in which archival autopsies and liver biopsies from human female recipients of male BM transplantation showed that Y chromosome–positive hepatocytes could be identified in liver specimens exhibiting highly variable engraftment frequencies [7, 8, 12].

These findings lead researchers to believe that HSCs had greater plasticity than once thought and fueled further efforts regarding the therapeutic potential of

HSCs for liver diseases. Using fumarylacetoacetate hydrolase (FAH)–deficient mice, an animal model of tyrosinemia type I, Lagasse and colleagues investigated the ability of BM cells to engraft and restore biochemical functions of the recipient liver [13]. In the first set of experiments, the authors transplanted 1×10^6 LacZ-expressing unfractionated BM cells into each of nine lethally irradiated $FAH^{-/-}$ recipient mice; by terminating treatment of 2-(2-nitro-4-trifluoromethylbenzyol)-1,3-cyclohexanedione (NTBC), which selects for donor cell engraftment by providing a proliferative advantage to cells that are wild type for FAH, it was found that four of nine mice survived. From 30% to 50% of the livers had donor-derived cells at 7 months posttransplantation, and all biochemical functions were improved. In the second experiment, transplanted small numbers (10, 50, 100, or 1000) of fluorescence-activated cell sorter–purified HSCs were transplanted into lethally irradiated $FAH^{-/-}$ mice along with 2×10^5 congenic adult bone marrow cells as a radioprotective dose. Donor-derived cells were detectable in the liver of the recipient animals, and coexpression of albumin and LacZ were detectable in some of the cells. On the other hand, engraftment was not detected in the liver of animals transplanted with a non-HSC population from BM. Subsequently, Krause et al. reported that transplantation of a single HSC in irradiated mice could engraft in multiple organs and differentiate into nonhematopoietic lineages [14].

The newly developed plasticity theory of HSCs was quickly challenged by the finding that such phenomena could simply result from cell fusion, as shown by two independent studies [15, 16]. Using an in vitro culture model, Terada et al. showed that bone marrow cells spontaneously fused with embryonic stem cells in the presence of interleukin-3 and adopted the phenotype of the fusion partner, which, without detailed genetic analysis, might be interpreted as dedifferentiation or transdifferentiation. Similarly, Ying et al. derived neurospheres from transgenic mice expressing either the G418 resistance gene or the green fluorescent protein (GFP) and cocultured with HT2 embryonic stem (ES) cells that carry the hygromycin phosphotransferase–herpes simplex virus thymidine kinase (Hytk) fusion gene. By selection with either G418 or puromycin, HT2 cells were removed, and colonies with ES cell–like morphology reemerged. Colonies recovered from 23 independent cocultures harbored transgenic markers from both the neurosphere and the ES cell, and metaphase spread showed a tetraploid chromosome karyotype. Cell fusion as the basis of hepatocytes arising from transplantation of HSCs in animals was then demonstrated in two independent studies [17, 18]. In one study, Wang et al. transplanted donor cells that are wild type for FAH into $FAH^{-/-}$ mice and then serially transplanted BM-derived hepatocytes (BMHs) into tertiary recipients and analyzed them cytogenetically. It was found that greater than 30% of metaphases in all mice with BMHs had karyotypes resulting from fusion between diploid donor and host cells, or diploid donor cells with tetraploid host hepatocytes. In addition, many cells were found to contain aneuploid karyotypes. By fluorescence in situ hybridization it was confirmed that almost all FAH^+ hepatocytes (18 of 19) also contained donor chromosomes. In the second study, by transplanting FAH^+ BM cells into $FAH^{-/-}$

mice, Vassilopoulos et al. dissected FAH$^+$ liver nodules and found that 43 of 43 nodules contained 12%–48% donor DNA and that FAH$^+$ hepatocytes in these nodules expressed both the donor and host alleles of β2-microglobulin. Subsequently, Alvarez-Dolado et al. used a Cre/lox recombination–based system in which Cre-expressing, BM-derived cells are cocultured with neurospheres derived from the R26R mouse line and in which the LacZ reporter gene is exclusively expressed after the excision of a loxP-flanked stop cassette by Cre-mediated recombination, to detect cell fusion in vitro and in vivo [19]. When BM-derived cells were cocultured with neurospheres in vitro, 1 to 2 per 80,000 cells were found to be β-gal$^+$ and to have two or more nuclei. To study cell fusion in vivo, the authors reconstituted the BM of lethally irradiated R26R mice with BM from mice constitutively expressing Cre recombinase and green fluorescence protein (GFP). In all animals, β-gal$^+$ cells were detected only in the brain, heart, and liver, and β-gal$^+$ cells coexpressed the donor marker GFP. Similarly, by reconstituting wild-type hosts with purified HSCs from GFP$^+$ transgenic mice, it was found that mobilized HSCs failed to differentiate into hepatocytes even in the presence of hepatic injuries such as carbon tetrachloride–induced toxicity or partial hepatectomy [20]. Camargo et al. found that HSC-derived hepatocytes primarily arise from mature myelomonocytic cells; using a Cre/lox-based strategy, they demonstrated that myeloid cells spontaneously fuse with host hepatocytes [21].

Although it has been determined by in vitro studies that HSCs are not the source of hepatic progenitors in BM, the presence of cells that can be induced to express liver-related marker genes has been shown [22]. Oh and colleagues showed that freshly isolated BM cells transcriptionally expressed high levels of c-met, low levels of alpha-fetoprotein (AFP), and undetectable levels of albumin. However, when BM cells were cultured in the presence of high levels of hepatocyte growth factor (HGF), cells upregulated the expression of albumin and AFP while downregulating c-met. By immunofluorescence staining, cells were found to be positive for albumin, AFP, cytokeratin-8 (CK-8), and cytokeratin-18 (CK-18) after 3 weeks of induction and exhibited morphologies of hepatocytes, characterized by a large nucleus, few nucleoli, and numerous cytoplasmic granules. Similar results were demonstrated by Miyazaki et al. In the presence of HGF and epidermal growth factor (EGF), mRNA transcripts of the late-stage hepatocyte markers tryptophan-2, 3-dioxygenase and tyrosine aminotransferase were detectable [23].

The first evidence of liver differentiation from a purified population of nonhepatic somatic stem cells was reported by Schwartz et al. [24]. Multipotent adult progenitor cells (MAPCs) derived from mouse, rat, and human BM were treated with a number of different factors, including HGF, fibroblast growth factor-1 (FGF-1), FGF-2, FGF-4, FGF-7, bone morphogenetic proteins (BMPs), oncostatin M (OSM), dimethyl sulfoxide (DMSO), and sodium butyrate in the presence of fibronectin, Matrigel, or collagen coating. The effect of cell density was also tested, given that differentiation required cell cycle arrest. Optimal differentiation to hepatocyte-like cells was observed when MAPCs were plated at 2.15×10^4 cells/cm^2 in the presence of FGF-4 and HGF on Matrigel. At 2 weeks postinduction, approximately 60% and 90% of murine and human cells, respectively, were positive for albumin,

CK-18, and hepatocyte nuclear factor (HNF)-3β by immunofluorescence analysis; 17.3% and 31.3% of murine and human cells, respectively, were binucleated. Supplementation of FGF-1, FGF-2, FGF-7, BMPs, and OSM did not increase the percentage of differentiation, while DMSO and sodium butyrate did not support hepatic differentiation. Cell density was found to play a significant role in differentiation; densities below 1.25×10^4 were inhibitory to hepatic differentiation. Expression of early markers such as HNF-3β and GATA-4 was detectable in the first week of differentiation, while after 21 days greater than 90% of cells were positive for late markers of differentiation, including HNF-1α, HNF-4, CK-18, and albumin. It was further demonstrated that differentiated cells exhibited in vitro metabolic functions characteristic of hepatocytes.

Although the demonstration of hepatic potential from an extrahepatic somatic stem cell was a significant breakthrough, the question of the existence of MAPCs remained a puzzle. The successful isolation of MAPCs is dependent on the cellular senescence of plate-adhering marrow stromal cells after extensive subculturing and, subsequently, the emergence of a novel cell population differing in surface immunophenotype, proliferative potential, and differentiation capacities [25]. The existence of such a population was controversial and was further complicated by the lack of reproducibility. However, these findings provided the foundation for a reevaluation of the differentiation capacity of mesoderm-derived multipotent somatic stem cells and, in particular, mesenchymal stem cells (MSCs).

3 Mesenchymal Stem Cells

MSCs have been widely studied over the last decade, and a detailed review of these cells is beyond the scope of this chapter. A general overview of MSCs is given here, and readers are referred to the excellent reviews in the literature for further details [26–28].

As early as a half-century ago, it was observed that bony tissue formation resulted when BM was transplanted under the renal capsule of mice, illustrating the presence of osteogenic precursors in BM [29]. These osteogenic precursors were later isolated by Friedenstein et al. based on the property of adherence to tissue culture plastic [30]. It was shown that such precursors retained osteogenic capacity even after extensive culture expansion. These cells are fibroblast-like in morphology and were later shown to be capable of differentiation into osteoblasts, chondrocytes, adipocytes, and myoblasts even after extensive culture passaging [31–39]. Given the heterogeneity nature of cell types and the supportive nature in cellular functions of the isolated population, these cells were referred to as marrow stromal cells.

A decade ago, Pittenger et al. described conditions for culture expansion of a cell population that we now generally refer to as MSCs that exhibit relatively uniform expression of a panel of cell surface antigens and the ability to differentiate into

bone, fat, and cartilage [40]. However, even such a population was functionally heterogeneous, and not all cells are functionally equivalent in differentiation potential [41]. Studies of multiple single cell–originating clones derived from MSCs showed a varying degree of lineage potency. Less than one third of the clones were capable of trilineage differentiation, approximately half of the clones were of bipotential, and the remainder were capable of commitment into only a single cell lineage. There is still yet to be an operational definition for MSCs, but there is a general consensus that MSCs are cells of nonhematopoietic and nonendothelial origin that express CD29, CD44, CD73, CD105, and CD166 and are capable of differentiating into bone, fat, and cartilage lineages of the limb-bud mesoderm.

BM was the first known source of MSCs, but numerous groups have reported the successful isolation of MSCs from virtually all postnatal organs and tissues, including synovium, muscle, brain, spleen, liver, kidney, lung, adipose tissue, thymus, pancreas, trabecular bone, periosteum, umbilical cord blood, amniotic fluid, placenta, scalp tissue, and peripheral blood [42–61]. The wide accessibility of MSCs from postnatal tissues implies an abundance of autologous cells for a variety of applications such as tissue engineering and cell therapy.

4 Hepatogenic Potential of Mesenchymal Stem Cells

Liver development begins from the ventral foregut endoderm, and early expression of AFP and albumin mRNA is detected prior to morphologic specification. Hepatocyte precursors proliferate and migrate toward cardiac mesoderm in the septum transversum. FGF-1, FGF-2, and FGF-8 secreted by the cardiac mesoderm induce commitment and expression of liver genes; this is followed by the vascularization of the liver bud and increases in liver mass [62, 63]. Several factors have been found to be essential in the formation of the liver bud, including HGF, c-met, c-jun, and Hlx [64–67], while other factors such as rel-A, N-myc, sek-1, and jumonji are critical in later stages of liver development [68–71]. MSCs are ideal candidates as an extrahepatic source of hepatocytes because they are known to express a number of growth factor receptors, including FGF receptors (FGFRs), including FGFR-1, and c-met, the receptor for HGF [72–76]. Both of these receptors play an important role in the molecular signaling during early development of the liver. FGFR-1 is required in the formation of the liver bud from the foregut endoderm, and disruption of this gene results in failure of the mesodermal and endodermal lineages, leading to early embryonic lethality [77]. Another pathway of importance to the formation of the liver bud is HGF/c-met signaling, and disruption of HGF and c-met is embryonically lethal and results in small liver formation [64, 65].

Our laboratory set out to investigate the hepatogenic potential of MSCs using clonally derived cells isolated from both BM and umbilical cord blood. On the basis of studies of embryonic development and liver regeneration, we designed a two-step protocol to effect the hepatic differentiation of MSCs, comprising an initial

induction phase and a later maturation phase [48, 78]. To induce transition of MSCs from a mesodermal lineage into an endodermal lineage, differentiation medium in the induction phase was supplemented with HGF, FGF-2, and nicotinamide [78]. HGF is a pleiotropic cytokine exhibiting mitogenic, morphogenic, motogenic, and antiapoptotic activities [79]. Upon treatment with induction medium the first response noted is an increase in cell numbers, even at near culture confluency, as well as a slight change in cell morphology. Cells become slightly broadened, resulting in increased cell–cell contact (Fig. 1), consistent with the finding that hepatogenic inductions are more effective at higher cell densities [24]. By treatment with induction medium, moderate expression of early and intermediate liver marker genes such as AFP, albumin, and CK-18 was induced, as well as low expression levels of several late marker genes, including alkaline phosphatase, tryptophan 2,3-dioxygenase, HNF-3β, cytochrome P450 1A1, and cytochrome P450 7A1 [80–84].

To induce maturation of the aforementioned committed hepatocyte-like cells into functional hepatocytes, medium in the maturation phase was supplemented with OSM, dexamethasone, and ITS+ premix supplement [78]. OSM is a pleiotropic cytokine of the interkeukin-6 group that transduces signals through the gp130 transmembrane protein and the Janus kinase–signal transducers and activators of transcription pathway. Studies have shown that OSM plays an important role in hepatic differentiation during fetal liver development [85, 86], as well as in the maturation of hepatocytes under in vitro culture by inhibiting cell proliferation [87–90]. MSCs have been shown to express OSM receptor, and treatment with OSM further induces and upregulates expression of late-stage and mature liver marker genes such as tyrosine aminotransferase, glucose 6-phosphatase, α1-antitrypsin, connexin-32, HNF4, cytochrome P450 2B6, and DPPIV [78, 82, 91–93]. Further cytoskeletal changes are induced by treatment of cells with maturation medium; cells lose the fibroblastoid morphology and cell bodies retract to become more polygonal (Fig. 1). Cells gradually develop a cuboidal morphology of epithelial cells in a time-dependent manner and accumulate cytoplasmic granules that are characteristic of hepatocytes. Not only do differentiated cells produce albumin, they also exhibit a variety of liver functions, including uptake of low-density lipoprotein, storage of glycogen, secretion of urea, and cytochrome enzymatic activities to metabolize xenobiotics [48, 78, 81].

In addition, a number of subsequent studies have reported various factors that enhance the commitment of MSCs into hepatocytes, including trichostatin A (TSA), 5-azacitidine, leukemia inhibitory factor (LIF), and activin A [83, 84, 94, 95]. It is conceivable that inhibition of histone deacetylase by TSA enhances expansion of the chromatin to allow increased transcription of the relevant genes upon stimulation with hepatic-inducing factors. TSA has also been reported to play an important role in maintaining the functional differentiation of primary cultured rat hepatocytes [96]. Pretreatment of MSCs with 5-azacitidine and incorporation into DNA would lead to inhibition of methyltransferase, thereby resulting in demethylation of DNA, which may enhance transcription of hepatic genes in the presence of inducing factors. LIF is a member of the interleukin-6 group of cytokines,

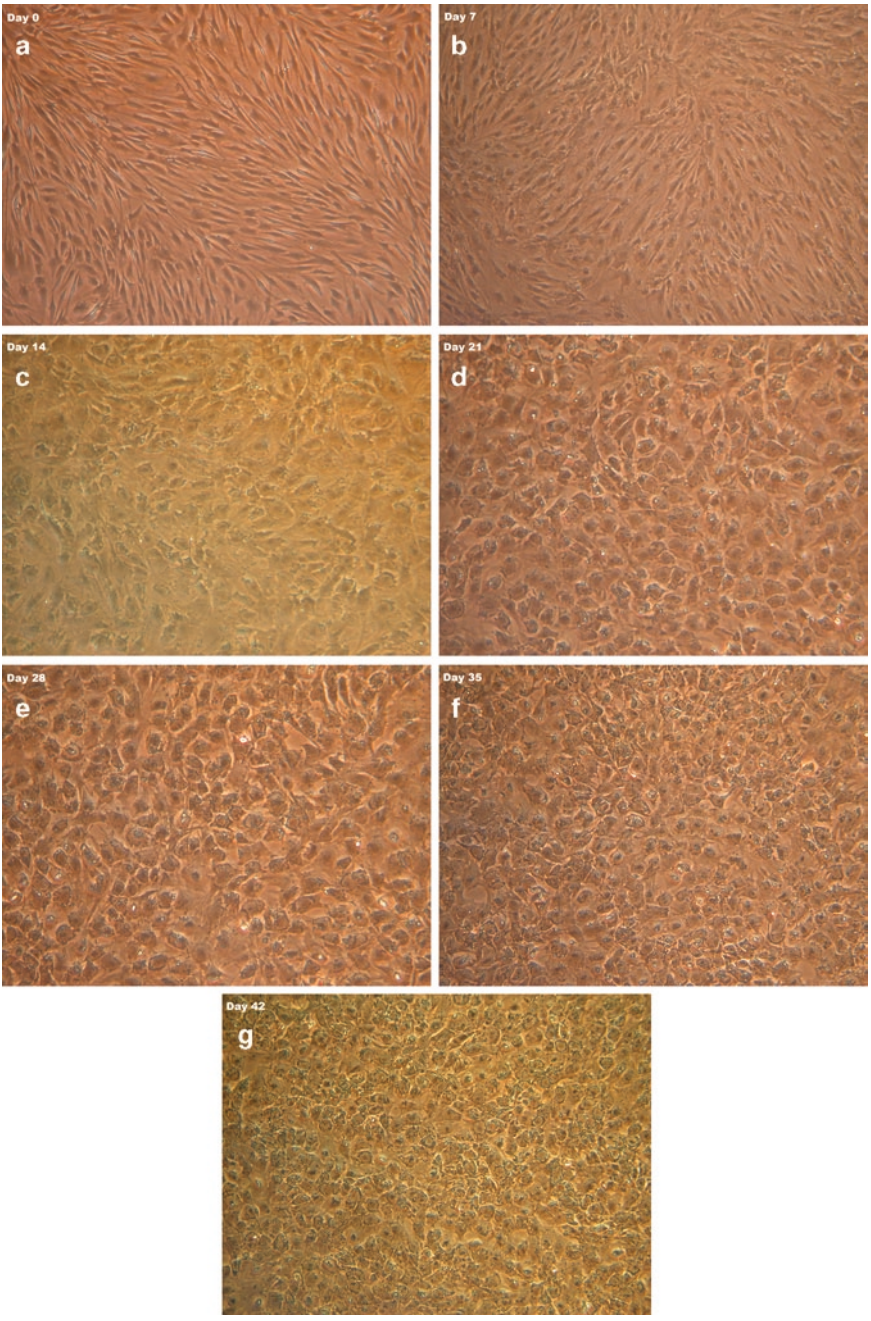

Fig. 1 Photomicrographs showing changes in the morphology of mesenchymal stem cells (MSCs) through hepatic differentiation. **a:** Undifferentiated MSCs. **b:** Hepatic induction 7 days. **c:** Hepatic induction 14 days. **d:** Hepatic induction 21 days. **e:** Hepatic induction 28 days. **f:** Hepatic induction 35 days. **g:** Hepatic induction 42 days

closely resembling OSM in structure and function, and, therefore, it is conceivable that it has similar hepatic inductive effects on MSCs.

The hepatic potential of MSCs was further confirmed by a number of animal studies. Using mice and sheep models of in utero transplantation, if was found that MSCs readily contributed to the development of the liver, among a variety of other tissues [97, 98]. MSCs transplanted into mice with sublethal liver injuries engrafted in the periportal regions of the liver and differentiated into functional hepatocytes without evidence of fusion with host hepatocytes [99, 100]. Chamberlain et al. demonstrated that intrahepatic injections of MSCs into fetal sheep resulted in donor-derived hepatocytes widely distributed throughout the liver parenchyma, whereas periportal distribution was observed when cells were injected intraperitoneally [101]. Similarly, MSC-derived hepatocytes infused into mice also showed engraftment in the liver and resulted in further functional maturation of donor cells [92].

5 Therapeutic Application of Mesenchymal Stem Cells for Liver Diseases

Liver transplantation is presently the only effective treatment for a variety of end-stage liver diseases. However, donor organs are extremely scarce, and most patients die before a suitable donor organ becomes available for transplantation. As an alternative to organ transplantation, hepatocyte transplantation was shown to be therapeutically effective and was thought to hold great promise. Even though hepatocyte cell numbers equivalent to 0.5%–3% of the liver parenchyma may be sufficient to restore partial liver function [102], the paucity of cadaveric liver renders the procurement of transplantable cells difficult [103]. After cryopreservation, viability has been approximated to be 65%–75%, and far less for cells that retain characteristic functions when thawed [104]. Upon infusion, less than 20%–30% of transplanted hepatocytes survive, and multiple transplantations are required to achieve liver repopulation [105–107]. These factors imply a need for ex vivo expansion for clinical application to be of potential effectiveness.

While hepatocytes have shown great replicative potential in vivo, their expandability in vitro is restricted by their limited lifespan and the rapid loss of hepatic functions under culture [108]. Long-term primary cultures of hepatocytes from various mammalian species have been studied extensively over the last three decades and, while improvements in culture conditions have been made to sustain characteristic hepatic functions, cultured hepatocytes show little replicative capacity in vitro [109–112]. It was not until the last decade that conditions enhancing the in vitro proliferative potential of human hepatocytes while retaining differentiated phenotypes have been reported [111,112]. Nevertheless, it is difficult to obtain large numbers of human hepatocytes for clinical applications. Furthermore, the documented growth potential of hepatocytes was based on experiments using murine hepatocytes, which are known to constitutively exhibit telomerase activity, while

human hepatocytes do not, and it is unclear whether adult human hepatocytes will possess the same degree of proliferative capacity [103]. In addition, other complications include pulmonary embolism, pulmonary hypertension, arrhythmias, potential development of heart failure, and the demand for lifelong immunosuppressive treatments for patients receiving transplantation [102, 105, 113].

The hepatogenic nature of MSCs makes it an invaluable extrahepatic source of transplantable cells. Not only are MSCs easily accessible from a variety of postnatal tissues, but they are also easily proliferated under in vitro culture to yield large numbers of cells, making possible the derivation of the magnitudes of hepatocytes required for transplantation. However, in animal studies, the frequencies of transplanted MSCs differentiating into hepatocytes were found to be relatively low overall, and only rare incidences up to 12.5% ± 3.5% were reported [101]. These findings cast doubt on whether MSCs possess sufficient therapeutic feasibility for the treatment of liver diseases.

Using a mouse model of chemically induced fulminant hepatic failure (FHF), our laboratory examined the therapeutic effect of transplantation of MSC-differentiated hepatocytes [114]. By administration of a lethal dose of carbon tetrachloride (CCl_4), we found that recipient mice developed submassive necrosis of the liver, leading to 100% lethality within 6 days in the absence of cell transplantation. However, a single transplantation of MDHs was effective in rescuing up to two thirds of mice from hepatic dysfunction, and rescued animals showed complete recovery of liver functions, as well as reconstitution of the liver parenchyma. These results suggest that MSCs may serve as an alternative source of transplantable hepatocytes. Of interest, MSCs demonstrated further value and applicability in the treatment of liver disease in the undifferentiated form. Transplanted MSCs not only showed up to 100% efficiency in rescuing mice from FHF, but also reduced the cell dose requirement by an order of magnitude. Transplanted cells readily engrafted recipient liver, but only a small fraction of cells differentiated into functional hepatocytes in the liver. By laser capture microdissection, human albumin-expressing cells were confirmed to be of donor origin, and this was further corroborated by fluorescence-activated cell sorting and Western blotting assays. It was found that antioxidative and paracrine effects conferred by donor MSCs played a significant role, promoting cell divisions of resident cells to effect rapid regeneration of the host tissue. In another study, injection of MSC-derived molecules partially reversed FHF in rats, further supporting the paracrine-mediated rescue of FHF [115]. The hepatoprotective effect of MSCs was corroborated by another study demonstrating that human umbilical cord–derived MSCs engrafted and differentiated into functional hepatocytes in an animal model of CCl_4-induced damage and enhanced liver recovery [116].

Unlike acute liver injury, liver fibrosis and cirrhosis, the most advanced stage of fibrosis, is a wound-healing response that involves fibroblasts and other cells. The sustained signals associated with chronic liver disease caused by infection, drugs, metabolic disorders, or immune attack are required for significant fibrosis to accumulate [117]. In animal models of chronic injury, liver fibrosis is induced in rats by multiple intraperitoneal injections of CCl_4 or dimethylnitrosamine (DMN). Zhao et al. induced liver fibrosis in rats for 6 weeks using CCl_4 and DMN and infused

bone marrow–derived MSCs intravenously at 3×10^6 per animal [118]. Animals infused with MSCs showed improved vigor, active food intake, and grooming activities, while control animals appeared extremely lethargic, exhibiting pale eyes and even labored respiration. By histologic and Masson trichrome staining, a significant reduction in inflammation, collagen accumulation, and fatty degeneration was evident in animals transplanted with MSCs. In addition, liver hypoxyproline content and serum concentrations of hyaluronic acid and laminin were reduced, and alanine aminotransferase (ALT) and total bilirubin levels were near normalized. These findings were also confirmed by other studies [119, 120]. Oyagi et al. reported that transplantation of HGF-treated bone marrow mesenchymal cells into CCl_4-injured rats returned serum albumin concentrations to normal levels and significantly suppressed serum levels of aspartate aminotransferase (AST) and ALT in the recipient animals [121]. Furthermore, histologic analysis by Azan staining revealed dramatic reduction in fibrosis compared with nontransplantation control. Consistent with these studies, Jung et al. reported that various markers of liver function, including AST, ALT, albumin, total serum protein, total bilirubin, and alkaline phosphatase, were improved in CCl_4-injured rats by infusion of MSCs [122]. By evaluation of cirrhosis-related factors, it was found that MSCs significantly inhibited the expression of transforming growth factor-beta1, type I collagen, and alpha-smooth muscle actin in the liver of CCl_4-induced cirrhotic rats. However, in one study, Carvalho et al. reported the absence of functional improvement or reduction in fibrosis by MSC transplantation in a rat model of severe chronic liver injury [123]. Severe chronic liver injury was established by injection of CCl_4 every other day for 15 weeks, and 1×10^7 isogenic rat MSCs were injected into the portal vein of each recipient animal. Histologic and biochemical function assay were performed prior to and at 1–2 months after transplantation. It was found that injection of both placebo and MSCs near-normalized serum concentrations of AST, ALT, and albumin but without statistical significance between placebo and MSC transplantation. The extent of liver fibrosis also appeared to have been alleviated by both placebo and MSCs but, again, without significant difference between the two groups.

6 Clinical Outcomes of Mesenchymal Stem Cells for the Treatment of Liver Diseases

Although detailed molecular mechanisms regulating the hepatic specification of MSCs are still being elucidated, preclinical investigations of the application of MSCs for liver diseases have rapidly emerged. Results from these studies have encouraged clinical investigations of the therapeutic benefit of MSCs for liver diseases.

Terai et al. first investigated the effect of bone marrow mononuclear cell (MNC) transplantation on patients with liver cirrhosis [124]. Autologous bone marrow were harvested from the ilium, and $5.20 \pm 0.63 \times 10^9$ MNCs separated from the bone marrow were infused into each patient. Liver functions were monitored by

blood examinations for 24 weeks, and significant improvements in serum albumin levels and total protein were observed at 24 weeks after cell transplantation. Child-Pugh scores demonstrated significant improvements in overall liver functions at 4 and 24 weeks after cell infusion. Expressions of AFP and proliferating cell nuclear antigen were found to significantly increase in liver biopsies after cell transplantation, suggesting an induction of hepatocyte proliferation by cell infusion. Serum pro–collagen III peptide level was monitored as a marker of liver fibrosis, but cell transplantation had only mediated a slight decrease without statistical significance. Levels of HGF were also slightly elevated, but the change was not significant. No adverse effects were observed.

Mohamadnejad et al. conducted two concurrent phase I trials on patients with decompensated liver cirrhosis. In one trial, autologous HSCs were infused, while in the other trial autologous MSCs were infused [125, 126]. In the HSC transplantation trial, patient 1 showed marginal improvement in serum albumin but without significant changes in other assays; patient 2 displayed reduced prothrombin time but worsened scores in the Model of End-Stage Liver Disease (MELD), total bilirubin, and serum creatinine; patient 3 exhibited improvements in serum albumin, prothrombin time, and MELD score; and patient 4 developed radiocontrast nephropathy and died of liver failure. The trial was terminated as a result of patient death [125]. On the other hand, transplantation of MSCs exhibited no adverse effects, and MELD scores of patients 1 and 4 improved by 4 and 3 points, respectively, and the quality of life of all four patients improved by the end of follow-up [126].

A phase I/II clinical study on the treatment of liver cirrhosis with autologous MSCs was completed recently [127]. Eight patients with MELD scores greater than or equal to 10 were enrolled, and bone marrow was harvested from the iliac crest. MSCs were isolated and culture expanded, and 30–50 $\times 10^6$ cells were infused into the peripheral or the portal vein. Functional evaluations were performed at baseline and at 1, 2, 4, 8, and 24 weeks posttransplantation. It was found that autologous MSC transplantation was well tolerated by all patients, and no adverse effects were seen. MELD scores decreased from 17.9 ± 5.6 to 10.7 ± 6.3 ($p < 0.05$); prothrombin complex from international normalized ratio decreased from 1.9 ± 0.4 to 1.4 ± 0.5 ($p < 0.05$); Serum creatinine decreased from 114 ± 35 to 80 ± 18 μmol/L ($p < 0.05$); no significant changes in serum albumin and total bilirubin were noted.

Clinical data on the therapeutic benefits of MSCs for liver diseases are limited, and more investigations are required. Nevertheless, the aforementioned trials have shown encouraging results and warrant further research into the application of MSCs for liver diseases. At present, three other clinical trials using MSCs to treat liver cirrhosis are being conducted (http://www.clinicaltrials.gov).

7 Regulatory Signaling Network of Liver Generation

Although a growing body of evidence has demonstrated that MSCs possess tremendous lineage plasticity to cross germ layer and differentiate into hepatocytes [24, 78], and clinical trials of MSCs for the treatment of liver diseases are currently

under investigation, little is known about the precise molecular mechanisms controlling this process.

Early studies revealed that the development of the embryonic liver requires a number of signals from mesodermal cells, and that repression of Wnt/β-catenin and FGF-4 signaling promotes liver induction [128]. In mouse, FGFs and BMPs are required to induce hepatic genes expression at E8.5, corresponding to about 3 weeks of human gestation [129]. Recently, Wandzioch and Zaret studied the roles of BMP, TGF-β, and FGF signaling pathways in the induction of pancreas and liver. It was found that the signaling networks of these pathways are dynamic and operate in parallel, and independently, on different downstream target genes that are important for cell fate specification [130].

Similarly, two independent studies have revealed a role of Wnt/β-catenin signals in hepatic fate specification of MSCs [83, 131]. Yoshida et al. reported that upon hepatic commitment of MSCs, the expression levels of many Wnt signal-related molecules were downregulated, including protein phosphatase 2 catalytic subunit, β-catenin (CTNNB1), Wnt-family genes, and Frizzled-family genes [83]. On the other hand, Wnt-inhibitory genes such as β-catenin interacting protein 1 (CTNNBIP1) and protein phosphatase regulatory subunit (PPP2R1B) were downregulated. In addition, a translocation of β-catenin was observed during hepatic differentiation, from predominant localization in the nucleus to the cell membrane and cytoplasm. Furthermore, RNA interference of Frizzled-8 induced expression of albumin, CCAAT-enhancer-binding protein alpha (C/EBPa), and cytochrome P450 family 1 member A1/2 and induced storage of glycogen, as well as translocation of β-catenin from the nucleus to the cytoplasm and cell membrane. Consistent with these findings, Ke and colleagues compared the expression of genes in MSCs cultured in the presence of damaged-liver extract with those cultured in the presence of normal-liver extract and found that Wnt/β-catenin signaling genes were downregulated during hepatic induction of MSCs [131]. Genes found to be downregulated include Wnt-1, Wnt-5a, Frizzled-1, Disheveled, glycogen syntheses kinase-3β, and β-catenin. Furthermore, inhibition of Wnt signaling with exogenous Dickkopf-1 induced earlier expression of albumin under hepatic inductive conditions.

Analysis of the transcriptome and signaling pathways in hepatic differentiation of MSCs revealed that 1639 genes exhibited an alteration of 10-fold or greater, of which 1252 genes were upregulated and 387 genes were downregulated [132]. By Gene Ontology classification, genes upregulated were involved in inflammatory response, complement activation, innate immune response, blood coagulation, adaptive immune response, response to chemical stimulus, circulation, hormone metabolism, lipid metabolism, steroid metabolism, cytolysis, response to xenobiotic stimulus, carboxylic acid metabolism, nitrogen compound metabolism, and fibrinolysis; genes downregulated were involved in cells division, cell cycle, chromosome segregation, organelle localization, and cytoskeleton organization and biogenesis. Moreover, regulators of the epithelial–mesenchymal transition such as Twist and Snail were downregulated; expression of mesenchymal markers such as N-cadherin and vimentin were downregulated; and epithelial markers such as E-cadherin and α-catenin were upregulated. These expression profiles suggest a pivotal role of mesenchymal–epithelial transition in the hepatic differentiation of MSCs.

8 MicroRNA

Not only do protein-coding genes participate in the regulatory network of hepatocyte generation but also a newly discovered noncoding RNA family termed microRNA (miRNA) has been demonstrated to be important in controlling cell behavior and differential fates and to contribute an additional layer of control in the regulatory network. miRNAs are genomically encoded, small, noncoding RNA that regulate gene expression by controlling either translation or stability of mRNAs through an RNA interference–like pathway. The first miRNA to be found was lin-4, in *Caenorhabditis elegans* [133, 134]; it functions as a trigger to regulate a cascade of gene expression posttranscriptionally that distinguishes one larval developmental stage from another [133]. The importance of miRNAs became apparent when they were identified from several organisms and their sequences were found to be phylogenetically conserved [135, 136]. According to the most recent release of the miBase Registry (13.0, released March 2008), there are around 9539 distinct mature miRNA sequences from 58 species, including 851 human miRNAs [137]. The human miRNAs comprise 1%–3% of the human genome [138–141]. miRNAs have been demonstrated to be involved in several physiologic processes, including aging, differentiation, hematopoiesis, and endocrine functions.

The biogenesis of miRNA is initiated by RNA polymerase II transcription [142–145]. The primary transcripts (pri-miRNAs) containing both a 5′ cap and a 3′ poly(A) tail are first cleaved to release a hairpin-like precursor (pre-miRNA) by a microprocessor complex that consists of a member RNase III family named Drosha and its cofactor DGCR8 (known as Pasha in flies and nematode) [146–149]. Pre-miRNA possesses a stem-loop structure with a 5′ phosphate and a 2-nucleotide (nt) 3′-overhang [149]. Because of the designation of the miRNA sequence, the progression of pri-miRNA is an important step in miRNA biogenesis to create one end of the molecule from long pri-miRNAs [150]. Following the initial processing in nucleus, the pre-miRNAs are exported to cytoplasm by the nuclear transport factor exportin-5 (Exp5) [151]. The exported pre-miRNAs are processed into 22-nt miRNA duplexes by a second RNase III enzyme, Dicer [152,153]. One strand of the Dicer-generated RNA duplex remains as a mature miRNA and is assembled into the effector complex to form miRNA-induced silencing complex (miRISC) or miRNPs [154–156]. Recruitment of miRNPs in plant requires extensive sequence complementarity and typically causes the cleavage of target mRNA [157]. In contrast, animal miRNAs exert the repression of translation by binding to imperfect complementary sites within the 3′ untranslated regions (3′-UTRs) of their target mRNAs. The putative mechanisms of repression of mRNA expression by miRNA include blocking of the initiation of translation, ribosome dropoff during elongation of translation, and the recruitment of miRNPs to processing bodies (P bodies), which contain untranslated mRNA and can serve as sites of mRNA degradation [158–162]. This type of gene expression control reveals a novel regulatory mechanism and affects a number of crucial cellular processes.

8.1 MicroRNAs in Stem Cells

One of the important biologic functions of miRNAs is the regulation of development. In *C. elegans*, mutations in *lin-4* and *let-7* result in heterochronic phenotypes [133, 163, 164]. In plants, miRNAs have been shown to target transcription factors that are involved in development [165, 166]. In *Drosophila melanogaster*, the gene *bantam* encoding miRNA is spatially and temporally expressed during development and controls cell proliferation and apoptosis [167, 168]. In mouse, a screening of miRNAs cloned from organs indicated that many of them were organ specific, consistent with roles in development [169, 170]. In complicated developmental processes, miRNA may fine tune or restrict cellular fate by targeting key transcription factors or important pathways [171]. A series genetic studies showed the requirement of miRNAs in maintenance of ES cell self-renewal and pluripotency. ES cells with genetic deletion of key miRNA processing enzymes DGCR8 or Dicer lose their pluripotency and display defective differentiation [172–175]. Dicer is required for both miRNA and small interfering RNA pathways, whereas DGCR8 participates only in miRNA biogenesis. Both DGCR8-deficient and Dicer-null ES cells showed similar effects on the expression of differentiation markers and reduced the kinetics of cell cycle progression [172, 175], suggesting that the function of Dicer is predominantly in the miRNA pathway in ES cells and the major function of miRNAs during the differentiation of ES cells is involved in the regulation of cell cycle progression [176].

Several reports revealed that a core regulatory circuitry of transcription controls the self-renewal and pluripotency properties of ES cells [177–181]. Oct4, Sox2, and Nanog function as central regulators to the transcriptional control hierarchy that determines the fate of ES cells [177, 182, 183]. Moreover, miRNAs, including miR-134, miR-296, miR-470, and miR-145, have been shown to modulate the pluripotency of ES cells by repressing the expression of Oct4, Sox2 and Nanog [184, 185]. In contrast, miR-294 can replace c-myc and work with Oct4, Sox2, and Klf4 to promote induced pluripotency [186]. Knowing that both protein-coding genes and noncoding genes are regulated by key transcription factors provides novel insights into the molecular mechanism of cell differentiation and reprogramming of ES cells.

Thus far, little has been reported on the role of miRNAs in MSCs, and its expression and function in the differentiation of MSCs remain largely unknown. Using microarrays and quantitative reverse transcription-polymerase chain reaction to detect the alteration of miRNAs during MSC differentiation, Laskshmipathy et al. showed that miR-38 and miR-663 are novel markers in chondrocytic differentiation of MSC [187]. In addition, the expressions of several miRNAs, including miR-24, let-7a, let-7b, let-7c, miR-138, and miR-320, were altered during osteogenic differentiation of MSCs. A subset of these miRNAs was further demonstrated to be regulated by platelet-derived growth factor signaling during osteogenesis [188]. Schoolmeesters et al. used a library of miRNA inhibitors to investigate the role of miRNAs in osteogenesis of MSCs. Their results showed that miR-148b, miR-27a,

and miR-489 play important roles during osteogenic differentiation of MSCs [189]. Apparently, the two groups identified different sets of osteogenesis-related miRNAs. This might be due to either the different experimental approaches or a variability of miRNA expression profiles between individual hMSC donors. The roles of miRNAs in hepatogenic differentiation of MSCs have not been uncovered yet.

8.2 MicroRNAs in Liver Development

In liver, miR-122 is the most abundant miRNA, and it is recognized as a liver-specific miRNA [170, 190]. A number of studies have revealed that miR-122 mediated cholesterol and lipid metabolism, suggesting the importance of miRNAs in liver [191–193]. Furthermore, the expression level of miR-122 is associated with liver diseases, such as hepatitis C infection, nonalcoholic steatohepatitis, and hepatocellular carcinoma [194–200]. Although miR-122 is involved in a broad range of physiologic and pathologic processes, little is known about the roles of miRNAs in the liver. Recently, the function of hepatocytes and the expression of miRNAs were examined in liver-specific Dicer-knockout mice. Mice with miRNA deficiency in the liver showed a normal phenotype, such as normal behavior, blood glucose, albumin, cholesterol, and bilirubin [201]. On the other hand, the conditional Dicer-deficient mice developed hepatic damage, including hepatocyte apoptosis, hepatocyte regeneration, and portal inflammation, as the mice aged. These results suggest an essential role for miRNAs in sustaining normal hepatic functions throughout the lifespan of the organism.

Two recent studies showed that the miR-30 family and the miR-23b cluster are involved in development of the liver [202, 203]. Repression of miR-30a function by an antisense oligo caused defective biliary morphogenesis in the zebrafish, demonstrating a functional role for miRNA in hepatic organogenesis [202]. High levels of miR-23b cluster, which comprises miR-23b, miR-27b, and miR-24, downregulated Smad 3, 4, and 5 to block TGF-β/BMP signaling pathways, resulting in the repression of bile duct genes and the promotion of hepatocyte proliferation. In contrast, lower levels of miR-23b cluster keep Smad protein stable and allowed TGF-β/BMP signaling pathways to activate, facilitating the differentiation of cholangiocyte and the formation of bile duct [203]. Taken together, the evidence shows that miRNA indeed plays a critical role in hepatogenic differentiation.

9 Conclusions

On the basis of tremendous efforts from various groups worldwide, it is clear that the differentiation potential of mesenchymal stem cells is much greater than previously thought. The ability to produce hepatic progenies certainly will further expand the usefulness of mesenchymal stem cells in clinical applications to treat

hepatic disorders. Although embryonic stem cells have been believed to be more pluripotent, use of mesenchymal stem cells clinically can avoid ethical issues as well as the risk of tumor formation. Of importance, the concept of plasticity needs to be revisited from the perspective of mesenchymal stem cells, which are harvested from mesodermal-derived tissues and can then differentiate into endodermal lineage progenies. This unique platform will serve as an excellent model to help elucidate the molecular machinery governing somatic stem cell plasticity.

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The Role of Time-Lapse Microscopy in Stem Cell Research and Therapy

Kevin E. Loewke and Renee A. Reijo Pera

Abstract Stem cell therapy holds enormous potential for treating a wide range of genetic and sporadic degenerative disorders. However, one of the major hurdles facing stem cell therapy is the ability to assess cell fate or outcome prior to transplantation. Recent studies have shown that time-lapse microscopy may be a useful tool to assess cell fate via observation of dynamic behavior at the single-cell and population levels. The ideal embodiment of time-lapse microscopy would be a high-throughput, noninvasive device that can identify stem cells that form nontumorigenic differentiated progeny capable of integration into mature tissues. Such technologies are on the horizon and hold promise for clinical and therapeutic applications.

Keywords Imaging • Time-lapse microscopy • Stem cells

1 Introduction

A stem cell is a cell that possesses the ability to self-renew and the capacity to differentiate [1]. Essentially, self-renewal is the process of going through multiple cell divisions in the undifferentiated state, while differentiation is the process of becoming more specialized and transforming stepwise to a specific cell type. There are several different types of stem cells, but here we focus primarily on embryonic stem cells and adult stem cells. Embryonic stem cells are derived from the inner cell

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mass of a 5- to 6-day old embryo, are pluripotent, and can differentiate into any of the three germ layers, the germ cell lineage, and extraembryonic cells. In contrast, adult stem cells are generally considered tissue and/or organ specific and have a more limited potency, and they can only differentiate into a closely related family of cells. In addition to these cell types, remarkable advances have been made in generating cells that possess properties similar to embryonic stem cells called induced pluripotent stem cells (iPSCs). These cells are produced by harvesting adult somatic cells and reprogramming them by introduction of a small set of genes [2, 3].

Stem cells have the potential to treat a broad range of genetically based and sporadic degenerative disorders [4]. The basic "formula" of stem cell therapy is that undifferentiated stem cells may be cultured in vitro, differentiated to specific cell types, and subsequently transplanted to recipients for regeneration of injured tissues and/or organs. Potential applications include the treatment of neurologic disorders such as Alzheimer and Parkinson diseases, vascular system disorders and heart diseases, muscular and skeletal disorders such as arthritis, and autoimmune diseases and cancers [5]. There is also significant interest in using stem cells for drug discovery by evaluating targets and novel therapeutics [6].

Several bottlenecks impede progress in moving pluripotent stem cells such as embryonic stem cells from basic bench science to clinical applications. To enable stem cell–based therapies, new techniques will be necessary to assess cell fate (i.e., viability, tumorigenicity, and differentiation state) prior to transplantation. Ideal techniques to overcome the bottleneck in cell fate prediction would be both high throughput and noninvasive. To illustrate this idea, consider the following scenario: A patient suffering from knee pain is found to have worn knee cartilage due to degenerative arthritis. The patient elects stem cell therapy as an alternative to total knee replacement, where small quantities of cartilage cells are grown in a laboratory and subsequently transplanted into the knee joints. In this situation, rather than grow a single tissue sample, it will be desirable to grow hundreds of cell samples simultaneously, monitor their differentiation, and select the optimal culture(s) for transplantation. This process will require the use of high-throughput technologies for successful implementation, especially for culture under strictly defined, clinically applicable conditions and for monitoring the processes of self-renewal, differentiation, and posttransplantation outcomes.

Time-lapse microscopy may be one of the cornerstone technologies for assessment of stem cells and outcomes as it can allow observation of dynamic events in a large number of cells at the single-cell level. Here, we review the state of time-lapse microscopy and its utility in stem cell research. Although significant advances have been made, the use of time-lapse microscopy for studying stem cell behavior is in its infancy. We propose future directions that encompass the unique advantages of time-lapse microscopy and its potential to be integrated with other technologies.

2 Current Methods in Stem Cell Research

2.1 Genomics and Proteomics

The process of stem cell differentiation can be characterized, at least in part, by intricate patterns and profiles of gene expression and protein activity. Accordingly, the most commonly used tools for studying stem cells have traditionally been gene expression profiling and proteomic analysis [7, 8]. Both of these are methods are high-throughput technologies that are capable of analyzing hundreds or thousands of samples simultaneously. A fundamental limitation, however, is that both require the sample to be destroyed. Accordingly, population-based studies can be used to obtain expression patterns as a function of developmental stage. This approach has been applied, for example, to characterize the development of preimplantation human embryos [9].

2.2 Live-Cell Imaging

Stem cell behavior has also been studied using live-cell imaging technologies [10]. Live-cell imaging can be categorized into two groups: in vivo molecular imaging and in vitro imaging. In vivo molecular imaging modalities include, for example, magnetic resonance imaging (MRI), positron emission tomography, and single photon emission computed tomography to visualize cellular function through the use of biomarkers [11]. The choice of imaging modality depends on the specific application and usually involves a tradeoff in terms of spatial resolution, temporal resolution, and sensitivity. Molecular imaging is most useful for tracking stem cell survival and differentiation after transplantation into the body [12, 13]. Most studies have been limited to animal models, and further work is needed for human clinical applications [14].

More recently, there has been an increased use of in vitro imaging techniques, and in particular time-lapse microscopy, to study stem cells. With time-lapse microscopy, cells are grown under controlled conditions and imaged over an extended period of time that can range from several hours to several weeks [10]. The time interval between successive image captures depends on the specific application, but typically images are collected every few minutes. For stem cell applications, this can allow for the observation of dynamic cellular events such as differentiation at the single-cell level. Some of the more popular imaging modalities for use in time lapse imaging include wide-field fluorescence microscopy and confocal fluorescence microscopy, which detect light emitted by fluorophores that have been previously attached to the cells of interest [15]. A disadvantage of these techniques, however, is that illumination of fluorescent molecules can result in phototoxicity, altered biologic properties, and ultimately cell death.

2.3 *Light Microscopy*

The simplest and least-invasive form of live-cell imaging is light microscopy. In order to optimize imaging and increase contrast without having to stain samples, different illumination techniques can be used including, for example, dark-field, phase contrast, and differential interference contrast (DIC) (Fig. 1). In dark-field microscopy, a hollow cone of light is focused on the sample by placing a circular aperture between the light source and condenser lens. The objective lens collects light that is scattered by the sample and rejects directly transmitted light, producing a bright image on a dark background. Dark-field illumination is a low-cost approach for contrast enhancement and works well for visualizing cell edges in transparent samples [16].

DIC and phase contrast are complementary techniques that enhance contrast in images of thin, transparent samples [16]. DIC works on the principle of interferometry, in which a prism is used to split polarized light into two beams that take different optical path lengths through the sample. The two beams interfere upon being recombined, producing a change in image brightness or darkness according to the optical path lengths. DIC can give a three-dimensional appearance to thin, transparent samples, although this not topographically accurate [17]. Phase contrast

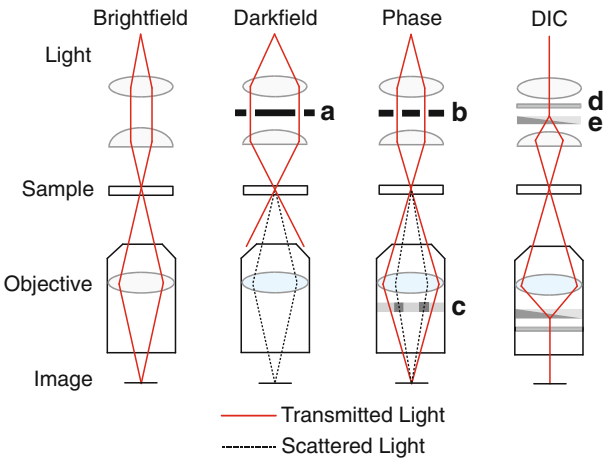

Fig. 1 Different illumination techniques for light microscopy. In brightfield, light is focused on the sample, collected by the objective, and focused to an image. In darkfield, an aperture (**a**) is placed before the condenser lens to create a hollow cone of light that is focused on the sample. The objective collects scattered light and rejects directly transmitted light, producing a bright image on a dark background. In phase contrast, an annulus (**b**) is placed before the condenser, and both the scattered light and directly transmitted light are collected and focused to an image. A phase plate (**c**) in the objective modulates the phase of transmitted light relative to the scattered light such that they interfere at the image plane to create contrast. In differential interference contrast (DIC), light is polarized with a filter (**d**) and split by a prism (**e**) to create two beams that take different optical path lengths through the sample. The beams interfere upon being recombined, producing contrast proportional to the optical path lengths

passes light through a condenser annulus to create a cone of light, similar to dark-field illumination. However, unlike dark-field illumination, both scattered light and directly transmitted light are collected and focused to the image plane. A phase plate in the objective lens modulates the phase of the transmitted light relative to the scattered light such that they interfere and create contrast. The annular rings transmit a small degree of diffracted light, which creates the commonly observed bright halos surrounding specimen boundaries [16]. Typically, DIC produces higher-resolution images than phase contrast since the objective and condenser apertures are not obstructed, allowing for full use of the numerical aperture. However, DIC is a more expensive approach and does not work well with plastic specimen carriers such as Petri dishes due to their interaction with the polarized light.

3 Applications of Time-Lapse Microscopy

3.1 Differentiation

Stem cell differentiation is the process by which an undifferentiated cell becomes specialized. Perhaps the most intuitive metric for characterizing differentiation is morphologic assessment, which includes tracking cell size, shape, configuration, and color (Fig. 2). For example, time-lapse phase contrast microscopy was

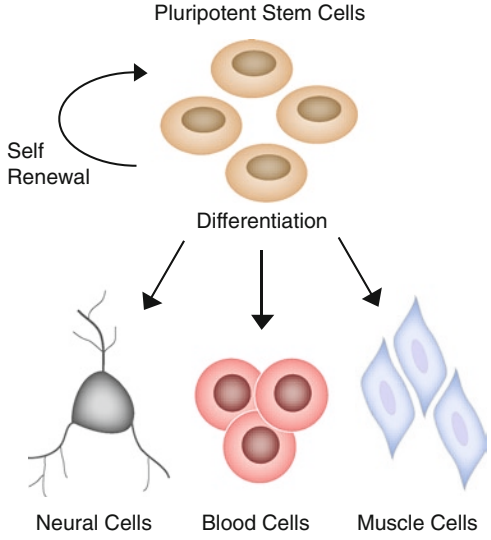

Fig. 2 Stem cell differentiation can be characterized in part by changes in morphology. For example, neural cells tend to form well-defined structures with thin radial processes, while blood cells tend to form round shapes and cluster together

used to observe the formation of primitive blood cell colonies from mouse blast colony–forming cells [18]. The process was characterized by two key morphologic events: the formation of tightly adherent clusters followed by the appearance of nonadherent round cells. Similarly, a combination of time-lapse phase contrast and wide-field fluorescence microscopy was used to observe blood cell generation at the single-cell level [19]. This was used to track the fates of mesodermal cells derived from mouse embryos and show that they can give rise to hemogenic endothelial cells. To identify differentiated blood cells, both morphologic and molecular markers were used.

In other efforts time-lapse phase contrast microscopy was used to determine whether biochemical manipulation could induce differentiation in mesenchymal stem cells (hMSCs) [20]. After transferring cells to a neural induction medium, it was observed that the hMSCs change temporarily from a fibroblastic morphology to adopt a pseudoneuronal morphology. Subsequent gene expression profiling showed that the morphologic changes were not representative of differentiation but rather of cytoskeletal collapse and cell shrinkage. Thus, this study suggested that a combination of morphologic analysis with independent measures such as gene expression profiling might be more optimal than examination of morphology on its own in classifying and characterizing differentiation.

3.2 Asymmetric Division

Stem cells balance the processes of self-renewal and differentiation through the establishment of asymmetric and symmetric divisions (Fig. 3). When stem cells divide asymmetrically, one daughter cell will maintain stem cell properties such as self-renewal while the other daughter cell differentiates. In symmetric cell division, both daughter cells will either remain as stem cells (symmetric renewal) or differentiate (symmetric commitment). Recently, a method was presented for identifying asymmetric cell division in mouse hematopoietic precursors

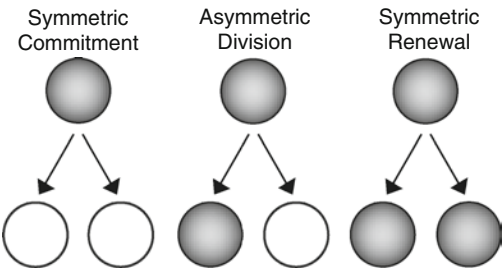

Fig. 3 Three types of stem cell division. In symmetric commitment, both daughter cells differentiate. In asymmetric division, only one daughter cell differentiates while the other daughter remains a stem cell. In symmetric renewal, both daughter cells remain as stem cells

using time-lapse fluorescence microscopy [21]. This study monitored dividing hematopoietic cells and their resulting daughter cells, and expression levels of green fluorescence protein (GFP) were obtained by measuring pixel intensities in the fluorescence images. Results indicated that differentiated daughter cells expressed lower levels of GFP than those that remained undifferentiated and that the balance between symmetric and asymmetric divisions could be modulated by extrinsic growth signals.

In other studies time-lapse phase contrast and fluorescent microscopy were used to track divisions in human embryonic stem cells [22]. Small stem cell colonies were monitored for a period of 5 days under four different culture conditions, three of which promoted distinct differentiation outcomes. Differentiation was identified through changes in morphology as well as reduced expression levels of a GFP-labeled transgene known to regulate pluripotency. Although it was predicted that both symmetric and asymmetric divisions would occur under different conditions, results suggested that human embryonic stem cells likely differentiated exclusively by symmetric cell division in each of the four conditions tested.

3.3 Fate Specification

Fate specification is the process of determining precisely when a stem cell differentiates (Fig. 4). This is of great importance to stem cell research as it can provide insight regarding cell potency, the frequency asymmetric divisions, and the maintenance of undifferentiated cells. Another method was presented for retrospective cell fate mapping of neural stem cells derived from the brain of an embryonic rat using time-lapse DIC microscopy [23]. Cell division and differentiation were monitored over a period of 6–8 days using a custom live-cell culture chamber and microscope. At the end of the experiment, immunostaining and analysis of cell morphology was performed to determine whether individual cells had differentiated into one of three

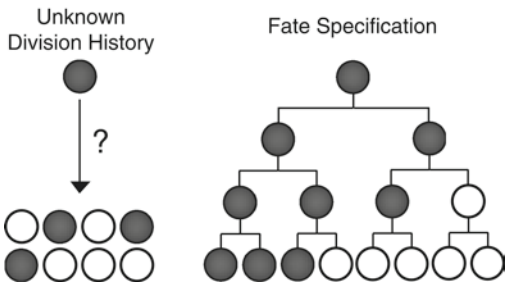

Fig. 4 If time-lapse data are not available, the division history of a group of cells is unknown. Time-lapse imaging can help to determine when fate is specified, which provides insight into potency and the balance of symmetric versus asymmetric divisions

possible cell types typically generated by neural stem cells. By manually tracking cell lineage in the time-lapse image data, it was possible to retrospectively identify when differentiation occurred as well as the potency of the unspecified cells.

3.4 Case Study: Time-Lapse Imaging of Human Embryogenesis

More recently, a combination of dark-field time-lapse microscopy and gene expression profiling was used to characterize early human embryo development [24]. In these studies, the authors monitored a total of 204 embryos in four experiments with multiple time-lapse microscopes for a period of up to 5 days (Fig. 5). At 24-hour intervals, one dish of embryos was removed from the imaging systems and collected as either single embryos or single blastomeres for subsequent gene expression analysis. The goal of this study was to document the growth of human embryos from zygote (fertilized egg) through the blastocyst stage, an early and definitive landmark of successful mammalian development. With blastocyst formation, the totipotent embryonic cells differentiate to either trophectoderm (extraembryonic) cells or the inner cell mass (cells of the embryo proper).

Via time-lapse image analysis, the authors retrospectively identified a set of dynamic imaging parameters that predict an embryo's potential to develop to the

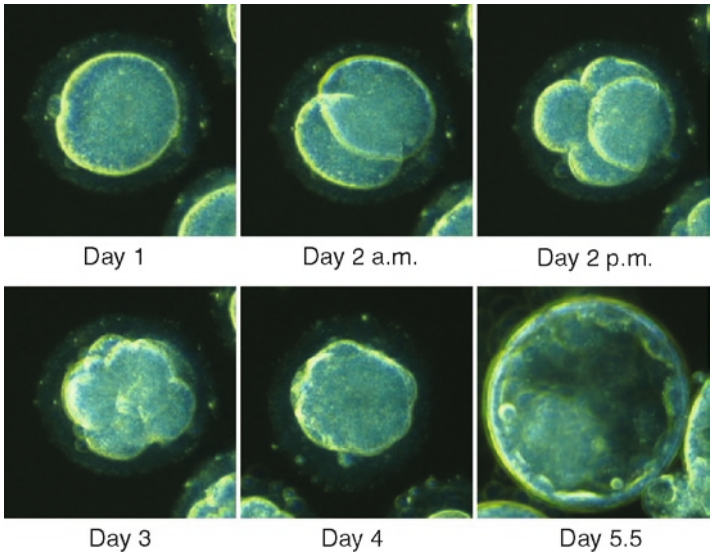

Fig. 5 Image sequence of human embryogenesis acquired with a time-lapse darkfield microscope [24]. The embryo starts as a zygote (fertilized egg) at day 1, undergoes a series of cell divisions, and turns into a blastocyst by day 5 to 6. Blastocyst is the first morphologic evidence of differentiation, where the totipotent embryonic cells differentiate to either trophectoderm (extraembryonic) cells or the inner cell mass (cells of the embryo proper)

blastocyst stage. The most robust parameters to predict developmental success were (1) the duration of the first cytokinesis, (2) the time interval between the first and the second mitosis, and (3) the time interval between the second and the third mitosis. These results have significant clinical implications since the parameters could potentially be used to prospectively identify the best embryos for transfer during in vitro fertilization procedures.

This study also investigated the correlation of time-lapse microscopy with gene expression profiling. In order to understand the molecular mechanisms behind normal and abnormal development, the authors performed gene expression profiling on groups of embryos at different developmental stages. They found a strong correlation between abnormal cytokinesis, as observed from the time-lapse image data, and aberrant expression of key genes required for cytokinesis and RNA processing, as well as other gene categories. The data indicated that human embryo fate is predictable and likely to be predetermined at least in part by inheritance of maternal transcripts."

4 Perspectives on Future Clinical Applications

While significant advances in stem cell research had been made, there are a number of issues that must be addressed before stem cell therapy can become a reality. Perhaps one of the most important needs is the ability to prospectively isolate cells. Prospective isolation is the concept of identifying or "predicting" the fate of a stem cell before it has committed [1] and has enormous utility in stem cell research and therapy since it could identify optimal cells for transplantation or testing of drugs and other therapeutics. Prospective isolation could also help to identify cells with tumorigenic potential, especially in the case of pluripotent embryonic stem cells or the more recent iPSCs. The importance of predicting tumorigenesis cannot be understated since even small numbers of tumorigenic cells can contaminate entire cell cultures and ultimately lead to the development of tumors in vivo. The mechanisms that cause tumorigenesis are largely unknown, although it has been linked to defects in the regulation of symmetric and asymmetric cell divisions [25].

Time-lapse microscopy is likely to play a significant role in enabling prospective isolation by enabling continuous observation at the single-cell level. However, many applications require additional information regarding the underlying biologic events taking place. It was suggested that this could be achieved by integrating imaging technologies with genomics and proteomics [26], although the manner in which these technologies would be combined remains unclear. In a recent interesting study image phenotypes were correlated with molecular phenotypes for characterizing normal and abnormal embryo development [24].

The idea of correlating imaging technology with gene expression profiling has also been investigated in other areas of medical imaging. Diehn et al. presented a method for correlating MRI images of glioblastoma with gene expression profiles of biopsied tissue samples [27]. Specifically, they measured image phenotypes such as tumor physiology, morphology, cellularity, and composition and correlated these

to gene expression "modules." These modules consisted of clusters of genes related to known biologic processes such as proliferation, overexpression, and hypoxia. They observed that a large fraction of the gene expression program could be reconstructed from a small number of imaging traits and were able to identify an imaging phenotype that is associated with overall survival of glioblastoma patients. A similar study was presented by Segal et al., in which dynamic imaging traits in computed tomography imaging were correlated with global gene expression programs in liver cancer [28].

The goal of these prior studies was to use imaging technologies to noninvasively assess the genetic and biochemical makeup of the living tissue. This is a powerful technique that could be extremely useful in stem cell therapy and prospective isolation. For example, the process of differentiation could be characterized a priori by correlating image phenotypes with molecular phenotypes in order to create a predictive model. In clinical applications, this predictive model could be used in combination with time-lapse image analysis to prospectively isolate single cells from larger colonies grown in a high-throughput manner. Of course, this process has many other challenges, such as directing cell decisions through environmental stimuli and determining the point at which to isolate cells for transplantation.

5 Conclusion

Before stem cell therapy can become a reality, new techniques will be needed to assess cell viability prior to transplantation. Recent studies have shown that time-lapse microscopy can address these needs by observing dynamic events at the single-cell level such as differentiation, asymmetric versus symmetric divisions, and fate specification. Time-lapse microscopy can be performed in a high-throughput and noninvasive or minimally invasive manner and has the potential to become a powerful predictive tool when combined with an understanding of the underlying biologic processes. Such technologies are currently under development and could be an enabling step toward new clinical and therapeutic applications.

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Part III
Stem Cells for Therapeutic Applications

Therapeutic Applications of Mesenchymal Stem/Multipotent Stromal Cells

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Abstract Stem cell therapies offer enormous hope for treating many tragic diseases and tissue defects. In particular, mesenchymal stem/multipotent stromal cells (MSCs) are capable of differentiating into multiple types of connective tissues (i.e., bone, cartilage, and even muscle and neuron) and have proangiogeneic and immunomodulatory effects. MSCs have potential utility for treating a variety of diseases and disorders, including graft versus host disease, problems related to organ transplantation, cardiovascular disease, brain and spinal cord injury, lung, liver, and kidney diseases, and skeletal injuries. This chapter summarizes the current status of therapeutic applications of MSCs. It begins by introducing the basics of MSCs and then focuses on their therapeutic potential, including mechanism of action, delivery routes, MSC homing, the current status of clinical trials, and potential challenges and safety issues. Finally, the chapter describes chemical approaches developed in the authors' laboratory to promote homing and engraftment of systemically infused MSCs within specific tissues.

Keywords Mesenchymal stem cell • Mesenchymal multipotent stromal cell • Mesenchymal stromal cell • MSC • Cell therapy • Immunomodulation

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1 Introduction

1.1 History and Definition

In 1976, Friedenstein et al. first identified mesenchymal stem/multipotent stromal cells (MSCs) when they isolated the cells from bone marrow by their tight adherence to the tissue culture plate [1]. Some of these cells appeared to have spindle-like morphology (Fig. 1) and were capable of forming single cell–derived colonies, which were defined by Friedenstein et al. as "colony forming units-fibroblastic" (CFUs-F). However, it is critical to note that not all adherent cells from bone marrow are capable of forming CFUs-F. In fact, often the population of cells harvested from bone marrow includes fibroblasts and endothelial cells, among others. MSCs have also been referred to as "bone marrow stromal cells" because of their role in creating bone marrow niche for maintaining hematopoietic stem cell (HSC) functions and their use as feeder layers for HSCs [2, 3]. Subsequently, researchers who focused on the multilineage differentiation potential of MSCs named them "mesenchymal stem cells" [4, 5]. To avoid confusion in defining these cells, the International Society for Cytotherapy Committee in 2006 suggested the name "multipotent mesenchymal stromal cells" [6, 7]. In this chapter, we use the abbreviation MSC.

1.2 Origins, Isolation, and In Vitro Culture

While MSCs are primarily isolated from bone marrow, it is now known that they exist in the connective tissues of many organs, such as adipose tissue [8], muscle [9], liver [10], lung [11], umbilical cord blood [12, 13], and amniotic fluid and

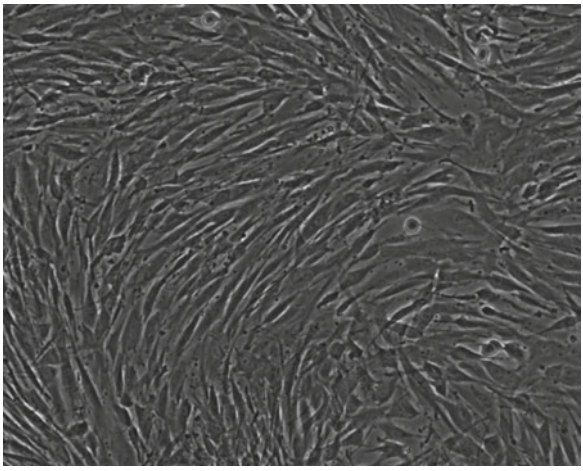

Fig. 1 A confluent monolayer of human bone marrow-derived MSCs in culture

amniotic membrane [14], among others [15–17]. In addition to human sources, MSCs can also be obtained from many other species. Note that the source of MSCs has an impact on their phenotype and functions [15].

The protocol for MSC isolation and culture has not yet been standardized. Traditionally, MSCs are isolated from bone marrow aspirates and separated from HSCs and other blood cells by their selective adherence to tissue culture plastics [1], which typically produces significant heterogeneity of the cell population. More recently, some researchers have used antibodies, such as STRO-1 antibody combined with CD106 (VCAM-1) antibody or CD146 (MUC18) antibody [18, 19], or DNA aptamers [20] to isolate MSCs. Regardless of the isolation methods, a largely heterogeneous population of cells is obtained due to the heterogeneous nature of MSCs and due to the lack of specific surface markers [21]. The isolated, adhered MSCs are typically maintained and expanded on tissue culture plates in an appropriate medium, which also varies from one laboratory to another. In our laboratory, we obtain MSCs from the Center for Gene Therapy at Tulane University, a facility funded by the National Institutes of Health/National Center for Research Resources for distribution of MSCs with standardized protocols for preparation and culture [7]. Specifically, we culture MSCs with α-Minimum Essential Medium supplemented with 15% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin at 37°C and 5% CO_2. The cells are routinely subcultured using trypsin–ethylenediaminetetraacetic acid solution or other nonenzyme cell dissociation solution when they reach approximately 70%–80% confluency [22]. While it is reported that MSCs can divide up to 40 times in culture [29], we typically use passages below 8 in our studies, which translates into fewer doublings (see later discussion). Note that, in addition to the isolation sources, the isolation methods, culture conditions (i.e., medium, incubation environments), passage number, donor age, and confluency of the passaged cells all have profound impact on the biologic functions of MSCs and therefore may affect their therapeutic efficacy [15].

1.3 Characterization

Given the heterogeneity of MSCs and the different isolation and culture methods described in the literature, it is difficult to compare and contrast the resulting outcomes [7]. To address this issue, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed minimal criteria for defining MSCs [6]. Specifically, to be qualified as MSCs, the cells must be able to (1) adhere to plastic in standard culture conditions, (2) express (at least 95%) CD105, CD73, and CD90 and not express (2% or less) CD45, CD34, CD14 or CD11b, CD79 or CD19, and HLA-DR, and (3) differentiate in vitro to osteoblasts, adipocytes, and chondroblasts. We give a detailed list of the human MSC surface markers and secreted molecules in Tables 1 and 2, respectively. Note that the listed markers are summarized from the literature [23–32] and often vary among reports, depending on the source, culture

Table 1 Expression of surface markers on human MSCs

Common Name	CD Locus	Detection
Adhesion molecules		
ALCAM (activated leukocyte cell adhesion molecule)	CD166	+
ICAM-1 (intercellular adhesion molecule-1)	CD54	+
ICAM-2	CD102	+
ICAM-3	CD50	+
E-Selectin	CD62E	–
L-Selectin	CD62L	+/–
P-Selectin	CD62P	–
LFA-3 (lymphocyte function–associated antigen-3)	CD58	+
Cadherin 5	CD144	–
PECAM-1 (platelet/endothelial cell adhesion molecule-1)	CD31	–
NCAM (neural cell adhesion molecule)	CD56	+/–
HCAM (homing-associated cell adhesion molecule)	CD44	+
VCAM (vascular cell adhesion molecule)	CD106	+/–
Growth factors and cytokine receptors		
IL-1R (α and β) (interleukin-1R)	CD121a, b	+
IL-2R	CD25	–
IL-3R	CD123	+
IL-4R	CD124	+/–
IL-6R	CD126	+
IL-7R	CD127	+
Interferon γ R	CDw119	+
TNF-α-1R (tumor necrosis factor-α-1R)	CD120a	+
TNF-α-2R	CD120b	+
TNFRSF8 (tumor necrosis factor receptor superfamily, member 8)	CD30	–
TNFRSF5	CD40	–
FGFR (fibroblast growth factor receptor)		+
PDGFRα (platelet-derived growth factor receptor α)	CD140a	+
PDGFRβ (platelet-derived growth factor receptor β)	CD140b	+
Transferrin receptor	CD71	+
C-kit receptor	CD117	–
EGFR-1 (HER-1) (early growth response factor-1)		+
EGR-4 (HER-2) (early growth response factor receptor-4)	CD340	–
Flk-1 (fetal liver kinase-1)		+/–
EGFR (epidermal growth factor receptor)		+
HGFR (hepatocyte growth factor receptor)		+
IGF1R (insulin-like growth factor-1 receptor)	CD221	+
VEGFR1 (vascular endothelial growth factor receptor 1)		+/–
VEGFR2	CD309	+/–
Tie-2 (tyrosine kinase with immunoglobulin-like and EGF-like domains)	CD202B	+/–
CCR1 (chemokine (C–C motif) receptor-1)	CD191	+
CCR2	CD192	+
CCR3	CD193	+

(continued)

Table 1 (continued)

Common Name	CD Locus	Detection
CCR4	CD194	+
CCR5	CD195	+
CCR6	CD196	+
CCR7	CD197	+
CCR8	CDw198	+
CCR9	CDw199	+
CCR10		+
CXCR1 Cytokine (C-X-C Motif) receptor-1	CD181	+
CXCR2	CD182	+
CXCR3-A/B	CD183	+
CXCR4	CD184	+/–
CXCR5	CD185	+
CXCR6	CD186	+
CXCR12		–
CX3CR1		+
XCD1		+
TLR2 (Toll-like receptor 2)	CD282	+
TLR3	CD283	+
TLR4	CD284	+
TLR5		+
TLR6	CD286	+
NGFR (nerve growth factor receptor)	CD271	+
Integrins		
VLA-α1 (very late antigen-α1)	CD49a	+
VLA-α2	CD49b	+
VLA-α3	CD49c	+
VLA-α4	CD49d	+/–
VLA-α5	CD49e	+
VLA-α6	CD49f	+
VLA-β chain	CD29	+
B4 integrin	CD104	+
LFA-1 α chain (leukocyte function–associated antigen-1)	CD11a	–
LFA-1 β chain	CD18	–
Vitronectin R α chain	CD51	–
Vitronectin R β chain	CD61	+
CR4 α chain	CD11c	–
Mac1	CD11b	–
Additional markers		
T6	CD1a	–
CD3 complex	CD3	–
T4,T8	CD4, CD8	–
Tetraspan	CD9	+
LPS receptor (lipopolysaccharide receptor)	CD14	–
Lewis X	CD15/CD34	–
Leukocyte common antigen	CD45	–

(continued)

Table 1 (continued)

Common Name	CD Locus	Detection
5′-Terminal nucleotidase	CD73	+
B7-1	CD80	–
HB-15	CD83	–
B7-2	CD86	–
Thy-1	CD90	+
Endoglin	CD105	+
MUC18	CD146	+
BST-1	CD157	+
STRO-1	Stromal antigen-1	+
α Smooth muscle actin		+
MAB1740		+
HLA class I (A, B, C) (human leukocyte antigen)		+ (upon IFN-γ stimulation)
HLA-DR		–
	CD19	–
	CD79α	–
Aminopeptidase N	CD13	+
CALLA (common acute lymphocytic leukemia)	CD10	+/–
TNFSF8	CD153	+/–
	CD154	–
Fas	CD95	+
Fas ligand	CD95L	+/–
TRAIL (TNF-related apoptosis-inducing ligand)		–
TRAIL-R		–
HB-EGF (heparin-binding epidermal growth factor)		–
Sca-1 (stem cell antigen-1)		–

+, positive; –, negative; +/–, low expression, or not sure if expressed.

Table 2 Cytokines, Growth Factors, and Other Molecules Secreted by MSCs Under Appropriate Conditions

IL-1 (interleukin-1)?, IL-6, IL-7, IL-8, IL-10?, IL-11, IL-22?, IL-14, IL-15, LIF (leukemia inhibitory factor), M-CSF (macrophage colony stimulating factor), G-CSF (granulocyte colony stimulating factor), GM-CSF (granulocyte-macrophage colony stimulating factor), Flt3 ligand (Fma-like tyrosine kinase 3 ligand), SCF (stem cell factor), SDF-1 (stromal cell–derived factor), VEGF (vascular endothelial growth factor), HIF-1α (hypoxia inducible factor-1α), angiopoeitin-1, plasminogen activator, bFGF (basic fibroblast growth factor), HGF (hepatocyte growth factor), IGF-1 (insulin-like growth factor-1), TGF-β (transforming growth factor), PGE2 (prostaglandin E2), NO (nitric oxide), IDO (indoleamine 2,3-dioxygenase), PIGF (placental growth factor), MCP-1 (monocyte chemoattractant protein-1), MMP-2 (matrix metalloproteinases-2), MMP-14, TNF-α–induced protein 6 (TSG6), collagen types I, III, IV, V, and VI, fibronectin, laminin, hyaluronan, and proteoglycans
The controversial markers are marked with a question mark.

conditions, and passage numbers. In particular, it is known that MSCs gradually lose some surface markers during continuous passaging [33, 34].

1.4 Multipotent Differentiation

MSCs are capable of self-renewal and differentiation into connective tissue cell types such as osteogenic, adipogenic, and chondrogenic cells (Fig. 2) both in vitro and in vivo (although there are no standardized methods to image host MSCs or examine the specific signals that regulate their differentiation in situ) [4–6, 29, 30, 35]. In addition, MSCs can differentiate into connective vascular smooth muscle–like stromal cells in the bone marrow niche supporting HSC functions [36]. More recently, it has been found that under appropriate conditions, MSCs are capable of differentiating to nonmesoderm lineages including muscle cells, neurons, and epithelial cells (Fig. 2) [15]. Note, however that the capacity of MSC differentiation to other lineages (ectoderm and endoderm), particularly in vivo, is still controversial.

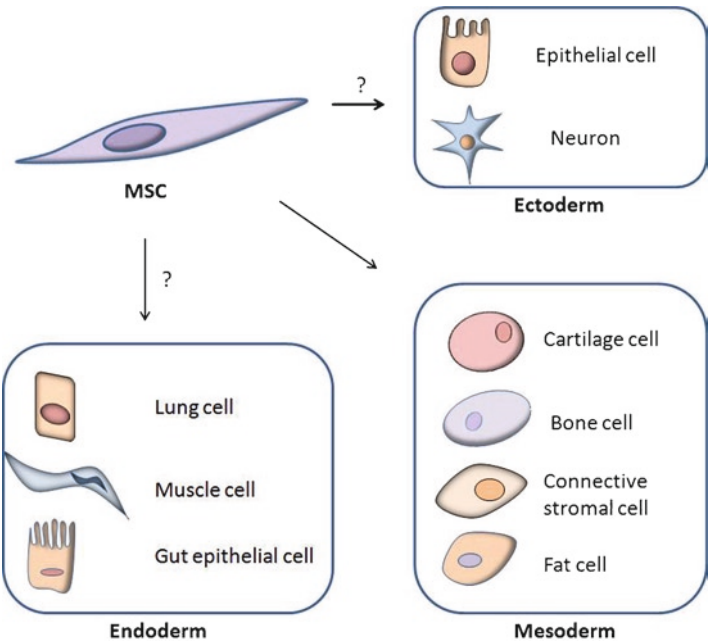

Fig. 2 Multipotent differentiation potential of MSCs to mesoderm cells (*arrow*) and nonmesoderm (ectoderm and endoderm) cells (*arrows with question marks*, due to controversy over in vivo results)

2 Therapeutic Applications

2.1 Therapeutic Mechanisms

Stem cell transplantation to repair/regenerate tissues and organs holds great promise for treating many diseases and disorders. HSC transplantation has been successfully used in the clinic for more than 30 years for treating blood diseases such as leukemia. MSCs represent a promising cell type that have already found utility in clinical practice for regeneration of bone tissue and may be useful for treatment of many other disorders due to their potential for multiple therapeutic effects as outlined in the following sections.

2.1.1 Tissue Regeneration Through Multilineage Differentiation

Transplanted MSCs have been shown to differentiate into cells that constitute the HSC niche, including bone marrow stromal cells, pericytes, myofibroblasts, osteoblasts, and endothelial cells. The differentiation potential of MSCs together with their paracrine actions on HSCs (see next subsection) potentiates enhanced engraftment and survival of HSC through cotransplantation [35, 36]. Moreover, the capability of differentiating into mesoderm tissues such as bone, fat, and cartilage makes MSCs particularly useful for skeletal tissue repair/regeneration of bone, cartilage, and tendon [37–41]. In such cases, culture-expanded MSCs can directly be implanted into injured sites in vivo, where MSCs differentiate to desirable tissues in situ; alternatively, in a traditional tissue engineering scenario, MSCs can be seeded on an appropriate scaffold, typically biodegradable polymers, and induced to differentiate in vitro [42]. Subsequently, the cell–scaffold construct is implanted to the injured sites, where newly implanted cells are gradually integrated with host cells while the scaffold dissolves. For instance, Petite and coworkers demonstrated the use of a coral scaffold seeded with in vitro–expanded MSCs to increase osteogenesis [43]. Ponticiello et al. showed that gelatin scaffolds loaded with MSCs and implanted in an osteochondral lesion in medial femoral condyle can differentiate into both cartilage and bone cells, which represents a promising approach for cartilage regeneration therapy [44]. However, it is critical to note that cells need to be within approximately 200 μm of the nearest blood vessel, the distance the nutrients can diffuse before being completely consumed; this ischemia quickly leads to necrosis [45] (cell and tissue death). Survival of implanted cells thus depends on new blood vessel formation to provide oxygen and nutrients.

Due to the remarkable plasticity of MSCs (i.e., differentiation to cells of both ectodermal and endodermal nature (Fig. 2), including cardiomyocytes, hepatocytes, neurons, skeletal muscle, pancreatic, epithelial cells, and epidermal-like cells [15]), it has been suggested they can be used for treating a wide range of diseases, including heart disease, renal disease, liver disease, and neurologic diseases, some of which have been demonstrated to be feasible in animal studies [46]. For instance, bone

marrow MSCs can differentiate into epithelial cells in the lung, liver, and skin in a mouse model [15]. Transplantation of MSCs into infarcted hearts has been shown to improve cardiac function due to the enhanced vascularization as MSCs differentiate into cardiomyocytes, endothelial cells, pericytes, and smooth muscle cells [47]. MSCs injected into the central nervous system (CNS) of newborn mice adopt morphologic and phenotypic characteristics of astrocytes and neurons [15, 48]. However, the in vivo differentiation of MSCs to nonmesoderm lineages is still very controversial, and the current results are often conflicting [15]. A recent study by Rose et al. [49] demonstrated that although MSCs can adopt a cardiogenic phenotype, via plasticity, they fail to differentiate to mature functional cardiomyocytes. This work is in direct contrast to an earlier report by Pijnappels et al. [50] demonstrating differentiation of MSCs to cardiomyocyte-like cells, whereby cells demonstrated cardiac action potentials. However, Pijnappels et al. used rat neonatal MSCs, whereas Rose and colleagues used adult mouse MSCs. Furthermore, some researchers have suggested that in these cases MSCs do not actually undergo real differentiation, but rather fuse with specialized differentiated cells [51]. For instance, bone marrow–derived MSCs have been shown to fuse spontaneously with neurons, cardiomyocytes, and hepatocytes, evidenced by the formation of multinucleated cells [52]. Several groups also suggested that engrafted bone marrow MSCs in heart tissue undergo fusion with endogenous cardiac cells, which had been previously thought to be MSC differentiation [53]. Similar questions have also been raised with regard to renal and neural repair models, where real differentiation or cell fusion is highly debatable [54]. Note, however, that the cell fusion hypothesis should not be accepted as a general pathway by which MSCs integrate with other cells, as there is compelling evidence demonstrating fuse-independent MSC differentiation [7]. The broad multilineage differentiation of MSCs to nonmesoderm tissue in vivo has been the topic of intense debate, and more research is clearly required to address this issue.

2.1.2 Paracrine Factors and Immunomodulatory Effects

In addition to multilineage differentiation, secretion of paracrine factors has been proposed to play a prominent role in MSC therapy [38, 54–56]. MSCs affect neighboring cells by both cell–cell contact and release of a wide range of cytokines and growth factors (Tables 1 and 2). It has long been recognized that they secrete bioactive molecules in the bone marrow HSC niche supporting hematopoiesis [3, 57, 58]. Some of these cytokines and growth factors, including VEGF, bFGF, HGF, and IGF-1, can enhance epithelial and endothelial proliferation and promote angiogenesis [59], which provides an additional mechanism by which MSCs repair tissues/organs such as heart, liver, and kidney. In other words, the therapeutic efficacy of infused MSCs somewhat relies on their potential local or systemic paracrine (or trophic) activity. For cardiac repair, for example, it is now accepted that the current MSC therapy assists the heart predominantly by facilitating endogenous repair processes (promotes endothelial cell proliferation and vascularization and inhibits apoptosis and excessive inflammation) instead of through regeneration of lost cardiac and vascular cells [53].

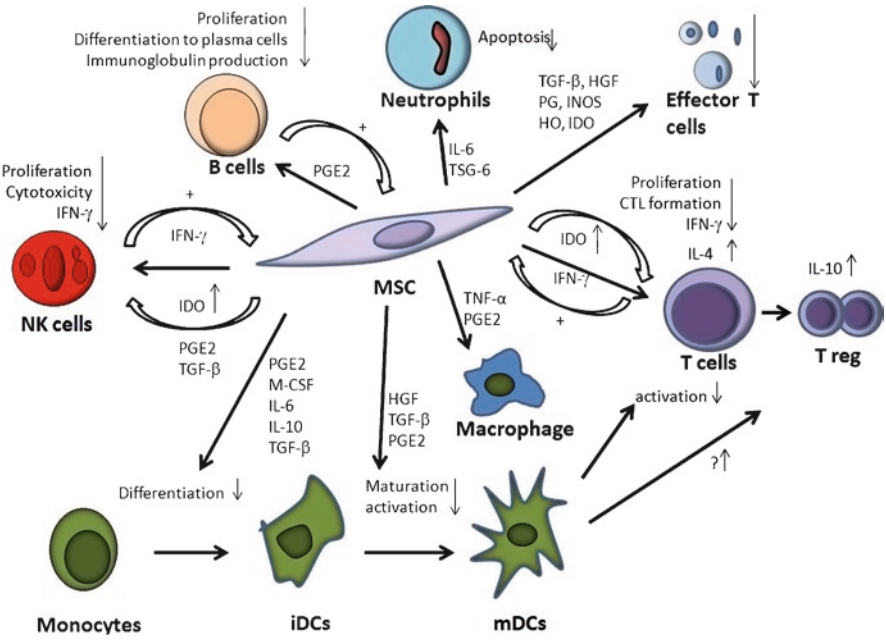

Fig. 3 Immunoregulatory effects of MSCs on immune cells. CTL, cytotoxic T cells; HO, heme oxygenase; iDCs, immature dendritic cells; INOS, inducible nitric oxide synthase; mDCs, mature dendritic cells; NK cells, natural killer cells; PG, prostaglandin; Treg, regulatory T cells. See Tables 1 and 2 for other abbreviations

Significantly, MSCs, which, as discussed, are immunologically privileged, have very potent immunosuppressive effects on a variety of immune cells via cell–cell contact and/or soluble factors (summarized in Fig. 3) [24, 55, 60–63]. MSCs have been found to suppress T lymphocyte (T cell) activation and proliferation in vitro and arguably in vivo [62]. The mechanism, which is not fully understood, may involve intercellular communication via soluble factors including TGF-β and HGF; blocking these factors by antibodies reduces the inhibitory effect from MSCs. Other studies found that the direct contact between MSCs and T cells, which modulates the expression of cytokine receptors and transduction molecules for cytokine signaling, also contributes significantly to the inhibition of T cell proliferation [62]. For instance, direct cell–cell contact is required for MSCs to induce forkhead box P3 $(FoxP3)^+$ and $CD25^+$ mRNA and protein expression in $CD4^+$ T cells [64]. This is not surprising, as MSCs do in fact express a large array of receptors that bind to T cells, including VCAM, ICAM-1, ALCAM, and LFA-3 [62]. MSCs have also been shown to exhibit potent immunomodulatory effects on neutrophils, dendritic cells, B cells, natural killer cells, monocytes, or macrophages via soluble factor/cell–cell contact mechanisms [62]. Interested readers are referred to some recent excellent reviews on this topic [24, 55, 60–63]. The immunomodulatory properties of MSCs make them an excellent candidate as immunosuppressive agents during HSC and solid-organ transplantation and treating graft versus host disease, inflammatory diseases, cancer, and other autoimmune diseases.

2.1.3 Genetically Engineered MSCs

The therapeutic utility of MSCs may be broadened by applying them as gene delivery vehicles [65–68]. Given the ability of MSCs to self-renew at a high proliferative rate and their unique capability to home to injured tissue and cancer, MSCs can be used as genetically modified vehicles to deliver genes to injured tissues and to tumors. Specifically, MSCs can be engineered to secrete a variety of therapeutic proteins in vivo that could potentially treat diseases and disorders, including neurologic disorders [69], blood disorders, vascular diseases, musculoskeletal diseases, and cancer [3]. For instance, genetically engineered MSCs that can "replace" mutant genes in genetic deficiencies have been applied to treat osteogenesis imperfecta [66,70]. Intravenous injection of MSCs transduced with the interferon β (IFN-β) gene (and therefore can produce IFN-β to inhibit tumor cell growth) into SCID mice with established MDA-MB-231 breast carcinoma led to prolonged survival compared to untreated control mice [66]. Furthermore, Sasportas et al. recently reported genetically engineered MSCs that express tumor necrosis factor apoptosis ligand-induced, caspase-mediated apoptosis in glioma cells, which represents a novel approach of using MSCs for cancer therapy [71].

2.2 Advantages of Using MSC as Therapeutic Cells

In addition to their potential to differentiate into tissue-specific cell types and to secrete paracrine factors to regulate the immune system, MSCs have other characteristics that make them clinically useful, which includes convenient isolation and expansion and a lack of ethical controversy concerning their use [72]. MSCs are immunologically privileged, and transplanted MSCs generate little immunogenicity, permitting allogeneic transplantation without use of immunosuppressive agents or rejection, as they do not express MHC class II and costimulatory molecules CD40, CD80, and CD86 [73, 74]. MSCs are also less prone to genetic abnormalities during in vitro passages, possessing a low risk for induction of malignancies, as compared to other types of stem cells, such as embryonic stem cells [72], although long-term safety concerns remain, as discussed later.

2.3 Delivery Routes

In addition to the timing of delivery and dose, the delivery route is known to be a key factor determining the success of MSC therapy [75]. MSCs can be injected locally to injured tissue, which, however may not be clinically feasible in many cases due to its potential invasiveness (e.g., into the heart or brain), and locally injected cells often die before significantly exerting the therapeutic effects due to diffusion limitations of nutrients and oxygen [75, 102]. Systemic administration is therefore a

great alternative, which typically includes intravenous (IV) injection, intra-arterial (IA) injection, and intracardiac (IC) injection [33, 75]. IV delivery is the least invasive; however, it has been shown that most (approximately 99%) of the administrated MSCs quickly accumulate in the lung (although they subsequently escape the lung and enter the bloodstream again), which may impair their therapeutic efficacy [75]. Of interest, recent work by Lee et al. [31] showed that the accumulation of MSCs in the lung is followed by redistribution of a fraction of the cells, while the others may remain to form emboli, leading to the release of paracrine factors. IC and IA delivery may reduce accumulation of MSCs within filtering organs such as the lung and therefore lead to higher engraftment rates than IV delivery in certain models, such as myocardial infarction and brain injury [75]. However, IC delivery is normally very invasive, and IA delivery may lead to increased probability of microvascular occlusions or "passive entrapment" [103]. Therefore, the appropriate administration route has to be carefully chosen for a given application.

The key to the therapeutic feasibility and success of systemically injected MSCs is that MSCs have been shown to selectively "home" to injured or inflamed organs/tissues [75, 76]. For example, several studies showed that transplanted MSCs can home to injured tissues in animal models, including myocardial injury [77] and acute renal failure [56]. However, the efficiency of homing is limited, and the precise homing mechanism of MSCs is still largely unknown. In particular, it is not known whether they undergo a leukocyte-like homing cascade, that is, rolling, firm adhesion, and transmigration [75]. Nonetheless, MSCs do express a range of cell adhesion molecules (i.e., integrins), cytokine and growth factor receptors, and matrix metalloproteinases (MMPs) (see Tables 1 and 2) that can contribute to their homing, chemotaxis, and transmigration. It has been shown that MSCs demonstrate coordinated rolling and adhesion behaviors on human umbilical vein endothelial cells via P-selectin and VCAM-1 [78]. However, the rolling velocities reported were approximately 100–500 μm/sec at shear stresses of 0.1–1.0 dynes/cm^2. To provide context, it is important to note that leukocyte rolling has been typically observed to be less than 5 μm/sec at shear stresses up to 4 dynes/cm^2 [79, 80]. Sackstein et al. recently confirmed the lack of a significant rolling response of MSCs on TNF-α–activated endothelium from 0.5 to 30 dynes/cm^2 [81]. Similar to leukocytes, MSCs can extravasate from the blood vessels into tissues probably due to their expression of surface adhesion molecules, chemokine receptors, and MMPs [33, 34, 82]. The chemotactic signals that guide MSCs to injured tissue have also been studied. The chemokine SDF-1 and its receptor CXCR4, which plays a dominant role in recruiting HSCs, had limited contribution to MSC homing because MSCs express little CXCR4 (or they lose this during culture expansion) [33,34]. Rather, it has been shown in vitro that MSC migration is under control of a large range of tyrosine kinase receptor growth factors, including PDGF-AB and IGF-1, and CC and CXC chemokines [34]. MSCs primed by TNF-α, an inflammatory cytokine, demonstrated more effective homing capacity toward the aforementioned chemokines than untreated MSCs due to the upregulation of some key cell membrane receptors, indicating that the mobilization and homing of MSCs to injured tissues may depend on the systemic and local inflammatory state [34].

2.4 Therapeutic Applications

Owing to their multipotent differentiation potential, paracrine action, immunomodulatory effects and other advantages mentioned earlier, MSCs hold great promise for the treatment of a variety of diseases and disorders [24, 26, 46, 51, 56, 60, 61, 83–89]. The diseases that can be potentially be treated by MSCs, ongoing clinic trial status, and major involved companies/hospitals/institutes are summarized in Table 3. Briefly, MSCs can be used to support HSC engraftment, inhibit immune response after organ transplantation, reduce manifestations of graft versus host disease, treat various autoimmune conditions and cancer, and repair heart, liver, lung, kidney, and CNS tissue [16, 24, 74, 90, 91]. They can be used as building blocks for artificially engineered tissues, including bone, cartilage, tendon, and muscle [37–41]. Furthermore, MSCs can be used as vehicles to deliver specific genes to target tissues, which represent one of the most promising therapeutic approaches using combined cell and gene therapy [65–68].

2.5 Challenges of MSC-Based Therapy and Safety Concerns

While promising, the use of MSCs in clinical practice is still in its infancy, and there are many challenges and issues that remain to be addressed [55, 84, 92, 93].

1. The source, isolation method, and culture conditions, as well as administration route, timing, and dose, need to be standardized to address the variability among studies and (pre)clinical trials [61, 94].
2. More studies are required to address key aspects of MSC biology, including their homing properties, the mechanism of their immunomodulatory effects, and the mechanisms that regulate their in vivo multilineage differentiation potential [75].
3. More efficient targeting strategies are required, that is, determining how to minimally invasively target MSCs to tissues of interest with high efficiency and how to reduce their nonspecific accumulation in the lung [7].
4. While the clinical trials thus far have shown the safety of using MSCs, long-term follow-up is required to ensure that MSC therapy is indeed safe. Moreover, the potential for MSCs to exacerbate malignancy is clearly a concern [95]. Furthermore, the safety of animal serum used in current MSC culture protocols is unknown [96].
5. Although MSCs are being considered to deliver genes or viruses to treat diseases such as cancer, MSCs can also potentiate cancer growth by promoting angiogenesis and metastasis [95]. Therefore, more studies are required to characterize the complex interactions of MSCs within the tumor microenvironment [55].

Table 3 Summary of clinical trials reported from clinicaltrials.gov using search term "Mesenchymal Stem Cell" or "Mesenchymal Stromal Cell" as of July 9, 2009

Disease		Status of Clinical Trial	Company/University
Hematopoietic stem cell (HSC) transplantation, graft versus host disease, hematologic malignancies, promotion of engraftment of HSC transplantation, myelodysplastic syndrome, leukemia		Completed	Osiris Therapeutics
		Phase II	University Hospital of Liege
		Phase I, II	University of Salamanca
		Phase I, II	Medipost Co. Ltd.
		Phase I, II	Christian Medical College
		Phase II	University Hospital of Liege
		Phase II	Jonsson Comprehensive Cancer Center
		Phase I	MD Anderson Cancer Center
		Phase I	Case Comprehensive Cancer Center
		Phase I, II	UMC Utrecht
		Phase I, II	Hadassah Medical Organization
Solid-organ transplantation	Kidney transplantation, chronic allograft nephropathy	Phase I, II	Fuzhou General Hospital
	Renal transplantation	Phase I, II	Leiden University Medical Center
	Liver transplantation, decompensated cirrhosis	Phase II	University of Tehran
	End-stage liver disease	Phase I, II	Shaheed Beheshti Medical University
	Kidney transplantation	Phase I, II	Mario Negri Institute for Pharmacological Research

Skeletal, muscle, and skin tissue repair	Articular cartilage defects, degenerative arthritis, chondral defects, osteochondral defects	Phase II, III	Cairo University
	Knee cartilage defects, osteoarthritis	Phase I	Royan Institute
	Degenerative disc disease, spondylolisthesis, spinal stenosis	Phase I, II	Mesoblast Ltd.
	Cartilage defects	Phase I	Ullevaal University Hospital
	Tibial fracture	Phase I, II	Hadassah Medical Organization
	Tibial fractures, open tibial shaft fractures	Phase I	University Hospital, Tours
	Necrosis	Completed	Erasme University Hospital
	Osteodysplasia	Completed	St. Jude Children's Research Hospital
	Multiple trauma	Phase I	University of Ulm
	Adult periodontitis	Completed	Translational Research Informatics Center
	Osteogenesis imperfecta	Completed	St. Jude Children's Research Hospital
	Recovery following partial medial meniscectomy	Completed	Osiris Therapeutics
	Tibia or femur pseudoarthrosis	Phase II	University Hospital, Clermont-Ferrand
	Wound healing, burn	Phase I	Ohio State University
	Bone neoplasms	Phase II, III	Emory University
	Refractory systemic lupus erythematosus	Phase I, II	Nanjing Medical University
	Diabetic wounds, venous stasis wounds	Phase I	Washington DC Veterans Affairs Medical Center
	Lupus nephritis	Phase I, II	Organ Transplant Institute, China
	Critical limb ischemia	Phase I, II	Stempeutics Research Pvt Ltd

(continued)

Table 3 (continued)

Disease		Status of Clinical Trial	Company/University
Cardiovascular diseases	Peripheral artery disease, peripheral vascular disease, critical limb ischemia	Phase I	Pluristem Ltd.
	Ischemic heart disease	Phase I, II	Rigshospitalet
	Heart failure	Phase II	Angioblast Systems
	Congestive heart failure	Phase II	National Heart, Lung, and Blood Institute
	Myocardial infarction	Phase I, II	Angioblast Systems
	Dilated cardiomyopathy	Phase II	Hospital Universitario Reina Sofia
	Congestive heart failure	Phase I, II	Rigshospitalet
	Heart disease, blocked arteries, coronary ischemia, coronary disease, coronary artery disease, coronary atherosclerosis	Phase II	TCA Cellular Therapy
	Dilated cardiomyopathy	Phase II	University Medical Centre, Ljubljana
	Myocardial ischemia, coronary heart disease	Completed	Rigshospitalet
	Ventricular dysfunction, left; ischemic heart failure	Phase I, II	University of Miami
	Heart failure, myocardial infarction, coronary artery disease	Phase II	Helsinki University
	Myocardial infarction	Phase I, II	Stempeutics Research Pvt Ltd
	Myocardial infarction	Phase I	Osiris Therapeutics
	Hypercholesterolemia, familial	Phase I	University of Tehran
	Pened Chest Surgery for Programmes Coronary Bypass	Phase I	Nantes University Hospital
	Ventricular dysfunction, left; patients undergoing cardiac surgery	Phase I, II	National Heart, Lung, and Blood Institute

Neurologic diseases and neuroimmunologic/ neurodegenerative diseases	Multiple sclerosis	Phase I, II	University of Cambridge
	Ischemic stroke	Phase II	University Hospital, Grenoble
	Stroke	Phase II	University of California, Irvine
	Osteonecrosis of the femoral head	Phase I	Fuzhou General Hospital
	Multiple sclerosis	Phase I	The Cleveland Clinic
	Chronic spinal cord injury	Phase I, II	Cairo University
	Multiple sclerosis	Phase I, II	Hadassah Medical Organization
	Multiple system atrophy	Phase II	Yonsei University
Liver	Liver cirrhosis	Phase I	Kanazawa University
Lung	Pulmonary disease, chronic obstructive pulmonary emphysema, chronic bronchitis	Phase II	Osiris Therapeutics
Crohn disease		Completed	Osiris Therapeutics
Diabetes	Type 2 diabetes mellitus	Phase I, II	Fuzhou General Hospital
	Cotransplantation of islet and MSCs in type 1 diabetic patients	Phase I, II	Fuzhou General Hospital
	Type 1 diabetes mellitus, insulin-dependent juvenile diabetes	Phase II	Osiris Therapeutics

3 Chemically Engineered MSCs with Homing Receptors: A Novel Approach to Promoting MSC Homing

As mentioned earlier, one of the biggest challenges in MSC therapy is to improve their targeting efficiency to the tissue of interest [7, 75]. While native MSCs may exhibit certain adhesion molecules and receptors to facilitate their homing, culture-expanded MSCs often lose these key receptors and therefore have extremely poor tissue-targeting efficiency (less than 1%) [75]. To address this challenge, a number of approaches have been developed. Retrovirus vectors encoding homing receptors such as CXCR4 [97] or the α4 subunit of the VLA-4-integrin [98] have been recently used to enhance homing and engraftment of MSCs. Alternatively, Sackstein et al. showed that the enzymatic engineering of surface glycans of MSCs enables them to home to bone more efficiently than unmodified MSCs [81]. Another approach involves the conjugation of antibodies to the cell surface via bispecific antibodies [99] or palmitated protein G or protein A, which permits cell-surface functionalization by potentially any antibody bearing an accessible Fc region [100].

We have developed a simple platform technology to chemically attach cell adhesion molecules to the cell surface to improve homing efficiency to specific tissues [101]. The ultimate goal is that systemically infused engineered MSCs will home specifically to target tissue via a stepwise leukocyte-like rolling, adhesion, and transmigration process (Fig. 4). Specifically, as shown in Fig. 5, the chemical approach

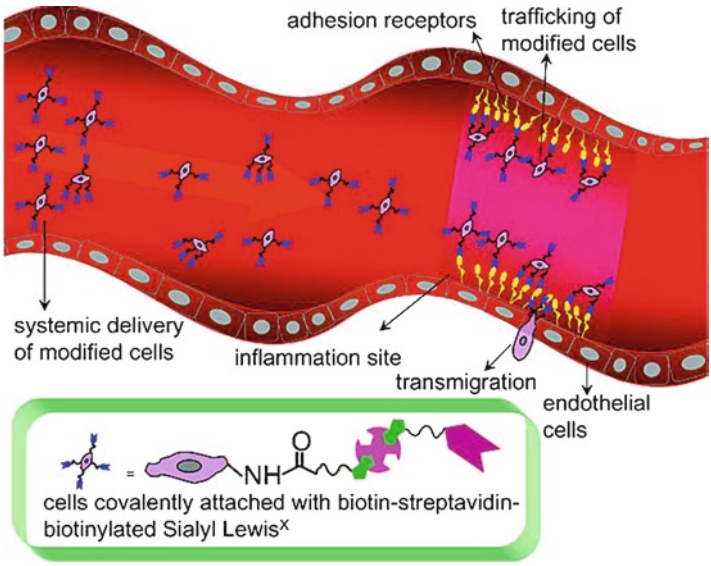

Fig. 4 Diagram of the homing process of systemically infused sialyl Lewis X–engineered MSCs to P-selectin–expressing endothelium under inflammatory conditions. (Reproduced from Sarkar, D., Vemula, P.K., Teo, G.S.L., et al. (2008). Chemical engineering of mesenchymal stem cells to induce a cell rolling response. *Bioconjug. Chem.* 19, 2105–2109; with permission from the American Chemical Society.)

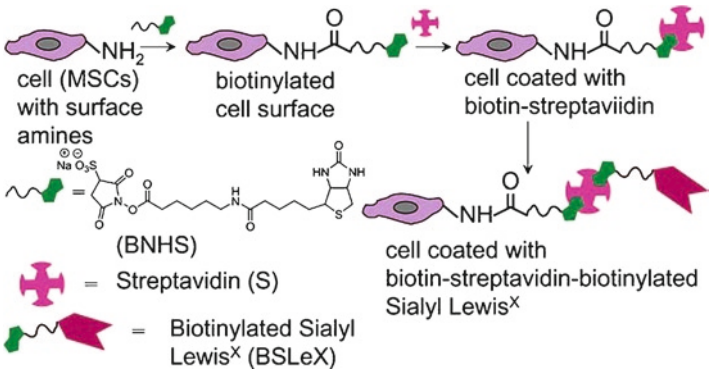

Fig. 5 Schematic illustration of preparation of sialyl Lewis X–engineered MSCs. (Reproduced from Sarkar, D., Vemula, P.K., Teo, G.S.L., et al. (2008). Chemical engineering of mesenchymal stem cells to induce a cell rolling response. *Bioconjug. Chem.* 19, 2105–2109; with permission from the American Chemical Society.)

involves a stepwise process including (1) treatment of cells with sulfonated biotinyl-*N*-hydroxysuccinimide to introduce biotin groups on the cell surface, (2) addition of streptavidin, which binds to the biotin on the cell surface and presents unoccupied binding sites, and (3) attachment of biotinylated targeting ligands. In our model system, a biotinylated cell rolling ligand, sialyl Lewis X (SLeX), is conjugated on the MSC surface. The SLeX-engineered MSCs exhibit a rolling response on a P-selectin–coated substrate under shear stress conditions [101], indicating their potential utility in targeting P-selectin–expressing endothelium in the bone marrow or at sites of inflammation. Of importance, the surface modification has little impact on the native phenotype of MSCs, including their multilineage differentiation capacity, viability, proliferation, and adhesion kinetics. Note that this approach to covalently modifying the cell surface and immobilizing required ligands is not limited to MSCs or the SLeX ligand. It should have broad implications on cell therapies that use systemic administration and require targeting of cells to specific tissues [101].

4 Conclusion and Perspectives

Owing to their multipotentiality, paracrine effects, and immunomodulatory properties, MSCs will likely find utility in treating a variety of diseases and disorders. The clinical trials for a number of MSC products in several disease conditions are being conducted or have just been completed. For instance, Osiris Therapeutics (Columbia, MD) has three products, Prochymal, Provacel, and Chondrogen, that are nearing the final testing stage of clinical trials to be used in the clinic for treating graft versus host disease, myocardial infarction, and knee injuries, among others. Although current trials may lead to novel therapies that may improve the lives of thousands of people around the world, it is critical to consider that mesenchymal

stem cell therapy is still in its infancy, and there are many challenges and issues remaining to be addressed. First, while no adverse effects from the use of MSCs have been reported in clinical trials, the long-term safety of MSC therapy is still unknown. The second issue is to standardize MSC preparation, manufacturing practice, and administration to avoid the high variability that has been observed within and between studies. Finally, more research has to be performed to address some of the current unknown aspects in MSC biology, such as their homing properties, the mechanism of their immunomodulatory effects, their in vivo multilineage differentiation potential, and their interplay with cancer cells.

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Gastrointestinal Stem Cells

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Abstract Stem cell research is advancing at an incredible pace, with new discoveries and clinical applications being reported from all over the world. Stem cells are functionally defined by their ability to self-renew and to differentiate into the cell lineages of their tissue of origin. Stem cells are self-sustaining and can replicate themselves for long periods of time. These characteristics make them very promising for treating debilitating disorders such as heart diseases, liver diseases, stroke, spinal injuries, Parkinson disease, Alzheimer disease, retinal degeneration, muscular dystrophy, diabetes mellitus, and so on. Stem cell therapy has generated interest in clinicians and the public. Clinical applications have been reported in heart diseases, spinal cord injury, ischemic limbs, retinal degeneration, and liver diseases. This chapter summarizes recent developments concerning gastrointestinal stem cells.

Keywords Stem cells • Liver • Autologous bone marrow • Crypts • iPS

1 Introduction

Stem cells are self-sustaining and can replicate themselves for long periods of time [1]. They can be classified into two major categories according to their developmental status: embryonic stem cells and adult stem cells. Embryonic stem cells are isolated from blastocyst [2]. Although this opens up the enticing possibility of "designer" tissue and organ engineering, it is clouded by the ethical issues that surround the use of these cells harvested from early human embryos. Adult stem cells are specialized cells found within many tissues of the body, where they function in tissue homeostasis and repair. They are precursor cells capable of differentiation into several different cells.

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They have been propagated from bone marrow, liver, brain, skin, skeletal muscle, adipose, cord blood, Wharton's jelly, and peripheral blood [3].

There is continuous and rapid renewal of the epithelial lining of the gastrointestinal (GI) tract. Several studies have demonstrated that continuous renewal of the GI lining occurs due to cells with high proliferative capacity anchored in specific locations along the GI epithelium [4]. During the last three decades knowledge of the cellular hierarchies of these proliferative stem cells has gradually increased, especially using animal models, and the renewal concept is well proved.

2 Gut Stem Cells

The GI tract is a rich repository of stem cells. Attempts have been made in mice to harvest neural stem cells from the GI tract. This has potential in the treatment of motility disorders such as achalasia, congenital hypertrophic pyloric stenosis, and diabetic gastroparesis [5]. Stem cell division serves two purposes: replenishing the stem cell compartment and generating transit-amplifying cells. Transit-amplifying cells undergo several rounds of division, and their progeny eventually execute the differentiation pathways characteristics of the epithelium.

2.1 Identification and Isolation of Esophageal Epithelial Stem Cells

In esophageal epithelium, which has a single differentiation pathway, tissue renewal is performed by the transit-amplifying cell population. Current studies carried out to study the proliferation of cells in esophageal epithelium using mitotic figures and immunohistochemical staining showed that cell proliferation takes place in the basal zone [6]. The cells in the interpapillary basal layer of the esophagus are candidates for esophageal epithelial stem cells. They proliferate in vivo, and their division yields one daughter cell that remains in an area of low proliferative activity (a putative stem cell) and one that enters an area of high proliferative activity (a putative transit-amplifying cell). Basal cells from the esophageal epithelium can be separated on the basis of their fluorescence-activated cell sorting profile [7].

2.2 Intestinal Stem Cells

In most tissues of the body, stem cells divide only rarely—perhaps once a month. That is not true of the rapidly dividing stem cells of the intestine. Their entire life, intestinal stem cells make tissue every day. Every 5 days, the intestinal lining is replaced in its entirety, leaving only the stem cells and their Paneth cell defenders constant.

The stem cells produce an impressive 200–300 g of new cells every day. The lining of the intestine is made up of peaks known as villi and valleys called crypts [8]. The crypts contain stem cells and so-called Paneth cells, which serve to protect the stem cells.

The intestinal stem cells can differentiate into four different cell lineages: absorptive enterocytes, mucin-secreting goblet cells, peptide hormone–secreting neuroendocrine cells, and microbicide-secreting Paneth cells. The majority of the cells in the villi are enterocytes, with a few goblet and neuroendocrine cells located at various intervals. These three lineages form and mature as they migrate up the crypt to the tip of the villus. During tissue homeostasis these cells are replaced and subsequently exfoliated into the intestinal lumen. Paneth cells differentiate as they travel down to the base of the crypt. The switch between a polyclonal crypt at birth and a monoclonal crypt after crypt fission in young animals suggests that several stem/progenitor cells reside within the initial crypts. However in adult intestine, only one stem/progenitor cell population appears to remain per crypt [9].

The enteric nervous system (ENS) is a complex network of interacting neurons and supporting glial cells that regulates intestinal motility, blood flow, and secretion. ENS neurons are located in two distinct anatomic regions: the myenteric plexus and the submucosal plexus. Myenteric plexus neurons send most of their axonal projections to the muscle layers of the intestine. Submucosal plexus neurons send the majority of their projections to the subepithelial region, including the area surrounding crypts.

In addition to being in close contact with enteric neurons and blood vessels, adult crypts are surrounded by a layer of specialized mesenchymal cells known as pericryptal fibroblasts (or subepithelial myofibroblasts). Tritiated-thymidine labeling studies have indicated that, like their overlying epithelial cells, pericryptal fibroblasts continuously migrate upward from the crypt base. This puts them in a strategic position to establish and maintain instructive communications with stem cells and their descendants [10].

Although the intestine is thought to have multipotent stem cells with the capacity to differentiate into enterocytes and goblet, Paneth, and neuroendocrine cells, the current biologic evidence for the existence of multipotent intestinal stem cells is not as well established as it is for other epithelia. This is primarily because of the paucity of cell surface markers or promoters specific for intestinal stem cells that would permit their isolation from their putative location in the crypt. There is a critical need for convenient biologic assays that allow intestinal stem cells to be identified based on their ability to give rise to descendant lineages ex vivo. Without such clonigenic assays, characterization of the cellular and molecular factors required to sustain growth or survival of stem cells or to generate descendant lineages will continue to be a slow and haphazard process. Thus, it is encouraging to read reports such as the one by Whitehead et al. [11] describing a technique for establishing in vitro growth of undifferentiated epithelial cells from disaggregated normal human and mouse colonic crypts.

Stem cells have been transplanted to treat refractory fistulas in patients with Crohn disease [12]. In the intestine, multipotent stem cells are thought to be housed in the crypt base. The work carried out by Krause et al. [13] using a single stem cell demonstrated differentiation to epithelial cells throughout most of the gastrointestinal tract. Tissue regeneration upon injury involves recruitment of epithelial stem cells to replace the damaged cells. The adult liver offers an example [14].

3 Liver Stem Cell Transplantation

Liver stem cells are capable of proliferating and differentiating into functionally specialized cells. The liver has a large regenerative capacity. In prolonged liver damage or inhibition of hepatocyte regeneration, the oval cells located in the canals of Herring can act as facultative stem cells [15,16]. The human hepatic stem cells (hHpSCs) are found in ductal plates in fetal liver and in canals of Hering in adult liver. The hHpSCs were isolated by using epithelial cell adhesion molecule (EpCAM) as a progenitor marker, and the cells positive for EpCAM were sorted by immunoselection. They constitute approximately 0.5%–2.5% of liver parenchyma of all donor ages. The self-renewal capacity of hHpSCs is indicated by phenotypic stability after expansion for greater than 150 population doublings in a serum-free, defined medium and with a doubling time of approximately 36 hours. The hHpSCs are positive for cytokeratins 8, 18, and 19, CD133/1, telomerase, CD44H, claudin 3, and albumin (weakly), and they are negative for α-fetoprotein (AFP), intercellular adhesion molecule (ICAM)-1, and markers of adult liver cells (cytochrome P450s), hematopoietic cells (CD45), and mesenchymal cells (vascular endothelial growth factor receptor and desmin). hHpSCs when cultured on STO feeders give rise to hepatoblasts with cordlike colony morphology and upregulation of AFP, cytochrome P450 3A7, and ICAM-1. The transplantation of EpCAM-positive cells or cultured hHPSCs into NOD/SCID mice results in mature liver tissue expressing human-specific proteins. The hHpSCs are good candidates for liver cell therapy.

Isolation of hepatic progenitors from a human source is a major challenge for the clinical application of this therapy. Recently hepatic progenitors have been isolated from the following sources:

Intrahepatic sources: Cadaver livers are a source of hepatic progenitors. Hepatocytes isolated from aborted human fetuses are another potential source.

Extrahepatic sources: These include autologous bone marrow [17], umbilical cord blood, Wharton's jelly, peripheral blood monocytes, and adipose tissue.

Liver stem cells can be transplanted through several routes: intraperitoneal and percutaneous intrahepatic artery catheterization in acute liver failure, intrahepatic route and portal vein or intrahepatic artery catheterization in patients with metabolic liver diseases, and through intrasplenic artery, hepatic artery catheterization, and portal vein catheterization in patients with chronic liver diseases. Attempts have been made to infuse cells from autologous bone marrow along with

granulocyte-stimulating factor. The preferred route is hepatic artery catheterization. At our center, 25 patients with Child-Pugh grade C cirrhosis of the liver have been given this therapy [18].

4 *In Vitro* Transdifferentiation of Adult Hepatic Stem Cells into Pancreatic Endocrine Hormone–Producing Cells

Liver and ventral pancreas arise from the same population of cells in the endoderm of the embryo. The location, growth factors, and cell adhesion molecule expressions are responsible for the differentiation of these cells into liver or pancreas. Several studies suggest that pancreas possesses the capacity to differentiate into liver cells [19]. When the liver cells are cultured in media containing high glucose, they can transdifferentiate into insulin-secreting cells, and these cells can self assemble to form three-dimensional islet cells like clusters that express pancreatic islet cell differentiation markers such as PDX-1, PAX-4, PAX-6, Nkx2.2 and Nkx6.1, insulin I, insulin II, glucose transporter 2, and glucagon. The transdifferentiated cells have significant implications for future therapies of diabetes.

5 Induced Pluripotent Cells

Cell transplantation based on induced pluripotent (iPS) cells has enormous clinical potential; however, a truly regenerative treatment would direct endogenous cells to participate in the repair of damaged tissues that cannot regenerate it and such nonphysiologic changes in differentiation status. iPS cells equivalent to embryonic stem cells has been harvested from human adult skin using genetic reprogramming. Human iPS cells, produced either by expression of *OCT4*, *SOX2*, *c-Myc*, and *KLF4* or by *OCT4*, *SOX2*, *NANOG*, and *LIN28*, are also remarkably similar to human embryonic stem (ES) cells. These cells are morphologically similar to human ES cells, express typical human ES cell–specific cell surface antigens and genes, and differentiate into multiple lineages in vitro.

The conversion of a somatic stem cell to a pluripotent stem cell has been done successfully, and researchers are progressing toward induction of nonphysiologic transitions between cell types by using a limited number of factors. Recently iPS cells were derived from liver, stomach, pancreatic β cells [20], and mature B cells [21]. Liver-derived iPS cells were shown to have an origin of albumin-expressing cells. Takashi et al. showed that iPS cells derived using a genetic marking system that permanently labels liver cells once they differentiate to express albumin, could be reprogrammed to so-called induced pluripotent cells that can contribute to all cell types in adult mouse. They also showed that the epithelial cells lining the stomach can generate iPS cells, and that doing so requires a less rigorous screening system than that used with cultured skin cells or fibroblasts [22].

6 Mesenchymal Stem Cells

Human mesenchymal stem/progenitor cells (MSCs) were isolated and characterized from first trimester fetal blood, liver, and bone marrow. MSCs are closely associated with hematopoietic stem cells in adult bone marrow, while first trimester fetal blood contains significant numbers of hematopoietic progenitors [23], and SCID repopulating cells [24] and fetal liver and bone marrow are well-known sites of active hematopoiesis during ontogeny [25]. They differentiate into adipocytes, osteocytes, and chondrocytes when cultured in differentiating media. The morphology, growth kinetics, and immunophenotype of MSCs isolated from first trimester fetal liver and bone marrow were comparable to those of fetal blood–derived MSCs. Cells derived from adult bone marrow and fetal bone marrow circulate in first trimester human blood and provide novel targets for gene therapy.

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Lung Epithelial Stem Cells

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Abstract The lung epithelium is structurally and functionally a complex tissue composed of different cell types. It is exposed to toxic agents and pathogens that can with time result in various lung diseases, including lung cancer. The major cell types in the proximal tracheobronchial part are basal cells, goblet cells, ciliated cells, and cells of the submucosal glands. Further down the bronchial tree, Clara cells replace basal cells. Neuroendocrine cells can be found spread throughout the bronchial tree and in cell clusters referred to as neuroendocrine bodies. The most distal part of the lung contains the type I and type II respiratory alveolar cells. The branching form of the lung epithelium necessitates that stem cells located within special niches down the respiratory tree maintain the structural and functional integrity of the lung during normal cellular turnover and during repair. The stem cell niche in the lung is poorly defined, but as in many other organs, stromal cells and extracellular matrix likely play a fundamental role in regulating stem cell activity. Basal cells and cells in the neck of the submucosal glands have been shown to contain stem cell characteristics in trachea and large bronchi. In the bronchioles a subpopulation of Clara cells, the so-called variant Clara cells, and bronchioalveolar stem cells can generate both epithelial cells of the bronchioles and alveolar type II cells that in turn can generate type I cells. Increased knowledge of the cellular context of the lung epithelium, including spatial location of endogenous stem cells and characterization of the stem cell niche, may in the near future have a major impact on the understanding and treatment of many lung diseases.

Keywords Lung development • Lung morphogenesis • Epithelial stem cells • Tracheobronchial zone • Stem cell niche

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1 Introduction

The respiratory epithelium has various functions, such as mediating gas exchange, forming a mechanical barrier, regulating lung fluid balance and metabolism, clearing inhaled agents, activating inflammatory cells in response to injury, and regulating airway smooth muscle function via secretion of numerous mediators. Due to its unique position toward the external environment the respiratory epithelium is continuously exposed to agents such as pollutants, viruses, and bacteria. This sustained stress can gradually lead to injury, resulting in various lung pathologies such as asthma, chronic obstructive pulmonary disease (COPD), pulmonary fibrosis, and lung cancer. To maintain a balanced homeostasis in tissues and organs, regulation of self-renewal and differentiation at the stem cell level is of major importance. The cellular composition of the stem cell niche and the molecular signals involved in fate decision of stem cells are being unraveled in many organs. This knowledge is rapidly being applied to the mechanisms behind tissue architecture and progression of various diseases. Better understanding of the cellular context in the lung, such as the spatial location of endogenous stem cells, the mechanism of cellular turnover, and the heterotypic cross-talk between the epithelium and the surrounding stroma is important to allow functional restoration of the respiratory epithelium following injury.

In this chapter we first briefly discus lung development and methods to track stem cells in vitro and in vivo. We then focus on the cellular hierarchy in the lung epithelium, the potential location of endogenous lung stem cells, and important extrinsic factors regulating stem cell fate. We also discuss current evidence indicating that stem cells are potential cancer-initiating cells in the lung. Finally, we review the current in vitro models available to study lung epithelial morphogenesis and cancer progression.

2 Lung Development and Cellular Turnover

Lung development starts by invasion of the ventral foregut endoderm into the surrounding mesenchyme. This occurs at embryonic day 9.5 in mice and in week 4 of embryo development in humans. Both the invasion and further growth occur through a continuous process of elongation and branching morphogenesis. The anatomy and the cellular complexity of various parts of the adult lung necessitate the presence of several stem cell or progenitor zones to replenish the normal cellular turnover and regenerate the functional epithelial cells after injury. It is likely that distinct types of lung cancer originate in these various progenitor or stem cell zones [1]. The respiratory system is continuously exposed to exogenous agents that damage the airway epithelium. Therefore, the epithelium undergoes constant cell turnover to replace damaged cells. Under normal conditions, this turnover is slow, but following injury, the turnover is rapidly increased, resulting in functional and structural restoration of the epithelial layer. There is an ongoing debate over the

cellular phenotype and spatial location of endogenous stem cells in the human lung. It appears that different regions of the respiratory system contain different stem cell niches that are responsible for local tissue maintenance and repair [2]. Most of our current knowledge regarding stem cells in the respiratory system is derived from mouse models. There is, however, an inherent species difference between mice and humans in terms of the complexity of branching processes and cellular composition in the respiratory system that needs to be taken into account.

Human lung epithelial stem cells have been much less studied than stem cells of the blood, gut, or skin. This is partially due to the challenging complexity of the lung and airways, which are characterized by many cell types, heterogenic structures and functions, and relatively slow cellular turnover. Furthermore, models mimicking lung development in vitro have been lacking [3]. An important current issue is to localize prospective stem cells and progenitor cells within the human lung and to define both the extrinsic and intrinsic regulatory elements controlling stem cell fate. This could help to understand in detail the molecular and cellular mechanisms of many lung diseases and to identify potential lung cancer–initiating cells.

3 Tracking Lung Epithelial Stem Cells in Vivo and in Vitro

A combination of immunohistochemistry and cell ablation strategies using injury models by injection or inhalation of specific toxic agents has been used to track lung stem cells and cell lineage development in mouse models. After exposure to toxic substances such as naphthalene, oxidants (NO_2), or sulfoxide and concurrent injections of nucleotide analogs such as bromodeoxyuridine or tritiated thymidine (^3H) it is possible to target the cell population responsible for epithelial restoration. Stem cells have considerable proliferative capacity but divide slowly. This slow cycling property is a characteristic that can be used to track stem cells through labeling of DNA using various labeled nucleotide analogs. Due to the faster cell proliferation in transient amplifying cells, nucleotide analogs are washed out faster in these cells than in slower-cycling stem cells. Stem cells are therefore often called labeling-retaining cells (LRC). This method has been pivotal for identification of stem/progenitor cells in the mouse lung. For obvious reasons injury models and cell labeling are not applicable to humans in vivo. Immunohistochemistry using known stem cell markers and markers of cell lineage differentiation are useful to prospectively identify the spatial location of candidate stem cells in both rodents and humans. Immunohistochemistry combined with flow cytometry or other cell separation assays are also useful to isolate candidate stem cells for structural or functional studies in vitro or for transplantation studies in animal models. Single-cell culture in low-attachment plates has been shown to preferentially facilitate growth of stem cells. In addition, three-dimensional cell culture models can allow the recapitulation of tissue morphogenesis in vitro. All of these methods and models are of major importance for studying lung stem cells, including their role in lung morphogenesis.

4 Cellular Context in the Lung Epithelium

The lung is composed of three distinct anatomic zones: (1) proximal cartilaginous tracheobronchi, (2) small, noncartilaginous bronchioles, and (3) distal respiratory alveolar zones. In the larger proximal trachea and bronchi the continuous epithelial sheet forms a pseudostratified layer that becomes a columnar and cuboidal single layer in the small distal bronchioles and finally cuboidal and flattened alveolar cells in the respiratory zone. The submucosal glands extend from the pseudostratified layer in the intercartilaginous regions of the larger bronchi. Each of these different zones in the lung epithelium is composed of several cell types with unique function and localization (Fig. 1).

4.1 Tracheobronchial Zone

The pseudostratified layer in the tracheobronchial zone contains ciliated surface cells, mucus-producing goblet cells, and basal cells [4]. These cells rest on a continuous basement membrane that separates the epithelium from the surrounding stroma. Ciliated epithelial cells are the predominant cell type in the proximal conducting zone. They are believed to arise from either basal or secretory cells and have until recently been thought to be terminally differentiated. Basal cells are ubiquitous in the conducting epithelium, but the number of these cells decreases with reduced airway size. They are firmly attached to the basement membrane but do not reach the airway surface. Basal cells are postulated to be candidate stem or progenitor cells necessary for epithelial maintenance and repair in this zone [4, 5]. Immunohistochemistry from human lung autopsies reveals that basal cells count for 51% of the proliferation compartment in the large airways [4], suggesting that they are likely to be either stem cells or transient amplifying cells. One of the first indications that basal cells might be the stem/or progenitor cells in the proximal airway epithelium came from studies in rats in which basal cells take up [^{3}H]thymidine that was later found in ciliated and goblet cells [6, 7]. p63, a marker for basal cells in various organs such as skin, mammary gland, and prostate [8], is also abundant in basal cells in the lung [5]. Daniely et al. demonstrated that knockout mice lacking expression of p63 were depleted of pulmonary basal cells and showed premature differentiation toward columnar and ciliated phenotype in the trachea [9]. These data suggest that p63 is an important regulatory molecule for maintenance of undifferentiated basal cells.

The submucosal glands (SMGs) extend from the epithelial surface in the trachea and large bronchi into the submucosa and contain serous- and mucus-producing cells. SMGs are the major secretory units lying beneath the epithelium of the cartilaginous airway [10]. These glands are believed to play a crucial role in normal lung function and innate immunity by producing and secreting antibacterial agents, mucus, and fluid into the lumen [10]. SMG dysfunction is also

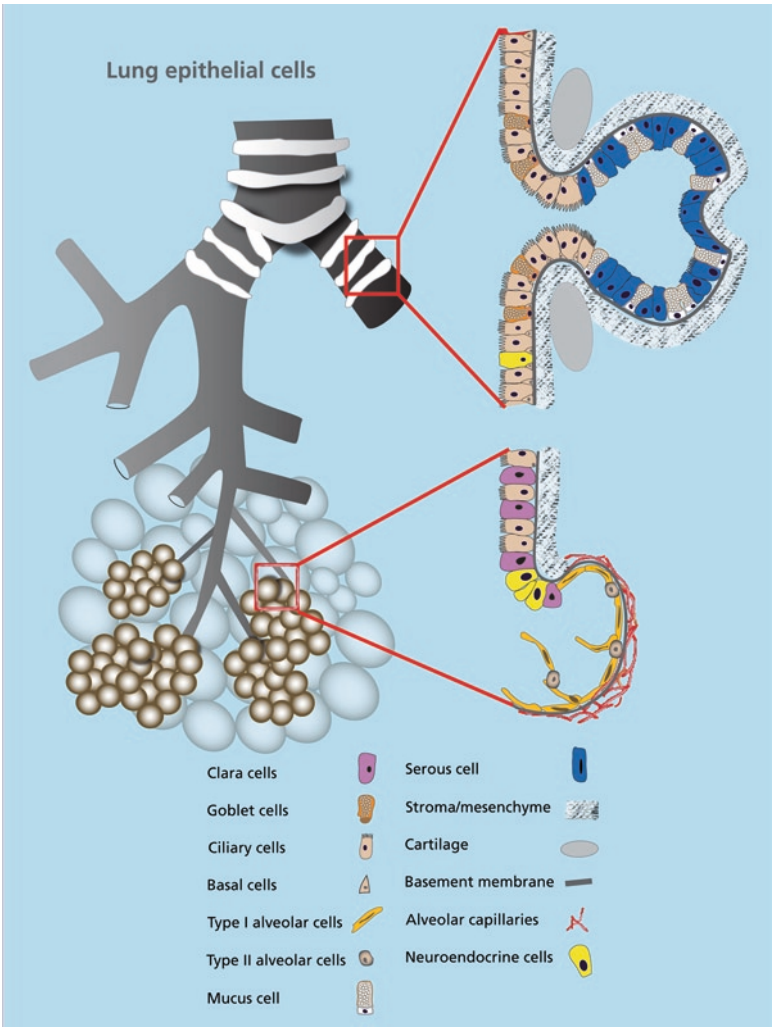

Fig. 1 Cellular context in the human respiratory epithelium. Schematic drawing of the tracheobronchial tree. The upper and lower details on the right depict the cellular components of the large and small airways, respectively. The cartilaginous tracheobronchial part (upper detail) is composed of ciliary cells, goblet cells, and basal cells. Submucosal glands (SMGs) are abundant in the large airways and contain serous and mucus cells. Bronchioles (lower detail) are composed of ciliary cells, Clara cells, and neuroendocrine cells that can also be found sparsely in the large airways. The distal alveolar part is composed of type I and type II alveolar cells. The alveolar epithelium is surrounded by a complex network of capillaries. The respiratory epithelium is separated from the collagenous stroma by a basement membrane throughout the airways. Basal cells in the bronchi and in the neck of the SMGs are considered to be candidate stem cells in the proximal airways. In the distal airways the bronchioalveolar zone contains candidate stem cells such as variant Clara cells

thought to play an important role in many lung diseases, such as cystic fibrosis, chronic bronchitis, and asthma (reviewed in ref. 11). Of interest, expansion of SMGs occurs in these diseases, leading to abnormal mucus production in the airways. Normally, SMGs are not found in distal airways such as bronchioles, but in diseases such as cystic fibrosis SMG hyperplasia occurs in bronchioles, resulting in mucus hyperproduction.

In rodents SMGs are sparse, but in human airways they are abundant [12]. In humans, SMGs develop during gestation when luminal epithelial cells invade the lamina propria of the proximal trachea [12]. Studies using retroviral tagging of human bronchial cells and subsequent transplantation of these cells onto denuded tracheal xenografts in immune-suppressed mice have shown that cells residing in the neck of the SMGs are able to regenerate both airway surface epithelial cells and SMGs [11]. Goblet cells are abundant in the tracheobronchial zone in humans. It is noteworthy, however, that in mice, Clara cells not goblet cells are present in the tracheobronchial part, further underscoring the differences between species. Following injury to the proximal trachea by sulfoxide or naphthalene, LRCs localize to the neck of the SMGs and to basal cells, indicating that these areas harbor stem or progenitor cells.

4.2 Bronchiolar Zone

The transition from the proximal bronchial part to bronchioles is accompanied by transitions from a pseudostratified layer in the large bronchi to a columnar and cuboidal layer in the bronchioles. Here most of the basal cells have also been replaced by Clara cells. Clara cells are thought to produce bronchiolar surfactant and metabolize xenobiotic compounds by the action of the P450 monooxygenase system. This property makes them vulnerable to naphthalene. Recent evidence suggests that these cells play an important stem cell role, serving as a progenitor for both ciliated and mucus-secreting cells [13]. A subpopulation of Clara cells (Clara variant or Clara^{v}) are resistant to naphthalene toxicity, and these cells show the ability to regenerate epithelial integrity in the damaged tissue by differentiating into mucus and ciliated cells [14]. The Clara^{v} cells are located in clusters in neuroepithelial bodies close to the bronchoalveolar junction [7]. Clara^{v} cells have a similar marker expression as the recently identified bronchioalveolar stem cells (BASCs). BASCs have been found in neuroepithelial bodies at the bronchioalveolar duct junction [15]. Clara^{v} cells and BASCs could thus be the same cell population, but this has not yet been proven. Clara^{v} cells express both the Clara cell marker CCSP (also known as CCA, CC10, uteroglobin, or Scgb1a1) and the type II alveolar marker pro–surfactant protein C [16]. Recent data suggest that Clara^{v} cells specifically contribute to bronchiolar and tracheal repair while having no role in alveolar maintenance, supporting a model in which distinct populations of epithelial progenitor or stem cells contribute to repair and renewal in different zones [13]. Clara^{v} cells and BASCs also

express the stem cell surface antigen sca-1 and CD34, which allow for purification and in vitro testing of these cells. In clonogenic assay in Matrigel (reconstituted basement membrane matrix) BASCs give rise to both Clara cells and alveolar type I and II cells [15]. The transcriptional regulation in BASCs is actively being probed. A recent study has shown the importance of Wnt signaling pathways in BASC regulation. Gata6, a known transcription factor in lung development and an inhibitor of the canonical Wnt pathway, regulates the temporal appearance and number of BASCs in the lung. In Gata6-null mice, BASCs show premature appearance and subsequent disappearance due to spontaneous differentiation. Of interest, the expansion of BASCs was the result of an increase in canonical Wnt signaling in lung epithelium upon loss of Gata6 [17]. Recently, Tesei et al. isolated BASC-like cells from human lung [18]. By seeding unsorted lung epithelial cells derived from lobectomy into low-attachment plates, commonly used to culture stem cell populations, they were able to grow colonies termed bronchospheres. Phenotypic characterization of these colonies revealed expression of CCSP and SP-A, indicating that these cells may share identity with Clara^{v} cells or BASCs in mice.

Pulmonary neuroendocrine cells (PNECs) are specialized epithelial cells found throughout the bronchial tree as solitary cells or in clusters (neuroepithelial bodies [NEBs]). NEBs are densely innervated groups of complex sensory airway receptors involved in the regulation of breathing. Together with their surrounding Clara-like cells, they exhibit stem cell potential through their capacity to regenerate depopulated areas of the epithelium following lung injury [19]. PNEC are rare cells that increase in number in chronic bronchitis.

4.3 Respiratory Alveolar Zone

In the most distal part of the respiratory tree, the bronchioli gradually transit into alveolar ducts and alveoli. This transition zone, referred to as the bronchiolar alveolar duct junction, has been identified in mice but not in humans. This most distal part of the lung is composed of type I and type II alveolar epithelial cells. Type II cells contain numerous secretory vesicles called lamellar bodies that are filled with surfactant protein, whereas type I cells express aquaporin-5. The type II cells constitute approximately 70% of the total alveolar cells. However, the flattened type I cells line approximately 90% of the alveolar surface area, while the abundant type II cells cover less than 10% due to their cuboidal shape [20]. Type I alveolar cells are responsible for gas exchange and are in close contact with microvessels. Type II alveolar cells control production of surfactant, regulate ion transport, and metabolize xenobiotics.

Type II cells are also believed to be progenitor cells for both type I and II, based on data from injury models showing that type II cells can restore functional alveoli after all type I cells have been destroyed [7].

5 Branching Regulators and Components of the Stem Cell Niche

The mesenchyme is known to play a major role in organogenesis and maintenance of tissue structure and function in many organs, such as the mammary gland [21], prostate [22], and lung [23]. The lung initially develops from endoderm in the fetal foregut in a process that is highly dependent on cross-talk between the endodermal cells and the underlying mesenchyme. Thyroid transcription factor (TTF-1) is the earliest known marker associated with commitment of endodermal cells to lung epithelial cell lineages. Outgrowth and branching of TTF-1–positive endodermal cells is stimulated by fibroblast growth factors (FGFs), in particular FGF10 expressed by the mesenchymal cells acting through FGF-receptor-2 (FGFR-2) on the invading epithelial cells [24]. TTF-1 and FGFR-2 are present in respiratory epithelial cells throughout lung morphogenesis and in adult tissue [25]. Many studies have shown a critical role for the mesenchyme in lung branching, although most studies are not able to discriminate the mesenchymal components mediating these effects. It has been shown that the mesenchyme can even instruct epithelial cells from other tissues to attain a pulmonary epithelial phenotype, showing the importance of heterotypic cellular interaction or cell–matrix interactions in this developmental process [12, 26, 27]. These studies use mouse models, focusing on the mouse embryo during development or lung tissue explants from fetal lung.

5.1 Branching Morphogenesis

Branching morphogenesis is one of the key developmental processes during lung development. A recent seminal paper indicated that this process can be reconstructed using three-dimensional patterning through a hierarchical model based on simple local modes of branching that occur in a highly regulated fashion throughout development. The authors proposed that these simple branching modes (only three different modes were present) were controlled genetically through master regulators [28]. They suggested that one of the developmental switches or regulators of this process might be the sprouty gene family, specifically sprouty-2. Branching morphogenesis in various tissues has been shown to be highly conserved between species and between different organs in the same species. The sprouty gene family has been suggested to be a major regulator of branching in many organs. Sprouty was originally isolated and cloned from *Drosophila melanogaster*, where disruption of sprouty function was shown to have a phenotype of markedly increased branching in the *Drosophila* airways (thus the name sprouty) [29]. During mouse development, sprouty-1, -2, and -4 are all expressed in the lung epithelium [30]. In addition, it has been shown specifically that murine sprouty-2 appears to be dynamically expressed in the peripheral endoderm in embryonic lung and downregulated in the clefts between new branches. Furthermore, they showed that targeted overexpression of

mSprouty2 in the peripheral lung epithelium in vivo resulted in diminished branching [31]. These developmental studies are supported by the fact that the sprouty-2 and -4 double knockouts have severe defects in lung morphogenesis lethal to the embryo [32]. The mechanism of action of sprouty in the lung is believed to be through negative regulation of FGF-10 signaling, a known mesenchymal-derived stimulator of lung development and branching morphogenesis [31]. Important aspects, such as the spatial and temporal analysis of sprouty function and detailed mapping on the branching mechanism in human lungs, awaits cell culture assays that can observe the branching process in an in vitro setting.

5.2 The Vascular Niche

In the adult lung, the mesenchymal tissue changes substantially from the proximal conducting part to the distal alveolar part. In the larger bronchi, cartilage tissue supports the epithelial tissue. Fibroblasts, smooth muscle cells, and large vessels are also prominent in the proximal part. In the distal bronchoalveolar zones the cartilage has disappeared, and microvessels are prominent, especially surrounding the alveoli. Recent studies using embryonic lung tissue cultured under the kidney capsule of immunocompromised mice suggest an interaction between blood vessels and alveolar formation [33]. Of interest, Yamamoto et al. have demonstrated that endothelium-derived hepatocyte growth factor is necessary for distal lung morphogenesis in mice, including formation of alveoli [34]. Recent data from various organs, such as the liver, pancreas, brain, and bone marrow, indicate that organ-specific endothelial cells are of major importance for fate control of stem cells and for organogenesis and tissue maintenance (reviewed in ref. 35). Lammert et al. showed that endothelial cells are important for both pancreas and liver organogenesis [36]. Similarly, endothelial cells were shown to be vital components in the stem cell niche in the nervous system [37] and in hematopoiesis [37]. It is thus becoming evident that endothelial cells are important regulators of stem cells in many organs and a crucial component for cell fate decisions and tissue morphogenesis. In the large airways LRCs occur in clusters in intercartilage regions that are highly vascularized and accompanied by nerves [38]. Indeed, it has been suggested that both endothelial cells and neurons play a vital role as part of the stem cell niche in this area.

5.3 Neuroendocrine Bodies

In the distal lung it has been suggested that neuroendocrine bodies (NEB) support Clara^{v} stem cells. Recently De Proost et al. demonstrated how NEB cells communicate with their cellular neighbors in the NEB microenvironment by releasing ATP, subsequently evoking purinergic activation of surrounding Clara-like cells [19]. This suggests that local paracrine purinergic signaling within this potential stem

cell niche may be important to normal airway function, airway epithelial regeneration after injury, and/or the pathogenesis of lung cancer. Small cell lung cancer has phenotypic traits of PNECs, indicating that it may originate from NEBs or PNECs outside NEBs.

It is clear that the mesenchymal tissue plays a vital role in lung morphogenesis and regulation of stem cell niche. In addition to the cellular components mentioned earlier, many other cell types, including smooth muscle cells, fibroblasts, chondrocytes, and immune cells, are believed to contribute to lung morphogenesis. Their direct role, however, in regulating stem cell niches is unknown, but such knowledge could have a substantial impact on our understanding of common diseases such as asthma, COPD, and lung cancer. It is therefore critical to isolate and characterize the function of each of these cellular components in the human lung in vitro with the aim of recapitulating in culture the critical aspects of lung morphogenesis.

6 Modeling Human Lung Morphogenesis in Vitro

Knowledge of lung development, tissue maintenance, and lung cancer is largely derived from animal models. Many elegant studies in the mouse have led to great insights into the developmental processes involved in the lung, such as the mechanism of branching morphogenesis that is typical for lung development (as well as many other epithelial organs; e.g., breast, kidney, pancreas, and prostate) [28, 39]. There is, however, a gap in knowledge regarding the cell linage relationship and function of stem cells in the human lung and the cellular origin of various types of lung cancer. This is partially due to the lack of representative cell lines and shortcomings of the cell culture systems available. The functional and structural complexity of the lung and the diversity of cell types found within particular zones require the development of reliable cell culture assays that capture the phenotypic traits of each cell type found within the lung epithelium. Such assays could help to define the origin of various lung diseases such as lung cancer and thereby open avenues for targeting specific defects at the cellular and molecular levels. In vitro models of human lung cells might benefit from some of the models and techniques used in other organ systems, such as the skin, mammary gland, prostate, salivary gland, and intestinal tract [40–44]. Thus, using skin stem cells and dermal fibroblasts, it has been possible to generate skin equivalents in culture [40]. In vitro modeling of the human mammary gland has led to valuable information regarding each identified cell type [45–47]. Furthermore, breast epithelia cells with stem cell characteristics can generate the branching structures of the mammary gland when cultured in three-dimensional cell culture [47]. Likewise, cultured human prostate and salivary gland progenitor cells have captured critical phenotypic traits of their in vivo counterparts [42, 43]. Of interest, data from the skin, mammary gland, salivary gland, and prostate models suggest that basal cells have stem cell characteristics as evidenced by their ability to regenerate in vivo–like phenotype in vitro. This has not yet been tested in culture with cells from the human lung and awaits

isolated primary cells with stem cell characteristics such as basal cells or cell lines that can capture critical aspects of lung morphogenesis in three-dimensional culture. Recently, Keith Mostov's group demonstrated that primary human alveolar type II cells generate cystlike structures in reconstituted basement membrane matrix. Generally, most morphogenetic processes require cell proliferation and/or cell death to form a hollow lumen. In contrast, type II cells move, collide and form alveolar-like cysts without any notable cell proliferation and/or apoptosis [48]. It was also recently shown that single cells from human lung tissues were able to form bronchospheres on low-attachment filter plates. These bronchospheres were composed of cells with high expression of stem cell markers and mixed phenotype of both Clara cells and alveolar type II cells [18]. It needs to be answered whether these cells are true stem cells and whether they are analogous to the Clara and/or BASCs in the mouse lung. However, taken together, data and experience on isolation, culture, and characterization of the various human lung cell types in vitro are limited.

6.1 Human Lung Epithelial Cell Lines

Immortalized human lung epithelial cell lines have been used to study function and tissue morphogenesis of the lung [49,50]. Given the complexity of bronchial epithelia, it is interesting that many available bronchial cell lines have not been precisely defined with respect to their cellular origin and lack critical characterization in terms of expression of differentiation markers [51]. The most-cited lung epithelial cell line, A549, is derived from a human bronchoalveolar cell carcinoma [52]. This cell line originally represents type II alveolar cells; however, it has been widely used to study lung biology in general, although the structure and function of malignant alveolar epithelial cells is quite different from those of normal bronchial epithelial cells. The human bronchial cell lines 16HBE14o-, Calu-3, and BEAS-2B have been successfully applied to study drug transport, metabolism, and delivery due to their ability to form tight junctions (TJs) (reviewed in ref. 51). The cell line 16HBE14o- was established by transformation with SV40 large T antigen of cultured human bronchial epithelial cells [53]. In contrast, the Calu-3 cell line was derived from bronchial adenocarcinoma [54] and the BEAS-2B line from normal human epithelial cells immortalized using the adenovirus 12–SV40 hybrid virus [55]. Calu-3 and 16HBE14o- cell lines have been identified as the most differentiated models available and have been used to study barrier function and the activity of TJ complexes [51]. Of interest, none of these cell lines has been shown to harbor stem cell characteristics. We have recently generated and described a novel bronchial epithelial cell line using immortalization of airway epithelial cells by transducing them with a retroviral construct carrying the E6/E7 oncogenes from human papillomavirus-16. The cell line, referred to as VA-10, has a basal cell phenotype and has stable phenotypic characteristics with regard to its ability to capture several important features of pulmonary epithelium in vivo [56]. The VA10

cell line shows some stem cell characteristics in culture. When cultured using an air–liquid interface they differentiate into a pseudostratified layer with p63-positive basal cells facing the filter and ciliated cystic fibrosis transmembrane conductance regulator–positive cells facing the surface [56]. The cell line has stable phenotypic characteristics, and initial results are promising with regard to their ability to mimic several important features of pulmonary epithelium, such as functionally active TJ complexes generating high transepithelial electrical resistance in suspended or air–liquid interface culture. The structural and functional epithelial integrity generated by VA10 cells in air–liquid culture is important for analyzing drug transport and cellular response to chemical agents as well as host–pathogen interactions in vitro [57, 58]. However, the disadvantages of air–liquid cultures include lack of SMGs, a characteristic feature of the tracheobronchial tree, and an inherent limitation to allowing branching morphogenesis, including alveolar formation.

7 Discussion and Future Perspectives

The airway tree is a complex organ composed of diverse cell types. The cellular heterogeneity has led to controversies regarding the presence of stem cells and their role in remodeling the respiratory system. This is of particular interest in light of the constant requirement for regeneration of airway epithelia in response to environmental insults and the delicate balance between functional repair on one hand and carcinogenesis or dysfunctional airways on the other. Recent studies have defined several candidate zones that appear to harbor stem or progenitor cells responsible for tissue maintenance and repair. Despite species differences, the majority of current concepts and hypotheses regarding stem cells in human respiratory epithelia come from studies in rodents. Such studies in mice have identified several stem cell niches mostly based on injury models in which a particular cell type can be ablated by injection or inhalation of toxic agents. In the tracheobronchial zone, SMGs and basal cells have been identified as stem cell niches. In the bronchioles it is likely that a subset of Clara cells, so-called variant cells or Clara^{v}, substitute for basal cells as candidate stem cells. Clara^{v} may share the same identity as BASCs, but further studies are required to clarify this possibility. In the alveolar zone, type II cells generate both type I and type II cells. In addition, mesenchymal components play a crucial role in maintenance of the stem cell niche. In this regard the vascular niche appears to protect and regulate lung epithelial stem cells in particular.

Many clinical studies and abundant data from in vitro models suggest that constant exposure of the airways to the environment produces epithelial injury. In contrast, data from studies focusing on airway epithelial regeneration are relatively scarce, which is interesting because it is likely that epithelial injury requires dynamic repair to prevent pathogenesis of various lung diseases. Therefore studies of airway epithelial regeneration, stem cell niches and differentiation in accurate in vitro models could hold the key to novel therapeutic options. Thus, understanding

the self-renewal, differentiation, and cell fate decisions in the various stem cell niches in the human lung promises to shed light on important disease processes and could unravel critical pathways amenable to therapeutic manipulation. However, such an ambitious approach would require dedicated multidisciplinary teams of clinical and basic scientists and the application of knowledge from other epithelial organs. It can be hoped that such cooperation will be encouraged by the scientific community in general.

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Placental-Derived Stem Cells: Potential Clinical Applications

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Abstract Placental-derived stem cells are easily accessible and do not have many of the limitations of embryonic stem cells and their derivatives for use in clinical trials. Preclinical animal studies using amnion and, more recently, human amnion epithelial cells (hAECs) have provided evidence of many exciting potential clinical applications. We have characterized hAECs derived from term gestational tissues and investigated their potential application in the treatment of adult and perinatal lung injury. Studies by our group have shown that hAECs display key features of pluripotent stem cells. They do not form teratomas after transplantation into the testes of immunodeficient mice, and they have restricted expression of major histocompatibility antigens in vitro. hAECs also appear to suppress lymphocyte proliferation. Collectively, these findings indicate that hAECs may elicit minimal immune recognition following transplantation to an allogeneic recipient in their native form. Our observations on the reparative effects of hAECs in adult models of lung injury have encouraged us to investigate their potential use as a cellular therapy in the treatment of bronchopulmonary dysplasia and respiratory distress syndrome of preterm infants. In an in utero ventilation model in fetal sheep, our preliminary results indicate that administration of hAECs reduces inflammation and fibrosis, thus providing a potential novel cellular therapy in the treatment of the very preterm infant. Our research on multipotential amnion-derived epithelial stem cells may well revolutionize the approach to the use of stem cells in clinical therapies.

Keywords Human amnion epithelial cells • Hematopoietic stem cells • Mesenchymal stem cells • Hepatic regeneration • Low-density-lipoprotein receptor

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1 Introduction of Stem Cell Sources

Adult or somatic stem cells have been isolated from most tissues in the body. The bone marrow, the traditional source of adult-derived stem cells, contains both hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). HSCs, derived from adult bone marrow, have been widely used clinically for more than 50 years in the treatment of hematologic malignancies such as leukemia [1] and, more recently, following chemotherapy [2, 3]. MSCs, also derived from adult bone marrow, are being used in clinical trials for the treatment of myocardial infarction and in preclinical studies for vascular and neurologic disorders, bone regeneration, spinal disc repair and replacement, and anticancer therapies [3–8]. In preclinical studies, they have been shown to improve myocardial function after acute myocardial infarction [9–12], cerebral function after cerebral infarction [13], lung damage [14] and to repair liver and joint damage [15]. In addition, the immunomodulatory properties of MSCs are being evaluated as an adjunct therapy in the resolution of graft versus host disease following organ transplantation [16].

As a source of stem cells, gestational tissue offers considerable advantages over other sources of stem cells such as bone marrow– or embryo-derived cells. There is a virtually unlimited potential supply of, and easy access to, such tissues, and minimal ethical and legal barriers are associated with their collection and use. They are mainly derived from pregnancy tissue such as the amniotic fluid [17], placenta [18], umbilical cord, including Wharton's jelly [19, 20] and fetal membranes, including the amnion and chorion [21–23] (Fig. 1a). Placental-derived stem cells also have the advantage of being obtained without the need for an invasive procedure such as bone marrow biopsy.

Early focus on gestational tissues as a source of stem cells arose from the isolation and use of HSCs and MSCs from umbilical cord blood [24]. Indeed, cord blood stem cell banking for autologous and allogeneic use is now well established [25]. It is only more recently that the placenta [26], the fetal membranes [27], and amniotic fluid [28] have all been proposed as other potential sources of stem cells for clinical application.

Investigators at the Children's Hospital Oakland Research Institute in California have developed a method of obtaining large numbers of HSCs from human term placenta for potential use in the same manner as cord blood–derived HSCs are used currently. This group reported for the first time that the human term placenta is a hematopoietic organ [29]. They have now demonstrated that human placentae could provide abundant amounts of $CD34^+$, $CD133^+$ colony-forming cells, as well as other primitive hematopoietic progenitors, suitable for transplantation in humans. The total number of live HSCs, or colony-forming units in culture, that could be obtained from placentae was an order of magnitude larger than the number of HSCs obtained from cord blood from the same source. HSCs, which maintain their differentiation capacity, as well as stromal stem cells that support long-term culture of hematopoietic cells, were harvested from perfusate of placenta following CXCR4 receptor blockade. Cells from fresh or cryopreserved placental tissue generated erythroid

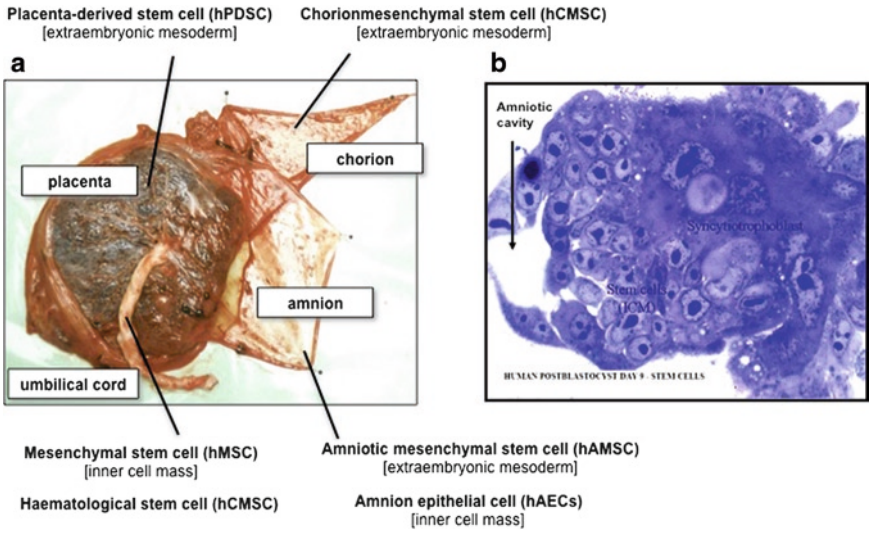

Fig. 1 **a:** Gestational tissue as a source of stem cells. Cell types with therapeutic potential can be derived from gestational tissue, including the placenta, umbilical cord, amniotic fluid, and fetal membranes, including the amnion, chorion, and Wharton's jelly. **b:** As some of these cell types develop from the inner cell mass (ICM) prior to gastrulation, these cells may share common properties with embryonic stem cells (courtesy of Prof. A. Henry Sathananthan, Monash University, Clayton VIC 3800 Australia)

and myeloid colonies in culture. Of importance, live HSCs were also obtained from whole cryopreserved placentae, offering the possibility of cost-efficient placental storage after minimal tissue handling or processing. Cells derived from placental tissue differentiated into all blood lineages in vitro. Animal experiments further demonstrated successful engraftment of placenta-derived HSCs, which reconstituted hematopoiesis and produced lymphoid cells after transplantation in immunodeficient mice [18]. Barcena et al. also recently reported the presence of hematopoietic cells in term human placenta [30].

These results suggest that human placenta could become an important new source of hematopoietic cells for allogeneic transplantation. Thus, it is highly likely that placental stem cells, like umbilical cord blood and bone marrow stem cells, could be used in the future to cure chronic blood-related disorders such as sickle cell disease, thalassemia, and leukemia.

If placenta is to be an alternative and more readily available source of clinically available stem cells, they will need to be fully characterized for optimal growth conditions and in vivo safety. MSCs have been isolated from human placenta and expanded and tested ex vivo using good manufacturing practice–compliant reagents [31, 32]. This study suggested that human placenta is an acceptable alternative source for human MSCs for clinical trials, and, indeed, such cells are being used in a clinical trial of adjunct therapy for graft versus host disease [32].

2 Development of Amnion Epithelium

Our group has an interest in cells derived from the amniotic membrane obtained from term pregnancies, so-called amnion epithelial cells (AECs). Following fertilization, the zygote undergoes a series of cell divisions to form a solid ball of cells (the morula) (Fig. 2a). After further divisions, the cells form a blastula, which consists of a spherical layer of cells surrounding a central fluid-filled cavity called

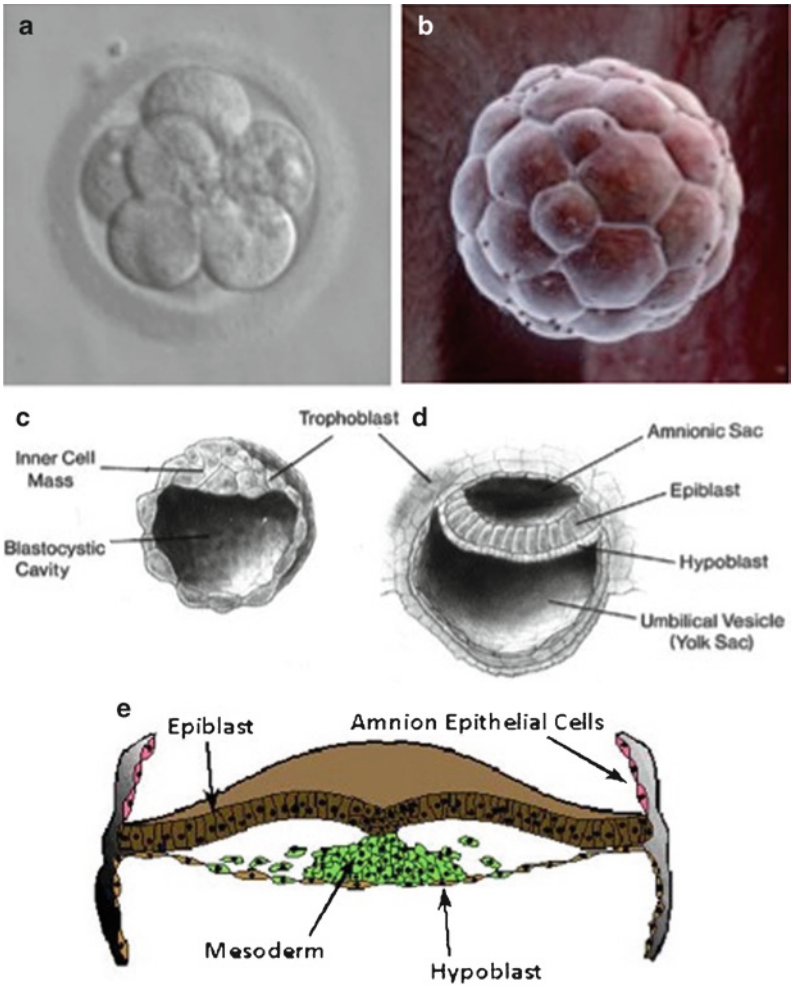

Fig. 2 Several stages of embryogenesis, starting with eight-cell morula **(a)**, complete blastocyst **(b)**, cross section of a blastocyst showing inner cell mass **(c)** and embryonic disc **(d)**, and a cross section of the embryonic disc, showing amnion epithelial cells, as well as the formation of the mesoderm by gastrulation **(e)**. (Adapted from http://en.wikipedia.org/wiki/File:Embryo,_8_cells.jpg, Langman's Medical Embryology Chapter. 3–5.)

the blastocoel (Fig. 2b). The blastocyst arises after compaction and comprises an inner cell mass, which subsequently forms the embryo, and an outer cell mass, or trophoblast, which forms the placenta (Fig. 2c). The cells of the inner cell mass at this stage of development are used to produce embryonic stem cells (ESCs). At 4–5 days post-fertilization, the inner cell mass becomes differentiated into two tissues: the hypoblast (primary endoderm), which will form most extraembryonic structures, and the epiblast (primary ectoderm), from which the embryo and some extraembryonic structures will develop (Fig. 2d). The formation of the embryonic disc (hypoblast and epiblast) divides the blastocyst into two chambers—the amniotic cavity and the yolk sac. The amniotic cavity is a fluid-filled chamber that cushions the developing embryo/fetus and helps to maintain fetal body temperature at a constant level. Prior to gastrulation, epiblast cells migrate along the walls of the amniotic cavity and form the amnion epithelium [33] (Fig. 2e). The migration of cells from the epiblast to the lining of the amniotic cavity occurs before the formation of the three primary germ layers.

3 Stem Cell Properties of Amnion Epithelial Cells

As AECs are derived from the epiblast prior to gastrulation, these cells may share common properties with ESCs. Embryonic stem cells are derived from the inner cell mass prior to the formation of the embryonic disc (Fig. 1b). ESCs have a potentially unlimited capacity for self-renewal and are pluripotent, that is, they can differentiate into the derivatives of the three primary germ layers. This differentiation potential is an important property that is common to ESCs and human amnion epithelial cells (hAECs) [34, 35]. Stem cells and cells with stem cell properties are usually defined according to their cell surface antigens and other specific markers. While hAECs are a heterogeneous population of cells, many groups have demonstrated that hAECs have a stem cell marker profile similar to that of human ESCs (Table 1). While "stemness" of a cell type can only really be defined by functional assays, expression of these markers suggests that hAECs may share some common properties with ESCs.

Pluripotency distinguishes ESCs from other stem cell types, such as MSCs, which, with a more limited differentiation repertoire, are described as multipotent. For example, MSCs have only been shown to be able to differentiate into limited number of cell types, such as bone, cartilage, muscle, and fat [5, 36]. More recently, it has been suggested that MSCs may also be capable of neural differentiation [37]. As hAECs are derived after the formation of the embryonic disc but before gastrulation, these cells might also be expected to display pluripotent differentiation potential. This expectation was originally supported by the creation of pluripotent embryonal carcinoma cells from embryonic ectoderm or epiblast [38]. Furthermore, more recent investigations have demonstrated that hAECs per se have the potential to differentiate to all three germ layers—ectoderm (neurons, glial cells, follicular epithelium, and skin epidermis), mesoderm (cardiomyocytes, myocytes, osteocytes,

Table 1 Stem cell marker profile of human amnion epithelial cells (hAECs), human embryonic stem cells (hESCs), and human mesenchymal stem cells (hMSCs)

Cell Surface Antigen	hAECs	hESCs	hMSCs	References
CD9	+	+	–	92, 93
CD117 (c-kit)	+/–	+/–	–	34, 94
SSEA-1	–	–	–	34, 95
SSEA-3	+	+	–	34, 95
SSEA-4	+	+	–	92, 96
STRO-1	–	–	+	97
TRA 1-60	+	+	–	34, 96
TRA 1-81	+	+	–	34, 95
Oct-3/4	+	+	–	34, 98
Nanog	+	+	–	34, 99
Sox-2	+	+	–	35, 100
GCTM-2	+	+	–	35, 96
TERT	–	+	+/–	34, 101, 102

and adipocytes), and endoderm (hepatocytes, pancreatic cells, and lung alveolar type I and type II cells) in vitro [34, 35, 39].

As hAECs are derived from the epiblast, otherwise known as the primary ectoderm, it may be expected that they would have a natural tendency to differentiate down ectodermal lineages, similar to what is seen with ESCs. This is supported by the expression of neural progenitor marker expression on naive primary hAECs. Sakuragawa et al., in initially describing the expression of neural progenitor neural and glial markers by hAECs, succeeded in inducing active acetylcholine and catecholamine metabolism in hAECs [40, 41]. Furthermore, dopamine-producing cells were produced from hAECs, providing increasing evidence that hAECs can be induced to form functional neuronal-like cells. Fliniaux and colleagues also investigated whether amnion epithelium could be transformed into skin and hair follicles by associating mouse amnion with mouse embryonic hair-forming dermis [39]. These associations were able to produce follicular epithelium and skin epidermis but not the associated dermal cells. This research has important practical potential for the tissue engineering of replacement skin for use in a variety of clinical settings, such as for the treatment of burn victims.

Thus far, there is only limited published evidence of hAEC differentiation into mesodermal lineages. Miki et al. [34] induced the expression of cardiac-specific genes and demonstrated positive immunostaining for the α-actin protein by culturing hAECs in media used to induce the cardiac differentiation of ESCs. This evidence is supported by our studies in which, using the same culture conditions, we detected the expression of troponin T (TNNT), a marker of differentiated cardiomyocytes [35]. This study also demonstrated that hAEC could differentiate down the myocyte, osteocyte, and adipocyte lineages using established methods to characterize hAEC differentiation, smooth muscle alpha-actin (ACTA2) expression for myocytes, von Kossa staining for calcified bone, and oil red O staining for neutral

triglycerides and lipids. To date, there is no published evidence for the differentiation of hAEC into chondrocytes.

For the treatment of fatal liver diseases such as viral cirrhosis, hepatocyte transplantation has been proposed as a novel alternative to whole-organ transplant. In 2000, Sakuragawa et al. showed evidence of synthesis and excretion of albumin by hAECs, as well as the expression of liver-specific markers, human serum albumin, and α-fetoprotein [42]. Takashima et al. added to this evidence with a comprehensive study analyzing the expression of a panel of hepatocyte-related genes [43]. They observed that only a subset of hepatocyte-related genes were expressed by hAECs. Stimulating hAECs with soluble factors known to regulate liver development induced hAECs to secrete albumin and α1-antitrypsin, as well as glycogen storage, all of which are typical functions of hepatocytes.

It has also been demonstrated that hAEC could differentiate into pancreatic β-like cells by stimulating hAECs in vitro with nicotinamide for 4 weeks [44]. Following stimulation, hAECs expressed insulin and glucose transporter-2 (GLUT-2) mRNA. These data were supported by studies demonstrating that freshly isolated hAECs express pancreas duodenum homeobox-1 (PDX-1) and that stimulation with nicotinamide induces the expression of paired box homeotic gene 6 (Pax-6), the NK2 transcription factor–related locus 2 (Nkx2.2) and the mature hormones insulin and glucagon. These observations suggest that hAECs may be an important cell source for the treatment of diabetes using cell replacement therapy.

The increasing rate of smoking-related lung disease, as well as genetic diseases such as cystic fibrosis, has prompted researchers to investigate the potential for cell therapy to treat these otherwise incurable diseases. A recent publication demonstrated the ability of a mixed population of fetal membrane–derived cells composed of approximately 50% mesenchymal cells and 50% epithelial cells to abrogate bleomycin-induced fibrosis in mice [45]. Recent investigations by our group have demonstrated that hAEC can be induced to develop a lung phenotype in vitro (Fig. 3).

4 Immunoregulatory Role of the Amnion

In addition to the multilineage differentiation potential of hAECs, evidence has been accumulated regarding the anti-inflammatory nature and low immunogenicity of hAECs. These properties make hAECs suitable candidates for allotransplantation. The immune properties of hAECs are likely due to their role in maternal tolerance to the fetal allograft. Tolerance is aided by the fetal tissues being antigenically immature, as well as the immunologic inertness of the mother. This acts to create immunologic separation of the mother and fetus [46]. Akle et al. were the first to test the theory of the "immune-privileged" amnion by transplanting amnion membrane, with epithelial cells intact, into seven healthy volunteers [47]. These studies did not find any evidence of acute rejection of the allografts. Amnion membrane has been used on skin wounds, burn injuries, and chronic leg ulcers and, more recently, to aid ocular surface reconstruction [48–51]. These procedures were carried out without

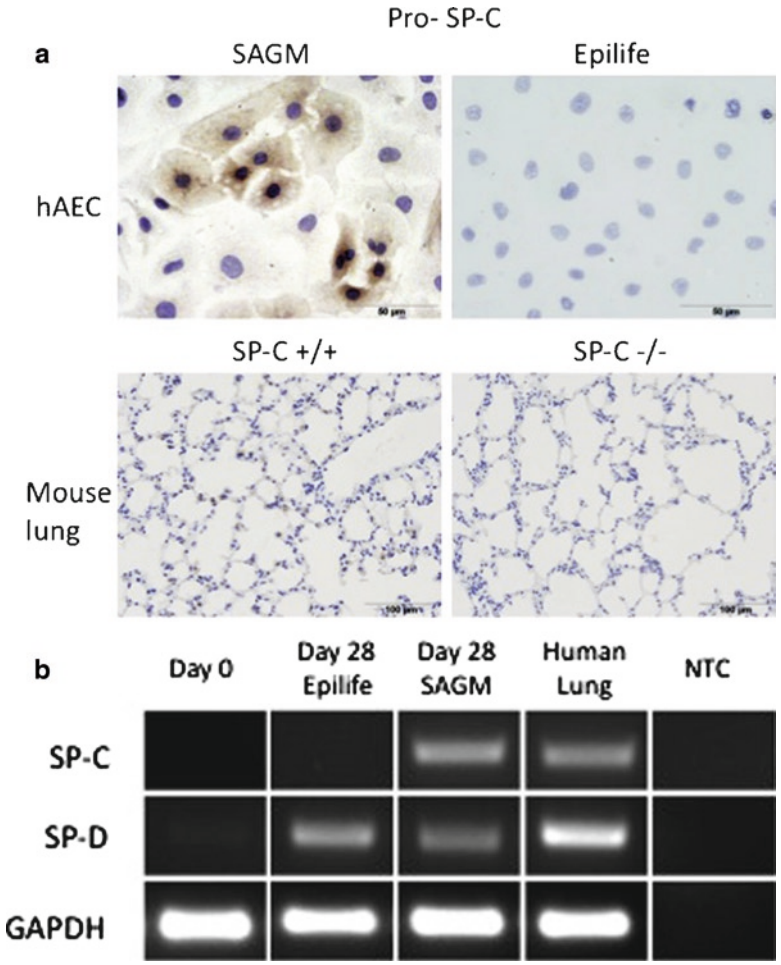

Fig. 3 Differentiation of human amnion epithelial cells (hAECs) into alveolar epithelial cells when cultured in small-airway growth medium (SAGM) in vitro. Following culture for 4 weeks in SAGM, hAECs produce pro–surfactant protein C (Pro-SP-C). When cultured for the same period in Epilife, hAECs do not produce Pro-SP-C. Lungs from SP-C$^{+/+}$ and SP-C$^{-/-}$ mice were used as positive and negative controls, respectively **(a)**. Expression of mRNA from SP-C and SP-D was also detected by polymerase chain reaction **(b)**

immunosuppression and did not induce acute immune rejection. Amnion membrane has also been transplanted into humans to attempt to correct lysosomal hydrolase deficiency [52, 53]. Although unsuccessful in significantly improving patient outcome, these studies did not show any signs of acute rejection.

In vitro biochemical analysis of the immune properties of amnion epithelium has been performed since 1982, with varying results. This is probably due to differing isolation techniques, as well as increasingly specific antibodies available as studies have progressed. Adinolfi et al. reported that HLA-A, -B, -C and -DR antigens and

β2-microglobulin could not be detected by immunofluorescence on freshly collected or cultured amniotic epithelial cells [54]. However, they did state that small quantities of these antigens could be detected using a sensitive radiobiologic technique [54]. In 1988 Hsi et al. identified varying levels of expression of HLA class I antigens on amnion epithelium using higher-affinity monoclonal antibodies [55, 56].

Immunohistochemical and fluorescence-based flow cytometry analysis determined that HLA class 1a antigens (HLA-A, -B, -C) were poorly expressed in the amnion; however, expression of HLA class Ib HLA-E and HLA-G was detected [57]. HLA-E and HLA-G may be involved in fetomaternal interactions because they are expressed abundantly in certain trophoblast populations and show limited and low-level expression in other tissues. As the HLA-G molecule has low polymorphism compared with class Ia antigens, immunogenicity against the fetus is not easily initiated by HLA-G expression at the fetomaternal interface. It is postulated that one of the roles of HLA-E and/or HLA-G is to engage NK cells and provide a negative signal to prevent NK killing of classic class I–negative cells. This theory is supported by data demonstrating that hAECs express HLA-G, but that expression decreases gradually during culture in vitro [58]. This study provided evidence that interferon-γ induction of HLA-G expression occurs in hAECs. HLA-G immunomodulatory properties could be exacerbated during inflammatory processes or inflammatory-associated diseases. We have recently independently verified the negligible expression of HLA class Ia and class II antigens by hAECs [35]. However, HLA expression did change as the cells were differentiated down different lineages in vitro. For example, differentiation down hepatic and pancreatic lineages induced HLA-A, -B, -C expression, while differentiation into cardiomyocytes did not [35].

The knowledge that amnion epithelium does not express HLA-A, -B, and -C antigens, together with the observations just cited, has prompted renewed enthusiasm about the use of amnion epithelium as a potential source of allografts. Amnionic membranes have been transplanted into the limbal area, intracorneal space, and under the kidney capsule of Lewis rats [59]. The authors of that study could only detect mild $CD4^+$ and $CD8^+$ T cell infiltration surrounding limbal and kidney grafts, compared to severe infiltration with the human skin graft controls.

After demonstrating that hAECs could successfully engraft and display microchimerism in bone marrow, brain, lung, and thymus when injected into newborn swine and neonatal rats, Bailo et al. investigated the immunologic reactivity of hAECs [60]. hAECs failed to induce immunologic reactions when cocultured with allogeneic or xenogeneic peripheral blood mononuclear cells (PBMCs). Furthermore, hAECs significantly suppressed the immune response of PBMCs when stimulated with another allogeneic cell source.

It has been proposed that soluble factors produced by hAEC have anti-inflammatory effects [61]. Topical application of hAEC culture supernatant to the cornea led to suppression of suture-induced neovascularization and the presence of fewer major histocompatibility complex (MHC) class II^+ antigen-presenting cells

after thermal cautery. Human interleukin-1ra protein was detected in the culture supernatant in significant concentrations, however, the primary factor(s) responsible for these suppressive effects remains unknown. Further investigations have determined that these secreted factors act to inhibit both the innate and adaptive immune systems [62]. Culture supernatant from hAECs significantly inhibited the chemotactic activity of neutrophils and macrophages toward macrophage inflammatory protein-2, a process essential for the innate immune response. This is likely due to presence of biologically active macrophage migration-inhibitory factor, a potent inhibitor of macrophage migration and NK cell–mediated cytolysis. The adaptive immune response was suppressed through the reduced proliferation of both T and B cells after mitogenic stimulation, possibly through the induction of apoptosis. This hypothesis was proposed after investigation revealed that hAECs express tumor necrosis factor-α, Fas ligand, and tumor necrosis factor–related apoptosis-inducing ligand, which are involved in mediating apoptosis, as well as macrophage migration-inhibitory factor, a potent inhibitor of macrophage migration and NK cell–mediated cytolysis [62].

5 Preclinical Animal Studies

5.1 Neural Disorders

Since the discovery that hAECs express markers of neural and glial cells and can be induced to secrete neurotransmitters, investigations have been undertaken to determine the potential of hAEC transplants to treat various neurologic disorders. Parkinson disease (PD) is characterized by a loss of dopamine neurons, resulting in the impairment of motor skills, speech, and other functions. hAECs transplanted into rat models of PD were found to survive without evidence for overgrowth 2 weeks postgrafting; however, it should be noted that rats were immunosuppressed during cell treatment [63,64]. In this study, the number of dopamine neurons was significantly increased in rats given the hAEC transplant as compared to that in control animals without the cell treatment. In vitro investigation demonstrated that treatment with conditioned medium derived from hAEC cultures enhanced the survival of dopamine neurons in serum-free cultures. Recently, PKH26-labeled hAECs have been transplanted into the stratum in a mouse model of PD [65]. The authors demonstrated that hAECs survive in the mouse brain and that intrastriatal transplantation of hAECs promotes neurologic functional recovery of the PD mice. However, this study did not detect any hAEC-derived neurons, as no PKH26/tyrosine hydroxylase double-positive cells were found, suggesting that engraftment and differentiation of hAECs are not required for functional recovery.

The therapeutic potential of hAECs to treat spinal cord injuries has been investigated by several groups. Revival of motor functions after traumatic paraplegia would be the ultimate goal of spinal cord injury repair. Sankar and Muthusamy performed microsurgical subpial total transection of the spinal cord of four

female bonnet monkeys [66]. Dioctadecyl indocarbocyanine–labeled hAECs were transplanted into the transection cavities of the spinal cord, and survival of transplanted cells after 15 and 60 days was examined. hAECs survived in the transplanted environment, supported the growth of host axons through them, and prevented the formation of glial scar at the cut ends of the transection cavity. Of importance, the authors stated that they did not detect any evidence of immune rejection. hAECs have also been transplanted into the injured spinal cord of rats to investigate whether the cells can improve the rats' hindlimb motor function [67]. hAECs survived for 8 weeks and integrated into the host spinal cord without immune rejection. Compared with the control group, hAEC-treated rats had a greater number of glial cells in the transaction cavity and had improved hindlimb motor function. However, behavioral testing is a very subjective method of assessment, and data resulting from this method should be treated with some caution.

5.2 Hepatic Regeneration

The technique of hepatocyte transplantation is limited by the shortage of suitable hepatocytes. As hAECs can be induced to express hepatocyte markers, secrete albumin and α1-antitrypsin, and exhibit glycogen storage, many investigators have proposed that these cells will provide a suitable alternative. β-Galactosidase–labeled hAECs have been transplanted into livers of uninjured SCID mice [42]. β-Galactosidase–labeled hAECs integrated into liver parenchyma 1–2 weeks posttransplantation. Human-specific gene analysis and immune reactivity to human albumin and α-fetoprotein demonstrated that hAECs survived and differentiated into hepatocyte-like cells in vivo. There was no detectable immune reaction to allotransplantation.

Expression of low-density-lipoprotein receptor (LDLR) was induced in hAECs by adenovirus transfection and used for transplants into a mouse model of familial hypercholesterolemia, a genetic disease characterized by high levels of low-density lipoprotein [68]. Treated animals had a significant decrease in serum cholesterol with eventual return to pretreatment level. hAECs migrated out of the sinusoids and into the hepatic parenchyma and expressed LDLR until at least 20 days post-transplantation. However, transplanted hAECs were significantly reduced in numbers at 10 days post-transplantation, with an increased inflammatory cell infiltration. This may be associated with xenograft rejection, and the role of the adenovirus in this process is unknown.

After determining that undigested amnion membrane secreted significantly greater amounts of albumin, Takashima et al. transplanted small pieces of amnion membrane into the peritoneal cavity of SCID mice and assessed the survival and secretion of albumin in vivo [43]. The transplanted amnion membrane survived in the peritoneal cavity for up to 2 weeks. With the use of a human-specific antibody, human albumin was detected in the ascites and serum of recipient mice.

This supports the hypothesis that transplanted amnion membrane/hAECs may be able to take on the role of functioning hepatocytes to treat liver disease.

5.3 Pancreatic Tissue Insulin Production

Current treatment for diabetes mellitus relies on daily multiple insulin injections or insulin pump placement or beta cell or whole-pancreas replacement. Islet transplantation as a possible therapy is limited by the scarcity of transplant material and the requirement for life-long immunosuppressive therapy. An alternative cell-based therapy would represent a major breakthrough in the management of this common chronic disorder. After demonstrating that hAECs can be stimulated to express insulin and glucose transporter-2 (GLUT-2) mRNA, Wei et al. investigated the potential for hAEC and human adult mesenchymal cells (hAMCs) to restore blood glucose levels in diabetic mice [44]. In mice receiving hAECs, blood glucose gradually decreased to normal levels from the first week to 1 month post-transplantation. The body weights of hAEC-treated mice also normalized compared to mice not receiving cells. Those animals receiving hAMC continued to lose weight and have hyperglycemia. These data were improved upon several years later by Chang et al. [69], who showed that insulin and glucagon can be secreted by hAECs. They used the same mouse model as Wei et al. [44] to demonstrate that transplanted hAECs form glandlike tissues, differentiate into insulin and glucagon-positive cells, and stably restore normoglycemia in diabetic mice. These two groups demonstrated that hAECs survive, differentiate, and contribute to glucose metabolism when transplanted into SCID mice induced to show symptoms of diabetes. Hou et al. [70] contributed to this interesting set of data by transplanting hAECs into immune-competent C57 mice induced to become diabetic. They observed similar results, with hAEC treated mice showing reduced hyperglycemia, normalized body weight, and maintenance of normoglycemia for up to 30 days. Significantly, these data concur with observations by other groups, even though the mouse model used was immune competent. This supports the immune-privileged status of hAECs and argues a strong case for the use of hAECs for the treatment of diabetes mellitus in humans.

6 Amnion Epithelial Cells in Lung Regeneration

Regenerative medicine aimed at maintaining, restoring, or enhancing tissue and organ function is intended to assist in the treatment of a number of human conditions ranging in severity from chronic to life threatening. Chronic lung diseases are a major global problem and are characterized by significant loss of lung tissue as a result of chronic inflammation, fibrosis and scarring, leading to the morbidity and mortality associated with these conditions [71–73]. Causes of lung disease include

tobacco smoking and exposure to industrial pollution. Current and potential methods of treatment have varied effectiveness and are not without side effects, which range from nausea to cardiac implications [74–77]. Hence there is increasing pressure for cell- and gene-based therapy for the treatment of chronic lung diseases.

In 2007, De Coppi and colleagues isolated cells from amniotic fluid representing 1% of the population of cells derived from amniocentesis [17]. These cells had properties similar to those of hAECs [17]. Carraro and colleagues [78] showed that these amniotic fluid–derived cells integrated into murine lung and differentiated into pulmonary lineages after injury of the lung, suggesting that amniotic fluid might provide a source of reparative cells for cell-based therapy of the lung.

Our group has recently demonstrated that hAECs can be induced to develop a lung phenotype in vitro (Fig. 3). In addition, a significant proportion of hAECs administered into bleomycin-injured, immune-compromised mice was located in the lung after 4 weeks and had adopted the specific morphology of alveolar epithelial cells. Administration of hAECs was shown to have anti-inflammatory effects and reduce the fibrosis that occurred following lung injury.

During our initial profiling of hAECs for stem cell markers, we [35], and now others [78], noted that freshly isolated hAECs express the thyroid transcription factor Nkx2.1 (also known as TTF-1). This transcription factor is one of the earliest lineage-specific markers of the developing lung and is involved in the regulation of branching morphogenesis. Of interest, however, primary hAECs did not express mRNA for surfactant protein-A, -B, -C, or -D, markers of the mature lung. These observations suggested to us that, while primary hAECs did not have a functional lung phenotype, it might be possible to direct differentiation down a lung lineage, with a view to using them in lung repair. To test this, we cultured primary hAECs in "small-airway growth medium" (SAGM); a medium that has been used to maintain primary culture of small airway cells to induce differentiation of ESCs into type II alveolar epithelial cells [79]. When cultured in SAGM for 4 weeks, hAECs produced surfactant proteins, as determined by immunohistochemistry and mRNA expression (Fig. 3). These data suggested that, in SAGM, hAECs differentiated into type II alveolar cells, as now reported by others [78].

We have examined the potential for primary hAECs to differentiate and repair injured lung in vivo. To do this, we used the established bleomycin-induced model of lung inflammation and fibrosis [80] in SCID mice. hAECs (2×10^6) were injected into the tail vein 24 hours following intranasal bleomycin (or saline in controls) and were identified 2 and 4 weeks postinjection through positive immunostaining for antihuman inner mitochondrial membrane (IMM) protein. While we could identify human cells in the bleomycin-injured lung at both 2 and 4 weeks (6 ± 0.4 cells per 100 cells counted), we could not find cells in control (saline-administered) lungs. Of importance, some of the IMM-positive cells (hAECs) had morphologic features of alveolar cells, consistent with in vivo differentiation.

After 2 weeks, we confirmed in vivo differentiation of the hAECs into alveolar cells by showing that cells retrieved from lung digests, using an antihuman CD29 Ab (human specific), were immunopositive for surfactant protein-A, -B, -C, and -D.

Most important, when injected into bleomycin-treated mice, whether 24 hours or 2 weeks after bleomycin, hAECs reduced inflammation and fibrosis and restored essentially normal lung architecture [103].

Most recently, we have extended our adult studies into fetal lung. Using two established models of fetal lung injury, we have shown that the effects of hAECs in the injured adult mouse lung are also seen in acute fetal sheep lung injury. Stuart Hooper and his group have recently reported this in utero model of ventilation-induced lung injury [81]. While in utero ventilation has been used before in term fetal sheep, this is the first use of this technique in the very preterm fetal lamb—equivalent to the 26- to 30-week human fetus—and the first to model the bronchopulmonary dysplasia (BPD)–like effects. Ventilation of the preterm lung in this model creates inflammation, alveolar arrest, and septal thickening very similar to what is seen in human infants with BPD [81], validating it as a suitable model for studying BPD and potential novel therapies. In our studies, the lungs of saline-treated animals, at 7 days after 12 hours of intrauterine ventilation, had fewer secondary septal crests, simplified distal airsacs, atelectatic areas, and abnormal collagen and elastin deposition, which are changes reminiscent of BPD and identical to those recently reported [81]. In contrast, in the animals receiving hAECs' all of these changes were mitigated, with near-normal lung architecture restored. As with our adult mouse studies, while we could retrieve human cells (hAECs) from injured lung 7 days after administration, we could not retrieve cells from any other tissue in those animals, nor from any tissue, including lungs, collected from control animals [104].

We have also administered hAECs to fetal sheep, concurrent with administration of intra-amniotic lipopolysaccharide (LPS), in an established model of in utero infection [82–87]. Intra-amniotic LPS induces chorioamnionitis and a systemic fetal inflammatory response, with the lung being the major target organ for that inflammation [87]. This leads to "accelerated maturation" characterized by induction of surfactant production, increased epithelial surface area and airspace volume and improved lung compliance. In human infants, these infection-related changes result in less acute respiratory distress but ultimately increase the risk of BPD due to alveolar arrest [88]. However, when hAECs were given with the LPS, the induction of mRNA for SP-A and SP-C by LPS was mitigated and lung compliance reduced (unpublished data, T. Mors). These preliminary observations suggest that the hAECs were exerting an anti-inflammatory effect, as has been shown in other tissues [89, 90], and this offers the possibility that hAECs may protect against subsequent "BPD" in this model.

7 Clinical Application of Amnion Epithelial Cells

The use of cells isolated from human term placenta for regenerative medicine represents a field of investigation that is still in its infancy but holds great promises on several fronts. Specifically, their plasticity and immune characteristics and the

lack of ethical barriers to their procurement make them ideal candidates as the basis of further research into disease treatment. Established methods for the isolation, cryopreservation and culture of hAECs involve the use of animal products. Current regulations for the use of human cells as a cell therapy require the isolation process to be performed with animal product–free reagents, enzymes and growth media.

We have shown that hAECs can be isolated and stored in a manner that will be suitable for use in the clinic. To achieve this we have developed methods to isolate, characterize and store hAECs so that they are suitable for human use in future cell therapies. These methods are comparable to the previously established animal product–containing methods, producing a yield of relatively pure hAECs with high viability [105]. In support of this, human amnion epithelial cells have a number of other important "safety" attributes that make them attractive as a cell therapy [91]. They maintain a normal karyotype in culture, have conserved long telomere lengths [17] (Fig. 4) and, unlike ESCs, do not form teratomas in vivo [35]. Furthermore, it has been proposed that hAECs may have immunoregulatory functions [89], an attractive property for transplant tissue. Indeed, as indicated previously, amniotic membranes have long been used in wound repair, burn injuries and corneal surgery without the need for immunosuppression or the development of immunorejection [89], suggesting a degree of immunoprivilege. Consistent with this, in vitro, hAECs suppress lymphocyte proliferation [90] and we have recently shown that undifferentiated hAECs have very low levels of HLA expression [35].

8 Conclusions

In summary, hAECs derived from term amnion would appear to be an ideal candidate for "stem cell" regenerative/reparative therapy. They have the molecular machinery consistent with pluripotent stem cell–like cells, they can be directed to differentiate down endodermal, mesodermal and ectodermal cell lineages in vitro and they have been shown, in selected disease models, to differentiate in vivo, resulting in tissue repair and regeneration. Their karyotypic stability, nontumorigenicity, immunoregulatory and immunoprivileged properties also afford a safety profile and allogeneic transplant potential unmatched by ESCs or adults stem cells. Their immunoregulatory functions may be fundamental to their role in injury repair. On a practical level, and unlike ESCs or adult stem cells, they are plentiful—we isolate 100–200 million cells from each term amnion—and are relatively inexpensive to collect, isolate and culture, while, unlike embryonic stem cells, they present no ethical challenges in their isolation, preparation and application.

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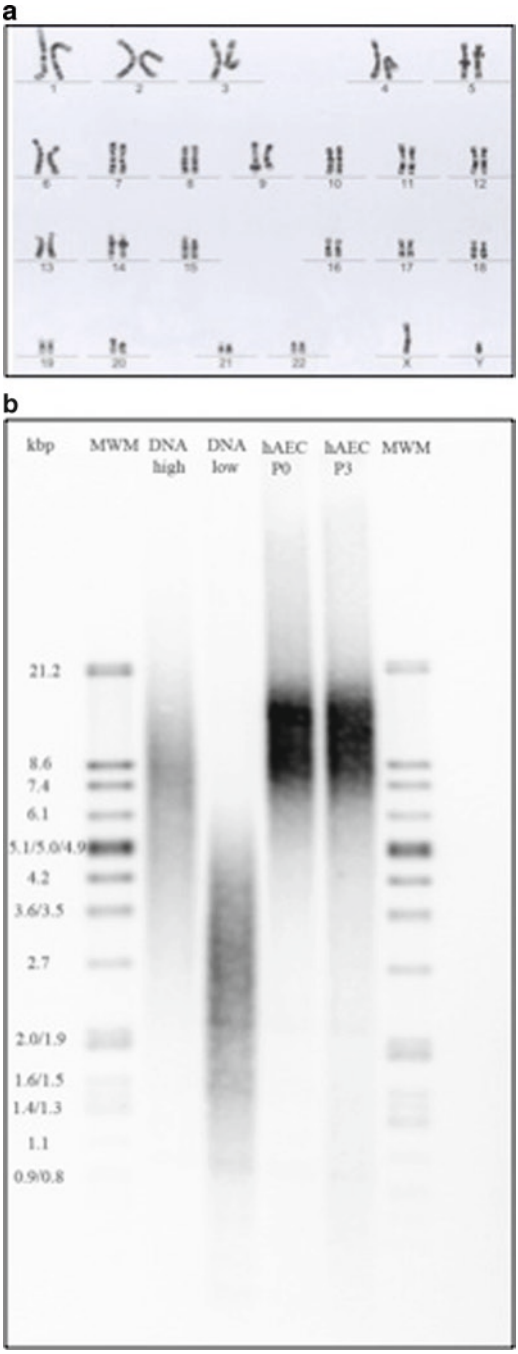

Fig. 4 Characterization of tumorigenic potential of human amnion epithelial cells (hAECs) in vitro. **a:** Giemsa-band karyogram showing chromosomes of passage 5 hAECs. Telomere lengths of hAECs are shown when freshly isolated (P0) and with passage 5 (P5). High-length control and low-length control telomere standards are provided in the assay kit. **b:** Average telomere lengths.

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Bone Marrow Cell Therapy for Acute Myocardial Infarction: A Clinical Trial Review

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Abstract Over the last decade, the use of adult stem cells to repair myocardial damage has been the focus of intense research. Bone marrow–derived progenitor cells (BMCs) have emerged as among the most promising sources for stem and progenitor cells. A large body of preclinical experiments has lent support to the use of BMCs to treat patients post myocardial infarction (MI). Many questions remain unanswered regarding the therapeutic potential and the mechanisms responsible for the observed effects. This chapter reviews the clinical application of BMCs in the post-MI setting and addresses the techniques, safety, and efficacy of some of the most relevant clinical studies to date.

Keywords Bone marrow–derived progenitor cells • Cardiomyocytes • Stem and progenitor cell therapy • Neovascularization

1 Introduction

Despite advances in reperfusion therapies for acute myocardial infarction (MI), many patients are left with large scars and significant adverse remodeling, underscoring the need for new approaches for myocardial preservation and regeneration [1–3]. Novel therapeutic approaches targeted to repairing myocardial damage have been the focus of intense research over the recent years. In vitro and in vivo research has confirmed that the adult bone marrow (BM) is a reservoir of cells with the potential for plasticity [4–8,10]. In addition, proof-of-concept preclinical experiments have been performed with bone marrow–derived progenitor cells (BMCs) supporting the use of these cells in clinical trials in post-MI patients

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[5–12]. Numerous mechanisms, some controversial, have been postulated to explain the likely beneficial effects of BMCs on cardiac function, and these include BMC transdifferentiation into new cardiomyocytes [5, 9], cell fusion [13], enhanced neovascularization [14], inhibition of apoptosis [15], and possible paracrine mechanisms [16, 17]. Even though the exact mechanism(s) remain controversial, results from these preclinical models have encouraged investigators to begin clinical trials of autologous bone marrow cell infusions in patients with ischemic heart disease. This chapter reviews some of the clinical trials of BMC therapy in patients post-MI. We first briefly outline the single-center, nonrandomized, proof-of-concept trials and then, in more detail, review the larger multicenter, randomized clinical trials. We also address safety and highlight some of the important differences among these trials that may have led to the variable efficacies in the reported endpoints.

2 Nonrandomized Clinical Trials: Proof-of-Concept and Safety

The initial wave of nonrandomized trials was meant to demonstrate the safety and feasibility of BMC therapy post-MI. We outline three of these trials as follows.

The first report was by Strauer et al. in 2002, who enrolled 10 patients in a single-center study and administered BM mononuclear cells (BMMNCs) via the intracoronary route in the infarct-related artery 5–9 days post-MI [18]. After 3 months of follow-up, no adverse side effects were observed, and in fact, the treated patients had significantly smaller infarct regions, greater infarct wall movement velocity, better stroke volume indexes, lower left ventricular (LV) end-systolic volumes (ESVs), and greater perfusion of the infarct region than the nonrandomized historical control patients. Subsequently, Assmus et al. also demonstrated the feasibility of infusing BMMNCs or ex vivo expanded peripheral blood (PBMNCs) after MI in 20 patients [19]. In comparison to baseline measures, statistically significant improvements were observed in ejection fraction (EF), regional wall motion in the infarct zone, ESV, wall motion score index, coronary artery blood flow reserve in the infarct-related artery, and cardiac viability assessed by positron emission tomography (PET). No significant differences were noted between the BMMNC and PBMNC groups. Moreover, no inflammatory response or arrhythmias were observed in either group during the follow-up period.

Fernandez-Aviles et al. studied 20 BMMNC-transplanted patients and 13 control subjects with acute anterior MI [20]. At 6 months posttherapy, magnetic resonance showed a decrease in the ESV, improvement of regional and global LV function, and increased thickness of the infarcted wall compared to baseline values. No changes were reported in the nonrandomized contemporary control group. Together, these and other small, nonrandomized studies [21, 22] provided the safety data that led to the undertaking of larger randomized and in some cases multicenter clinical trials.

3 Randomized Trials: Time to Address Efficacy

Several randomized clinical trials have been conducted using BMC therapy post-MI. Table 1 summarizes the major clinical studies published to date. A selected number of these studies will be reviewed in this section. Most of these studies used BMMNC delivered into the infarct-related artery after arterial patency was achieved as first described by Strauer et al. [18]. It should be noted that comparing the studies to each other is difficult, as they vary in a number of significant ways, including patient selection, methods of BMMNC isolation, timing and number of cells delivered, imaging studies used, and duration of follow-up. One of the earliest randomized clinical trials was published in 2006 by Ge et al. [23]. This was a small study of only 20 patients, but it showed that BMMNC therapy, as evaluated by echocardiography, enhanced LVEF significantly compared to the control group from 1 week to 6 months after acute MI. Left ventricular diastolic diameters remained unchanged in the BMMNC group but were significantly enlarged in the control group, and the myocardial perfusion defect score was also significantly decreased in the cell therapy group but unchanged in the control group [23].

Huang et al. then reported on a larger study of 40 patients using magnetic resonance imaging (MRI) as their imaging modality of choice [24]. At 6 months, LVEF was significantly higher than at baseline in both cell therapy and control groups, but the absolute change of LVEF in the BMMNC group was significantly higher than that in the control group (Table 1). Moreover, the myocardial lesion area of the BMMNC group decreased more significantly than in the control group. Nevertheless, there was no difference in change of LV end-diastolic volume (EDV) between the two groups. Similar results in terms of evolution of LVEF were presented by Huikuri et al. [25]. The absolute LVEF was not different between groups at 6 months of follow-up, but the absolute change of global LVEF was greater in the BMMNC group than in the placebo group by echocardiography and angiography (7.1 ± 12.3 vs. $1.2 \pm 11.5\%$, $p = 0.05$) (Table 1). Unlike the previous trials, Janssens et al. [26] reported that there was no significant difference in the absolute change of the LVEF with cell therapy compared with control post-MI. However, BMMNC therapy appeared to be associated with a significant reduction in infarct size ($p = 0.036$) and a better recovery of regional systolic function. The authors suggested that quantitative assessment of regional systolic function might be more sensitive than global LV ejection fraction for the evaluation of BMMNC therapy after MI [27].

Schachinger et al. conducted the largest clinical trial using BMC post-MI to date. The double-blind, placebo-controlled, randomized multicenter trial—the Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction (REPAIR-AMI) trial—was designed to investigate whether intracoronary infusion of enriched BMMNCs is associated with improved global left ventricular function in patients with MI treated with state-of-the-art methods [31, 32]. REPAIR-AMI included 204 patients randomized to receive an intracoronary infusion of BMMNC or placebo medium into the infarct artery. After 4-month follow-up, mean LVEF

Table 1 Randomized clinical trials: study design and efficacy

Trial	Sample size	Method	Cell preparation	Timing from MI	Cell dose	Primary endpoint	Follow-up (mo)	Baseline LVEF (%)	Follow-up LVEF (%)	ΔLVEF (%)	*p* value (ΔLVEF)
Ruan et al. [46]	BM: 9 Control: 11	ECHO	Not stated	Within first 15 hr	Not stated	Not stated	6	BM: 53.4 ± 8.9 Control: 53.5 ± 5.8	BM: 59.3 ± 12.9 Control: 50.3 ± 8.3	Not available	–
Janssens et al. [26,27]	BM: 33 Control: 34	MRI	Gradient centrifugation	24–48 hr	$3 \pm 1.28 \times 10^8$	Changes on LVEF	4	BM: 48.5 ± 7.2 Control: 46.9 ± 8.2	BM: 51.8 ± 8.8 Control: 49.1 ± 10.7	BM: 3.4 ± 6.9 Control: 2.2 ± 7.3	NS
Meyer et al. [32] (BOOST)	BM: 30 Control: 30	MRI	Gradient centrifugation	Mean 6 d	$2.46 \pm 0.94 \times 10^9$	Changes in global LVEF	6 and 18	BM: 50 ± 10 Control: 51.3 ± 9.3	BM: 38.9 ± 10.3 Control: 41.3 ± 9	BM: NA Control: NA	NS
Ge et al. [23]	BM: 10 Control: 10	SPECT, ECHO	Gradient centrifugation	Within 15 hr	4×10^7	LVEF, LVED, internal diameter, myocardial perfusion defect	6	BM: 53.8 ± 9.2 Control: 58.2 ± 7.5	BM: 58.6 ± 9.9 Control: 56.3 ± 3.5	Not available	–
Huang et al. [24]	BM: 20 Control: 20	Angiography	Gradient centrifugation	24–48 hr	$1.8 \pm 4.2 \times 10^8$	Not stated	6	BM: 44.5 ± 7.1 Control: 43.4 ± 6.7	BM: 51.5 ± 5.2 Control: 47.9 ± 6.7	BM: 6.95 ± 3.33 Control: 4.5 ± 1.68	0.047

Lunde et al. [29,43] (ASTAMI)	BM: 50 Control: 51	SPECT, ECHO, MRI	Gradient centrifugation	Median of 6 d	0.54–1.3 $\times 10^8$	Changes on LVEF	6 and 12	BM: 45.7 ± 9.4 Control: 46.9 ± 9.6	BM: 48.8 ± 10.7 Control: 49 ± 9.5	BM: 3.1 ± 7.9 Control: 2.1 ± 9.2	NS
Schachinger et al. [42,44] (REPAIR-AMI)	BM: 101 Control: 103	Angiography	Gradient centrifugation	3–6 d	2.36 ± 41.74 $\times 10^8$	Changes in LVEF	4 and 12	BM: 48.3 ± 9.2 Control: 46.9 ± 10.4	BM: 53.8 ± 10.2 Control: 49.9 ± 13	BM: 5 ± 7.3 Control: 3 ± 6.5	0.01
Meluzin et al. [30,31]	BM HD: 20 BM LD: 20 Control: 20	SPECT	Gradient centrifugation	Mean 7 d	0.9–2 $\times 10^8$ and 0.9–2 $\times 10^7$	Change in regional systolic function of the infarcted wall	3, 6, and 12	HD: 40 ± 2 LD: 41 ± 2 Control: 40 ± 2	Not reported	HD: 7 ± 2 LD: 4 ± 1 Control: 0 ± 2	0.027[a]
Penicka et al. [47]	BM: 17 Control: 10	SPECT, ECHO	Not stated	9 d (range 4–11 d)	26.4 $\times 10^8$	Not stated	4 and 12	BM: 39 ± 6 Control: 39 ± 4	BM: 45 ± 9 Control: 47 ± 7	BM: 15.4 Control: 20.5	NS
Huikuri et al. [25] (FINCELL)	BM: 39 Control: 38	Angiography, ECHO	Gradient centrifugation	3–5 d	4.02 ± 1.96 $\times 10^8$	Changes on LVEF	6	BM: 56 ± 10 Control: 57 ± 10	BM: 60 ± 8 Control: 56 ± 10	BM: 4 ± 11.3 Control: –1.4 ± 10.1	0.03

(continued)

Table 1 (continued)

Trial	Sample size	Method	Cell preparation	Timing from MI	Cell dose	Primary endpoint	Follow-up (mo)	Baseline LVEF (%)	Follow-up LVEF (%)	ΔLVEF (%)	*p* value (ΔLVEF)
Mouquet et al. (2008)[b] (BONAMI)	BM: 52 Control: 49	SPECT, RNA, ECHO	Gradient centrifugation	9.3 ± 1.7 d	1×10^6	Myocardial viability; LVEF	3	BM: NA Control: NA	BM: 53.8 ± 10.2 Control: 49.9 ± 13	BM: 5.5 ± 7.3 Control: 3 ± 6.5	NS
van der Laan et al. [33] (HEBE)	BM: 67 PB: 62 Control: 60	MRI	Gradient centrifugation	3–8 d	NA	Changes in regional myocardial function	4	NA	NA	BM: 3.8 PB: 4.2 Control: 4	NS

ASTAMI, Autologous Stem cell Transplantation in Acute Myocardial Infarction; BM, bone marrow-derived mononuclear cells; BONAMI, Bone Marrow in Acute Myocardial Infarction; ECHO, echocardiography; HD, high dose; LD, low dose; LVED, left ventricle end-diastolic; LVEF, left ventricle ejection fraction; MRI, magnetic resonance imaging; PB, peripheral blood mononuclear cells; REPAI-AMI, Remodeling in Acute Myocardial Infarction; RNA, radionuclide angiography; SPECT, single photon emission computed tomography.

[a]HD vs. control.

[b]Data presented at the American Heart Association Scientific Sessions November 2008, New Orleans, Louisiana.

increased from 47% to 50% in the placebo group and from 48 to 54% in the group given BMMNC. The mean absolute change in favor of BMMNC treatment was 2.5% ($p = 0.01$; Table 1). There was also a significant reduction in clinical events (prespecified cumulative endpoint of death, myocardial infarction, or necessity for revascularization) after 12 months in the treatment group compared with the placebo group [respectively, 24% ($n = 24$) vs. 41% ($n = 42$); $p = 0.009$]. Likewise, the combined endpoints of death, recurrence of myocardial infarction, and rehospitalization for heart failure were significantly reduced in the patients receiving intracoronary BMMNC administration [2% ($n = 2$) vs. 12% ($n = 12$); $p = 0.006$]. The benefits of functional improvement were achieved in patients with larger infarcts (EF < 49%) (change in LVEF 7.5% vs. 2.5%, $p = 0.002$) and those who received the cell therapy after 5 days post-MI (change in LVEF 7% vs. 1.9%, $p = 0.004$), and, of interest, there was no benefit if the infarct was small (baseline EF greater than 49%) or if the therapy was delivered within 5 days post-MI. Of note, the LVEF reported in this study was measured by LV angiography. Recently, the same group published an MRI substudy of 54 patients (27 BMMNC, 27 placebo). They demonstrated that only in patients with a baseline EF of 48.9% or less was BMMNC administration associated with a significantly improved EF (+6.6%, $p = 0.01$), reduced LVEDV increase ($p = 0.02$), and stabilization of LVESV ($p = 0.01$) at 12 months [28].

Lunde et al. also conducted a randomized study that included patients with large anterior MI, but they reported no differences between groups for the variables of LV function at 6 and 12 months as measured by echocardiography, single photon emission computed tomography (SPECT), or MRI in the Autologous Stem cell Transplantation in Acute Myocardial Infarction (ASTAMI) trial [29] (Table 1). Moreover, when the patient population from this study was dichotomized according to the median value for LVEF at baseline (45%), no significant treatment effects were noted on LVEF, LVEDV, or wall motion score index. In contrast to the REPAIR-AMI trial, the authors concluded that injection of BMMNC after anterior wall MI using the ASTAMI trial study protocol, which was different, especially in terms of the cell isolation method, than the protocol used in the REPAIR-AMI trial (see later discussion), did not improve LV function compared to a randomized control group. An interesting trial was reported by Meluzin et al. evaluating two doses of BMCs (high, $0.9–2 \times 10^8$, and low, $0.9–2 \times 10^7$) compared to control [30, 31]. Doppler tissue imaging and gated technetium-99m sestamibi SPECT were performed before cell transplantation and at 3, 6, and 12 months. At 3 months, regional myocardial function of the infarcted wall was improved in a dose-dependent manner, but at 12 months, the changes from baseline values no longer differed significantly. In contrast, the posttransplant improvements in LVEF by 6%, 7%, and 7% at 3, 6, and 12 months, respectively, were preserved in the high-dose cell therapy group and remained significantly better than in controls. The low-dose group also improved LVEF over time, but this was not superior to control.

The BOOST (bone marrow transfer to enhance ST-elevation infarct regeneration) trial conducted by Meyer et al. has the longest follow-up of 18 months after cell therapy post-MI to date [32]. At 6 months, the increase in global LVEF measured by MRI was significantly greater in the BMMNC group than in the

control group ($p = 0.0026$); however, global LVEF change at 18 months was not significantly enhanced in the BMMNC group compared with the control group (Table 1; $p = 0.27$). Of interest, the speed to LVEF recovery over the entire course of 18 months was significantly higher in the BMMNC transfer group ($p = 0.001$). The mean global LVEF in the control group increased by 0.7% and 3.1% after 6 and 18 months, respectively, while in the BMMNC group, it increased by 6.7% and 5.9%, respectively. In addition, a transient improvement in the wall motion in the infarct border was noted in the BMMNC transfer group after 6 months ($p = 0.01$), but this was not sustained at 18 months.

One of the most recent trials is the Bone Marrow in Acute Myocardial Infarction (BONAMI) trial (data reported by Frederic Mouquet et al. at the American Heart Association Scientific Sessions November 2008, New Orleans, Louisiana). In this study of 101 patients with acute MI, only those patients with a low LVEF (36.3% ± 6.9%) and regional impairment of myocardial viability were enrolled in six academic hospitals (control group, $n = 49$; or a BMMNC group, $n = 52$). At 3 months, myocardial perfusion score improved in 34% of patients in the cell therapy group as compared to only 16% in the control group ($p = 0.06$). However, no difference was observed for LVEF between BMMNC and control groups as assessed by either radionuclide angiography (38.9% ± 10.3% vs. 41.3% ± 9.0%, $p = 0.6$) or echocardiography (39.1% ± 10.2% vs. 41.5% ± 8.8%, $p = 0.7$). In addition, no difference was observed for scar size with MRI (30.9% ± 13.9% vs. 27.7% ± 9.5%, $p = 0.6$).

Finally, the HEBE trial [33] randomized 200 patients within 12 hours of symptom onset to intracoronary BMMNC infusion ($n = 69$), PBMNC infusion ($n = 66$), or primary percutaneous coronary intervention alone ($n = 65$). At 4 months, there was no statistically significant difference between groups in the primary endpoint of change in regional myocardial function measured by MRI. Similarly, none of the secondary endpoints—systolic wall thickening, LVEF, and LVEDV—showed significant differences between groups. However, when only patients with baseline LVEDV above the median of 96 mL/m^2 were considered in a post hoc analysis, a significant improvement in this parameter was seen in patients infused with bone marrow cells ($p = 0.04$ vs. controls).

4 Randomized Clinical Trials: Mixed Results from Mixed Protocols?

Despite the numerous clinical trials using BMCs post-MI, many questions remain unanswered, and the absolute benefits of cell-based therapy remain controversial. Overall, BMMNC seems to have at best a modest effect on the short-term LVEF post-therapy, with similar favorable trends on end-systolic and end-diastolic volumes and myocardial scar size [34,35]. Many of the studies have significant limitations in terms of their small sample size and design, and there are many differences among trials that make comparison of the results very challenging. In many of these trials, LVEF has been the primary endpoint, and the methods used to evaluate this have varied

(echocardiography, SPECT, left ventricular angiography, MRI). The trials also diverge in many other ways, including the clinical characteristics of the patients enrolled, the size and type of the infarct on admission, the methods of cell isolation, the number and timing of cells delivered, and the time points for follow-up assessments of outcome.

5 Safety Issues

The published trials are too small to reliably evaluate the effects of BMC therapy on mortality and morbidity post-MI. Overall, BMC transplantation appears safe, but future larger and longer-term studies will need to be conducted to more confidently address these important questions [34, 35]. Most of the studies thus far have reported no safety concerns using BMC post-MI except for two studies: One was the trial by Penicka et al., which was terminated prematurely and reported an unexpected occurrence of serious complications in the BMC group even though the adverse events did not seem to be directly related to the BMC procedure [29] (one patient had a ventricular septal rupture before the injection of BMC, underwent emergency surgery, and died 3 months later from severe heart failure; one patient suffered stent thrombosis with reinfarction immediately after BMC harvesting; another patient had biliary carcinoma diagnosed 6 weeks after BMC therapy and died 2 months later; and one patient suffered reinfarction due to coronary occlusion distal to the implanted stent 9 months after randomization).

The second trial was a cohort study by Bartunek et al. [36] demonstrating that intracoronary infusion of enriched $CD133^+$ BMC post-MI was associated with a higher incidence of in-stent restenosis and de novo coronary lesions in the infarct-related arteries. The latter authors are now conducting a randomized trial (SELECT-MI, ClinicalTrials.gov Identifier NCT00529932) to better define the risk and mechanisms of potential side effects on the epicardial coronary circulation where proangiogenic cells may also stimulate atherogenesis [37].

6 Conclusion

Stem/progenitor cell therapy for cardiac disease is still in its early years. The publication of multiple clinical trials suggests that BMMNC treatment post-MI seems to be safe and may lead to a modest improvement in left ventricular function over conventional therapies in the short term after cell delivery. Many challenges remain to be elucidated, including the inclusion criteria for which patients would get the most benefit, the optimal cell type(s) (e.g., mesenchymal cells, adipose-derived cells, native cardiac progenitor cells, embryonic stem cells, and induced pluripotent cells), methods of cell processing, timing and dose of cell delivery, and issues surrounding cell retention and engraftment of stem cells in the heart after delivery. Ideally, cell therapy would provide a readily available and well-characterized cell

type(s) capable of homing to the infarct zone and altering the infarction healing process to limit or prevent ventricular remodeling. The potential mechanism(s) of stem cell benefit on cardiac function needs to be further investigated in the preclinical small- and large-animal settings. In addition, despite the benefit of avoiding immune reactions, the use of autologous cells has the limitation of individual variability between patients and, more important, the risk of impairment of cell function related to cardiovascular disease and risk factors [38–40].

Further knowledge with regard to cell processing is also critical before cell therapy becomes widely available. A carefully conducted study comparing cell processing methods from the two larger clinical trials using BMMNC [41] (REPAIR-AMI vs. ASTAMI) [42, 43] revealed major differences in the phenotype and function of BMMNC, which might have contributed to the differences in the clinical outcomes posttherapy. The ideal timing for cell therapy is still controversial. Currently, the data suggest that avoiding the early phase of inflammation (e.g., within the first 5 days of MI) but administering the cells before scar formation results in the greatest benefit [34, 44]. In addition, whether multiple rounds of cell therapy post-MI would have additive clinical effects has not yet been investigated.

Lastly, it is well known that the cell retention and engraftment rates after cell delivery are very low [45]. The modest or transient effects after cell therapy may partly be related to this issue. As outlined earlier, higher cell dosing appears to be associated with better outcome, which may be due to a higher rate of engraftment, but this is speculative [35]. The low rates of engraftment seem to be associated with both an early mechanical loss due to cell leakage and possibly cell death caused by biologic processes [38]. Potential alternatives using biomaterials and cell engineering techniques that will increase cell retention and engraftment are in development, and future clinical studies will need to be conducted to evaluate their safety and efficacy. In summary, we are in the early days of stem cell therapy to treat ischemic heart disease. Much more work remains to be performed to better understand the potential of cell therapy post-MI.

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Stem Cell Transplantation to the Heart

Michael J. Mann

Abstract Cardiovascular disease remains the most potent killer in all developed societies. Heart failure is the most common cause of hospitalization in the United States. Despite impressive improvements in the interventional and medical therapy of ischemic coronary artery disease, this disorder remains the most common cause of heart failure. Given the severe limitation on the availability of and the long-term morbidity associated with cardiac transplantation, a means of preventing or reversing the loss of functional myocardium in end-stage heart disease is an important translational target. This chapter discusses the progress of cardiac transplantation and the development of possible clinical targets, as well as clinical trials in humans.

Keywords Myoblasts • Cardiomyopathy • Myocardial infarction • Xenotransplantation

1 Introduction

Cardiovascular disease remains the most potent killer in all developed societies. Although risk factor identification and modification, both through lifestyle and dietary changes and through pharmacotherapies such as the statins, have had remarkable success in lowering mortality from myocardial infarction [1], heart failure persists at epidemic levels in our aging population. In fact, heart failure is the most common cause of hospitalization in the United States and is the greatest contributor to U.S. health care costs [2, 3].

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It is no wonder, then, that a means for halting or even reversing the relentless progression of heart failure remains a highly sought after goal of translational cardiovascular researchers. A handful of pharmacologic strategies, in particular, the manipulation of neurohumoral axes such as the renin-angiotensin and adrenergic signaling systems, have had limited success in slowing the progression of pathologic ventricular remodeling. No conventional treatment to date, however, has realized the holy grail of "reverse remodeling" that might allow a failing and structurally deranged heart to transition back toward more efficient and healthful structure and cardiac pump function.

Despite impressive improvements in the interventional and medical therapy of ischemic coronary artery disease, this disorder remains the most common cause of heart failure. In fact, improvement in survival after myocardial infarction (MI) may be an important contributing factor to the current incidence of heart failure. Nonfatal acute MI results in a loss of pump function by the infarcted region of the myocardium; over time, the resulting stress on the remaining, so-called remote myocardium also results in an insidious process of myocyte apoptosis, myocardial fibrosis, and ventricular dilation in that region that contributes heavily to the evolution of chronic post-MI heart failure. Chronic myocardial ischemia, resulting from undiagnosed or untreatable coronary occlusive disease, also results in a progressive dilated cardiomyopathy and eventual failure.

Given the severe limitation on the availability of and the long-term morbidity associated with cardiac transplantation, a means of preventing or reversing the loss of functional myocardium in end-stage heart disease is an important translational target. Until the last decade, the myocardium was believed to be incapable of regeneration, comprising a limited number of terminally differentiated cells much like the central nervous system does. The discovery in the late 1990s of the ability of adult stem cells to differentiate along a cardiac myocyte lineage and to become incorporated into adult myocardium revolutionized this thinking overnight. Suddenly researchers and clinicians allowed themselves to consider the replacement of lost myocardium in an infarcted or remodeled heart, and the race to realize such an exciting and previously unimaginable goal was launched.

Nearly a decade later, however, we know only a very limited amount more about how to achieve this exciting goal than we did after the first exciting reports of improvement with myoblast and progenitor/stem cell transplantation. To be sure, the 2920 articles referenced in the National Institutes of Health's Medline database under the keywords "cardiac stem cell transplantation" since 1996 (roughly 120 between 1996 and 1999, close to 800 from 2000 through 2004, and approximately 2000 since 2005) have taught us much about different candidate cell types for cardiac cell transplantation, about different potential methods of introducing cells into the adult myocardium, and about early human application of the simplest therapeutic strategies. The sum total of this knowledge indicates, however, that although preclinical models suggest that delivery of stem cells may reduce the adverse sequelae of acute and possibly chronic myocardial ischemia, the mechanisms of this benefit are largely unknown. It is therefore not surprising that human clinical application of these simplest of preclinical strategies has not demonstrated any

clear-cut benefit to human patients. Although the disappointment of the euphoric enthusiasm for cardiac stem cell therapy has dampened what was likely the most superficial and least valuable scientific and clinical interest in this nascent field, a maturity of thought is now carrying this important investigational area forward, which is still believed by many to hold a tremendous promise for future generations of patients with heart disease.

2 "Stem Cells" and Candidate Cells for Cardiac Cell Therapy

The most widely accepted definition of a stem cell is a cell capable of both self-renewal and pluripotent differentiation into different mature cell types. The definitive prototype stem cell may always be the embryonic stem cell of the early blastula, the daughter cells of which, by definition, are capable of generating all of the differentiated cells of the eventual adult organism. Stem cells have long been known to exist in certain adult tissues, the best studied of which—the hematopoietic stem cell of adult bone marrow—has been used for decades in autologous and allogeneic transplantation. The discovery of undifferentiated, self-renewing stem cells in numerous adult tissues, also established in the late 1990s, on the other hand, had an enormous impact on the acceptance of a much broader potential for cell-based regenerative therapies.

A review of the diverse array of progenitor cells and stem cells that have been identified for these hypothesized regenerative therapies is beyond this discussion of cardiac applications of possible stem cell therapies. The following brief synopsis instead touches upon the array of cell types that have been studied in the context of cardiac cell transplantation. Some of these cells have discrete, identifiable phenotypes; others are truly heterogeneous mixtures of cells identified by their means of collection and isolation, by their behavior in in vitro culture, and, in some instances, by a limited characterization of expression of cell surface markers. Essentially every cell type discussed has demonstrated some form of histologic and/or functional benefit in an experimental, preclinical model; many have had multiple demonstrations of preclinical efficacy; and in some cases positive findings in the hands of one research group have been difficult to duplicate by others. The preclinical examples described here are therefore intended to provide a sense of the basic research upon which early clinical studies have been based but are not intended as an exhaustive catalogue of preclinical cardiac cell therapy data.

2.1 Skeletal Myoblasts

Skeletal myoblasts are cells that are committed to myocyte differentiation, including the formation of electrically coupled syncytia, but, unlike mature myocytes, retain the capacity for proliferation [4]. Skeletal myoblasts can be isolated from

adult muscle biopsies and can be expanded in vitro, making them good candidates for autologous donation in anticipation of cardiac cell transplantation. This isolation and expansion strategy avoids both problems of immunologic rejection associated with allogeneic cell transfer and the ethical issues associated with potential fetal/embryonic cell therapy. Skeletal myoblasts are also relatively resistant to ischemia [5], such as that found in regions of chronic coronary artery occlusion or in postinfarction scar. On the other hand, the requirement for weeks of cell culture essentially precludes application in urgent or emergent settings such as acute myocardial infarction. It is not surprising, then, that early clinical applications of skeletal myoblast transplantation have been considered in the setting of elective coronary revascularization or the treatment of chronic MI/coronary ischemia.

Autologous or isogenic skeletal myoblast transplantation has been reported in chronic MI models in both rodents and larger animals such as sheep [6–9]. The number of surviving myoblasts has varied in different reports and has been correlated to the degree of benefit observed [8]. Surviving myoblast implants have been found to undergo some degree of transdifferentiation, including the expression of cardiac-specific contractile proteins [8, 9]. Specific benefits after injection in and around areas of previous scar formation have included a reduction in post-MI ventricular dilation and improvement in left ventricular (LV) function [6, 7, 9].

2.2 Bone Marrow–Derived Stem Cells

Although an obvious choice for transplantation of hematopoietic precursor cells, the pluripotent potential of adult bone marrow–derived stem cells was found to include myocyte differentiation in the mid-1990s [10, 11]. In particular, in vitro treatment of bone marrow–derived cells with 5-azacytidine was used to encourage myocyte, and in some studies, cardiac myocyte phenotypic differentiation [12, 13]. These observations encouraged researchers to transplant bone marrow–derived cells in the setting of preclinical models of myocardial infarction [14, 15]. Whereas earlier reports underscored the difficulty in translating the survival of individual cells within the myocardial scar to actual functional benefit, generally observed only after in vitro induction of myocyte differentiation [14], a landmark report by Orlic et al. [15] claimed the regeneration of up to 68% of the infarcted myocardial wall within 9 days of injection of minimally selected bone marrow–derived cells 3–5 hours after coronary ligation in mice. Although the donor cells in this study were sorted from the total bone marrow cell population based on a lineage-negative, *c-kit*–positive phenotype via fluorescent-activated cell sorting, this study was among the most prominent preclinical reports that served as a rationale for early clinical trials involving the cardiac transplantation of essentially unsorted bone marrow–derived cells (see the later section Early Clinical Targets and Initial Human Clinical Trials).

Although credited with generating tremendous interest in cardiac stem cell therapy, the findings of Orlic et al. also proved among the most controversial and

difficult to repeat. In fact, numerous studies subsequently demonstrated that bone marrow–derived cells, largely hematopoietic in nature, injected into areas of acute myocardial infarction do not differentiate into myocyte lineages and do not contribute to myocardial regeneration or improved recovery after MI [16, 17]. To make matters worse, studies documenting donor cell fusion with preexisting myocytes [18, 19] also called into question earlier reports of in vivo cardiac myocyte differentiation by bone marrow–derived and other stem cells since these newer studies provided an alternative mechanism for the observation of donor-specific genetic markers in mature cardiac myocytes that had previously been assumed to be sufficient evidence of actual myocyte differentiation after transplantation.

Given the growing suspicion that the benefit observed in studies of bone marrow–derived and other stem cell transplantation does not reflect the generation of new myocardial muscle derived from donor cells, alternative mechanisms of benefit have been proposed and explored in preclinical models. Among these hypothesized mechanisms is the instigation of enhanced angiogenesis or neovascularization of the damaged myocardium as a result of cell transplantation [20–22]. Such an effect on angiogenesis could result from secretion of angiogenic factors by the donor cells or by angiogenic differentiation by a subpopulation of those cells. Recent studies have explored mechanisms by which the proangiogenic capacity of bone marrow–derived cells can be enhanced, such as the in vitro treatment of these cells with an enhancer of nitric oxide synthase activity [23].

2.3 Adult Mesenchymal Stem Cells

A working concept has evolved, particularly among translational scientists focused on potential regenerative therapies, of a "mesenchymal stem cell" that can be developed as a target for cardiac and other cell transplantation applications [11, 24–27]. At the core of this concept are observations that a subset of adult stem cells is particularly susceptible to biochemical induction of differentiation into a variety of cell types associated with the embryonic mesenchymal lineage, such as adipocytes, osteoblasts, and myocytes. These cells have been isolated, in turn, from a variety of adult tissue types, such a bone marrow and fat. Although certain patterns of cell surface marker expression have been associated with this stem cell subpopulation—for example, expression of CD29 and SCA1 but not of CD34, CD45, or CD11b [28]—mesenchymal stem cells do not represent a purified cell type, but rather a heterogeneous collection of cells that share a programmed developmental pathway. As such, the most common method for isolation of these cells, particularly from adult bone marrow, has been simple differential plating on plastic plates: mesenchymal stem cells will plate down within 24–48 hours, while the majority of bone marrow–derived cells that follow a hematopoietic lineage remain in suspension and can be washed away [25]. Although mesenchymal stem cells tend to adopt a spindle cell shape over time in cell culture, their phenotypic characteristics and their patterns of cell surface marker expression, will change over time, even in the absence of a

specific chemical stimulation for tissue type differentiation. Of interest, my laboratory and those of other researchers have reported that phenotypic characteristics of bone marrow–derived mesenchymal stem cells differ significantly from species to species; these differences may have significant implications for translation of preclinical models into human application. Whereas the exponential growth phase is reached within 2 weeks in rat and human cells, for example, the lag phase can last as long as 8 weeks in mice [29, 30]

Despite the nebulous nature of these potential donor cells, they possess a number of distinct advantages as candidates for cardiac regenerative cell therapy. First, if they truly represent a mesenchymal lineage, they would already share that lineage with the cardiac myocytes into which some researchers still hope the cells will differentiate after transplantation in vivo. Furthermore, many have come to believe that cardiac cell transplantation may succeed not via the simple generation of new cardiac myocytes, but through the instigation of complex tissue regeneration that includes multiple cell types of mesenchymal origin, including vascular cells and even fibroblasts. In addition, years of research have led to a repeated observation that mesenchymal stem cells possess a capacity for immunomodulation and immunologic privilege, such that immunosuppression may not be required even when an allogeneic source of mesenchymal stem cells is used for transplantation [28]. This last feature is particularly important when considering the development of "off-the-shelf" therapies both for standardization and for use in emergent settings.

Successful reduction of infarct size and improvement in LV function with mesenchymal stem cell injection just after experimental MI and in dilated cardiomyopathy in rodents [24, 31, 32] led to studies of post-MI mesenchymal cell transplantation in larger-animal models [33–36]. Histologic evidence of cardiac differentiation and improvement in macroscopic and microscopic myocardial wall structure were accompanied by improvement in LV function in porcine models [33–35], whereas cardiac function and blood flow to the region of cell transplantation were improved in sheep [36]. Different reports produced contrasting claims with regard to the actual contribution of donor cells or their progeny to new myocardial muscle or vascular tissue, although nearly all researchers agree that even when such differentiation is suggested the actual number of transplant-derived cells is very small, generally lack functional electrical coupling to host cells, and do not likely contribute greatly in a direct manner to either improved pump function or coronary blood flow [37].

2.4 Fetal Cardiac Myoblasts/Embryonic Stem Cells

Among the earliest reports of successful cardiac cell transplantation was one that involved the injection of fetal cardiac myocytes by Snoopa et al. into mouse myocardium [38]. Donor cells not only proliferated in vivo, but also were found to form intercalated disks with host cells, suggesting functional electrical integration. Subsequent studies suggested therapeutic benefit when fetal cardiac cells were

injected in scar tissue after experimental MI in rodents [39–41]. Fetal cardiac myocytes may represent the most logical source of cells for actual donor cell regeneration of lost myocardium since these are cells that are already committed and programmed for functional adult cardiac myocyte differentiation yet have not lost their natural proliferative potential. In addition, it has also been reported that these cells can contribute to the other hypothesized mechanisms of benefit from cardiac cell transplantation, such as enhanced neovascularization of scar tissue via secretion of angiogenic factors [42].

It is unlikely, however, that fetal cardiac cell transplantation will become a feasible clinical strategy for humans in the foreseeable future. Researchers have instead focused on the feasibility of embryonic stem cell transplantation as a means of improving the survival, proliferation, and successful cardiac differentiation of donor cells compared to the results that have generally been observed with adult donor stem cells. It is not surprising that embryonic stem cell engraftment has been documented after injection into infarcted rodent hearts and has been associated with improvements in cardiac recovery and function [43]; similar results have even been reported with injection of human embryonic cells into immunosuppressed rodents [44, 45]. Other reports, on the other hand, have suggested that cells derived from embryonic stem cell injection may not form functional, integrated myocardium but may instead lead to teratoma formation [46, 47]. Intense research toward the development of politically and socially acceptable sources of therapeutic embryonic stem cells continues, and pluripotent stem cells induced from human fibroblasts have already been shown to have the capacity to adopt genetic and phenotypic features of cardiac myocytes [48]. These cells, however, are not yet free of some of the same limitations associated with the use of fetal cells for transplantation. While rejection of these relatively immunologically immature cells may be muted, for example, it is not yet clear that immunosuppression would not be necessary to preserve any benefit derived from human allogeneic embryonic stem cell transplantation in the heart [49, 50].

2.5 Endothelial Progenitor Cells

Disappointment over the inability of different research groups to consistently demonstrate the generation of functional myocardium from the progeny of transplanted stem cells in the heart led to a substantial shift in the focus of translational researchers in the mid-2000s. An indirect mechanism of benefit from cell transplantation was needed to sustain interest in the relatively consistent empiric findings of benefit in preclinical models and to make efforts at translation of those empiric findings more likely to succeed. Angiogenesis and neovascularization of the diseased myocardium provided a ready-to-use mechanism of benefit since therapeutic angiogenesis, based on either molecular- or gene-based approaches, had captured the imagination of these same researchers less than a decade previously. It is curious, however, that the collective memory of the cardiovascular research

community did not instigate greater skepticism of this hypothesized mechanism. Very potent direct methods for induction of neovascularization in preclinical models had had a devastatingly unsuccessful early push into disappointing early clinical trials that are now largely viewed as having been premature; it is unclear why the more indirect angiogenesis hypothesized to accompany stem cell transplantation would be expected to enjoy greater success in the daunting venue of early clinical application.

Nevertheless, interest in cell transplantation–related angiogenesis led researchers to explore the use of endothelial progenitor cells themselves as candidate donors for cardiac cell therapy. Much like the explanation of the person who looks for lost coins under a street lamp because "the light is better," advocates of this approach have pointed to the ready availability of these cells from peripheral blood and the resultant feasibility of autologous cell donation [51,52]. Both myocardial ischemia and therapeutic cytokine administration have been found to increase the number of circulating endothelial progenitors, and in vitro expansion of these cells has further facilitated experimental donor cell acquisition [51,53–55]. Because these cells are believed to home to areas of ischemia within the myocardium, intravenous delivery has been associated with significant improvements in cardiac structure and function [54, 55]. Furthermore, there have even been reports of these cells transdifferentiating into cardiac myocytes [56, 57]. Despite this near-perfect donor cell profile of EPCs, researchers in this area remained concerned about two related findings: ex vivo expansion of endothelial progenitors, an almost certain requirement for human application, results in a marked diminishment of their therapeutic potential as donor cells. Even more disappointing has been the observation that endothelial progenitor cells isolated and expanded from older patients and those with significant atherosclerotic disease, precisely the patient population to be targeted by this therapeutic strategy, are significantly less active as donor cells than are those isolated from younger, healthy individuals [58].

2.6 Cardiac Stem/Progenitor Cells

Documentation of cardiac cell proliferation after MI by Beltrami et al. [59] led to the subsequent identification of pluripotent stem cells within the adult heart that seem particularly well disposed toward cardiac cell differentiation [60–63]. Given the limitations associated with all other potential donor cell types, these cells have catapulted to the top of many researchers' lists of cardiac cell therapy hopefuls. As with nearly every other cell type tested, there have been early reports of structural and functional benefit associated with the injection of cardiac-derived stem cells in both small- and large-animal models [64, 65]. As with other promising cell types, it remains to be seen whether the harvest and expansion of these cells, particularly from patients with already diseased myocardium, will truly provide a practical foundation for autologous human cardiac stem cell transplantation.

3 Preclinical Models and Methods of Delivery

3.1 Reproducible "Positive" Findings

After roughly a decade of increasingly intense research into myocardial stem cell transplantation, one unquestionable conclusion is that it is possible to influence the structural and functional outcome of post-MI cardiomyopathy through the injection of multiple types of progenitor or stem cells into or near the area of myocardial infarction at the time of, or shortly after, the acute coronary event. It may also be true that cell injection in the context of a more mature cardiomyopathy may result in functional improvement, but the evidence here is less consistent and therefore less compelling.

It is also true that in addition to a wide variety of candidate cell types for cardiac cell transplantation, researchers have established a number of successful means of delivery of these cells to the injured and/or dysfunctional myocardium.The direct injection of cells into a specified region of the heart can place donor cells exactly where they are believed to be needed. This direct transplantation is perhaps most straightforward in the context of an open surgical procedure, such as an adjunctive therapy at the time of surgical coronary bypass. Alternatively, cell injection may be accomplished via a minimally invasive thoracoscopic approach. An even less invasive approach for direct injection is also feasible through a transventricular catheter-based injection. Although the localization of injections is less straightforward with this approach, percutaneous systems for the electrical mapping of ischemic myocardium, such as the Biosense NOGA system, are well developed [66–68].

Remarkably, it has been feasible to deliver various cell suspensions to target areas of the myocardium via intracoronary infusion without the instigation of widespread coronary occlusion by the donor cells. It is further noteworthy that donor cells have been found to migrate across the coronary bed and to successfully engraft. This approach must be performed with great care and with attention to the "concentration" of cells and the resultant viscosity of the injectate. Whereas application of this approach after percutaneous revascularization, particularly with coronary stent placement, is a particularly attractive strategy for delivery to the area of ischemia and infarction, limitations are encountered when culprit vessels remain occluded. A retrograde approach via the coronary sinus and the venous coronary circulation may be of use in these clinical circumstances.

Finally, simple intravenous infusion of stem cells, and particularly of endothelial progenitor cells, has been studied as a safe and clinically feasible means of achieving cardiac regeneration. This approach, of course, depends on a homing mechanism in the donor cells and therefore may be limited to stem and progenitor populations, such as cardiac-derived stem cells, that are already endowed with a cardiac predisposition.

3.2 Mechanistic Understanding and Other Limitations

With the exception of a handful of studies, primarily those involving transplantation of fetal cardiac tissue, the generation of new functional, electrically integrated myocardial mass from donor-derived cells has not been consistently demonstrated in preclinical studies of cardiac cell transplantation. The empiric benefits observed must therefore be explained by other mechanisms if clinical translation of these findings is to be realized.

The two most prominent mechanisms that have been explored experimentally are the "paracrine" and "angiogenesis" theories. As discussed briefly earlier, the latter hypothesis has found support in the documentation of improved blood flow after the transplantation of various donor cell types into ischemic myocardium compared to controls. Since the survival and proliferation of donor cells are as limited in the context of generating new vascular cells as they are in the context of generating new cardiac myocytes, it is likely that even an "angiogenic" effect of stem cell transplantation is likely to result not from the growth of donor cell–derived blood vessels, but from a paracrine effect on the host myocardium. It is therefore likely that these two competing hypotheses actually accordion down into one potential explanation. In fact, the possible "paracrine" effects of cardiac cell transplantation may include enhanced myocardial protection, enhanced neovascularization, inhibition of pathologic cardiac remodeling, and improvement in cardiac myocyte metabolism and contractility [37].

Although a number of studies have attempted to identify putative paracrine factors that might be released by donor cells to instigate the beneficial effects of experimental stem cell transplantation in the heart [69–72], this bottom-up analysis will likely prove a daunting task. Although genomic expression analysis can be applied to in vitro cell cultures, there is no guarantee that a similar pattern of expression will be observed after the cells are injected into a harsh in vivo environment of myocardial ischemia. Genomic analysis of tissue lysates after injection pose the challenge of identifying the cell type providing the source for each identified transcript. In situ reverse transcription-polymerase chain reaction, on the other hand, can only be applied to preidentified target factors and is therefore quite limited in scope. Although genetic modification of donor cells in an experimental setting may help to identify critical individual paracrine factors, it is more likely that the paracrine effect of donor cells represent a complex orchestration of upregulation and downregulation of multiple signaling molecules in the extracellular myocardial milieu.

Given the daunting prospect of establishing a paracrine mechanism by studying the fate or secretory activity of donor cells, my laboratory has instead focused on the biologic impact of cell transplantation on the host myocardium since this is most likely the site of origin of the eventual benefit of donor cell delivery. A pattern of selective prosurvival signaling that might be capable of inducing and supporting a physiologic, adaptive hypertrophic response was observed in the surviving myocardium of mice subjected to coronary occlusion and early mesenchymal stem cell transplantation [29]. This change in signaling compared to controls that did not

receive cell therapy was observed in the ventricular wall that was remote both to the infarction and the site of actual cell injection, suggesting that the presence of the cells does result in a global, likely paracrine modification of myocyte biology.

4 Early Clinical Targets and Initial Human Clinical Trials

Despite the uncertainties of mechanism and the publication of conflicting data in some key areas of research, experimental human application of cardiac stem cell transplantation began almost as soon as the ink was dry on initial preclinical reports. To date, well more than 100 studies have been undertaken worldwide of some form or other of cardiac cell transplantation. The results of studies involving more than 1000 patients who have undergone the delivery of blood- or bone marrow–derived cells early after acute MI have been reported, and meta-analyses have been performed [73–75]. Similarly, hundreds of patients have been enrolled in both uncontrolled and controlled trials that have examined the efficacy of muscle biopsy–derived myoblast transplantation [76].

4.1 Therapy for Acute Myocardial Infarction

The most successful clinical studies to date in human cardiac cell transplantation have involved the delivery of bone marrow–derived mononuclear cells within the first week after acute MI. Although the preclinical data for this approach have not been as plentiful as those for other cell preparations, such as cultured mesenchymal stem cells, the absence of a requirement for extended in vitro cell culture made this strategy easy to implement in a clearly definable patient population. Although at least 13 randomized studies have been performed, the only clearly promising data have come from the group led by Zeiher and Dimmeler at the University of Frankfurt. Following up on initial smaller-scale studies, they reported results from the Remodeling in Acute Myocardial Infarction (REPAIR-AMI) study in 2006. Directed by subpopulation analyses from the earlier studies, this trial randomized 204 patients who were successfully revascularized via stent placement after an acute, ST-segment elevation MI but who had a residual deficit in LV function as reflected in an LV ejection fraction of 45% or less. Patients underwent harvest of bone marrow between days 3 and 6 post-MI, followed by coronary reinfusion of mononuclear cells that were obtained via Ficoll gradient centrifugation of the aspirates in a central lab and were suspended overnight in medium supplemented with their own serum. Control patients underwent bone marrow harvest followed by reinfusion of suspension medium alone. At 4 months, patients receiving the mononuclear cells had a modestly greater improvement in ejection fraction compared to controls (5.5% ± 7.3% vs. 3.0% ± 6.5%, $p = 0.01$), and at 1 year the

composite primary endpoint of death, recurrent MI, or revascularization was reduced by 40% ($p = 0.01$).

Enthusiasm for this modest improvement in clinical status with mononuclear cell therapy after MI has been somewhat dampened by an onslaught of confusing and conflicting results from other studies. The most prominent of these was the Autologous Stem cell Transplantation in Acute Myocardial Infarction trial, in which 100 patients were randomized to a similar bone marrow–derived mononuclear cell therapy early after MI at two centers in Norway [77]. This study was completely negative, although the REPAIR-AMI investigators pointed to differences in the two groups' handling of the marrow-derived cells as the explanation for the conflicting results. At least four other randomized studies reported between 2006 and 2008 either failed to meet their primary endpoints or had mixed results [76, 78, 79]. Several meta-analysis compiling data from 800 to 1000 patients have indicated an improvement in LV ejection fraction on the order of 3% and a similarly modest 3% reduction in infarct size [73, 75].

Two other cell types have also been studied in the context of intracoronary infusion after MI. A trial involving progenitor cells expressing the cell surface marker CD133 that has been associated with endothelial cell progenitors was stopped early when higher-than-expected rates of in-stent restenosis led to a concern that these proangiogenic cells could also stimulate acceleration of intimal hyperplasia [80]. One randomized study of 69 patients reported an improvement in LV function and perfusion at 3 months after post-MI infusion of mesenchymal stem cells compared to saline injection. Other methods have also been used to deliver mesenchymal stem cells. One commercial enterprise is promoting the clinical development of an off-the-shelf preparation of mesenchymal stem cells isolated from healthy donors and has organized a Phase II study of intravenous delivery within 1 week of acute MI.

4.2 Therapy for Chronic Angina or Heart Failure

Less extensive clinical efforts have begun in other potential applications of cardiac cell transplantation. Two randomized studies are looking at the delivery of either $CD34^+$ endothelial progenitors or fat-derived stem cells in patients with refractory angina [76]. A number of studies have looked at the delivery of bone marrow–derived cells in conjunction with bypass surgery for the treatment of chronic heart failure [81–83], all with disappointing results. In this setting, it was a nonrandomized study of $CD133^+$ cells that showed the most promise for reversal of chronic cardiomyopathy [84]. Other small-scale studies have also begun to look at either direct injection or coronary infusion of bone marrow–derived circulating progenitor cells, but data are too premature to draw any substantial conclusions.

Finally, one other area of cardiac cell therapy has undergone a substantial amount of early clinical investigation: that of skeletal myoblast transplantation. The first small-scale reports of efficacy with surgical delivery of these cells to areas of

unrevascularized failing myocardium during bypass of other regions were tempered by the observation of an increased rate of ventricular arrhythmias [85]. Subsequent studies of myoblast injection have therefore been undertaken in conjunction with implantation of automatic defibrillators. Ninety-seven patients were treated in a randomized fashion in the Myoblast Autologous Grafting in Ischemic Cardiomyopathy trial of similar myoblast injection at the time of coronary bypass of other regions of the heart; this study, however, failed to document an improvement in LV function [86]. The results of one study of 47 patients divided between optimal medical therapy and catheter-based delivery of myoblasts to chronically failing hearts have been similarly disappointing [76].

5 Future Directions and Conclusions

Like therapeutic angiogenesis of the mid-1990s and cardiovascular gene therapy before that, potential stem cell therapies for heart disease erupted into the awareness of cardiovascular researchers and clinicians in the early part of the 2000s with the roar of hope for new treatment paradigms. Having learned little from those previous disappointing experiences, however, many in the cardiovascular community jumped again at the prospect of immediate clinical translation at the expense of rigorous scientific definition of the opportunities and the limitations at hand. It is ironic, although not surprising, that basic scientists met with disappointment in attempting to verify the initial, simplistic presumed mechanism of action of cell transplantation at the same time that larger-scale clinical trials met with disappointment in simplistic clinical translation.

The initial euphoria surrounding cardiac stem cell therapy emerged primarily from the previously unavailable possibility for regeneration of lost myocardium. It is further ironic, therefore, that the best suggestion of success in early clinical trials came not in the treatment of existing cardiomyopathy or chronic MI, in which the generation of new myocardium is truly needed, but in the treatment of acute myocardial infarction, for which many excellent therapies already exist and in which myocardial preservation may be more important than myocardial regeneration. In fact, many have viewed this early clinical experience as further demonstration that the deposition of stem cells into injured myocardium is not a means by which new myocardium may be generated but is a vehicle for modulation of host myocyte biology that limits the detrimental effect of the acute infarct.

The magnitude of benefits observed in human cardiac cell therapy trials has been of marginal clinical significance at best. It is therefore understandable that small differences between trial design and execution could result in conflicting and mixed results. True success in clinical translation of more robust preclinical observations will almost certainly depend on a greater understanding of the mechanisms of those benefits and a corresponding opportunity to amplify the human realization of those mechanisms. This deeper comprehension will greatly enable the identification of ideal cell types for different clinical scenarios, as well as optimal delivery strategies and timing.

Ongoing research will continue to improve both our understanding of developmental cardiac myocyte biology [87] and our ability to enhance the therapeutic properties of donor stem and progenitor cells. Early experiments have already documented improvement in the functional benefit of transplantation through genetic and chemical modification of donor cells [14, 88]. These modifications may enhance the capacity of donor cells to proliferate and differentiate in the host myocardium, but earliest applications may simply allow greater numbers of transplanted cells to survive. Along these lines, bioengineers are developing delivery vehicles that may support the survival of transplanted cells and provided an enhanced microscopic milieu within the harsh, ischemic host myocardium.

It has been said that xenotransplantation of solid organs is just around the corner…and always will be. The same may be said some day of therapeutic angiogenesis and of cardiovascular gene therapies. Although daunting challenges remain in the difficult road toward human clinical translation, many researchers remain optimistic that cell-based therapies for cardiovascular disease represent a more realistic and achievable goal. Tremendous discipline, however, will be required of translational researchers as the need for rigorous mechanistic analysis becomes paramount in the face of unforeseen mysteries and as unrealistic expectations for immediate success inevitably dampen the enthusiasm of the public and, more important, of funding agencies. The promise of true myocardial regeneration remains too great for any opportunity to be overlooked, and it is this promise that will drive both the inspiration and the perspiration required for its eventual realization.

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Adult Neural Progenitor Cells and Cell Replacement Therapy for Huntington Disease

Bronwen Connor

Abstract Huntington disease (HD) is an autosomal dominant genetic neurodegenerative disorder caused by an expansion mutation of a naturally occurring trinucleotide (CAG) repeat in exon 1 of the IT15 gene, which encodes a 350-kDa protein termed Huntingtin. This results in the progressive degeneration of (γ-aminobutyric acid) GABAergic medium spiny projection neurons in the basal ganglia. With no efficient treatment available in the clinic to alleviate or compensate for the progressive neuronal cell loss in HD, novel treatment strategies such as endogenous cell replacement therapy need to be investigated. This chapter provides an overview of what is known about the response of endogenous adult neural progenitor cells to neurodegeneration in the HD brain, the mechanisms by which this response may occur, and how this knowledge may be translated into effective therapeutic strategies.

Keywords Huntington disease • Adult neurogenesis • Adult neural progenitor cells • Basal ganglia • Striatum

1 Introduction

Evidence accumulated over the last several decades has dispelled the long-held dogma that the adult mammalian brain cannot generate new neurons. Neural progenitor cells have been identified in both the forebrain subventricular (SVZ)-olfactory bulb pathway and the hippocampal dentate gyrus of the adult mammalian brain, including the human brain [1–6]. The SVZ is organized as an extensive network of chains of migrating neuroblasts (type A cells) that travel through glial tubes formed by processes of slowly proliferating glial fibrillary acidic protein (GFAP)–positive

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cells (type B cells). Clusters of rapidly dividing, immature progenitors (type C cells) are scattered along the network of migrating chains [7, 8]. SVZ neuronal precursors in both the rodent, nonhuman primate, and human brain migrate long distances via the rostral migratory stream (RMS) to their final destination in the olfactory bulb, where they differentiate into granule and periglomular neurons [9–13]. In contrast to the extensive migration undertaken by neurons destined for the olfactory bulb, dentate gyrus granule neurons are born locally in the SGZ, a germinal layer between the dentate gyrus and the hilus [1, 3, 4, 6, 14]. Within the SGZ, GFAP-positive cells (type B cells) divide to give rise to immature type D cells, which then generate granule neurons [15, 16]. Of interest, type D cells divide less frequently and are more differentiated than the transit amplifying type C cells in the SVZ.

Neurogenesis in the adult brain can be divided into three phases: (1) proliferation when new cells are generated, (2) migration toward a target area, and (3) terminal differentiation into a specific phenotype. Over the last decade, research has begun to shed light on mechanisms that regulate neurogenesis in the adult brain. Of interest, adult neurogenesis is not static, but its rate may fluctuate in response to environmental change. Evidence from in vitro and in vivo studies has demonstrated that neurogenesis can be regulated by a range of growth and neurotrophic factors, neurotransmitters, and hormones, as well as by a wide variety of attractive and repulsive chemotropic factors [17, 18]. Neurogenesis has also been shown to be altered by the presence of cell death induced by brain injury or disease [17–20]. In the SGZ of the adult rodent brain, progenitor cells have been observed to respond to a range of injuries, including excitotoxic or mechanical lesions, focal ischemic injury, and chemoconvulsant-induced seizure activity, by increasing neurogenesis [21–24]. An increase in SVZ progenitor cell proliferation has also been observed in various injury models, including aspiration or transection lesions of the forebrain, inflammatory or chemical demyelination, percussion trauma, chemoconvulsant-induced seizure activity, and focal ischemic injury of the adult rodent brain [24–33]. Furthermore, studies by Parent et al. [32, 33] and Arvidsson et al. [31] demonstrated an increase in SVZ neurogenesis following focal ischemic injury or chemoconvulsant-induced seizure activity, leading to the migration of neuroblasts from the SVZ to damaged areas of the striatum. These observations have led to the exciting hypothesis that endogenous progenitor cells may be able to generate new neurons to replace cells lost through brain injury or neurodegenerative disease.

Huntington disease (HD) is a prime candidate for augmentation of SVZ neurogenesis in response to neuronal cell loss due to the close proximity of the SVZ to the adjacent caudate putamen. HD is an autosomal dominant genetic disorder caused by an expansion mutation of a naturally occurring trinucleotide (CAG) repeat in exon 1 of the IT15 gene, which encodes a 350-kDa protein termed Huntingtin [34]. This results in the progressive degeneration of (γ-aminobutyric acid) GABAergic medium spiny projection neurons in the basal ganglia. With no efficient treatment available in the clinic to alleviate or compensate for neuronal cell loss in HD, novel treatment strategies such as endogenous cell replacement therapy need to be investigated. This chapter provides an overview of what is known about the response of endogenous adult neural progenitor

cells to neurodegeneration in the HD brain, the mechanisms by which this response may occur, and how this knowledge may be translated into effective therapeutic strategies.

2 Neurogenesis in the Adult Human Huntington Disease Brain

The neuropathology of HD is marked at both the gross and microscopic levels. The principal gross change seen in HD is severe bilateral atrophy of the caudate putamen and neocortex. The microscopic neuropathologic changes are most pronounced at the basal ganglia, which consists of four large subcortical nuclei: the caudate nucleus, globus pallidus, subthalamic nucleus, and substantia nigra. Neurochemical studies of HD brains demonstrate a reduction in the levels of GABA and calbindin in the caudate nucleus resulting from the loss of medium-sized spiny projection neurons. This is accompanied by depletions of substance P, dynorphin, and enkephalin. In contrast, medium-sized aspiny neurons that colocalize the neuropeptides somatostatin and neuropeptide Y and the larger, aspiny interneurons containing choline acetyl transferase are relatively spared. The neuropathologic progression of HD has allowed for an ascending grading scale (0–4) to be implemented on the basis of neuronal degeneration [35]. HD grade 0 brain has a grossly normal, convex caudate nucleus, but microscopically there is already loss of projection neurons. At grade 4, the caudate nucleus is markedly concave, and there is a 95% loss of projection neurons from the caudate nucleus. Neurochemically, striatopallidal neurons that colocalize GABA and enkephalin are affected in the early grades of the disease (grade 0), followed by neurodegeneration of striatonigral neurons that colocalize GABA and substance P in more intermediate stages (grades 1–2). Finally, striatopallidal neurons that colocalize GABA and substance P are affected in the higher grades of HD (grades 3–4).

In 2003, Curtis et al. [36] demonstrated for the first time upregulation of progenitor cell proliferation and neurogenesis in the SVZ of the HD human brain. This was achieved by examining the number of proliferating progenitor cells in the SVZ of postmortem normal and HD human brains using antibodies to the cell cycle marker proliferating cell nuclear antigen (PCNA), which labels cells in the S phase of cell division. While a small number of PCNA-positive cells were detected in the SVZ of normal human brains, the number of PCNA-positive cells was considerably increased in HD, as demonstrated by a significant increase in the thickness of the SVZ and number of PCNA-positive cells in HD compared to normal human brains. The progressive and massive cell death that occurs in HD appears to cause the SVZ to become substantially thicker (2.8-fold increase) compared to normal [37, 38]. The cellular composition of the SVZ is also altered, and the number of proliferating type A, B, and C cells is increased 2.6-fold overall. Of interest, in the HD brain, despite an approximate 50% increase in the number of type A and C cells compared to normal, the largest increase is in the number of type B cells: the ratio A:B:C

shifts from 1:3:1 in the normal brain to 1:7.5:1 in the HD brain [37, 38]. Furthermore, the grade of PCNA staining in HD cases significantly correlated with the HD neuropathologic grade and the number of CAG repeats in the expanded allele of the IT15 gene. However no correlation was found between the PCNA grade and the age, sex, or postmortem delay of the cases [36]. Double-label immunofluorescent techniques using antibodies against βIII tubulin, which labels neurons early in development, and the glial cell marker GFAP were performed to determine the fate of PCNA-positive cells in the SVZ of HD human brains. PCNA-positive cells in the SVZ of HD human brains were observed to colocalize with the neuronal marker βIII tubulin. The PCNA/βIII tubulin–positive cells were located mainly in the deeper regions of the SVZ adjacent to the caudate nucleus and comprised on the order of 5% of the PCNA-positive cells in the SVZ [36]. In addition, a large number of PCNA-positive cells in the SVZ of HD human brains colocalized with GFAP, demonstrating a glial phenotype. The PCNA/GFAP–positive cells were identified predominantly in the more superficial region of the SVZ adjacent to the ependymal layer and made up approximately 50% of the PCNA-positive cells in the SVZ [36]. These results demonstrate that the HD human brain has the potential to repair itself.

3 Neurogenesis in the Excitotoxic Rodent Model of Huntington Disease

In agreement with the findings made in the postmortem human HD brain, SVZ progenitor cell proliferation and neurogenesis has also been observed to be enhanced in the quinolinic acid (QA) lesion rodent model of HD [39, 40]. It has been suggested that metabolic compromise and oxidative damage leading to secondary excitotoxicity and cell death may be implicated in the neuropathology of HD [41]. Consistent with this hypothesis, the selective neuropathology seen in the human HD brain can be mimicked in experimental animals by direct striatal injection of the excitatory amino acid agonist QA [42, 43]. As a result, lesioning of the striatum in rodents using QA has been extensively used as an animal model of HD. Although the QA lesion model reflects an acute, nongenetic model of HD, examining the response of SVZ progenitor cells in a model mimicking the selective neuronal cell loss observed in the human HD brain provides an opportunity to elucidate key environmental cues regulating compensatory SVZ neurogenesis. Using the QA lesion rodent model, it has been demonstrated that progenitor cells not only proliferate in response to selective striatal cell loss, but they also migrate from the SVZ toward the site of cell loss, where they generate new striatal neurons [39, 40]. Cell proliferation and neurogenesis were assessed with bromodeoxyuridine (BrdU) labeling and immunocytochemistry for cell type–specific markers. BrdU labeling demonstrated increased cell proliferation in the SVZ ipsilateral to the QA-lesioned striatum, resulting in expansion of the SVZ in the lesioned

hemisphere [39, 40]. A time-course study [39] revealed that SVZ progenitor cell proliferation had begun 1 day following QA lesioning and was significantly increased compared to sham-lesioned animals from 1 to 14 days post QA lesion, with maximum increase observed at day 7 postlesion. SVZ progenitor cell proliferation declined to a lower, but still increased, level of proliferation by day 28 post QA. These changes were associated with an increase in cells in the anterior SVZ ipsilateral to the lesioned striatum expressing the antigenic marker for SVZ neuroblasts, doublecortin (Dcx) [39, 40]. A large number of Dcx-positive neuroblasts were observed in both the transition zone and the lesion core of the QA lesioned striatum, and they demonstrated morphologic characteristics of both migrating and nonmigrating cells. In contrast, Dcx was expressed only in the SVZ and RMS of the sham-lesioned and normal rat brain, with no Dcx-positive cells observed in the striatum [39, 40]. Within the QA-lesioned striatum, a subpopulation of newly generated cells expressed markers for immature and mature neurons, including phenotypic markers of striatal medium spiny neurons (DARPP32) and interneurons (parvalbumin and neuropeptide Y) [39, 40].

The temporal correlation between progenitor cell proliferation and neuroblast migratory response into the damaged striatum was further examined following QA lesioning of the adult rat striatum [44]. Retroviral labeling of SVZ-derived progenitor cells demonstrated that cell loss in the QA-lesioned striatum increased progenitor cell migration through the RMS for up to 30 days postlesion. In addition, a population of dividing cells originating from the SVZ generated Dcx-positive neuroblasts that migrated into the damaged striatum in response to cell loss invoked by the QA lesion. Quantification of BrdU-labeled cells coexpressing Dcx revealed that the majority of cells present in the damaged striatum were generated from progenitor cells dividing within 2 days either prior to or following the QA lesion. In contrast, cells dividing 2 or more days following QA lesioning migrated into the striatum and exhibited a glial phenotype [44]. The acute and transient migratory response exhibited by SVZ-derived progenitor cells following QA lesioning may reflect changes in environmental cues expressed in the damaged striatum over time. Of interest, the temporal profile of neuroblast migration observed in the QA-lesioned striatum correlates with both the time course and extent of GABAergic medium spiny striatal cell loss following QA lesioning [42–45]. This may be explained by the activation of resident microglia following striatal cell death. Activated resident microglia are observed in the lesion core within 12 hours of QA injection and are able to recruit blood-borne microglia by 3–5 days following QA injection [46–50]. Activated astrocytes and microglia are also prominent pathologic features in the HD human brain [35]. The ability of activated microglia to direct the migration of neural progenitor cells has been previously identified [51] and is most likely due to microglial release of chemokines. In agreement, the chemokines MCP-1, MIP-1α, and GRO-α were found to be significantly upregulated in the striatum 2–3 days following QA-induced lesioning, correlating with maximum SVZ-derived progenitor cell recruitment into the lesioned striatum [52]. Furthermore, MCP-1, MIP-1α, and GRO-α act as potent chemoattractants for SVZ-derived progenitor cells in vitro

[52]. Overall, the results obtained in the QA lesion model correspond with the observations made in the human HD brain [36] and suggest that the response of SVZ progenitor cells to QA lesioning reflects the temporal dynamics of cell loss and microglia activation in the damaged striatum.

4 Neurogenesis in Transgenic Models of Huntington Disease

The response of SVZ progenitor cells to chronic neurodegeneration has also been examined in a range of HD transgenic mouse models. The most commonly studied transgenic mice are the R6/1 and R6/2 lines, which carry exon 1 of the human HD gene, with 115 and 150 CAG repeats, respectively. In human HD, CAG repeat lengths in this range lead to severe symptomatology and death in early infancy; the most common HD CAG repeat range is 40–55 repeats, and this usually results in disease onset at 40 years of age or older. A number of studies using either the R6/1 or the R6/2 transgenic line demonstrate a reduction in cell proliferation and neurogenesis in the hippocampus [53–58]. In the R6/1 mouse model, the survival of BrdU-positive cells was reduced in the dentate gyrus. In addition, the number of Dcx-positive cells and the total number of new neurons were greatly reduced compared to wild-type mice. In contrast, the proportion of BrdU-positive cells differentiating into mature neurons was not significantly different between genotypes [54]. In both wild-type and R6/1 mice, housing in an enriched environmental increased the number of BrdU- and Dcx-positive cells and also generated longer neurites and increased the migration of Dcx-positive cells [54], demonstrating that hippocampal progenitor cells in R6/1 mice continued to be responsive to changes in the microenvironment induced by enrichment. Similarly, in the dentate gyrus of the R6/2 mouse model, progenitor cell proliferation and morphology were compromised compared to wild-type mice but not differentiation or survival [56]. However, neurogenesis failed to be upregulated in the dentate gyrus of R6/2 mice in response either to seizure activity [56] or physical activity [58]. Of interest, however, no difference was observed in the in vitro growth of adult neural progenitor cells between genotypes [56]. These results suggest that decreased neurogenesis might be responsible, in part, for the hippocampal deficits observed in these mice. Furthermore, the abnormality in hippocampal neurogenesis observed in the transgenic mouse models may not be attributable to an intrinsic impairment of neural progenitor cells but may be attributable to the environment in which the cell is located.

One of the most significant findings, however, from the studies undertaken in the R6/1 and R6/2 transgenic mouse models is the lack of significant change in SVZ neurogenesis observed in these models [53–57]. In both the R6/1 and R6/2 models, no significant change in SVZ proliferation has been observed compared to wild-type mice. Furthermore, Moraes et al. [55] observed impairment in neuroblast migration through the RMS to the olfactory bulb in R6/2 mice. This was characterized by an accumulation of cells in the caudal RMS but no alteration in polysialic acid-NCAM expression and/or cell death. These observations are in stark contrast

with the upregulation of progenitor cell proliferation, migration, and neurogenesis observed in the SVZ in both the HD human brain and the QA-lesioned rat brain [39, 40, 44, 59]. The reason for this discrepancy is unknown, but it may be that the stimulus for SVZ proliferation in humans and the QA lesion model is neurodegeneration in the adjacent caudate nucleus/striatum, which is minimal in both the R6/1 and R6/2 transgenic lines. It may also reflect intrinsic species differences and the shortened lifespan of the R6/2 and R6/1 transgenic lines. In conflict with other studies however, Batista et al. [60] reported an expansion of SVZ progenitor cells in the R6/2 mouse model of HD. Using the in vitro neurosphere assay as an index of progenitor cell number in vivo and to assess proliferation kinetics in vitro, they demonstrated that disease progression in the R6/2 model led to an increase in SVZ-derived progenitor cell number. Furthermore, once SVZ progenitor cell proliferation was induced in vivo, it could be maintained during in vitro passaging of progenitor cells. However, this was not reproduced in presymptomatic R6/2 mice. Basista et al. [60] also demonstrated that a subpopulation of SVZ progenitor cells in R6/2 mice redirected their normal migration through the RMS to the olfactory bulb and instead migrated into the striatum, potentially in response to neurodegeneration. The results obtained by Batista et al. [60] using the R6/2 model more closely replicate the observations made in the human HD brain; however, they have not been further reproduced.

5 Mechanism of Neurogenesis in Huntington Disease

While it is apparent that alteration in progenitor cell proliferation and neurogenesis in the HD brain reflect changes in the microenvironment as a result of neurodegeneration and/or Huntingtin mutation, the exact signals involved in regulating these changes are poorly understood. A range of growth and neurotrophic factors, neurotransmitters, and hormones have been shown to regulate adult neurogenesis. Basal expression of these factors may be altered in response to apoptotic cell death [61] and the activation of astrocytes and/or microglia [51, 62–66]. More specific to HD, however, SVZ neurogenesis may be induced by loss of the neurotransmitter GABA following degeneration of the GABAergic medium spiny projection neurons. The neurotransmitter GABA has been identified as playing a crucial role in regulating several steps of neurogenesis [67]. $GABA_A$ receptors are expressed on neural progenitor cells and their progeny in both juvenile and adult animals, and, in contrast to mature neurons, GABA acts to depolarize immature cells in the brain during the first 2–3 weeks of neuronal development [67]. Depolarization of neural progenitor cells by GABA acting through the $GABA_A$ receptor has been shown to inhibit proliferation by limiting the progression of neural progenitor cells through cell cycle [68, 69]. GABA has also been shown to increase the expression of the neuronal differentiation factor NeuroD in nestin-expressing hippocampal neural progenitor cells [70]. Of interest, the $GABA_A$ receptor subunit $\gamma 2$, which is involved in the desensitization of the receptor complex to GABA, is more enriched in the SVZ than other regions of the brain and has been shown to be significantly increased in the HD brain [38].

In addition, neuropeptide Y (NPY) may also play a role in regulating SVZ progenitor cell proliferation and neurogenesis in HD. Medium-sized aspiny neurons expressing NPY are relatively spared in HD [35], and in the human brain, the SVZ is enriched in NPY-positive cells [38]. In rodents, NPY promotes progenitor proliferation, as evidenced by a 50% reduction in progenitor cells in NPY knockout mice [71]. Furthermore, intracerebral injection of NPY stimulates the proliferation of SVZ progenitor cells in the mouse brain [72]. Using knockout mice and NPY-receptor agonists and antagonists, this proliferative effect has been shown to be mediated by the NPY Y1 receptor subtype, which is highly expressed in the SVZ [72]. NPY-positive cells also express other transmitters, such as nitric oxide synthase, which has been shown in rodents to influence progenitor cell proliferation, migration, and neurite outgrowth [73, 74]. Therefore, the loss of GABAergic medium spiny projection neurons and/or modulation of $GABA_A$ receptor subunit composition in the SVZ, combined with maintenance of NPY expression, may result in the upregulation of SVZ progenitor cell proliferation and neurogenesis observed after QA lesioning and in the HD human brain.

6 Enhancing Neurogenesis in Huntington Disease

Confirmation that neurogenesis occurs in the adult brain has led to investigations examining the potential for therapeutic manipulation of endogenous progenitor cells for the treatment of neurologic diseases, such as HD. While upregulation of SVZ progenitor cell proliferation and neurogenesis has been demonstrated in HD, the level of SVZ neurogenesis observed is insufficient to counteract the progressive cell loss occurring in the HD adult brain. Consequently, for endogenous neural progenitor cells to be useful therapeutically, methods need to be developed to augment neurogenesis and direct the migration of progenitor cells to specific areas of neuronal cell loss. This may be achieved by the targeting of endogenous progenitor cells for directed mobilization and differentiation using mitogenic or trophic growth factors as well as chemotrophic factors that affect cell proliferation, migration, and differentiation. In addition, a number of current drug therapies have been demonstrated to regulate various aspects of neurogenesis, suggesting that they may provide a mechanism of therapeutic manipulation [75, 76].

Previous studies have suggested that HD pathogenesis may be mediated in part by a loss of brain-derived neurotrophic factor (BDNF) [77–79]. In addition, BDNF appears to play a major role in regulating the survival and fate of adult neural progenitor cells. In vitro, BDNF has been shown to promote both neuronal differentiation and the survival of newly generated daughter cells [80, 81]. In vivo, BDNF delivery to SVZ-derived adult neural progenitor cells increased neuronal recruitment to the olfactory bulb [82] and resulted in ectopic addition of newly generated neurons, expressing markers of GABAergic medium spiny striatal neurons, to the striatum of the normal adult rat brain [83, 84]. These observations were extended into the QA lesion HD model in a study by Henry et al. [85] in which the effect of

BDNF on basal and QA-induced SVZ neurogenesis was compared in relation to progenitor cell distribution and levels of neuronal differentiation and survival. BDNF was overexpressed in the SVZ via recombinant adeno-associated virus ($AAV_{1/2}$) gene delivery, and newly generated cells were identified using BrdU labeling. In the normal brain, BDNF overexpression significantly increased BrdU-positive cell numbers in the RMS, indicating enhanced progenitor cell migration. Following QA lesioning, a reduction in BrdU immunoreactivity was observed in the SVZ, which was restored to basal levels following overexpression of BDNF. Most significantly, BDNF enhanced the recruitment of progenitor cells to the QA-lesioned striatum and promoted neuronal differentiation in both the normal and the QA-lesioned striatum. This suggests that BDNF augments the recruitment, neuronal differentiation, and survival of progenitor cells in both neurogenic and nonneurogenic regions of the normal or QA-lesioned brain [85].

In a similar study, Cho and colleagues [86] examined whether the combined overexpression of BDNF and Noggin could recruit new striatal neurons in the R6/2 HD transgenic mouse model. Injection of adenoviral BDNF and adenoviral Noggin (AdBDNF/AdNoggin) into R6/2 mice resulted in the recruitment of BrdU/betaIII-tubulin–positive neurons in the striatum, which developed as DARPP-32–positive and GABAergic medium spiny neurons that expressed either enkephalin or substance P and extended fibers to the globus pallidus. Only R6/2 mice treated with AdBDNF/AdNoggin exhibited Dcx-positive neuroblasts in the striatum. Furthermore, AdBDNF/AdNoggin–treated R6/2 mice exhibited sustained rotarod performance and open-field activity and survived longer compared to AdNull-treated or untreated R6/2 mice. Intraventicular infusion of the mitotic inhibitor Ara-C completely blocked the performance and survival effects of AdBDNF/AdNoggin, suggesting that the benefits of BDNF and Noggin were derived from neuronal regeneration.

The growth factor FGF2 is an essential mitogen for multipotent neural progenitor maintenance and proliferation in vitro [87–90]. In vivo delivery of FGF2 into the adult rodent results in an increase in proliferating cells in the hippocampus, SVZ, and striatum and a subsequent increase in the number of neurons migrating from the SVZ to the olfactory bulb [91–95]. Jin and colleagues [96] therefore examined the neurogenic effect of subcutaneous fibroblast growth factor-2 (FGF-2) delivery to HD transgenic R6/2 mice from 8 weeks of age until death. FGF-2 increased the number of proliferating cells in the SVZ by approximately 30% in wild-type mice and by approximately 150% in R6/2 mice. FGF-2 also induced the recruitment of new neurons into the striatum and cerebral cortex of R6/2 mice. In the striatum, these new neurons exhibited the phenotypic characteristics of DARPP-32–positive medium spiny striatal neurons. In addition to its neurogenic effects, FGF-2 was also neuroprotective, reduced polyglutamate aggregate formation, improved motor performance, and extended the lifespan of R6/2 mice. These results suggest that FGF-2 may provide both neuroprotective and regenerative effects for the treatment of HD.

Several studies have also investigated the effect of antidepressant agents on hippocampal neurogenesis in animal models of HD. In addition to progressive

motor and cognitive symptoms, patients affected with HD often display psychiatric symptoms, including depression. Given that transgenic HD mice have decreased hippocampal cell proliferation and that a deficit in neurogenesis has been postulated as an underlying cause of depression [97–99], decreased hippocampal neurogenesis may contribute to the depressive symptoms of cognitive decline in HD. Postnatal neurogenesis has been shown to be stimulated by a number of antidepressant interventions, in particular, selective serotonin reuptake inhibitors (SSRIs) [100, 101]. In order to examine the effect of the SSRI fluoxetine on hippocampal neurogenesis and cognitive and psychiatric function in HD, Grote et al. [102] treated HD transgenic R6/1 mice with fluoxetine from 10 to 20 weeks of age. They observed that treatment of R6/1 mice with fluoxetine increased cognitive function and reversed a depressive phenotype compared to untreated mice. In addition, fluoxetine treatment rescued the deficit in hippocampal neurogenesis and volume loss in the dentate gyrus observed in untreated R6/1 mice. Specifically, fluoxetine treatment resulted in a significant increase in the number of new BrdU/NeuN–labeled neurons in the hippocampus but had no effect on cell proliferation in the dentate gyrus, suggesting that fluoxetine promotes neuronal differentiation and/or enhances the survival of new neurons rather than stimulating cell proliferation. Similarly, Peng et al. [103] demonstrated that treatment of HD transgenic R6/2 mice with the SSRI sertraline daily from 6 weeks of age prolonged survival, improved motor performance, and reduced striatal atrophy compared to untreated R6/2 mice. In contrast to the results by Grote et al. [102], sertraline was shown to increase cell proliferation in the dentate gyrus of the hippocampus as well as promote cell survival in R6/2 mice. However, sertraline had no effect on the percentage of newly generated hippocampal neurons. Corresponding to these observations, sertraline was also observed to attenuate the deficit in BDNF levels seen in untreated R6/2 HD mice. SSRIs such as fluoxetine and sertraline have been shown to stimulate the cAMP-responsive element binding protein (CREB) and increase the production of BNDF [104–113]. As both BDNF and CREB play a role in regulating adult neurogenesis [105, 114, 115], it can be proposed that antidepressant agents such as the SSRIs may provide a mechanism to augment neurogenesis in the HD brain through the enhancement of BDNF or CREB.

7 Concluding Remarks

Research indicates that neurogenesis is altered in the adult HD brain. While not examined yet in the human HD brain, studies using a range of HD transgenic mouse models have demonstrated a reduction in hippocampal neurogenesis, which may be associated with the cognitive and psychiatric symptoms experience by HD patients. Furthermore, in both the human HD brain and the QA lesion model, SVZ cell proliferation and striatal neurogenesis are upregulated, indicating that the HD brain has the potential to undergo regeneration in response to neurodegeneration. However, in conflict with the observation of upregulated SVZ cell proliferation in the HD

human brain, the majority of studies undertaken in HD transgenic mouse models do not report a change in SVZ proliferation from basal levels. While this disparity between the HD human brain and HD transgenic models is difficult to reconcile, it may represent the requirement for a sufficient level of cell loss to occur in the caudate putamen/striatum in order for SVZ-derived neurogenesis to be stimulated. Therefore, while HD transgenic models provide the best construct validity, they lack sufficient cell loss to stimulate and therefore investigate the mechanism of SVZ-derived neurogenesis in HD. Further development of HD transgenic models exhibiting more extensive neurodegeneration may help to reconcile the differences currently observed.

The possibility to "harness" the regenerative potential of the adult brain for the treatment of neurologic disorders such as HD is very exciting. However, the amount of neuronal cell replacement from endogenous progenitor cells in the diseased brain without additional manipulation is minimal, and strategies need to be developed to enhance this process in order to make it functionally relevant. In order to achieve this, we must identify key mechanisms by which adult neurogenesis is regulated in both the normal and the diseased human brain. As endogenous cell death cues are already present in the HD brain, and with an increasing knowledge of the mechanisms by which growth factors, neurotrophic factors, and neurotransmitters regulate neurogenesis, mechanisms could be developed by which to "encourage" new cells to replace dysfunctional or degenerating neurons in the early symptomatic stages of the disease process, thereby resulting in a novel therapeutic strategy for the treatment of HD.

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Migration of Transplanted Neural Stem Cells in Experimental Models of Neurodegenerative Diseases

Nathaniel W. Hartman, Laura B. Grabel, and Janice R. Naegele

Abstract Significant progress has been made toward development of stem cell–based therapies to treat neurodegenerative diseases. Transplanted cell populations can incorporate and, in some cases, restore function in rodent models. Therapeutic efficacy is dependent upon the ability to both direct the migration of endogenous or transplanted progenitors to locations of cellular degeneration and promote the maturation of desired cell types. A more complete understanding of the molecular and cellular guidance cues for neuronal and glial migration in the embryonic and adult brain is necessary for developing effective cell replacement therapies that direct newly introduced stem cells to the specific brain structures devastated by degeneration. When compared with currently utilized treatments, the ability to physically replace previously degenerated neurons within neural circuits is more likely to provide functional recovery. Therefore, stem cell therapies targeted for the treatment of adult brain injuries must invoke the mechanisms that govern normal migration of neural progenitors during embryogenesis and adult neurogenesis. In addition, these new cells must overcome the barriers to migration that develop after traumatic injury to the adult brain and spinal cord. This chapter reviews current knowledge regarding neuronal and glial migration in development and adulthood, as well as the factors that promote or limit cell migration in degenerative disorders, including demyelinating diseases, stroke, and epilepsy.

Keywords Transplantation • Migration • Neurodegenerative diseases • Cell-based therapies

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1 Introduction

In the last few years, there has been a tremendous surge of interest in neural stem cell–based therapies for treating neurologic disorders. The cells for brain repair may be derived from endogenous neural stem cell (NSC) populations or exogenous sources, such as embryonic stem (ES) cells. For these therapies to be effective it is imperative to be able to recruit transplanted neural progenitors to the damaged sites. Directing the migration of either endogenous or transplanted progenitors to the locations of degeneration may be necessary.

More extensive knowledge of the mechanisms of neuronal and glial migration in the developing and adult brain will facilitate the development of cell replacement therapies that target new cells to precisely those areas devastated by degeneration. An ability to direct the migration of NSCs to specific sites within neural circuits will facilitate incorporation of these cells and functional recovery. Therefore, stem cell therapies for treating adult brain injuries will need to invoke the mechanisms that guide normal migration of neural progenitors during embryogenesis and adult neurogenesis and overcome the barriers to migration that form after traumatic injury to the adult brain and spinal cord.

2 Modes and Mechanics of Migration

In the developing brain, newly formed neurons exit the germinal zones and disperse to their destinations by migrating radially along a radial glia scaffold or tangentially utilizing cell–cell contacts and adhesive guidance cues. The directionality and specific targeting of migrating neurons are governed at multiple levels, including chemoattractant and chemorepellent guidance molecules in the environment of the migrating neuron and specific interactions with other cells and the extracellular matrix [1]. As discussed later, many of the same molecules controlling axonal guidance turn out to be used for guiding neuronal migration [2].

Cellular motility mechanisms, including those associated with neuronal movement, have been well established. Upon binding to cell surface receptors, a series of downstream signaling events lead first to the spatially appropriate rearrangement of the cytoskeleton and distribution of intercellular and cell–extracellular matrix adhesions [3]. The front or leading edge of the cell, rich in migratory structures such as lamellipodia and the Arp2/3 complex, mediates actin polymerization, which promotes extension of the membrane in the direction of migration [4]. Just behind the leading edge, more stable focal adhesions and complexes form and provide contact with the underlying extracellular matrix. An increased turnover rate of focal adhesions and complexes is associated with faster migration. Downstream events include phosphorylation and activation of myosin, which, when combined with actin stress fibers, provide the contractile force to coordinate forward movement. In the nervous system, as elsewhere, cells have been observed migrating individually and in groups.

For each specific type of nervous system cell, the direction and distance of migration are determined by the relative strength of attractant and repellent cues, the expression of cell surface receptors for these molecules, and extracellular matrix components. The ability to adapt to a changing environment is also thought to critically depend upon transcriptional regulation of cell surface guidance receptors in a precise spatial and temporal manner, although our understanding of how this occurs is still very incomplete [2].

3 Migration in the Forebrain

3.1 Embryogenesis

Cell migration is key to establishing the intricate and interconnected structures of the mammalian central nervous system (CNS) (Fig. 1). At early stages of neural tube formation in the developing brain, a network of radial glia links the two surfaces of the neuroepithelium (NE)—the ventricular zone (VZ) lining the cerebral

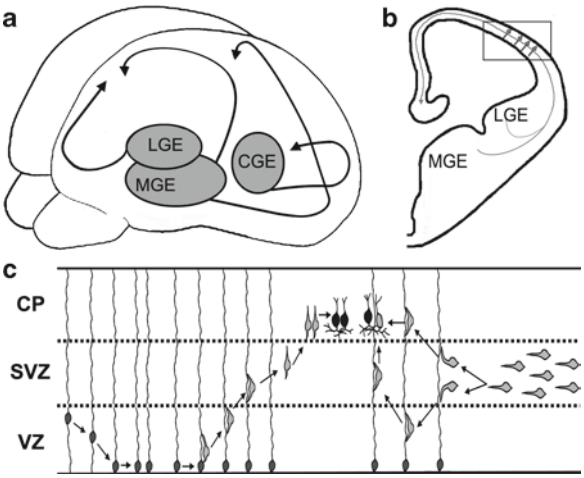

Fig. 1 Migration patterns in the embryonic forebrain. **a:** Interneurons arising from the subpallium, including the medial (MGE), lateral (LGE), and caudal (CGE) ganglionic eminences, follow tangential routes as they migrate into the pallium of the developing brain. **b:** As interneurons reach the developing cortex, they cross paths with radially migrating neuroblasts in the developing cortex (*box*). **c:** Radial glia in the developing cortex divide in the ventricular zone (VZ), where they can give rise to two daughter radial glial cells or a migrating neuroblast, which migrates into the subventricular zone (SVZ) and differentiates into a cortical neuron in the cortical plate (CP). Tangentially migrating interneurons also use the radial glial scaffold to migrate out from the SVZ to mature and integrate into the cortex. Panels A and B are modified from ref. 128

ventricles, and the pia, formed by the innermost layer of the meninges. This physical scaffold guides young neuroblasts in their radial migrations to their final destinations [5]. In the forebrain, the radial glial cells are also NSCs that undergo mitosis to produce new neurons and glial cells [6]. In the initial stages of CNS formation, the NE expands and radial glial cells arise in the VZ. Each radial glial cell has a soma in the VZ and extends a process that reaches the pial surface [7]. Studies suggest that Cajal–Retzius (CR) cells, a transient population that quickly migrates to the pial surface, have a prominent influence in the laminar organization of the cortex by producing guidance cues for radially migrating neuroblasts [8, 12]. Radial glia cells undergo symmetric divisions to give rise to additional radial glia cells during stages of VZ expansion. Subsequently, they commence asymmetric division and produce one daughter cell that migrates toward the pial surface and one radial glial/stem cell that re-enters the cell cycle. The projection neurons that form layers two to six in the cortex assemble in an inside–outside pattern, such that the projection neurons that become the deeper cortical layers are born earliest and migrate the shortest distance, while those that form the more superficial layers are born last and must migrate past the early-born cells to reach their ultimate laminar positions [9, 10].

Once the deep layers of the cerebral cortex have been generated, a secondary germinal matrix called the subventricular zone (SVZ) is formed. Neural progenitors from the VZ migrate a short distance into the SVZ, detach from the radial glia, and subsequently undergo several rounds of symmetric or asymmetric division to produce projection neurons destined for the superficial cortical layers or glial cells. As described later, the SVZ is maintained beyond fetal life, existing as a stem cell niche known to give rise to olfactory bulb interneurons and glia in the adult forebrain. At late embryonic stages in the rodent, a population of radial glial cells migrates away from the SVZ of the lateral ventricles into the developing hippocampus, initially forming a tertiary germinal matrix in the dentate hilus, which subsequently retracts to form the subgranular zone (SGZ) [11]. While the cortical SVZ contains pluripotent NSCs, the radial glial cells in the SGZ are more limited multipotent progenitors that provide new dentate granule neurons and oligodendrocytes for the dentate gyrus of the hippocampus [13, 14].

Migration of the projection neurons of the cerebral cortex from the NE is thought to be controlled by the expression of the basic helix-loop-helix transcription factors neurogenin1 (Ngn1) and neurogenin2 (Ngn2), which can simultaneously alter the expression of several downstream genes directly linked to migration [15]. Studies in the migrating cortical projection neurons of Ngn2 mutants have shown that Ngn1/2 can upregulate the expression of doublecortin (DCX) and p35 while downregulating the expression of RhoA, a GTPase that alters motility of the cytoskeleton [15]. Microtubule-associated proteins, such as DCX, as well as connexins are associated with the proper migration of both cortical projection neurons and interneurons [16]. To ensure migration to the correct laminar positions, cortical neurons transcriptionally control the expression of Brn1 and Brn2, the class III POU-domain transcription factors. These transcription factors, in turn, regulate the

expression of reelin, a cell surface–associated glycoprotein, its intracellular adaptor protein Dab1, and cyclin-dependent kinase 5 (Cdk5) [18].

In contrast to the projection neurons of the developing cerebral cortex and hippocampus, the forebrain GABAergic (GABA, γ-aminobutyric acid) interneuron populations originate in the ventral forebrain, or subpallium, in a series of transient bulges known as ganglionic eminences (GEs), which appear along the ventral walls of the telencephalic vesicles. The GE is the source not only of GABAergic interneurons, but also oligodendrocytes, which form axonal myelin sheaths necessary for formation and maintenance of the corpus callosum and long-range fiber tracts. The cells originating in the GE do not migrate along the radial glia, but use tangential migration followed by inward or outward radial migration patterns.

The GE-derived progenitors follow three migratory routes from the ventral telencephalon to reach the striatum and the dorsal telencephalon [19, 20]. The first of these routes involves deep movement to reach the developing striatum. These migrating cells form tight clusters, or chains, of migrating neuroblasts, similar to the chain migration observed in the rostral migratory stream (RMS). A second superficial migratory route is formed by interneurons traveling in the cortical marginal zone beneath the meninges [17, 21, 22]. Upon arriving in the cerebral cortex, interneurons begin to invade the cortical plate and migrate to their proper laminar positions [23]. Immature GABAergic interneuron progenitors destined for the hippocampus also originate in the GE, migrating tangentially toward the medial hem of the cerebral cortex, which forms the dentate NE [19]. These progenitors give rise to all of the interneurons in the adult hippocampus.

Considering the striking differences in their respective origins and migratory routes to the telencephalon, it is not surprising that the molecular mechanisms controlling interneuron migration also differ from those that control the migration of cortical projection neurons. The medial ganglionic eminence (MGE) gives rise to interneurons that populate the striatum as well as the cerebral cortex, and recent studies showed that the expression of receptors for semarphorin-3A and semaphorin-3F, known as neuropilin-1 and neuropilin-2, dictate the different routes taken by these two interneuron populations. The interneurons migrating past the striatum into the cerebral cortex express these repellent molecules, while those that invade the striatum do not [17]. In addition, the homeodomain transcription factor Nkx2-1 influences the ability of interneurons to respond to semaphorins. Expressed early on during specification of MGE-derived interneurons, Nkx2-1 is downregulated in migrating cortical interneurons, but expression remains high in MGE-derived striatal interneurons [2]. Induction of Nkx2-1 expression in MGE-derived interneurons prevented their migration into the cortex [24]. These studies showed that Nkx2-1 expression controls the sorting of striatal and cortical interneurons during migration by negatively regulating the expression of neuropilins. In addition, Dlx1 and Dlx2 homeodomain transcription factors are two additional regulators of neuropilin-2, although the exact repression mechanisms remain unclear [25], and Dlx1/2-deficient mice show striking deficits in interneuron migration [21, 26, 27].

3.2 *Adult Neurogenesis*

Initially controversial, neurogenesis in the adult mammalian brain is an ongoing process that has been well characterized in several brain regions, including the dentate gyrus of the hippocampus and the olfactory bulbs (Fig. 2) [28, 29]. The best-described NSCs present in the adult brain are restricted to two tightly regulated niches in the SVZ of the lateral ventricles and the SGZ in the dentate gyrus just below the granule cell layer (GCL) [30, 31]. These neurogenic zones are closely associated with vasculature, an important source of growth factors known to regulate neurogenesis in both the adult and the embryonic brain [32–34].

In addition to producing forebrain GABAergic interneurons, progenitors from the lateral ganglionic eminence migrate into the anterior SVZ, where they continue to produce olfactory interneuron precursors in the postnatal brain [20]. These neural precursors migrate rostrally from the SVZ to the olfactory bulb by chain migration within the RMS. Ensheathed in astrocytes, the RMS restricts the migration of SVZ progenitors to a narrow route. However, in vitro data suggest that some chain migration of SVZ progenitors may still occur without the ensheathing astrocytes [35, 36]. After reaching the olfactory bulb, SVZ progenitors differentiate into several interneuron subtypes, including the granule cells in GCL and periglomerular cells in the glomerular layer [37].

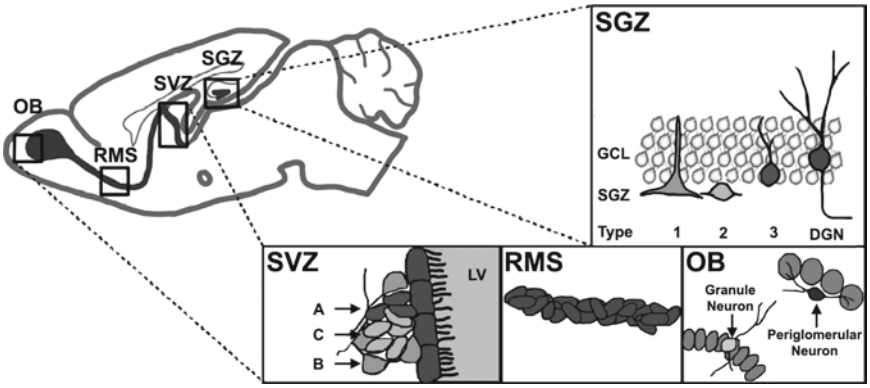

Fig. 2 Migration patterns of neural progenitors in the adult brain. In the mammalian brain, the two neural stem cell niches are the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus. Type 1 cells in the SGZ are astrocyte-like neural stem cells that give rise to type 2 cells, a faster-proliferating intermediate stem cell. As type 2 cells divide, they give rise to a migrating neuroblast (type 3) that migrates into the granule cell layer (GCL) and matures into a dentate granule neuron (DGN). B cells, the neural stem cells of the SVZ, divide to give rise to a transient-amplifying C cell that then differentiates into a migrating neuroblast, the A cell. These neuroblasts enter the rostral migratory stream (RMS), where they exhibit chain migration toward their destination in the olfactory bulb (OB). Once the neuroblasts reach the OB, they differentiate into two types of inhibitory interneurons, either granule or periglomerular neurons. Modified from refs. 129 and 130

The stem cell niche of the SGZ is comprised of three cell types. Type 1 cells comprise an early precursor population and have the morphology of radial glia, extending processes through GCL [38, 39]. Type 1 cells divide slowly, expressing the astrocytic marker GFAP, the intermediate filament nestin, BLBP, and Sox2 but not the postmitotic astrocyte marker S100β [40]. Type 1 cells can give rise to type 2 cells, a faster-proliferating intermediate astrocyte-like stem cell in the SGZ, which have short processes and do not send radial extensions through the GCL. At this stage of hippocampal neurogenesis, GABA supplies an excitatory input, promoting the differentiation of type 2 cells [15]. The transitional stage from NSC to postmitotic neuron occurs in type 3 cells. As these cells migrate into the GCL, they express the migrating neuroblast markers DCX and PSA-NCAM, as well as markers of the neuronal lineage, but lack astrocytic markers [41, 42]. Synaptic input onto new cells is initially only GABAergic and excitatory. However, beginning 2 weeks after cell division, excitatory glutamatergic synapses are formed on these new cells, and they extend dendritic spines. At about this stage, the GABAergic input becomes inhibitory due to changes in chloride ion conductance [43]. Finally, as the apical arborizations mature, newly born granule neurons functionally incorporate into the hippocampal circuit, extending their mossy fiber axons through the hilus to targets in CA3 [44, 45]. The entire process of adult neurogenesis in the SGZ, from the birth of a type 2 cell to migration into the GCL and finally to full incorporation into the hippocampus, occurs over the course of approximately 1 month.

4 Migration of Transplanted Cells in Models of Neurodegenerative Diseases

The extent and direction of migration of transplanted cells depend upon the conditions of the host. Relative to other types of brain insults, damage resulting from strokes or demyelinating diseases is typically associated with a strong inflammatory response and microglial activation. To be successful in these conditions, the transplanted cells must migrate distances of several millimeters. In temporal lobe epilepsy stemming from traumatic brain injury or prolonged febrile convulsions, neurodegeneration may be more circumscribed and limited to the entorhinal cortex and hippocampus. To replace specific hippocampal cell types damaged by seizures, it would be advantageous to direct migration of grafted cells to specific sites. Disruption of the blood–brain barrier and increased inflammation is thought to further impair survival and integration of transplanted cells, but microglia and reactive astrocytes have also been shown to release cytokines that act as chemoattractants. To direct stem cell migration into the injured regions of the brain, a better understanding is needed of the local host brain environment in different neurologic disorders. Studies suggest that there are substantial differences in the expression of different molecules influencing migration, depending upon the location and extent of neurologic damage (Table 1).

Table 1 Selected factors influencing the migration of neural stem cells

Migratory cue	Notes	Ref.
Growth factors		
BDNF	Stimulates migration of ES-derived glial progenitors	61
VEGF	Stimulates migration of SVZ cultured NSCs	111
PDGF	Stimulates migration of ES-derived glial progenitors	61
Chemorepellants		
Semaphorins	Repels endogenous migrating OPCs and interneurons	115
Netrins	Repels endogenous migrating OPCs	17, 68
Reelin	Repels endogenous migrating neuroblasts in the developing cortex and hippocampus	69
Chemokines		
CXCL 12	Guides transplanted hESNPs to infarct in models of ischemia	81
MCP-1	Simulates migration of adult-derived NSCs	88
Cell adhesion/motility		
PSA-NCAM	Cell migration aberrations in loss-of-function experiments	42
DCX	Linked to proper migration of cortical neuroblasts and interneurons	15
Connexins	Loss of cortical lamination in loss-of-function experiments	16

Many factors guide the migration of neural stem cells (NSCs) both in the central nervous system and in vitro, including brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF). In addition, chemorepellants, chemokines such as stromal-derived factor 1a (CXCL12), and monocyte chemotactic protein-1 (MCP-1) are thought to be important influences on cell migration. Additional molecules that influence cell adhesion and motility include polysialated neural cell adhesion molecule (PSA-NCAM), doublecortin (DCX), and connexins.

4.1 Demyelinating Diseases

Demyelinating diseases can either be acute, as in the case of spinal cord injury (SCI), or chronic, as in the case of multiple sclerosis (MS). Whether the cause of demyelination is viral, genetic, or trauma related, inflammation and astroglial scarring may impede migration. SCI occurs in two phases—primary and secondary. The primary phase is characterized by the initial injury, which disrupts axons and blood vessels, resulting in a secondary phase of edema, ischemia, inflammation, astroglial scarring, and cell death (both from apoptosis and necrosis). Despite evidence that central neurons are capable of extensive regeneration, growing axons are blocked from reconnecting distal to the lesion by astroglial scarring and the production of molecules that prevent axonal growth. Provided these hurdles can be overcome, cell replacement therapies may offer a novel treatment approach.

Initial studies transplanted either terminally differentiated CNS neurons or glial cells into rodent models of SCI to either replace the destroyed tissue or initiate

endogenous axonal elongation, respectively [46, 47]. While the capacity for regeneration and axonal elongation was limited when differentiated cells were transplanted, the axons penetrated damaged tissues and formed new synaptic connections [48]. Obtaining large numbers of neural or glial progenitor cells from ES cell lines is now possible [49]. Graft incorporation and more extensive repair of the damage could theoretically improve because progenitor cells show a greater capacity for cell migration in in vitro studies performed in brain slice preparations [50].

After they are transplanted, NSCs derived from fetal brains do not integrate extensively into the host tissue but do home to the area of injury. However, fetus-derived NSCs can migrate up to 10 mm along white matter tracts in the brains of host animals [51]. Extensive migration was also observed when oligodendrocyte progenitor cells (OPCs) derived from hES cells were transplanted 1 week after injury to the spinal cord [52]. The length of time between injury and stem cell transplantation is critical; when OPCs were transplanted 10 months after the SCI, their ability to migrate was significantly reduced. As endogenous oligodendrocyte migration is retarded in a mouse model of autoimmune encephalomyelitis, astroglial scarring in the secondary phase could be preventing the migration of the transplanted cells, as well as their integration into the host tissue [53]. After the initial damage, the time window for transplantation to treat SCI may be very short, at least before glial scars form during the secondary phase.

Cell-based therapies are promising approaches for treating global demyelinating diseases such as MS. A hallmark feature of MS is widespread demyelination; stem cell therapy for this class of disorders aims to supply populations of oligodendrocytes that migrate extensively and remyelinate fiber tracts in the CNS. Animal models of global demyelinating diseases fall into three categories: genetic, pharmacologically induced, or the infection of myelin-reactive T lymphocytes (for further review, see ref. 54). Inducing MS by experimental autoimmune encephalomyelitis (EAE) leads to inflammation in the CNS and widespread demyelination. In addition, a genetic mouse model of MS has been developed that displays myelin deficiency due to loss of myelin basic protein, resulting in seizures and reduced lifespan [55].

By grafting human ES cell–derived neural progenitors (hESNPs) in a pharmacologically induced model of EAE, researchers were able to achieve a neuroprotective effect, most likely due to an immunosuppressive mechanism, as remyelination from the grafted cells was very sparse [56]. When OPCs derived from hES cells were transplanted into another genetic model of MS, one occurring due to a spontaneous mutation in *shiverer* mice, remyelination of the CNS was observed, with functional recovery [57]. Previous work showed that, although OPCs can achieve migration rates of 1 mm/day, gray matter can hinder their dispersion [58]. Therefore, by transplanting OPCs directly into white matter tracts in the forebrain and spinal cord, the researchers were able to achieve extensive migration of grafted cells (Table 2).

Demyelination, whether in SCI or MS, is associated with inflammation. The invasion of T cells to the lesion site may influence the migration of transplanted OPCs. What is known about the factors that influence OPC graft migration is inferred from developmental studies of endogenous OPCs and in vitro migration assays. Two important chemoattractants are platelet-derived growth factor (PDGF) and fibroblast

Table 2 Migration of transplanted cells in rodent models of demyelinating diseases, stroke, and epilepsy can be influenced by the source of the cells and the location of the transplant

Disease model		Cell source	Injection site	Notes	Ref.
Demyelinating diseases	SCI	Fetal mNSC	Spinal cord	Cells migrate to penumbra of damage and up to 10 mm throughout CNS	51
	SCI	hOPC	Spinal cord	Cells migrate toward lesion and restore some mobility	52
	MS, induced	hESNP	ICV	Cells migrate toward lesion with little integration or remyelination	56
	MS, genetic	hOPC	Forebrain and spinal cord	Cells migrate throughout CNS and display extensive remyelination	57
Stroke	MCAO	Fetal hNSC	Cortex	Cells migrate towards infarct, up to 1.2 mm in distance	76
	MCAO	Adult SVZ mNSC	Cortex	Enriched environment aids in transplanted cell survival and migration	78
	MCAO	mESNP	Ipsolateral: cortex contralateral: striatum	Cells migrate toward infarct; however, cells transplanted to contralateral striatum do not reach damaged tissue	80
	MCAO	hESNP	Striatum	Cells migrate from injection site in the infarct core up to 0.2 mm	77
	MCAO	hESNP	Contralateral cortex	Cells migrate toward infarct displaying chain migration	81
Epilepsy	KA, ICV	Fetal mNSC	Hippocampus	Cells differentiate into astrocytes and neurons but display no migration	123
	Pilocarpine	mESNP	Hippocampus	Cells do not migrate from injection site but do extend long, ramified projections	126
	KA, systemic	mESNP	Hippocampus	Cells migrate anterior to posterior in the SGZ of the upper blade	124
	KA, systemic	hESNP	Hippocampus	Cells migrate anterior to posterior in the SGZ of the upper blade	125
	Pilocarpine	Fetal hNSC	Intravenous	Cells migrate to the hippocampus, amygdala, subiculum, and piriform complex	127

CNS, central nervous system; ESNP, embryonic stem cell–derived neural progenitor; h, human; ICV, intracerebral ventricular injection; KA, kainic acid; m, mouse; MCAO, middle cerebral arterial occlusion; MS, multiple sclerosis; NSC, neural stem cell; OPC oligodendrocyte progenitor cell; SCI, spinal cord injury; SVZ subventricular zone.

growth factor 2 (FGF2). During development, PDGF is expressed by neurons in the developing forebrain, while OPCs express the PDGFR-α ligand [59]. In vitro migration studies show that PDGF induces the migration of oligodendrocytes without affecting proliferation [60,61]. In addition, mice that are deficient for PDGF-α exhibit tremors and reduced myelination resulting from dysregulated migration rather than a deficit in oligodendrocyte proliferation or differentiation [62]. It remains unclear how PDGF regulates migration, but it is known that the downstream signals from the PDGF-α receptor signal through Fyn tyrosine kinase and Cdk5 [63]. Both PDGF and FGF2 are necessary for the efficient differentiation of ES cells into OPCs [64,65]. FGF2 is a potent mitogen for OPCs, maintaining them in an immature state that is more conducive to migration [66]. In light of the extensive migration reported in these studies, it is no surprise that these molecules are expressed at sites of demyelination [67]. While the evidence suggests that increases in PDGF and FGF2 not only control proliferation but also regulate migration of transplanted OPCs, *in vivo* support for this hypothesis has not yet been obtained.

In addition to PDGF/FGF2 signaling, semaphorin 3A (sema 3A) and netrin-1 are involved in OPC migration. Instead of acting as chemoattractants, these molecules are thought to provide the stop signal for migrating OPCs. In the developing retina, the intensities of sem3A and netrin-1 increase in the caudal direction and can halt OPC migration [68]. Netrin-1 also increases the dispersal of OPCs from the ventral floor of the spinal cord during embryogenesis [69]. In early stages of demyelination, netrin-1 may be upregulated in areas of inflammation [70]. Netrin-1 could then serve as both a stop signal and a maturation signal for OPCs transplanted to the site of damage [71]. As inflammation in demyelinating diseases increases, netrin-1 may be upregulated, leading to leukocyte recruitment and the inhibition of axonal elongation [72]. Neither netrin-1 nor sema 3A mRNA is observed in the glial scar that forms, but rather, the proteins are located at the edge, decreasing the ability of endogenous OPCs and neuronal axons to navigate the damaged area [73]. These data then suggest that in the case of SCI, the most efficient route for cell-based therapies is a short window directly following injury when the expression of chemoattractant molecules is high and the expression of chemorepellant molecules is low and does not prohibit migration into the sites of damage.

4.2 Stroke

Ischemic stroke results in extensive damage to the cortex and striatum, as well as a potentially fatal breakdown of the blood–brain barrier. Current therapies are effective only when applied within the first few hours following the stroke, necessitating the development of novel strategies to rescue infarct tissue long after the initial insult [74]. Without treatment, patients may suffer from severe neurologic problems, including reduced motor coordination and cognitive ability. Researchers have modeled ischemic stroke in rodents through the use of arterial occlusion, which induces forebrain damage that is associated with an infarct core and a penumbra.

Ischemic stroke can be broken down into three basic phases: the acute, subacute, and delayed phases. During the acute phase, infarct formation occurs within minutes of ischemic onset and is a direct result of oxygen loss and energy failure. The subacute phase occurs during the next 4–6 hours, during which the infarct core expands into the penumbra. Lastly, the delayed phase of injury develops over the course of 1 week, leading to inflammation and subsequent apoptosis, both of which have been shown to greatly expand the area of damage [75]. It is this last phase that is targeted by ESNP therapies with the hope that replacing the cells lost during the extensive neural damage will restore function.

ESNPs can be readily derived in large numbers and retain the potential to proliferate and differentiate into the desired subtypes. In addition, ESNPs have the ability to migrate great distances into damaged tissue, displaying a tremendous potential for a cell-based approach to treating ischemic stroke. Most transplantation studies in models of ischemic stroke focus on delivering cells either directly to the infarct core or near the penumbra (Table 2). Cells are usually injected a week following arterial occlusion, with a post mortem analysis anywhere from 1 week to several months later. In general, transplanted NSCs derived either from adult stem cells, fetal progenitors, or ES cells display a stereotypic migration toward the infarct core [76]. Cells transplanted to the infarct core in the striatum migrate out from the injection bolus and differentiate [77]. These transplanted cells do not migrate extensively throughout the entire area of damage; however, some level of motor function is restored. With the use of expanded mouse adult SVZ NSCs, cells transplanted to the cortex and corpus callosum migrate through the white matter tracts and enter the penumbra of the ischemic damage [78]. Of interest, an enriched environment did not alter transplanted cell survival or migration as has been seen in endogenous neural progenitors in previous studies [79]. Migration extent and honing are even greater in studies utilizing hESNPs. and hNSCs Grafted hESNPs in the contralateral cortex are able to migrate across the corpus callosum toward the infarct, using a form of chain migration [80, 81].

The secondary wave of cell death in ischemic stroke ignites a large neuroinflammatory response. As microglia invades the infarct, reactive astrogliosis is upregulated, causing inflammatory cytokine release and weakening of the blood–brain barrier. Together with microglia, infiltrating leukocytes produce chemokines, such as monocyte chemoattractant protein (MCP-1 or CCL2) and stromal-derived factor-1 (SDF-1 or CXCL12). These cytokines are potent chemoattractants for NSCs. Ischemic insults increase neurogenesis in the adult brain in both the SVZ and the SGZ. Proliferation in the SGZ of the dentate gyrus as well as the parenchyma of the hippocampus increases following stroke. SGZ progenitors exit the neuroproliferative zone and migrate into the adjacent GCL, where they differentiate into granule neurons [82]. Newly born cells arising from the parenchyma migrate into the pyramidal layer, where they differentiate into CA1 neurons and create synaptic inputs to the hippocampal circuit [83]. In addition to the SGZ, migration of endogenous NSCs from the SVZ extends into the striatum, where the NSCs differentiate into mature neurons and astrocytes [84]. CXCL12, in part, regulates this migration, as blockade of its receptor, CXCR4, suppresses progenitor migration from the

SVZ [85]. Chemokines released during inflammation in ischemic stroke may provide the initial cues for endogenous as well as transplanted cell migration toward the infarct core.

MCP-1 mRNA levels are elevated starting 6 hours post arterial occlusion, and expression remains high for several days [86]. Microglia are found at the core of the damage, along with GFAP-positive astrocytes. Both the astrocytes and the Mac-1α–positive microglia in the infarct express MCP-1 [87]. *In vitro*, NSCs migrate robustly toward a source of MCP-1 [88, 89]. When adult SVZ progenitors are transplanted onto hippocampal slice cultures derived from MCP-1–knockout mice, they fail to migrate toward a site of induced inflammation [90]. Moreover, when NSCs that lack the MCP-1 receptor CCR2 transplanted to the ipsolateral cortex, there is a significant reduction in migration to the ischemic striatum [91].

CXCL12, a known chemotactic signal for hematopoietic cells, is an important regulator of migration during development of the early brain. CR cells, a transient neuronal population in the marginal zone, maintain the radial glia scaffold that allows for the proper lamination and migration of neuroblasts to form the cortex. CXCL12 expression in the meninges directs the migration of CR cells, which express CXCR4, to the pial surface [12]. CXCL12 was shown to direct the tangential migration patterns of cortical interneurons from the, GEs, as well as the formation of the DG during development [92, 93]. Neural progenitors of the adult brain, in both the SVZ and the SGZ, express CXCR4, and in vitro studies show that CXCL12 can induce their migration [94]. In addition, as ischemic damage increases, SVZ neural progenitors proliferate and migrate toward the infarct. Blocking the CXCL12/CXCR4 pathway decreases the migratory response [85]. When this molecular pathway is obstructed, hESNPs transplanted to the contralateral cortex in ischemic rodents fail to migrate to the infarct [81]. These data suggest that future cell-based treatments for stroke can use chemokines to direct migration into the affected area. It should be noted that chemokine expression is reduced as astroglial scarring forms in the ischemic brain. If ESNPs use the chemokines expressed in the infarct to direct their migration after transplantation, the window of opportunity for treatment may be less than a month after the initial stroke, much narrower than expected.

4.3 Epilepsy

Many factors, including traumatic brain injury, viral infection, and genetic mutations, can lead to the complex spontaneous seizures that define mesial temporal lobe epilepsy (TLE). While many forms of epilepsy are treatable with antiepileptic medication or diet restrictions, current treatments for TLE often involve the surgical excision of the epileptic focus [95]. Surgical excision may involve removing a broad area of cortex, including structures such as the hippocampal formation, a structure required for encoding declarative memory. If the seizure foci are located

bilaterally in the temporal lobes, removal of both is not possible without complete loss of declarative memory. The debilitating and invasive effects of current treatments have driven the derivation of cell-based therapies, which hold the potential to restore aberrant neural wiring, replace dead cell populations, and reduce the severity and frequency of spontaneous seizures.

In both chemoconvulsant and kindling models of epilepsy that give rise to spontaneous seizures, stereotypic cell death is observed in the hilus of the DG and the pyramidal cell layers [96]. Inhibitory, somatostatin-positive hilar interneurons account for the majority of the dying cells in the hilus [97, 98]. The somatostatin-positive interneurons send their axons from the hilus into the dentate GCL, forming synaptic contacts with dentate granule neurons. The loss of this cell population drives a reduction in the overall inhibition of dentate granule neurons, adding to the hyperexcitability of the hippocampal circuit [98]. In addition to interneuron cell death, dentate granule neurons also undergo mossy fiber sprouting, a form of aberrant axonal collaterals, that projects to the inner third of the dentate GCL instead of the pyramidal cell targets in CA3 [99]. These aberrant connections may further increase dentate granule neuron excitability, thereby facilitating seizure activity in the hippocampus.

Seizures upregulate adult neurogenesis in the SGZ of the DG in models of TLE [100]. While proliferation in the SGZ increases, recent research suggests that these newly born neurons may contribute to the pathology of the disease through erroneous migration and the formation of improper synaptic connections [101, 102]. Newly born dentate granule neurons displaying normal migration activity extend errant basal dendrites through the GCL in addition to sending the normal apical dendrites into the molecular layer. Furthermore, the axonal projections of these GCL neurons tend to branch more frequently, forming connections in the GCL [103]. Migration of the neural progenitor population into the dentate hilus is also observed, and this seizure-induced aberration may be due to increased proliferation and dispersion of the DCX-positive neuroblasts [104]. The cells that migrate from the SGZ into the hilus do not differentiate into hilar interneurons; instead they become ectopic dentate granule neurons that synapse back onto cells in the GCL. These ectopic granule cells receive synaptic input from the GCL, adding excitation into the hippocampal circuit and increasing the probability of a seizure episode [105].

The inflammatory response that is upregulated following seizures may promote the erroneous migration of SGZ NSCs into the hilus. Microglia and reactive astrogliosis is observed throughout the hippocampus during the first week following the administration of either kainite or pilocarpine [106, 107]. Microglial invasion of the hippocampus following seizures coincides with the increased expression of CXCL12 in the DG as well as the pyramidal layers [108]. As CXCL12 is known to influence the migration of endogenous and transplanted neural progenitors following ischemic insult [81, 85], chemokines may also contribute to aberrant migration patterns in the epileptic hippocampus.

In addition to chemoattractants, the postseizure environment of the hippocampus alters the expression of many chemorepellants, molecules involved in cell cycle,

control, and neurotrophic factors, including brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor [109–111]. BDNF overexpression alone is sufficient to induce ectopic migration of neural progenitors into the dentate hilus [112]. In addition to an increase in growth factors, research has shown that viral-mediated knockdown of Cdk5 also leads to aberrant migration [113]. In addition, reeler mice display ectopic granule cells and a highly excitable hippocampus [114]. Reelin, released by CR cells in the molecular layer of the developing DG and subsequently by adult hilar somatostatin-positive interneurons, serves as a stop signal for dentate granule cell migration [115]. As the somatostatin-positive interneurons preferentially die following seizures, reelin expression is lost. The increase of both neurotrophic factors and the downregulation of chemorepulsive cues may contribute to the aberrant migration observed in the postseizure hippocampus.

There has been moderate success in the suppression of seizures following engraftment of fetal cholinergic neurons in a kindling model of epilepsy [116, 117]. In this case, fetal tissue transplanted to the amygdala, piriform cortex, and hippocampus elicited some protection when compared to sham grafts, perhaps by the innervation of GABAergic interneurons. Engraftment of GABA-producing cells into the substantia nigra (SNr) reduced seizure susceptibility in both kindling and chemoconvulsant rodent models [118–120]. In addition, fetal-derived GABAergic interneurons also display the ability to reduce seizure frequency when transplanted into the SNr [121]. Finally, using the chemoconvulsant kainate model, spontaneous seizures were reduced when fetal GABAergic interneurons were transplanted into the rat hippocampus following status epilepticus [122].

While these studies demonstrate the ability of transplanted cells to reduce seizure severity and susceptibility, neither reconstruction of damaged tissue nor long-range migration by transplanted cells has been observed (Table 2). Although differentiation into all three cell types of the CNS occurs, long-range migration is not observed when NSCs derived from fetal hippocampal tissue are transplanted into the lesioned aged hippocampus [123]. Both mESNPs and hESNPs transplanted into the hippocampus of kainate-treated mice migrate through the SGZ of the DG, but not to the damaged CA3 region, whereas engraftment to the fimbria results in oligodendrocytes but not migration into the pyramidal cell layers or the DG [124,125]. Moreover, mESNPs transplanted into the hippocampus of rats following pilocarpine-induced seizures do not migrate extensively from the graft core but do functionally incorporate into the host tissue and display long, ramified extensions [126]. On the other hand, when NSCs derived from human fetal tissue are intravenously injected into pilocarpine-treated rodents, they exhibit migration into the hippocampus, amygdala, and other brain regions, differentiating into many cell types, including GABAergic hilar interneurons [127]. The honing of these grafted cells closely resembles the previously reported pattern of microglial activity and CXCL12 immunolabeling [108]. These data suggest that transplanted cell migration is highly restricted when the cells are delivered directly into the hippocampus but that the cells themselves still retain migratory capability. Until researchers gain further understanding regarding the guidance cues governing the migration and

integration of transplanted cells intended to repopulate damaged areas and attenuate spontaneous seizure activity, cell-based therapies will continue to lie beyond the horizon.

5 Conclusions

We have reviewed the current literature on the molecular and cellular mechanisms guiding neuronal and glial migration, as well as some of the factors known to promote or limit cellular migration in degenerative disorders and epilepsy. There is conclusive evidence that when transplanted into rodent models of neurodegenerative diseases, NSCs retain their ability to migrate. Responding to endogenous cues, transplanted cells can migrate toward areas of damage in models of demyelinating disease, ischemic stroke, and, to a lesser degree, TLE. Cells transplanted soon after an initial traumatic brain injury migrate more extensively in the CNS. This directed migration is in part due to the neuroinflammatory response observed after the initial brain trauma. Neuroinflammatory responses may promote the migration of transplanted cells by upregulating chemokines and growth factors. Alternatively, these responses may impose limitations on the ability of transplanted cells to incorporate into neural circuits due to formation of astroglial scars. By gaining a better understanding of the differences in host brain responses to injury over time and through studies of the transcriptional regulation of neuronal migration, it may be possible in the future to repopulate damaged neural circuits by regulating the ability of transplanted cells to navigate through the adult brain and spinal cord.

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Prospects for Neural Stem Cell Therapy of Alzheimer Disease

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Abstract Alzheimer disease (AD) is an incurable, degenerative, and terminal disease. Stem cellular therapeutics has been considered as offering the potential for possible intervention or cure. There is, however, an underlining complexity of AD in terms of its diversity in clinical presentation together with uncertainty over the molecular events associated with its onset. Neural stem cells cultured in vitro as neurospheres or adherent cultures may offer potential cellular resources. Nevertheless, AD with its heterogeneity in presentation and diffuse pathology remains a challenging cellular therapeutic target.

Keywords Neural stem cells • Alzheimer disease • Neurospheres • β-Amyloid neurodegeneration

1 Introduction

Alzheimer disease (AD) is an incurable, degenerative, and terminal disease first described by Alois Alzheimer in 1906 [1]. Patients with AD can be categorized as either those with early-onset (familial) AD or, more commonly, late-onset (non-familial) AD. Early-onset AD is caused in the majority through mutations in the amyloid precursor protein (APP) and accounts for less than 5% of cases. Late-onset AD is most often diagnosed in people older than 65 years of age, with the earliest symptoms sometimes mistaken as being part of the natural aging process. Typically these early symptoms often include short-term memory loss and clumsiness. For the late-onset forms of the disease there is no definitive cause, but there are a number of

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risk factors, most notably age. The risk of Alzheimer disease doubles above the age of 65 years, and by the age of 85 years there is a 50% risk of having the disease (http://www.alz.org/alzheimers_disease_causes_risk_factors.asp). Other risk factors include having a previous head injury, having a family member with AD, cardiovascular disease, and apolipoprotein (Apo) allele status (see later discussion). AD is typically diagnosed via the use of standardized behavioral assessments and cognitive tests. Diagnosis of AD can be assisted via medical imaging to help exclude other cerebral pathology. Much effort has gone into the development of better in vivo imaging of the abnormal β-amyloid deposits by the use of Pittsburgh compound B positron emission tomography as part of the diagnosis [2]. Confirmation of AD occurs at post-mortem by histologic examination of brain tissue [3].

Although there are many common symptoms, each Alzheimer patient can manifest the condition in many different ways. In general, symptoms proceed from early-stage dementia to advanced dementia and death. Symptoms of advanced disease include irritability, aggression, confusion, mood swings, language breakdown, long-term memory loss, and withdrawal [4]. Prognosis for any individual patient is difficult to assess due to differences in the extent of the disease at diagnosis. However, life expectancy following diagnosis is approximately 7 years [5]. Death is associated with an eventual accumulation of a generalized functional loss and is usually caused by some indirect effect on the weakened body such as pneumonia. Autopsy reveals a characteristic loss of neurons and synapses resulting in degeneration in temporal and parietal lobes as well as parts of the frontal cortex and cingulate gyrus [3].

AD is a great social and economic burden [6]. The main caregiver is often the spouse or a close relative, often suffering considerable self-sacrifice and expense. The direct and indirect costs of caring for Alzheimer patients can be considerable [7, 8], and it has been estimated that the United States spends $100 billion each year on AD-associated costs [6].

2 The Biology of Alzheimer Disease

The progressive cognitive decline seen in AD is characterized by the loss of synapses, the formation of neurofibrillary tangles, and the deposition of neuritic plaques composed of aggregated β-amyloid (Aβ) in the neocortex and the limbic system [9, 10]. Synaptic loss during the course of Alzheimer disease occurs early in the progression of the disease and in two phases. The first involves loss of plasticity, and the second involves aberrant sprouting and neuritic disorganization, which leads to neurodegeneration. This precedes the neuronal loss that is due to plaques and tangle formation.

Apoε4 is currently the only validated risk factor for late-onset (nonfamilial) Alzheimer disease (for review see ref. 11). Apoε is a protein required for the catabolism of triglyceride-rich lipoproteins and is specified by three possible alleles, Apoε2, Apoε3, and Apoε4. Apoε2 is associated with type III hyperlipoproteinemia.

Apoε3 is the neutral form with no associated risk factors for disease (55% of the population is homozygous for ε3). Having two Apoε4 alleles (1%–2% of the population) is associated with 20 times increased risk of developing the late-onset, nonfamilial form of AD. How these two facts are related was unknown until recently, when was postulated that Apoε enhances the proteolytic breakdown of β-amyloid and the ε4 isoform is less efficient at catalyzing the reaction than the other isoforms, potentially leading to increased deposition of plaques [12].

One of the first hypotheses put forward for the causes of Alzheimer disease was the cholinergic hypothesis. This was based on the fact that there is degeneration of the cholinergic neurons in the basal forebrain in patients with AD. These cholinergic neurons provide widespread innervations to the cortex and play a role in various cognitive functions, including memory. The loss of these neurons causes cognitive impairment. This led to the development of drugs that target the cholinergic system, the best-known of these being donepezil (Aricept). This drug inhibits the breakdown of acetylcholine by inhibiting the acetylcholinesterase and thus relies on there being sufficient cholinergic neurons remaining for the drug to act on. In fact there have been disappointing results for donepezil, with only modest improvements in cognition and no improvement in quality of life [13].

More recently research into causes of AD and treatments has focused on Aβ and tau protein. Aβ is a peptide of 39–43 amino acids and is the major constituent of senile plaques that are found in the aged brain and that are a hallmark of the AD brain. Aβ is produced by sequential cleavage of APP, a protein present in all neuronal membranes and whose role is not clear. Cleavage of APP first by β-secretase produces a truncated APP still anchored into the cell membrane. Subsequent cleavage with γ-secretase cleaves within the membrane, releasing the soluble Aβ peptide. γ-Secretase can cleave at Val^{711} or Ile^{713} to produce $A\beta_{1–42}$ or $A\beta_{1–40}$, respectively. Of the two forms, $A\beta_{1–40}$ is the more common, but $A\beta_{1–42}$ is more fibrillogenic and associated with early-onset AD. Some cases of early onset AD are linked to specific mutations around the β- and γ-secretase cleavage sites—for example, the Swedish APP670/671 and London APP717 mutations [14–16]. Generally these mutations increase the amount of Aβ peptides produced or the proportion of the longer $A\beta_{1–42}$, which is more likely to aggregate and is more toxic than the shorter $A\beta_{1–40}$.

The second major hallmark of AD is the intracellular accumulation of microtubule-associated protein (MAP)–associated hyperphosphorylated tau. The "tau hypothesis" suggests that AD is driven by neurofibrillary tangles that arise as a result of either excess phosphorylation or a reduction in dephosphorylation. Tau was first isolated as a protein that copurifies with tubulin and promotes microtubule formation in vitro. Microtubules are key regulators of neuronal morphology via formation of axonal and dendritic processes (for review see ref. 17). They play a crucial role in both maintaining structure and in cellular trafficking, especially of mitochondria, synaptic vesicle proteins, ion channels, and receptors to and from presynaptic and postsynaptic sites. Synapses are particularly vulnerable to impairments in transport, and any blockages in transport can lead to malfunctions in synaptic transmission and ultimately synaptic degeneration—one of the early signs of AD.

Oligomers of tau form into pre-tangles that assemble into insoluble filaments and then into tangles. The phosphorylation state of tau plays a key role in its affinity for both tubulin and for signaling molecules and thus could affect both microtubule dynamics and downstream signaling [18]. Hyperphosphorylated tau isolated from AD brain has a lower microtubule-promoting activity in vitro and sequesters normal tau, MAP1, and MAP2, causing the inhibition of assembly and the promotion of disassembly. This depletion of normal microtubules and presence of abnormal microtubules are likely to impair microtubule-based transport, and this will have major implications for the health of the cell. How Aβ and tau are related in AD is still being debated. Unlike the situation with Aβ, where there are known mutations associated with familial AD, there are no direct genetic links between tau and AD; mutations in tau are associated with frontotemporal dementia but not AD [19–21]. It has been postulated that excess Aβ is the causative agent, and this event is upstream of tangle formation; however, it is not clear how excess Aβ could lead to tau aggregation.

The development of new medicines for Alzheimer disease requires the development of disease-relevant models, both in vitro and in animal models. AD is a disease that develops over decades, and therefore trying to model this in a culture dish over a period of days or weeks or in the much shorter lifespans of small-animal models remains challenging. Initial in vitro models used in AD research were primitive and involved adding high concentrations of Aβ peptides to cultured neurons. Initially, fresh peptides were added, but more recently peptides have been incubated at 37°C to induce aggregation similar to what is seen in vivo. These studies, however, used concentrations of peptides in the high-micromolar range, and in some cases the concentration was potentially high enough to cause cell death by preventing exchange of gases and nutrients rather than having a direct biologic effect. More recently these models have been used to look for subtler changes, for example, the effects of Aβ on synaptic biology [22].

In vivo most research has centered on developing transgenic mouse models; however simpler organisms, such as *Caenorhabditis elegans*, *Drosophila* (for review see ref. 23), and zebrafish have also been used, which have distinct advantages over more complex rodent or primate models. In particular, they have a much shorter development and lifespan than higher vertebrates, making them attractive as experimental model systems. These model organisms possess homologs of genes involved in both the amyloid pathway and tau, and in addition these can be easily deleted and replaced with the human or mutated forms. Zebrafish can have genes deleted or modified by injection of the morpholino antisense oligonucleotides, mRNA, or transgenes and is small enough to be grown in 96-well plates, allowing relatively high throughput screening to be carried out on a whole (vertebrate) animal. Their translucent embryos allow imaging of disease progression at both cellular and subcellular levels. Compounds can be added directly to the water and are absorbed through the skin, and a variety of readouts can be measured, including altered mobility and changes in fluorescence. In a recent study, Paquet et al. [24] successfully generated zebrafish that overexpress fluorescently labeled human tau P301L, a mutated form of human tau linked to frontotemporal dementia. The animals were

used for compound screening, which resulted in the discovery of AR-534, a novel GSK-3β inhibitor, showing that it is possible to successfully screen for active drugs using a whole-animal model.

The homolog of APP in *Drosophila* is Appl, but it does not contain the peptide Aβ. However, deletions of Appl do cause defects in locomotor behavior, a phenotype that can be rescued by expressing the human APP [25]. Expression of the toxic $A\beta_{1-42}$ in *Drosophila* leads to diffuse extracellular amyloid, impaired olfactory associative learning, and neurodegeneration [26]. *Drosophila* also has a homolog of presenilin, and mutations in this protein cause phenotypes similar to notch mutants. Other components of the presenilin complex, including Aph1, nicastrin, and Pen-2, are also present in *Drosophila*. Another key molecule associated with AD, tau, has also been reported in *Drosophila* [27]. Overexpression of human tau in *Drosophila* sensory neurons causes abnormalities, including swelling and axonal degeneration [28]. Thus, all the key proteins implicated in AD are present in the fly and can be studied and modified using the advanced genetics available in this system.

Despite the experimental advantages of these model organisms, there is a desire to have mammalian models that would allow the study of the consequences of specific mutations on higher-order brain function. The creation of transgenic animals that show all of the pathology of AD (Aβ deposits, neurofibrillary tangles, neurodegeneration, and neuroinflammation) is essential for both our understanding of the disease progression and pathology and for the evaluation of novel therapeutic agents. There are no naturally occurring small-animal models of Alzheimer disease. The first transgenic mice were developed expressing the entire human APP gene, human APP751, Aβ, or the C-terminal fragment of APP. All of these models showed only mild neuropathologic changes and few or no Aβ deposits. Subsequently, mice were created that overexpressed mutant forms of human APP, and these did show age-dependent, AD-like pathology [29, 30]. The discovery of presenilin as a component of the γ-secretase complex spurred the creation of a number of transgenic mouse lines. In spite of the fact that mutations associated with PS1 are associated with a form of familial AD [31], transgenic mice expressing either wild-type or mutated PS1 failed to develop substantial AD pathology. Breeding of the PS1 mice with the APP mice created animals with accelerated neuropathology [32]. The PDAPP mouse model expresses the human APP717 under the PDGF β-subunit promoter and has neuropathologic features characteristic of those observed in Alzheimer disease. Specifically, these include amyloid plaques, dystrophic neurites, activated glia, and loss of synapses in the hippocampus and frontal cortex, two regions of the brain that are particularly affected in Alzheimer disease [33]. The development of tau transgenic animals has confirmed the connection between tau and synaptic loss. Mice expressing P301S human tau [34] show hippocampal synaptic loss prior to the formation of neurofibrillary tangles. Loss of synaptic proteins can be detected as young as 3 months of age, and evaluation of the brains at 6 months (prior to neuronal loss and neurofibrillary tangle formation) showed impairments in synaptic transmission, presynaptic function and long-term potentiation in comparison with wild-type controls. For a more extensive review of relevant transgenic mouse models of human AD see Woodruff-Pak [35].

3 Neural Stem Cells

The overall encouraging outcomes of clinical trials in which human fetal mesencephalic tissue was transplanted into Parkinson disease patients [36, 37], together with the rapid pace of progress in the stem cell field, have raised awareness and expectations for future stem cell transplantation therapies for the treatment of Alzheimer disease. However, there are no reports of clinical trials using fetal brain tissue or stem cell transplants in AD patients. The reason for this mostly likely lies in the underlining complexity of AD in terms of its diversity in clinical presentation and uncertainty over the molecular events associated with its onset. Compared to the well-defined loss of ventral midbrain dopaminergic neurons in Parkinson disease, for example, the pathology of AD is diffuse, affecting multiple neuronal subtypes in different brain regions, leading to debate over which neuronal cell type would be the best to use in clinical trials. Nevertheless, stem cell research continues to be a major driver in the search for an AD cellular therapeutic.

Somatic stem cells, for example, neural stem cells, are self-renewing, multipotential cells with the developmental capacity to give rise to all major cell types of a particular tissue, as opposed to progenitor cells, which are committed and restricted to a specific lineage fate. The three principal cell types into which multipotent neural stem cells can develop in vivo and in vitro are neurons, astrocytes, and oligodendrocytes. The development of enzymatic single-cell suspension methods of the fetal brain, initially used for the generation of primary neuronal and mixed glial cultures, enabled the breakthrough discovery that neural stem cells (NSCs) can be isolated from fetal or adult rodent central nervous system tissue and expanded as free-floating cell spheroids called “neurospheres” in the presence of growth factors [38–40]. Soon thereafter, long-term neurosphere NSC cultures were reported from the human fetal brain [41–44]. The neurosphere culture system takes advantage of the ability of NSCs to avoid anoikis and grow anchorage independently, whereas other differentiated cells and committed progenitors die off without attachment. This, together with the action of epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2) on stem cell proliferation, results over time in NSC-enriched culture. It was suggested that for human neurosphere cultures, besides EGF and FGF-2, the addition of leukemia inhibitory factor further enhances their expansion [43]. The neurosphere system has proven invaluable in exploring NSC biology. The sphere cultures are heterogeneous, however, containing differentiating glial and neuronal cells in the center, surrounded by nestin-positive progenitors and NSC [45]. Significant enrichment of sphere-initiating NSCs in neurosphere cultures was achieved by positive selection with the prominin/CD133 cell surface marker during the isolation process [44]. Subsequently, the LeX/ssea-1 surface protein was suggested as another marker to enrich for NSC before the generation of neurosphere cultures [46].

The establishment of long-term, symmetrically self-renewing, adherent NSC cultures from both rodent and human central nervous system in the presence of EGF and FGF-2 has reduced the heterogeneity of cultured neural cells even further [47–49].

The homogeneity of the adherent cultures also allows for meaningful global expression analysis of unimmortalized NSCs. Adherent NSCs display diagnostic profiles of neurogenic radial glia NSCs, the main source of cortical neurons during embryonic brain development [50, 51]. Although the existence of dividing cells in the adult brain was first demonstrated more than 40 years ago [52], it was not until much more recently that the existence of neural stem cells and the occurrence of neurogenesis in the adult mammalian brain, including human, were widely accepted and the prospective adult astroglial-like NSC was described in detail [53, 54].

Two main observations made in AD patients have led to the hypothesis that defects in NSCs and subsequent neurogenesis might be involved in the progression of the disease. First, odor identification is pathologically affected in AD patients, and even healthy individuals with the Apoε4 allele showed impairment compared with allele-negative controls [55, 56]. Second, the hippocampal formation is one of the regions in the AD brain that is most heavily burdened with amyloid plaques. Notably, the two recognized regions of adult neurogenesis are the subventricular zone (SVZ), replenishing the neurons of the olfactory bulb, and the dentate gyrus of the hippocampus, in which neurogenesis is suggested to be important for memory tasks that are impaired in AD [57, 58]. Therefore, an area of intense research effort, with conflicting results, concerns how soluble sAPP and Aβ affect proliferation and differentiation of neural stem cells and adult neurogenesis. An early in vitro study indicated that purified soluble forms of APP promoted the proliferation of NSCs rather than inhibiting it [59]. This principal finding was confirmed by experiments in vivo, which showed that sAPP binds to EGF-responsive NSCs in the SVZ of the adult brain and acts as cofactor to stimulate the proliferation of these cells [60, 61]. However, other researchers reported that sAPP induced differentiation of human NSC into astrocyes and reduced the number of generated neurons, presumably mediated by the gp130 and notch signaling pathways [62, 63]. Other than for soluble APP, which appears to have attributes of a growth factor, in another in vitro study neurogenic effects of Aβ peptide on hippocampal and striatal NSCs were also demonstrated. This activity was carried by Aβ42 and not Aβ40 and by oligomers rather than fibrils [64]. To further examine this subject, the rate of neurogenesis in numerous transgenic mouse AD models was investigated, and the results so far have been inconsistent. Initially two studies using APP_{Swe} single-transgenic mouse lines reported decreased neurogenesis in the aged hippocampus [65, 66], whereas with a single-transgenic, double Swedish and Indiana APP mutant model a twofold increase in neurogenesis was found [67]. Ermini et al. [68] demonstrated that the observed effects on neurogenesis are dependent on both the transgenic mouse model and the age of analysis. In single-transgenic APP23 mice overexpressing APP_{Swe} at very high levels (sevenfold over endogenous APP) they found a significant increase in neurogenesis compared to controls at the age of 25 months, which could be interpreted as a compensatory response to neuronal cell death in the hippocampus. In contrast, double-transgenic, 8-month-old APP/PS1 mice exhibited decreased neurogenesis. Crucially, by crossing these mice with a nestin-GFP reporter strain, they demonstrated that this deficit already occurs at the level of quiescent astrocyte-like adult stem cells and

not only through diminishing of neuronal differentiation from NSCs. Suggested mechanisms for the reduced neurogenesis from NSCs in AD transgenic mice include alterations of the gp130 and notch pathways in APP23 mice and increased BMP4 and reduced noggin expression levels in APP/PS1 mice [62, 63, 69, 70]. Of interest, triple-transgenic 3× Tg-AD mice, which harbor mutant genes for APP, presenilin-1, and tau, the mouse model with the best-known manifestation of cognitive decline, also had an impairment in hippocampal neurogenesis, which occurred earlier in females than in males, reflecting the higher prevalence of AD in women than in men [71–73].

There are very few publications describing the utility of NSCs after transplantation into transgenic mouse AD models. The group of Kiminobu Sugaya, whose research into the gliogenic effects of APP on NSCs was described earlier, reported that treatment with the cholinesterase inhibitor phenserine, known to reduce APP levels in vitro and in vivo, significantly increased the neuronal differentiation of implanted human NSCs in the hippocampus and cortex of APP23 mice [69]. This opens up a strategy in which pharmaceutical reduction of APP levels could improve neuronal replacement approaches with transplantation of NSCs. However, examples of acute brain injuries or other neurodegenerative diseases have shown that neuroreplacement is not necessarily required for therapeutic effects of NSC transplantation, which are instead mediated by growth factor secretion or immunomodulation. Indeed, a recent study demonstrated that NSCs transplanted into 3× Tg AD mice rescued spatial learning and memory deficits via a growth factor secretion bystander effect [74]. Consistent with the Sugaya group's findings, only a small percentage of the transplanted NSCs differentiated into neurons, but growth factors secreted from the graft improved cognition via stimulating the formation of hippocampal synapses. Brain-derived neurotrophic factor (BDNF) was identified as playing a key role in this process. BDNF levels are decreased in brain and cerebrospinal fluid of patients with AD [75]. The major source of growth factor production secreted into the cerebrospinal fluid and therefore available to nourish NSCs in the SVZ are the epithelial cells of the choroid plexus, made up of modified ependymal cells, and it has therefore been hypothesized that aging and thinning of the choroid plexus epithelium concurrent with a declining growth factor secretion may be an underlying cause of neurodegenerative diseases, including AD [76–79].

4 Future Directions/Conclusions

The social and economic impacts of AD disease will continue to drive research and development activities into cellular therapeutic approaches to finding a cure. This will only be possible with a continuing effort to study and understand the molecular and cellular defects that lead to the disease. Progress in neural stem cell research is offering greater understanding and better-tuned cellular resources for potential cellular therapeutics. Nevertheless, AD with its heterogeneity in presentation and diffuse pathology remains a challenging cellular therapeutic target. Despite this, the

stem cell therapeutic research related to AD will continue to yield many insights and resources that in the short term could be used for drug-screening strategies with a longer-term view to cellular therapeutic strategies.

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Part IV
Nuclear Reprogramming and Induced Pluripotent Stem Cells

Nuclear Transfer Embryonic Stem Cells as a New Tool for Basic Biology

Sayaka Wakayama, Eiji Mizutani, and Teruhiko Wakayama

Abstract Recently, induced pluripotent stem (iPS) cells have become a very hot topic, and the popularity of nuclear transfer embryonic stem (ntES) cells has declined due to ethical problems in addition to the extremely difficult techniques required. However, ntES cells have a unique potential: some research designs can be approached only with this procedure and cannot be replaced by any other procedure, including iPS cell technology.

We previously showed that ntES cells can be established with a tenfold higher success rate than the production rate of cloned mice, including a variety of mouse genotypes and cell types, even though it may be more difficult to generate the clones directly. Moreover, ntES cells were identical to the ES cells derived by fertilization in terms of their global gene expression and their differentiation potential. In addition, several reports have demonstrated that ntES cells can be used in regenerative medicine, such as in the treatment of Parkinson disease or of immunodeficient mice. In addition, by combining the cloning and the ntES cell techniques, this approach could be applied to fertility treatments using somatic cells instead of gametes. Furthermore, live mice now can be generated from frozen dead bodies, suggesting that extinct animals, such as the mammoth, can be possibly resurrected by this technology if nondamaged nuclei are retrieved from the permafrost. Thus, although there remain many cloning issues in ntES technology, it has the potential to become a powerful new research tool with broad-based applications in the study of basic biology.

Keywords Clone • Nuclear transfer • ntES cell • Reprogramming

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1 Introduction

Since it was first reported in 1997 [1], somatic cell cloning has been demonstrated in at least 17 mammalian species. While cloning efficiencies can range from 0% to 20%, efficiency rates of just 1%–2% are typical in mice (i.e., 1 or 2 live offspring are produced per 100 initial embryos). Moreover, many abnormalities in mice cloned from somatic cells have been reported, including abnormal gene expression in embryos [2–4], abnormal placenta [5], obesity [6, 7], and early death [8]. Such abnormalities notwithstanding, success in generating cloned offspring has still opened new avenues of investigation.

For regenerative medicine, embryonic stem (ES) cells derived from fertilized blastocysts and the progeny of such cells inevitably face the risk of immunorejection upon transplantation. Therefore, it has been proposed that ES cells self-derived from embryos cloned from the host patient's cell nuclei represent a potential solution to such rejection, as any replacement cells would be genetically identical to the host's somatic cell nuclei [9–11]. We and others have shown that nuclear transfer–derived ES cell lines possess the same potential as ES cells from fertilized blastocysts [12, 13]. To distinguish nuclear transfer–derived ES cell lines from those lines derived from fertilized embryos, the former are referred to as nuclear transfer ES (ntES) cell lines [14]. However, ntES cells have raised ethical objections concerning human cells, as fresh oocytes must be donated by healthy women, and the cloned embryos would be deprived of their potential to develop into a complete human being. To avoid these objections, several approaches have already been attempted, and some problems have been successfully solved. For example, aged oocytes from failed in vitro fertilization (IVF) were used instead of fresh donated oocytes [15]. The strongest candidate is the induced pluripotent stem (iPS) cell, which has been established directly from somatic cells by retroviral infection rather than by nuclear transfer [16, 17], and therefore there is no need for embryo production or destruction. However, iPS cells require genetic modification, at least in current methodology.

Use of iPS cells carries no ethical problems and is a relatively easy technique, whereas use of ntES cells has serious ethical issues and is an extremely difficult technique. Therefore, use of ntES cells in preliminary medical research is waning. However, ntES cell techniques can still be applied to basic biologic research. For example, ntES cell techniques can be applied to characterize very rare and specialized cell types in the body, such as olfactory neurons [18, 19]. In theory, 10 donor cells can sufficiently establish one ntES cell line, and, once established, these cells will propagate indefinitely. Another example is the generation of offspring from a somatic cell of an infertile mouse by combining cloning with ntES technology [20]. Moreover, cloned offspring could potentially be generated even from nuclei of dead donor cells or bodies, such as from an extinct frozen animal [21]. Currently, only ntES technology is available for this purpose because all other techniques require a significant number of live donor cells. This review describes the features of cloning and ntES cell technology for both basic and applied investigations.

2 Animal Cloning

The first reproducible cloning of a mammal (sheep) was achieved by transferring the eight-cell-stage embryonic nuclei into recipient unfertilized eggs (oocytes) [22]. It is probable that in mammalian cloning, the oocyte represents a more viable recipient than the zygote [23]. Thereafter, techniques of mammalian cloning were developed further, and more-mature cells were selected as donors. Ten years later, Campbell et al. reported cloned sheep derived from an established embryonic cell line [24]. Using these techniques, the same group later reported the first somatic cell–cloned animal [1]. To date, among mammalian species, mouse [25], cattle [26], goat [27], pig [28, 29], gaur [30], mouflon [31], domestic cat [32], rabbit [33], horse [34], mule [35], rat [36], African wildcat [37], dog [38], ferret [39], wolf [40], and red deer [41] have been successfully generated as cloned animals.

However, we still do not understand the nature of genomic reprogramming, and the cloning success rate is less than 10%, irrespective of animal species. Many attempts have been reported on how improve this low success rate of cloning, but none has succeeded in improving it significantly. A recent molecular analysis of cloned embryos revealed abnormal epigenetic modifications such as DNA methylation and histone modification [42–45]. Therefore, the prevention of epigenetic errors is expected to improve the success rate of animal cloning. There are a number of promising reports concerning 5-azacytidine, an inhibitor of DNA methylation, and trichostatin A (TSA), a histone deacetylase inhibitor (HDACi) [46]. However, those drugs are very toxic, and each drug must be pharmacologically examined for its appropriate exposure, timing, concentration, and duration.

We recently optimized the conditions for TSA in a study in which the addition of 5–50 nM TSA for 10 hours following oocyte activation led to a greater-than-fivefold increase in the success rate of mouse cloning and a twofold increase in the establishment rate of ntES cell lines (Fig. 1) [47]. We also found that TSA can be used to produce cloned mice even from an outbred “unclonable” strain [48]. Because TSA has a strong toxicity, we searched for a less toxic reagent and found that another HDACi, Scriptide, can be use to produce cloned mice from all of the important inbred mouse strains, such as C57BL/6 and C3H/He, which until now have been unsuccessful even with TSA [49]. Thus, our findings provide a new approach for practical improvements in mouse cloning techniques, as well as new insights into the reprogramming of somatic cell nuclei.

3 Nuclear Transfer Embryonic Stem Cells

The first success in generating ES-like cell lines from somatic cells via nuclear transfer was initially performed in the cow [50] and then subsequently in the mouse [51, 52]. These ES-like cell lines are believed to possess the same capacities for unlimited differentiation and self-renewal as conventional ES cell lines derived from normal embryos produced by fertilization. We previously showed that those nuclear transfer–derived, ES-like cell lines are capable of differentiating into all three germ

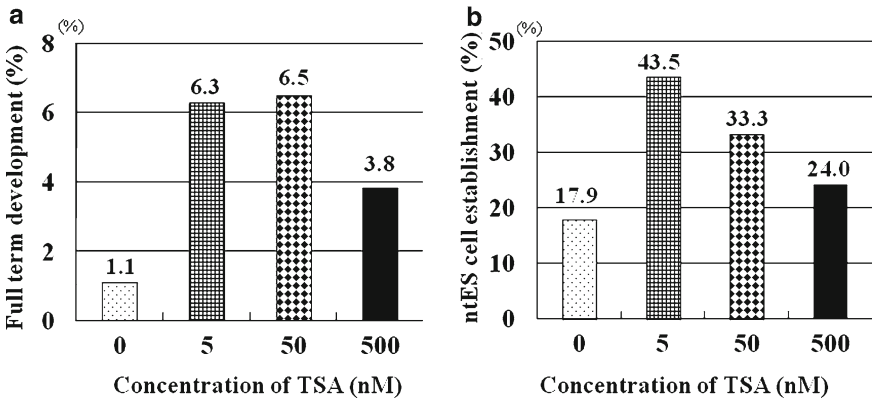

Fig. 1 Effect of trichostatin A (TSA) treatment on cloned embryo development to full term and on nuclear transfer embryonic stem (ntES) cell establishment. **a:** The success rates for obtaining full-term cloned mice were significantly improved when cloned embryos were cultured with 5–50 nM TSA for 10 hours. **b:** ntES cell establishment similarly improved as with cloning mice

layers in vitro or into spermatozoa and oocytes in chimeric mice [14]. This was the first demonstration that ES-like cells have the same potential as ES cells from fertilized blastocysts. To distinguish nuclear transfer–derived ES cell lines from those derived from fertilized embryos, the former are referred to as ntES cell lines [14]. The ntES cell lines can be established with success rates 10 times higher than rates from reproductive cloning (Fig. 2a) [12, 14, 53, 54]. Therapeutic cloning is thus at least an order of magnitude more successful than reproductive cloning [11].

3.1 Establishment of Nuclear Transfer Embryonic Stem Cell Lines from Individuals

It has been proposed that ntES cells derived from a host patient's cells represent a potential solution to the problem of immunorejection, as any replacement cell would be genetically identical to the host's own cell [9–11, 55, 56]. Therefore, it is important to know the possible genotypic effects of a particular animal strain or sex of the donor nucleus. Such factors often affect the successful full-term development of cloned animals [57, 58]. In contrast to cloning mice, ntES cell lines can be established regardless of the mouse genotype, cell type, or sex. These conditions of donor nuclei give a higher success rate (Fig. 2b). When inbred and F1 genotypes were compared, the rate of development to the blastocyst stage and the frequency of ntES cell derivation were significantly better when F1 cumulus cells were used as the donor nucleus. This difference was only seen, however, when the data were compared from reconstructed oocytes, suggesting that the overall success rate appears possibly to depend primarily on preimplantation development; once the embryos have reached the blastocyst stage, the genotype differences are no longer significant [54]. Moreover, it has been shown that even differentiated neurons and lymphocyte cells

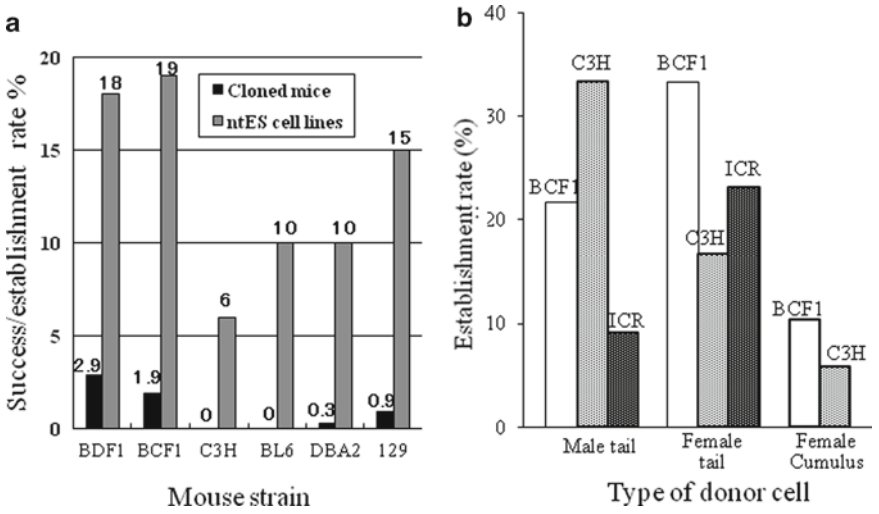

Fig. 2 Comparison of the establishment rate of nuclear transfer embryonic stem (ntES) cell lines between full-term offspring of donor cell type. **a:** The rate of ntES cell establishment is nearly 10 times higher than the rate of producing cloned mice. Of interest, ntES cell lines can be established from even "unclonable" mouse strains such as C3H or C57BL/6 (BL6). These data were obtained before discovering the effect of trichostatin A. **b:** The establishment rate between strain and sex exhibited no difference, but when it was compared between tail cells and cumulus cells, the tail cells usually displayed the better rate

can be used to establish an ntES cell line (0.1%–2%), although in previous studies these cells failed to produce any cloned offspring [14, 18, 19, 59].

3.2 Normality of Nuclear Transfer Embryonic Stem Cells

It remains unclear whether these somatically derived ntES cells are identical to the ES cells derived from normally fertilized embryos. In chimeric mice, most ntES cell lines differentiate into germ cells [12, 14, 54], which is the strongest evidence to date that these cells have the same potential as ES cells. In contrast, most, if not all, cloned animals present critical fetal and postnatal abnormalities, perhaps resulting from the defects or from an incomplete genomic reprogramming [2–4], as mentioned earlier. As ntES cell lines are established using the same procedures, it is possible that they will also exhibit epigenetic defects. Thus, either most ntES cell lines have been established from cloned embryos with negligible reproductive potential, or most cloned embryos die during or after implantation because of abnormal placental development, which does not involve ES cells. If these ntES cell lines have any inherent abnormalities, such as epigenetic defects, there may be potential risks in their clinical use, and the scientific discoveries based on these ntES cells might be of limited utility. For example, we have found that the potential of ntES cell nuclei for creating full-term cloned offspring by second nuclear transfer differed among the ntES cell lines, even when the cell lines were derived from

the same individual at the same time [53, 60]. Thus, ntES cell lines show different potentials not only according to their genetic background, but also by any epigenetic differences between each line, even among those from the same individual.

To evaluate the characteristics of ntES cells, we examined the pluripotency and karyotype, physical damage of DNA by nuclear transfer, similarity of ntES cells by tissue-dependent and differentially methylated region assay, similarity of ntES cells by global gene expression analysis, and differentiation potential into functional embryonic tissues by tetraploid chimera formation assay (Table 1) [12, 54]. Of significance, we demonstrated that these ntES cells were identical to fertilized ES cells in all experiments. The DNA microarray profiles demonstrated that ntES cells and control fertilized ES cells derived in the our laboratory are similar to each other but are different from other commercially available ES cell lines, such as E14 and ESVJ. The insignificant differences were minor, probably resulting from the subtle variations to be expected in sample-to-sample preparations. Other groups have also demonstrated that bovine and mouse cloned embryonic cells, respectively, both closely resemble naturally fertilized embryos, using global gene expression profiles [13, 61]. In addition, the sequential ntES cell establishment data indicate that the process of nuclear transfer or the physical treatment itself did not cause the abnormalities in the ntES cell lines, even when nuclear transfer was repeated 10 times [12]. Moreover, the establishment rate of the re-ntES cells increased with the increase in the number of transfer repeats. These data suggest that early-generation ntES cell lines may still have some epigenetic abnormalities and that these differences were corrected by serial nuclear transfer and/or re-reprogramming.

3.3 Why Are Nuclear Transfer Embryonic Stem Cells Normal?

To establish an ntES cell line, a cloned blastocyst must be cultured for longer than 1 month in vitro. During this period, survival of the cloned embryo and the early-stage derivative is independent of most development-related gene expression and placental development; it might be able to proceed by reprogramming gradually to obtain the normal pattern of gene expression. Another possibility is that some of the cloned embryos possess only a few normally reprogrammed cells [2, 4], and these few cells are insufficient to sustain a viable fetus to term. However, viable ntES cells can be established even from these few normal cells during a period of 1 month of culture. Alternatively, in reproductive cloning, the cloned embryos must express all of the appropriate genes, and they exhibit relatively normal placental development immediately after implantation. This more stringent set of criteria may account for the higher developmental failure rates of presumably incompletely reprogrammed cloned embryos following implantation. At the very least, the data suggest that this therapeutic cloning approach appears to be a powerful and reliable method for establishing ES cells that display almost identical biologic characteristics to normal fertilized ES cells.

Table 1 Comparison of cell marker, gene expression, genetic and epigenetic normality, and differentiation potential between embryonic stem cells, nuclear transfer embryonic stem cells, and aged fertilization failure nuclear transfer embryonic stem cells

	Nuclear normality		Gene expression normality[a]			Epigenetic normality	Differentiation potential	
Cell type	Chromosome damage	DNA damage	ES cell marker	Microarray	HiCEP	DNA methylation	In vitro	4N Chimera
ES	None	None	All positive			Normal	Good	Live offspring
ntES	None	None	All positive	No difference	No difference	Normal	Good	Live offspring
AFF-ntES	None	None	All positive	ND	ND	ND	Good	Live offspring

AFF-ntES, aged fertilization failure nuclear transfer embryonic stem; ES, embryonic stem; HiCEP, high-coverage expression profiling; ntES, nuclear transfer embryonic stem.

[a]Comparison between ES and ntES cells.

4 Ethical Issues in Using Nuclear Transfer Embryonic Stem Cells

4.1 A General Attempt to Avoid Ethical Problems

Among the ethical objections to human ntES cell research are the following: (1) fresh oocytes must be harvested from healthy women, and (2) the embryos are denied their potential to develop into offspring. Several different approaches have been proposed to overcome these ethical objections, and these approaches can be categorized into the following three methodologies:

Category 1: Use lethally vulnerable embryos or use embryos without destroying them. For example, parthenogenetically activated embryos can be used to establish ES cell lines without the destruction of "normal" embryos because parthenogenetically activated embryos lack a paternal genome and invariably die [62–64]. However, this method can only be used for healthy women, and the differentiation potential of such ES cells is poor. To generate patient-specific ES cells, genetically altered donor nuclei have been employed for nuclear transfer in a mouse model [65] in which the cloned embryos lack a gene essential for trophoblast development. This can prevent implantation without interfering with ES cell potency.

Recently, Egli et al. successfully established ntES cell lines by using enucleated polyspermic mouse zygotes, which also cannot develop due to a triploid phenotype [66]. Alternatively, ES cell lines have been established nondestructively by removing single blastomeres from fertilized embryos [67]. For this approach, biopsied blastomeres were cocultured with other ES cells, and 2% of blastomeres were converted to ES cells. We also tried a similar experiment with a simpler method (in which blastomeres were just cultured in new ES medium), and the establishment rate was 10 times higher than in previous reports. Moreover, our results suggest that the single cells of any stage of embryo or polar bodies can be potentially converted into ES cells without any special treatment (Fig. 3) [68]. However, this can only be applied to subsequent generations, not to treatment of the patient.

Category 2: Reprogram somatic cells without the use oocytes. One reprogramming approach is cell fusion between pluripotent stem cells (e.g., ES cells) and somatic cells [69,70]. Although such fused cells become tetraploid, somatic cell nuclei are significantly reprogrammed and exhibit pluripotency. Another approach uses iPS cell lines that have been established directly from somatic cells by retroviral infection rather than by nuclear transfer [16,17]. In both situations, there is no requirement for embryo production or destruction, although it does require the genetic modification of the cell lines. However, Verlinsky et al. have developed a novel method they call "stembrid technology" [71–73], which is based on the hybridization of adult somatic cells with enucleated cells of human ES (hES) cells, leading to a pluripotent state without any contamination from the hES cell nucleus. Using this technique, they have established a hES cell line repository that includes over 100 cell lines with a normal genotype [72].

Category 3: Create oocytes from ES cells or use the oocytes of other species, which has shown great promise toward addressing the chronic scarcity of human

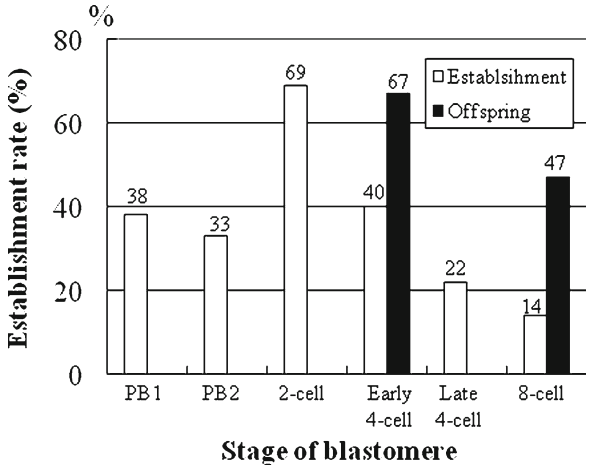

Fig. 3 Embryonic stem (ES) cell establishment from polar bodies (PBs) or single blastomeres and the production of offspring from biopsied embryos. Newly developed ES cell culture media can support ES cell derivation from single blastomeres of two-cell- to eight-cell-stage embryos with a significantly higher establishment rate. Moreover, the remaining biopsied embryos can develop to full term just the same as an intact embryo when they were transferred into recipient females. Polar bodies were use as donor nuclei, and embryos were obtained when reconstructed oocytes were parthenogenetically activated

oocytes for use in nuclear transfer studies. These studies can be accomplished either through the artificial creation of oocytes by in vitro differentiation [74] or through nonhuman oocytes, such as those of the cow or rabbit [75,76], which have the potential to be reprogrammed using human somatic cell nuclei to establish human ntES cell lines. However, fundamental questions remain as to whether artificial oocytes have the same developmental potential as natural oocytes and whether ntES cells and their rabbit mitochondria–containing derivatives would be tolerated by a human host immune system upon transplantation.

Thus, several approaches have now been reported, all of which have merits and demerits for establishing cell lines. We have also tried the following two approaches to avoid or at least to minimize the ethical problems associated with ES cell establishment.

5 Improving the Differentiation Potential of Parthenogenetic Embryonic Stem Cells by Nuclear Transfer

Parthenogenesis is the process by which an oocyte develops into an embryo without being fertilized by a spermatozoon. Although such embryos lack the potential to develop to full term, they can be used to establish parthenogenetic embryonic stem (pES) cells for autologous cell therapy in females without the need to

destroy the normally competent embryos. Such pES cells may be more acceptable ethically for the treatment of human patients. For primates, it has already been demonstrated that pES cells can be differentiated into all three embryonic germ cell lines in vitro [62]. However, there are several negative reports on the application of pES cells. When chimeric mouse offspring were generated from either parthenogenetic or fertilized embryos, the offspring manifested postnatal growth retardation. Moreover, the cell lineages of the parthenogenetic cells were restricted, particularly in the formation of mesoderm and endoderm [77–80]. To resolve and to improve the differentiation potential, we tried to reestablish pES cells using nuclear transfer and ntES techniques. As a result, the in vivo differentiation potentials of nuclear transfer pES (nt-pES) cells improved significantly (twofold to fivefold) compared to the original pES cells, as determined by the production of chimeric mice [64]. We compared the gene expression profiles between pES and nt-pES cell lines by DNA microarray analysis and by allele-specific DNA methylation analysis. Although changes in the expression levels were very low, with some particular genes, such as the paternally expressed imprinted gene *U2af1-rs1*, which is not expressed in the original pES cell, the expression level and the differentially methylated regions were significantly increased in all nt-pES cells. Surprisingly, this was also observed in the conventionally produced ntES cells, which has never been reported previously [63]. So far, nuclear transfer techniques have required additional oocytes. However, in this study, only eight oocytes per line were required for an establishment (a mean of 12.4% from oocytes) because of the high success rate of the nt-pES cell line growths. Although these nuclei maintained parthenogenetic phenotypes, these cloned embryos are still destined to perish, so ES cell derivation may still be performed without any concern for destroying potential individuals. This would help to overcome the ethical objections to sacrificing normal embryos. In turn, we have also established a useful tool for investigating the effect of epigenetic modifications because these nt-pES cells permit the possibility of investigating the roles of imprinted genes.

6 Establishing Nuclear Transfer Embryonic Stem Cell Lines from Aged Mouse Oocytes That Failed to Fertilize

Human IVF is now routinely performed in infertility clinics with a high degree of success (60%–70% in humans, greater than 70% in mice) [81–83], but some oocytes still fail to fertilize for unknown reasons. This failure might be because the oocytes have innate defects rather than because of sperm dysfunction [84]. These unfertilized oocytes continue to age in vitro, and they often show abnormalities such as metaphase spindle disassembly [85–87]. However, if such aged fertilization failure (AFF) oocytes could be used as recipient oocytes to generate ntES cell lines, then this would reduce or even eliminate any ethical concerns over oocyte donation and embryo destruction.

Although in vitro development of cloned embryos to the blastocyst stage was significantly lower in the AFF oocytes than in fresh oocytes, the rate of establishment of ntES cell lines from blastocysts was similar or even higher than those of fresh oocytes [15]. The entire established cell lines stained positive for the ES cell-specific markers alkaline phosphatase, Oct3/4, and Nanog, and all karyotypes were within the normal ranges.

For practical use in humans, as nuclear transfer is inefficient, it might become necessary to transport AFF oocytes from other clinics in order to increase the number of oocytes available. Mouse oocytes can survive functionally when stored at room temperature, but not at 37°C or 4°C [86]. Surprisingly, even when oocytes are stored at room temperature for 1 day, they still retain their capacity for genomic reprogramming, and they still can be used to establish ntES cell lines from several different mouse strains. Thus, in theory, AFF human oocytes could be pooled among nearby clinics by simple storage at room temperature.

On the other hand, if these cloned embryos have the potential to develop to full term, a second ethical problem, that is, "the embryos are denied their potential to develop into established ntES cell lines," might arguably remain. Therefore, we also examined the full-term developmental potential of cloned embryos produced from AFF oocytes. None of these cloned embryos developed to full term when AFF oocytes were used. It appears the genomic reprogramming potential of AFF oocytes is sufficient to generate ntES cells but insufficient for full-term development. Thus, AFF oocytes might serve as a basic science tool. For example, to identify potential reprogramming factors, fresh and AFF oocytes can be compared [15].

7 Applications of Nuclear Transfer Embryonic Stem Cell Techniques

7.1 Therapeutic Medicine

The ntES cells are genetically identical to the donor and are potentially effective for therapeutic applications. Therefore, therapeutic cloning may improve the treatment of neurodegenerative diseases, blood disorders, or diabetes since therapy for these diseases is currently limited by the availability and immunocompatibility of tissue transplants [9–11, 56].

We have demonstrated that dopaminergic and serotonergic neurons can be generated from ntES cells derived from tail-tip cells [14]. Furthermore, Rideout et al. reported that therapeutic cloning combined with gene therapy enabled them to treat a form of combined immune deficiency in mice [56]. They made one ntES cell line from an immune-deficient mutant mouse. First, the ntES cell with mutated alleles was repaired by homologous recombination, thereby restoring normal gene structure. They then transplanted those ntES cells into a different immunodeficient mouse. In addition, Barberi et al. reported that mouse-tail– or cumulus cell–derived

ntES cells could be differentiated into neural cells at even higher efficiencies than fertilization-derived ES cells [55].

Similarly, we perfected regenerative medicine using a mouse model in which a donor mouse was cured using its own ntES cells derived from its own somatic cells [88]. In this experiment, a total of 24 mice were rendered parkinsonian-like via intrastriatal 6-hydroxydopamine injections in New York. After confirming the disease, tail-tips were collected and shipped from New York to Japan. By then performing nuclear transfers, we could establish a total of 187 ntES cell lines from all individuals, with a minimum of one line per mouse. All ntES cells were shipped to New York, and cells were differentiated into midbrain dopamine neurons, and each mouse received dopamine neuron progeny obtained from an ntES cell line derived from its own tail fibroblast donor nuclei. Several months later, most mice grafted with matched ntES cell–derived dopamine neurons showed a significant amelioration of the parkinsonian phenotype in all behavioral tests without any immunologic response [88]. These data demonstrate for the first time the feasibility of treating individual parkinsonian mice via therapeutic cloning and further suggest considerable therapeutic potential for the future.

7.2 A New Tool for Basic Biology

We propose that ntES techniques can be applied to the biologic sciences as a novel investigative tool. Some potential investigations could be done only with this technique and not by iPS technology. For example, the ntES cell techniques can be applied to the characterization of very rare cells in the body. Once ntES cells from these rare cell nuclei are established, the cells can proliferate indefinitely.

It is hypothesized that a single odorant receptor gene chosen from thousands is facilitated by DNA rearrangements in olfactory sensory neurons, such as in lymphocyte nuclei. Yet this cannot be demonstrated due to the very low number of specific, differentiated cells. Li et al. and Eggan et al. generated ntES cells from the nucleus of a single olfactory sensory neuron and then demonstrated that the odorant receptor gene choice is reset by nuclear transfer and is not accompanied by genomic alterations [18,19].

Similarly, monoclonal mice have been generated from ntES cells derived from B and T lymphocyte nuclei [59]. In this case, due to the extreme difficulty of mouse cloning directly from lymphocyte nuclei, the authors combined two different methods—nuclear transfer and tetraploid complementation—in which ntES cells were injected into the tetraploid blastocyst. As a result, almost all parts of the chimeric offspring, including the germ cells, originated from the ntES cells. The offspring were referred to as the ES mouse [89, 90] or the clonal mouse [91]. After mating this clonal mouse, the authors finally succeeded in obtaining monoclonal mice by germline transmission.

On the other hand, ntES cell techniques can be used to assess tumorigenic potential. The ntES cell lines have also been established from embryonic carcinoma cells or melanoma cell nuclei, but chimeric mice from ntES cells developed cancers with higher frequency. It has been demonstrated that nonreprogrammable genetic modifications

define the tumorigenic potential [92, 93] and therefore would also be an appropriate application that requires ntES cells.

7.3 Producing Offspring from Individual Mice

The genetically modified mouse is a powerful tool for research in the fields of medicine and biology. Detrimentally, however, in one large-scale ethyl-nitrosourea mutagenesis study, infertility was listed as a phenotypic trait in more than half of the mutants described [94]. This is a challenge worth the undertaking, as the ability to maintain such types of mutant mice as genetic resources would afford numerous advantages crucial to research in human infertility and to the biology of reproduction. Unfortunately, the success rate of somatic cell cloning is very low. Even in cases in which the cloning of a sterile mouse is successful, due to the nonreproductive nature of the phenotype, it will still be necessary to clone all subsequent generations. This potentially represents another significant barrier, as the success rate of repeated cloning from cloned mice decreases for each successive generation after the first nuclear transfer [95].

On the other hand, the ntES cell establishment rate is nearly 10 times higher than the success rate of cloned mice, even from "unclonable" mouse strains. Unfortunately, the conversion from somatic cells to ntES cells does not improve the overall success rate of cloning by the second round of nuclear transfer [14, 54, 60]. However, we recommend the establishment of ntES cell lines at the same time in order to preserve the donor genome because these lines can then be used as an unlimited source of donor nuclei for nuclear transfer.

For example, generally, due to the low success rate of cloned mice, only few individual donor mice succeeded to generate their clone. However, we were ultimately able to obtain cloned mice from six of seven individuals by using either somatic cells or ntES cells [53]. As another example, senescent mice are often infertile, and the cloning success rate decreases with age, making it almost impossible to produce cloned progeny directly from such animals. However, we succeeded in establishing ntES cell lines from all aged mice regardless of sex or strain. The cloned mice were obtained from these ntES cell by the second nuclear transfer. In addition, healthy offspring were obtained from all aged donors via germline transmission of the ntES cell in chimeric mice [60]. This technique is thus applicable for the propagation of a variety of animals, regardless of age or fertile potential.

7.4 Preserving Unique but Infertile Mutant Mouse Genes

We have found a mutant, hermaphroditic, sterile mouse in our ICR mouse-breeding colony. Unfortunately, ICR is one of the most difficult strains to use in cloning [48, 96]. However, ntES cell lines from a tail-tip fibroblast of the mutant infertile

mouse have still been successfully established. Although the mutant mouse died accidentally soon after tail-tip biopsy, we have attempted to make cloned mice from ntES cell nuclei, but again, we could not obtain full-term offspring.

As the offspring from the ntES cell lines were not obtained by conventional nuclear transfer, we have tried to make chimeric mice by using either diploid embryos or tetraploid embryos. Using the tetraploid complementation method, we obtained two clonal mice from ntES cells, which were phenotypically male and were proven infertile. On the basis of the diploid chimera method, the animal with the highest contribution of ntES cells in terms of the coat color was infertile, but one diploid chimeric male transmitted most of its mutant mouse genes to the next generation via the ntES cells. Thus, ntES cells can maintain the mutant gene, but neither cloning nor tetraploid complementation chimera construction could rescue the lineage of the original infertile hermaphrodite male [20].

7.5 The Possibility of Resurrecting an Extinct Animal

Cloning animals by nuclear transfer provides an opportunity to preserve any endangered mammalian species. However, the "resurrection" of extinct species (such as the woolly mammoth) is thought to be impractical because no live cells are available. Therefore, we developed a new nuclear transfer method that allows us to use naked nuclei collected from dead cells rather than live cells, such as freeze-thawed [21] or freeze-dried cells [97]. Using this technique, we have attempted to produce cloned mice from bodies kept frozen at −20°C for up to 16 years without any cryoprotection (Fig. 4a). These conditions are almost similar to the condition of a frozen body discovered from permafrost. Based on the use of brain nuclei as nuclear donors, cloned embryos could develop to blastocysts at the same success rate as fresh cells.

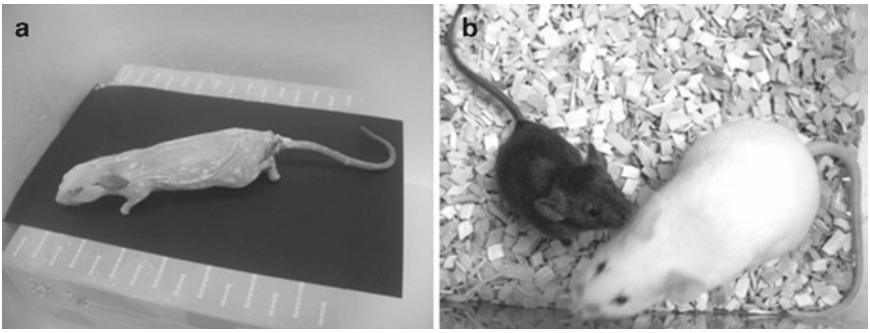

Fig. 4 Cloned mice from a frozen dead body. **a:** As a model of extinct animals retrieved from permafrost, we used frozen dead mice as donors, which were kept in a −20°C freezer for 16 years. **b:** Healthy cloned mice were obtained by a second round of nuclear transfer using these nuclear transfer ES (ntES) cell lines

Although we could not produce cloned offspring from the somatic cell, several ntES cell lines with normal karyotype were established from most organ cell nuclei. Finally, healthy cloned mice were produced from these ntES cells by the second round of nuclear transfer (Fig. 4b) [21]. Thus, nuclear transfer techniques could be used to resurrect animals or to maintain genome stocks from tissues that have been frozen or even frozen and dried for prolonged periods.

8 Conclusion

Recently, several new methods have been proposed to create patient-specific ES cells or iPS cells that would not elicit ethical objections, so that ntES cells might come to be seen as an old-fashioned technology. Thus far, however, ntES cells are pluripotent stem cells that are the most similar to fertilized-derived ES cells without any genetic modification of the original donor nucleus. It is still too early to judge which of these approaches is the most promising. Moreover, as mentioned earlier, the ntES cell has many unique advantages, all of which can be applied as a new tool for the study of basic biology. For regenerative medicine, it seems clear that a deeper understanding of nuclear reprogramming will be necessary, and we believe that the nuclear transfer technique is one of best tools for solving the mechanisms of reprogramming. Once it became clear, it will allow us to generate better patient-specific ES cell lines without any genetic modifications.

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Pluripotent Stem Cells in Reproductive Medicine: Formation of the Human Germ Line *in Vitro*

Sofia Gkountela, Anne Lindgren, and Amander T. Clark

Abstract The germ cell lineage and germ line inheritance have fascinated biologists for centuries. Clinically, the function of germ cells in sexually reproducing organisms is to ensure reproductive fitness and to guard against extinction. However, the consequence of abnormal germ cell development can lead to devastating outcomes, including germ cell tumors, spontaneous recurrent miscarriage, fetal demise, infant morbidity and mortality, and birth defects. To overcome abnormal germ cell development, it has been proposed that germ cells could be generated from pluripotent stem cells in vitro. The technology for creating germ cells in this manner remains theoretical. However, recent advances suggest that the field is progressing toward this goal. In particular, the identification, isolation, and characterization of the initial step in human germ cell development has recently been reported by multiple groups. Furthermore, the birth of live, healthy, fertile young has now been achieved following fertilization of murine in vitro–derived germ cells. This remarkable achievement in the mouse has stemmed from years of studies aimed at understanding the first step in murine germ cell development, the formation of primordial germ cells (PGCs). This chapter describes the events in human PGC development in vivo and how this information should instruct PGC differentiation from human pluripotent stem cells in vitro.

Keywords Primordial germ cells • Human embryonic stem cells • Nuclear remodeling • Chromatin dynamics

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1 Introduction

The human germ line is primarily responsible for passing genetic information between generations. Lack of a germ line is not lethal. However, abnormalities in the formation of this lineage can be devastating. For example, abnormal germ cell differentiation and function during fetal life can lead to infertility or germ cell tumorigenesis in one generation. Alternatively, fertilization of abnormal germ cells can result in spontaneous miscarriage, fetal demise, infant morbidity, mortality, and birth defects in the next generation.

In recent years, there has been considerable interest in the generation of germ cells from pluripotent stem cells in vitro. The major use of this tool will be as a cell-based model to interrogate molecular events that regulate fetal germ cell development and to pinpoint molecular causes of poor reproductive outcomes due to defects in the germ line. There is also interest in the potential use of in vitro–derived germ cells in regenerative medicine to treat infertility. The current state of the art for generating germ cells from pluripotent stem cells is still under construction and is many years away from being used clinically. Specifically, the methods for differentiating human pluripotent stem cells toward the germ line in vitro are currently at step 1, the primordial germ cell (PGC) step, in a five-step program outlining the major events in germ cell differentiation (Fig. 1). Although PGCs are a fetal cell type, the establishment of these cells sets the foundation upon which all future steps of germ cell development are built. Therefore, careful analysis of PGC identity during human germ cell development in vivo, and in parallel with differentiation in vitro, will be essential to ensuring a quality cell-based product for investigation in the lab and further differentiation toward a haploid gamete for regenerative medicine.

Contemporary analysis of murine PGC development has revealed that a dynamic series of nuclear and chromatin remodeling events is accomplished during the PGC stage [1, 3]. Major outcomes of this remodeling include the complete erasure of CpG methylation from imprinted loci in both male and female PGCs, global DNA demethylation, and, in females, reactivation of the X chromosome [4–6]. After nuclear remodeling, male and female PGCs become gonocytes and are fated to enter quiescence (G0) or meiosis, respectively, which effectively ends the PGC period. In humans, these orderly changes in chromatin architecture during PGC development have never been described. This is most likely due to the difficulty in

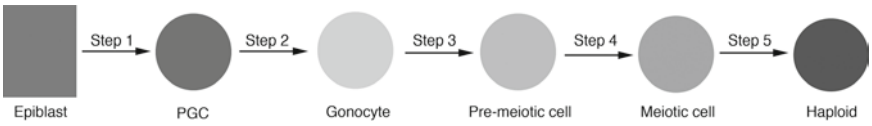

Fig. 1 Schematic representation of germ cell development. The premeiotic germ cell step refers to the stage before meiosis. In males, this involves formation of intermediate spermatogonia, prespermatogonia, and spermatogonia. The identity of similar intermediate stages in female germ cell development is not known

accessing human fetal tissue during this early time point in pregnancy. Therefore, the differentiation of PGCs from human pluripotent stem cells (human embryonic stem cells or human induced pluripotent stem cells) has the potential to fill this void in our understanding. Furthermore, differentiation of PGCs in vitro could also enable the technology to test hypotheses regarding the mechanisms underlying the essential nuclear remodeling steps in PGC development. In this chapter our goal is to introduce the current state of the art of PGC differentiation in vitro and the future outlook toward regenerative medicine treating infertility. Treatment of infertility will require the generation of a haploid gamete. No study to date has convincingly showed that human pluripotent stem cell–derived germ cells can effectively undergo meiosis and recombination, a requirement to move from step 4 to step 5 (Fig. 1). Furthermore, the formation of gonocytes or premeiotic cells in steps 2 and 3 requires the interaction with gonadal somatic cells, which have not been well described in vitro (Fig. 1). In contrast, the formation of the initial definitive germ cell, the PGC, in culture is very promising. However, to better understand the formation of PGCs in vitro, more characterization and comparison either to human PGCs in vivo or to the mouse system will be required.

2 Genesis of Human Germ Cells

The earliest stages of human fetal germ cell development were described in the late 1940s and early 1950s using histology and ultrastructural analysis. The earliest developmental time point at which human PGCs have been described is their localization outside the developing fetus in a rudimentary embryonic structure called the yolk sac [7]. Extrapolation to the murine model indicates that PGCs are not specified in this structure, and instead are derived directly from the epiblast at the time of implantation due to exogenous signaling by BMP4 [8, 9]. From the yolk sac, human PGCs migrate into the embryo, ultimately colonizing the primitive human gonad at approximately 4–5 weeks postconception (Fig. 2).

There is no consensus for the nomenclature of human developmental timing in the primary literature. For example, “postconception” is used to describe the timing of fetal development from the time of fertilization. In contrast, “stage of pregnancy” is used to date fetal development beginning from the last menstrual period. Usually, “gestational age” is used interchangeably with stage of pregnancy. However, if gestational age is defined as being 2 weeks prior to the recall of the last menstrual period, then gestational age is equivalent to postconception. In some cases, developmental landmarks such as fetal length or limb development are used to assist in fetal staging and can normalize these discrepancies [10]. However, in many cases these landmarks are not available, as they are destroyed during tissue procurement. With these caveats, a discrepancy in developmental timing between scientific reports may vary by 2–3 weeks of human fetal life. In our lab we use gestational age as an indicator of weeks postconception. Fig. 3 shows an example of the specimens acquired by our lab. In particular, our library includes fetal male and female

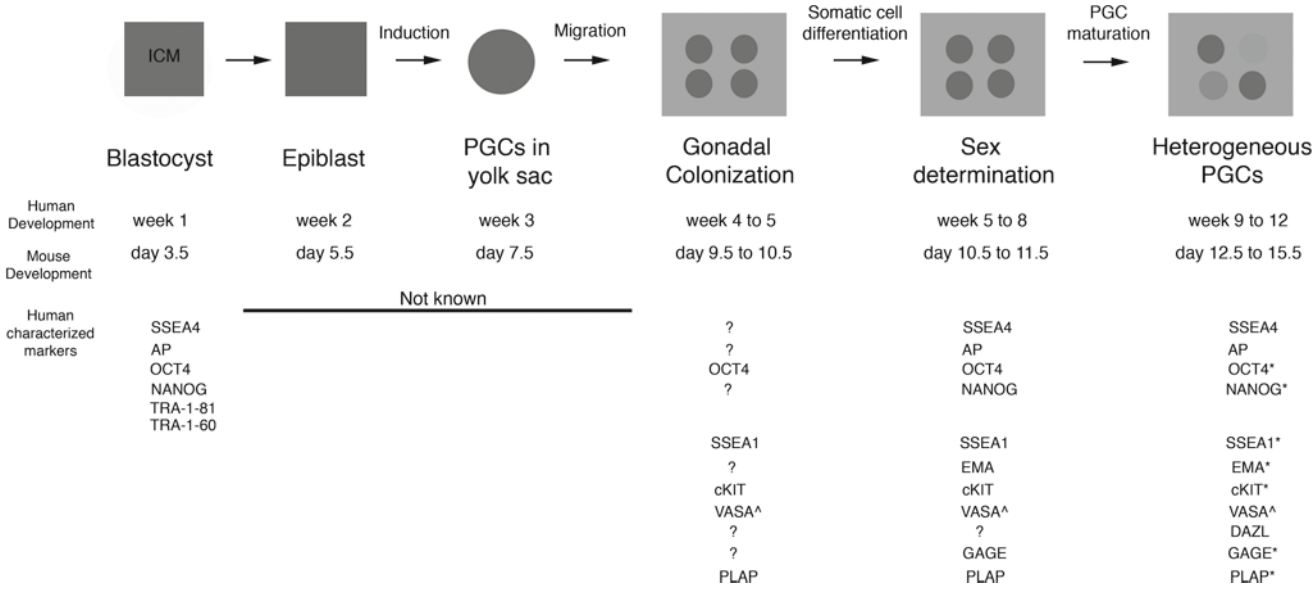

Fig. 2 Time line of human primordial germ cell (PGC) formation showing the major stages occurring in the first 12 weeks postconception. Shown are weeks postconception for human development and the corresponding embryonic day postcoitum in mouse development at each stage. Listed under each stage are PGC markers characterized on human fetal gonads in vivo. Markers prior to 4 weeks of gestation have not been described due to unavailability of tissue (this period is often prior to pregnancy being realized). Asterisks indicate that this PGC marker is found on subpopulations of PGCs. Carets indicate a discrepancy between reports, with VASA being absent by Western blot of whole-fetal gonads [39] but present by immunofluorescence or immunohistochemistry [31, 34, 35, 40]. Question marks indicate that marker has not been evaluated at this stage. Marker analysis was compiled from the cited publications [10, 20, 30, 34, 35, 39, 40]. ICM, inner cell mass

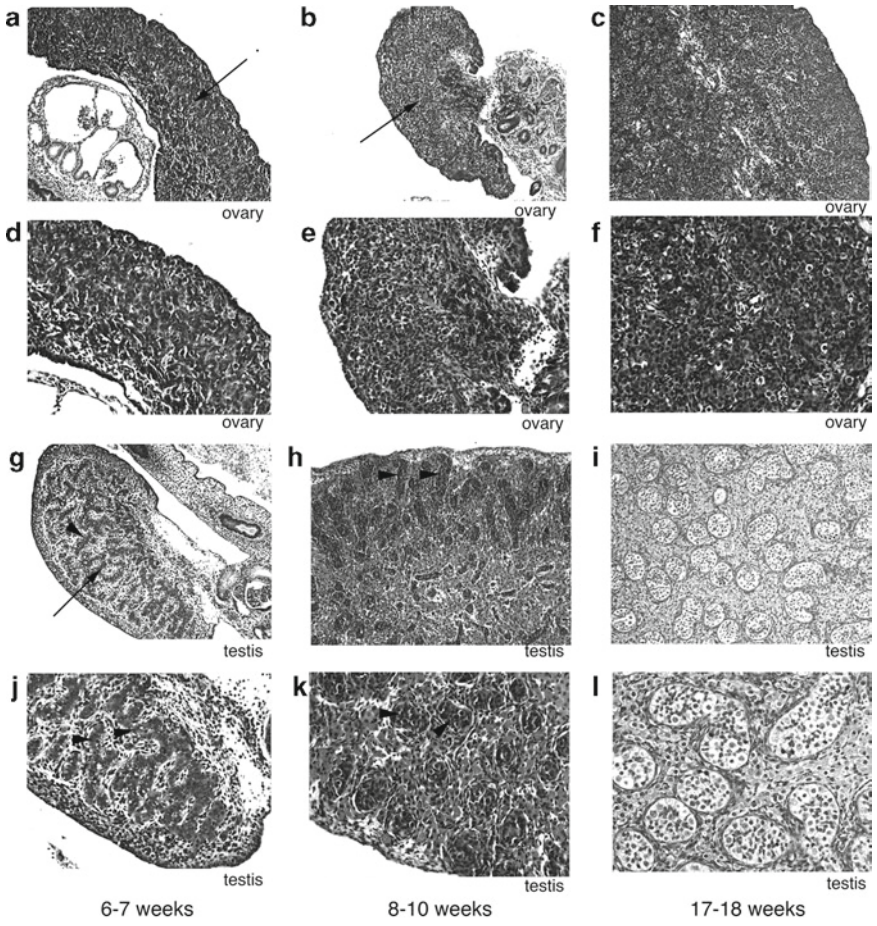

Fig. 3 Histologic sections of human fetal gonads from 6 weeks postconception to 18 weeks postconception. **a–f:** Human fetal ovaries. **g–i:** Human fetal testes. Panels A–C and G–I are at 100× magnification; panels D–F and J–L are at 200× magnification. Arrows show gonad in panels A, C, and H. Arrowheads indicate testicular cords in panels G, H, J, and K. The letter M denotes the mesonephros, which is closely associated with the human gonad in the first 6–10 weeks of gestation (panels A, B, D, and G)

gonads from 6 to 7 weeks postconception to as late as 18 weeks postconception, which can be used for studies on human germ line formation (Fig. 3).

In the first 2 weeks after germ cell specification (weeks 2–4 postconception), PGC differentiation occurs in the absence of a gonad. Once male and female PGCs enter the gonad, they continue to migrate as the organ remodels. By ultrastructural analysis, PGCs during this period have been described as “ameboid-like” on account of their migratory behavior. PGCs retain this description until approximately week 7 postconception [11]. The timing of PGC maturation into gonocytes (Fig. 1, step 2) is not entirely clear because the molecular identity that distinguishes

PGCs from gonocytes in humans remains ambiguous. A recent comprehensive review of fetal gonocytes from mice, rats, and humans suggests that gonocyte differentiation occurs at 7 weeks postconception [12]. This timing would agree with ultrastructural descriptions that somatic cells enclose the PGCs around this time and the PGCs lose their ameboid-like features [11].

The molecular signals that induce the formation of the somatic gonadal anlage prior to 4 weeks postconception are also unknown. Ultrastructural descriptions of week 4.5 fetal gonads demonstrate that they are composed of poorly differentiated mesothelial cells that are actively proliferating [11]. Distinct subpopulations of gonadal somatic cells are visible between 5 and 6 weeks postconception [13–16]. On the basis of simple histology, the fetal ovary and testis have remarkably different morphology at 6–7 weeks (Fig. 3). Therefore, it could be envisaged that the somatic compartments of the gonad could be molecularly specified even before these morphologic changes are observed. In males, steroid production requires the activity of Leydig cells, which become a distinct cell type at 7–8 weeks postconception [11, 17]. Despite their primitive appearance, these cells are apparently functional, as steroid levels in the developing testis begin to increase from 8 weeks postconception [18].

Granulosa cells are the somatic cell types responsible for supporting the differentiating oogonia and oocytes. These cells are identified by ultrastructure at 7 weeks of gestation, similar in timing to the appearance of Sertoli cells in males [11]. The primitive granulosa cells have been described as having long cytoplasmic processes that envelope the developing female PGCs as they migrate through the gonadal anlage [11]. By 9 weeks postconception, meiotic cells can be identified at the caudal end of the gonad closest to the mesonephros [19]. At this time, meiotic germ cells in the female gonad are relatively rare, with most germ cell types still corresponding to primitive mitotic PGCs. From 9 weeks, meiosis proceeds in a wave from medulla to cortex until approximately 13 weeks postconception. During this time, many of the dividing oogonia remain connected by intracellular bridges, suggesting synchrony to the cellular divisions prior to entering meiotic arrest [11].

Stereologic measurements of human fetal ovaries and testes at 6–7 and 9 weeks postconception have determined the numbers of somatic cells and PGCs in a single gonad. The female gonad contains approximately 10 times as many PGCs at both 6–7 and 9 weeks postconception than the male gonad (Table 1) [15, 19]. This stereologic measurement also agrees with reports that enclosure of male germ cells in the seminiferous cords is associated with reduced mitotic index in the germ line [11]. The somatic compartment of the ovary is smaller than that of the testis at 6–7 weeks postconception (Table 1). However, by 9 weeks postconception the number of somatic cells equalizes between the sexes. Taken together, the ratio of somatic cells to germ cells in the ovary from weeks 6 to 9 remains relatively constant at 6:1. In contrast, the ratio of somatic cells to germ cells in the fetal testis changes from 175:1 at 7 weeks to 58:1 at 9 weeks postconception (Table 1). It is not clear whether this change will be an important consideration as we recreate the gonadal niche for male PGC differentiation in vitro into gonocytes and premeiotic germ cells.

Table 1 Stereologic measurements of fetal ovary and testis

Sex	Weeks postfertilization	Number of somatic cells	Number of germ cells	Ratio somatic: Germ cells
Female	6–7	165,000	26,000	6:1
Male	6–7	526,000	3,000	175:1
Female	9	1.5×10^6	250,000	6:1
Male	9	1.74×10^6	30,000	58:1

At 9 weeks postconception, there is obvious heterogeneity within the germ cell population of both male and female gonads (Fig. 2). Whether this heterogeneity is found in germ cells prior to 9 weeks has not been sufficiently addressed due to technical difficulties with fetal dissection and gonadal isolation at these time points. With current germ line markers, migrating and colonizing human PGCs appear relatively homogeneous prior to weeks 7–8 postconception (Fig. 2). However, after this time many subpopulations can be observed based upon studies using multiple germ line markers in the same histologic section. This heterogeneity is most clearly illustrated in male germ cell differentiation after 12 weeks of gestation, where three populations of male germ cells are described, including gonocytes, and two types of premeiotic cells called intermediate cells and prespermatogonia [10, 20]. More-advanced stages of human germ cell development such as those described by Gaskell et al. [10] and the molecular events that coordinate the differentiation of steps 2–6 to create haploid gametes are important areas of discussion but are beyond the scope of this chapter.

Taken together, specification of human PGCs, together with the formation of the human fetal gonad and the integration of germ cells into the gonad, is a well-orchestrated and dynamic process. How epigenetic changes during PGC development coordinate with these events will be an important avenue for investigation and critical to understanding appropriate development for PGC differentiation from pluripotent stem cells in vitro.

3 Nuclear Remodeling and Chromatin Dynamics in Primordial Germ Cells

The PGC period is defined by significant nuclear and chromatin remodeling, which has been most comprehensively studied in the mouse. Murine PGC specification results in the formation of a cluster of 40 PGCs at the base of an extraembryonic structure outside of the embryo proper called the allantois. This is most likely the equivalent cell type observed by Witshi in 1948 (Fig. 2). Originally these definitive PGCs were derived from approximately four to six cells expressing the transcriptional repressor PR domain containing 1 (Prdm1) that emerged from the Wnt3 primed epiblast [9, 21, 22]. Loss of Prdm1 results in fewer specified PGCs and abnormal expression of genes that are associated with mesoderm differentiation [21]. Therefore Prdm1 is considered one of the master regulators of PGC

specification. A second PR domain–containing protein (Prdm14) is also required for PGC specification and is involved in regulating the reexpression of the pluripotency program in the germ line as well as the switch from global histone H3K9 dimethylation to H3K27 trimethylation [23].

Once specified, these 40 PGCs proliferate and migrate into the embryo and toward the gonad, entering the genital ridges at e9.5–e10.0. By e11.5, the PGCs have completely colonized the gonads, and within the next 24 hours undergo massive germ line reprogramming involving loss of linker histones, genome-wide demethylation, including loss of methylation at imprinted genes, and reactivation of the X chromosome in female germ cells [1, 2, 4–6]. For an excellent review of epigenetic remodeling in murine PGC development during this period refer to Hayashi and Surani [24]. By extrapolation, these fundamental events must be occurring in human PGCs sometime between weeks 5 and 8 postconception; however, the exact timing remains to be accurately determined (Fig. 2). Therefore, to effectively study this developmental stage it will be imperative to build an in vitro model that recapitulates in vivo human PGC formation.

4 Current State of the Art for Generating Primordial Germ Cells from Human Pluripotent Cells

PGCs do not emerge directly from the inner cell mass (ICM) of the blastocyst. Instead, human PGCs are presumably, based on the murine model, induced in the epiblast some time during the second week postconception (Fig. 2). Given that human embryonic stem cells (hESCs) are hypothesized to more closely resemble epiblast cells than ICM, the specification of PGCs in vitro should be a relatively rapid event. Furthermore, it could be hypothesized that the subsequent differentiation of PGCs to an equivalent cell type of a late-migrating, early-colonizing PGC in vivo would only require 2–3 weeks of differentiation. Even though hESCs have an epiblast-like morphology when cultured under self-renewing conditions, hESCs also express many genes known to be associated with PGC development, including deleted in azoospermia (DAZL), NANOS, PUMILIO, and DPPA3 [25]. The expression of these germ cell–specific genes does not necessarily indicate that hESCs and PGCs are equivalent cell types. However, it does suggest that the partial expression of a PGC program in undifferentiated hESCs may accelerate the pace of PGC fate choice in vitro.

Differentiation of human PGCs from hESCs has been performed by a number of groups (Table 2). The initial studies validating the use of pluripotent stem cells as a model to differentiate germ line used simple marker analysis to show that PGCs existed in a population of differentiating cells [25]. In the original description differentiation was performed using embryoid bodies (EBs) in media containing 20% fetal bovine serum (FBS) in the absence of fibroblast growth factor 2. In this work the gene VASA, an RNA helicase uniquely expressed in germ cells, was transcribed between days 3 and 5 of differentiation. Evaluation of EBs

after 2–3 weeks of differentiation revealed that VASA-positive cells localized in clusters within EBs, suggesting the presence of a local niche. Furthermore, addition of BMP2, 4, and 7 to the media from the start of differentiation increased the proportion of VASA-positive cells present in EBs [26]. Following these studies, other groups also showed evidence for germ cell development by reporting increased germ cell–specific gene expression changes that correlate with modifications in culture conditions [27,28]. However the isolation of presumptive PGCs from differentiating hESCs was not performed in these studies.

Recently, new methods for differentiating and isolating PGCs from human pluripotent stem cells, including human induced pluripotent stem cells, have been published [29–31]. The complexity of differentiating PGCs from human embryonic stem cells (hESCs) was best illustrated in the work of Bucay et al. [29], which revealed that the method used to routinely passage hESCs under self-renewing conditions significantly impacts the ultimate differentiation of PGCs in vitro. For example, hESCs are routinely cultured as tight clusters with well-defined borders often on a cell-based supporting layer of mouse embryonic fibroblasts (Fig. 4). When the colonies are at density (almost touching), the hESCs are split using a method that breaks the colony into smaller pieces. This means that hESCs are often cultured as heterogeneous mixtures with highly variable colony sizes (Figs. 4a, b). Furthermore, because colony size is difficult to control, the larger colonies can initiate random nondirected differentiation characteristically at their edges (Fig. 4c). In addition, in any given culture there is usually a small fraction of colonies that are fully differentiated (Fig. 4d). The work of Bucay et al. showed that by simply changing the passaging technique to maintain the small colonies corresponding to either 11–20 or 21–30 cells per colony and changing the media once between passages, cells positive for CXCR4 are induced [29]. Isolation of these CXCR4-positive cells showed that they were enriched in germ cell–specific markers such as cKIT and PRDM1 [29]. CXCR4 is a known marker of murine PGCs in vivo [32]. However, the stage at which CXCR4 is expressed in human PGCs has not been determined. Although the mouse has been extremely informative in guiding identification of the human germ line, there have been exceptions in which mouse and human PGC development are different. For example, expression of SOX2 is found in murine but not human PGCs [33]. Therefore, the use of human gonadal tissue to validate markers for human PGC formation should be incorporated into the experimental design wherever possible when generating and validating new markers of PGC formation in vitro.

In parallel to the aforementioned study, a second group independently showed that human PGCs could be differentiated using two-dimensional differentiation on gelatin [30] (Table 2). However, in these studies, instead of using CXCR4 as a marker, the surface sugar SSEA1 was used. SSEA1 is expressed on the surface of male and female PGCs starting from 5 weeks of gestation (the earliest time point evaluated to date for this surface marker) [31, 34, 35]. SSEA1 remains expressed on subpopulations of PGCs until 13 weeks postconception in female and male fetal gonads (Fig. 2). Between weeks 8 and 13 there is significant discordance in expression of germ cell genes between individual PGCs in both male and female gonads

Table 2 Primary works describing human primordial germ cell differentiation from pluripotent stem cells in vitro

Method	Cell line	Media	Marker	Time of differentiation (d)	Isolation of PGC	Reference
EBs	HSF-6, HSF-1, H9	20% FBS	VASA	Up to 21	No	25
EBs	H9	20% FBS + BMP2, 4, 7	VASA	1–3	No	26
Adherent on MEFs	BGO1	20% KSR	VASA, OCT4	Up to 30	No	27
Adherent culture	NTU1, NTU2	20% FBS + hLIF	cKIT, DPPA3, VASA, GDF9	Up to 21	No	28
Adherent on gelatin and EBs	H9, NCL1	20% FBS	SSEA1	1–28	Yes	30
Adherent on gelatin	HSF-6, H9	20% KSR + FGF2, then 10% FBS in complex media + nicotinamide	CXCR4	6, then 10	Yes	29
Adherent on primary hFGS	HSF-6, HSF-1, H9, hIPS1,[a] hIPS2[a]	20% FBS	SSEA1 plus cKIT	7	Yes	31

BMP, bone morphogenetic protein; EB, embryoid body; FBS, fetal bovine serum; FGF2, fibroblast growth factor 2; hFGS, human fetal gonadal stromal cells; hLIF, human leukemia inhibitory factor; KSR, knockout serum replacer; MEF, mouse embryonic fibroblasts; PGC, primordial germ cell.

[a]First description of PGC formation from human induced pluripotent stem cells.

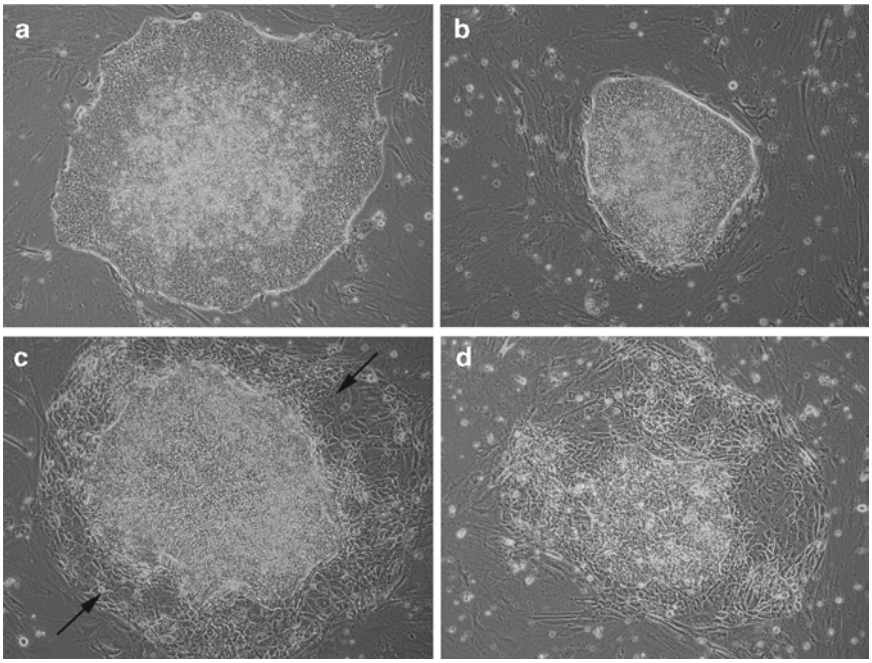

Fig. 4 Human embryonic stem cells (hESCs) are cultured as heterogeneous colonies under self-renewing conditions. All micrographs are taken at the same magnification of the hESC line HSF-1 (20×). **a:** A large, undifferentiated hESC colony with well-defined borders. **b:** A smaller, undifferentiated colony with well-defined borders. **c:** A colony that has differentiated around the outside (*arrows*), whereas the inner portion of the colony continues to display morphologic characteristics of an undifferentiated state. **d:** A fully differentiated colony. In any given culture all types of heterogeneity exist.

due to the asynchrony in differentiation during this period. However, in females at 13 weeks postconception, SSEA1 is absent from oocytes that have entered meiosis. Furthermore, female meiotic germ cells are also negative for cKIT and OCT4 [35]. In the male, SSEA1 expression peaks on PGCs at 8 weeks postconception and is reduced to background together with OCT4 and NANOG as development progresses. In contrast, the expression of cKIT on germ cells in males significantly increases in number after this period. Therefore, sorting for positive cells expressing SSEA1, cKIT, OCT4, and placental-like alkaline phosphatase from male or female hESC lines should theoretically yield the PGC population (Fig. 2). Furthermore, negative selection for the surface markers Tra-1-81 and Tra-1-60, which are found on undifferentiated hESCs but not PGCs, may also be a method for isolating a highly enriched population of PGCs (Fig. 2) [35]. Taken together, these suggest that using SSEA1 alone would enrich for PGCs. However, given that SSEA1 expression has been reported on many other fetal cell types, including neural stem cells, epithelia of the reproductive tract, and renal tubules [36], the risk of using only SSEA1 expression in a nondirected differentiation strategy would include the possibility of

contaminating the SSEA1-positive population with other non–germ line cell types.

Using adherent differentiation on gelatin, Tilgner showed that SSEA1-positive cells correspond to 5% of the population after 3 weeks of differentiation in media containing 20% FBS [30]. These SSEA1-positive cells were also positive for SSEA4, a second marker that is found on both human PGCs and somatic cells of the fetal gonad in vivo (Fig. 2). Molecular analysis of sorted SSEA1-positive cells 3 weeks after differentiation revealed expression of germ cell genes including VASA, as well as loss of DNA methylation at the imprinted genes H19 and IGF2, together with chromatin changes at some key loci [30]. Of interest, a small group of haploid cells was identified in the SSEA1-positive population at 3 weeks, and meiosis-specific mRNAs, SCP1 and SCP3, were enriched in these cells compared to the SSEA1-negative cells. These SSEA1-positive meiotic cells in vitro are most likely precociously differentiating PGCs, as it is clear that in vivo meiotic cells are SSEA1 negative.

In the most recent study published by our group, PGC differentiation was evaluated using double labeling for two surface markers, SSEA1 and cKIT [31]. We chose to evaluate PGC formation after 7 days of differentiation to capture the earliest phases of human germ cell development in vitro when the PGC population would still be relatively homogeneous. Prior to initiating differentiation, we evaluated the utility of these two surface markers by first staining human fetal gonads from 5 to 9 weeks postconception and confirming germ cell–specific coexpression with OCT4 and VASA [31]. We also performed flow analysis for SSEA1 and cKIT using isolated human fetal gonads at 7 weeks postconception and found that SSEA1 and cKIT colocalized in human fetal gonads in vivo. For our pluripotent stem cell differentiation system, we chose a two-dimensional culture method similar to those of Bucay et al. [29] and Tilgner et al. [30] in 20% FBS. However, we initiated hESC differentiation directly on human fetal gonadal stromal cells that were isolated as primary cells from a 10-week postconception human fetal testis. Our hypothesis was that supplying a gonadal supporting layer during differentiation would have a positive effect on promoting a fate choice toward PGCs. This is because a partial PGC program is found in undifferentiated hESCs cultured under self-renewing conditions. We found that differentiation on primary gonadal supporting cells for 7 days resulted in a yield of 5%–10% of cells that are double positive for SSEA1 and cKIT. Molecular analysis of these sorted cells indicated enrichment in germ cell markers such as VASA, PRDM1, and DPPA3 and repression of somatic markers such as the HOX genes. The early stage of human PGC differentiation by this method is best illustrated by the finding that CpG DNA demethylation had started but ranged from only 12% to 48% loss of methylated CpGs at a given imprinted loci. Extrapolating these data to mouse development in vivo would imply that at 7 days of hESC differentiation, the SSEA1 and cKIT double-positive cells correspond to PGCs in the migration stage (prior to 4–5 weeks of gestation). This result suggests that the gonadal cells are not contributing novel factors to promote differentiation toward a gonadal-stage PGC in vitro during the first 7 days of differentiation. We have evidence to support this assumption, as differentiation on

primary nongonadal human cells (fetal liver and placenta) still supports the emergence of SSEA1 and cKIT double-positive cells in vitro. However, the percentage of positive cells was twofold lower on these nongonadal cell types [31]. In future studies it will be interesting to determine what the factors are in gonadal stromal cells that improve PGC formation over nongonadal cell types. Furthermore, characterization of the molecular identity of PGCs acquired following differentiation under multiple conditions will be necessary to evaluate the importance of these different niches during the initial phase of PGC differentiation in vitro.

5 Is Formation of Haploid Gametes from Human Pluripotent Cells a Reality or a Myth?

Although the state of the art is currently at step 1 in germ cell formation in vitro, it is expected that in the future, the haploid gamete at step 5 will be achieved (Fig. 1). In April 2008, the Hinxton Group, an International Consortium on Stem Cells, Ethics and Law, met in Cambridge, England, to discuss the science, ethics, and policy challenges of pluripotent stem cell–derived gametes. The result of this meeting was a consensus statement outlining a set of principled recommendations for how research ought to proceed (http://www.hinxtongroup.org/au_pscdg.html). Notes and follow-up to this meeting are given by Mathews et al. [37]. At the time of this meeting, the technology for differentiating haploid gametes from pluripotent stem cells was not available. The technology remains theoretical. However, recent achievements in the nonhuman model indicate that the generation of viable functional gametes from pluripotent stem cells in a dish will be a reality in the future [9]. The burden of proof for correct germ cell formation is the birth of live young following fertilization of an in vitro–derived gamete. An even more stringent and necessary requirement is that the animal also be healthy and fertile [9]. This test is reasonable and essential in the murine model. However, the method for ensuring that this same level of quality is achieved from human in vitro–derived germ cells is currently under discussion. This is because of the sensitive ethical issue related to the generation of human embryos as a functional test for in vitro–derived gamete quality. Scientifically, however, there appear to be two major bottlenecks in the formation of in vitro gametes. The most challenging hurdles are the appropriate reprogramming of PGCs during fetal life to create the sex-specific gametes (Fig. 1, steps 2 and 3) and creation of a haploid genome through the process of meiosis and especially homologous recombination (Fig. 1, steps 4 and 5) [38]. The challenges of meiosis are beyond the scope of this chapter. However, chromatin remodeling in the germ line may be addressed starting from undifferentiated hESCs cultured under self-renewing conditions. Our work using gonadal stromal cells showed that although PGC chromatin remodeling is initiated at imprinted genes during in vitro PGC formation, the presence of an abnormal chromatin signature in the self-renewing state prevents correct reprogramming in the primitive PGCs at day 7 of differentiation [31]. It remains to be seen whether this represents a delay in reprogramming that

could be corrected by increased time in differentiation conditions. Alternatively, the chromatin state of some self-renewing ESC lines makes them useless for the formation of quality PGCs and gametes because the chromatin at key loci cannot be accurately remodeled.

6 Conclusions

Generation of haploid human germ cells from pluripotent stem cells will be a remarkable achievement. At the present, the initial step in human germ cell development, the primordial germ cell (PGC), is the most promising platform from which to build further differentiation strategies to achieve this goal. However, more work is required to ensure that PGCs formed in vitro closely recapitulate the PGCs that form in vivo. So far, insufficient comparisons have been made to human PGCs in the majority of studies, and this information is essential to ensuring that PGC development proceeds as expected. Furthermore, the technology for differentiating PGCs and the methods for identifying them in culture are not consistent between groups. In addition, the burden of proof for demonstrating PGC formation in vitro is becoming increasingly stringent, to the point where it should no longer be considered acceptable to show germ cell–specific markers within a population as being evidence of PGC formation without isolation of the presumptive PGCs. Future work on human PGC differentiation in vitro needs to reconcile the various methods of PGC formation that have recently been reported. Ideally, this would be performed using multiple cell lines and multiple sites in order to determine the most robust method among independent laboratories. After this point, the field can move forward into the complex task of differentiating steps 2–5 and ultimately creating the haploid gamete.

In 2009 Kee et al. [41] reported that hESCs can be used to generate haploid sperm with 2% efficiency. This was achieved by over expressing three cytoplasmic RNA binding proteins, Deleted in Azoospermia (DAZ), DAZ Like (DAZL) and BOULE. It will be important in future studies to determine the efficiency of this remarkable achievement in a cohort of cell lines including hIPS cells, and to begin the discussion of how to best test functional capacity and quality of in vitro derived haploid cells.

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Prospects for Induced Pluripotent Stem Cell Therapy for Diabetes

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Abstract Although diabetes can be managed clinically with the use of insulin injections, it remains an incurable and inconvenient disorder. In the long term it is associated with a number of clinical complications, and there is a desire to see new methodologies to replace defective cells and provide a lasting normality without the need for drug treatment. Pluripotent stem cells offer the potential to generate any cell of the body as a source of cells for cellular therapeutic approaches. There remain many issues, however, that must be addressed before this type of treatment could become a reality. Induced pluripotent stem (iPS) cells offer a possibility of circumventing the ethical and immunologic response issues associated with the use of embryonic stem cells. There is, however, a need for a greater understanding of the underlying biology of diabetes and iPS cells for this form of therapy to become a reality.

Keywords Diabetes • Stem cells • Therapeutics • Review

1 The Impact of Diabetes

Diabetes mellitus (DM) is a metabolic disease that affects an estimated 170 million people worldwide [1]. It is characterized by high blood glucose levels caused by the progressive failure of the insulin-secreting β cells of the islets of Langerhans in the pancreas and can be subdivided into two subclasses. In type I (insulin-dependent) DM, pancreatic β cells are progressively destroyed, probably by environmental triggers that activate autoimmune mechanisms in genetically susceptible individuals [2]. This is evident with β cell mass being reduced by approximately 70%–80% at the time of diagnosis of the condition [3]. Type II (non–insulin-dependent) DM is characterized by impaired insulin secretion caused by defects in

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the β cells resulting in an inability to appropriately increase insulin secretion in response to rising glucose levels. This occurs alongside increased glucose production by the liver and a decrease in tissue (especially in skeletal muscle) response to the secreted insulin [4]. Consequently, β cell apoptosis results from the chronic hyperglycemia that occurs. Although the interplay between these two pathogenic pathways is not fully understood, studies have suggested that a degree of mild insulin resistance might occur long before glucose intolerance develops and prior to β cell dysfunction [4]. Currently, type I DM affects 3–4 people per 1000 in the United Kingdom by the age of 20 years, and the incidence of both types of diabetes is rising steadily [5]. Diabetic treatment regimens require constant attention to diet and exercise, regular blood tests, and up to four daily injections of insulin.

Normally blood glucose levels are kept within a relatively tight window (3.5–7.0 nmol/L). Although levels rise following ingestion of a meal, they return to this baseline level 2–3 hours later as a result of endocrine pancreatic secretion of insulin. Plasma levels of insulin generally follow a similar pattern. In type I DM, the lack of functional pancreatic β cells leads to failure of this normal response to rising blood glucose levels, resulting in long-term health consequences as described later, and it may even be potentially fatal in the short term. Although blood sugar levels can adequately be controlled by the subcutaneous injection of insulin in order to relieve symptoms and prevent the development of diabetic ketoacidosis, this form of therapy does not provide a particularly physiologic insulin profile. As a result, one of the major difficulties with current regimens is insulin excess potentially leading to episodes of hypoglycemia.

Advances in pharmacologic techniques, especially those involving recombinant DNA technologies, have resulted in the generation of insulin analogs with improved pharmacokinetic profiles when used as subcutaneous preparations. Preparations such as insulin glargine crystallize in the alkaline environment of the subcutaneous injection site and result in prolonged release compared to more acute-acting types of insulin and therefore provide less of a nonphysiologic insulin peak following administration [6]. These preparations, however, often require additional administration of short-acting insulin treatment following meals. Continuous subcutaneous insulin infusion has been regarded as a possible answer to the problems associated with long-acting insulins and is currently being used by an estimated 20% of patients with type 1 DM in the United States [7]. Despite its efficacy, this therapy still requires continued blood glucose monitoring and adjustment of infusion rates, together with a substantial training program for the user if he or she is to maintain a degree of personal independence. Nevertheless, it still is not possible to effectively provide exogenous therapeutic insulin that mirrors the normal physiologic response.

Current therapy still only gives limited protection against late complications of diabetes, and although it permits a relatively normal lifestyle, it is by no means a cure. It is likely that 30% of children with DM will go on to develop irreversible renal disease and that more than 50% will require laser therapy to protect their vision. It has therefore become increasingly apparent that new therapeutic options

in the treatment of diabetes are desperately needed. The ideal therapy would allow effective cell replacement to restore the patient's normal physiologic insulin secretion without having to resort to repeated invasive monitoring and exogenous insulin administration by injection.

2 Pathophysiology and Complications of Diabetes

Although for the most part, DM complications are as a result of chronically elevated glucose levels, type I DM can lead to acute diabetic ketoacidosis due to severe hyperketonemia, resulting in a life-threatening metabolic acidosis with mortality as high as 5%–10%. Similarly, in type II DM, hyperglycemia can result in the development of the hyperosmolar nonketotic state with a massively elevated plasma osmolality, with mortality rates exceeding 30% in the older population [8]. Chronic DM complications can be considered as having two main classifications: microvascular and macrovascular. The microvascular complications include nephropathy, neuropathy, and retinopathy and can be considered as representing damage to the body's microcirculation, these tissues being heavily dependent on their microvascular blood supply. The basement membrane of these vessels becomes thickened, resulting in a diabetic microangiopathy with altered vessel function and impaired ability to deliver the required oxygen and nutrients to meet the target tissues' requirements [9]. Eventually this results in complete loss of microvessels with subsequent end-tissue damage. Endothelial cells in the retina, neurons, and mesangial cells in the renal glomerulus are also sensitive to hyperglycemia-induced molecular responses [9]. Microvascular complications may to some degree be controlled by strict adherence to normoglycemia.

Macrovascular complications principally result from atherosclerotic vascular damage to the cerebral, cardiac, and peripheral vasculature, with resultant increased risk of stroke, cardiac ischemia, and peripheral vascular disease, respectively. This atherosclerosis may be the result of multiple different mechanisms, generally resulting in the activation of several proinflammatory and proatherosclerotic genes in the endothelial lining of blood vessels. Atherosclerosis often behaves more aggressively in DM than in nondiabetic patients [8]. Generally the progression of atherosclerosis is relatively unaffected by diabetic control measures.

3 Stem Cells

For some time DM has been considered to be a potential candidate disease for medical cellular therapeutic intervention [10]. In particular, stem cell therapeutics has been proposed as a means of β cell replacement. This includes the potential use

of cells differentiated from human embryonic stem (hES) cells lines. hES cells are isolated from the inner cell mass of the developing human blastocysts sourced from excess embryos produced as a result of in vitro fertilization [11]. These cells are immortal, capable of unlimited self-renewal, and therefore offer an infinite supply of scalable cells for transplantation. As these cells possess the ability to differentiate into cells from all of the three germ layers, they have the potential to generate all cell types from all tissues of the body. There are, however, a number of concerns over the use of hES cells as a tool for therapeutic transplantation. First are the ethical concerns inherent in the use and disposal of human embryos. This has caused heated debate and regulation over the use of these cells. Second, hES cells are tumorigenic, giving rise to teratoma formation in animal models. Any cells for transplantation differentiated from these cells would therefore need to be completely free of any of the hES cells from which they were derived. Finally, differences in major and minor histocompatibility complexes (MHCs) present as antigens on any form of graft tissue cells can trigger an immune response from the host that will result in the rejection of the graft by the host. Although self-renewing hES cells generally express low levels of MHC antigens, these levels are gradually upregulated during their subsequent differentiation to specific tissues cell types, leading to concerns about the possibility of inducing graft versus host responses from tissue derived from this type of cell. This will necessitate the creation of a bank of qualified and histocompatibility-typed hES cell lines for transplantation to address these issues.

The ability to generate a supply of pluripotent stem cells directly from the somatic cells of affected individuals would provide the basis of autologous transplantation regimes in what has been loosely termed “patient-specific cellular therapeutics.” This would avoid the risk of immune rejection or requirement for long-term immunosuppressive therapies, as somatic cells from the patient’s body would be treated in a manner to create stem cells than resemble embryonic stem cells. These cells could be subsequently differentiated into the required tissue type (possibly after correction of any genetic defects) and be used to treat the patient’s defective tissue. In addition to avoiding any issue with graft versus host reactions, this type of treatment would circumvent the ethical concerns associated with use of embryo-derived cell types. Several methods have been considered in order to induce somatic cells to revert to an embryonic state, making them suitable for further differentiation. Strategies including nuclear transfer, cellular fusion, and induced reprogramming with defined factors have all been employed.

Nuclear transfer was first described in 1952 by Briggs and King [12], who demonstrated the creation of normal hatched *Rana pipiens* tadpoles following the transfer of nuclei from blastocysts into enucleated eggs. Although many examples of successful embryonic nuclear transfer have been reported, it proved difficult to successfully accomplish nuclear transfer from a differentiated mammalian adult cell. A major breakthrough was accomplished by Wilmut’s group with the creation of Dolly the sheep from nuclei derived from cultured adult mammary gland cells [13]. Other mammals have since been successfully cloned, including mice, cows, goats, pigs, rabbits, and cats (for review see ref. 14). While the production of adult animals

from this method remains somewhat inefficient, in contrast the frequency of the derivation of ES cells from blastocysts created by nuclear transfer appears similar to that of ES derivation from natural conceptus [15]. This has lead to a theoretical possibility of creating "patient-specific" ES cells through the transfer of somatic cell nuclei from a patient into a human oocyte. Although breakthroughs in this area have been reported and retracted [16], nuclear transfer is likely to remain an active area of stem cell therapeutic research.

The main barrier to using nuclear transfer to generate "patient-specific" embryonic stem cells is the limitations associated with the access to donated human oocytes. An alternative approach that has been considered is reprogramming via the fusion of somatic cells with previously isolated hES cells. This rationale is an extension of that involved with standard cloning, but in this case via the use of an existing hES cell. The first demonstration of this technique involving human ES cells was by Cowan et al. in 2005 [17], who demonstrated the fusion of human fibroblasts with hES cells, which resulted in hybrid cells with similar morphology, growth rates, and antigenic expression to those of hES cells. The original fibroblasts were marked with antibiotic resistance genes via retroviral transduction in order to allow easy selection of any resultant hybrid cells. The hybrid cells were found to be tetraploid and to contain both somatic and hES cell chromosomes. The main limitation of the therapeutic use of these cells is thus due to the continued presence of the hES chromosomes. This issue has been addressed to a degree by the development of techniques to eliminate specific chromosomes from the resultant hybrid cells. Matsumura et al. demonstrated such a technique with the removal of specific ESC-derived chromosomes from fused hybrid cells using genetic targeting techniques [18].

There still remains a lot of work to be done on ES cell fusion techniques if this technique is to provide a viable clinical resource. There is still a strong possibility of rejection following transplantation of tissues generated from these cells due to persistent expression of ES cell antigens in the resultant hybrid cells [19]. Improved techniques capable of removing the entire complement of ES cell chromosomes and avoiding recombination between host and transferred chromosomes are needed.

3.1 Induced Pluripotent Stem Cells: Cellular Reprogramming by Defined Factors

It has long been considered that ES cells may contain specific and dominant genes or factors that enable them to self-renew and maintain their pluripotent state. Indeed the ES fusion techniques listed previously support this hypothesis, as ES cell/somatic cell fusion can result in cells that maintain a pluripotent phenotype via the reprogramming of the somatic chromosomes [20]. Following on from this hypothesis, it was considered possible to induce and maintain pluripotency by the introduction or activation of specific factors within somatic cells. The first study that employed this theory was published in 2006 by Takahashi and Yamanaka [21].

In this study, they systematically overexpressed genes in mouse embryonic fibroblasts (MEFs) that were known to be expressed in ES cells in order to assess their ability to induce pluripotency. An assay based on the expression of drug resistance linked to the expression of the *Fbx15* gene was used to assess the effects of each of the factors when introduced to the fibroblasts by retroviral transduction. No single factor was capable of inducing pluripotency; however, when pooled, the overexpression of these genes led to multiple ES-like cell colonies being produced. By gradually reducing the number of factors used, four factors were ultimately determined to be key modulators of reprogramming to these ES-like cells. These genes were Oct3/4, Klf4, Sox2, and c-Myc. The resultant ES-like cells were designated induced pluripotent stem (iPS) cells. These iPS cells were analyzed by reverse transcription-polymerase chain reaction and DNA microarray in order to compare marker gene expression and were found to express the majority of specific ES cell marker genes, although microarray analysis demonstrated that there are some differences from embryonic stem cells. The pluripotency of these cells was confirmed by teratoma formation, where the cells differentiated into all three germ layers, with evidence of neural tissue, cartilage, and columnar epithelium. In these particular studies a major issue was the inability of the created mouse iPS cells to produce adult chimera mice and subsequent germline transmission, leading to concerns that the cells were not fully reprogrammed.

In 2007, three groups generated iPS cells that were capable of adult and germline chimeras [22–24]. These groups used Oct4 and Nanog activation to select for pluripotency, resulting in cells that were both epigenetically and biologically indistinguishable from normal ES cells. The Meissner method [22] involved picking colonies based upon morphology and eGFP expression instead of using drug resistance as in the Takahashi model [21]. Meissner et al. also demonstrated the creation of an iPS cell line from genetically unmodified mice that was capable of generating chimeric mice. Wernig et al. used MEFs carrying a neomycin resistance marker present in either the endogenous Oct4 or Nanog [24]. This group also demonstrated that although pluripotency is initially established by the virally transduced factors, this pluripotency is largely maintained by the activity of endogenous pluripotency factors, as the viral factors are largely silenced by de novo methylation. Okita et al. [23] demonstrated iPS generation using the four retroviral factors following selection for Nanog. As with the other groups, these cell colonies were capable of generating adult chimera mice and could be transmitted through the germline. Around 20% of the adult chimeras formed from these cells developed tumors, attributable to the reactivation of the c-Myc retrovirus. This propensity would obviously limit the potential clinical applications if the foregoing methods were used to generate iPS cells to be differentiated to transplantable cells.

Soon after these studies, iPS cells were generated from human fibroblasts. Takahashi and Yamanaka again used the same four factors and a retroviral transduction method in order to induce pluripotency in adult human dermal fibroblasts [25]. In this case, to increase the transduction efficiency, a lentiviral vector was used to introduce the mouse retroviral receptor Slc7a1 into the fibroblasts prior to retroviral

induction. This resulted in the formation of ES-like cell colonies at day 30. The human iPS cells created in this way were morphologically similar to ES cells, and in addition had similar surface markers, gene expression, telomerase activity, in vitro differentiation, and teratoma formation. There was evidence that the retrovirus expression was strongly silenced following transduction; however, there also was evidence of at least 20 retroviral integration sites per clone (around three to six per factor). Park et al. [26] isolated iPS cells from human ES cell–derived fetal fibroblasts (in this case differentiated ES cells expressing GFP and neomycin resistance genes integrated into the Oct4 locus). The cells were infected with a cocktail of retroviral supernatants from the four factors. Following this, they generated iPS cells from primary fetal tissue, as well as from adult fibroblasts. In these studies Oct and Sox appeared to be essential for reprogramming with either Klf4 or c-Myc, enhancing the efficiency of colony formation. Yu et al. [27] also successfully demonstrated the reprogramming of human somatic cells to pluripotency but accomplished this by using Oct, Sox, Nanog, and Lin28.

Oct4 and Sox2 therefore appear to play a pivotal role in the generation of iPS cells, with other factors being interchangeable. Huangfu et al. [28] successfully generated iPS cells from primary human fibroblasts using retroviruses expressing only Oct4 and Sox2 in an optimized protocol exposing the cells to valproic acid (a histone deacetylase inhibitor shown to improve reprogramming efficiency in MEFs). This potentially has clinical application advantages due to the elimination of two oncogenes from the transduction process. In 2009, Kim et al. [29] demonstrated that a single factor could induce the formation of iPS cells by using only Oct4. In this case adult mouse neural stem cells were used as the source cell type. This single-factor induction has not yet been reported in human cells, although this certainly will be a major area of research.

Other reprogramming methods have been successfully implemented following the original retroviral methods. Virus-based reprogramming methods results in varying degrees of viral integrations into the somatic genome. This obviously carries with it safety concerns over endogenous gene activation or inactivation, as well as transgene persistence or reactivation after differentiation. A single iPS clone can have 20–40 viral integration sites, and although it might be possible to perform whole-genome sequencing to map these sites, it may be difficult to be completely sure of their clinical safety [30]. Since the initial retroviral-mediated generation of iPS cells, several groups have successfully employed other methods of reprogramming somatic cells to the pluripotent state, including plasmids [31], lentiviruses [32], excisable transgenes [33, 34], small molecules, and recombinant proteins [35].

iPS cells created by plasmid transfection have been reported by Okita et al. [31], who used two individual plasmid vectors containing the CAG promoter, one containing Oct4, Klf4, and Sox2 and the other containing c-Myc. Using a transfection protocol targeting embryonic fibroblasts on days 1, 3, 5, and 7, they successfully created mouse iPS cells, with no evidence of genomic integration of the plasmid DNA. These clones successfully generated teratomas and chimeric mice. Soldner et al. [32] used doxycycline-inducible lentiviral vectors of the four reprogramming factors that were excisable with Cre recombinase. Using this method,

they generated 16 clones with no evidence of integration of any of the viral reprogramming factors.

Recently, two groups have used excisable transgene technology to allow virus-free integration of the programming factors, followed by their subsequent removal [33, 34]. Both groups used the 2A peptide sequence from foot and mouth disease virus to link the sequences of c-Myc, Klf4, Oct4, and Sox2, which allows for efficient multiprotein expression. Transduction to the pluripotent stem cell state was first achieved by introduction of the single vector 2A-linked system. The exogenous factors were then removed by transient *Cre* transfection, as the reprogramming cassette was flanked by *loxP* sites. A piggyback (PB) transposon was then used to deliver the reprogramming factors under the control of a doxycycline-inducible system. The PB system then allows removal of the transgene, thus creating iPS cells with no trace of reprogramming factors once the exogenous expression is no longer required.

Zhou et al. [35] recently described the generation of iPS cells using recombinant proteins. This technique therefore avoids the use of any genetic material, potentially reducing the likelihood of unexpected genetic modifications to the somatic genome. In this case, the reprogramming proteins are delivered directly into the cell, as opposed to relying on the cell to transcribe the proteins itself. This study used *Escherichia coli* to generate inclusion bodies containing the proteins, which were then purified. The resultant proteins were then used to reprogram MEFs, resulting in iPS cells. There are potential advantages to this technique, as there is no requirement to analyze and select the resultant colonies based upon the existence of integration sites in the somatic genome. It has also recently been reported that certain small molecules can be used to both enhance the reprogramming process and, in some cases, replace specific factors. Shi et al. [36, 37] demonstrated small-molecule combinations that significantly enhance the reprogramming process (using only Oct4/Klf4) in MEFs. Other groups also showed enhanced efficiency with the additional use of small molecules that are known to inhibit specific pathways within the cell [28, 38]. There is a drive to generate iPS cells using small-molecule techniques alone.

3.2 β Cells from Induced Pluripotent Stem Cells

The ability of ES cells to generate cells of any tissues has made them a candidate to create functional β cells that could be potentially used as transplantable tissue for diabetic patients. Initial attempts at this type of differentiation using mouse ESCs used a transfection and selection method to screen for insulin-secreting ES cell clones capable of restoring normoglycemia in diabetic mouse models [39]. Lumelsky et al. [40] selected cells positive for nestin (a filament protein found in neural precursors) following embryoid body formation. Following further differentiation, cells that resembled islet cells were produced. However, repeated analysis of such protocols by other groups have suggested that these cells may not be capable of de novo insulin synthesis to the same degree as normal β cells and in fact are principally secreting insulin absorbed from the culture media.

It became clear that a more stepwise approach to β cell generation would be required that may reflect the changes seen in normal human development. In these protocols the initial differentiation step is designed to drive differentiation toward the formation of definitive endoderm. Developmental studies have revealed that the formation of endoderm during normal fetal development is instigated by Nodal (a member of the transforming growth factor-β). Using activin A, which is closely related to Nodal, two groups successfully generated endodermal cells [41,42]. Following this, one of these groups [43] continued with a stepwise differentiation protocol based on normal development through definitive endoderm, primitive gut tube, posterior foregut, pancreatic precursor, and endocrine cell. The cells generated by this protocol contained similar amounts of glucose to normal adult β cells. When exposed to secretory stimuli, these cells were also capable of releasing insulin. However, the lack of response of these cells to glucose suggested these cells may be akin to immature fetal β cells rather than mature β cells found in the adult. Jiang et al. [44] continued to develop a stepwise approach from definitive endoderm in serum-free conditions. This protocol resulted in the production of insulin-producing, islet-like clusters containing cells representative of ductal, exocrine, and endocrine pancreas. These β-like cells contained secretory granules and responded to an in vitro glucose-stimulated release assay. In 2008, Kroon et al. [10] successfully generated human endoderm that was capable of producing insulin that was responsive to glucose in such a way as to achieve normoglycemia in a diabetic mouse model. In this case, the endoderm was implanted into mice prior to terminal β cell differentiation. These mice were then rendered diabetic by the destruction of their natural β cells. Animals with the grafted endoderm maintained normal blood glucose levels, while control animals became hyperglycemic. If the grafted animals had the grafts removed, they too were rendered hyperglycemic. Although this research is extremely promising, more than 15% of animals developed tumors in the graft, raising safety concerns if this was translated in humans.

There have been two successful reports of insulin-producing cells generated from iPSCs [45, 46]. Tateishi et al. [45] used retrovirally induced iPS cells sourced from human skin cells. These cells were then subjected to a protocol almost identical to that of Jiang et al. [44]. This resulted in the formation of glucose-sensitive, insulin-secreting cells. Following this, Zhang et al. [46] also published a protocol similar to that of Jiang et al. to differentiate ES and iPS cells in a stepwise fashion, resulting in around 25% of cells positive for insulin. These cells secreted C peptide in response to glucose stimulation in vitro.

4 Future Directions

Significant steps have been made toward the use of stem cell technologies to generate β cells as a potential therapy for patients suffering from diabetes. However, there remain many hurdles to overcome in generating a safe clinical application from this technology. iPS cell technology may provide the potential for ethically

acceptable replacement tissue that is free of the problem of immunologic-mediated rejection. Studies demonstrate that iPS cells clearly have the potential to differentiate into β cells, although better and more efficient techniques, as well as more robust and more extensive characterization of the differentiated cells, must be addressed. Despite these advances, the possibility of tumor formation caused by contaminating residual stem cells after differentiation that may lead to teratoma formation remains a concern. Moreover, the inherent risk of aberrations caused by reprogramming must be carefully studied. If retroviral integration is used, then there are concerns about the effects of these integrations on the host genome, as well as the use of oncogenic viruses. To this end, even greater importance will be placed on studies to develop safer methodologies of reprogramming that are already well underway. This rapidly developing iPS technology offers a promising route to finding a cure for this widespread, expensive, inconvenient, and eventually destructive disease. Clearly this approach would have to be coupled with endeavors to obtain a comprehensive understanding of the factors responsible for the onset of diabetes in any particular patient. Even if β cells for transplantation can be efficiently made from stem cells, a greater understanding is required about both autoimmune reactions that lead to β cell destruction and those inherent β cell defects that lead to reduced functionality in order for this technology to be applied.

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Keratinocyte-Induced Pluripotent Stem Cells: From Hair to Where?

Trond Aasen and Juan Carlos Izpisúa Belmonte

Abstract The generation of induced pluripotent stem (iPS) cells has spawned unprecedented opportunities for investigating the molecular mechanisms that underlie cellular pluripotency and reprogramming, as well as for obtaining patient-specific cells for future clinical applications. However, both prospects are hampered by the low efficiency of the reprogramming process. The skin keratinocyte is one cell type that is easily obtainable and can be reprogrammed to pluripotency by retroviral transduction with Oct4, Sox2, Klf4, and c-Myc. Keratinocyte-derived iPS cells appear indistinguishable from human embryonic stem cells in colony morphology, growth properties, expression of pluripotency-associated transcription factors, and surface markers, as well as in their capacity to differentiate both in vitro and in vivo. Notably, reprogramming keratinocytes to iPS cells is in excess of 100-fold more efficient and twofold faster than that of fibroblasts. In addition, keratinocytes can be cultured from a single plucked hair from adult individuals, permitting generation of iPS cells without the need for invasive biopsies. This chapter summarizes iPS cell technology, with focus on keratinocyte reprogramming and how this cell type has allowed expansion of the molecular tools aiding the understanding, practicability, and future implications of the iPS field.

Keywords Keratinocytes • Reprogramming • Induced pluripotent stem cells • Embryonic stem cells • Pluripotency • Differentiation • Therapy • Background

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1 Embryonic Stem Cells

Embryonic stem (ES) cells can be derived from the inner cell mass of an early stage embryo—the blastocyst. Mouse ES cells were first described in 1981 [1], and in 1998 James Thomson and colleagues at the University of Wisconsin–Madison were able to derive the first human ES cells [2]. ES cells are characterized by having unlimited capacity for self-renewal and being pluripotent, that is, having the ability to differentiate into all derivatives and cells of the three primary germ layers: ectoderm, endoderm, and mesoderm. Due to these features, ES cell therapy has been proposed for regenerative medicine and tissue replacement after injury or disease. However, no approved medical treatments using ES cells have been established, although in January 2009, the U.S. Food and Drug Administration approved the first clinical trial to treat patients with spinal cord injury. Adult stem cells, such as cord blood stem cells, on the other hand, have been used successfully to treat several specific diseases [3], but there are many limitations, and these cells are only capable of differentiating into certain cell types. Besides the ethical concerns of ES cell derivation and therapy, there is also a technical problem of graft versus host disease associated with allogeneic stem cell transplantation.

2 Induced Pluripotency: The Savior of All?

Early work on somatic cell nuclear transfer (SCNT), most famously the cloning of Dolly the sheep [4], showed that differentiated somatic cells can, under certain circumstances, revert back to a pluripotent state. In 2006, Takahashi and Yamanaka devised an elegant set of experiments to achieve direct reprogramming of somatic cells using retroviruses to express 24 candidate reprogramming factors in mouse fibroblasts. Upon introduction of only four transcription factors—Oct3/4, Sox2, Klf4, and c-Myc (OSKM)—the cells could acquire properties similar to those of ES cells [5]. In 2007, Yamanaka and several other investigators reported generation of induced pluripotent stem (iPS) cells from human fibroblasts after forced expression of the same four factors [6,7] (OSKM) or by using OS together with Lin-28 and Nanog [8]. These iPS cells are molecularly nearly identical to ES cells, including self-renewal capacity, gene expression profile, promoter methylation pattern, doubling time, embryoid body formation, in vitro differentiation, teratoma formation, and viable chimera formation (mouse). The generation of iPS cells has revolutionized the field investigating the molecular mechanisms that underlie cellular pluripotency and reprogramming. More important, iPS cells allow generation of patient-specific cells, thus avoiding ethical and host-rejection issues associated with classic ES cell technology. This has spawned enormous interest and promise for future clinical applications

(Fig. 1). However, the process of reprogramming is slow and inefficient, and the permanent integration of retroviral vectors into the genome is one of the problems that can limit the use of iPS cells for therapeutic applications [9]. Indeed, the full extent of whether iPS cells can replace ES cells in every aspect is still being debated, and partial reprogramming or "over-reprogramming" poses challenges. It is thought that the process of iPS cell generation is highly stochastic and depends on many factors, including age, type, and origin of the cells used. Thus, it remains to be demonstrated what the ultimate source of cells will be in regard to both availability and applicability after reprogramming and re-differentiation.

Recently, we explored the possibility of using ectodermal skin keratinocytes as a starting population for iPS cell generation [10]. As an easily accessible cell type, we speculated that it would be easier to reprogram than fibroblasts (see later discussion), thus allowing progress not only in elucidating the molecular mechanisms, but also toward the generation of safe and integration-free iPS cells.

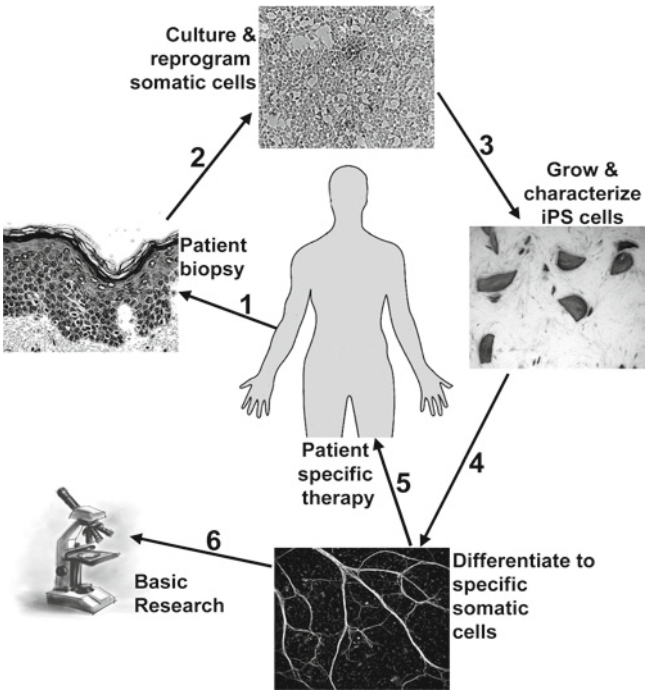

Fig. 1 Patient-specific induced pluripotent stem cells. A simple punch biopsy or a plucked hair allows culture and reprogramming of adult somatic keratinocytes into pluripotent stem cells. These cells have unlimited self-renewal and are capable of differentiating into any cell type, thus spawning unprecedented opportunities for cell replacement therapy, as well as providing new tools for basic research

3 Keratinocyte-Derived Induced Pluripotent Stem Cells

3.1 Background

The skin is the largest organ of the body and has a variety of functions, ranging from barrier homeostasis to the sense of touch [11]. It is a complex organ arranged in three layers: the mesodermal-derived hypodermis and the dermis, which are highly collagenous and fibroblast-rich layers, and the ectodermal-derived outer covering of skin called the epidermis (and its associated appendages of eccrine sweat glands and hair follicles), which consists mainly of stratifying keratinocytes [11,12]. The epidermis can be divided into four major layers where proliferating keratinocytes in the basal layer, attached to an underlying basement membrane, move upward as they undergo terminal differentiation, forming a continually shedding but protective barrier (Fig. 2a). It is now clear that the epidermis is regenerated from multipotent stem cells situated in several specialized regions such as the hair follicle (including the bulge, an area of the outer root sheath), the sebaceous gland, and in the interfollicular epidermis [11, 13].

3.2 Generation

Keratinocytes from human foreskin or punch biopsies can be cultured in serum-free and low-calcium medium, which facilitates a highly proliferative undifferentiated

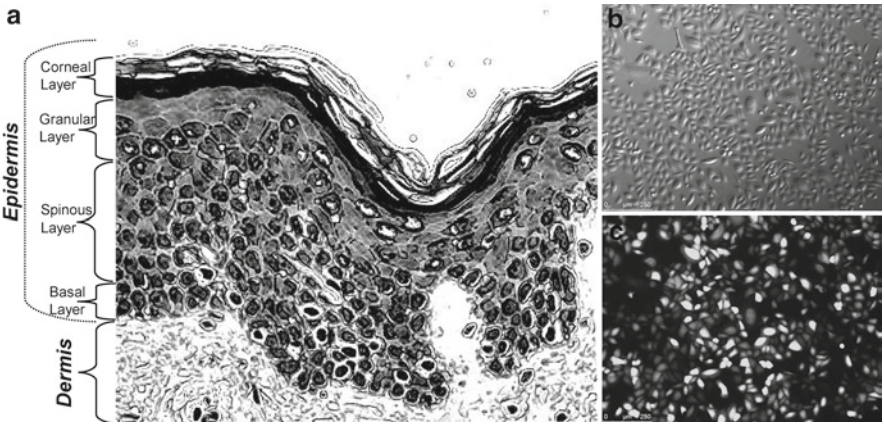

Fig. 2 Human epidermis and keratinocytes. **a**: The typical structure of the epidermis, the outer covering of skin, consisting mainly of stratifying keratinocytes that differentiate as they move upward from the basal layer. Below the epidermis is a collagen-rich dermis containing many cell types, including fibroblasts. **b:** Human keratinocytes can be cultured in a serum-free defined medium allowing rapid proliferation and maintaining an undifferentiated phenotype. **c:** High efficiency of retroviral infectivity can be achieved under these conditions, as illustrated here by green fluorescent protein fluorescence

state (Fig. 2b). Under these culture conditions, only keratin-positive keratinocytes grow, which readily differentiate upon addition of physiological levels of calcium and can form a fully differentiated stratified epidermis within a week [14]. Retroviral transduction of these keratinocytes with green fluorescent protein (GFP) using two 45-minute spinfections 24 hours apart results in nearly 100% infection of undifferentiated cells (Fig. 2c). This infection protocol can be used to transduce keratinocytes with retroviruses encoding Oct4, Sox2, Klf4, and c-Myc [10]. Typically, 50,000 keratinocytes (passages 1–5) are seeded (day 0) and infected on days 1 and 2 with a 1:1:1:1 mixture of retroviruses. Cells are trypsinized on day 3 or 4 and seeded onto a layer of irradiated mouse embryonic fibroblasts in ES cell medium. Within 2–3 days (6–7 days postinfection), several hundred small, tight cell colonies appear that grow rapidly and, by day 10 postinfection, display a typical human ES (hES) cell–like morphology (tight colonies of cells with large nuclei-to-cytoplasmic ratio and prominent nucleoli; Fig. 3c). Clumps of differentiated nonproliferating cells (Fig. 3a), as well as some colonies with distinct morphology, including typical keratinocyte colonies (Fig. 3b), all easily distinguishable from hES cell–like colonies, can also be observed.

Transduction of keratinocytes with single factors or GFP does not result in the formation of cell colonies if seeded at the same density, except after expression of c-Myc, which generates large numbers of colonies but with distinct keratinocyte morphology. Generation of keratinocyte-derived iPS (KiPS) colonies is also not observed with combinations of two or three factors, except when using the specific combination of Oct4, Sox2, and Klf4. The number of colonies in the absence of c-Myc is much lower and their appearance is delayed, but nevertheless sufficient colonies can be generated, which is important, as this transcription factor is a known oncogene. Indeed, 20% of the chimeric mice generated from OSKM-derived mouse iPS cells developed cancer. Nakagawa et al. also generated iPS cells without c-Myc, and the resulting chimeras did not develop cancer [15]. However, there are still many concerns in relation to cancer. The other transcription factors may also have oncogenic activity, and retroviral insertion can activate proto-oncogenes or inactivate tumor suppressor genes, whereas over-reprogramming or partial reprogramming can also change the expression profile of important genes regulating cell behavior in relation to cancer. Moreover, failure to completely redifferentiate all the pluripotent cells before transplantation is a further concern, a problem also present in therapy using standard ES cells.

3.3 Characterization

Mechanical passaging onto feeders allows expansion and characterization [10]. KiPS colonies have hES cell–like morphology (Fig. 3d) and stain strongly positive for alkaline phosphatase (AP) activity. KiPS cells express genes and cell surface markers characteristic of hES cells, including Nanog, Oct4, Sox2, Rex1, Cripto, Connexin43, IGF1 receptor, SSEA3, SSEA4, Tra-1–60, and Tra-1–81, and completely

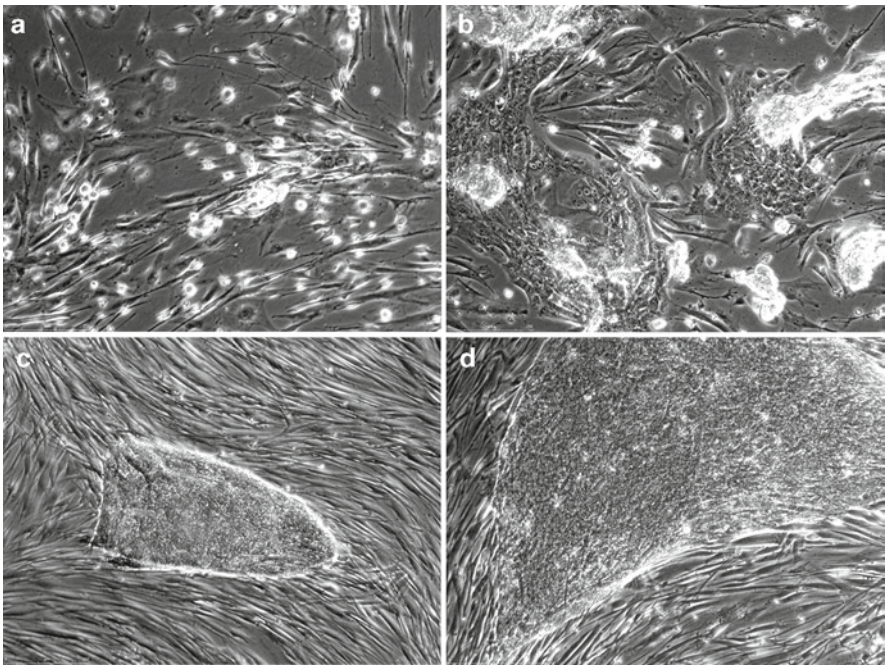

Fig. 3 Keratinocyte reprogramming process. **a:** Human keratinocytes seeded at low density in human embryonic stem cell medium on top of irradiated fibroblast feeder cells tend to stop growing and differentiate into clumps of keratin. **b:** Some keratinocytes survive, particularly if expressing c-Myc or seeded at higher density, but these cells display typical keratinocyte morphology and tend to differentiate over time. **c:** Example of an early, 12-day postinfection, induced pluripotent stem cell–like colony displaying typical features, such as high nuclear-to-cytoplasmic ratio and tight borders. **d:** Example of bona fide keratinocyte-derived induced pluripotent stem cell colony on feeders

lose keratinocyte-specific markers such as Keratin 14. Overall, the expression of stem cell markers in KiPS cells is indistinguishable from that of hES cell lines maintained under similar conditions [10].

Southern blot analysis of genomic DNA can be used to confirm the independent origin of iPS cell lines, and polymerase chain reaction of genomic DNA can demonstrate integration of the transcription factors. Of interest, the number of retroviral integrations in KiPS cells [10] is clearly lower than that of iPS cells generated from human fibroblasts [6] and more similar to iPS cells from mouse hepatocytes and gastric epithelial cells [16].

It has been suggested that the correct generation of iPS cells requires silencing of the retroviral transgenes [17]. This is also the case for KiPS cells. As with fibroblasts [18], the occasional incomplete silencing of transgenic expression in KiPS cells is associated with deficient reprogramming, as evidenced by their tendency to spontaneously differentiate and their failure to activate the endogenous expression of ES-associated transcription factors such as Oct4, Sox2, Nanog, and Cripto.

Thus, for further support of proper epigenetic reprogramming of keratinocytes or any other cell type into a pluripotent state, demethylation of the promoters of pluripotency genes such as Oct4 and Nanog should be demonstrated.

3.4 Differentiation

One of the key features of pluripotent stem cells, and the reason why they are so useful for both basic studies and therapy, is their capacity to differentiate into cell derivatives of all three embryonic germ layers. In theory, these cells can be differentiated into any of the more than 220 cell types that exist in the human body. All KiPS cell lines tested have been capable of differentiating into endoderm, mesoderm, and ectoderm derivatives. Furthermore, highly efficient differentiation into specific cell types such as dopaminergic neurons and beating cardiomyocytes has demonstrated that KiPS cells have the potential to achieve complete cellular differentiation, an essential feature for transplantation purposes. The ability to give rise to teratomas upon injection into immunocompromised mice is another essential test for the quality of iPS and ES cells. All KiPS cell lines tested readily generate complex intratesticular teratomas comprising structures and tissues derived from the three embryonic germ layers, expressing a number of markers.

Thus, in conclusion, it is evident that human keratinocytes, similar to fibroblasts, can be successfully reprogrammed to pluripotency by retroviral transduction with defined factors.

3.5 Properties and Efficiency

When reprogramming keratinocytes, colonies can be identified as early as 10 days postinfection, whereas colonies of iPS cells generated by retroviral transduction of human fibroblasts with the same four factors appear after 21–25 days postinfection [6,18]. In addition to speed, reprogramming appears more efficient, although the reason for this higher efficiency is unknown. Using the same retroviral supernatants to transduce primary dermal fibroblasts and epidermal keratinocytes isolated from the same foreskin biopsy in parallel, an overall keratinocyte reprogramming efficiency close to 1% is observed [10], whereas an efficiency of less than 0.01% is achieved [6] using fibroblasts. Thus, reprogramming of human keratinocytes is more than 100-fold more efficient than that of fibroblasts. Using GFP-encoding retroviral supernatants to infect keratinocytes and fibroblasts in parallel, we do not detect significant differences in the percentage of transduced cells nor in the median intensity of GFP fluorescence. Moreover, the protein levels of the four transcription factors do not appear to differ significantly between fibroblasts and keratinocytes transduced in parallel with the same retroviral supernatants, clearly indicating that the higher efficiency of keratinocyte reprogramming cannot be

accounted for by a higher susceptibility to retroviral transduction. In support of this, we have shown that KiPS cells display fewer retroviral integrations than fibroblast-derived iPS cells [10], which suggests that there are intrinsic differences between the two cell types that may account for the higher reprogramming efficiency of keratinocytes. In the mouse, hepatocytes and gastric epithelial cells also appear to be more easily reprogrammed and require fewer retroviral integrations than fibroblasts [16], although the mechanism(s) responsible for this difference have not been explored [19].

Comparing different cell types, we have shown that keratinocytes express much higher levels of c-Myc and Klf4 than fibroblasts, hES cells, or KiPS cells [10], suggesting the possibility that the elevated levels of endogenous expression of these factors may render keratinocytes especially susceptible to reprogramming. In this respect, it has been shown that Klf4 blocks the proliferation–differentiation switch of basal keratinocytes [20], whereas c-Myc stimulates exit from the adult stem cell compartment to drive cells into a proliferative mode toward the epidermal and sebaceous gland lineage [21] while inducing major global histone modifications at the same time [22]. Of interest, c-Myc has also been shown to induce high levels of telomerase activity in keratinocytes [23], as well as to repress terminal differentiation in response to a high-calcium switch [24], a circumstance that occurs during keratinocyte reprogramming when changing to ES cell culture media. Moreover, keratinocytes, unlike fibroblasts, display high expression levels of certain stem cell markers [25], such as CD24, suggesting that the transcriptional profile of keratinocytes is more similar to that of ES cells than fibroblasts are to ES cells. In this respect, keratinocytes appear to have some intrinsic plasticity, as underscored by the fact that transient expression of Oct4 alone is sufficient to induce Sox2, Nanog, Utf1, and Rex1 expression, enabling these cells to differentiate into neuronal cells [26]. Indeed, the expression levels of an array of genes related to stem cell identity, growth, or differentiation in keratinocytes, fibroblasts, hES cells, and KiPS cells by real-time reverse transcription-polymerase chain reaction clearly indicate that the transcriptional profile of keratinocytes is significantly more similar to that of hES and KiPS cells than fibroblasts are to hES and KiPS cells [10]. Future research will be needed to identify the exact transcriptional status of different cell types that favor reprogramming, as well as the epigenetic features that constrain or facilitate this process. We have already performed genome-wide transcriptional profiling of primary keratinocytes and fibroblasts, hES cell lines, and KiPS cell lines, which clearly indicates that KiPS cells cluster together with the hES cell lines, with fibroblasts and keratinocytes as outgroups [10]. Of importance, the overall transcriptional profile of KiPS cells appears indistinguishable from that of hES cells, in the sense that the differences between KiPS cell lines and hES cell lines are in the same range as the differences among different hES cell lines. In addition, unsupervised clustering has identified a number of transcripts that are expressed in keratinocytes, KiPS cells, and hES cells but not in fibroblasts. Some of these genes may explain why keratinocytes appear to reprogram more easily. It is also possible that epithelial cells are more amenable to reprogramming because, unlike fibroblasts, they do not need to undergo a mesenchymal-to-epithelial transition during reprogramming into iPS cells.

In addition to fibroblasts, mouse iPS cells have been generated from a number of cell types, including hepatocytes and gastric epithelial cells [16], pancreatic cells [27], neural stem cells [28], and B lymphocytes [29]. These studies have uncovered that certain cell types may be better for complete or faster reprogramming, perhaps with a reduced risk of teratoma formation. In addition, it may be easier to generate pancreatic β cells and hepatocytes from iPS cells derived from somatic cells of endodermal origin such as gastric epithelial cells, for example. An increasing number human somatic cell types, including blood progenitors [30], are also being used as sources for iPS cell generation, allowing a more detailed study of the role of the origin of the cell. Notably, iPS cells derived from mouse hepatocytes [16] or human keratinocytes [10] have fewer retroviral integration sites than do iPS cells derived from fibroblasts. A full study of the ideal starting population of somatic cells is warranted. A comparison showing the known advantages and disadvantages between fibroblasts and keratinocytes is summarized in Table 1.

3.6 Hair-Derived Keratinocyte-Derived Induced Pluripotent Stem Cells

Keratinocyte reprogramming is much more efficient than that of fibroblast reprogramming and is useful for studying and understanding the mechanism of reprogramming. It is also advantageous to be able to generate iPS cells in a manner that is not invasive or stressful to the patient and also is easy to perform. Due to the high efficiency of keratinocyte reprogramming, it is possible to generate KiPS cells from

Table 1 Comparison of the advantages and disadvantages of keratinocytes versus fibroblasts as starting populations for induced pluripotent stem cell technology

Fibroblasts	Keratinocytes
+	+
• Easy to culture • Many disease cell lines are banked • Can be obtained by simple biopsy	• Epithelial cell type as embryonic stem cells • Fewer retroviral insertions • Obtained by biopsy or plucking hair • More efficient reprogramming • Faster reprogramming • No partial reprogramming states have been reported
–	–
• Require mesenchymal-to-epithelial transition • High number of retroviral insertions are observed • Inefficient reprogramming • Slower reprogramming • Partial reprogrammed lines are often observed	• Fewer disease cell lines are banked • More difficult to maintain in culture long term

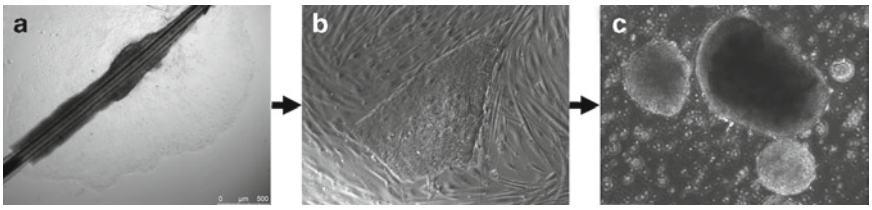

Fig. 4 From hair to induced pluripotent stem cell to any cell. **a:** Human keratinocytes can be cultured from a single plucked human hair. **b:** These cells can be infected with retroviruses expressing the reprogramming factors Oct3/4, Sox2, Klf4, and c-Myc, resulting in direct induced pluripotent stem (iPS) cell colonies. **c:** Example of embryoid bodies derived from hair-derived iPS cells, which under defined conditions can differentiate into cells of all three embryonic germ layers.

plucked human hair [10]. A single plucked hair can be cultured in hES cell medium, resulting in keratinocytes proliferating out of the outer root sheath area of the plucked hair (Fig. 4a). Keratinocytes isolated in this way can be split and subjected to retroviral infection and reprogramming. The resulting KiPS cell colonies (Fig. 4b) can be expanded after mechanical picking, stain strongly for AP activity, and display colony morphology, growth characteristics, and expression of pluripotency-associated transcription factors and surface markers indistinguishable from those of hES cells or KiPS cells from epidermal keratinocytes. Similar to other KiPS cells, these cells can generate embryoid bodies (Fig. 4c) with the ability to differentiate into derivatives of all three embryonic germ layers [10].

Previous reports have uncovered the existence of multipotent stem cells in the bulge [31, 32] or dermal papilla [33] areas of mouse and human hair follicles, and these cells may offer new avenues for future therapy. However, these cells grow as self-renewing spheres in hES medium, whereas hair-derived cells from the outer root sheath display a typical epithelial morphology of keratinocytes and differentiate completely upon passaging in the presence of hES medium (as with epidermal keratinocytes). Thus, true iPS cell generation from plucked hair has so far only been achieved using keratinocytes from the outer root sheath and retroviral transduction using all four factors. Extensive experiments using fewer reprogramming factors and other cell types from plucked hair remain to be investigated in detail.

4 Conclusions

The system of induced reprogramming of keratinocytes to pluripotency should provide a valuable experimental model for investigating the bases of cellular reprogramming and pluripotency. Moreover, it provides both a practical and advantageous alternative for the generation of patient- and disease-specific pluripotent stem cells.

The technology of direct cell reprogramming removes two important obstacles associated with human ES cells: immune rejection after transplantation and ethical

concerns regarding the use of human embryos. However, the clinical application of iPS cells still faces many obstacles, some shared with ES cells, such as teratoma formation. Viral integration is a particular problem in respect to cancer, but recent years have seen numerous reports with improvements of the reprogramming technology. Thus, it is now possible to reprogram cells using nonintegrative plasmids [34] and excisable transposons and piggyback constructs [35, 36] or even by adding the OSKM factors directly as recombinant proteins [37, 38]. In the near future, it is essential to perform detailed analyses of these new iPS lines compared to traditional ES cell lines, which remain the gold standard. A detailed study of the differences between iPS cells derived from alternative somatic cell sources would also be important. Apart from the safety issues, a challenge that remains in using cells for therapeutic purposes is establishing efficient protocols, in terms of both quality and quantity, for differentiating these cells into a specific cell type such as blood cells, neuronal cells, or cardiac cells.

From hair to where? Only the future will know, but a new avenue for treating severe diseases and certain cancers has excited the scientific community. The technical race for iPS cell generation has peaked, but the race for applying these cells to answer basic research questions and for therapeutic regenerative medicine is only in its infancy.

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Generation and Characterization of Induced Pluripotent Stem Cells from Pig

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Abstract Successful establishment of pluripotent embryonic stem cells from ungulates, especially pigs, is an important but challenging endeavor. The pig is an attractive species for creating pluripotent cell lines because, unlike the currently preferred mouse model, the pig resembles the human quite closely in size, anatomy, and physiology. This chapter describes the derivation of induced pluripotent stem cells (iPSCs) from pig fibroblasts by means of retroviral transduction of the same four reprogramming genes, *OCT4, SOX2, KLF4*, and *c-MYC*, used by others to create iPSCs from mouse and human somatic cells. Besides describing technical aspects of porcine iPSC (piPSC) generation, which includes (1) virus preparation and transduction of porcine fibroblasts, (2) selection of reprogrammed cells and their properties, and (3) transcriptome profile of the cells, we discuss the extent to which our cells resemble and differ from the piPSCs generated by other groups. The piPSCs afford advantages in developing clinical models for human regenerative medicine and for testing the safety and efficacy of "personalized" transplants, and they may also have application in agriculture.

Keywords Embryonic stem cells • Induced pig pluripotent stem cells • Porcine fibroblasts • Transcriptome • Stemness

1 Introduction

1.1 Embryonic Stem Cells

Embryonic stem cells (ESCs) were first established from the inner cell mass (ICM) of day 3.5 mouse embryos. ESCs, like the ICM precursor cells, are truly pluripotent,

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in the sense that they retain the potential to differentiate into all the primary cell types found in the body. In addition, under appropriate culture conditions, ESCs proliferate indefinitely [1, 2]. These defining attributes qualified murine ESCs (mESCs) as ideal candidates for introducing mutations either by "knocking out" or "knocking in" genes by homologous recombination. The resultant cells can be reintroduced into a new recipient blastocyst to give rise to chimeric mice that, in turn, can pass on the mutated gene through their germline [3, 4]. Therefore, with the discovery of ESCs, the mouse was elevated even further as a model for biomedical research to the extent that other animal models have gradually become sidelined [5].

Following the initial success of mESC derivation, similar lines have also been established in monkeys and humans [6]. The description of human ESCs (hESCs) just more than a decade ago attracted considerable excitement because of the potential use of such cells in regenerative medicine. Although ethical barriers continue to preclude many applications in hESCs that are feasible with mESCs, the human cells hold enormous therapeutic promise in tissue repair and replacement and for gene therapy [7]. However, many roadblocks persist before these applications can be realized, including the safety of the grafted cells and how to test the technical aspects of any proposed therapy. To this effect, ESCs from animals whose anatomy and physiology better resemble those of the human than do those of the mouse and that are a cheaper and more convenient alternative to primates are highly desirable [8–10]. The mouse, because of its small body size, marked physiologic differences from human, and shorter lifespan, is not an appealing model for many kinds of applications. In addition, ESCs from mouse and human differ in their properties and general phenotype, and it is unclear whether they have equivalent developmental capability [11]. For example, murine and human ESCs display many presently unexplained differences in culture requirements and in the cell signaling pathways that govern self-renewal and pluripotency [11]. Human and murine ESCs also differ in their display of cell-surface antigens. For example, the carbohydrate antigen SSEA1 is strongly displayed on mESCs but absent in hESCs, while SSEA3, SSEA4, TRA1-60, and TRA1-81 are strictly human [12] (Table 1). Human ESCs are also much more prone to spontaneous differentiation and divide more slowly than their mouse counterparts, making their culture and maintenance much more demanding [13,14].

Therefore, the quest remains for stem cells from alternative species that afford advantages over the existing mouse and monkey models. In this regard, pluripotent stem cell lines from pig, a species that is a well-established model of choice for biomedical and agriculture applications, would be of considerable advantage [8–10]. A goal would be to use pigs to test the safety and efficacy of transplantation in advance of conducting human trials.

1.2 Pluripotent Stem Cells from Pig

For reasons yet to be fully understood, the isolation of ESCs from pig has been unfruitful. Despite intensive efforts by multiple groups over 15 years, the establishment

Table 1 Comparison of pluripotent marker expression in porcine induced pluripotent stem cells with mouse embryonic stem cells and induced pluripotent stem cells, rat embryonic stem cells, human embryonic stem cells and induced pluripotent stem cells, and pig inner cell mass/primordial germ cells

	mESCs	miPSCs	Rat ESCs	Pig ICM/PGCs	piPSCs	hESCs	hiPSCs
POU5F1	+	+	+	+	+	+	+
SOX2	+	+	+	+	+	+	+
NANOG	+	+	+	+[a]	+	+	+
AP	+	+	+	+	+	+	+
SSEA1	+	+	+	+	+	–	–
SSEA3	–	–	–	Unknown	–	+	+
SSEA4	–	–	–	Unknown[b]	–/+[c]	+	+
TRA-1-60	–	–	–	Unknown	–	+	+
TRA-1-81	–	–	–	Unknown	–	+	+

hECS, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells; ICM/PGCs, inner cell mass/primordial germ cells; mECSs, mouse embryonic stem cells; miPSCs, mouse induced pluripotent stem cells; piPSCs, porcine Iinduced pluripotent stem cells.

[a] NANOG gene expression is not restricted to pluripotent cells in the pig; it is also detectable in some adult somatic tissues [42].

[b] Pig amniotic fluid–derived mesenchymal cells cultured for transplantation express SSEA4 and OCT4 [43].

[c] We observed weak SSEA4-positive staining some colonies, but the intensity of the signal was weaker than that of hESC (H9 cells) that were used as controls.

of ESCs from pig has not been successful. There are a number of plausible explanations for these failures, including the choice of wrong stage of embryo as the starting material, contaminations from rapidly growing and resilient endoderm and trophectoderm cells, and, most important, inappropriate culture conditions. Many of these topics have been the focus of recent excellent reviews [8, 10, 15, 16]. Despite these many failures, there have been reports of a few cell lines that have met some of the criteria of pluripotency, such as an ability to be maintained in culture for prolonged periods [17], form teratomas, and, in one instance, give rise to chimeras, although the contribution to the offspring born was minor [18]. However, most of the cell lines reported over the last 20 years have been poorly characterized, so that their pluripotent nature remain in doubt. Similarly, attempts to establish pluripotent lines from germ cells have proved equally futile [19]. Consequently, this field of research still awaits a major breakthrough.

1.3 Induced Pluripotent Stem Cells Are Ideal Alternatives to Embryonic Stem Cells

Several recent papers have reported the generation of induced pluripotent stem cells (iPSCs) from somatic cells of mouse and human [20–25]. In most of these examples, the same four reprogramming genes (*OCT4*, *SOX2*, *KLF4*, and *c-MYC*) used by Takahashi and Yamanaka in their pioneering publication were able to establish

iPSCs from both species [23, 24]. The cells produced all of the characteristics of ESCs in morphology, proliferative capacity, surface antigens, overall gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity, although they were clearly not identical to ICM-derived ESCs. The "induced" cells, however, could differentiate into the three main germ layers (ectoderm, mesoderm, and endoderm) and, in the case of murine cells, provide chimeras and even generate whole animals [26, 27].

In addition to mouse and human, iPSCs have been derived from rat and monkey, making the reprogramming approach a proven and reliable system for deriving "stem cell–like" pluripotent cells. The recent establishment of porcine iPSCs (piPSCs) for which authentic ESCs are not currently available, although not a conceptual or technical breakthrough, was a valuable step forward for testing the potential utility of stem cells in human medicine and agriculture [28–30]. In addition to the advantages associated with ESCs, piPSCs will also allow establishment of "personalized" stem cells for transplantation. If pluripotent cells can be created from a neonatal animal and these cells successfully directed along particular pathways of differentiation, it would be possible to transplant the cells into the same animal from which the founder cells originated. The ability of these transplanted cells to be incorporated into damaged organs or tissues could be measured and the functionality and stability of the grafts assessed over time. Of equal importance, a study on pigs will provide confidence that stem cell transplantation can be performed safely without the risk of cancer occurring over a period of years from adolescence to more mature age. The model may also provide a means for establishing pig-to-human transplants (xenotransplantation) of genetically modified tissues.

2 Technical Aspects Involved in the Generation and Characterization of Induced Pluripotent Stem Cells from Porcine Fibroblasts

2.1 Lentiviral Preparation and Transduction

To create piPSCs, we employed human *OCT4* (h*OCT4*), h*SOX2*, h*KLF4*, and h*c-MYC* (OSKM) reprogramming genes similar to the ones used by other groups. The open reading frames of the genes were cloned into FUGW (SKM) and SIN18.cPPT.hEF1a.EGFP.WPRE (O) lentiviral vectors by using *Bam*HI, *Eco*RI [31], and *Bam*HI, *Sal*I sites, respectively (Fig. 1a). We have yet to investigate whether the porcine pluripotent genes are in any way superior to their human counterparts in remodeling somatic cells. Although porcine and human OCT4 and SOX2 show a high level of sequence identity at the amino acid level (greater than 93%), the same is not true for KLF4 and c-MYC, which only share about 85% and 90% identity, respectively. However, we speculate that these differences will not significantly

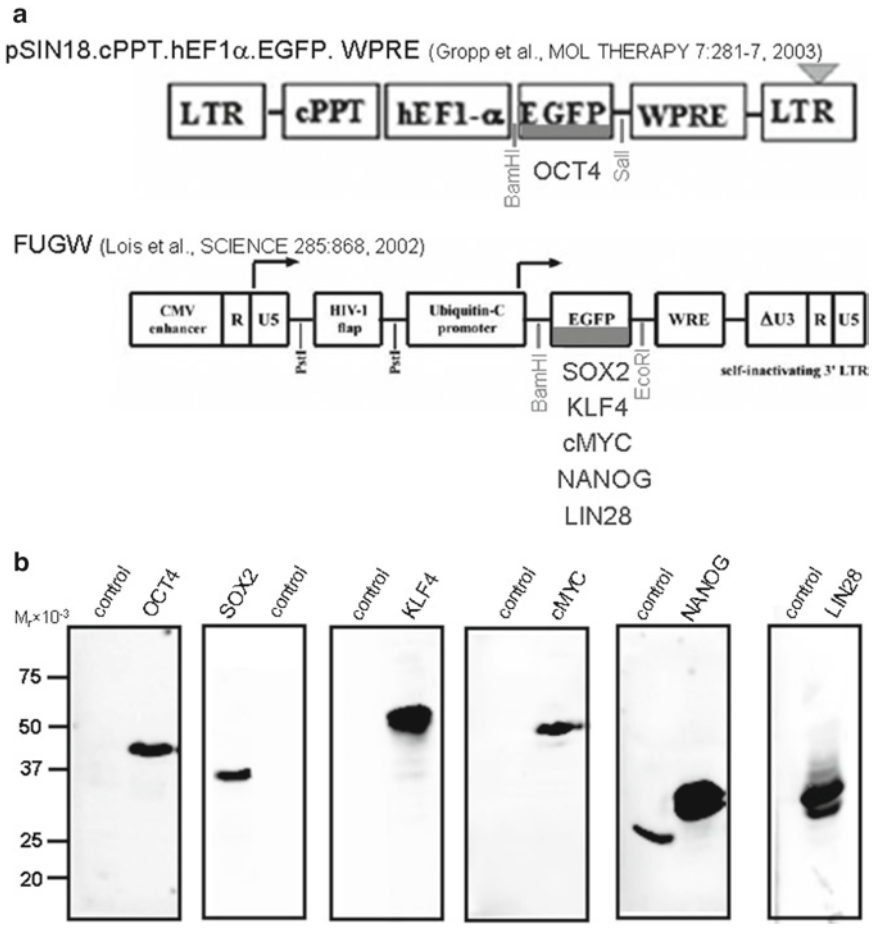

Fig. 1 **a:** Schematic of lentiviral expression plasmids used for porcine induced pluripotent stem cell derivation. The cDNAs for human *OCT4*, *SOX2*, *KLF4*, and *cMYC* were cloned into the region of lentiviral plasmid encoding enhanced green fluorescent protein (EGFP). **b:** Expression of reprogramming gene products from retroviral expression plasmids transfected packaging cell line 293FT. After centrifugation (16,060 × *g*, 3 minutes), soluble protein (100 μg/lane) was analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein in the gels was transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). Equal amount of lysates from 293FT cells transfected with a second lentiviral vector (pSico) were used as control. (Figure S1, with permission) [29]

impact the outcome. Virus production and harvest procedures conformed to the method described in the Lentivector Expression Systems user manual (System Biosciences, Mountain View, CA; http://www.systembio.com/downloads/Manual_LentivectorExpressionSystem_5_070613_web.pdf). Briefly, viruses incorporating

each gene were produced in 293FT cells (Invitrogen, Carlsbad, CA) by transfection with each lentiviral vector (FUGW or pSIN18) along with VSV-G envelope (pMD2.G) and packaging vectors (psPAX2) [32]. 293FT cells in 10-cm plates (6×10^6 cells) were transfected with 5.0 μg of lentiviral vector (FUGW or pSIN18), 1.7 μg of pMD2.G, and 3.3 μg of psPAX2 using Lipofectamine-Plus reagents (Invitrogen). Beginning 2 days after transfection, the supernatants were collected every day over a period of 3 days and the cells re-fed each time. The supernatants for each individual virus were pooled and filtered through a 0.45-μm cellulose acetate filter and stored at −80°C until use.

Protein expression from each viral vector was examined on lysates of the transfected 293FT cells by Western blotting with appropriate antibody reagents (Fig. 1b). Once the expression from each lentiviral vector was confirmed, the titer was also estimated by procedures described elsewhere [33]. We chose porcine fetal fibroblasts (PFFs) derived at day 34 of pregnancy and expressing enhanced green fluorescent protein as target cells for reprogramming because the reprogrammed cells could then be localized in chimeras and other types of mixed-cell populations (Fig. 2a). The PFFs were seeded at a density of 1×10^5 cells per 35-mm dish and infected with the OSKM mixture of pseudoviruses each with a multiplicity of infection of approximately 5. On the day following infection, the cells were dispersed with trypsin and transferred to 10-cm plates preseeded with irradiated mouse

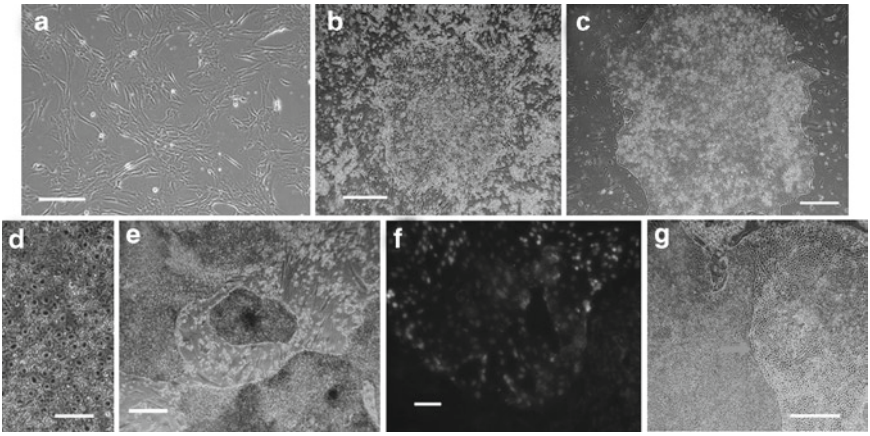

Fig. 2 Porcine induced pluripotent stem cell (piPSC) colonies derived from porcine fetal fibroblasts (PFFs). **a:** Phase contrast image of PFFs. **b:** Phase contrast image of granulated piPSCs similar to mouse and human iPSCs begin to emerge approximately 3 weeks following viral infection. **c:** A representative piPSC colony after serial passage conforming to human embryonic stem cells shown at low magnification (panels A–C: *bar*, 500 μm). **d:** Shown at higher magnification (*bar*, 50 μm). The piPSC colonies **(e)** express alkaline phosphatase (AP) and **(f)** display nuclear localization of OCT4 (nuclear) and surface SSEA1 (cytoplasm) (*bar*, 100 μm). **g:** Some of the piPSC colonies have a disposition for spontaneous differentiation as evidenced by the markup (*arrow*) on the right side of the colony. The differentiated cells exhibit cobblestone morphology with a relatively low nucleus-to-cytoplasm ratio [29]. (From Figure 1, with permission.)

embryonic fibroblasts (MEFs) at a density of 2×10^4 cells/cm^2 to provide a "feeder layer." The cells were maintained in both 4% and 20% O_2 conditions in a culture medium standardized for hESCs [34], which contained 4 ng/mL recombinant human FGF2, a growth factor necessary for sustaining hESC pluripotency.

3 Selection of Reprogrammed Cells and Their Properties

About 3 days following transfection, small foci of loosely packed "granular" cells appeared, especially under 20% O_2 conditions, which were further expanded into larger colonies. These cells stained weakly for OCT4 and were negative for alkaline phosphatase (AP) and SSEA1. Therefore, we concluded that the granular cells were not iPSCs but "partially reprogrammed" or transformed cells of some kind whose phenotype awaits further characterization. The partially reprogrammed "granular" cells noted in our experiments have also been reported by other research groups who employed mouse and human cells [23, 24], and they probably represent usual byproducts of a stochastic reprogramming process. However, by day 22 (Fig. 2b), well-formed colonies that were later found to be the verifiable iPSCs began to emerge. These colonies were mechanically dissociated with the tip of a fine glass pipette and then drawn into the plastic tip of a P-200 pipette before transference into fresh medium in 24-well plates coated with irradiated MEFs. After approximately 4 days, well-developed colonies resembling those in Fig. 2c became visible. This procedure, followed by subsequent passage, has allowed large numbers of clonal "lines" to be created, each derived from an original colony on the initial plate. We have developed 96 clonal lines with typical ESC-like morphology (Fig. 2c, d) under 4% O_2 conditions and 65 under 20% O_2 conditions. An additional 26 colonies with a different phenotype have also been collected. These appear to express pig trophoblast (placenta) markers and will not be discussed further here. The fact that the stem cell–like piPSCs required almost 3 weeks after viral infection to appear is consistent with data from other laboratories that employed similar methods to reprogram somatic cells [20, 22–25]. It is unclear whether these secondary colonies arose secondarily from the smaller granular cells or from progressive reprogramming of a different subset of the cells originally infected with the lentiviruses.

4 Transcriptome Profile of Porcine Induced Pluripotent Stem Cells

Induced pluripotent cells exhibit a "stemness" profile similar although not identical to that that of ESCs [20, 22–25]. Thus, defining a deeper transcriptome profile of piPSCs may have value in defining whether or not the derived cell lines possess the full spectrum of genes normally associated with pluripotency. In this regard, the Porcine Genome Array from Affymetrix (Santa Clara, CA), which contains 23,937 probe sets allowing interrogation of 23,256 transcripts and constitutes the most sensitive and reproducible microarray currently available for swine genomic studies

[35], was utilized. Due to a recent annotation effort [36] (GEO accession number GPL6472) approximately 82% of the probe sets have now been annotated, making the array much more useful than before. In addition, an unpublished annotated file from Dr. Christopher K. Tuggle (Iowa State University, Ames, IA) was also appended to make the annotation process more robust. In addition to its value in defining the phenotype of piPSCs, this chip could be useful for comparing the transcript profiles of different piPSC lines, for example, to define common genes and consistent differences among lines, and to compare the piPSCs with the cells from which they were derived. In addition, it could be employed to follow the molecular events that accompany piPSC differentiation; for example, the cells appear to express pig trophoblast markers.

The microarray data were analyzed by the GeneSpring GX analysis software suite (Agilent Technologies, Santa Clara, CA). Pairwise comparisons between piPSCs and PFFs revealed that 8015 genes showed an acceptable fold change of 1.3 or above ($p < 0.05$), of which 4297 were upregulated in piPSCs compared to PFFs, while the remainder were downregulated. Hierarchical clustering of these differentially regulated genes resulted in close grouping of all three piPSC clones, branching distinctly from the founder fibroblast lines (Fig. 3c). However, among the three piPSCs analyzed, one of the piPSC clones (ID6) was found to be an outlier. Several genes that are associated with the pluripotent state in human and mouse, such as *SOX2* and *NANOG*, were either not represented or annotated on the porcine genome array. However, of those pluripotent genes that were represented on the array, several, such as *CDH1*, *PODXL*, *LIN-28*, *GCNF*, *TNAP*, *GNL3*, *POU5F1*, *CD9*, *ZFP42*, and *UTF1*, were found to be upregulated in piPSCs compared to PFFs. The $\log_2$ fold changes in expression values of these genes are shown in Fig. 3d. However, *c-MYC* and *KLF4* showed considerable downregulation compared to PFF cells (approximately 97% and 75%, respectively), an observation that was verified by our reverse transcription-polymerase chain reaction (RT-PCR) analysis (Fig. 3b, d). Of interest, *n-MYC* showed a 102-fold upregulation in piPSCs compared to PFFs (Fig. 3d).

In order to verify the results from our microarrays, the upregulation of key endogenous pluripotent genes such as *OCT4*, *SOX2*, *NANOG*, *TDGF1*, and *TERT* was confirmed by RT-PCR with porcine gene–specific primers (Fig. 3b). In addition, the expression from the integrated human transgenes was also investigated with human-specific primers. The data revealed persistence of expression from all four exogenous transgenes (Fig. 3a).

In piPSCs, in addition to the upregulation of key pluripotent genes, the differentiation-associated fibroblast-specific genes *COL6A2* and *THY1* were found to be downregulated. The significance of this is that these genes are among the earliest fibroblast genes that are downregulated during the reprogramming process. Furthermore, besides the canonical pluripotent genes, other candidate genes whose promoters are “bound” by OSK transcription factors in completely reprogrammed murine iPSCs [37] and were either upregulated or downregulated compared to partially reprogrammed cells showed similar expression patterns in piPSCs, with the exception of *BMP4* and *PDLIM1*. This additional line of evidence further ratifies our piPSC lines as comprising a completely reprogrammed cell type.

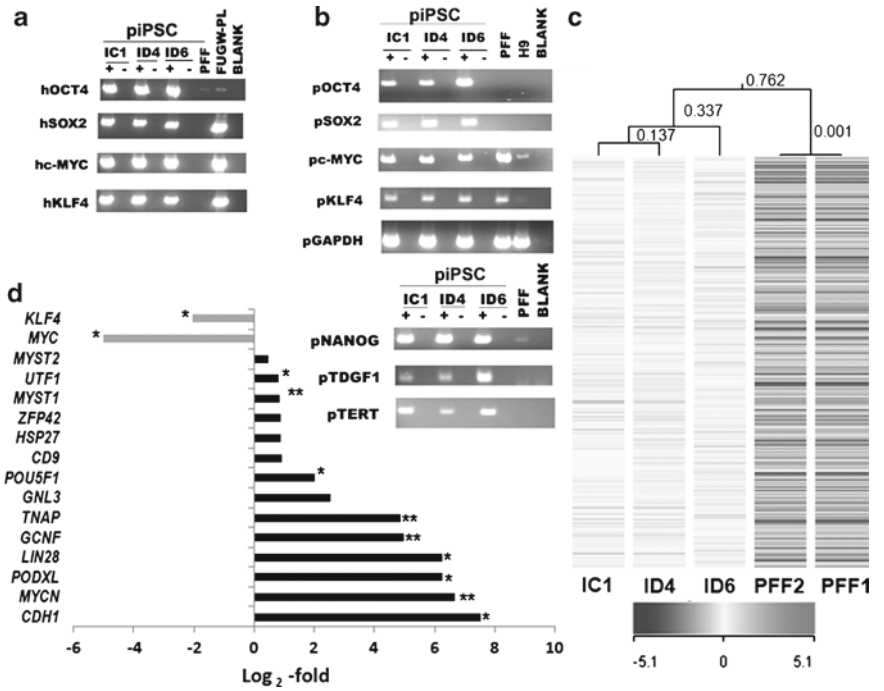

Fig. 3 **a:** Polymerase chain reaction (PCR) analysis for expression of four human genes introduced into the porcine fibroblasts by retroviral infection that were still expressed in the reprogrammed porcine induced pluripotent stem cell (piPSC) sample with (+) reverse transcription (RT) but not in the control sample without (−) RT. **b:** RT-PCR analysis for expression of selected pluripotency genes in piPSCs, porcine fetal fibroblasts (PFFs), and H9 human embryonic stem cells (hESCs). The primers were chosen for their specificity toward the porcine (p) genes rather than their human orthologs, but those for *pc-MYC* and *pKLF4* showed cross-reactivity. **c:** Hierarchical clustering of microarray data for three piPSC lines (IC1, ID4, and ID6) and two PFF cells (1 and 2) by using the Pearson-centered single-linkage rule. All the genes (n = 8015) that displayed a fold change of 1.3 or greater of their normalized expression between piPSCs and PFFs and a p value of ≤0.05 were used in the analysis. The values next to the branches represent Pearson distances. **d:** Fold differences ($\log_2$) in expression of select genes between the piPSCs and PFFs. Bars on the right-hand side of the axis and shown in black represent those genes that were upregulated in piPSCs compared to PFFs, while the bars on the left side of the axis in light gray were downregulated genes (*$p \le 0.05$, **$p \le 0.01$) [29]. (From Fig. S2 and 2 with permission.)

5 Comparison of Porcine Induced Pluripotent Stem Cells Generated in Different Laboratories

Within a few weeks of each other, three reports appeared on the generation of piPSCs: ours and two from groups in China [28,30]. While we used PFFs from a commercial pig strain, Esteban et al. used fetal fibroblasts of a Tibetan miniature

Table 2 Comparison of the porcine iinduced pluripotent stem cells produced by esteban et al. [28], Ezashi et al. [29], and Wu et al. [30]

		Esteban et al. [28]	Wu et al. [30]	Ezashi et al. [29]
Reprogramming factors		Mouse and human OSKM	Human OSKM	Human OSKM
Medium in which cultured		DMEM/FBS with FGF2 mESC medium with LIF	hESC medium with doxycycline[a]	hESC medium with FGF2
Pluripotent markers (endogenous)	AP	+	+	+
	TERT	+ (mRNA)	+ (enzyme activity)	+ (enzyme activity)
	OCT4	?	+	+
	SOX2	+	+	+
	NANOG	+	+	+
	Lin-28	+	+	+
	REX1	+	+	+
	CDH1	?	+	+
	DNMT3b	?	+	+
Surface markers	SSEA1	?	−	+
	SSEA3	?	+	−
	SSEA4	+	+	±[b]
	TRA-1-60	+	+	−
	TRA-1-81	?	+	−
Loss of fibroblast markers	Col6a2	?	?	+
	Thy1	?	?	+
Tests of pluripotency	Teratoma	+	+	+
	Embryoid body	?	+	+

A question mark indicates that the experiment was either not performed or not reported. AP, alkaline phosphatase; DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum; FGF2, fibroblast growth factor-2; LIF, leukemia inhibitory factor; OSKM, the combination of four genes, *POU5F1* (*OCT4*), *SOX2*, *KLF4*, and *c-MYC*.

[a] The lentiviral vectors employed are doxycycline inducible.

[b] In our immunofluorescence experiments the staining for SSEA4 was inconclusive. The cells stained positively for SSEA4, but the intensity of the signal was weaker than that of human embryonic stem cells (hESCs) (H9 cells) that were used as controls. It is curious that, unlike Ezashi et al. [29], Wu et al. [30] failed to note SSEA1 staining in porcine induced pluripotent stem cells (piPSCs). In addition, they reported expression of other surface markers (SSEA3, SSEA4, TRA-1-60, and TRA-1-81) for which no reference control staining was provided. In this regard, the piPSCs reported by Wu et al. were identical to hESCs in their cell surface markers.

pig, and Wu et al. used both PFFs and primary bone marrow cells from the fetus of a domestic pig. Table 2 summarizes the general approaches employed and the characteristics of the different piPSCs obtained. The same four reprogramming factors were used by all three groups, although Wu et al. modified the vectors for doxycycline-inducible expression. The piPSCs established by all three groups met the usual criteria for ESCs, including expression of a full complement of markers for pluripotency (including AP and TERT) and teratoma formation. The ability of the cells to differentiate into embryoid bodies was demonstrated by two of the groups.

Despite many shared similarities, interesting differences emerged, especially in terms of cell surface markers (see Table 2). Overall, however, the cells resembled hESCs more than mESCs in their growth properties and culture requirements. In particular, they did not require leukemia inhibitory factor but were dependent upon FGF2 to maintain their undifferentiated state. Of importance, none of the groups reported the ability of the cells to become incorporated into chimeras and to contribute to the germline. In this sense they resembled murine lines established from epiblast rather more than those from ICM [38].

6 Conclusions and Perspectives

Porcine iPSCs provide great promise in biomedical and agricultural research. They have an advantage over ESCs in transplantation studies because of the possibility of generating "patient-specific" stem cells that can be grafted back into the same animal from which they were derived. Swine, an animal whose size, anatomy, and physiology better resemble those of human than those of mouse, will provide a means to test transplantation technologies for their safety and efficiency in grafts established over a relatively long span of time and hence a superior model for transplantation research than most other species, especially mouse. Therefore, the derivation of piPSCs is a significant milestone in transplantation biology. The piPSC so far described pass the usual tests for pluripotency and resemble hESC morphologically, in growth characteristics, and in gene expression. The continued expression of the exogenous reprogramming genes from integrated retroviral vectors remains a cause of concern, as they may elevate the risk of spontaneous teratoma or embryonic carcinoma formation, as *c-MYC* and *KLF4* are oncogenes. Moreover, the continued expression of these genes characteristic of an undifferentiated state may interfere with the ability of these cells to undergo normal development [39]. To overcome these drawbacks, the transgenes will either need to be deleted or effectively silenced after the cells have been reprogrammed. Alternatively, the use of nonviral-mediated delivery of reprogramming genes [40] or direct delivery of reprogramming proteins [41] or small molecules will need to be explored.

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Induced Pluripotent Stem Cells: On the Road Toward Clinical Applications

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Abstract Somatic cells can now be reprogrammed back to an embryonic stem cell–like pluripotent state by forced expression of external factors, generating induced pluripotent stem (iPS) cells. These iPS cells resemble embryonic stem cells (ESC) at many different levels, as measured by multiple in vitro to in vivo assays. In particular, full-term live, viable, and fertile mice were recently generated from iPS cells through the most stringent test for pluripotency, the tetraploid complementation assay. This demonstration of the true pluripotency of these iPS cells indicates that they are an ideal alternative to ESCs for future studies and applications such as in regenerative medicine, disease investigation, and pharmaceutical development.

Keywords Induced pluripotent stem cells • Chimera generation • Teratoma formation • Germline transmission

1 Introduction

Human embryonic stem cells and induced pluripotent stem cells (iPSCs) provide great promise for the development of clinical applications of cell-based therapies in regenerative medicine, as well as for improvement of in vitro disease modeling and drug screening. For the last three decades, researchers have been studying embryonic stem cell (ESC) characteristics and have focused on deriving many specific cell types, such as cardiomyocytes [1, 2], hepatocytes [3], dopaminergic neurons [4–6], and β islet cells [7] from mouse embryonic stem cells and later using human ESCs.

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The first description of iPSCs in 2006 [8] provided attractive alternatives to ESC systems, and their derivation and differentiation are being studied along with safety evaluations [9–11] in the hope of moving them toward clinical applications as with ESCs. The true pluripotency of iPSCs as compared to ESCs has been the central question as this technology starts its journey on the road to regenerative medicine.

2 Induced Pluripotent Stem Cells Offer Great Therapeutic Potential

Since the establishment of ESCs in vitro by Thomson's group in 1998 [12], ESCs have shown great promise for clinical therapy and for regenerative medicine. iPSCs apparently present a good alternative to ESCs.

One of the biggest challenges in using ESCs is immune rejection after transplantation into patients for clinical therapy. iPSCs provide a great alternative since they can avoid immune rejection if they are derived from a patient's somatic cells. In addition, they have the following advantages when compared to ESCs.

3 Induced Pluripotent Stem Cells May Bypass Some of the Ethical Obstacles Presented by Embryonic Stem Cells

To avoid immune rejection of ESCs, the concept of therapeutic cloning was introduced [13]. This requires the creation of reconstructed embryos by somatic nuclear transfer from a patient's cells into enucleated host oocytes to derive patient-specific ES cells. Not only is this very hard to achieve in humans, the ethical controversy presented by using human oocytes has shadowed human ESC research for decades and still exists. Moreover, some people who object to somatic cell nuclear transfer believe that creating embryos with the intention of destroying them in the process of deriving ES cells violates respect for nascent human life [14].

iPSCs present an attractive alternative since they can avoid immune rejection if they are derived from patients' somatic cells. The iPSC procedure forces expression of selected transcription factors to dedifferentiate somatic cells into pluripotent cells with almost all the features of ESCs. This allows the creation of pluripotent cells directly from somatic cells of patients, avoiding the problem of histocompatibility or immune rejection, while bypassing the ethical problems presented by ESC derivation. On the other hand, since iPSCs are very easy to derive, we must carefully consider other issues in human clinical applications. The key points were described by Cyranoski [15].

4 Induced Pluripotent Stem Cell–Derived Cell Types Have Promising Therapeutic Potential

The generation of autologous iPSCs has sparked enthusiasm for applying this technology in regenerative medicine. The first step is to evaluate the potential of differentiation of iPSCs into specific cell types, which has been studied in a few labs. The results from studies using iPSCs for cell-based therapy are promising, and many cell types, such as cardiovascular cells [2, 16–19], hematopoietic cells [20, 21], neurons [5, 6, 22, 23], retinal cells [24, 25], and islet β cells [7, 26], have been generated from iPSCs and have similar potential to ESCs.

Many human diseases, such as myocardial infarction, diabetes, retinal degeneration, and spinal cord injury, occur because of cell loss, degeneration, and injury. Theoretically, with the transplantation of specific cells created from autologous iPSCs, the cells that are lacking can be replenished and replaced by cells with the defects corrected, thereby relieving a patient's symptoms. The transplantation of iPSC–derived cells has shown promising therapeutic effects in a few animal disease models, for example, in a rat model for Parkinson disease [5] and a mouse model for acute myocardial infarction [27].

In addition, iPSC-derived cells also have been shown to be effective for cell therapy of genetic diseases, like Fanconi anemia and sickle cell anemia [28]. With the use of a humanized sickle cell anemia mouse model, upon correction of the genetic defect, mice can be rescued after transplantation with hematopoietic progenitors obtained in vitro from autologous iPSCs [21]. With regard to humans, although direct evidence for the therapeutic potential of patient-specific iPSCs is lacking, the derivation of disease-corrected hematopoietic progenitors from Fanconi anemia iPSCs is on the forefront of applications in regenerative medicine [28].

5 Induced Pluripotent Stem Cells Offer Good Models for Personalized Medicine

iPSCs can also provide attractive cellular tools to screen new drugs in vitro and investigate drug effects at the individual level. Development of new drugs is costly and can require huge resources before achieving any success. Human ESCs are called upon when assay systems with high human relevance for drug searching are strongly needed. iPSCs could likewise allow the screening of drugs in the general population, but in addition could be a huge asset for tailoring personalized medicine. For example, patient-specific, iPSC-derived cardiomyocytes can be used in pharmacogenetic studies to test the effects of cardioactive drugs on beating frequency and contractility, which may be different across various patient ethnic populations [16].

6 Characteristics of Induced Pluripotent Stem Cells

6.1 In Vitro Studies of Induced Pluripotent Stem Cells

6.1.1 General Properties of Induced Pluripotent Stem Cells and Embryonic Stem Cells

The first generations of both mouse iPSCs and human iPSCs mimicked ES cells in morphology, as they grew rapidly and formed clones whose edges were smooth and refractive after 3–4 days of culturing on feeder cells. Karyotypes of iPSCs are usually normal and similar to those of ESCs. iPSCs show gene expression profiles similar to those of ESCs for commonly known pluripotency genes and surface markers, and they also present nearly identical overall DNA methylation patterns. Regarding developmental potentials of iPSCs or ESCs in vitro, they both can form embryoid bodies (EBs) when cultured in ES medium without leukemia inhibitory factor and are able to differentiate into many different cell lineages, such as cardiovascular cells, hematopoietic cells, neurons, and so on. In vivo studies of iPSCs are discussed later.

7 Genetic and Epigenetic Properties of Induced Pluripotent Stem Cells

The gene expression profiles of iPSCs were shown to be somewhat different from those of ESCs, and the significant differences appear to lie in differentiation genes [29, 30]. The methylation status of the iPSCs, particularly at the promoters of differentiation genes, were shown to be different from ESCs by one study [31].

The first-generation iPSCs were indistinguishable from ESCs by morphology and teratoma formation. Although they expressed most of the ESC marker genes (such as Oct4, Nanog, and SSEA1), these iPSCs appeared to have different global gene expression and DNA methylation patterns from those of their ESC partners [8]. Notably, such incomplete reprogramming of somatic cells to iPSCs were considered to result in those pluripotent cells being refractory to differentiation and thus could create many obstacles for medical and pharmaceutical applications.

Later studies in which iPSCs were derived by selection for Oct4 or Nanog activation showed patterns closer to ESCs in DNA methylation, gene expression, and chromatin states, in addition to being germline competent by producing chimeric mouse with germline transmission [9, 32]. However, these iPSCs can only support embryo development until embryonic day 14.5 when injected into tetraploid blastocysts (see later discussion) [32]. This phenomenon indicates that the second-generation iPS cells that can self-renew and differentiate into certain cell lineages are only partially reprogrammed. These differences compared to ESCs were thought to result from differential promoter binding by reprogramming factors [32].

8 In Vivo Functional Studies of Induced Pluripotent Stem Cells

8.1 The First Step: Teratoma Formation and Chimera Generation

There are several criteria representing different reprogramming levels, and their determination relies on different molecular and functional standards when we evaluate the pluripotency of cells. The potential to produce teratomas containing tissues from all three germ layers is the least stringent criterion of pluripotency. The iPSCs first described by Takahashi and Yamanaka in 2006 were capable of forming teratomas of all three germ layers [8].

A more stringent criterion of pluripotency is chimera formation, especially with germline transmission. Initially, iPSCs were shown to form chimeras upon injection into mouse normal diploid blastocysts, but their ability to contribute to the germline was not determined. In 2007, Wernig et al. [32] first reported viable diploid chimeras using reprogrammed mouse fibroblasts with germline contribution, although they still did not achieve live birth upon tetraploid complementation. Okita et al. [9] also achieved diploid chimeras derived from iPSCs with germline contribution induced from fibroblasts and demonstrated the epigenetic similarity between iPSCs and ESCs. In 2008, Aoi et al. [33] induced adult mouse hepatocytes and gastric epithelial cells into iPSCs, and they showed an ability to contribute to the germline of diploid chimeras and demonstrated that retroviral integration is dispensable for iPSCs. Kim et al. [34] reported chimeras achieved from iPSCs with germline contributions derived from adult mouse neural stem cells, using exogenous Oct4 together with either Klf4 or c-Myc. Subsequently, Eminli et al. [35] used neural progenitor cells, which expressed high levels of endogenous Sox2, to generate iPSCs, and their iPSCs also contributed to viable chimeras. The ability to form chimeric mice and demonstrate germline transmission using iPSCs further demonstrated their pluripotency compared to ESCs.

9 True Pluripotency: Tetraploid Complementation

The most stringent criterion for testing pluripotency is an assay called tetraploid complementation. By injecting iPSCs into blastocyst donors that were previously fused at the two-cell stage so that they are unable develop into a live animal, the resulting animal must consist of all iPSCs. Earlier reports showed that the embryos produced from iPSCs through tetraploid complementation can survive to embryonic day 14.5 or further, but it is difficult to get full-term live mice [9, 10]. Since the development of embryos beyond day 14.5 involves the terminal differentiation of many tissues, this suggests that production of progenitor cells from iPSCs may occur normally, but the progenitors fail terminal differentiation. For some time, it was not known whether iPSC clones could produce viable mice through tetraploid

complementation or whether reprogramming in iPSCs is complete [36]. By using better selection markers or improving the iPSC induction system, iPSCs were shown to have increasingly ESC-like gene expression and epigenetic modification patterns compared with the first-generation iPSCs reported in 2006. In 2009 Zhao et al. [37] described for the first time the generation of viable, and fertile, iPSC mice through tetraploid complementation, a result independently confirmed by Boland et al. [38].

Zhao et al. [37] used several iPSC lines that were generated from mouse embryonic fibroblast (MEF) cells by virus infecting the four "Yamanaka factors" carrying the Oct4 (also known as Pou5f1)–enhanced green fluorescent protein, and they generated 37 iPSC lines expressing specific pluripotency markers; many of them produced chimeric mice with germline transmission. Three of the 37 iPS cell lines also produced viable, live-born offspring by tetraploid complementation, and many of these mice were fertile and generated numerous second- and third-generation offspring. This was achieved by culturing the infected fibroblast cells with knockout serum replacement medium instead of fetal bovine serum for iPSC derivation, and improved efficiency was observed for iPSC formation, as well as greater competency for tetraploid complementation when cell lines were derived at an earlier time point.

Detailed analysis of these iPSC lines, as well as the live progeny generated from them, included in vivo and in vitro strategies (Table 1). ESC-specific markers and karyotypes, EB formation, gene expression, epigenetic markers, and global transcription patterns were analyzed. Epigenetic methylation analysis was performed for the Oct4 and Nanog promoter regions, which showed identical patterns for the iPSCs and ESCs. For in vivo assays, from the least to the most stringent assays for pluripotency, teratoma formation, chimeric mice production with germline formation, and tetraploid complementation were tested and all determined to be positive. The results of chimeric mouse formation with germline transmission from injecting iPS cells into normal 2N blastocysts is illustrated in Fig. 1B. After iPSCs (from mice with black coats) were injected into tetraploid blastocysts from mice with white coats made by electrofusion of two-cell embryos, they were transferred to pseudopregnant recipient females, and 4N complementation (4N-comp) mice with a clearly black coat were generated (Fig. 1C). To identify clearly the lineage of the cells and the 4N-comp mice, a variety of tests were used, including simple sequence length polymorphism analyses (Fig. 1F) and unique microsatellite polymerase chain reaction (PCR) for characterization of iPSC mice through tetraploid complementation (Fig. 1E), reverse PCR, and Southern blot analysis for validation of genomic integration of transgenes.

Boland et al. [38] generated live-born adult mice derived entirely from iPSCs by doxorubicin-induced, lentiviral-mediated reprogramming of MEFs. They obtained 21 colonies from doxorubicin-plus-valproic acid–treated cells, referred to as iPSC lines. They generated iPSC lines that contributed to chimera with germline formation, and four of six iPSC lines generated live, fertile mice.

These two groups along with others [39] validated that iPS cells could be fully pluripotent to generate all types of cells in the body. This provides hope for the broad

Table 1 Commonly Used Strategies for Characterization of Induced Pluripotent Stem Cells

	Assays	Results
In vitro assays	Molecular marker	Positive for AP,[a,b] Oct4,[a,b] Nanog,[a,b] SSEA-1,[a] SSEA-3,[b] SSEA-4,[b] GCTM-2[b]
	Karyotype	Normal
	Erythroid body formation	Positive
	In vitro differentiation	Can differentiate into three germ layers
	Gene expression	Similar to embryonic stem cells
	Epigenetic modification	Almost completely reset their H3K27 methylation patterns
	Global transcription analysis	Similar to embryonic stem cells but with a small subset of genes of distinct expression
In vivo assays	Teratoma	Differentiated into three germ layers (e.g., neurons, striated muscles, minor salivary gland, etc. [37])
	Chimera formation	Chimeric mouse (2N) produced
	Chimera with germline formation	Chimeric mouse (2N) with germline produced
	Tetraploid complementation	4N complementation mice generated

AP, alkaline phosphatase.
[a] Murine
[b] Human.

application of iPSC techniques in the field of regenerative medicine. That said, a system of long-term testing of these "all-iPSC" progeny is still needed to evaluate the practicality of using iPSCs for clinical interventions for disease treatment.

10 Induced Pluripotent Stem Cells: From Bench to Bedside

Since the iPSCs have passed the most stringent criterion of pluripotency—tetraploid complementation—and successfully produced full-term, fertile mice, demonstrating that they are very similar to embryonic stem cells in vitro and in vivo, there is a more promising future for the application of iPSCs to clinical therapy. The general strategy of applying iPSCs in cell-based therapy involves (1) reprogramming of defective somatic cells into iPSCs, (2) repair of the genetic defect through homologous recombination [21], (3) in vitro differentiation of repaired iPSCs into specific cell types, and (4) transplantation of these cells back into the patient. All of these steps need to be strictly evaluated before clinical application, which may have a long way to go, but with the tremendous progress in iPSC research in the last few years, it has a bright future in disease investigation, pharmaceutical development, and regenerative medicine.

After demonstrating the feasibility of generating iPSCs with full pluripotency, ongoing iPSC research is focusing on improving the efficiency of generating iPSCs,

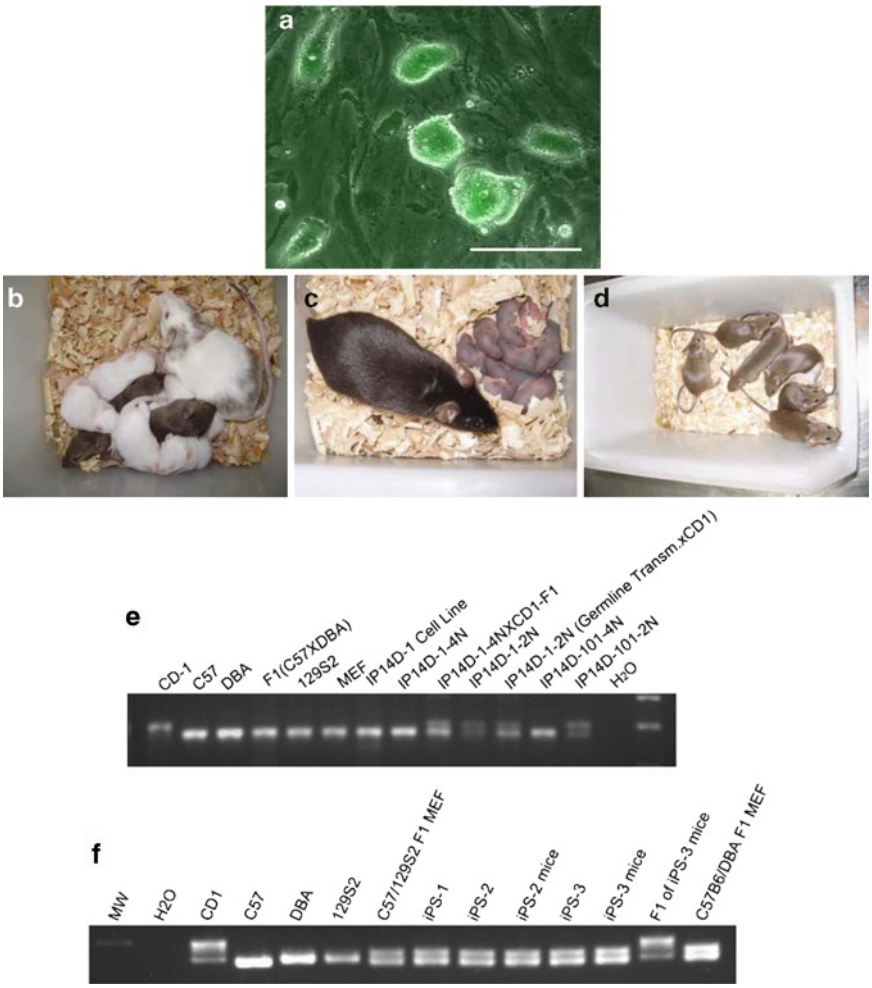

Fig. 1 Induced pluripotent stem cell (iPSC) characterization. **a:** Morphology of one iPSC line (IP14D-1) at passage 14. Bar = 100 μm. **b:** iPSC chimera mouse and its offspring, demonstrating germline transmission. The chimeric mouse (shown in the upper right) was generated by injecting IP14D-1 cells (brown coat) into a blastocyst (white coat), and this mouse's progeny, after mating with the CD-1 mouse, are either brown or white coated, depending on the lineage of the chimera's germ cell. **c:** iPSC-tetraploid mouse with a uniformly black coat at 7 months and its live progeny. **d:** Progeny of iPSC-tetraploid mouse (these F1 pups of an iPSC-tetraploid mouse mated with a CD-1 mouse have uniformly brown coat color). **e:** Confirmation of iPSC chimera and tetraploid mice using microsatellite markers. The data show that these mice are generated from the corresponding iPSC line. **f:** Lineage identification of different strains of mice by simple sequence length polymorphism analysis, showing that the 4N mice have polymorphic DNA patterns identical to those of the parental MEF cells. (Panels B–E are reprinted from Zhao, X.Y., W. Li, Z. Lv, et al. (2009). iPS cells produce viable mice through tetraploid complementation. *Nature*, 461, 86–90, Fig. 2h, f, g, d, respectively, with permission)

exploring new cell origins for iPSC induction, and the safety measures necessary before any clinical intervention. First, iPSC induction efficiency from somatic cells is still very low. Small-molecule compounds may improve the efficiency of iPSC induction by either inhibiting differentiation pathways and activating signal pathways that help the iPSC obtain pluripotency [40–42] or changing the epigenetic status [43] to generate iPSCs. Other new ways to improve the efficiency of iPSC induction involve reducing the O_2 concentration [44]. The iPSC reprogramming process is believed to be different from the oocyte cytoplasm reprogramming involved in nuclear transfer [45,46]. Although the detailed mechanism is unknown, some interesting results have provided direction, such as implying that cancer suppressor genes likely participate in the process; when p53 is inhibited, the efficiency of iPSC induction is high, and associated chromatin remodeling is also involved [47].

Second, iPSCs derived from MEFs have true pluripotency, but embryonic tissues are not a feasible source for human iPSC derivation. Finding the most easily obtainable starting material for high-quality iPSCs is important, and researchers have generated iPSCs from liver and stomach cells [33], pancreatic beta cells [48], and mature B lymphocytes [49].

Last and most important is the safety of using iPSCs in therapeutic applications. Earlier studies reported the generation of tumors [8] thought to result from viral integration of the iPSC factors. Thus, many studies have since been dedicated to making the system safer by decreasing the possibilities of tumorigenesis by different strategies. One example is substituting the small molecules described previously instead of viral integration for induction. In another example, after removal of the virus DNA sequences in human iPSCs, the gene expression patterns of the human iPSCs were shown to more resemble embryonic stem cells [50]. Several groups have generated virus-free systems that should also improve the safety of iPSCs [51, 52], and the Yamanaka group demonstrated that high-quality iPSCs can be produced without the oncogene Myc and did not form tumors during this study [53]. Because full-term, fertile mice have been produced through tetraploid complementation from “all-iPSCs,” we now have substantially greater capacity to investigate the developmental process and its abnormalities at an organismal and microscopic scale using these iPSC mice and their progeny. This will provide valuable information about the potential uses of human iPSCs and their long-term effects spanning multiple generations.

11 Summary and Prospects

In recent years, iPSC studies have progressed very rapidly. We now know that iPSCs not only resemble ESCs by in vitro assays, but they also possess true pluripotency equal to ESCs as demonstrated by generation of viable, fertile mice through tetraploid complementation. There are still small differences between the two cell types. Elucidating these differences could be very important in the study

of fundamental mechanisms and clinical applications in the stem cell field. In January 2009, the first human trial using embryonic stem cells as a cell transplantation therapy to treat spinal cord injury was approved by the U.S. Food and Drug Administration. With the advantages of relieving issues of immune rejection and avoiding major ethical controversies presented by ESCs, it is hoped that high-quality iPSCs can be generated that can complement ESCs to achieve great progress in regenerative medicine, especially personalized medicine, and pharmaceutical applications in the near future.

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Direct Reprogramming of Human Neural Stem Cells by the Single Transcription Factor OCT4

Jeong Beom Kim, Holm Zaehres, and Hans R. Schöler

Abstract Reprogramming of human somatic cells represents a valuable tool for enhancing our understanding of the mechanisms underlying the acquisition of pluripotency and for realizing the potential to generate patient-specific pluripotent stem cells. Induced pluripotent stem (iPS) cells have been generated from mouse and human somatic cells by the ectopic expression of four transcription factors (*OCT4, SOX2*, c-*MYC*, and *KLF4*). Recently, we reported that *Oct4* acting singly is sufficient to directly reprogram adult mouse neural stem cells (NSCs) into iPS cells. Furthermore, we found that the generation of one-factor (1F) human iPS cells from human NSCs is possible by the ectopic expression of *OCT4* alone. These human NSC-derived 1F iPS cells are indistinguishable at the molecular level from human embryonic stem cells (ESCs) and can differentiate into cells of all three germ lineages both in vitro and in vivo. These findings demonstrate that the transcription factor OCT4 is sufficient to reprogram mouse and human NSCs into pluripotent cells. This chapter focuses on the generation of iPS cells from mouse or human NSCs and uses biologic concepts to compare NSCs and pluripotent stem cells such as ESCs and iPS cells.

Keywords iPS • Neural stem cells • Reprogramming • OCT4

1 Introduction

Embryonic stem cells (ESCs) derived from the inner cell mass of mammalian blastocysts are able to grow indefinitely while maintaining a state of pluripotency but also have the potential to differentiate into cells of all three germ layers [1–3]. Understanding the properties of murine and human ESCs (mESCs and hESCs,

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respectively) may spur development of cell replacement therapies for diseases and damaged tissues, such as Parkinson disease, spinal cord injury, and diabetes [3]. Stem cell research aims to develop methods and strategies for the management of genetic and degenerative disorders by the provision of an abundant supply of transplantable cells. Widespread interest into the utility of ESCs stems from their ability to generate all cell types of an organism [4].

The use of the transcription factors Oct4, Sox2, Klf4, and c-Myc can lead to induction of pluripotency in mouse and human fibroblasts [5–13]. These generated ESC-like induced pluripotent stem (iPS) cells are very similar to ESCs with respect to genetic, epigenetic, and developmental criteria. The ultimate utility of reprogrammed somatic cells lies in the generation of pluripotent cell lines specific to individual patients, obviating the need for obtaining pluripotent cells from human embryos.

The molecular mechanisms underlying reprogramming are still far from being understood due to several variables, such as the number of exogenous factors required and the heterogeneity of target cells. To simplify this scenario, we selected neural stem cells (NSCs) as a cell model for reprogramming, using different combinations of transcription factors [14]. Various combinations of transcription factors support the generation of iPS cells, with *OCT4* being an integral factor. We first demonstrated that *Oct4* and *Klf4* are sufficient to induce pluripotency in adult mouse NSCs [14]. By omitting *Klf4*, we further demonstrated that Oct4 is not only essential but also sufficient to induce pluripotency in mouse and human NSCs [15, 16]. These studies provide the first evidence that a single transcription factor can convert somatic cells into pluripotent cells with potential to develop into derivatives of all three germ layers both in vitro and in vivo. The generation of iPS cells by a single transcription factor—that is, one-factor (1F) iPS cell generation—should enhance our understanding of somatic cell reprogramming in general and generation of patient-specific pluripotent stem cells in particular. iPS cells generated using the single transcription factor Oct4 should be associated with fewer genomic integrations and have no oncogenic potential compared with iPS cells generated using four transcription factors. In this chapter, we provide insight into the reprogramming of mouse and human NSCs by the transcription factor Oct4 and discuss advantages, from the biologic, technical, and clinical applicability perspectives, of using NSCs as the starting cell population.

2 Background

2.1 Generation of Induced Pluripotent Stem Cells from Human Neural Stem Cells by OCT4 Alone

The utility of patient-specific iPS cells is limited by two issues inherent to the reprogramming strategy: (1) the restrictive passage or heterogeneity of primary human cells, which hinders the generation of iPS cells from a small starting cell

population or may result in irregular reprogramming efficiency, and (2) the questionable clinical applicability of retrovirus-mediated iPS cells due to the integration of viral transgenes into the somatic cell genome, particularly of c-*Myc* and *Klf4* oncogenes. Viral integrations can give rise to insertional mutagenesis and unpredictable genetic dysfunction [8]. Indeed, reactivation of the c-Myc retrovirus contributes to tumor formation in chimeric mice derived from iPS cells [8, 17].

To circumvent these limitations, that is, to eliminate the presence of oncogenes and reduce integration, we previously developed an approach involving the use of NSCs, rather than differentiated cells, as the starting cell population.

Our analyses of the endogenous level of the four transcription factors showed that mouse NSC expressed approximately twofold higher levels of *Sox2*, tenfold higher levels of c-*Myc*, and eightfold lower levels of *Klf4* than ESCs [14] (Fig. 1). They do not require Sox2, Klf4, and c-Myc transduction to be reprogrammed to pluripotency, which we recently demonstrated when generating iPS cells from mouse NSCs by Oct4 and Klf4, or Oct4 alone [14, 15].

Using Oct4-green fluorescent protein expression as a marker for reprogramming, we observed that the time required to complete reprogramming (i.e., duration of reprogramming) was dependent on both the number (four, three, or two) and the combination of the pluripotency-inducing factors (Oct4, Sox2, Klf4, and c-Myc) [14]. The more transcription factors used, the earlier were NSCs reprogrammed at higher efficiency. In addition, we confirmed that mouse NSCs could not be reprogrammed in the absence of Oct4, regardless of the combination of reprogramming factors used [14] (Table 1).

Similarly, human iPS cells could be generated from hNSCs by *OCT4* alone. These human iPS cells are very similar to embryo-derived hESCs, exhibiting a

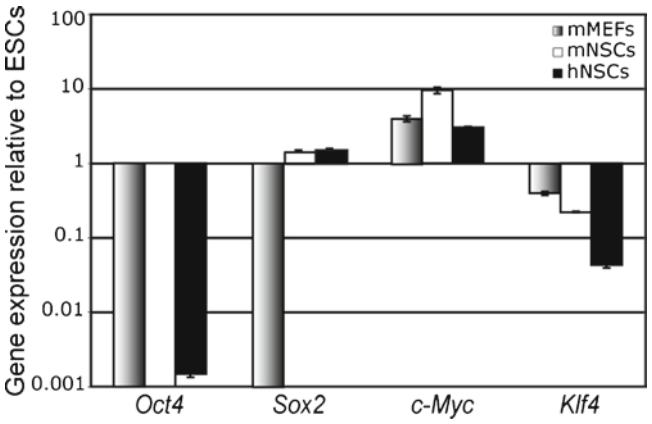

Fig. 1 Endogenous expression levels of the four factors Oct4, Sox2, Klf4, and c-Myc in mouse embryonic fibroblasts (MEFs) and mouse and human neural stem cells (NSCs) based on quantitative polymerase chain reaction (PCR) analysis of endogenous expression of the four factors in NSCs and MEFs. RNA levels were determined by quantitative real-time PCR using primers specific for endogenous transcripts

Table 1 Comparison of Estimated Reprogramming Efficiencies for Several Initial Populations

Initial Population	Number of Infected Factors	Number of Starting Population	Number of iPSC Colonies	Infection Efficiency (%)	Reprogramming Efficiency (%)
MEFs-Fbx 15 [8,10]	4	80,000	4–125	50	0.01–0.5
MEFs-Nanog [8,10]	4	80,000	47–1,800	50	0.001–0.03
MEFs-Oct4 [12]	4	100,000	8	10	0.08
MEFs-Nanog [12]	4	100,000	5	10	0.05
MEFs [7]	4	100,000	52	10	0.5
Hepatic-Fbx 15 [26]	4	80,000	83	4	2.6
Hepatic-Fbx 15 [26]	3	80,000	61	9	0.8
mNSCs [14]	4	50,000	73 ± 11	4	3.5 ± 0.5
mNSCs [14]	2 (OK)	50,000	11 ± 2	21	0.11 ± 0.02
mNSCs [15]	1 (O)	50,000	3	45	0.013
hNSCs [16]	2 (OK)	50,000	3	—[a]	0.006
hNSCs [16]	1 (O)	50,000	2	—[a]	0.004

Hepatic, hepatocytes; hNSCs, human neural stem cells; iPSC, induced pluripotent stem cell; MEFs, mouse embryonic fibroblasts; mNSCs, mouse neural stem cells; O: OCT4; OK: OCT4 and KLF4.

[a]Reprogramming efficiency is calculated by starting cell number.

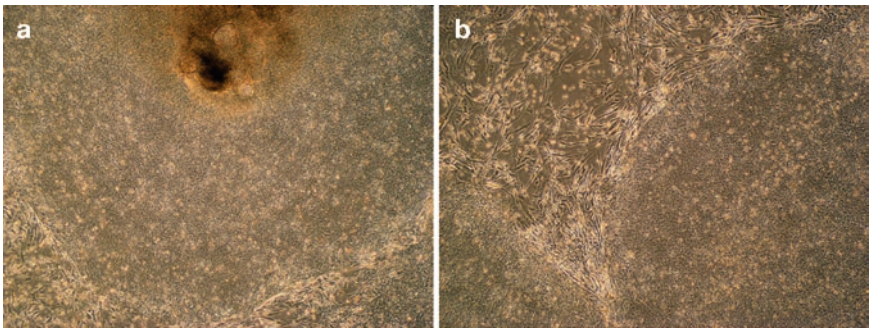

Fig. 2 One-factor human neural stem cell–derived induced pluripotent stem (iPS) cell colony formation. **a:** The human embryonic stem (ES) cell–like colony outgrowing from neural rosette-like center. **b:** Typical human ES cell–like iPS colony growing on a feeder layer

closely clustering global gene expression pattern and the same differentiation potential. During reprogramming we found a neural ectodermal morphology with an hESC-like morphology that may resemble an intermediate state; human NSCs may be dedifferentiated to the pluripotent state through neural rosettes (Fig. 2). As in the NSC culture condition, human NSCs cultivated in monolayer express higher endogenous levels of *SOX2* and *MYC* and lower endogenous levels of *KLF4* than ESCs and can be reprogrammed to a pluripotent cell state using two transcription factors (*OCT4* and *KLF4*) or one (*OCT4*) [16] (Fig. 1).

2.2 *Endogenous Expression of Pluripotency Markers*

Our initial hypothesis that the number of reprogramming factors can be reduced when using somatic cells that endogenously express appropriate levels of complementing factors could also be verified for human fibroblasts.

Two transcription factors (OCT4 and SOX2) are sufficient to generate iPS cells from human neonatal fibroblasts [18], a fully differentiated somatic cell that is already expressing KLF4 and c-MYC. One report notes that a combination of OCT4, SOX2, NANOG, and LIN28 is sufficient to reprogram primary human fibroblasts in the absence of KLF4 and c-MYC transduction [19].

NANOG is one of the core pluripotency genes. Even without exogenous transduction it is expressed in human iPS cells at the same level as in human ESC cells [16, 20–22].

The sequential expression of marker genes, such as alkaline phosphatase (AP) and stage-specific embryonic antigen-1 (SSEA-1), has been shown to represent an intermediate reprogramming stage preceding the expression of *Oct4* or *Nanog* during reprogramming [12, 20]. Of interest, mouse NSCs exhibit endogenous SSEA-1 expression and AP activity [21, 22] (Fig. 3).

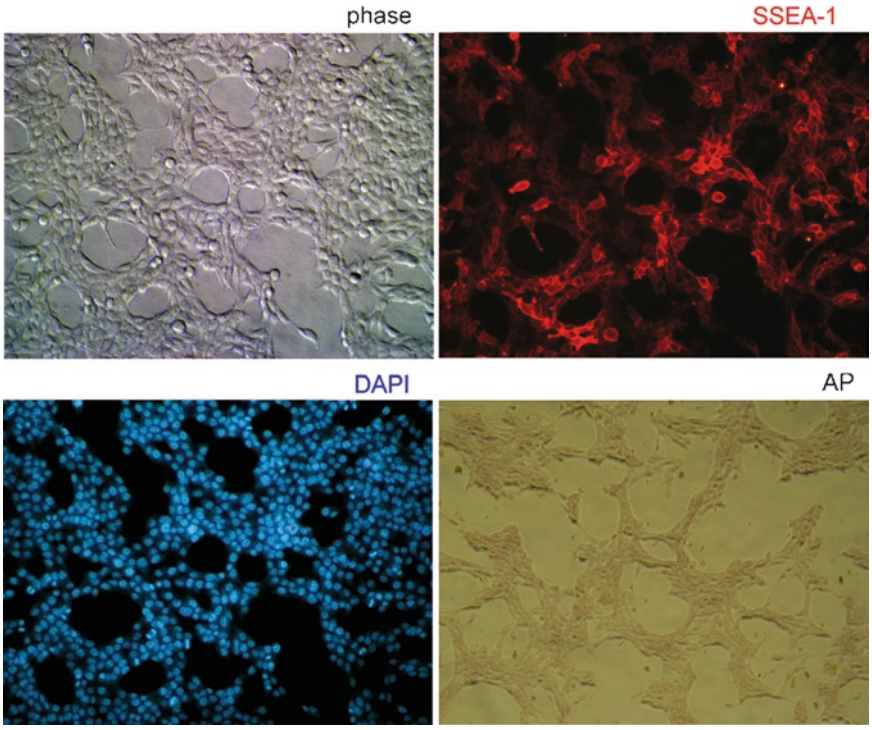

Fig. 3 Expression of stage-specific embryonic antigen-1 (SSEA-1) and alkaline phosphatase (AP), representing intermediate reprogramming markers, in neural stem cells (NSCs). NSCs express both SSEA-1 **(upper right)** and AP **(bottom right)**. The cells were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) **(bottom left)**

We therefore considered whether NSCs might represent a more advanced stage of reprogramming through which terminally differentiated cells have to go to subsequently acquire pluripotency. Consistent with this notion is the fact that NSCs are reprogrammed earlier and more efficiently than fibroblasts.

The resultant iPS cells were capable of differentiating into cells of all three germ lineages.

2.3 Comparison of the Transcriptional Profiles

The mechanisms regulating self-renewal and cell fate decisions in mammalian stem cells are still poorly understood. Two reports compared the gene expression profiles of mouse ESCs, NSCs, and hematopoietic stem cells, all of which seem to share a common genetic program that may be important for "stemness" [23, 24]. Transcriptional-profiling studies indicate that ESCs and NSCs share a number of

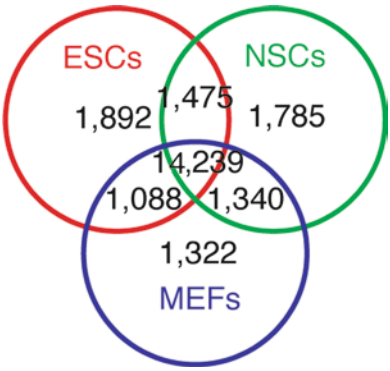

Fig. 4 Overlapping gene expression in embryonic stem cells (ESCs), neural stem cells (NSCs), and mouse embryonic fibroblasts (MEFs). Venn diagram detailing shared and distinct gene expression in each population

similarities at the transcriptional level and can enhance our understanding of stem cell biology and the nature of stem cells.

We investigated the use of NSCs and mouse embryonic fibroblasts (MEFs) as a starting cell population by comparatively analyzing the transcriptional profiles of ESCs, NSCs, and MEFs by microarray (Fig. 4). The analysis was intended to identify genes enriched in each individual cell population and then to compare the resulting sets of products. We found an overlap of 15,714 genes (84%) between ESC-enriched genes and NSC-enriched genes and of 15,327 genes (81.9%) between ESC-enriched genes and MEF-enriched genes. However, there is no significant difference between NSCs and MEFs compared with ESCs. One might expect an overlap between ESCs and MEFs, as both cell populations are derived from embryos. Nevertheless, NSCs (multipotent cells) more closely resemble ESCs (pluripotent cells) than MEFs (fully differentiated cells) with respect to enriched genes in the hierarchic clustering [14, 15]. These results demonstrate that the transcriptome pattern of NSCs is more similar to that of ESCs than that of MEFs, which can be attributed to higher reprogramming efficiency and earlier reprogramming kinetics. In summary, we determined the molecular similarities between two distinct stem cell populations: NSCs and ESCs. The similarities in microarray analyses defined a common stem cell molecular signature. It is likely that a hallmark property of stem cells, such as the ability to balance self-renewal and differentiation, is governed by shared molecular mechanisms.

3 Conclusions

The molecular and functional similarity between human NSC-derived 1F iPS cells and ES cells makes in vitro reprogramming an attractive approach to generating patient-specific stem cells for the study and potential treatment of degenerative diseases. This study has several implications: (1) OCT4 alone can induce pluripotency in human NSCs. 1F iPS cells could be differentiated into lineage-committed populations, including germ cells, leading to germline transmission. (2) Human iPS

cells can be generated without the oncogenic factors c-MYC and KLF4. Since reactivation of the c-Myc virus may cause tumor formation [8], iPS cells have been generated without infection of the c-Myc oncogene [18, 25]. However, the presence of KLF4, another oncogenic factor, in the reprogramming combination may also cause tumor formation. (3) Reducing the number of factors needed to reprogram somatic cells reduces the likelihood of retroviral insertional mutagenesis. Analysis of previously described iPS cells revealed the presence of up to 20 retroviral integrations using the four transcription factors [12, 26]. In this study, we demonstrated that human 1F iPS cells contain three integrations of only the *OCT4* transgene. (4) The use of NSCs—which endogenously express Sox2, c-Myc, and Klf4, as well as AP and SSEA-1—as a target cell population for reprogramming provides a unique opportunity to study the mechanisms underlying the generation of iPS cells, as NSCs can be reprogrammed by four, three, two, or one factor.

In conclusion, OCT4 alone is sufficient to induce pluripotency in human NSCs without the need of the oncogenes c-*Myc* or *Klf4*, attesting to its crucial role in the process of somatic cell reprogramming. Our results support the hypothesis that NSCs represent an intermediate state between differentiated and pluripotent cells. The one-factor–induced human iPS cells resemble human ESCs cells in pluripotency and can thus differentiate into cells of all three germ layers, as demonstrated by in vitro and in vivo analyses, global gene expression profiles, and epigenetic states. Our studies provide insight into the mechanisms governing the reprogramming of somatic cells to pluripotency. Therapeutic use of iPS cells would be safer and more practical if we could generate 1F iPS cells by nonviral means such as via recombinant proteins and/or small molecules.

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Part V
Tissue Engineering

Stem Cells and Biomaterials: The Tissue Engineering Approach

Stefania Antonini, Angelo Vescovi, and Fabrizio Gelain

Summary Regenerative medicine is an interdisciplinary field of medical science that brings together the principles of tissue engineering and the life sciences to develop biologic "components" for the maintenance, regeneration, and replacement of tissue and organs. The principal components of regenerative medicine are cells, scaffolds, and specific physical, chemical, and biochemical signals that replicate the natural microenvironments within the body.

Keywords Regenerative medicine • Tissue engineering • Stem cells • Biomaterials

1 Cell Sources

One of the most important objectives of regenerative medicine is to obtain a sufficient number of cells that maintain their phenotype and their biologic activities. A variety of cells have been used for tissue engineering. The first works used mature primary cells directly isolated from explants of adult tissue. Tissue-specific differentiated autologous cells can be harvested from individual biopsies, cultured ex vivo for their expansion, and then implanted into the damaged tissue [1]. Several preclinical and clinical applications are being explored, including chondrocytes for cartilage repair, keratinocytes and dermal fibroblasts for burn repair, retinal pigment epithelial cells for age-related macular degeneration, and Schwann cells to restore myelin in the central nervous system [2]. Primary cells can be derived from the patient tissue, avoiding immunologic rejection, but in general they are postmitotic and thus have limited proliferative potential; moreover, during the expansion period in culture, the cells can dedifferentiate and acquire inappropriate phenotypic

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characteristics [3]. Due to these limitations, it is necessary to find and develop alternative sources of cells for regenerative medicine, and stem cells seem to provide solutions to most of the problems typical of mature primary cells.

The common hallmarks of stem cells are their unlimited self-renewal capacity for proliferation and their high multilineage differentiation potential, which confer on them a fundamental role during embryo/fetal development and throughout adult life. According to their developmental potential, stem cells can be divided in different categories: totipotent, pluripotent, multipotent, or unipotent. Totipotent stem cells can be found in the early stages of embryo development, after the very first cell divisions; these cells are able to differentiate in all the embryo tissues and in the trophoblast. After the segregation phase, the embryo, now called a blastocyst, contains a cluster of cells, the inner cell mass, from which the embryo develops, and embryonic stem cells (ESCs) are isolated; ESCs are defined as pluripotent because they can differentiate into cells of the three germinal layers (ectoderm, mesoderm, endoderm) but not in the trophectoderm lineage. Lower down in the hierarchical tree there are the multipotent stem cells, isolated from specific niches in many adult tissue and organs, which can produce a limited range of differentiated cell lineages. Finally, there are the unipotent stem cells, also defined as committed progenitors because can generate only one specific cell type and have more limited proliferative potential.

2 Pluripotent Stem Cells

ESCs (Fig. 1a) are the most plastic cell source for regenerative medicine [4, 5]. They were first isolated in the 1980s by Evans and Kaufman from mouse blastocyst [6]; it took another 17 years before their isolation from human embryos [7]. Apart from being a simple model for studying early developmental processes and differentiation, the isolation of ESCs, in particular from human blastocysts, represented an important boost for regenerative medicine. A unique set of transcription factors ensures self-renewal and pluripotency and simultaneously suppresses differentiation [8]. This ensemble can be maintained in vitro using specific culture conditions and specific cytokines; conversely, by changing the culture environment, one can induce ESCs to differentiate into all cells of the three primary germ layers: ectoderm, mesoderm, and endoderm [9]. For clinical application, efforts must be addressed to define good manufacturing practices (GMPs) in order to validate protocols for the derivation, culture, and differentiation of human ESC lines [10]. Moreover, since it was demonstrated that human ESCs can express nonhuman antigens during growth in the presence of animal feeder cells or animal-derived components [11], it is necessary to culture human ESC lines in defined and humanized conditions. Various solutions have been tried, including the use of allogeneic human fibroblasts or human ESC-derived cells as xeno-free feeder layers [12] and protocols for feeder-independent culture conditions in defined medium [13].

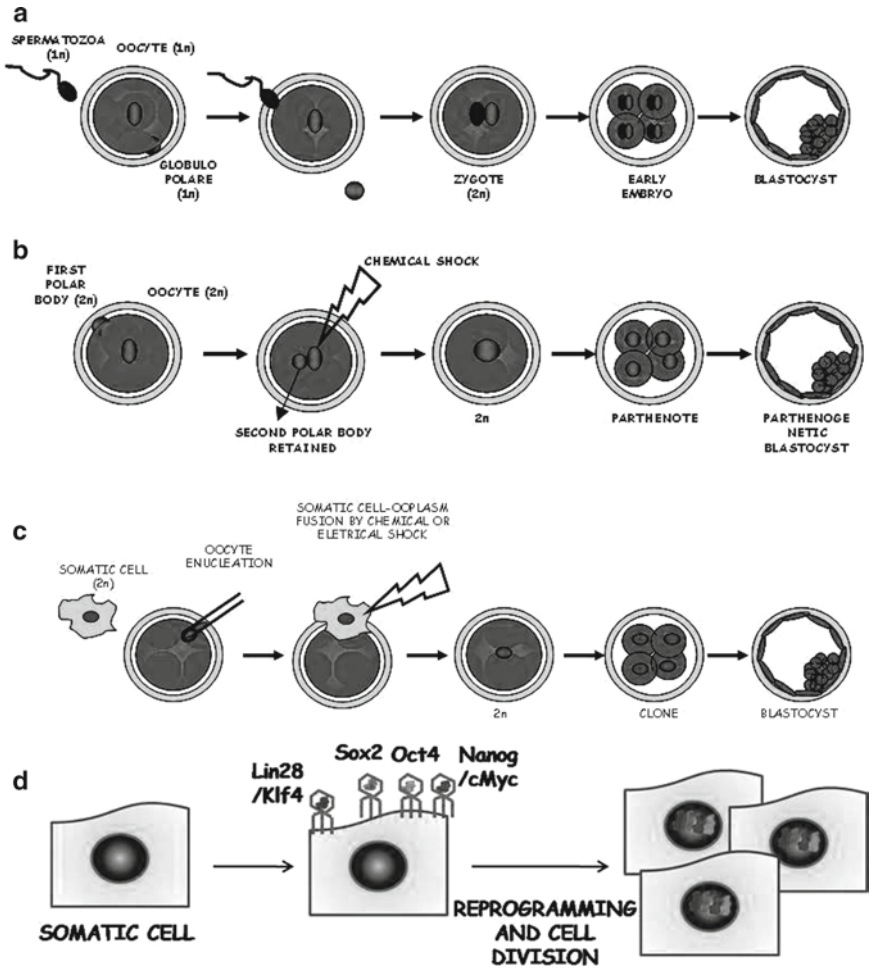

Fig. 1 Sources of pluripotent stem cells for regenerative medicine. **a:** In vitro–derived embryonic stem cells. **b:** Parthenogenetic embryonic stem cells. **c:** Embryonic stem cells obtained from cloned embryos. **d:** Induced pluripotent cells (iPSCs)

Clinical trials and therapies require the ability to culture large quantities of ESCs under GMP conditions; thus it is reasonable to state that development of scalable human embryonic stem cell systems remains in its infancy. Recently, a robotic system for plating, feeding, and harvesting ESCs was developed that gives growth and differentiation rates similar to those of the normal culture methods [14]. An alternative is suspension culture expansion, which potentially offers a more scalable system than adherent plate-based culture [15]. These systems have been developed with murine ESCs, so the translation of these findings to human ESCs may be complicated by differences between the two cell types.

Finally, the derivation and use of human embryonic stem cells is hindered by ethical concerns regarding the destruction of normal human embryos. For this reason, scientists are looking for alternative protocols in order to derive embryonic stem cells that do not require embryo destruction. Lines can be created from arrested embryos derived by in vitro fertilization procedures, albeit at low efficiency [16]. Alternatively, Chung et al. [17] derived human ESC lines using single-cell biopsies from embryos at the morula stage, but given the labor-intensive nature of this process and the risk that blastomere biopsy may pose to the development of the embryo, it is unlikely that this protocol will become a widespread strategy for generating patient-specific stem cell lines. Blastocysts produced by chemical-induced parthenogenesis—asexual development of an unfertilized oocyte (Fig. 1c)—can be used to derive ESCs lines [18, 19]. Although parthenogenesis results in a high incidence of genetic abnormalities (i.e., deleterious recessive mutations in the donor oocyte), this method does not require embryo destruction and may be suitable for generating human leukocyte antigen–matched lines that could be banked for patient-specific regenerative therapies.

Other possibilities relate to the reprogramming of adult somatic cells in order to obtain patient-specific and immune-compatible embryonic or embryonic-like stem cells. In addition to reducing the ethical concerns surrounding human embryonic stem cells, these novel methods aim at developing patient-specific pluripotent cell lines and generating lines suitable for clinical applications.

Somatic cell nuclear transfer (SCNT), or therapeutic cloning, comprises the transfer of the nucleus of a somatic cells into an enucleated donor oocyte whose ooplasm contains a pattern of still-undefined factors that are able to erase the somatic epigenome, making possible the beginning of a new developmental program (Fig. 1c) [20, 21]. From the cloned embryo at the blastocyst stage it is possible to isolate ESCs that carry the patient genome, but the therapeutic application of SCNT is hindered by both the low efficiency of the technique and ethical issue [22].

Recently, scientists have developed different protocols that allow obtaining pluripotent cells from mature cells without using embryos [23–25]. Among these methods, a promising new source of patient-specific pluripotent cells is the induced pluripotent stem cell (iPSC) obtained from adult somatic cells reprogrammed directly to a state of pluripotency through the ectopic expression of defined transcription factors (Fig. 1d) [26]. iPSCs have been derived in various ways and from different murine and human cell types [27]. They possess morphologic, molecular, and developmental features that closely resemble those of blastocyst-derived ESCs. Direct reprogramming provides a way of generating a sufficient number of autologous pluripotent stem cells that could be used for regenerative and therapeutic purposes, as demonstrated in some mouse models [28, 29]. Although the exact mechanisms of reprogramming remain unknown, only a few challenges, including generation of retrovirus integration–free human iPSCs, remain before iPSCs can be used routinely in regenerative medicine.

One of the major hurdles to the successful and safe clinical use of pluripotent cells, regardless of their origin, is the possibility of teratoma or teratocarcinoma formation in the recipient owing to the presence of residual undifferentiated pluripotent cells in transplants.

3 Adult Stem Cells

Adult stem cells represent represent a valid alternative to embryonic/pluripotent stem cells. Every tissue of the adult organism maintains a population of putatively slowly cycling stem cells that maintain homeostasis of the tissue and respond to injury in pathologic situations. These cells are regulated and supported by the surrounding microenvironment, referred to as the stem cell "niche" (Fig. 2). The niche includes all cellular and noncellular components that interact in order to control the adult stem cells, and these interactions can often be broken down into one of two major categories: physical contact and diffusible factors.

Adult stem cells have attracted great interest for their use in regenerative medicine [30]. They appear to originate during tissue ontogeny and persist in specialized niches, where they remain quiescent; however, they can undergo proliferation and differentiation into more-mature and more-specialized cells following change in the

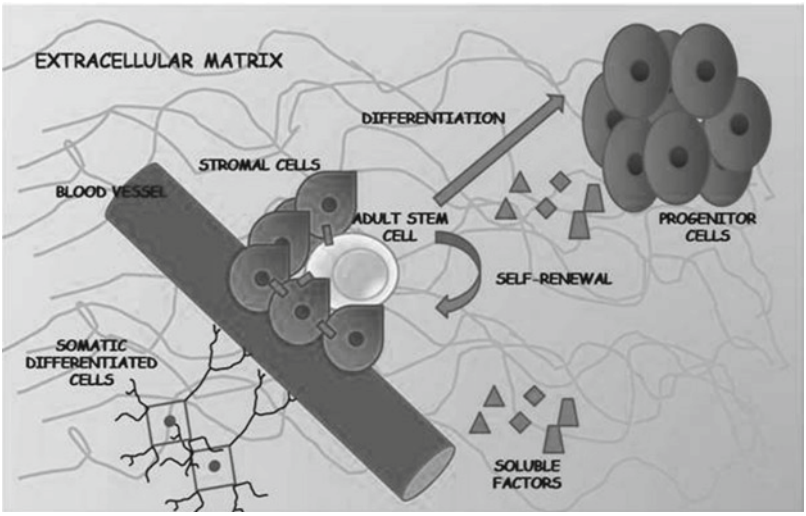

Fig. 2 Adult stem cells reside in specific microenvironments of adult tissues called "niches" where cellular and noncellular components, including stromal cells, soluble factors, and extracellular matrix proteins, can influence adult stem cell behavior, inducing their self-renewal or differentiation

microenvironment of their niches in both physiologic and pathologic conditions [31]. Traditionally, adult stem cells were viewed as cells committed to produce mature cells only from the tissue of origin; various reports challenged this dogma by demonstrating that adult stem cells can give rise to other cell types typical of other tissue, demonstrating that they are able of changing their cell fate under certain conditions [32]. This concept of "stem cell plasticity" defies the basic developmental biology principle of lineage restriction during morphogenesis, but it represents a considerable step forward in therapeutics [33].

The reciprocal interactions of adult stem cells with neighboring cells of the niche via the formation of adherent junctions and the secretion of soluble factors might contribute to their restricted mobility and their quiescent or activated state within a niche [34]. Some niches are clearly well defined within their respective tissues, and their stem cells have been identified quite precisely by their anatomic localization and their morphology; niches have been found in different tissues, including bone marrow, heart, nervous system, gut, skin, and adipose tissue [35].

4 Scaffolds and Microenvironments

The therapeutic application of stem cells is a promising and rapidly emerging branch of regenerative medicine in which stem cell–based treatment could be applied to treat and cure many aggressive and lethal diseases in human. The success of cell transplantation therapies is not only influenced by the protocols for the isolation and differentiation of stem cells, it also depends on the method by which cells are engrafted and induced to functionally integrate into the affected tissue. In fact, cells are located in three-dimensional (3D) microenvironments in vivo, where they are surrounded by other cells and by the extracellular matrix (ECM), whose components, such as collagen, elastin, and laminin, are organized in nanostructures (fibers, triple helixes, etc.) with specific bioactive motifs that regulate the cell homeostasis. It is therefore essential to develop scaffolds that create synthetic microenvironments providing 3D supports in order to control and direct the cellular behavior and promote specific cell interactions. Properties affecting a scaffold's utility include biocompatibility (nonimmunogenic and cytotoxic); porosity and pore size (to facilitate oxygen, nutrient, and waste transfer, as well as tissue integration and rapid vascularization); appropriate surface chemistry (including pH and surface charge) to favor cellular adhesion, differentiation, and proliferation, controlled biodegradability, and bioresorbability; mechanical properties; and the ability to integrate in the implantation site and promote cell–substrate interaction [36–38].

When designing a scaffold for tissue engineering the choice of the biomaterial is of crucial importance. Biomaterials investigated in regenerative applications can be divided into natural, synthetic, and semisynthetic polymers (Fig. 3) [39].

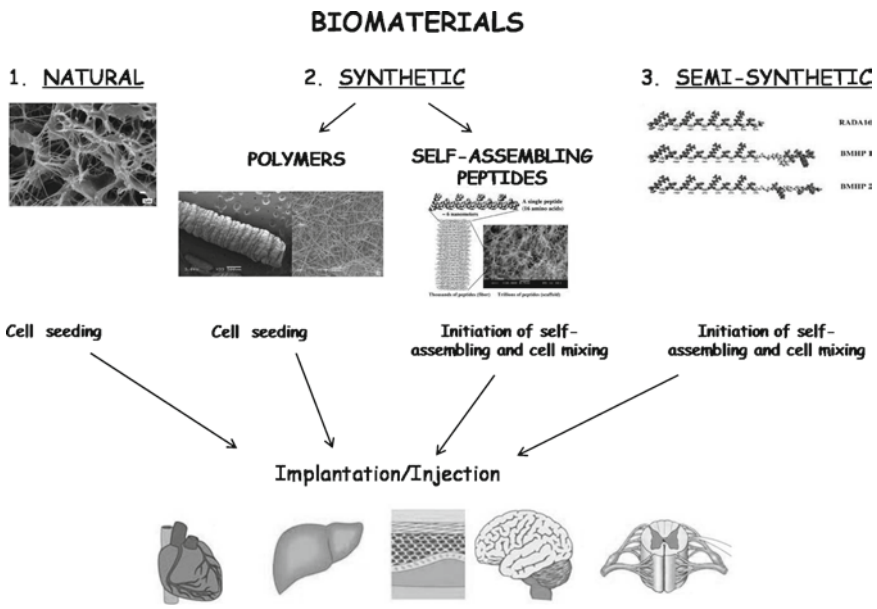

Fig. 3 Biomaterials and scaffolding approaches in tissue engineering

5 Natural Biomaterials

Natural materials are obtained from natural sources such as collagen and proteins of the extracellular matrix or alginates and exhibit similar properties to those of the tissues they are replacing. Collagen, a triple-helix protein, is one of the major components of the extracellular matrix. It provides support for connective tissue such as skin, tendons, and bone and also interacts with cells in connective tissues and provides signals for cell adhesion, migration, proliferation, differentiation, and survival. Collagen is characterized by high mechanical strength, good biocompatibility, low antigenicity, and the ability to be cross-linked; it may be used in crude form or processed into porous sponges, gels, and sheets and cross-linked with chemicals to make it stronger or alter its degradation rate [40]. The use of collagen matrix has been reported in several studies in peripheral nerve and spinal cord regeneration [41, 42] and in tendon regeneration [43, 44]. Other extracellular matrix molecules have also been used to prepare surface for the culture and differentiation of stem cells; for example, Matrigel, a complex mixture of laminin, collagen IV, and heparin sulfate, is largely used in in vitro culture systems [45]. Alginates isolated from brown sea algae form gels in the presence of bivalent cations such as calcium; they are biocompatible and inexpensive and for these reasons they have been tested in tissue engineering applications, even if they must undergo extensive purification to prevent immune responses after implantation [46, 47]. Moreover, the mechanical

weakness and the poor cell adhesion stimuli in the case of alginate scaffolds make this material not optimal for regenerative medicine applications unless it is mixed with other strengthening materials such as chitosan [40].

Despite their advantages, natural components face several challenges, as they can induce inflammatory response because of undefined factors that cannot be eliminated by purification prior to implantation and pathogen transfer [48]. Other problems are the significant degree of variability among different lots and the need for large-scale sources, particularly if human proteins are involved.

6 Synthetic Biomaterials

Materials composed of synthetic components offer evident advantages, such as low immunogenicity, reproducibility, and tailorability of their mechanical and biochemical properties [49, 50]. These materials can be combined by reacting together to produce a wide range of scaffolds exhibiting a mixture of the properties that are unique to each individual material [47, 51]. Optimizing these materials may lead to the development of reproducible, scalable, nontoxic, and nonimmunogenic materials for expansion and differentiation, as well as in vivo implantation. An important class of biomaterials is the class of poly(α-hydroxy esters), bioresorbable synthetic polymers that can be prepared in 3D scaffolds that biodegrade in the presence of water and carbonic anhydride and show minor inflammatory response [52]. High-porosity scaffolds based on these biomaterials are often made of microfibers and micropores, which result in similar or even bigger cell sizes; this results in microenvironments in which cells grow in a two-dimensional system. In order to culture cells in a true 3D microenvironment, the dimensions must be significantly reduced to nanometer dimensions.

Electrospinning represents an attractive technique for processing polymeric biomaterials into nanofibers. This technique offers the opportunity for control over the thickness and composition of the nanofibers along with porosity of the nanofiber meshes, using a relatively simple experimental setup. In electrospinning, a strong electrical field is applied to a droplet formed from a polymer solution. The material to be electrospun is first dissolved in a suitable solvent to obtain a viscous solution. The solution is passed through a spinneret, and a high-voltage supply is used to charge the solution. At a critical voltage, the repulsive forces of the charged solution particles result in a jet of solution erupting from the tip of the spinneret. Conventional electrospinning produces nanofibers that are randomly oriented, while the use of an electric field on the charged polymer solution makes it possible to control jet trajectory, enabling the production of oriented nanofibers that can be useful in the design of scaffolds for tissue engineering. The electrospinning process is economical and simple, yields continuous fibers and hollow fibers, and is versatile enough to be applied to a variety of materials. Among the synthetic polymers intensively explored for the fabrication of nanofibers are poly(glycolic acid) [53], poly(lactic acid) [54], poly(lactic-co-glycolic acid), and poly(L-lactic acid) [56, 57]. In order to improve the stability and mechanical properties of scaffolds,

researchers have tested several tubular nerve guides that have shown negligible inflammatory response made of poly(l-lactic acid)–caprolactone [58] and poly(l-lactic acid)–poly(Σ-caprolactone) blends [59].

Other synthetic materials considered attractive scaffolds for tissue engineering are the hydrogels—insoluble hydrophilic polymers that have high water content and tissue-like mechanical properties. Self-assembling peptides belong to this class of biomaterials. The majority of the developed self-assembling peptides present hydrophilic heads with hydrophobic tails or periodic repeats of alternations of hydrophilic and hydrophobic amino acids and spontaneously form β-sheet structures [38]. Under physiologic conditions, ions screen the charged residues, and hydrophobic residues of different β sheets can pack together via their hydrophobic interactions in water, giving layered β-sheet nanofibers [60]; alternatively, β-sheet structures can give rise to cylindrical nanofibers [61]. Self-assembling peptide scaffolds have a nanostructure with thin fibers (4–15 nm in diameter), and so they resemble ECM-derived substrates such as Matrigel [62]. The formation of a scaffold and its mechanical properties are influenced by several factors, such as hydrophobicity and the length of the peptide sequence: approximately, the higher the hydrophobic residue content and the longer the length of the peptide sequence, the stiffer is the scaffold [63–67]. One of the evident advantages of self-assembling peptides is that they are composed of natural amino acids, and so their degradation products can be reused by the body without eliciting a severe immune response [68–70]. These peptide scaffolds can be commercially synthesized and custom-tailored, and they can be inexpensively and quickly modified at the single–amino acid level. Furthermore, self-assembling peptide scaffolds have recently become powerful tools for regenerative medicine for repair of myocardial infarction [69], to stop bleeding [71], and to repair nervous tissue [70], as well as to serve as useful medical devices for slow drug release [72]. Self-assembling peptides may be useful bioreabsorbable scaffolds for tissue engineering applications; however, they have some drawbacks. Unlike electrospun matrices, self-assembled scaffold nanostructures are mainly randomly oriented in three dimensions: thus, randomly oriented monolayers have been obtained, despite the fact that further efforts have been undertaken to influence the formation of self-assembling structures via electromagnetic fields or microfluidic chambers. This issue may be crucial in tissues in which a particular regenerated cytoarchitecture has to be achieved at both the nanoscale and the microscale. Another disadvantage of synthetic materials is the lack of cell-recognition signals, and so they present few cellular interactions. Toward this end, efforts are being made to incorporate cell-adhesion peptide motifs into synthetic biomaterials.

7 Semisynthetic Biomaterials

The combination of synthetic materials with cell-recognition sites naturally found in living systems is very attractive. These hybrids materials could possess both the favorable properties of synthetic materials and specific biologic activities.

As to self-assembling peptides, the simplest method for incorporating functional motifs found in ECM proteins is to synthesize them sequentially along with the self-assembling sequences [62]; usually a spacer, comprising two or more glycines, is added in order to have a flexible and adequate exposure of the motifs to the cells. In solutions with neutral pH, the functionalized sequences self-assemble to give nanofibers flanked with the added motifs, creating a microenvironment with specific biologic stimuli [62, 73]. For example, using the RADA16 self-assembling peptide as a self-assembling "core" [74], we conjugated a range of bioactive peptides, including fibronectin, collagen VI, and laminin, as well as two bone marrow–homing peptides (BMHPs), BMHP1 and BMHP2 [62]. Adult mouse neural stem cells (NSCs) were cultured on these surfaces and assayed for adhesion level, viability, and differentiation. Of interest, the BMHPs supported greater adhesion than all the other peptides. The BMHPs seemed to induce differentiation nonspecifically, as there were approximately equal proportions of neurons, glia, and undifferentiated cells at 7 days in culture. However, gene expression profiling array experiments showed selective gene expression, possibly involved in NSC adhesion and differentiation. These results show very promising approaches for the use of these functionalized hydrogels in nervous system regeneration.

Synthetic materials other than self-assembling peptides have been blended with natural components. For example, Koh et al. [75] enhanced neurite outgrowth using nanostructure scaffolds of poly(l-lactic acid) coupled with lamin, a component of the ECM that is continuously secreted after nerve injury and plays a crucial role in cell migration, differentiation, and axonal growth. Other ECM proteins such as collagen have also been used in conjunction with synthetic polymers. Mahoney et al. [76] cultured rat embryonic forebrain–derived neural progenitor cells on a triblock copolymer consisting of poly(ethylene glycol) flanked by poly(lactic acid) blocks. The cells grown on this material in the presence of the cytokine fibroblast growth factor-2 exhibited increased cell growth, although the addition of collagen only to the cell suspension prior to cell plating had no real effect. These studies collectively demonstrate that natural components can provide biochemical signals necessary to support cell attachment, proliferation, and differentiation when blended with a synthetic substrate. Starting from these semisynthetic materials, it should be possible to develop fully synthetic materials that avoid some of the challenges of using isolated proteins, as these can potentially be replaced with recombinant or synthetic signals [39].

8 Conclusions

Electrospun constructs and self-assembling peptides, both characterized by their nanoscale architecture, have achieved widespread use in regenerative medicine. Self-assembling peptides alone cannot provide directed spatial guidance to regenerating cells. On the other hand, electrospun scaffolds have been adopted in regenerative

approaches targeting musculoskeletal and nervous system injuries [59, 77] due to the ability to spatially guide tissue regeneration and tailor mechanical properties through the choice of scaffold constituents and textures. A possible goal is a synergistic strategy using the tailorability and biocompatibility provided by self-assembling peptides and spatial orientation and high diffusive properties of microscale and nanoscale electrospun guidance channels.

Tissue engineering has emerged as an excellent approach for the repair/regeneration of damaged tissues, with the potential to circumvent the limitations of autologous and allogeneic tissue repair. Significant effort is still necessary to achieve a better understanding of stem cell biology and an optimal microenvironment capable of stimulating transplanted cells and host tissue regenerative "response" by developing functionalized scaffolds and choosing the appropriate set of cytokines to be slowly delivered locally. Indeed, although considerable progress has been made toward understanding embryonic stem cells, currently we cannot claim they can be used safely in clinical applications. At the same time, a number of challenges remain in the design of materials that are nonimmunogenic, scalable, mechanically tunable, and bioactive in their presentation of key regulatory signals to cells.

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Microtechnology for Stem Cell Culture

Elena Serena, Elisa Cimetta, Camilla Luni, and Nicola Elvassore

Abstract Advances in stem cell research in recent decades have been aided by progress in the development of novel technologies aimed at biological systems. At the same time mimicking stem cell niches in vitro has become crucial for both basic stem cell research and the development of innovative therapies based on stem cells. Innovative microscale technologies can contribute to our quantitative understanding of how phenomena at the microscale can determine stem cell behavior based on our increasing ability to control culture conditions and the throughput of data while reducing times and costs. In particular, microtechnologies must be designed and developed to capture the complexity of cell–substrate, cell–cell, and cell–soluble environment interactions considering the characteristic time and length scales of biological phenomena. While acknowledging the advantages of applying these technologies to stem cell culture, this chapter focuses on issues related to the control and mimicking of microenvironmental cues of the stem cell niche, such as substrate properties, cell topology, the soluble environment, and the electrophysiology.

Keywords Stem cells • Stem cell niche • Microtechnology • Patterning • Microfluidics

1 Introduction

The scientific community has become increasingly aware of the importance of the concept of the stem cell niche and of accurately control how to its temporal evolution in vitro [1]. Innovative technology can enhance our ability to gain new

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insights into stem cells and, in particular, contribute to quantitative understanding of how phenomena at the microscale can determine stem cell behavior at higher levels of complexity [2]. To explore the potential use of novel microtechnology in stem cell research, fundamental principles of biological and cellular stem cell functions have been analyzed with reference to their characteristic time and length scales. Cells in their native tissues reside in an extremely complex environment made up of other cell types, extracellular matrix proteins, and structural templates guiding their orientation and maintaining the three-dimensional (3D) architecture. In addition, one also has to consider the intricate network of short- and long-range communications between single cells and/or tissues that occurs through a vast cascade of signaling pathways and regulates their behavior [3–7]. Such complexity of cellular functions and their response to internal events and environmental perturbations or stimuli develop in both time and space with characteristic scales spanning orders of magnitude [8, 9].

Figure 1a shows a simple schematization of the components of the in vitro cell microenvironment constituting the so-called niche, which is mainly given by (1) the substrate, (2) the soluble environment, and (3) their electrical properties. The cells adhere to a specific substrate, which is defined by a set of physicchemical properties that include, for example, chemical composition, stiffness, and surface topology. The cells are immersed in a soluble environment composed of a complex ensemble of factors that can be either produced by the cells (endogenous factors) or by other cells in the surroundings (exogenous factors). In particular, the temporal evolution of the composition of both endogenous and exogenous factors within the microenvironment surrounding the cell is strictly related to cell local density and cell topology. Finally, another important component of the stem cell niche is given by electrophysiological stimuli, which are particularly relevant for electrically excitable cells. Figure 1b shows approximate ranges for both space and time scales of some relevant biological phenomena. It is evident that biological phenomena cannot be simply considered as the sum of "building blocks" whose final structure recapitulates the function or behavior of a cell or tissue. Each level is tightly interconnected with all the others, and phenomena occurring on smaller scales influence the large-scale phenomena. For example, in the case of electrically excitable cells or tissues, intracellular events triggered by small-ion dynamics can affect the whole single-cell or even cell cluster behavior in terms of contractile properties and/or gene transcription [10].

Conventional stem cell cultures are typically performed in Petri dishes, multiwell plates, or T-flasks in volume ranges from microliters to several milliliters. While they are simple and widely used devices, the culture conditions in these systems show some intrinsic limitations in relation to both the temporal evolution and the constitutive characteristics of the stem cell niche. First, the medium is stagnant over the cells, and the transport of metabolites, gases, and signaling molecules from the bulk to the cell surface is slowly driven only by diffusion, producing a time-dependent environment. Thus, the culture system's inherent properties can be accurately defined only at the beginning, whereas its temporal evolution is hardly controllable and often unpredictable. Moreover, the batchwise operation associated

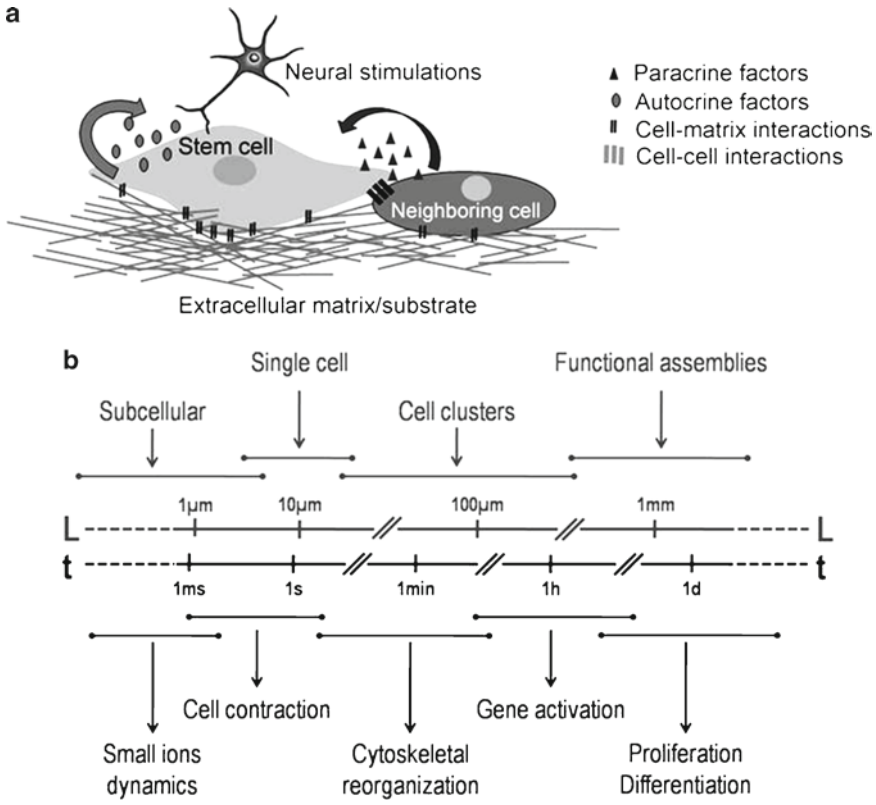

Fig. 1 The stem cell niche. **a:** Schematic representation of the stem cell niche, where matrix/substrate interactions, cell–cell contact, and soluble factors direct cell fate. **b:** Length and time scales for some relevant biological phenomena

with periodic medium changes does not allow stationary culture conditions or the generation of precise temporal patterns of biochemical stimulation.

On the other hand, the ability to control the local cellular environment is also not straightforward in conventional systems. For instance, the stiffness of the cell culture surface (typically made of glass or polystyrene), which is extremely relevant in stem cell differentiation [11], cannot be properly tuned, even when a surface functionalization technique is used.

Cell density and cell topology are key factors in stem cell differentiation and proliferation, but they are hardly controlled in a Petri dish [12]. In fact, after manual seeding, cells spread according to a random two-dimensional spatial distribution, which is scarcely repeatable and produces uncontrollable cell–cell interactions; this aspect is particularly relevant when cell coculture is required to have a given cell–cell pattern. Finally, electrical stimulation is totally neglected in conventional cultures. These observations lead to the impelling necessity to develop novel technologies for studying stem cell biology under controllable conditions able to

capture the complexity of the native environment and, at the same time, meet the demand for increased throughput [13]. To this aim, miniaturization of the culture systems is an important step toward accurate control of the cultured stem cells and tissues [14–16]. Microscale technologies are of paramount importance in successfully controlling stem cell cultures, allowing for the precise definition of the local microenvironment surrounding the cells and responding to the aforementioned specific requirements for accurately mimicking the natural niche encountered in vivo. In addition, a major advantage introduced by microscale technologies resides in the feasibility of producing parallel high-throughput systems and simultaneously acquiring vast amounts of information at affordable operative costs [17, 18].

These technologies have a wide field of application:

- Basic science knowledge on normal and pathologic stem cell behavior, both at the cellular and the molecular level. Phenomena such as stem cell activation, proliferation, differentiation, and cell cycle progression can be investigated in a defined microenvironment by systematic experimental efforts.
- Precise control over stem cell differentiation, which enables the development of cell constructs as reliable in vitro models for the development and screening of new therapeutic strategies and drugs.
- The transfer of biological knowledge for production of clinical-grade stem cells on a larger scale. Once the critical operating parameters can be identified, the repeatability of the experimental conditions and the safety of the process increase.
- The development of small-scale, highly reliable diagnostic technology.

Benefits from microscale technologies can derive from an accurate investigation of the following specific aspects:

1. Cell–substrate interactions through micrometric thin films of biomaterials: mechanical properties of the substrate, surface morphology, and surface functionalization with specific cell-binding ligands (peptides, proteins, etc.) have been demonstrated to be important cues that can substantially affect the cellular response and development.
2. Cell–cell interactions: cell pattern and topology are extremely relevant in stem cell culture and biology since their control allows the investigation of specific effects of cell organization and cell–cell cross-talk at the single-cell level, in cell clusters, and in multicellular coculture systems.
3. Cell–soluble environment interactions: the miniaturized and specific control of the soluble environment over the cells allows the investigation of precise concentration profiles, enabling the simultaneous investigation of several conditions with great reduction of experimental time and cost.
4. Cell–electrical field coupling: microelectrode-based technology is the basis of both exogenous cell electrical stimulation and the recording of biochemical and physiological signals in the sensor field.

The next sections present an overview of the principles and applications of microtechnology as they apply to the relevant stem cell issues just cited.

2 Microstructured Substrate and Cell Topology

Mammalian cells integrate and respond to a combination of factors in the microscale environment, such as the mechanical and chemical properties of substrates, substrate surface morphology, and the overall cell topology, which strongly affects cell–cell interactions. Substrate mechanical properties can be properly designed by physical or chemical deposition of micrometric thin layers of soft biomaterials. Among different biomaterials, hydrogels have recently captured attention in the field of tissue engineering because of their high water content, biocompatibility, and elastic properties, resembling those of native tissues. It is worth underlining that the elastic properties can be finely controlled and modulated by changing the amount of polymer and cross-linker ratio during the production process. The stiffness of the hydrogels affects cell behavior at the cytoskeletal level and the transcriptional activity of stem cells such as mesenchymal [19–21]. Hydrogels, such as poly(ethylene glycol) and derivatives, have been widely used as substrates for cell culture and for encapsulating living cells [22, 23]. Their nonfouling surface hinders cell adhesion, thus allowing the formation of suspension cultures of embryoid bodies, cell aggregates derived from embryonic stem cells, which initiate differentiation and recapitulate embryonic development to a limited extent [24]. On the other hand, in order to enhance and promote cell attachment, a wide variety of copolymers, composed of a synthetic backbone and grafted biomolecules (or vice versa), such as fibrinogen [25], hyaluronic acid [26], or short peptide sequences such as RGD [27], have been developed. These biomimetic substrates have been proposed and tested to reproduce biological cues required for stem cell differentiation.

Besides cell–substrate interactions, the control of cell topology is of paramount importance for studying stem cell differentiation, which requires a particular cell template, and for controlling cell–cell interactions. For example, this latter aspect could be extremely relevant when a specific cell pattern of two different cell types is required in a coculture system. To this aim, two different methods based on different principles have been widely developed and applied: (1) “physical” methods based on microstructured surface morphology and (2) “biochemical” methods based on micropatterning of functionalized surfaces.

In the first case, topologic control over cell culture can be achieved through the use of substrates (typically, synthetic polymeric membranes) whose surface has a three-dimensional morphology: nanopatterned gratings, microgrooves, and microtextures [28–30]. Cell adhesion is thus guided by the three-dimensional architecture imposed. These substrates are produced by the soft-lithography technique (see later discussion for definition and methodologies), and have found several applications, including cardiomyocyte cultures, where topographic and electrical cues on a single chip were incorporated [31], osteoblast differentiation [32], and liver bioreactor [33]. Microwell arrays allow the culture of a single or a cluster of stem cells in a defined microenvironment and facilitate experiments designed to dissect the interplay of soluble and insoluble molecules that comprise the stem cell niche, allowing analyses of fate changes of large numbers of single cells in a high-throughput manner [34].

On the other hand, an alternative strategy to control cell culture topology is the selective deposition of adhesion proteins (protein patterning) onto cell-repellent surfaces. Many patterning techniques have been proposed and widely reviewed over the last few years [35–39]; a more detailed list of such techniques will be given later. Protein patterning allows the deposition of adhesion proteins with a resolution of approximately 1 μm. Immobilization on the surface occurs through physical or chemical adsorption and allows proteins to maintain their activity without suffering from major denaturation phenomena. Consequently, cell adhesion is controlled with micrometric precision (Fig. 2a, b). The main advantage of this technique resides in the possibility of guiding cell adhesion with such high fidelity, the surface used being cell repellent, allowing the study of the effects of specific topologies on stem cell behavior. Patterns of proteins have been created on glass or polystyrene surfaces via microcontact printing (μCP) [40], and laminin lanes have been patterned using the same technique on polymeric films for the subsequent adhesion of cardiomyocytes [41–43]. The patterning can be designed to mimic the topology of specific tissues and thus to facilitate stem cell differentiation toward a specific lineage [44–46]. It can also be used in cocultures to control cell–cell contact and interaction at the microscale by topologically organizing two different cell types [47–49] (Fig. 2c).

As frequently argued, performing stem cell cultures onto surfaces, which are, 2D systems, does not mimic with high fidelity the natural 3D architecture of in vivo tissues. In this light, Albrecht and colleagues explored the role of microscale organization of tissues in three dimensions through the integration of coculture and topologic cell organization in a 3D model [50], and they demonstrated that chondrocyte biosynthesis specifically modulates microscale organization.

Another application of micropatterning aims at studying, in a high-throughput fashion, the effect of the extracellular matrix (ECM) on stem cell behavior (Fig. 2d). Microarray platforms of ECM proteins were developed to screen their effects on stem cells in a combinatorial and parallel fashion. A conventional DNA robotic spotter has been modified for the immobilization of specific ECM components (collagen I, collagen III, collagen IV, laminin, and fibronectin) or combinations of these molecules on hydrogel surfaces [51]. The developed array was used to study the commitment of mouse embryonic stem (ES) cells toward an early hepatic fate, leading to the identification of several combinations of ECM components that synergistically impact ES cell differentiation and hepatocyte function. This platform was further developed to analyze the effects of interactions between ECM components and soluble growth factors on stem cell fate, resulting in a multiwell microarray platform that allows 1200 simultaneous experiments on 240 unique signaling microenvironments. These studies were consistent with results previously reported in the literature, confirming the reliability of the platform. Similarly, a microarray of biomaterials has been developed and used for the high-throughput and rapid synthesis and screening of combinatorial libraries of photopolymerizable biomaterials to identify compositions that influence human ES cell attachment, growth, and differentiation [52]. More than 1700 human ES cell–material interactions were simultaneously characterized, and a multitude of unexpected effects were identified, offering new levels of control over human ES cell behavior.

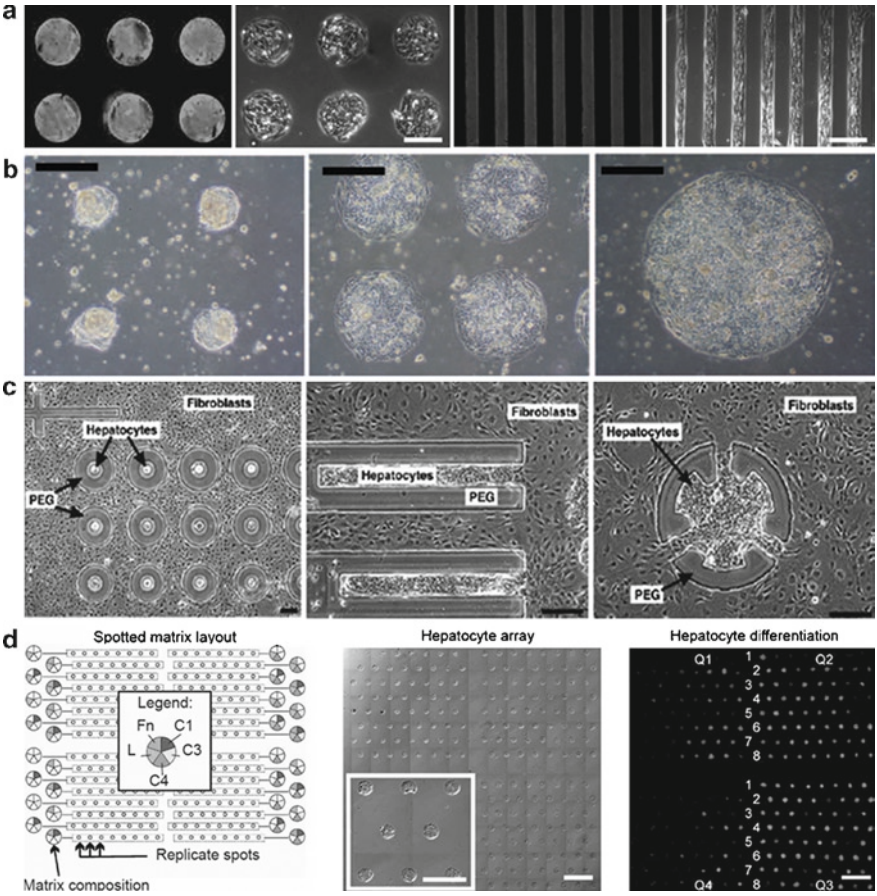

Fig. 2 Micropatterning and microstructured cultures. **a:** Examples of microcontact printing of laminin and resulting mouse satellite cell culture in 300-μm-diameter circular dots and 75-μm-wide, 125-μm-spaced parallel lanes. Scale bars, 300 μm. **b:** Human embryonic stem cell colonies patterned at diameters of 200, 400, and 800 μm. Scale bars, 250 μm. **c:** Phase contrast images of hepatocyte–fibroblast cocultures. Scale bars, 200 μm. **d:** Schematic representation of the layout of the extracellular matrix array; bright field of hepatocytes cultured onto the array; immunofluorescence of albumin (a marker of differentiated hepatocyte function). Scale bar, 1 mm; inset scale bar, 500 μm

3 Soluble Environment Control

Another aspect of the stem cell niche involves the soluble local microenvironment. Metabolites, gases, and growth factors are transported between the bulk of the culture medium and the cell surface in either direction. In standard culture systems these components are exclusively delivered by a mechanism of diffusion. When cells uptake or release a specific chemical component at a higher rate than that of the diffusion transport process, a concentration gradient is produced from the cell surface to the bulk of the culture medium. In other words, when diffusive mass

transport has a characteristic time larger than that of cell uptake or release, a depletion or accumulation of that component occurs at the cell surface [53]. Note that the two processes occur in series. Furthermore, if cell density in culture is not uniform, as is the case in standard culture systems, the concentration gradients surrounding single cells can intersect, and larger-scale regions of accumulation or depletion of that component are produced. These phenomena affect the homogeneity of the cell population and the definition of the actual local concentration at the cell surface, complicating insight into cell behavior.

In perfused systems, the cell culture is performed under precise control of the conditions, and convection occurs as a further mechanism of mass transport from the bulk of the culture medium to the cell surface and vice versa. Convection takes place in parallel with respect to diffusion, and it is the dominant mass transport mechanism only when its characteristic time, τ_C, is smaller than the diffusion characteristic time, τ_D, meaning that the ratio τ_C/τ_D is less than 1. The prevalence of either mechanism is dependent on the specific geometry of the culture system and on the operating conditions. The left panel of Fig. 3a schematizes a typical micro-perfused culture chamber. As an example, assuming that the inlet medium flow rate, Q, is fixed, it is possible to decide to grow the cells at the bottom of the well under either the diffusive or the convective regime. In fact, τ_C is proportional to the cross section area of the well, $d.h$, where d and h are the well width and height, respectively, and τ_D is proportional to the square of well height, h^2. Thus, their ratio, τ_C/τ_D, is proportional to d/h. This is the most important geometric variable that needs to be accurately selected for establishing the desired mass transport regime (either diffusive or convective) within micro-perfused systems [53]. Typically, for $Q = 1$ μL/min and $d/h = 1$, h must be in the range of few millimeters or less to obtain a diffusion-limited regime (right panel of Fig. 3a).

Despite its simplicity, the system in Fig. 3a shows all the potential of micro-perfused devices in defining the microenvironment in direct contact with the cells. Furthermore, as shown in the foregoing example, it is possible to regulate the mass transport rates at which different components reach the cell surface by choosing the appropriate mass transport regime. This technical solution allows stationary culture conditions if the medium inlet composition is kept constant or defined temporal patterns of biochemical stimulation if an upstream system of microvalves temporally regulates the composition of the inflow medium.

Focusing to our interest in the microscale, we move to the field of stem cell culture integrated with microfluidics: “the science and technology of systems that process or manipulate small amounts of fluids, using channels with dimensions of tens to hundreds of micrometers” [54]. At the relevant scale for microflows—that is, inside the microchannels and culture chambers of microfluidic devices—the dominating forces change and differ from the better-known macroscale ones. Excellent descriptions can be found in the literature [55–59], which extensively analyze the physics of fluids at the microscale and the nanoscale and illustrate the most relevant applications, giving insights into the operating principles for system configurations of possible interest. Particular attention has also focused on the accurate description and characterization of the dominant phenomena within microfluidic bioreactors,

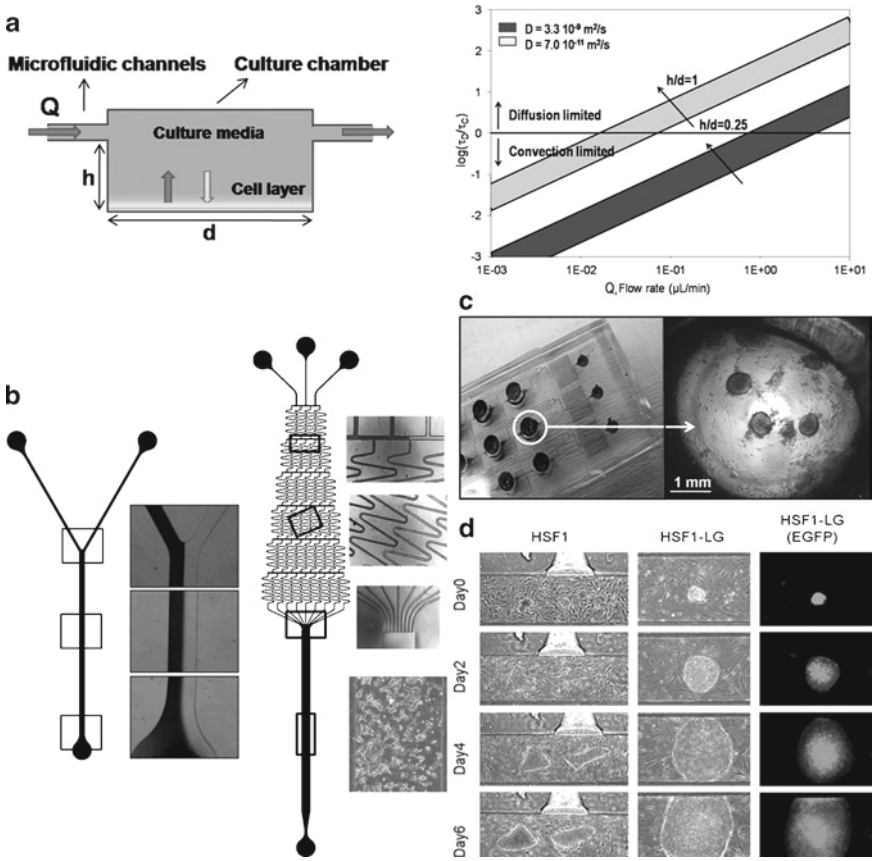

Fig. 3 Soluble environment control. **a (Left):** A schematic representation of a typical culture chamber with the microfluidic channels for fluid perfusion. The characteristic dimensions are its width, *d*, and height, *h* (distance between the bottom of the chamber and the microfluidic channels). (**Right)** Graph exemplifying the results of computational modeling of the averaged properties of the system and in particular of the diffusion and convection time constants as a function of medium flow rate and culture chamber geometry. Results are presented for two chemical species representative of a small metabolite (higher diffusion coefficient, *D*) and of a larger growth factor (lower *D*). The existence of two distinct mass transport regimes is evident—one dominated by molecular diffusion and the other by convection. **b:** Two examples of microfluidic platforms capable of recreating defined and compartmentalized concentration patterns on adherent cell populations within a microchannel. The microbioreactors designs and images of their validation are presented. **c:** An image of a microbioreactor array platform made up of three independent rows, each composed of four individual culture chambers (4 mm diameter), that was used for the culture of human embryonic stem cell (hESC) colonies (image on the right). **d:** Results of a long-term culture of hESC colonies within a microfluidic channel

giving the design specifications for important operating parameters and the principles for their optimization at the microscale level [53]. The feasible control over the operating parameters allows the application of complex and well-defined patterns

of stimulation not only, as already noted, in terms of temporal concentration profiles, but also of spatial dynamic perturbations, such as precise and localized concentration gradients or steep compartments (Fig. 3b). Examples include studies of chemotaxis in cell culture systems using hydrogels of differential compositions [60], short-term perfusion of graded concentrations of regulatory factors over seeded cells [61], and the assembly of membrane-based diffusion chips [62]. However, one must be well aware that the use of such microfluidic gradient generators is generally associated with hydrodynamic shear stresses exerted on cultured cells, which arise from the small characteristic dimensions of microfluidic channels [63].

Examples of applications of microfluidic platforms for lab-on-a-chip applications have been extensively reviewed [64, 65] and point to the advantages deriving from the miniaturization, integration, and automation of biochemical assays while giving more insight into the fabrication processes and properties of the materials that are typically used [66– 68]. Since its first appearance decades ago, microfluidics has been adapted to a multiplicity of different applications. The recent literature reflects increased interest in interfacing microfluidic devices with biological systems [54, 69–72] and in their use in drug discovery process [73–75] and molecular detection [76]. High-tech platforms involving integrated microdevices such as microvalves, injectors, pumps, or mixers [77] are also being considered for use in live-cell experimentation. Microscale technologies have been designed for applications ranging from studies at a single-cell level [78] to the recreation of more complex 3D structures [79–82] and the development of diagnostics platforms for clinical and medical research [83]. Concerning a specific application for this last-mentioned purpose, Toner and Irimia [84] reviewed the use such devices for blood manipulation at the microscale, highlighting the importance of having small, portable, high-throughput but yet reliable and ready-to-use instruments for specific blood analysis tasks. In addition, microfluidics has been adopted for tissue engineering purposes, and examples exist of applications involving basal lamina, vascular tissue, liver, bone, cartilage, and neurons [85]. The most interesting and relevant recent applications have seen the use of microfluidic devices in studies on human embryonic stem cells [86–88] (Fig. 3c, d).

Research is being conducted to improve the soluble environment of cell cultures at larger scales (hundreds of milliliters) using suspension bioreactors in which stirring is mechanically provided to improve the transport of metabolites, gases, and growth factors [89–91]. These devices have been used to culture stem cells that can grow in suspension as single cells or as cell clusters, such as hematopoietic stem cells [92–95], neural stem cells [96], or embryonic stem cells [97]. The potential of these bioreactors has also been extended to other types of cells that are suspended after encapsulation in gelatin microspheres [98] or inside porous polymeric microcarriers [99–101]. These large-scale devices have been limited in use because of the high costs and the large number of cells needed [91]. In perspective, scaling down these systems to stirred microliter bioreactors, taking advantages of knowledge of microfluidic stirring mechanisms already studied for other applications, such as buoyancy-driven thermoconvection and electrokinetic flow [102–104], would be extremely useful.

4 Electrical Stimulation and Recording

Endogenous electrical fields play a key role in several biological processes (cell differentiation, movement, and growth). They are present in developing and regenerating animal tissues, both in the extracellular space and in cell cytoplasm, and have magnitudes that range from few to several hundreds of millivolts per millimeter [105–107]. Electrical stimulation and recording of excitable tissues (brain, muscles, peripheral nerves, sensory system) are subjects of interest in electrophysiology and clinical functional electrical stimulation [108]. Consequently, the integration of electrical stimulation devices with stem cell cultures could be a powerful tool for studying the effects of an exogenous electric field on stem cell biology and give important insights into cell physiology.

The exposure of stem cells to neurophysiologic stimulation can be achieved through coculture with neuronal tissue [109, 110], the modulation of ion channel expression [111], or the generation of an electrical field between electrodes, the latter being the most efficacious, as it allows the fine control of crucial parameters such as amplitude, duration, and frequency of the imposed pulse [112]. The need to increase the high-throughput characteristics of experiments has led to the development of miniaturized and arrayed platforms [113]. Among these systems, microelectrode arrays are the most used and have been proven to be a valuable tool for detecting and measuring extracellular potentials for excitable cell types such as cardiomyocytes and neurons [31, 114–118] (Fig. 4a). In general, microelectrodes allow chemical measurements in spatial and temporal domains that were previously inaccessible [119].

In addition, microelectrode platforms can be used as biosensors for recording signals released from the cells, thus making it possible to monitor and record changes in defined physiologic and biochemical signals for applications ranging from pharmacology, cell biology, and toxicology to drug discovery [120, 121] (Fig. 4b). Several examples in the literature illustrate basic rules dictating their operating performance [122], and demonstrate the ease of use in effective signal recording [123, 124]. The most advanced systems integrate nanostructured materials, such as carbon nanotubes, within the sensing elements, thus greatly improving the performance of the devices [125, 126].

5 Overview of Microtechnology

As well described in works cited earlier [55, 58, 59], a multiplicity of techniques has been applied to gain micrometric control over culture systems. One of the first techniques applied to fabricate microelectromechanical systems is silicon or glass micromachining. However, although this method is still extensively used for applications involving strong solvents or high temperatures, its use for biologically studies is limited due to its high costs, difficult realization, and limited compatibility of the

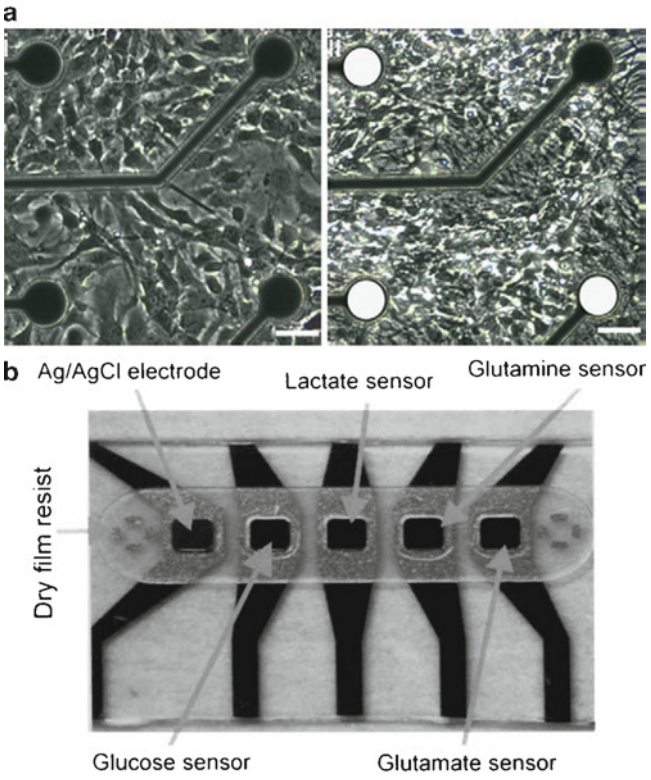

Fig. 4 Electrical stimulation and recording. **a:** Embryonic stem cell–derived neurons at different time points after initiating the differentiation. Phase contrast images of embryonic stem cell–derived neurons on microelectrode arrays at 7 (left) and 28 days (right) after initiating the differentiation. **b:** An example of an integrated multielectrode biosensor array that allows detection of glucose, lactate, glutamine, and glutamate

materials (i.e., silicon is not transparent). Lithographic techniques, and especially photolithography, are the most commonly used to fabricate microscale features on glass or silicon substrates to be subsequently used as masters for obtaining the final devices. Since the early 90s, when Whitesides and others initiated the exploration of its use in the field of biological studies [36, 127–129], soft lithography has been exploited for its many advantages, including ease of application, low cost, high versatility, and materials properties. Soft lithography is commonly used to create structures on surfaces for controlling cell–substrate interactions. It basically covers a group of techniques with the common feature that at some stage of the process an elastomeric (soft) material is used to create the desired structures. Such elastomeric devices—typically poly(dimethyl siloxane) (PDMS)—can be obtained through replica molding on the microstructured silicon masters. Photolithography and PDMS replica molding are the leading techniques for the production of microfluidic platforms and microbioreactors.

Similarly, μCP, first described by Whitesides and Kumar in 1993 [130], is a technique for micropatterned substrate production that consists in using a microstructured elastomeric stamp with relief features to transfer an "inked" material (adhesion proteins in the case of cell culture) onto a substrate. Similar to μCP, other techniques, such as microtransfer molding, micromolding in capillaries, and solvent-assisted micromolding, use microstructured PDMS replica masters to transfer its features onto other materials. Alternatively, microscale features could be created on supporting materials via selective chemical vapor deposition, wet or dry etching processes, or more advanced micromachining processes, such as bulk and surface micromachining, microstereolithography, or laser-mediated ablations. We again refer the reader elsewhere for a more detailed description of all these techniques [59].

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Using Lab-on-a-Chip Technologies for Stem Cell Biology

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Abstract The introduction of microtechnology and microfluidic platforms for cell culture can dramatically enhance the pace of stem cell research. With the use of microfluidic-based techniques, extracellular microenvironments can be controlled in a precise manner, and their influence on various cellular behaviors can be studied. Microfluidic devices made of transparent materials allow real-time and high-throughput monitoring of cell functions and cell fate by using fluorescence microscopy and other optical techniques. This chapter gives a perspective on the considerable capability of microfluidic devices, which remain an underutilized technology for stem cell research. It provides stem cell researchers with a brief review of basic microtechnology and the application of microfluidics to stem cell research, as well as highlights to engineers the peculiarities of stem cell culture and experimental capabilities of microfluidics. In addition, it provides insights into creating integrated, modular, and easy-to-use microfluidic devices to perturb stem cells with biochemico/mechanical stimuli in a precise, controlled, combinatorial, and high-throughput fashion.

Keywords Lab-on-a-chip • Stem cells • Microfabrication • Extracellular matrix • Microfluidic platforms • Micropatterning

1 Introduction

Stem cells reside in a complex biochemical and mechanical milieu and are subjected to varied cues in the form of cell–cell, cell–matrix, and autocrine signaling that affects stem cell behaviors and functions [1]. Conventional laboratory techniques

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for experimenting with stem cells include tissue culture in plastic or glass cover slips, gradient generation using micropipetting, differentiation and self-renewal in tissue culture dishes or multiwell plates, chemotaxis using Boyden chambers, hanging droplet methods, treatment with conditioned media, and other methods [2, 3]. These techniques can only crudely mimic the *in vivo* microenvironment experienced by embryonic or adult stem cells. Experimental methods for systematically altering or perturbing the local cellular environment and studying the ensuing cellular behavior suffer from lack of reproducibility, imprecise spatial and temporal control, and expensive use of bulk reagents, growth factors, antibodies, and cells [4]. With the advent of microfluidic platforms that mimic the *in vivo* complexity of tissue organization and composition, stem cells can now be cultured in well-defined microenvironments and biochemico/mechanical and physical cues can be provided in a spatiotemporally controlled and reproducible manner [5]. Besides miniaturization of conventional experimental methods, microfluidics allows harnessing the advantages of physics at the small scale to perform studies not implementable at the macroscale, including the formation of stable and complex gradients and precise control of shear stress. In addition, microtechnology provides a method for performing large-scale observations of stem cell phenotypes and high-throughput cellular screening in response to a combination of biochemical stimuli (e.g., soluble factors, cytokines, small-molecule reagents), extracellular matrix (ECM) interactions, heterotypic and homotypic cell–cell interactions, and metabolites, as well as mechanical (e.g., substratum rigidity, substratum topography, shear stress) and physical stimuli (e.g., oxygen tension, pH, temperature) [5, 6].

2 Microfluidic Lab-on-a-Chip Meets Stem Cell Biology

With the rapid expansion of stem cell research and investigation, microfluidics has emerged as an important tool for complementing or replacing existing laboratory techniques. The term microfluidics refers to the technology involving small amounts of fluids (10^{-18}–10^{-9} L) perfusing engineered devices of varied complexity. Alteration of physical phenomena observed in the microworld, frequently defying everyday intuition, experience, and common sense, provide new methods for manipulating and controlling experimental conditions. With this innovative technology, experiments can be conducted with much greater control over the chemical contents of the cellular environment, increased spatial and temporal resolution, and higher throughput, as well as with compatibility with numerous imaging and quantification techniques, while using significantly smaller amounts of reagents and cells than conventional laboratory techniques. However, before delving deeper into how these advantages arise, we explain a few basic physical principles governing the behavior of fluids and small objects in the microworld.

2.1 Physics of the Microscale

How physics operates at different size ranges can be simplistically explained using scaling laws [7]. These laws indicate that in the micrometer range, or *microscale*, inertial forces like gravity, which dominate our experiences in the macroworld, become less important than the more subtly experienced phenomena prominent in the microworld, such as surface tension, adhesion, viscosity, and friction [8]. Within fluids, in particular, viscous force dominates the inertial force and dampens any movement of a particle in small dimensions. Within the realm of microfluidics, all flows are essentially *laminar*. This means that microscopic fluid streams travel in a continuous smooth and predictable fashion, and a small particle moves within the fluid following the flow trajectory, distinct from the frequent emergence of turbulent flow at the macroscopic scale [50]. In particular, microfluidics also mimics the physiologic context of fluid flow present in microvasculature. A consequence of this is that transport of dissolved molecules between any two laminar streams can occur only through passive diffusion rather than convection. Therefore, in most applications, the experimental control of a microfluidic experiment is largely defined by the associated diffusion processes and their potential effects on cells immersed in or adjacent to flow.

Due to the laminar flow property of fluids at the microscale, fluid flow can essentially be treated analogously to the electric current in a circuit. The relevant equations for the physics of fluids at the microscale are given in Table 1. In fact, fluids present much more interesting ways to harness physics at a smaller scale than electronics due to the dramatic onset of the importance of noninertial physical phenomena as scales lower to micrometers [8, 9].

Table 1 Physics at the Microscale

Resistance (rectangular channel)	$R = \frac{12\mu L}{Wh^3}$	L = channel length, W = channel width, μ = fluid viscosity; $\mu_{\text{water,20°C}} = 1.13 \times 10^{-3}$ Pa s
Series resistance	$R_T = R_1 + R_2 + \cdots + R_n$	
Parallel resistance	$\frac{1}{R_T} = \frac{1}{R_1} + \frac{1}{R_2} + \cdots + \frac{1}{R_n}$	
Volumetric flow	$Q = \frac{Wh\Delta P}{R}$	ΔP = change in pressure between inlet and outlet, R = resistance
Diffusion	$J = -D\frac{\partial \phi}{\partial x}$	J = flux
Mass diffusion length	$L_d = \sqrt{4Dt}$	D = diffusivity, t = time
Reynolds number	$Re = \frac{\rho \upsilon D_h}{\mu}$	ρ = density of the fluid, υ = kinematic viscosity, D_h = diameter

2.2 Fabrication and Working with Microfluidic Devices

Most microfluidic platforms for experimental analysis of stem cells are fabricated by a process called *soft lithography*, explained in Fig. 1 (left). Lithography involves creating an embossed pattern containing small features on substrates such as silicon or glass by coating them with a photosensitive chemical, or photoresist, that alters its chemical properties upon exposure to ultraviolet (UV) light. The pattern is created by controlling UV light exposure, usually using precisely defined *masks*. For soft lithography, the embossed pattern containing the microscale features created by the photoresist is used as a mold, and the replica of the pattern can be transferred to a softer elastomer, typically made of polydimethylsiloxane (PDMS) [10]. PDMS offers many advantages for stem cell analyses. It is nontoxic to cells, impermeable to liquids, permeable to nonpolar atmospheric gases, and optically transparent, making it easily adaptable to standard optical imaging technologies, such as fluorescence microscopy [11]. A standard microfluidic chip would be made up of a patterned slab of PDMS that is reversibly bonded to a glass cover slip, forming a chamber of microscopic dimensions for culturing stem cells. The microfluidic chamber housing cells may be between 100 and 1,000 μm in its dimensions, can be

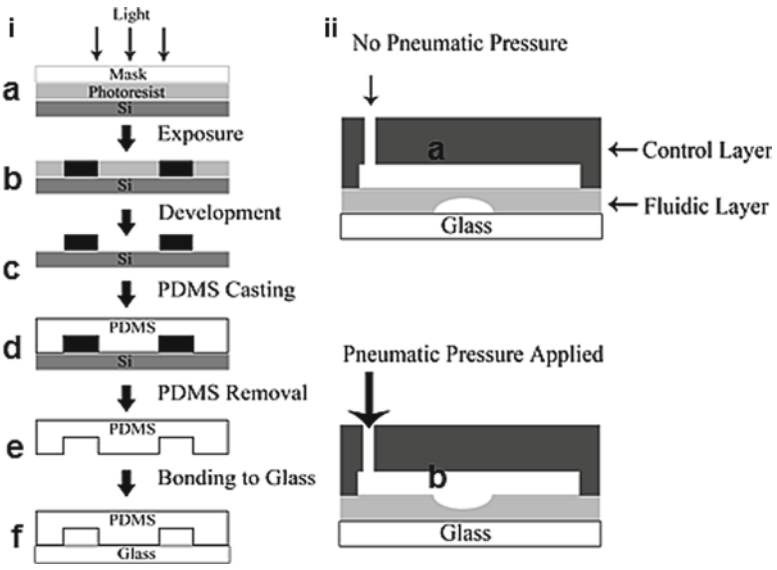

Fig. 1 **Left:** Soft lithography. **a:** Ultraviolet (UV) light is cast onto a high-resolution transparency, called the mask, with the desired pattern overlaid on a silicon (Si) wafer with photoresist. **b:** UV exposure polymerizes the photoresist. **c:** The remaining photoresist is developed away to create an embossed pattern. **d:** Polydimethylsiloxane (PDMS) is cast on the embossed pattern. **e, f:** It is polymerized and bonded to a glass cover slip. **Right:** Hydropneumatic valve control. **a:** A two-layered device contains cells in the lower fluidic layer and water in the control layer. **b:** The control layer can be externally subjected to high pressure to depress the elastic PDMS membrane and close the fluidic layer

coated with appropriate ECM proteins, and is completely filled with bathing fluids, such as cell media. Cells and solutions can be introduced into the device by using ports connected to an external fluid container (e.g., a syringe) and be subjected to desired perturbation by controlling differences in externally applied pressure.

Alternatively, fluid flow can be generated and maintained by using various types of pumps. Manual or automated hydropneumatic valves and other valve types can be used to provide temporal and spatial control of the fluid flow by using relatively high external pressure (30–60 psi) on a thin PDMS membrane that reversibly closes the channel [explained in Fig. 1 (right)] [11, 12]. Manifolds of such valves can be created in a single device, yielding complexity of control comparable to that with some integrated electronic chips. This setup can be interfaced to a computer to automate the valve control by writing a custom program for an experiment. In newer designs, the PDMS-based microfluidic devices can be bonded to a cover slip already containing an adherent cell culture or histologic explants using vacuum or magnetic forces [13, 14].

3 Lab-on-a-Chip Technology for Investigating Stem Cell Biology

In theory, a microfluidic device can be designed to perform any multistep, conventional biochemical experiment on a single platform, although this may lead to increased associated device and experiment complexity. However, even simple designs allow one to conduct tasks not easily accomplished previously, for example, generating gradients of soluble materials or automatically exposing cells to different reagents in a stepwise, time-dependent manner [4, 15]. These advantages have allowed microfluidic technology to be used for various biologic assays relevant to stem cell research, including genetic analyses, single-cell assays, coculture studies, and cell patterning investigations. Several applications of this technology for stem cell research are described in greater detail in the following sections.

3.1 Engineering Stem Cell Microenvironment in Microfluidic Devices

Stem cells interact with a variety of morphogens, growth factors, and cytokines through autocrine or paracrine signaling that can affect their growth, survival, differentiation, and other phenotypic factors [16, 17]. In addition, stem cells respond to various mechanical stimuli or alterations of the local gas content that might also affect various systemic cellular properties. One of the biggest advantages of using microfluidics for stem cells research is the capability to create high-throughput experiments relying on highly defined microenvironments for providing a physiologic context to the cells and to control experimental conditions in a precise manner [4, 5].

4 Generation of Soluble Gradients

In vivo, cells frequently reside in spatial gradients of various stimulants. Within the gradients, they frequently react by either differential responses to local stimulant doses or directed movement or growth defined by the direction of the gradients. Three key limitations in stimulating stem cells with relevant soluble factors in conventional tissue culture methods are (1) imprecise control of the concentration due to depletion of factors or temporal and spatial instability of gradients, (2) lack of combinatorial interactions involving two or more factors with different gradient profiles, and (3) difficulty of creating gradients robustly and reproducibly. With the use of the laminar flow property of fluids in microfluidic devices, stem cells can be exposed to stable gradients of multiple factors for prolonged periods of time.

Figure 2 (left) shows an illustrative gradient-generating device, colloquially referred to as the "Christmas tree." By changing the design of the upstream diffusion-based mixer, the "Christmas tree," a linear, logarithmic, U-shaped, N-shaped, or any other complex gradient shape can be generated and maintained [18]. Combinatorial gradients involving multiple factors or a combination of soluble and insoluble factors can also be created in two-dimensional surfaces or even in three-dimensional gels [17]. Such microfluidically generated gradients of various morphogens have been used to investigate stem cell phenotypes both in dose-response and chemotactic studies [19–21]. In similar devices, human neural stem cells were shown to exhibit differential migration and differentiation toward an increased concentration of epidermal growth factor and platelet-derived growth factor [22]. Recent advances make it possible to make gradients of any arbitrary profile, which can be tuned during the experiment. Furthermore, these systems are now available through customized upstream modular components that can be integrated by simple connections for use by nonspecialists [23].

5 Generation of Insoluble Gradients

In addition to gradients of growth factors and cytokines, microfluidic devices can be used to generate gradients of ECM molecules [24]. On the basis of the "Christmas tree" design just described, aqueous solutions containing relevant ECM molecules can be flown into the cell chamber to create the desired gradient by ECM deposition onto the glass from the gradient-containing solution [25]. These gradients can be combined with gradients of soluble factors, creating a combinatorially complex microenvironment better mimicking the complexity of tissues. The design shown in Fig. 2 (left), created by Wang et al., allows formation of a linear gradient of ECM molecules (e.g., laminin) along with a complex "N-shape" gradient of soluble factors [26].

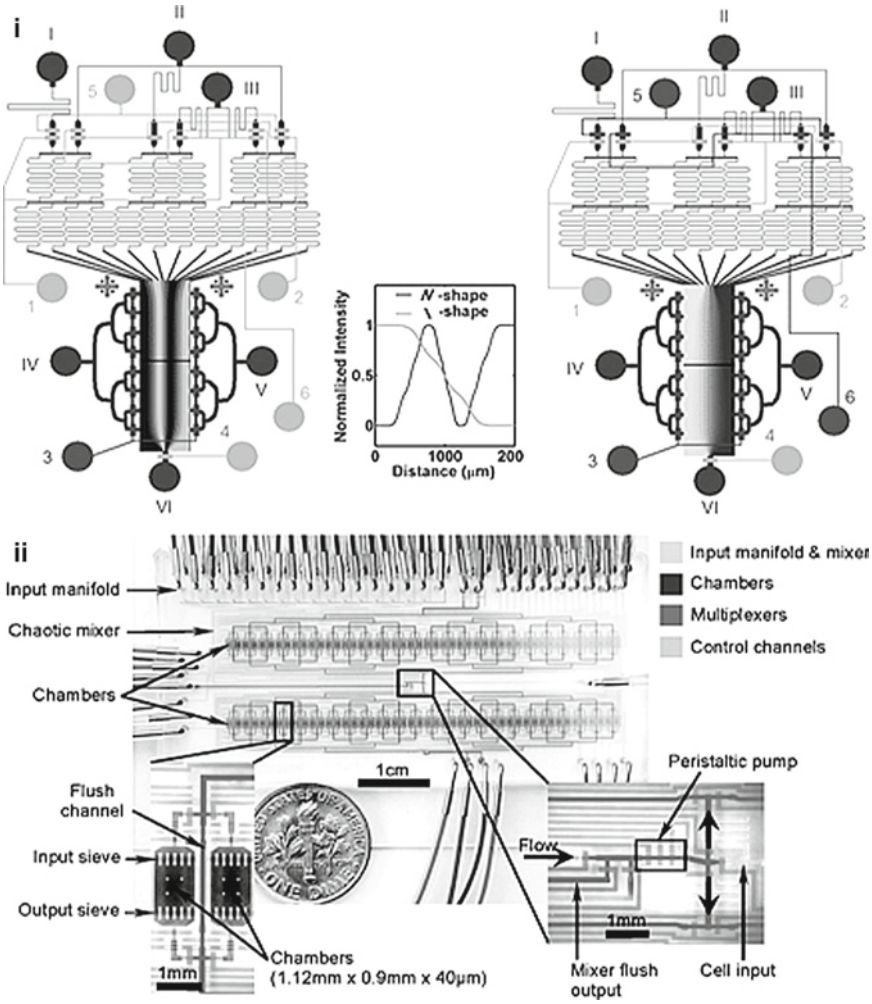

Fig. 2 **Top:** Gradient generation. A "Christmas tree"–based device for generating combinatorial gradients of soluble and insoluble factors. The left device shows the laminin coating in an N-shaped gradient, and the right device shows the shape gradient of the brain-derived neurotrophic factor. Gradients are illustrated from FemLab simulations. (From Wang et al. 2007 [26], with permission from the Royal Society of Chemistry.) **Bottom:** High-throughput screen. Cell culture screening system with 96 different, individually addressable chambers. The left inset shows two culture chambers, and the right inset shows an input multiplexer. (From Gomez-Sjoberg et al. 2007 [42], with permission from the Americal Chemical Society.)

6 Generation of Gaseous Gradients

Most stem cell niches exist in hypoxic environments, and hypoxia plays a central role in signaling in stem cells, affecting cell survival, migration, homing to the site of injury, and differentiation [27]. Recently, the permeability of PDMS

to gases has been harnessed to create microfluidic devices with differential O_2 tension [28]. A second-layer, O_2 perfusion system with high pressures of N_2 and O_2 can be used to create various degrees of hypoxia for cells. This device can help in mimicking the hypoxic conditions that are present in the stem cell niche and allow real-time perturbation of O_2 tension to study hypoxia signaling.

7 Control of Substratum Rigidity

The role of mechanical forces in affecting stem cell fate and rigidity-induced durotaxis has recently been discovered. Mesenchymal stem cells cultured on polyacrylamide gel differentiated into neurons, muscle cells, and osteoblasts, depending on the rigidity of the substratum [29–31]. Microfluidics has been used to create a gradient of rigidity by varying the concentration of the cross-linker using the "Christmas tree" design described earlier combined with photopolymerization of the polymer [29]. This presents yet another method for controlling the microenvironment of stem cells.

8 Three-Dimensional Stem Cell Culture

Primary cells can frequently lose their phenotypes when cultured on two-dimensional plastic or glass surfaces [32]. The three-dimensional (3D) environment created by hydrogels has been used extensively for directed differentiation of stem cells; however, due to the low diffusivity of nutrients and oxygen, the size of the hydrogel cultures is limited [33]. Microchannel-based poly(ethylene glycol) diacrylate gels encapsulating human embryonic stem cells exhibited increased vascularization and adipogenesis after implantation in mice [34]. The high surface-to-volume ratio achieved in microfluidics can be used to create hydrogels within the channels. Ong et al. developed 3D cell culture microfluidic devices and showed that cells preserve their native phenotype in a 3D environment [35]. Laminar flow has also been used to create threads of poly(lactic-co-glycolic acid) microfibers into a 3D scaffold within a microfluidic chip that enhanced axon elongation of neural stem cells [36].

9 Control of Shear Stress

Many stem cell types experience shear stress in their natural environment or respond to shear during migration [37]. However, creating conditions for defined shear stress is difficult in the absence of microfluidics. Microfluidics mimics

tissue vasculature in its very scale and has been used routinely in the field of biomicrorheology, which investigates the role of shear stress in endothelial stem cell differentiation, mesenchymal cell homing, and cell adhesion [38–41]. It takes a simple microfluidic channel seeded with endothelial cells to create an in vitro model of a blood vessel. Recently, Voldman's group described a logarithmically perfused, long-term cell culture for investigating the effect of flow rate on murine embryonic stem (mES) cells [20] [see Fig. 3 (top)]. It was found that mES cells, perhaps against expectations, form larger colonies when exposed to larger shear stress. Such devices can provide a platform for investigating the roles of shear stress and paracrine signaling in stem cell survival, differentiation, and proliferation.

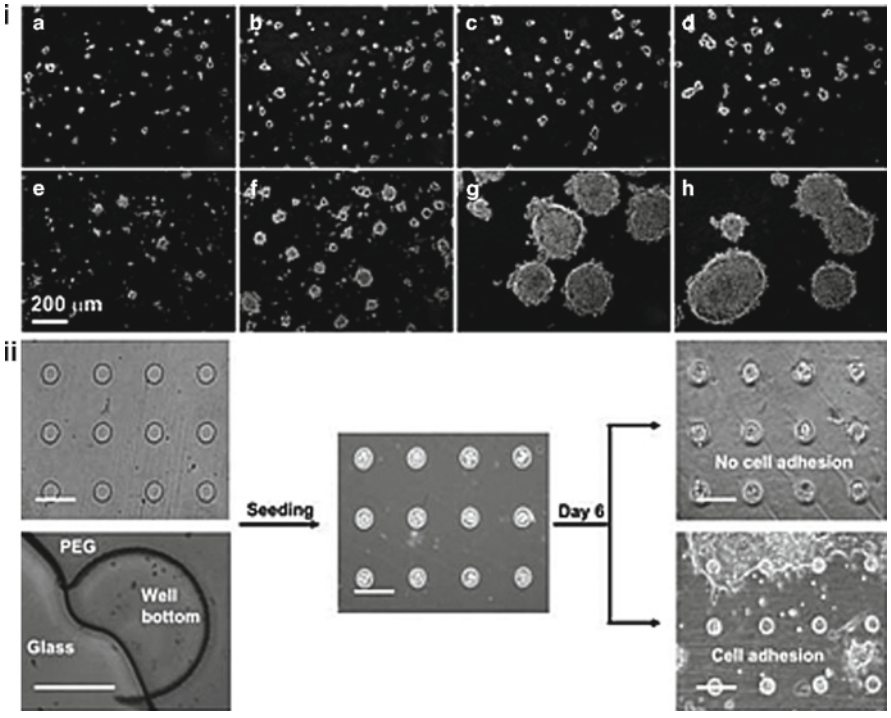

Fig. 3 **Top:** Embryonic stem cells in microfluidics. Mouse embryonic stem (mES) cells grown for 4 days inside a microfluidic device with logarithmically scaled shear stress. (From Kim et al. 2006 [20], with permission from the Royal Society of Chemistry with permission from the Royal Society of Chemistry.) **Bottom:** Micropatterning of stem cells. mES cells form embryoid bodies on microwells. **Left:** The poly(ethylene glycol) (PEG) microwells. **Middle:** Seeded mES cells. **Right:** mES cells after 6 days of growth. (From Moeller et al. 2008 [57], with permission from Elsevier.)

9.1 High-Throughput Screening of Differentiating and Other Factors in Stem Cells

An important advantage of miniaturization offered by microfluidic platforms is the ability to perform massive parallelization of experiments that, although mostly similar, can have variation of some relevant experimental parameters. For instance, cells coming from the same batch can be exposed to the same environmental conditions with the exception of variable dose of a growth factor or another relevant stimulus. The laminar flow in microfluidics provides relatively robust and simple ways to use multiplexing to perform multiple-parameter variation on stem cells.

Stem cells cultured in microfluidic devices can be subjected to a large number of potential stimuli with varying concentrations and times of stimulation with reliable precision. Hong and Quake demonstrated a microfluidic platform with 2056 valves and 256 cell compartments combined with 256 observable compartments [12]. The same group created a versatile, high-throughput microfluidic long-term cell culture to create arbitrary media formulations in 96 chambers [see Figure 2 (right)]. Arbitrary cell density, media composition, and feeding time can be automatically controlled with a computer program [42]. Melin et al. also created a high-throughput device to perform large-scale, multiparameter experiments on embryo cultures [43]. "High-content screening," a term coined by Cellnomics (Pittsburgh, PA), has been used to develop numerous microfluidic devices for in vitro analysis of cell behavior under a combinatorial set of physiologic stimuli [44]. Using the high-content screening, Desbordes et al. identified various compounds regulating stem cell renewal and differentiation [45]. Ungrin et al. used microfluidics to create a reliable high-throughput device for creating multicellular organization from hES cells [46]. Future applications for high-throughput analysis of stem cells might include dynamic analysis of signal transduction using fluorescent probes, screening and optimization of differentiation conditions, and drug screening.

9.2 Deducing Signal Transduction Pathways in Microfluidic Devices

Signal transduction pathways are involved in controlling all cellular processes, including differentiation, cell renewal, migration, proliferation, cell shape, and metabolism. Stem cells are affected by autocrine and paracrine signaling, cell–cell interactions, and cell–ECM interactions. For example, leukemia inhibitory factor controls self-renewal and prevents differentiation of mES cells [47]. Notch signaling between neighboring cells prevents neural stem cells in subventricular zone from differentiation into glia or putative neuroblasts [48]. In addition to the lack of knowledge of specific players involved in various signaling pathways, kinetic interactions between them are poorly understood. Furthermore, since tissue patterning and morphogenesis is an important process during development, there is a need to unravel the signaling involved.

Standard laboratory techniques present significant challenges in investigating signaling pathways. Studies are usually limited to population studies that average behaviors of large numbers of cells. Existing single-cell assays require multiple permutations to study different conditions. Control of the microenvironment becomes difficult because autocrine and paracrine signaling and morphogen degradation interfere with external stimulation. Furthermore, stability of any gradient in a nonlaminar flow is difficult to maintain and is often unpredictable. In addition, to study cell–cell signaling, precise cell positioning becomes very important.

Due to low volumes and laminar and continuous flow of fluid, microfluidics offers better control of the cellular microenvironment. Pneumatic valves can be created at high density and can be reliably controlled in an automated fashion, rendering temporal control of stimulations easy to achieve. Cells can be stimulated with one or a combination of growth factors for different exposure durations. Subcellular localization of downstream signaling molecules can be observed under a microscope using live-cell fluorescent probes or immunostaining, which can also be completely integrated within the same chip. Cheong et al. presented a immunocytochemistry-based microfluidic platform for studying 32 different conditions on adherent cell cultures with an internally designed temporal control of stimulations to probe various signaling pathways [49]. The platform is automatically controlled with no human intervention required for cell seeding, stimulations at different time intervals, imaging, or data analysis. Ellison et al. presented a microfluidic method for investigating the role of autocrine and paracrine signaling in mES cells [8]. Recently, a dielectrophoresis-based microfluidic platform determined a complementary method for characterizing neural stem cell phenotypes [51]. On the basis of cell markers, it was found that neural stem cells differ, preceding differentiation, from astrocytes and neurons by their frequency-dependent dipole movement. This illustrates the entirely new methodologies that microfluidics brings to the experimentalist's bench for probing stem cells.

9.3 Control of Spatial Arrangement and Topography of Stem Cells

Stem cells reside in a complex multicellular environment containing a structured ECM that supports stromal cells, vascular tissue, and other cell types that actively interact with the stem cells to regulate differentiation and self-renewal [1]. Microtechnology can help to decouple the effect of these homotropic or heterotropic interactions from other biochemical stimuli by creating precisely defined patterns with one or many cell types interacting in relatively controlled manner. Microfabricated PDMS wells of defined sizes have been used to form embryoid bodies of consistent and reproducible sizes from human embryonic stem (hES) cells [46]. These wells were created using the soft lithography techniques described earlier. In another study, it was shown that the size of microfabricated fibronectin islands influenced hES cell differentiation [52], showing

significant reduction in the expression of Oct4, Sox2, and Nanog when cultured on islands of diameter 200 μm versus larger islands of diameter 400–800 μm. ECM islands can be created using PDMS stencils created by soft lithography by a process called microcontact printing in which a PDMS mold with desired features is dipped in ECM solution and placed on a glass cover slip to create a replica of the parent pattern [52]. Mesenchymal stem cells cultured on fibronectin islands with higher mechanical stress or on flexible micropost arrays with higher traction force differentiated into osteocytes, in contrast to adipogenesis observed on patterns with lower mechanical stress or lower traction force [53]. Dielectrophoresis has also been used to precisely control the patterning of cells within a microfluidic chip [54].

Patterning has also been used to study heterotropic interactions between stem cells and other cell types. By using the laminar flow property of fluids, one can seed different cell types into a microfluidic device to study their interactions [55]. Microwell-patterned substrates have been used to study interactions between human embryonic stem cells with mouse embryonic fibroblasts [56]. An ECM-patterned, multicell system by Khademhosseini and others shown in Fig. 4 (right) was used to create islands of ES cells surrounded by fibroblasts. In a novel technique, neural stem cells were patterned and differentiated onto a designed matrix of cell-adhesive structures on top of cell-repellant surfaces to mediate axonal guidance and formation of synaptic junctions [58]. Markx et al. developed a microfluidic device to mimic the hematon, a 3D aggregation of cells that forms the niche for the production of all blood cells, using dielectrophoretic manipulation to create a layered niche in which mES cells were seeded on top of Jurkat cells, which were on top of AC3 stromal cells, which were on top of osteoblast cells in order to recreate a hematon-like structure [59].

Recently accumulating evidence indicates that topography plays a significant role in affecting stem cell phenotypes, including cell fate, migration, shape, and morphology [60]. PDMS- or hydrogel-based topographic structures can be created using soft lithography techniques and integrated within microfluidic chips to decouple the effect of topography and biochemical factors.

10 Conclusions and Outlook

Microfluidics has brought a whole gamut of conventional laboratory techniques to a single chip, introducing both sequential integration of multiple steps and massive parallelization of similar techniques for high-throughput experimentation. This is commonly referred to as the “lab-on-a-chip” approach. Stem cell research stands to gain from microfluidics in two fundamental ways: (1) miniaturization of nearly all possible experimental techniques allows rapid screening of phenotypic effects in cells in response to multiple conditions, conserves precious cells, reagents, and chemicals, and allows variation of parameters in a high-throughput and controlled fashion; and (2) various aspects of physics of the microscale allow many physiologically

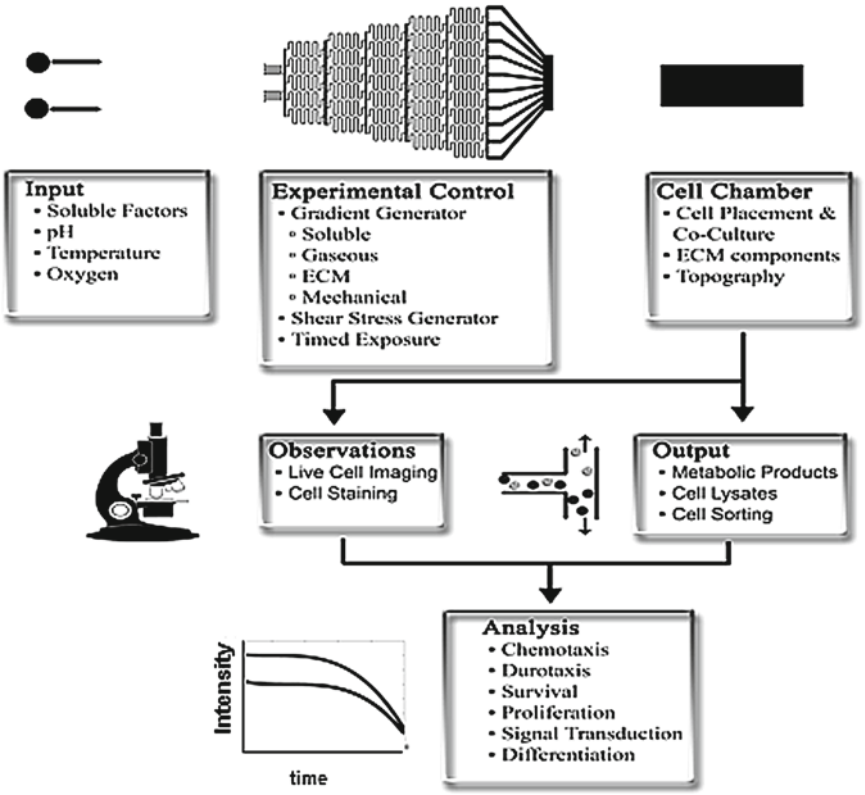

Fig. 4 Integrated microfluidics. Schematic showing the ability of microfluidics to precisely control the biochemico/mechanical and physical microenvironment of cultured stem cells and automate observations and analysis. Microfluidics in a modular format can allow connection of small and characterized components to form complex "fluidic circuits" that can perform experiments in a lab-on-chip manner. ECM, extracellular matrix

relevant conditions to be achieved in an *in vitro* setting to create precise microenvironment for stem cells and for performing experiments that were impossible or difficult at a macroscale.

Arguably, the greatest advantages of microfluidics will be fully realized when this technology is used in a modular manner in tightly integrated fluidic systems in which each module can be treated as a fully characterized, separate unit with defined input and output ports [23]. This framework, analogous to modular electric circuits as conceptualized in Fig. 4, is within reach and will allow easy-to-assemble, simple, characterized fluidic modules to create complicated fluidic networks for conducting experiments including substratum engineering, cell seeding, biochemical gradient generation, timely controlled stimulation, microscopy, cell lysis, genetic or proteomic analysis, and computational analysis.

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The Development of Small Molecules and Growth Supplements to Control the Differentiation of Stem Cells and the Formation of Neural Tissues

Victoria B. Christie, Daniel J. Maltman, Andy Whiting, Todd B. Marder, and Stefan A. Przyborski

Abstract The ability to direct the differentiation of mammalian stem cells down specific neural lineages is of significant value for basic research into the mechanisms that control neural development and the production of neural cell types for drug screening and potential therapeutic applications. An in-depth knowledge of the key signaling pathways that regulate stem cell renewal and neural differentiation is required to achieve this goal. This chapter reviews research using small molecules to control the differentiation of stem cells into neural phenotypes, mentioning in particular work on pluripotent stem cells and adult neuroprogenitor cells. The production of specific neural derivatives from stem cells in a reliable and consistent manner will depend on the development of standard operating procedures that incorporate the use of defined reagents including synthetic compounds. An example is highlighted by the development of a stable synthetic retinoid that acts as a potent inducer of neurogenesis resulting in enhanced neural development and decreased variability. Such compounds can be included in novel growth supplement formulations and protocols to further guide the differentiation of specific neural subpopulations. Collectively, these research tools will advance the use of stem cells in investigative research and contribute to the potential therapeutic application of stem cell biology.

Keywords Neural differentiation • Stem cells • Small molecules • Culture protocols

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1 Introduction

To be able to direct and define, specifically, the differentiation of mammalian stem cells has been one of the major challenges in stem cell science since the isolation of murine embryonic stem (ES) cells from the blastocyst in 1981 [1, 2]. Embryonic stem cells can self-renew and can also form all tissues derived from the three germ layers within the embryo, namely the endoderm, mesoderm, and ectoderm. While ES cells have this capacity to differentiate, scientists have found it difficult to direct in a homogeneous manner stem cell differentiation down specific cellular lineages *in vitro*. For this goal to be achieved, an in-depth knowledge of the key signaling pathways controlling stem cell renewal and differentiation is required. One approach scientists are taking to advance knowledge of these key areas is the study of small-molecule action in models of stem cell differentiation. This chapter reviews investigations using small molecules to control the differentiation of models of stem cells into neural phenotypes. We examine how studies involving ES cells, embryonal carcinoma (EC) cells, and neural stem cells have advanced our understanding of cellular differentiation. Finally, the chapter discusses how small molecules are able to influence stem cell differentiation in a controlled way and shows that by accurately following differentiation protocols incorporating such molecules as neural supplements, specific neuronal cell phenotypes can be induced. Ultimately, progress in this area has the potential to lead to new approaches for treatment of neurological disorders.

2 Neural Development in the Mammalian Embryo

During embryo development small molecules play an essential role in the control of developmental processes, including neurogenesis. Generally, small molecules act as morphogens, reproducibly and accurately modulating cellular differentiation by acting on specific signaling pathways in a dose-dependent manner. Many studies investigating these developmental processes have allowed scientists to recapitulate partially these processes *in vitro* with the aid of both naturally available and newly synthesized compounds that mimic the action of these signaling molecules.

Neurogenesis in the embryo is initiated during gastrulation, and in mammals this process is predominantly regulated by four main molecular pathways and the signaling molecules associated with them, namely, the fibroblast growth factor (FGF), bone morphogenetic protein (BMP), wnt-β-catenin, and Notch signaling pathways. In brief, the embryo initially divides into three distinct layers—the endoderm, mesoderm, and ectoderm. It is the ectoderm that gives rise to the outer epithelium of the body and the neural tube and the neural crest, leading to development of the central nervous system (CNS) [3]. FGF signaling is a key pathway involved in formation of neuroectoderm, acting first alone and later alongside the BMP pathway. BMP signaling actually inhibits neuroectodermal formation. Active BMPs bind to receptors

and activate genes specific for epidermal differentiation. During neurogenesis BMP action is blocked by both FGF signaling and the BMP antagonists noggin, chordin, and follistatin, which are released from the notochord. The inhibition of BMPs blocks epidermal differentiation and promotes neural differentiation; this process is termed the "default model" of neurogenesis [4]. Both the wnt-β-catenin and Notch signaling pathways play important roles in early neural cell fate specification, activating specific genes associated with this [5]. Initially, as the neural tube forms, it gives rise to cells that are associated with the forebrain only. It is only after a transformation signal is received from the paraxial mesoderm that this anterior neural tissue is converted into the more posterior cell types representing the midbrain, hindbrain, and spinal cord, respectively. These can all be identified by the expression of specific markers [6]. Signaling pathways involved in this transformation process include the retinoic acid receptor, FGF, and sonic hedgehog (Shh) pathways, which work closely together to pattern the new neural tissues [7] (Fig. 1). Indeed, these morphogens, particularly retinoic acid, are routinely incorporated into many differentiation protocols *in vitro* to induce neural cell fate specification and differentiation by cultured stem cells and neuroprogenitors.

3 *In Vitro* Models of Neural Differentiation

Many investigations into neural development incorporate the use of cell culture model systems. While ES cells are considered to be the most potent cell type in terms of their development potential, they remain a challenge to culture, requiring either feeder layers or highly defined culture systems to keep them from spontaneously differentiating. Their use is also surrounded by many legal and ethical issues, inhibiting some institutions from using them. Due to these concerns some research groups have opted for alternative cell types to carry out basic research, which tend to be simplified models of neurogenesis that can later be translated to ES cell systems. Examples of non-ES cell types that are commonly used include EC cells,

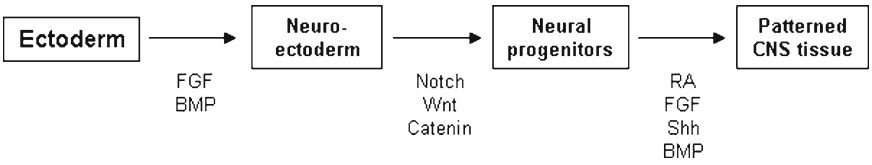

Fig. 1 Schematic representation of the various stages and possible signaling pathways understood to be involved in embryonic stem cell development toward patterned, differentiated central nervous tissue. Initially, stem cells form ectoderm, which is instructed to become the neuroectoderm by regulation of the fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) signaling pathways. Following signals from the Notch and Wnt-catenin pathways, neuroectoderm cells form committed neural progenitors, which further differentiate into the different mature neural cell subtypes found within the central nervous system. This final cell fate determination is predominantly controlled by the retinoic acid (RA), FGF, sonic hedgehog (Shh) and BMP signaling pathways

neonatal and adult neural progenitor cells, and more recently induced pluripotent stem (iPS) cells. Each of these cell types has been shown to respond to small-molecule-induced neural differentiation in a consistent and relevant manner.

4 Embryonal Carcinoma Cells as Robust Models of Embryonic Stem Cell Neural Differentiation

One of the earliest neural differentiation models involved the use of EC stem cells. EC stem cells are obtained from teratocarcinomas, a form of germ cell tumor. Teratocarcinomas contain both differentiated tissue and undifferentiated epithelial cells, which give rise to the malignant nature of these tumor types [8]. It is these undifferentiated epithelial cells that are termed EC stem cells. The relationship between EC cells and cells found in the embryo was first analyzed in the 1970s with the aid of cell surface markers [9]. Evidence of the close relationship between EC and ES cells came when studies outlined the ability of murine EC cells to be incorporated into developing murine embryos. Chimeric offspring were produced containing the EC cells, which had differentiated into many different tissue types [10]. It was also shown that the EC component within these chimeric mice was capable of extending to the germline, potentially bringing the relationship of EC cells to ES cells even closer [11].

One of the first and best-known human EC cell lines is the TERA2 cell line, isolated from a lung metastasis originating from a testicular germ cell tumor [12]. However, the TERA2 lineage was impure and only contained approximately 1%–2% EC cells, making study of these cells difficult. To overcome this problem, numerous cloned lines were derived, all of which showed varying abilities to differentiate upon instruction. One of the best-studied and most robust cloned lines was created by Andrews et al. in 1984, termed NTERA2 [13]. This clonal line responded to culture with the small molecule all-*trans*-retinoic acid (ATRA) by forming neurons. Another, more recent clone, TERA2.cl.SP12, was created in 2001 by immunomagnetic isolation and subsequent single-cell selection and was shown to have enhanced neuronal ability [14]. TERA2.cl.SP12 EC stem cells differentiate in response to ATRA, downregulating stem cell markers and upregulating cell surface antigens associated with neural differentiation, such as A2B5 and VINIS-53 [15]. After 21 days of culture in the presence of ATRA, TERA2.cl.SP12 cells differentiate into a heterogeneous population containing many mature functioning neurons, making them a useful model system for the *in vitro* study of neurogenesis [16].

Research continues using EC stem cells as models of neural development despite their malignant phenotype. This is partly because *in vitro* differentiation by these cells has been shown to recapitulate certain developmental processes, and they are viewed as a useful research tool. EC cells are seen as a robust and simplified model of ES cells and are often routinely used for basic research in preference over ES cells to address certain questions due to ease of culture, inexpensive use of reagents, and lack of ethical constraints.

5 Adult Neural Progenitor Cells

Until relatively recently it was thought that the CNS in mammalian adults was incapable of regeneration or repair due to an inherently inhibitory environment, in part due to glial scar formation, the presence of inhibitory factors such as Nogo A, and the lack of neurogenesis. However, papers refuting this dogma have been published. For example, in 1998, Eriksson et al. [17] reported neurogenesis within the dentate gyrus of the hippocampus in human adults, which was linked to neural turnover associated with learning and memory. To date, this is the only major region of the adult CNS in which this occurs, as corroborated by studies using carbon dating [18]. These studies exploited changes in the atmospheric ^{14}C levels from above-ground nuclear weapon testing in the 1950s to date neuronal DNA samples obtained from specific brain regions. Therefore, although specific regions of the human adult brain can regenerate, most cannot, which is the primary reason for the devastating and irreversible effects of neurological disorders and disease. Adult neural progenitor cell lines are therefore very important research tools for understanding small-molecule-induced adult neurogenesis and creation of treatment plans for neurological disorders. Animal cell lines have been successfully created from these region-specific neural progenitor cells—for example, the adult rat hippocampal progenitor cell line created at the Salk Institute [19]. This cell line was shown to proliferate in the presence of bFGF and to differentiate into neural cell types in response to culture with ATRA [20]. Most model systems are derived from animal models and not human tissues due to the obvious inability of ethically obtaining living adult "normal" brain material. However, a recent paper described techniques for deriving cultures of neural progenitor cells from human adult brains after death [21], which could prove a promising source of cells for the study of human adult neurogenesis.

6 Induced Pluripotent Stem Cells

The development of iPS cell technology has provided new opportunities for creating pluripotent stem cell lines from somatic tissues. In 2006, Takahashi and Yamanaka created the first mouse iPS cell line by inserting only four genes (*c-Myc*, *Klf4*, *Oct4*, and *Sox2*) into a mouse somatic adult cell, which resulted in cellular reprogramming and the formation of a pluripotent stem cell–like phenotype [22]. These cells may have significant therapeutic potential, enabling scientists to take cells from a patient's body and reprogram them *in vitro*, then differentiate the iPS cells into the desired cell type with small molecules for transplantation into the donor patient without risk of immune rejection. While this objective may still be some way off, scientists have already created iPS cells from patients with neurodegenerative disorders and differentiated them into neurons [23, 24]. Indeed, in mouse models of Parkinson disease, neurons generated from iPS cells were transplanted

into the brain and were shown to integrate with host tissue, forming functional neurons and inducing levels of functional recovery [25].

7 Inclusion of Small Molecules in Protocols for Neural Differentiation *in Vitro*

It is recognized that a number of key signaling molecules play critical roles in neurogenesis *in vivo*. Consequently, many neural differentiation protocols commonly incorporate similar molecules in order to recapitulate neurogenic effects *in vitro*. The addition of specific small molecules at specific concentrations at certain time points has allowed the directed differentiation of stem cells along the defined neurogenic pathways—for example, the induction of differentiation of pluripotent stem cells into neural precursors, subtype-selective neural progenitors, and finally to maturation into functional neurons [16, 26] (Fig. 2). In addition, most modern cell culture protocols employ commercial neural supplements, which contain various small molecules and support proteins. The advantage of administering small molecules into the culture system is that their concentration can be precisely controlled. Small molecules can be manipulated to target specific protein families and signaling pathways, allowing specific properties of cells to be investigated. Generally, their administration elicits a rapid cellular response, either inhibiting or activating the pathway in question, which in some cases can be reversible.

In recent years, there has also been a strong incentive to develop novel alternative molecules that display enhanced neuronal potency and/or stability under cell culture conditions. In this context, we discuss the current status and limitations of certain small molecules currently used in neural cell culture. Furthermore, we describe recent progress in the field of novel bioactive small-molecule chemistry and the development of new and enhanced neural growth supplements.

8 Natural Small Molecules

Examples of small molecules naturally associated with neural development that have been used to instruct neural differentiation by cultured stem cells include ATRA, sonic hedgehog, cytidine analogs, histone-deacetylase inhibitors, and protein kinase inhibitors. All of these molecules are able to enter individual cells, where they elicit a biological response, either by binding to nuclear receptors (ATRA), interacting with histone or DNA-modifying enzymes (cytidine analogs and histone-deacetylase inhibitors), or directly interacting with specific signaling pathways (sonic hedgehog and protein kinase inhibitors). Of these natural compounds, ATRA is most commonly incorporated into neural differentiation protocols. ATRA has routinely been used as a general inducer of stem cell differentiation, resulting in the formation of neural derivatives. ATRA has also been used to

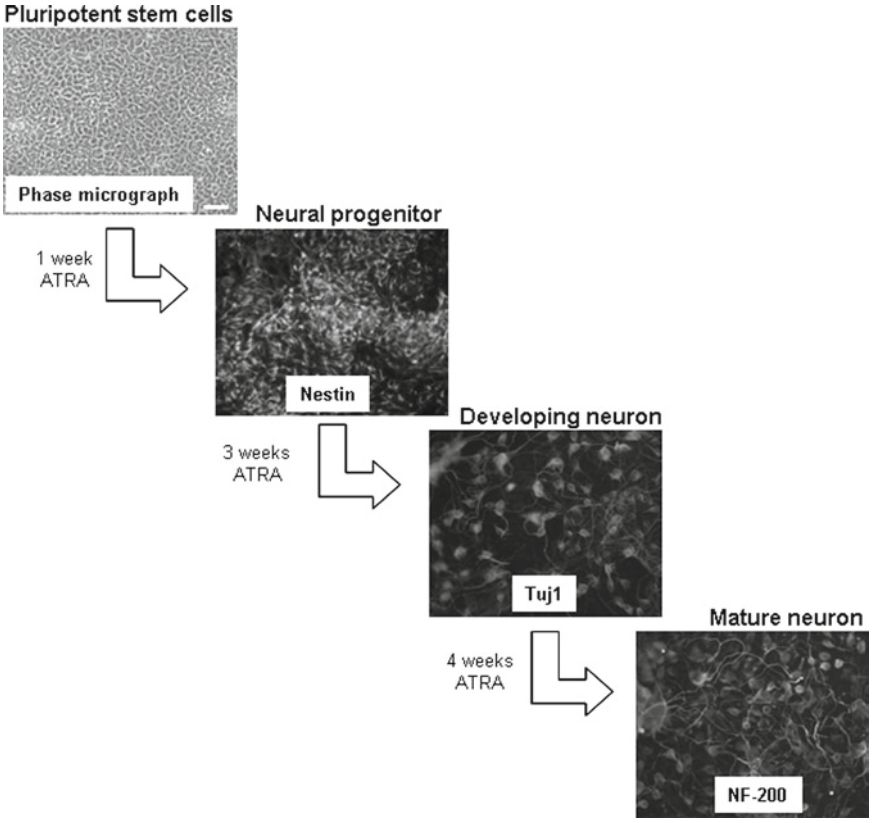

Fig. 2 The differentiation of pluripotent embryonal carcinoma stem cells can be followed using immunocytochemical analysis over a time course. Following a 4-week differentiation program incorporating the small molecule all-*trans* retinoic acid (ATRA, 10 μM), human pluripotent stem cells exposed to ATRA express markers of neural progenitors (nestin) after 7 days, markers of developing neurons such as beta-III-tubulin (Tuj1) appear after 2–3 weeks, and markers of mature neurons, including neurofilament 200 (NF-200), are expressed after 3–4 weeks in culture. This protein expression profile closely mirrors the developmental process of neurogenesis reported *in vivo*

differentiate and pattern ES cells into more specified neural cell types, including motor neurons [27, 28].

9 Limitations Associated with All-*trans*-Retinoic Acid Use *in Vitro*

Although natural small molecules are important for developmental studies, their use is not without its limitations. A prime example is ATRA, which, as noted earlier, is critically involved in numerous regulatory processes throughout both

embryonic and adult neural development. ATRA's prominent role in neurogenesis is reflected in its extensive use to promote neural differentiation *in vitro* in a broad range of cell models. As a consequence, the extensive use of ATRA *in vitro* has contributed significantly to our understanding of its mode of action and biological activity. However, a commonly overlooked problem associated with ATRA is its structural instability, resulting in a tendency to undergo considerable isomerization and degradation when exposed to light or heat [29–32]. For example, the five conjugated double bonds that give ATRA its excellent chromophore properties, efficiently absorbing light in the region of 300–400 nm, render the molecule extremely susceptible to photoisomerization. Consequently, ATRA can readily degrade into a mixture of several different retinoic acid isomers and degradation products under normal laboratory conditions. Previous studies have shown that during the preparation and maintenance of cell cultures containing ATRA, the concentration levels decrease markedly over time, an apparent consequence of its degradation, as well as of cellular metabolism [33]. This issue of compound stability is therefore an important consideration when reagents are being routinely used for cell culture applications, and this feature is often overlooked by cell biologists. Due to instability, there is an unavoidable variation in actual ATRA concentration being applied during an investigation, which can depend on a number of factors, such as how often or how long ATRA stocks have been thawed out and/or exposed to light during use. Cellular differentiation is responsive to ATRA concentration, and certain doses of ATRA are required to induce specific neuronal phenotypes. Accordingly, any discrepancy in concentration accuracy could potentially elicit dramatically different biological results. This is further compounded by the fact that some of the degradation products of isomerized ATRA are known to elicit differing biological activities. For example, known ATRA isomers differentially affect the ability of embryonic stem cells to differentiate into defined neuronal products [33, 34]. Evidence of these limitations has prompted the synthesis of more stable synthetic alternatives to ATRA for *in vitro* use in the investigation of neurogenesis and reproducible production of neural derivatives from stem cells.

10 Synthetic Small Molecules

There are two main approaches to the synthesis of new biologically active small molecules: (1) a targeted approach in which specific existing compounds are structurally modified/improved, and (2) a shot-gun approach of functionally screening chemical libraries against a specific biological activity.

In the case of overcoming the limitations associated with ATRA instability, a targeted approach to small-molecule synthesis may be adopted. Essentially, a specific problem with a small molecule is identified, and novel synthetic compounds are designed and synthesized to overcome it. For example, using available literature and chemical modeling programs, our research group synthesized isometric analogs

of ATRA with the aim of improving retinoid stability when handled under standard laboratory conditions. One compound analyzed, termed EC23, was a previously known synthetic retinoid that, like ATRA, activates all of the retinoic acid receptors [35]. On this basis, EC23 was examined on a range of stem cell–based model systems to evaluate biological responses, and these were compared against the actions of the natural ATRA counterpart [36]. Indeed, the small molecule EC23 was observed to induce enhanced neurogenesis in all cell model systems tested and appeared to do so via the activation of key components of the retinoic acid receptor pathway [37] (Fig. 3). Another example of a targeted approach is the synthesis of chondroitin sulfate glycosaminoglycans, which show enhanced reproducibility in inducing neuronal differentiation of hippocampal progenitor cells [38]. These small molecules have been implicated in neuronal development and spinal cord injury, and molecules enabling the induction of more reproducible results will greatly improve this area of research. In summary, such targeted approaches have resulted in the synthesis

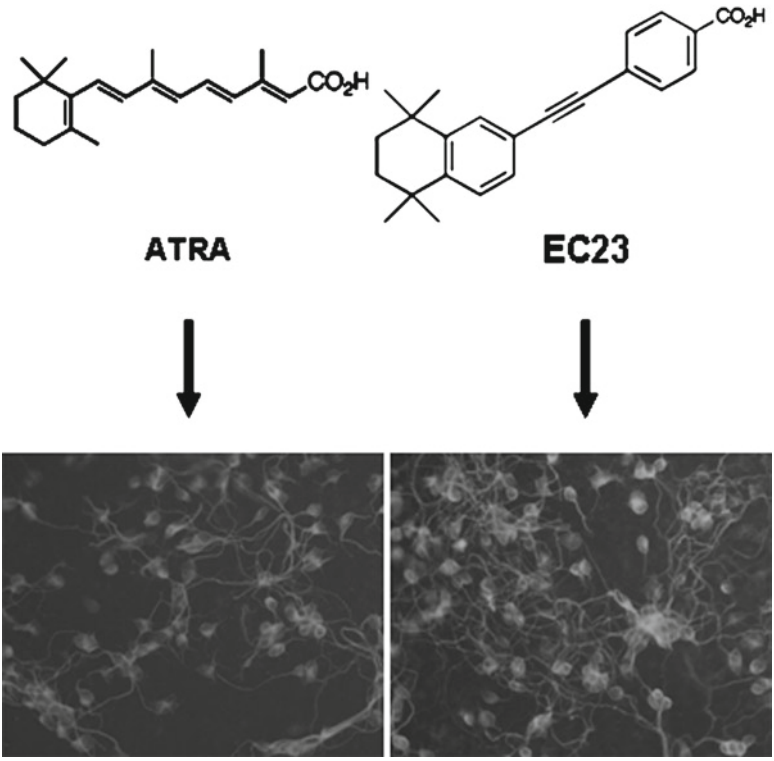

Fig. 3 The synthetic small molecule EC23 was developed as a stable, potential alternative to the natural retinoid all-*trans* retinoic acid (ATRA) for *in vitro* use as a modulator of cell differentiation. Biological analysis of compound activity on cultures of human pluripotent stem cells demonstrated the ability of 1 μM EC23 to induce neuronal differentiation after 3 weeks in culture at a comparable level to that seen with ATRA. Micrographs show immunologic staining of neurons for the neuronal marker beta-III-tubulin (Tuj1)

of small molecules that have been shown to be attractive alternatives to their natural counterparts for *in vitro* use in the study of stem cell–derived neurons.

The use of chemical libraries associated with biological screening is also a useful approach to identifying biologically active small molecules (Fig. 4). This approach allows for a higher-throughput analysis of a basic biological question. Many chemical libraries have been established that routinely contain hundreds of thousands of small molecules and focus on structure–activity relationships between these compounds and targeted specific signaling pathways [39]. Using this approach, scientists have been able to identify small molecules that actually inhibit cells from differentiating, that is, keep the cells in an undifferentiated/stem-like state. Pluripotin was developed in 2006 and was shown to keep mouse stem cells in an undifferentiated state by inhibiting both ERK1 and rasGAP proteins, which were known differentiation-inducing compounds [40]. Small molecules that modulate neuronal differentiation have also been identified. Neuropathiazol was identified from a large chemical library by high-content, image-based analysis of neuronal markers. It has been shown to induce reproducible and robust neuronal differentiation of primary multipotent hippocampal progenitor cells [41]. Another high-throughput screen identified the orphan ligand phosphoserine as an inducer of neurogenesis in primary human embryonic stem cell–derived neural progenitors [42]. This then led to the further discovery of a novel role for the metabotropic glutamate receptor 4 in neural cell regulation.

Taken together, these studies indicate the potential of small molecules in the field of neuronal development and how targeted screens are enhancing the knowledge in this area. A main goal of such research is, eventually, to achieve a better understanding of the control of neurogenesis, which will aid both basic research and development of treatments for neurological disorders.

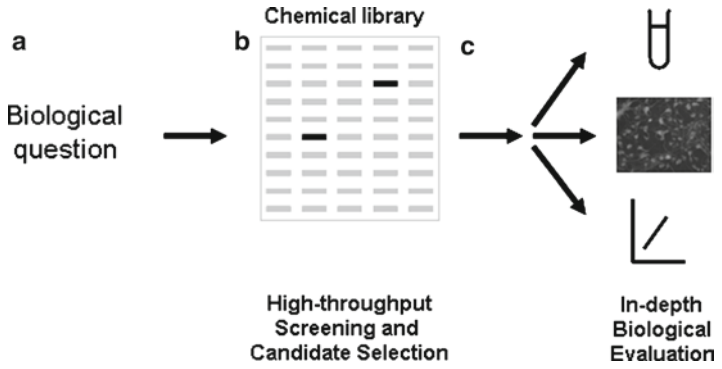

Fig. 4 Biological screening of small molecules. **a:** First, a biological problem or question is approached using a simple biological screen. **b:** This screen is used to test hundreds of thousands of small molecules, from which positive candidates can be selected for further analysis. **c:** Typically, a small number of candidate molecules are highlighted during the high-throughput screening process, and these are put forward for more in-depth biological analysis

11 Neural Supplements

Many protocols to induce the differentiation of stem cells into neural derivatives are carried out in serum-free conditions. Indeed, differentiation studies on mouse ES cells showed that enhanced neuronal differentiation was achieved in serum-free defined conditions [43]. This was attributed to the presence of neural inhibitory factors, such as BMPs, in culture serum. However, for cells to be grown in serum-free conditions, the culture medium needs to be supplemented with a variety of factors. Two of the most common neural supplements are N2 and B27. Both contain five key molecules, namely transferrin, insulin, putrescine, progesterone, and selenite, with B27 also containing vitamins, hormones, fatty acids, and antioxidants. All of the five base components have been shown to be important for maintaining neural progenitor cell growth, neuronal survival, and neuroprotective properties (for a recent review see ref. 44). B27 was developed in 1993 as an improved neural supplement allowing enhanced survival of neuronal cells [45]. However, recent reports have outlined limitations in the reproducibility of the B27 manufacturing process, indicating batch-to-batch variability, which significantly effects neuronal differentiation [46]. This has prompted the development of new neural supplements, such as NS21, that target the long-term survival of neuronal cell cultures [47]. This need for better-defined and better-controlled supplements is linked with the synthesis of novel small molecules that have enhanced stability and biological activity as discussed earlier. Inclusion of such compounds into novel growth supplement formulations is an area of significant interest and commercial value with the view to create robust standard operating procedures for the reproducible and consistent differentiation of cultured neural cells.

12 Use of Small Molecules for the Treatment of Neurological Disorders

Following the isolation and culture of human embryonic stem cells by Thomson et al. in 1998 [48], there has been significant effort to evaluate the feasibility of taking advantage of this cell type to create new, improved methods of cell-based therapy, drug development, and treatment plans. The rationale behind this concept is that if homogeneous populations of specific cell types can be generated *in vitro* in a controlled manner, then the resulting differentiated cells could be transplanted into damaged tissues as a way of repairing them, thereby forming the basis of a new therapeutic approach. Furthermore, cultured stem cells could also be manipulated to release soluble factors or small molecules that could influence cells in the targeted area toward self-renewal or, in the case of cancerous tissue, induce programmed cell death (Fig. 5). These techniques could potentially be implemented for a huge variety of both common and rare human diseases, such as certain types of cancer and degenerative diseases.

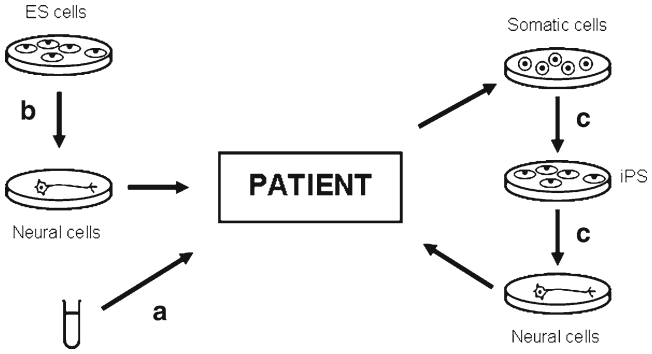

Fig. 5 Small-molecule involvement in potential neurological treatment programs. **a:** The small molecules themselves could be administered as a treatment to encourage endogenous cells to self-repair damaged neural tissues. **b:** Small molecules could be used to terminally differentiate embryonic stem (ES) cells into neurons prior to transplantation into the affected area. **c:** Somatic cells obtained from the patient could first be reprogrammed, perhaps using small molecules, into induced pluripotent stem cells (iPS cells). These iPS cells could in turn be induced to differentiate into neurons, using alternative small molecules, before transplantation back into the patient

One area that has the potential to benefit greatly from stem cell therapies is the brain and spinal cord. At present, many neurological disorders and injuries remain untreatable, leaving the patient with a much-compromised quality of life. Screens of small molecules that are influenced by a specific disease state could potentially improve the diagnostics of many neurological disorders. For example, it has been shown that the neuronal marker *N*-acetylaspartate is significantly reduced in the motor cortex and corticospinal tract regions in the brains of amyotrophic lateral sclerosis patients [49, 50]. The degree of loss of this marker correlates with the clinical indicators of affected patients, indicating that the small molecule is directly related to the progress of the disease state. Other studies have identified small molecules that promote neural repair. Often in the damaged CNS, neuritogenesis is inhibited by molecules associated with the glial scar, which suppresses axon regeneration. On the basis of a small-molecule approach, local administration of epidermal growth factor receptor inhibitors was found to promote significant regeneration within the damaged adult CNS, overcoming such axonal suppression [51]. In models of brain cancer, small molecules were identified that inhibited the proliferation of neural progenitor cells and were shown to have potent effects on the progression of certain neural tumors [52]. Each of these examples demonstrates how small molecules can be used to interact with known signaling pathways to influence cell behavior and potentially improve certain disease states.

13 Conclusions and Future Perspectives

Stem cells from embryonic, neonatal, and adult tissues have proved to be important research tools for investigating neural development. Small molecules have played a large part in the research associated with neuronal differentiation of stem cells.

Natural compounds incorporated into culture medium and specific neural supplements enhance the neuronal cell output. However, there are certain limitations to these approaches when using either natural small-molecule supplementation or the widely used commercial neural supplements. Synthetic small molecules can be designed with a specific biological outcome in mind and can exhibit very tightly regulated biological responses. More small molecules from targeted/shotgun screening projects have led to a greater understanding and control of neurogenesis *in vitro* but also contribute significantly to the development of new methods to control cell differentiation and the creation of improved culture reagents. Through the collaborative interactions between biologists and chemists, there is enormous potential to develop innovative approaches to control cell differentiation responses for the benefit of basic research and potential therapeutic applications.

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Long-Term Propagation of Neural Stem Cells: Focus on Three-Dimensional Culture Systems and Mitogenic Factors

Rikke K. Andersen, Jens Zimmer, and Morten Meyer

Abstract The number of neurons that can be derived from neural stem cells (NSCs) in culture depends particularly on the age of the donor, region of origin, mitogens used for propagation, and longevity in culture. As the cellular microenvironment provides essential regulatory factors to NSCs, this chapter focuses on three-dimensional culture systems for long-term propagation of fetal NSCs and mitogens commonly used for maintenance of their proliferative capacity and neurogenic potential.

Keywords Neurospheres • Neural tissue spheres • Mitogen • Stem cell niche

1 Introduction

Over the last two decades, neural stem cell (NSC) research has provided new and unexpected insights into the plasticity and regenerative potential of the brain. With established isolation procedures and standardized methods for in vitro propagation, NSCs hold great promise in regenerative therapy. Within a few years cells derived from NSCs are expected to be used for cell replacement therapy in neurodegenerative diseases like Parkinson and Huntington diseases, where defined populations of dopaminergic and GABAergic neurons are lost. Certain practical problems, however, remain to be solved before larger-scale clinical trials will be possible. One major issue is the need for highly standardized protocols for propagation and efficient generation of large numbers of transplantable cells with defined and sustained characteristics.

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Propagation and passaging for longer periods in culture are very often necessary to obtain sufficient numbers of cells, which then should retain the capacity to differentiate into functional neurons. Despite the lifelong presence of NSCs in the brain, there is a progressive reduction in proliferation and neurogenesis with increasing age [1, 2]. This is also reflected in stem cell cultures maintained for longer periods, in which the number of neurons that can be derived depends on the age of the donor, region of origin, and mitogens used for propagation. As the cellular microenvironment provides essential regulatory factors for NSCs, this chapter focuses on three-dimensional (3D) culture systems for long-term propagation of fetal NSCs and mitogens commonly used for maintenance of their proliferative capacity and neurogenic potential.

2 Neural Stem Cells and Their Differentiation Potential

By definition, stem cells should be able to self-renew in vivo or in vitro an indefinite number of times without significant changes in properties displayed at the initial isolation [3–5]. Proliferation and ability to self-renew are usually demonstrated during culturing by the formation of secondary clones derived from single cells in response to mitogens [5–10]. Furthermore, depending on stage in the differential hierarchy, neural stem cells or progenitor cells can give rise to a wider or more limited array of mature, functional progeny, as shown in vitro and in vivo [4, 8, 11].

NSCs can be isolated from immature and adult mammalian brain and spinal cord [12, 13], and they are classified as multipotent when able to differentiate into the three major cell types of the adult central nervous system, namely neurons, astroglia, and oligodendroglia [3, 5, 14, 15]. In contrast to NSCs, so-called neural progenitor cells do not necessarily self-renew (limited expansion potential), and they are often unipotent or bipotent, and as such produce only a very limited number of different neural phenotypes [16]. In this chapter the term neural precursor cell will be used as a common denominator for neural stem cells and progenitor cells when the nature and proliferative capacity of the cells in question are uncertain or not well defined.

3 Central Nervous Areas Used for Isolation of Neural Precursor Cells

Multipotential NSCs can be isolated from the subventricular zone (SVZ) lining the lateral ventricles in the telencephalic (forebrain) parts of both developing and mature brain [6,17–22] (for reviews, see refs. 15, 21, and 23). This neurogenic zone is far most prominent and active in the forebrain [24], but it can be identified as far caudally as the fourth ventricle [25]. Another proliferative area in the adult brain is

the recently described [26] subcallosal zone (SCZ), associated with obliterated hemispheric parts of the lateral ventricles and located between the white matter of the corpus callosum and the hippocampus. This region gives rise to glial precursors exclusively [26]. In addition, the subgranular zone (SGZ) of the hippocampal dentate gyrus is neurogenic both during development and in the adult brain, containing neural precursors that can give rise to neurons, astroglia, and oligodendroglia [27]. Neural precursors have been isolated from many other regions of the adult central nervous system, including septum, striatum, cortex, spinal cord, optic nerve, mesencephalon (substantia nigra), cerebellum, and subcortical white matter [14, 22, 28–36], although other studies suggest that some of these findings may be attributed to technical misinterpretations [37, 38]. In line with this, astrocytes derived from less-neurogenic brain regions such as the cerebral cortex, cerebellum, and spinal cord during the first two postnatal weeks can all form multipotent neurospheres, while only SVZ astrocytes retain this ability in the adult [39].

The many negative in vitro studies reporting absence of cells with proliferative neurosphere-forming ability in the brain and spinal cord may reflect an inadequate, site-specific mitogenic stimulation of the cells. This is supported by the finding that NSCs isolated from the adult spinal cord require simultaneous stimulation by both epidermal growth factor (EGF) and basic fibroblast growth factor (FGF2) to undergo self-renewal in vitro [22]. Forebrain and spinal cord neural precursors also behave differently when expanded in EGF, FGF2, heparin, and ciliary neurotrophic factor (CNTF), just as only forebrain precursors maintain their neurogenic potential during long-term culture [40].

4 Three-Dimensional Culture Systems for Propagation of Neural Stem Cells

Rupture of tissue is a common procedure for obtaining NSCs for propagation either as free-floating, sphere-forming cells or as attached, dispersed cell cultures. Most techniques, however, employ harsh dissociation procedures during passaging by often weekly mechanical, chemical, or enzymatic dissociation into single-cell suspensions. The way of dissociating and handling of cells affects the cellular content of the cultures. It has thus been found that mechanical dissociation results in fewer cells with chromosomal abnormalities after many passages than dissociation in nonenzymatic buffer or enzyme-containing dissociation media [41–43].

5 Neurospheres

Neurospheres (NSs) were first introduced by Reynolds and Weiss in 1992, who generated free-floating clusters of cells derived from adult mouse striata [6]. The ability of cells to form NSs was also used in a so-called NS assay (NSA) to distinguish and

separate NSCs able to form NSs from other cells not displaying this ability [44, 45] and as a model system for neurogenesis and neural development [44, 46–49].

The growing NSs are heterogeneous cell clusters that actually may contain relatively few NSCs, able to propagate as NS-forming cells after dissociation, together with more fate-restricted neural progenitor cells plus variable numbers of more-differentiated or even mature neural cell types [7, 50–54]. The fraction of self-renewing NSCs can constitute up to 30% of the total cell population, but is usually less than 5% [55, 56], illustrating that the cells are relatively quiescent [20, 57] with a doubling time of greater than 6 days and sometimes even greater than 15 days [17, 58]. Spheres of considerable size normally require at least 2 weeks of proliferation to develop [59], and establishment of NSs from human NSCs plated as single cells can require up to 8 weeks [60]. Once established, NSs are maintained relatively small (approximately 100–200 μm in diameter) due to restriction in supply of nutrients and oxygen to the inner cell mass in larger aggregates. The distance-dependent potential difference in supply of culture supplements may induce a compartmentalized, layered appearance of the NSs, with an inner, differentiated part and an outer, undifferentiated part [61]. Cell divisions are thus mainly found in the superficial parts of the NSs, where cells expressing neural stem cell markers like Notch1 and nestin are also located [61].

Neural precursor cells from rodents and humans can also be grown as monolayer cultures attached to various surfaces [62–65]. In line with the 3D culture system described here, freshly isolated and dissociated cells require plating at high densities (approximately 10,000 cells/cm^2) to allow cell contact–mediated factors to operate and in order to proliferate [31, 51].

6 Neural Tissue-Spheres

An alternative method for propagation of neural precursor cells is by way of neural tissue-spheres (NTS) [66], which in essence are free-floating, small tissue-explant cultures. One major difference between the NSs described earlier and NTSs is the fact that the tissue-spheres are never disrupted into cell suspensions for passaging but instead are only mechanically divided into quarters for further propagation (Fig. 1). The NTS technique advances a similar method [42] that was gentler than the NSA [6,52] for producing free-floating cellular spheres in vitro. Compared to other methods, the NTS method is accordingly expected to better preserve the microenvironment of the precursor cells. Since common elements of the stem cell niche include cell–cell interactions, somatic cell signaling, the extracellular matrix, and in some cases a basement membrane [67], the behavior of cells maintained as NTSs thus potentially is more reminiscent of their in situ counterparts. By means of the NTS method rat neural precursor cells can be propagated long term [66], which has otherwise been difficult [12, 68, 69]. This indicates that the method is suitable for investigations of cells from more-susceptible brain regions, which do not tolerate lack of yet-unknown factors in the culture settings.

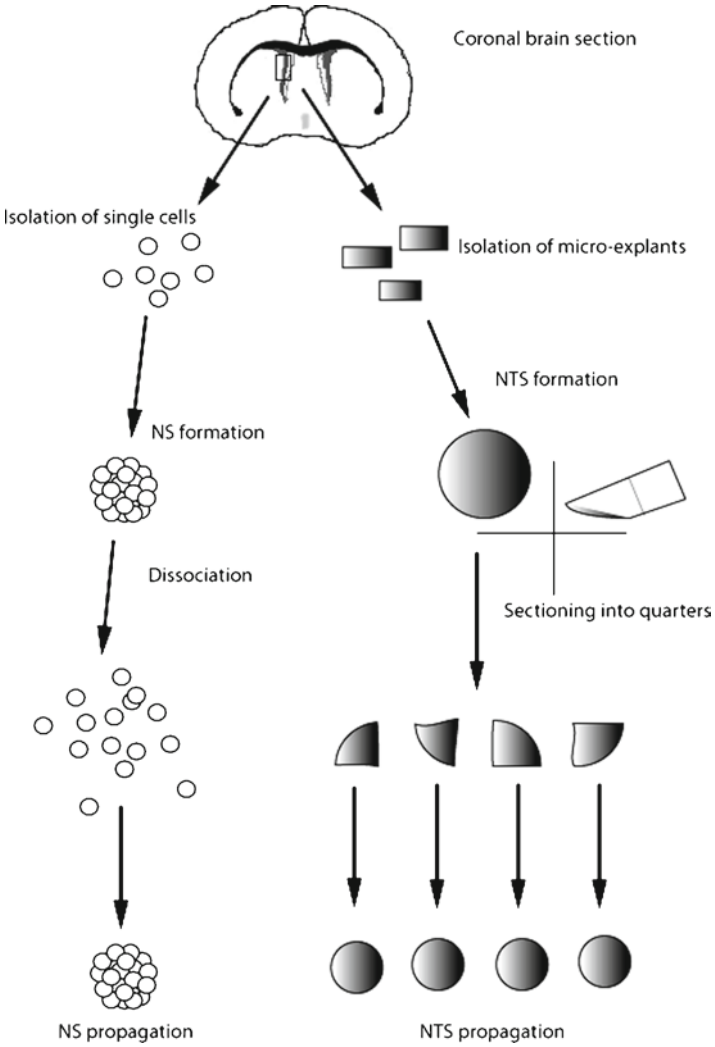

Fig. 1 Schematic illustration of propagation of stem cells in three-dimensional cultures. Cells or tissue blocks are isolated (here shown for the rostral part of the lateral ventricles) and either triturated into single cells or maintained as microexplants (cells in situ). After a period in culture, cells will form neurospheres (NS), or tissue blocks will round up to spherical structures called neural tissue-spheres (NTS). For passaging, when a given size is attained, NSs are dissociated mechanically, chemically, or enzymatically and reseeded to produce the next generation of NSs. For passaging of NTSs, spheres are cut into quarters by a scalpel knife, with a new generation of NTSs forming from the quarters

Furthermore, by taking use of 96-well plates, NTSs can be propagated in such a way that the fate of individual cultures from precisely defined regions can be monitored throughout the propagation period. In this way it was found that neural

precursor cells able to maintain NTS propagation for several generations and over long time are present throughout the forebrain SVZ but with regional differences and characteristics [66]. NTSs may thus be used for experimental investigations of microenvironmental factors that regulate NSC proliferation and lineage-specific differentiation.

7 Long-Term Propagation of Neural Stem Cells in Three-Dimensional Culture

Both NSCs and progenitor cells can divide in culture, while stem cells alone have the capacity for extensive self-renewal, that is, the ability to be passaged more than six times [70]. NSCs eventually undergo senescence [10, 71, 72], and mitotic crisis has been reported on many occasions [10, 71–74]. Rat cells in general go into crisis after approximately 4–5 weeks in vitro [1, 68, 75, 76], and human precursor cells become senescent or fail to respond to proliferative signals within 1 year [42, 73, 77], although a few studies have reported successful long-term culture past this time point [12, 78].

Comparison between studies of propagation of neural precursor cells is, in general, difficult due to the use of various protocols for subculturing, differences in cell densities for seeding and components in the growth medium, and different donor ages, species, and brain regions (Table 1). For example, a recent study [79] compared 20 studies using the NSA for propagation of tumor and nontumor stem cells, and none of the studies used exactly the same culture conditions. For illustration of similarities and diversities among studies dealing with long-term propagation of NSs, in casu culturing for more than 119 days [40], see Table 1. Some of the employed regulating factors and compounds presented in Table 1 are also dealt with later.

8 Mitogens Used for Propagation of Neural Stem Cells

Proteins and peptides known as neurotrophic factors or growth factors fall into several groups or families based on structure and receptors. Common to them are the property of being able to support the survival of particular subtypes of neural cells.

9 Epidermal Growth Factor and Fibroblast Growth Factor 2

In particular, EGF and FGF2 are widely used for epigenetic propagation of stem cells in vitro, alone, together, or in combination with other factors [18, 46, 80]. FGF2-responsive cells are in general found much earlier in development than EGF-responsive cells. The two factors have been reported to have a synergistic

Table 1 Selected studies on long-term propagation of neural precursor cells

Species	CNS region	Donor age[a]	Culture setting and number of cells	Passaging technique and time interval	Mitogens	Growth conditions	Culture period	Observations and differentiation potential	Ref.
Rat	Striatum + SVZ	Adult	NS (attached) 10,000/mL	Trituration 1–2 wk	EGF (20 ng/mL) FGF2 (20 ng/mL)	NS-A medium, 2% B27, TIPPS	P10-40 (P50 ~1 yr)	Cultures died when detached. Single-cell multipotency. High proportion of CNPase-positive cells in differentiated cultures.	153
Rat	DG OB	Adult	Monolayer, then NS Density not stated	Initial digestion with papain, trypsination before NS	EGF (20 ng/mL) FGF2 (20 ng/mL) PDGF-AB (20 ng/mL) (LIF 10 ng/mL)	Fibronectin coated 10% BIT-9500	>P35 (14 wk)	Test of 5 different media. DG cells detached in LIF. LIF did not affect OB cell proliferation. Ir in proliferating NS: Nestin, sox-2, β-tubulin, GFAP, NSE, CNPase.	3
Rat Human	Cortex Striatum VM hCb Human thalamus	ED14 6–21 wk	NS 2–400,000/mL	Chopping (7) 14 d	EGF (20 ng/mL) FGF2 (20 ng/mL) Heparin (5 μg/mL) >4 wk EGF only	2% B27, then 1% N2 > first passage	Senesced at 5 wk 250 d	Slower growth of VM cp to CX and STR. Different morphology of neurons derived from different regions.	69
Mouse	Cortex Mes	ED16	NS split 1:3 in 6-well plates Monolayer (PO/Fbn 10^4/cm^2)	Trituration Accutase 21 d	EGF (20 ng/mL) FGF2 (20 ng/mL)	3% oxygen	1 yr	Bipotent (no cell staining for oligodendrocytes but microarray indicates upregulation over time). Mesencephalic neural progenitors propagate faster and for extended periods in low oxygen with maintenance of telomerase activity.	154

(continued)

Table 1 (continued)

Species	CNS region	Donor age[a]	Culture setting and number of cells	Passaging technique and time interval	Mitogens	Growth conditions	Culture period	Observations and differentiation potential	Ref.
Mouse	SVZ RE1 RE2	Adult P15	NS 200/cm^2 in 35-mm Petri dishes 0.35–1 × 10^4/cm^2	Mechanical dissociation 4–6 d	Setup: EGF, FGF2, EGF/FGF2 EGF (20 ng/mL) FGF2 (10 ng/mL)	TIPPS	6 mo (P23)	Multipotent. No growth difference between GFs. Heterogeneity in relation to rostrocaudal axis: Higher numbers of oligodendrocytes from RE1. Slower growth of cells from RE2.	155
Mouse	Striatum	Adult	NS 3500/cm^2 in 35-mm Petri dishes	Mechanical dissociation 8–10 d	Setup: EGF, FGF2, EGF/FGF2 EGF (20 ng/mL) FGF2 (20 ng/mL) Heparin (2 μg/mL)	Insulin, progesterone	6 mo	Multipotent. Slower expansion rate in FGF2 than EGF, which is slower than EGF/FGF2 combined.	18
Human (mouse)	Striatal eminence	10 wk ED14	NS 200,000/mL	Initial trituration, subsequent passages, McIlwan chopper	EGF (20 ng/mL) FGF2 (20 ng/mL) Heparin (5 μg/mL)	2% B27 until first passage, then 1% N2	2 wk 4 mo	4 or 20 wk survival. No neurons after short-term survival. No cells in long-term grafts.	115
Human	Cortex	7–21 wk	NS 200,000/mL Subsequently 50 NS/T75 flask	Initial trituration + filter, then McIlwan chopper 14 d	EGF (20 ng/mL) FGF2 (20 ng/mL) Heparin (5 μg/mL) FGF2/heparin >28–35 d	B27 N2 from first passage	150 d	Bipotent at late passages (no oligodendrocytes). Mitotic, but nonexpanding at 6 mo.	42

Human	Cortex	8–13 wk	NS 200,000/mL	Initial trituration, subsequently chopping passaged biweekly	EGF (20 ng/mL) FGF2 (20 ng/mL) Heparin (5 μg/mL) >2 wk only EGF >20 wk EGF/LIF (10 ng/mL)	2% B27 until first passage, then 1% N2	53 wk (110 PDs ~75 wk)	Senescence before 30 wk (40 PDs) w/o LIF. Senescence at 110 PDs with LIF. Presented data from 53 wk.	97
Human	Cortex	7.5–8 wk	NS (neural stem) split 1:3-5	Trypsin, EDTA 2–7 d	EGF (10 ng/mL) FGF2 (10 ng/mL)	NS-A medium, N2, B27	P158	Homogeneous adherent cultures. Long-term maintenance of neurogenic potential. Neuronal differentiation in transplants >4 wks survival.	64
Human	Forebrain	7–10 wk	NS 2 × 10^6/mL	Trypsin, EDTA 14–21 d	EGF (20 ng/mL) FGF2 (20 ng/mL) LIF (10 ng/mL)	B27 50% conditioned medium (reuse)	252 d	Multipotent. Effective expansion for 200 d, then slowing of growth rate. No noticeable loss of proliferative capacity and differential potential.	77
Human	Forebrain	5–11 wk	NS 100,000/mL	Mechanical dissociation 7–30 d	EGF (20 ng/mL) FGF2 (20 ng/mL) Heparin (2 μg/mL) LIF (10 ng/mL)	N2 medium	P17-35 (175–371 d)	Multipotent but fewer neurons at later passages. Increased growth rate and prolonged growth with LIF. Expansion not EGF dependent in the presence of LIF. No difference in growth rate between gestational ages.	12

(continued)

Table 1 (continued)

Species	CNS region	Donor age[a]	Culture setting and number of cells	Passaging technique and time interval	Mitogens	Growth conditions	Culture period	Observations and differentiation potential	Ref.
Human	Forebrain	6.5 wk 8.5 wk 9.0 wk	NS 100,000/mL	Mechanical trituration 7- to 10-d intervals	EGF (20 ng/mL) FGF2 (20 ng/mL) LIF (10 ng/mL) Heparin (2 μg/mL)	N2 medium	P36 (max 360 d)	Survival for more than 1 yr after transplantation, integration of neurons.	156
Human	Forebrain	7 wk	NS 5 × 10^6/mL	Trypsin/EDTA 14 d	EGF (20 ng/mL) FGF2 (20 ng/mL) LIF (10 ng/mL)	NPBM medium 50% conditioned medium (reuse)	24 wk	Multipotent after transplantation to ischemic gerbils. 8% graft survival >28 d.	157
Human	Forebrain (Di + Tel) SC	4.5–12 wk 4.5–9.5 wk	NS 100–150,000/mL	Trituration 7–25 d	EGF (20 ng/mL) FGF2 (20 ng/mL) CNTF (10 ng/mL) Heparin (2 μg/mL)	1% N2	P10 Forebrain: 151–228 d SC: 154–245 d	Cellular composition not affected by gestational age, but by region. Ir in proliferating NS: Nestin, β-tubulin double-labeling, GFAP.	40
Human	Forebrain (Di-Tel) + 11 fetuses	10.5 wk	Adherent PLL 10^3/cm^2 8 wk, then 10^4/cm^2	Trituration 10–14 d	EGF (20 ng/mL) FGF2 (10 ng/mL)	TIPPS	P54 (>2 yr)	Multipotent. HNS.1 cell line later established by Villa et al. (2000).	78
Human	Forebrain Midbrain	6 wk 8 wk 9 wk	NS 400,000/mL	Trituration 18–27 d	EGF (20 ng/mL) FGF2 (20 ng/mL)	TIPPS 3% oxygen	11 mo	Proliferation rates markedly decreased after 8–9 mo. No growth in EGF w/o FGF2. Induction of low number of TH-positive cells in mesNS (but not forebrain) with cytokine cocktail.	104

Human	Tel Di Mes Rb	9 wk	NS	Mechanical dissociation, cutting with microscissors at 1.0 mm in diameter	EGF (10 ng/mL) FGF2 (20 ng/mL) LIF (10 ng/mL)	N2	150 d	Decreasing numbrt of TH^+ cells, increasing number of $GFAP^+$cells with culture. Morphologic difference of TH-positive neurons from Tel/Rb and Di/Mes.	158
Human	Tel Di Mes Cb Pons/med SC	13 wk	NS 200,000/ mL	Trypsin/ EDTA 7–8 d	LIF (10 ng/mL) FGF2 (20 ng/mL) Heparin (8 μg/ mL)	1% N2	1 yr 11 mo 9 mo 7 mo 4 mo 6 mo	Forebrain showed faster growth rates than midbrain and hindbrain. Reduction in neuronal emergence. Cultures maintain regional specification. Ir in proliferating NS: Nestin, GFAP, beta-tubulin.	107

Cb, cerebellum; CNTF, ciliary neurotrophic factor; DG, dentate gyrus; Di, diencephalon; ED, embryonic day; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; Fbn, fibronectin; FGF2, fibroblast growth factor 2; h, human; Ir, immunoreactivity; LIF, leukemia inhibitory factor; Med, medulla; Mes, mesencephalon; NS, neurospheres; OB, olfactory bulb; P, passage; PD, population doubling; PLL, poly-L-lysine; PO, polyornithine; Rb, rhombencephalon; RE, rostral extension; SC, spinal cord; SVZ, subventricular zone; Tel, telencephalon; TIPPS, transferrin, insulin, putrescine, progesterone, selenite; VM, ventral mesencephalon.

[a]Date postconceptum.

effect on proliferation of fetal rodent striatum and mesencephalon [68, 81], while others have found that this is not the case for cultures derived from rat or mouse embryos at later developmental stages (E16–20) and have even suggested that the factor combination is less effective than EGF alone [1].

While the presence of both EGF and FGF appears excessive in rodents [3, 18], human cells require both EGF and FGF2 [6, 7, 12, 47, 78]. Removal of EGF from human cultures results in massive cell death within 24 hours [64], as well as rapid decrease in human NTS expansion (R. K. Andersen, unpublished data). In addition, others have shown that the combination of the two factors provided the best conditions for generating NSs of rodent origin [47, 53] and that EGF is required for expansion of homogeneous mouse NS cell cultures with efficient suppression of differentiation and apoptosis [65].

10 Fibroblast Growth Factor 2

FGF2 appears to be unable to induce NS formation at clonal cell density without the presence of serum [82,83]. This might reflect a fast degradation of FGF2 in the absence of its cofactor heparin [51] or other glycosaminoglycans. Many studies have established cell cultures in vitro using EGF and FGF2 without addition of heparin. Heparin is apparently not necessary for initial propagation of cells; for example, FGF2 is critical for the division of E14 rat neural precursor cells, while heparin is only required for maximal effect [51]. However, most neural cell cultures appear to have a critical point at which the addition of heparin is needed for continued propagation. FGF2-responsive neural precursors stop dividing after 3 weeks of expansion unless heparin is added to the culture medium [84]. This timing appears to correspond to the in vitro elimination of cells with more-differentiated characteristics at the time of isolation [85].

The method of propagation also seems to play a major role for FGF2 activity, since cells attached to a substrate or cells grown at high cell density are less dependent on exogenous heparin due to greater cell–substrate and cell–cell contact or a continued endogenous production of heparin-like molecules under these conditions [51]. In line with this, our studies have shown that human ventral mesencephalic precursor cells propagated as NTSs maintained their proliferative capacity for as long as 525 days in the presence of EGF and FGF2 without addition of heparin (R. K. Andersen, unpublished data).

11 Leukemia Inhibitory Factor

Leukemia inhibitory factor (LIF) belongs to the interleukin-6 family of cytokines, which also includes CNTF [86–89]. Group members show differences in biologic effect but signal through the same transmembrane protein subunit, glycoprotein130 (gp130). Mice lacking gp130 or LIF receptor (LIFR) die during development or

shortly after birth with severe deficits in motor neuron and glial cell populations [90, 91]. In early experiments, LIF was found to be necessary and sufficient to sustain the ability of embryonic stem (ES) cells to self-renew [92]. Gp130 signaling via LIF enhances ES cell viability by suppressing differentiation by phosphorylation and activation of STAT3 [87, 91, 93], and STAT3 alone is necessary to support self-renewal of mouse ES cells [89]. In contrast to mouse ES cells, LIF does not seem quite as essential for human and rat ES cells [94]. This discrepancy may simply be due to different temporal cell-surface expression of gp130 and LIFR [95], as LIF (and CNTF) can support proliferation of human embryonic NSCs [96, 97], indicating increased responsiveness later during development. LIF appears to have a concentration-dependent effect on cells, driving cells to differentiate at higher concentrations, while even in very low doses it has an inhibitory effect on proliferation [88]. Mimicking diapause, the effect of LIFR signaling in the adult SVZ may thus primarily act on the maintenance of the self-renewing potency of NSCs [98] by acting as an inhibitor of spontaneous differentiation [92, 99].

LIFR/gp130-mediated signaling is necessary for the maintenance of NPCs in vivo [98], and it is possible that LIF act primarily as a permissive factor to maintain cell survival under minimal culture conditions [100]. The effect of LIF may also be region dependent since LIF seems necessary for continuous propagation of cells derived from fetal ventral mesencephalon ([101]; R. K. Andersen,unpublished data), while exerting other functions in different regions (e.g., SVZ), where this role is likely fulfilled by other growth factors. Similar findings have been provided in a study by Dictus et al. [3] in which LIF exerted an effect on hippocampal cells but not cells derived from the olfactory bulb.

12 Neurogenesis in Vitro: Regional and Developmental Differences

Despite the lifelong presence of NSCs in specific regions of the brain, aging is accompanied by a progressive reduction in proliferation and neurogenesis [1, 2]. This can be exemplified by the fact that mouse cortical precursor cells isolated at early embryonic stages (E10–11) give rise to more neurons than cells isolated at perinatal stages, which tend to differentiate into astrocytes under the same culture conditions [48]. This obvious difference is also reflected in rodent cell cultures maintained for longer periods in vitro [16, 69], and, in particular, ventral mesencephalic precursors seem to lose their neurogenic potential during culturing [102, 103], although a low number of dopaminergic neurons can still be induced in human cultures after 3–8 months of propagation [104]. On the other hand, neurogenic potential can be maintained by subpopulations of cells from rat forebrain SVZ and ventral mesencephalon even after long-term propagation when maintained in a 3D environment [66, 101]. There is an ongoing debate about whether regional characteristics in NS cultures derived from distinct regions of the fetal brain are at least partially maintained [69, 105–110] or not [110–115]. Regionality particularly seems to be lost after long-term propagation [114, 115], but

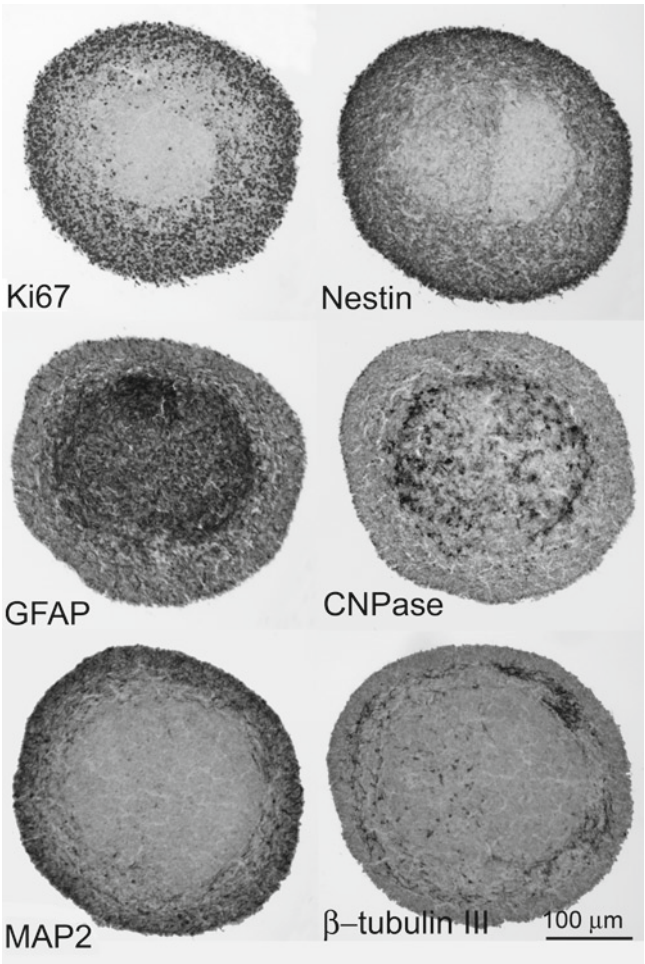

Fig. 2 Immunohistochemical staining of serial sections of a neural tissue-sphere. Immunohistochemical staining of serial sections of a neural tissue-sphere (NTS) derived from newborn rat lateral ventricle wall. NTSs propagated for several generations in culture often attain a stratified organization, as also seen for neurospheres (NSs). Toward the surface there is an outer layer of actively proliferating cells positive for the proliferation marker Ki67 and the marker for stemlike cells, nestin. Below that, the core of the sphere has a more differentiated appearance, with cells positive for the glial markers GFAP (astrocytes) and CNPase (oligodendrocytes). Cells expressing markers for newly developed (β-tubulin III) and more mature (MAP2) neurons are preferentially located in an intermediate layer toward and in the superficial layer

gene expression profiles can also be changed in cells cultured for just three passages (8–9 days, loss of dorsal markers) [111].

During culture, both NSs and NTSs develop characteristic layers or compartments with an outer proliferative zone and an inner, more differentiated core (Fig. 2). The outer, actively dividing compartments usually show immunoreactivity for

nestin, an intermediate filament often associated with stem cell potential [116], indicating a sustained potential to generate neurons. This was the case for SVZ-derived but not ventral mesencephalic NTSs [101], which is consistent with the suggestion that neurogenesis in the adult ventral mesencephalon is an extremely rare event [37].

13 Neurogenic Priming During Propagation: Influence of Leukemia Inhibitory Factor

Neuronal outcomes also depend on growth factor applications, as cultures grown in FGF2 at all times generate a higher percentage of neurons than EGF cultures regardless of the developmental age of the tissue [6, 47, 51, 81, 83, 117–120]. Of note, however, heparin can increase the number of neurons arising from EGF-responsive NSs [51]. The LIF family of cytokines acts to promote differentiation and/or survival of many different cell types, including astrocytes [46, 121–123], oligodendrocytes [121, 124, 125], and specific types of neurons [121–123, 126]. The effect of LIF during cellular differentiation also seems to depend on the developmental stage of the organism [127], exemplified by the observation that LIF stimulates dorsal root ganglion neuron differentiation during embryonic development but may act more as a survival factor for these neurons during postnatal development [128].

Whether LIF directly instructs neuronal differentiation or increase neuronal numbers indirectly by eliciting survival signals is yet to be clarified, since studies support both hypotheses. Proteins of the Bcl-2 family play a key role in controlling the activation of caspases, which are the proteases that dismantle the cell during apoptosis. Bcl-2–related proteins fall into two groups that generally either repress apoptosis (Bcl-2 and Bcl-x_L) or promote apoptosis (Bax, Bcl-x_s, Bak, and Bad) [129]. By inducing phosphorylation of Bad, LIF reduces the heterodimerization of Bad with Bcl-xL [130] or Bcl-2 [131], leading to increased survival of cells. In line with this, human fetal NSCs derived from either diencephalon or cortex generate both neurons and glia to a greater extent when LIF is added to the differentiation medium [126].

It appears that LIF has two different actions, depending on the developmental stage of embryonic precursor cells—more specifically, survival or maintenance of cells at early brain development and astrocytic differentiation at late development [132, 133]. This dual action is, in turn, possibly dependent on the duration and extent of activation of the p42/p44 MAPK [133, 134], and the EGF-receptor has furthermore been observed to regulate the competence of progenitors to interpret LIF as an astrocyte-inducing signal [46, 120, 135]. It has, on the other hand, been suggested that MAPK signaling promotes neurogenesis, whereas activation of Jak-STAT signaling promotes astroglial generation [136], but that when both pathways are activated, NSCs remain in an undifferentiated, proliferative state [88].

LIF is a potent inducer of astrocyte differentiation via STAT3 activation, but early embryonic neuroepithelial stem cells do not differentiate in response to LIF [67]. This results from CpG methylation of a STAT3-binding element in the GFAP promoter [137], which prevents STAT3 binding. At later developmental ages, the STAT3 element is demethylated, and the cells can differentiate into astrocytes in response to LIF [137]. The same mechanism could account for the growth-promoting effect of LIF in ES cells and cells from early developmental stages, while at later developmental ages LIF induces astrocytic and neurogenic differentiation and concomitant growth inhibition.

Several studies have reported that LIF and CNTF affect survival of various classes of neurons from different regions [128, 138, 139] and that LIF in combination with EGF and FGF2 produces more neurons (upon differentiation) than parallel cultures without LIF [12, 110, 126, 140]. In addition, for mouse ES cells, where LIF normally is included for proliferation, addition of LIF has been shown to increase the number of neuron-containing embryoid bodies by acting via the STAT3 signaling pathway [141]. Whether the effect of LIF is due to survival or a direct effect on proliferation has not been determined. A previous study reported more $GFAP^+$, $MAP2^+$, and $O4^+$ cells in LIF cultures [110] but not at later passages, indicating a time-limited effect of LIF or an impact on survival of primary cells. If LIF mainly affects neuronal outcome by differentiating cells, this would result in an initial increase in neurons but a later absence due to elimination of mature cells during long-term culture. In agreement with this, our results show that LIF transiently increases the number of neurons in both rat SVZ [101] and human ventral mesencephalic cultures (R. K. Andersen, unpublished data). In vivo studies have shown that endogenous LIF expression after injury is rapid and transient [86], indicating that chronic exposure to LIF is not physiologic and might even have a negative effect. Indeed, LIF differs in its effect on cells depending on the developmental state of the cells [127]. Both a soluble and an extracellular matrix-associated form of LIF exist [142], and in the mouse there are alternative copies of the first exon of the LIF gene transcribed from separate upstream promoter regions, raising the possibility that differential spatial and temporal regulation of the expression of the two forms of LIF may occur [143]. Low concentrations of LIF together with other additives has been shown to induce dopaminergic differentiation in EGF- and FGF2-, long-term expanded, rat or human fetal mesencephalic cultures, while this cocktail did not have an effect in corresponding forebrain cultures, further suggesting lineage restriction along the rostral-caudal axis of human brain early during ontogenesis [104, 144, 145].

14 Conclusion

Experimental isolation, propagation, and differentiation of NSCs provides a number of opportunities to study the regulation of neurogenesis and gliogenesis [26]. In pursuing these opportunities the employment of 3D culture systems is increasing.

One example is the use of semisolid restrictive substances to reveal the true potential of isolated cells [59]. Others, as dealt with in this chapter, are using organotypic microexplants of known neurogenic parts of the brain. Models allowing studies of a structured, organotypic cellular microenvironment are important for unraveling cellular interactions as part of tissue homeostasis, including the coexistence of NSCs and nonstem cells in their niche. One aspect, among the others mentioned, is the understanding of the interplay between the microenvironment and tumor stem cells derived from resident neural precursor cells sustaining oncogenic mutations [146–148].

A better understanding of microenvironmental cues regulating cell fate is also important for development of safe and successful cell replacement therapies when the grafted cells typically will be exposed to a host cellular environment expressing variable degrees of pathology [8, 149–152]. Increased knowledge about the microenvironment and stem cell niches is, moreover, mandatory for the development of therapies targeting the patient's endogenous NSCs and stem cell niches for the purpose of improving reparative responsiveness to ongoing neurodegenerative diseases.

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Part VI
Regenerative Medicine

Stem Cells and Regenerative Medicine in Urology

Anthony Atala

Abstract Organ transplantation remains a mainstay of treatment for patients with severely compromised organ function. Despite initiatives to increase the availability of transplant organs, however, the number of patients in need of treatment still far exceeds the organ supply, and this is expected to worsen as the global population ages. In the last two decades, as a response to the needs of these patients, scientists have attempted to grow native and stem cells, engineer tissues, and design treatment modalities using regenerative medicine techniques for virtually every tissue of the human body. This chapter reviews some of these techniques and describes the progress that has been achieved in the field of urologic regenerative medicine.

Keywords Organ transplantation • Biomaterials • Progenitor cells • Bladder regeneration • Urethra

1 Introduction

The use of one body part for another or the exchange of parts from one person to another has been mentioned in the literature for centuries, and these concepts have captured the imagination of many physicians and scientists through the years. In the early 1900s, investigations into organ transplantation began in earnest, and the maintenance of organs in culture became a major area of inquiry. Charles Lindbergh, the first pilot to successfully fly across the Atlantic, worked with Alexis Carrel, a Nobel Prize winner in the field of medicine, to investigate the potential of keeping organs alive ex vivo long term, and this research led to a number of new discoveries [1].

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The field of urology was the earliest to benefit from these new transplantation techniques. The kidney was the first entire organ to be replaced in a human, in 1954, in a procedure performed by Joseph Murray, who later received the Nobel Prize for his work [2]. However, this initial effort was performed using identical twins, and thus the immunologic barrier to allotransplantation was not yet addressed. In 1959, Murray performed a nonrelated kidney transplantation from a non–genetically identical individual into a patient. This transplant marked a new era in medicine, and transplantation became a viable therapy for failures in various organ systems. However, immunosuppressant protocols are inadequate, and physicians still lack the ability to monitor and control rejection. In addition, a severe organ donor shortage exists, and these drawbacks have opened the door for other alternatives.

Synthetic materials have been introduced to replace or rebuild diseased tissues in the human body. The advent of new man-made materials, such as tetrafluoroethylene (Teflon) and silicone, created a wide array of devices that could be applied for human use. However, although these devices can provide for structural replacement, the functional component of the original tissue cannot be achieved using these materials. As a result, researchers turned to the biologic sciences (cell biology, molecular biology, and biochemistry) to develop new techniques for cell harvesting, culture, and expansion. Studies of the extracellular matrix and its interaction with cells and with growth factors and their ligands led to increased understanding of cell and tissue growth and differentiation. The fields of tissue engineering and regenerative medicine were born.

This chapter reviews some of these tissue engineering and regenerative medicine techniques and describes the progress that has been achieved in the field of urologic regenerative medicine.

2 Components of Regenerative Medicine and Techniques for Urologic Applications

Regenerative medicine follows the principles of cell transplantation, materials science, and engineering toward the development of biologic strategies that can restore and maintain normal function. Regenerative medicine strategies typically fall into one of three categories: cell-based therapies, the use of biomaterials (scaffolds) alone, in which the body's natural ability to regenerate is used to orient or direct new tissue growth, and the use of scaffolds seeded with cells to create tissue substitutes.

2.1 Biomaterials

Synthetic materials have been used widely for urologic reconstruction. The most common type of synthetic prosthesis for urologic applications is made of silicone. Silicone prostheses have been used for the treatment of urinary incontinence with

the artificial urinary sphincter and detachable balloon system, for treatment of vesicoureteral reflux with silicone microparticles, and for impotence with penile prostheses [3–6]. There has also been a major effort directed toward the construction of artificial bladders made with silicone. In some disease states, such as urinary incontinence or vesicoureteral reflux, artificial agents (Teflon paste, glass microparticles) have been used as injectable bulking substances; however, these substances are not entirely biocompatible [7].

For regenerative medicine purposes, there are clear advantages in using degradable, biocompatible materials that can function as cell delivery vehicles and/or provide the structural parameters needed for tissue replacement. Biomaterials in genitourinary regenerative medicine function as an artificial extracellular matrix (ECM) and elicit biologic and mechanical functions of native ECM found in tissues in the body. Native ECM brings cells together into tissue, controls the tissue structure, and regulates the cell phenotype [8]. Biomaterials facilitate the localization and delivery of cells and/or bioactive factors (e.g., cell adhesion peptides, growth factors) to desired sites in the body, define a three-dimensional space for the formation of new tissues with appropriate structure, and guide the development of new tissues with appropriate function [9]. Direct injection of cell suspensions without biomaterial matrices has been used in some cases [10,11], but it is difficult to control the localization of transplanted cells. In addition, most mammalian cell types are anchorage dependent and die if not provided with a cell-adhesion substrate.

The design and selection of the biomaterial are critical in the development of engineered genitourinary tissues. Generally, the ideal biomaterial should be biocompatible, promote cellular interaction and tissue development, and possess proper mechanical and physical properties. In addition, the selected biomaterial should be biodegradable and bioresorbable, so that as new tissue develops, the material will degrade without hindering the regenerative process. Generally, three classes of biomaterials have been used for engineering of genitourinary tissues: naturally derived materials, such as collagen and alginate; acellular tissue matrices, such as bladder submucosa and small-intestinal submucosa; and synthetic polymers, such as polyglycolic acid (PGA), polylactic acid (PLA), and poly(lactic-co-glycolic acid) (PLGA). These classes of biomaterials have been tested with regard to their biocompatibility with primary human urothelial and bladder muscle cells [12]. Naturally derived materials and acellular tissue matrices have the potential advantage of biologic recognition. Synthetic polymers can be produced reproducibly on a large scale with controlled properties of strength, degradation rate, and microstructure.

Collagen is the most abundant and ubiquitous structural protein in the body, and it may be readily purified from both animal and human tissues with an enzyme treatment and salt/acid extraction [13]. Collagen has long been known to exhibit minimal inflammatory and antigenic responses [14], and it has been approved by the U.S. Food and Drug Administration (FDA) for many types of medical applications, including wound dressings and artificial skin [15]. Intermolecular cross-linking reduces the degradation rate by making the collagen molecules less susceptible to an enzymatic attack. Intermolecular cross-linking

can be accomplished by various physical (e.g., ultraviolet radiation, dehydrothermal treatment) or chemical (e.g., glutaraldehyde, formaldehyde, carbodiimides) techniques [13]. Collagen contains cell-adhesion domain sequences (e.g., RGD) that exhibit specific cellular interactions. This may help to retain the phenotype and activity of many types of cells, including fibroblasts [16] and chondrocytes [17]. This material can be processed into a wide variety of structures, such as sponges, fibers, and films [18–20].

Recently, natural materials such as alginate and collagen have been used as "bioinks" in a newly developed bioprinting technique based on inkjet technology [21,22]. Using this technology, these scaffold materials can be "printed" into a desired scaffold shape using a modified inkjet printer. In addition, several groups have shown that living cells can also be printed using this technology [23,24]. This exciting technique can be modified so that a three-dimensional construct containing a precise arrangement of cells, growth factors, and extracellular matrix material can be printed [25–27]. Such constructs may eventually be implanted into a host to serve as the backbone for a new tissue or organ.

Acellular tissue matrices are collagen-rich matrices prepared by removing cellular components from tissues. The matrices are often prepared by mechanical and chemical manipulation of a segment of bladder tissue [28–31]. The matrices slowly degrade after implantation and are replaced and remodeled by ECM proteins synthesized and secreted by transplanted or ingrowing cells. Acellular tissue matrices have been proven to support cell ingrowth and regeneration of genitourinary tissues, including urethra and bladder, with no evidence of immunogenic rejection [31, 32]. Because the structures of the proteins (e.g., collagen, elastin) in acellular matrices are well conserved and normally arranged, the mechanical properties of the acellular matrices are not significantly different from those of native bladder submucosa [28].

Polyesters of naturally occurring α-hydroxy acids, including PGA, PLA, and PLGA, are widely used in regenerative medicine. These polymers have gained FDA approval for human use in a variety of applications, including sutures [33]. The degradation products of PGA, PLA, and PLGA are nontoxic, natural metabolites that are eventually eliminated from the body in the form of carbon dioxide and water [33]. Because these polymers are thermoplastics, they can easily be formed into a three-dimensional scaffold with a desired microstructure, gross shape, and dimension by various techniques, including molding, extrusion [34], solvent casting [35], phase separation techniques, and gas foaming techniques [36]. More recently, techniques such as electrospinning have been used to quickly create highly porous scaffolds in various conformations [37–40].

2.2 Native Targeted Progenitor Cells

In the past, one of the limitations of applying cell-based regenerative medicine techniques to organ replacement was the inherent difficulty of growing certain human cell types in large quantities. Native targeted progenitor cells, or native cells,

are tissue-specific, unipotent cells derived from most organs. The advantage of these cells is that they are already programmed to become the cell type needed, without any extralineage differentiation. By noting the location of the progenitor cells, as well as by exploring the conditions that promote differentiation and/or self-renewal, it has been possible to overcome some of the obstacles that limit cell expansion in vitro. One example is the urothelial cell. Urothelial cells could be grown in the laboratory setting in the past, but only with limited success. It was believed that urothelial cells had a natural senescence that was hard to overcome. Several protocols have been developed over the last two decades that have improved urothelial growth and expansion [41–44]. A system of urothelial cell harvesting was developed that does not use any enzymes or serum and has a large expansion potential. Using these methods of cell culture, it is possible to expand a urothelial strain from a single specimen that initially covers a surface area of 1 cm^2 to one covering a surface area of 4202 m^2 (the equivalent area of one football field) within 8 weeks [41].

An additional advantage in using native cells is that they can be obtained from the specific organ to be regenerated, expanded, and used in the same patient without rejection, in an autologous manner. [30, 41, 45–60].

Bladder, ureter, and renal pelvis cells can be harvested, cultured, and expanded in a similar fashion. Normal human bladder epithelial and muscle cells can be efficiently harvested from surgical material, extensively expanded in culture, and their differentiation characteristics, growth requirements, and other biologic properties studied [41, 43, 44, 54, 55, 61–68]. Major advances in cell culture techniques have been made within the last decade, and these techniques use a number of autologous cell types possible for clinical application.

2.3 Stem Cells and Other Pluripotent Cell Types

Pluripotent human stem cells are envisioned to be an ideal source of cells for many regenerative medicine applications, as they can differentiate into nearly any replacement tissue in the body. Many techniques for generating stem cells have been studied over the last few decades. Some of these techniques have yielded promising results, but others require further research. The main techniques are discussed in detail here, and their advantages and limitations are summarized in Table 1.

Embryonic stem cells exhibit two remarkable properties: the ability to proliferate in an undifferentiated but still pluripotent state (self-renewal), and the ability to differentiate into a large number of specialized cell types [69]. They can be isolated from the inner cell mass of the embryo during the blastocyst stage, which occurs 5 days postfertilization. These cells have been maintained in the undifferentiated state for at least 80 passages when grown using current published protocols [70]. In addition, many protocols for differentiation into specific cell types in culture have been published. However, many uses of these cells are currently banned in the United States due to the ethical dilemmas that are associated with the manipulation of embryos in culture.

Table 1 Summary of Alternate Methods for Generating Pluripotent Stem Cells

Method	Advantages	Limitations
Somatic cell nuclear transfer	Customized stem cells Has been shown to work in nonhuman primates	Requires oocytes Has not been shown to work in humans
Single-cell embryo biopsy	Patient-specific to embryo Does not destroy or create embryos Has been done in humans	Allogeneic cell types Is not known if single cells are totipotent Requires coculturing with a previously established human embryonic stem cell line
Arrested embryos	Cells obtained from discarded embryos Has been done in humans	Allogeneic cell types Quality of cell lines might be questionable
Altered nuclear transfer	Customized stem cells	Ethical issues surround embryos with no potential Modified genome Has not been done with human cells
Reprogramming	Customized stem cells No embryos or oocytes needed Has been done with human cells	Retroviral transduction Oncogenes

Nuclear transfer, or cloning, can serve as another source of pluripotent "stem" cells that could possibly be used for regenerative medicine therapies. Transfer of a somatic cell nucleus into an enucleated oocyte can produce an embryo that is genetically identical to the donor nucleus. This process is used to generate blastocysts that are explanted and grown in culture, rather than in utero, to produce embryonic stem cell lines (Fig. 1). These autologous stem cells have the potential to become almost any type of cell in the adult body and thus would be useful in tissue and organ replacement applications [71]. Therefore, nuclear transfer may provide an alternative source of transplantable cells that are identical to the patient's cells.

Recently, exciting reports of the successful transformation of adult cells into pluripotent stem cells through a type of genetic "reprogramming" have been published. Reprogramming is a technique that involves dedifferentiation of adult somatic cells to produce patient-specific pluripotent stem cells without the use of embryos. Cells generated by reprogramming would be genetically identical to the somatic cells (and thus the patient who donated these cells) and would not be rejected. Yamanaka was the first to discover that mouse embryonic fibroblasts and adult mouse fibroblasts could be reprogrammed into an "induced pluripotent state (iPS)" [72]. The resultant iPS cells possessed the immortal growth characteristics of self-renewing embryonic stem (ES) cells, expressed genes specific for ES cells, and generated embryoid bodies in vitro and teratomas in vivo. Reprogramming is still poorly understood, but the implications of this technique for regenerative medicine are exciting.

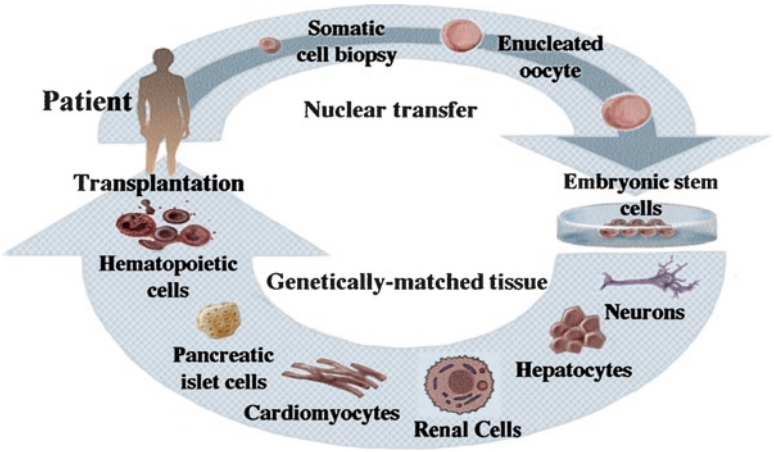

Fig. 1 Therapeutic cloning strategy and its application to the engineering of tissues and organs

Adult stem cells also have the advantage of avoiding some of the ethical issues associated with embryonic cells, and, unlike embryonic cells, they do not transdifferentiate into a malignant phenotype, so there is a diminished risk of teratoma formation should the cells be implanted in vivo. Adult stem cells, especially hematopoietic stem cells, are the best understood cell type in stem cell biology [73]. However, adult stem cell research remains an area of intense study, as the potential for therapy may be applicable to a myriad of degenerative disorders. Within the last decade, adult stem cell populations have been found in many adult tissues other than the bone marrow and the gastrointestinal tract, including brain [74,75], skin [76], and muscle [77]. Many other types of adult stem cells have been identified in organs throughout the body and are thought to serve as the primary repair entities for their corresponding organs [78]. The discovery of such tissue-specific progenitors has opened up new avenues for research. However, adult stem cells are limited for clinical use because expansion to the large quantities needed for tissue engineering is difficult.

Fetal stem cells derived from amniotic fluid and placentas have recently been described and represent a novel source of stem cells. The cells are obtained either from amniocentesis or chorionic villous sampling in the developing fetus or from the placenta at the time of birth. The amniotic fluid and placental membrane contain a heterogeneous population of cell types derived from the developing fetus [79, 80]. Cells found in this heterogeneous population include mesenchymal stem cells [81, 82]. In addition, the isolation of multipotent human and mouse amniotic fluid and placental–derived stem (AFPS) cells that are capable of extensive self-renewal and give rise to cells from all three germ layers was reported in 2007 [83]. AFPS cells represent approximately 1% of the cells found in the amniotic fluid and placenta and can be induced to differentiate into cells representing each embryonic germ layer, including cells of adipogenic, osteogenic, myogenic, endothelial, neural-like, and hepatic lineages. In addition to the differentiated AFPS cells

expressing lineage-specific markers, such cells can have specialized functions. Cells of the hepatic lineage secreted urea and α-fetoprotein, while osteogenic cells produced mineralized calcium. In this respect, they meet a commonly accepted criterion for multipotent stem cells, without implying that they can generate every adult tissue. Since the discovery of the AFPS cells, other groups have published work on the potential of the cells to differentiate to other lineages, such as cartilage [84], kidney [85], and lung [86]. Muscle differentiated AFPS cells were also noted to prevent compensatory bladder hypertrophy in a cryoinjured rodent bladder model [87].

3 Regenerative Medicine for Major Urologic Structures

3.1 Urethra

Various strategies have been proposed over the years for the regeneration of urethral tissue. Woven meshes of PGA without cells [88, 89] or with cells [45] were used to regenerate urethras in various animal models. Naturally derived collagen-based materials, such as bladder-derived acellular submucosa [31] and an acellular urethral submucosa [90], have also been tried experimentally in various animal models for urethral reconstruction.

The bladder submucosa matrix [31] proved to be a suitable graft for repair of urethral defects in rabbits. The neourethras demonstrated a normal urothelial luminal lining and organized muscle bundles. These results were confirmed clinically in a series of patients with a history of failed hypospadias reconstruction, in which the urethral defects were repaired with human bladder acellular collagen matrices [50]. The neourethras were created by anastomosing the matrix in an onlay fashion to the urethral plate. The size of the created neourethra ranged from 5 to 15 cm. After a 3-year follow-up, three of the four patients had a successful outcome with regard to cosmetic appearance and function (Fig. 2). One patient who had a 15-cm neourethra created developed a subglanular fistula. The acellular collagen–based matrix eliminated the necessity of performing additional surgical procedures for graft harvesting, and both operative time and the potential morbidity from the harvest procedure were decreased. Similar results were obtained in pediatric and adult patients with primary urethral stricture disease using the same collagen matrices [91]. Another study in 30 patients with recurrent stricture disease showed that a healthy urethral bed (two or fewer prior urethral surgeries) was needed for successful urethral reconstruction using the acellular collage–based grafts [92]. More than 200 pediatric and adult patients with urethral disease have been successfully treated in an onlay manner with a bladder-derived, collagen-based matrix. One of its advantages over nongenital tissue grafts used for urethroplasty is that the material is “off the shelf.” This eliminates the necessity of additional surgical procedures for graft harvesting,

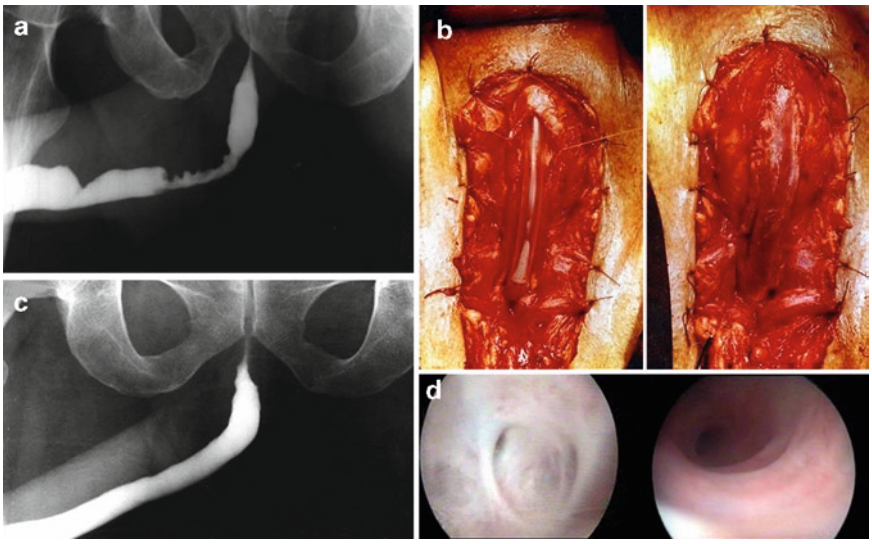

Fig. 2 Urethrogram 6 months after surgery in a patient who had a portion of his urethra replaced with the use of regenerative medicine techniques

which may decrease operative time, as well as the potential morbidity due to the harvest procedure.

The foregoing techniques, using nonseeded acellular matrices, were applied experimentally and clinically in a successful manner for onlay urethral repairs. However, when tubularized urethral repairs were attempted experimentally, adequate urethral tissue regeneration was not achieved and complications ensued, such as graft contracture and stricture formation [93]. Autologous rabbit bladder epithelial and smooth muscle cells were grown and seeded onto preconfigured tubular matrices. Entire urethra segments were resected, and urethroplasties were performed with tubularized collagen matrices either seeded with cells or without cells. The tubularized collagen matrices seeded with autologous cells formed new tissue that was histologically similar to native urethra. The tubularized collagen matrices without cells led to poor tissue development, fibrosis, and stricture formation. These findings were confirmed clinically. A clinical trial using tubularized, nonseeded small intestinal submucosa (SIS; see later discussion) for urethral stricture repair was performed in eight evaluable patients. Two patients with short inflammatory strictures maintained urethral patency. Stricture recurrence developed in the other six patients within 3 months of surgery [94]. Other cell types have also been tried experimentally in acellular bladder collagen matrices, including foreskin epidermal cells and oral keratinocytes [95, 96]. Vascular endothelial growth factor gene–modified urothelial cells have also been used experimentally for urethral reconstruction [97].

3.2 Bladder

Currently, gastrointestinal segments are commonly used as tissues for bladder replacement or repair. However, gastrointestinal tissues are designed to absorb specific solutes, whereas bladder tissue is designed for the excretion of solutes. When gastrointestinal tissue is in contact with the urinary tract, multiple complications may ensue, such as infection, metabolic disturbances, urolithiasis, perforation, increased mucus production, and malignancy [98–101]. Because of the problems encountered with the use of gastrointestinal segments, numerous investigators have attempted alternative reconstructive procedures for bladder replacement or repair. These include autoaugmentation [102,103] and ureterocystoplasty [104–106]. In addition, alternate methods for bladder reconstruction have been explored, including regenerative medicine approaches.

3.2.1 Matrices for Bladder Regeneration

Over the last few decades, several bladder wall substitutes have been created with both synthetic and organic materials. Synthetic materials that have been tested in experimental and clinical settings include polyvinyl sponge, Teflon, Vicryl (PGA) matrices, and silicone. Most of these attempts have failed because of mechanical, structural, functional, or biocompatibility problems. Usually, permanent synthetic materials used for bladder reconstruction succumb to mechanical failure and urinary stone formation, and use of degradable materials leads to fibroblast deposition, scarring, graft contracture, and a reduced reservoir volume over time [52,107].

Recently, there has been increased interest in the use of various collagen-based matrices for bladder regeneration. Nonseeded allogeneic acellular bladder matrices have served as scaffolds for the ingrowth of host bladder wall components. The matrices are prepared by mechanically and chemically removing all cellular components from bladder tissue [29,30,32,108,109]. The matrices serve as vehicles for partial bladder regeneration, and relevant antigenicity is not evident.

Cell-seeded allogeneic acellular bladder matrices were used for bladder augmentation in dogs [30]. The regenerated bladder tissues contained a normal cellular organization consisting of urothelium and smooth muscle and exhibited a normal compliance. Biomaterials preloaded with cells before their implantation showed better tissue regeneration than biomaterials implanted without cells, in which case tissue regeneration depended solely on ingrowth of the surrounding tissue. The bladders showed a significant increase (100%) in capacity when augmented with scaffolds seeded with cells compared to scaffolds without cells (30%). The acellular collagen matrices can be enhanced with growth factors to improve bladder regeneration [110].

SIS, a biodegradable, acellular, xenogeneic collagen-based tissue-matrix graft, was first described by Badylak and colleagues in the 1980s as an acellular matrix for tissue replacement in the vascular field [111]. It has been shown to promote regeneration of a variety of host tissues, including blood vessels and ligaments [112]. The matrix is derived from pig small intestine in which the mucosa is mechanically

removed from the inner surface and the serosa and muscular layer are removed from the outer surface. Animal studies have shown that the nonseeded SIS matrix used for bladder augmentation is able to regenerate in vivo [113,114]. Histologically, the transitional layer was the same as that of the native bladder tissue, but, as with other nonseeded collagen matrices used experimentally, the muscle layer was not fully developed. A large amount of collagen was interspersed among a smaller number of muscle bundles. A computer-assisted image analysis demonstrated a decreased muscle-to-collagen ratio with loss of the normal architecture in the SIS-regenerated bladders. In vitro contractility studies performed on the SIS-regenerated dog bladders showed a decrease in maximal contractile response by 50% from those of normal bladder tissues. Expression of muscarinic, purinergic, and alpha-adrenergic receptors and functional cholinergic and purinergic innervation were demonstrated [114]. Cholinergic and purinergic innervation also occurred in rats [115].

Bladder augmentation using laparoscopic techniques was performed on minipigs with porcine bowel acellular tissue matrix, human placental membranes, or porcine SIS. At 12 weeks postoperatively the grafts had contracted to 70%, 65%, and 60% of their original sizes, respectively, and histologically the grafts showed predominantly only mucosal regeneration [116]. The same group evaluated the long-term results of laparoscopic hemicystectomy and bladder replacement with SIS with ureteral reimplantation into the SIS material in minipigs. Histopathology studies after 1 year showed muscle at the graft periphery and center, but it consisted of small, fused bundles with significant fibrosis. Nerves were present at the graft periphery and center but they were decreased in number. Compared to primary bladder closure after hemicystectomy, no advantage in bladder capacity or compliance was documented. [117]. More recently, bladder regeneration has been shown to be more reliable when the SIS was derived from the distal ileum [118].

3.2.2 Regenerative Medicine for Bladder Using Cell Transplantation

Regenerative medicine with selective cell transplantation may provide a means to create functional new bladder segments [51]. The success of cell transplantation strategies for bladder reconstruction depends on the ability to use donor tissue efficiently and to provide the right conditions for long-term survival, differentiation, and growth. Various cell sources have been explored for bladder regeneration. Native cells are currently preferable due to their autologous source, because of which they can be used without rejection [41]. It has been shown experimentally that the bladder neck and trigone area has a higher concentration of urothelial progenitor cells [119], and these cells are localized in the basal region [120].

Human urothelial and muscle cells can be expanded in vitro, seeded onto polymer scaffolds, and allowed to attach and form sheets of cells. The cell–polymer scaffold can then be implanted in vivo. Histologic analysis indicated that viable cells were able to self-assemble back into their respective tissue types and would retain their native phenotype [47]. These experiments demonstrated for the first

time that composite, layered tissue-engineered structures could be created de novo. Before this study, only nonlayered structures had been created in the field of regenerative medicine.

In order to determine the effects of implanting engineered tissues in continuity with the urinary tract, animal models of bladder augmentation were used [30]. Partial cystectomies were performed in dogs. The animals were divided into two experimental groups. One group had their bladder augmented with a nonseeded, bladder-derived collagen matrix, and the second group had their bladder augmented with a cell-seeded construct. The bladders augmented with matrices seeded with cells showed a 100% increase in capacity compared with bladders augmented with cell-free matrices, which showed only a 30% increase in capacity. Most of the free grafts (without cells) used for bladder replacement in the past were able to show adequate histology in terms of a well-developed urothelial layer, but they were associated with an abnormal muscular layer that varied in terms of its full development [52,107].

On the basis of this study, it was hypothesized that building the three-dimensional bladder constructs in vitro before implantation would facilitate the eventual terminal differentiation of the cells after implantation in vivo and would minimize the inflammatory response toward the matrix, thus avoiding graft contracture and shrinkage. To better address the functional parameters of tissue-engineered bladders, an animal model was designed that required a subtotal cystectomy with subsequent replacement with a tissue-engineered organ [59]. Cystectomy-only and nonseeded controls maintained average capacities of 22% and 46% of preoperative values, respectively. An average bladder capacity of 95% of the original precystectomy volume was achieved in the cell-seeded, tissue-engineered bladder replacements. These findings were confirmed radiographically. The subtotal cystectomy reservoirs that were not reconstructed and the polymer-only reconstructed bladders showed a marked decrease in bladder compliance (10% and 42% total compliance, respectively). The compliance of the cell-seeded, tissue-engineered bladders showed almost no difference from preoperative values that were measured when the native bladder was present (106%).

Histologically, the nonseeded scaffold bladders presented a pattern of normal urothelial cells with a thickened fibrotic submucosa and a thin layer of muscle fibers. The retrieved tissue-engineered bladders showed a normal cellular organization, consisting of a trilayer of urothelium, submucosa, and muscle. Immunocytochemical analyses confirmed the muscle and urothelial phenotype. S-100 staining indicated the presence of neural structures [59]. These studies, performed with polyglycolic acid based–scaffolds, have been repeated by other investigators, showing similar results in large numbers of animals long term [121,122]. The strategy of using biodegradable scaffolds with cells can be pursued without concerns for local or systemic toxicity [123]. However, not all scaffolds perform well if a large portion of the bladder needs replacement. In a study using SIS for subtotal bladder replacement in dogs, both the unseeded and cell-seeded experimental groups showed graft shrinkage and poor results [124]. The type of scaffold used is critical for the success of these technologies. The use of bioreactors, in which mechanical stimulation is

started at the time of organ production, has also been proposed as an important parameter for success [125].

A clinical study involving engineered bladder tissue for cystoplasty reconstruction was conducted starting in 1998. A small pilot study of seven patients was reported, using a collagen scaffold seeded with cells either with or without omentum coverage, or a combined PGA-collagen scaffold seeded with cells and omental coverage (Fig. 3). The patients reconstructed with the engineered bladder tissue created with the PGA-collagen cell-seeded scaffolds with omental coverage showed increased compliance, decreased end-filling pressures, increased capacities, and longer dry periods over time (Fig. 4) [126]. It is clear from this experience that the engineered bladders continued their improvement with time, mirroring their continued development. Although the experience is promising in terms of showing that engineered tissues can be implanted safely, it is just a start in terms of accomplishing the goal of

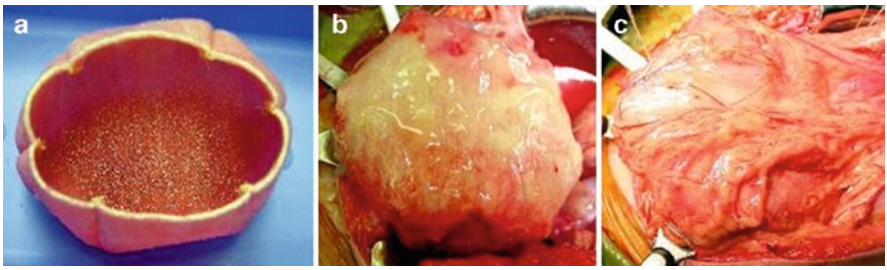

Fig. 3 Construction of engineered bladder. **a:** Scaffold material seeded with cells for use in bladder repair. **b:** The seeded scaffold is anastomosed to native bladder with running 4-0 polyglycolic sutures. **c:** Implant covered with fibrin glue and omentum

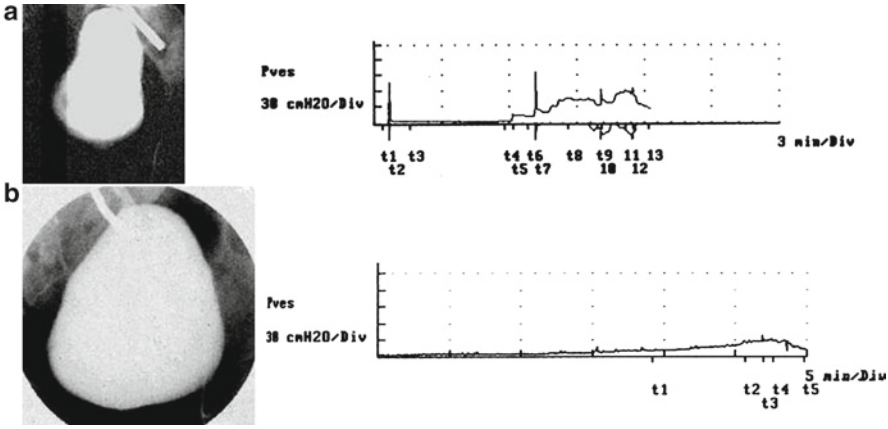

Fig. 4 Cystograms and urodynamic studies of a patient before and after implantation of the tissue-engineered bladder. **a:** Preoperative results indicate an irregular bladder in the cystogram and abnormal bladder pressures as the bladder is filled via urodynamic study. **b:** Postoperatively, findings are significantly improved

engineering fully functional bladders. This was a limited clinical experience, and the technology is not yet ready for wide dissemination, as further experimental and clinical studies are required. FDA phase 2 studies have now been completed.

From the foregoing studies, it is evident, as with the urethral studies, that the use of cell-seeded matrices is superior to the use of nonseeded matrices for the creation of engineered bladder tissues. Although advances have been made with the engineering of bladder tissues, many challenges remain. Current research in many centers is aimed at the development of biologically active and "smart" biomaterials that may improve tissue regeneration.

3.3 Kidney

Although the kidney was the first organ to be substituted by an artificial device and the first successfully transplanted organ [2], current modalities of treatment are far from satisfactory. Renal tissue is arguably one of the most difficult tissues to replicate in the laboratory. The kidney is a complex organ, and the unique structural and cellular heterogeneity present within this organ creates many challenges. The system of nephrons and collecting ducts within the kidney is composed of multiple functionally and morphologically distinct segments. For this reason, appropriate conditions must be provided to ensure the long-term survival, differentiation, and growth of many types of cells. Efforts in the area of kidney tissue regeneration have focused on the development of a reliable cell source [127–132]. Moreover, optimal growth conditions have been extensively investigated to provide adequate enrichment to achieve stable renal cell expansion systems [133–136].

Isolation of particular cell types that produce specific factors may be a good approach for selective cell therapies. For example, cells that produce erythropoietin have been isolated in culture, and these cells could eventually be used to treat anemia that results from end-stage renal failure [137]. Other, more ambitious approaches involve working toward the goal of total renal function replacement. To create kidney tissue that would deliver full renal function, a culture containing all of the cell types rising the functional nephron units should be used. Optimal culture conditions to nurture renal cells have been extensively studied, and cells grown under these conditions have been reported to maintain their cellular characteristics [138]. Cells obtained through the initial process of nuclear transfer were retrieved and expanded from cloned tissue. Moreover, renal cells placed in a three-dimensional culture environment are able to reconstitute into renal structures.

Recent investigative efforts in the search for a reliable cell source have been expanded to stem and progenitor cells. Use of these cells for tissue regeneration is attractive due to their ability to differentiate and mature into specific cell types needed. This is particularly useful in instances in which primary renal cells are unavailable due to extensive tissue damage. Bone marrow–derived human mesenchymal stem cells have been shown to be a potential source due to their ability to

differentiate into several cell lineages [127, 128, 131]. These cells have been shown to participate in kidney development when they are placed in a rat embryonic niche that allows for continued exposure to a repertoire of nephrogenic signals [132]. These cells, however, were found to contribute mainly to regeneration of damaged glomerular endothelial cells after injury. The major cell source of kidney regeneration was found to originate from intrarenal cells in an ischemic renal injury model [130]. Systemic administration of bone marrow–derived mesenchymal stem cells in mice has led to prevention of nephropathy in a diabetic model, the acceleration of acute renal injury, and angiogenesis and vasculogenesis in a renal injury model [139, 140].

Another potential cell source for kidney regeneration is circulating stem cells, which have been shown to transform into tubular and glomerular epithelial cells, podocytes, mesangial cells, and interstitial cells after renal injury [128, 129, 141–145]. Other stem cell types, such as human embryonic stem cells [146] and the more recently discovered human amniotic fluid and placental stem cells can also lead to renal differentiation [85]. These observations suggest that controlling stem and progenitor cell differentiation may lead to successful regeneration of kidney tissues.

Although isolated renal cells are able to retain their phenotypic and functional characteristics in culture, transplantation of these cells in vivo may not result in structural remodeling. In addition, cell or tissue components cannot be implanted in large volumes due to limited diffusion of oxygen and nutrients [147]. Thus, a cell-support matrix, preferably one that encourages angiogenesis, is necessary to allow diffusion across the entire implant. A variety of synthetic and naturally derived materials has been examined in order to determine the ideal support structures for regeneration [49, 59, 91, 126, 148]. Biodegradable synthetic materials, such as polylactic and polyglycolic acid polymers, have been used to provide structural support for cells. Synthetic materials can be easily fabricated and configured in a controlled manner, which make them attractive options for regenerative medicine. However, naturally derived materials, such as collagen, laminin, and fibronectin, are much more biocompatible and provide a similar extracellular matrix environment to normal tissue. For this reason, collagen-based scaffolds have been used increasingly in many applications [91, 93, 126, 149, 150].

The feasibility of achieving renal cell growth, expansion, and in vivo reconstitution using regenerative medicine techniques was investigated [49]. Donor rabbit kidney cells, including distal tubules, glomeruli, and proximal tubules, were plated separately in vitro, and after expansion they were seeded onto biodegradable polyglycolic acid scaffolds and implanted subcutaneously into host athymic mice. This included implants of proximal tubular cells, glomeruli, distal tubular cells, and a mixture of all three cell types. Histologic examination demonstrated progressive formation and organization of the nephron segments within the polymer fibers with time. Bromodeoxyuridine incorporation into renal cell DNA was confirmed. These results demonstrated that renal-specific cells can be successfully harvested and cultured and can subsequently attach to artificial biodegradable polymers. However,

it was unclear whether the tubular structures reconstituted de novo from dispersed renal elements or if they merely represented fragments of donor tubules that survived the original dissociation and culture processes intact. Further investigation was conducted in order to examine the process [151]. Mouse renal cells were harvested and expanded in culture. Subsequently, single isolated cells were seeded on biodegradable polymers and implanted into immune-competent syngeneic hosts. Renal epithelial cells were observed to reconstitute into tubular structures in vivo. Sequential analyses of the retrieved implants over time demonstrated that renal epithelial cells first organized into a cordlike structure with a solid center. Subsequent canalization into a hollow tube could be seen by 2 weeks. Histologic examination with nephron segment–specific lactins showed successful reconstitution of proximal tubules, distal tubules, loop of Henle, collecting tubules, and collecting ducts. These results showed that single suspended cells are capable of reconstituting into tubular structures, with homogeneous cell types within each tubule.

In a subsequent study mouse renal cells were harvested, expanded in culture, and seeded onto a tubular device constructed from polycarbonate [152]. The tubular device was connected at one end to a silastic catheter, which terminated into a reservoir. The device was implanted subcutaneously in athymic mice. Histologic examination of the implanted device demonstrated extensive vascularization as well as formation of glomeruli and highly organized tubule-like structures. Immunocytochemical staining with antiosteopontin antibody, which is secreted by proximal and distal tubular cells and the cells of the thin ascending loop of Henle, stained the tubular sections. Immunohistochemical staining for alkaline phosphatase stained proximal tubule–like structures. Uniform staining for fibronectin in the extracellular matrix of newly formed tubes was observed. The fluid collected from the reservoir was yellow and contained 66 mg/dL uric acid (as compared to 2 mg/dL in plasma), suggesting that these tubules are capable of unidirectional secretion and concentration of uric acid. The creatinine assay performed on the collected fluid showed 8.2-fold increase in concentration as compared to serum. These results demonstrated that single cells form multicellular structures can become organized into functional renal units that are able to excrete high levels of solutes through a urine-like fluid [152].

To determine whether renal tissue could be formed using an alternative cell source, nuclear transplantation (therapeutic cloning) was performed to generate histocompatible tissues, and the feasibility of engineering syngeneic renal tissues in vivo using these cloned cells was investigated [138] (Fig. 5). Nuclear material from bovine dermal fibroblasts was transferred into unfertilized enucleated donor bovine eggs. Renal cells from the cloned embryos were harvested, expanded in vitro, and seeded onto three-dimensional renal devices. The devices were implanted into the back of the same steer from which the cells were cloned and were retrieved 12 weeks later. This process produced functioning renal units. Urine production and viability were demonstrated after transplantation back into the nuclear donor animal. Chemical analysis suggested unidirectional secretion and concentration of urea nitrogen and creatinine. Microscopic analysis revealed

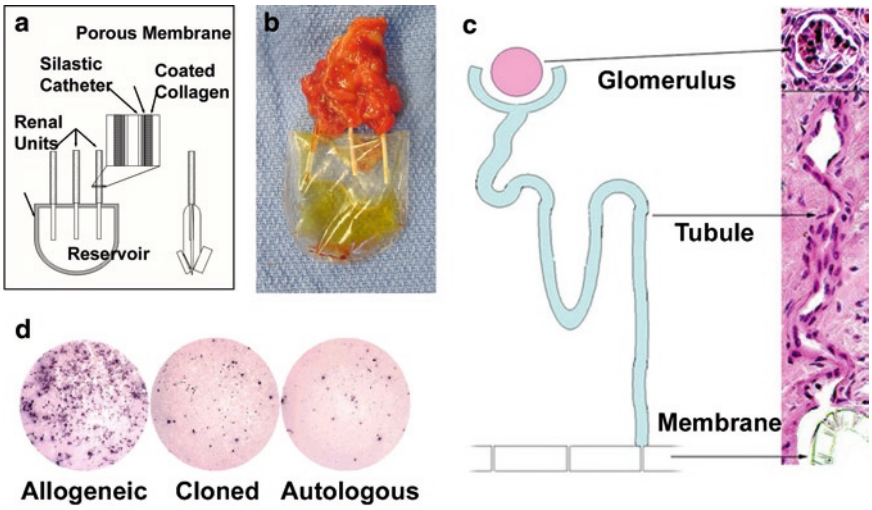

Fig. 5 Production of kidney tissue by therapeutic cloning and regenerative medicine. **a:** The tissue-engineered renal unit. **b:** Renal unit seeded with cloned cells, 3 months after implantation, showing the accumulation of urine-like fluid. **c:** There was a clear unidirectional continuity between the mature glomeruli, their tubules, and the polycarbonate membrane. **d:** ELISpot analyses of the frequencies of T cells that secrete interferon-gamma after primary and secondary stimulation with allogeneic renal cells, cloned renal cells, or nuclear donor fibroblasts

formation of organized glomeruli and tubular structures. Immunohistochemical and reverse transcription-polymerase chain reaction (RT-PCR) analysis confirmed the expression of renal mRNA and proteins. These studies demonstrated that cells derived from nuclear transfer can be successfully harvested, expanded in culture, and transplanted in vivo with the use of biodegradable scaffolds on which the single suspended cells can organize into tissue structures that are genetically identical to those of the host. These studies were the first demonstration of the use of therapeutic cloning for regeneration of tissues in vivo.

A naturally derived tissue matrix with existing three-dimensional kidney architecture would be preferable to the artificial matrix used in the foregoing experiments because it would allow for transplantation of a larger number of cells, resulting in greater renal tissue volumes. Thus, an acellular collagen–based kidney matrix that is similar to the native renal architecture, was developed. In a subsequent study it was investigated whether these collagen-based matrices could accommodate large volumes of renal cells and form kidney structures in vivo [153]. Acellular collagen matrices, derived from porcine kidneys, were obtained through a multistep decellularization process. During this process, serial evaluation of the matrix for cellular remnants was performed using histochemistry, scanning electron microscopy, and RT-PCR. Mouse renal cells were harvested, grown, and seeded on decellularized collagen matrices. Cell–matrix constructs grown in vitro and

implanted in the subcutaneous space of 20 athymic mice were analyzed over time. Renal cells seeded on the matrix adhered to the inner surface and proliferated to confluency by 7 days after seeding. Renal tubular and glomerulus–like structures were observed 8 weeks after implantation.

4 Conclusions

Regenerative medicine efforts are being undertaken for every type of tissue and organ within the urinary system. Most of the efforts expended in the genitourinary field have occurred within the last two decades. Regenerative medicine strategies involve the use of biomaterials alone, as has been accomplished clinically in patients with urethral disease; biomaterials with cells, as is currently being tested clinically with bladder and vaginal tissues; and cell therapies alone, as those applied clinically for urinary incontinence, initially with chondrocytes and now with muscle cells. Regenerative medicine is a multidisciplinary field that requires expertise in a wide variety of scientific disciplines, including cell and molecular biology, physiology, pharmacology, chemical engineering, biomaterials, nanotechnology, and clinical sciences. Although modest clinical success has been achieved in specific areas, the field is still in its infancy. Long-term studies are still essential to ensure safety and efficacy before these technologies have widespread clinical application.

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Muscle-Derived Stem Cells: A Model for Stem Cell Therapy in Regenerative Medicine

Burhan Gharaibeh, Lauren Drowley, and Johnny Huard

Abstract Our research group has shown that a population of muscle-derived stem cells (MDSCs) isolated by a modified preplate technique from murine postnatal skeletal muscle displays a high transplantation capacity in both skeletal and cardiac muscles. The ability of MDSCs to proliferate in vivo for an extended period of time, combined with their strong capacity for self-renewal, resistance to stress, ability to undergo multilineage differentiation, ability to induce neovascularization and the paracrine effects that they have on the host, at least partially explains the high regenerative capacity of these cells in vivo. Although the true origin of MDSCs is unclear, the similarity to human blood vessel–derived stem cells suggests a putative origin from the vascular wall. This chapter reviews current knowledge on the utility of the MDSCs to improve the healing of various musculoskeletal tissues and injured cardiac muscle and lists the potential clinical applications based on this knowledge. We propose that these cells are a successful model for the use in regenerative medicine but emphasize that other factors, such as age and sex of the host and donor cells, should be considered.

Keywords Stem cells • Skeletal muscle • Cardiac muscle • Bone • Cartilage • Cell therapy • Gene therapy • Paracrine effect • Tissue engineering • Regenerative medicine • Skeletal Muscle–Derived Stem Cells

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1 Background

Stem cells are characterized by their self-renewing ability, high proliferation capacity, and ability to differentiate into multiple lineages. Stem cells are used by the body to replace lost or damaged tissue and are thus a potential tool for therapeutic treatment after injury. They can be found in many tissues, including embryonic, fetal, and postnatal tissues. They have been isolated from several adult sources, including bone marrow and the hematopoietic compartment, adipose tissue, cardiac and skeletal muscles, and numerous other organs [1,2]. One of the most easily accessible and abundant sources of stem cells is postnatal skeletal muscle, which has the added benefit of avoiding potential ethical and legal concerns associated with the use of fetal and embryonic tissues. Several different progenitor cell types have been derived from the muscle compartment, including side population cells, satellite cells, and other cells distinguished by characteristic surface marker profiles and other phenotypic characteristics. The differences and similarities among these skeletal muscle stem cells are beyond the scope of this chapter, and we refer the reader to one of several publications that review data on skeletal muscle–derived cells [3–7]. In this chapter, we focus on muscle-derived stem cells (MDSCs) as described originally by our research group [8–10] and list the results obtained using them for the treatment of bone, cartilage, skeletal muscle, and heart defects.

2 Isolation of Murine Muscle-Derived Stem Cells

MDSCs have been isolated based on their adhesion characteristics to collagen-coated flasks using a modification of a method known as the preplate technique [8,10]. In the preplating process, a skeletal muscle biopsy is mechanically cut to fine pieces, which are subsequently exposed to a series of enzymatic digestions with dispase, collagenase, and trypsin followed by hypodermic needle aspirations (Fig. 1). The resulting single-cell suspension is plated onto a series of collagen-coated flasks, and the cells are then passaged on a daily basis for at least 6 days to new flasks. The cells that take the longest to adhere contain the MDSC population, and since they are a slowly adhering fraction of cells, they are known as SACs. The SACs are a heterogeneous population, and thus the marker profile for the MDSCs is not restricted to a few cell markers; however, we have used a series of "replates" to obtain an SAC population that is highly enriched with MDSCs [10].

MDSCs are found at a ratio of approximately 1 per 100,000 cells, showing that they are a rare cell population within muscle. These cells can be passaged to greater than 250 population doublings without any significant changes to their cell characteristics, and they maintain a stable karyotype that has no numerical or structural abnormalities [11]. This ability to expand the cells without a loss of stem cell characteristics indicates a self-renewal ability and is why MDSCs are considered very promising cells for future cell therapeutic strategies.

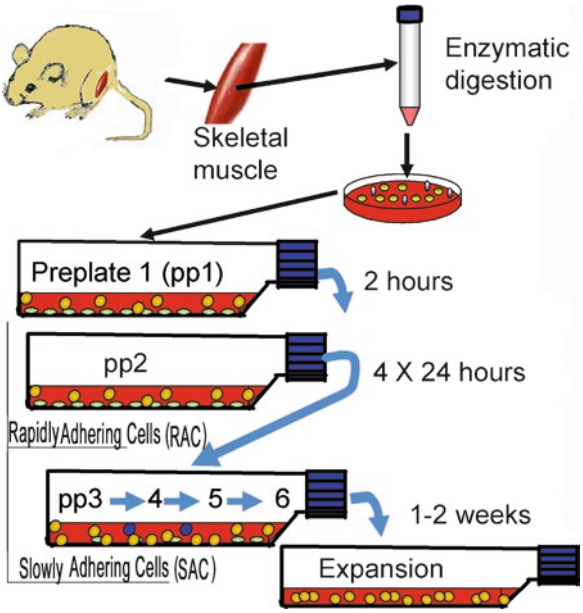

Fig. 1 Schematic of the modified preplate technique used for isolating muscle-derived stem cells. Cells are isolated from a skeletal muscle biopsy, and the single-cell suspension is serially plated onto collagen-coated flasks to separate the cells by their adhesion characteristics

We have found that the preplate technique can also be used with some modifications to isolate populations of muscle-derived cells from other species, including from human skeletal muscle biopsies [10]. Furthermore, one can further purify MDSC populations into subsets of cells using flow-activated cell sorting (FACS) for certain cell surface markers of interest.

Data obtained by our group show a proof of concept that a small biopsy taken from a patient's muscle could be used to isolate autologous cells in sufficient numbers to deliver into a clinical environment where they could be used to treat damaged tissues. This therapeutic strategy is the basis for clinical trials underway in Canada to treat stress urinary incontinence [12].

3 Characteristics and Origin of Muscle-Derived Stem Cells

Muscle progenitor cells have been isolated using a variety of isolation techniques and conditions reported earlier. MDSCs isolated by our group are a population of cells that were found to have specific morphology and behaviors in vitro [8,13] and typically express high levels of the cell surface markers Stem cell antigen-1 (Sca-1) and cluster of differentiation 34 (CD34), while they are negative for CD45 and

express very low levels of vimentin, a fibroblastic marker, desmin, and other markers of terminal muscle differentiation [8].

On the basis of surface marker profile data and other published results, we believe that the MDSCs are a likely earlier progenitors than to satellite cells and other myogenic cells found within skeletal muscle [14, 15]; therefore, we believe that the progenitor ontogeny of skeletal muscle cells is as follows: MDSCs can differentiate into committed myogenic cells (satellite cells) or they can self-renew; the satellite cells differentiate further into myoblasts, which can undergo division and then fuse with preexisting myofibers or fuse together to give rise to multinucleated myofibers [6, 15]. Satellite cells express Pax-7, and once they become activated and begin proliferating, they begin to express MyoD. The cells then begin to differentiate into myoblasts and begin expressing myogenin and lose their expression of Pax-7. Fully differentiated myotubes are positive for markers that include desmin, fast skeletal myosin heavy chain, MyoD, and myogenin. The mature muscle cell is the multinucleated muscle fiber that is characterized by the expression of many different muscle markers, including myf5, MRF4, MyoD, and myogenin (Fig. 2). Myofibers are surrounded by satellite cells that are within the basal lamina but outside the sarcolemma [16]. In situ immunocytochemistry of muscle sections shows that Sca-1–positive cells reside outside the basal lamina [17], while we found [8] these cells within the basal lamina. These results show that these cells are dynamic in nature and change both position and marker profile once a muscle injury occurs [6].

Recently, our group has isolated, by immunohistochemistry and FACS, a blood vessel–derived cell population from human skeletal muscle [18]. The human cells express myoendothelial and pericytes markers, are multipotent, and have high regenerative abilities and a high resistance to oxidative stress [18–20]. The murine-derived MDSCs seem to share marker profiles (positive for von Willebrand factor, VE-cadherin [CD144], and CD34 and negative for CD45) [10]. They also have similar multipotent capacities to human myoendothelial and perivascular cells,

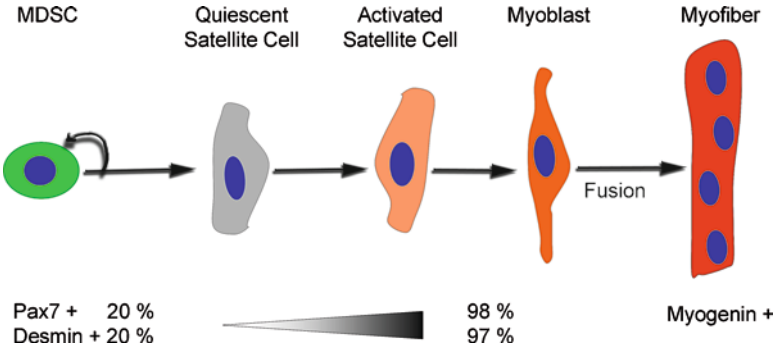

Fig. 2 Stages of myogenesis. Muscle-derived stem cells (MDSCs) are hypothesized to be the most primitive cell population in the muscle and express low levels of muscle markers, while myofibers are the most mature cells and express high levels of muscle markers

suggesting a similar developmental lineage relationship [18]. MDSCs also promote angiogenesis through vascular endothelial growth factor (VEGF) secretion [21–24]. The resultant neovascularization could possibly provide a new source of stem cells.

4 Differentiation Capabilities of Muscle-Derived Stem Cells

In vitro and in vivo studies provided evidence for the stem cell nature of MDSCs. MDSCs differentiate into multiple lineages and regenerate bone, cartilage, muscle, nerve, and cardiac tissue. In a lethally irradiated mouse model, systemic delivery of a subpopulation of MDSCs obtained from a dystrophic mouse resulted in the repopulation of the recipients' bone marrow and differentiation into a hematopoietic lineage [25]. It is noteworthy, however, that in animal models, MDSCs do improve the function of injured organs (e.g., infracted heart) [21,26,27] but do not necessarily proliferate in large numbers or differentiate into the host tissue in a significant manner. The mechanisms by which these cells improve the function of injured organs (particularly the heart) without engrafting in large numbers and differentiating into host tissue remains to be elucidated. We believe that the cells exert paracrine effects on the host tissue and induce regeneration by modulating local environmental responses. We discuss this point in more detail later.

5 Cellular Therapy Using Muscle-Derived Stem Cells

MDSCs are particularly attractive for cell therapy purposes because they are easy to isolate, are derived from postnatal skeletal muscle, are able to self-renew, and can differentiate down multiple lineages. The other important reason why muscle stem cells will potentially be a great tool for regenerative medicine is the potential for autologous transplantation, which would bypass the need for immunosuppression. There are many prospective uses for MDSC therapy, and investigations have shown promising results in bone, skeletal, and cardiac muscles and nerve [22, 26, 28–31]. We give specific examples in the following sections.

6 Gene Delivery Using Muscle-Derived Stem Cells

MDSCs can also be used effectively to deliver certain genes because they eliminate the need for repeated administration of the desired gene(s) or the gene product(s) in therapeutic strategies. Our lab has extensively researched the use of MDSCs to deliver dystrophin. MDSCs transduced to express dystrophin were effective in

restoring dystrophin expression and muscle function after implantation in *mdx* mice, a murine model of Duchenne muscular dystrophy [32]. MDSCs were also able to restore dystrophin at a much higher rate than myoblast populations, and their superior engraftment lead to higher regenerative indices. MDSCs have been used to deliver other genes of interest, such as those for bone morphogenetic proteins (BMP2 and BMP4), which enhanced the ability of MDSCs to differentiate toward an osteogenic lineage and improve bone healing in calvarial and long-bone defects [9, 33–36]. Furthermore, MDSCs transduced with a BMP4 antagonist, Noggin, were able to modulate the growth of bone in a calvarial defect [37] and inhibit heterotopic ossification in mice [38]. MDSCs have also been engineered to express VEGF using a retroviral vector and have been shown to aid in the neovascularization of skeletal and cardiac muscles [24, 26]. They have further been shown to aid in the regeneration of denser, healthier bone in a long-bone and calvarial defect model in mice [29, 37].

7 Role of Muscle-Derived Stem Cells in Muscle Regeneration

After skeletal muscle injury, there is a well-defined process of healing, which includes an interdependent cascade of events that involves MDSCs (Fig. 3). The muscle first begins to degenerate, and this is consequently followed by a rapid inflammatory response. The muscle then begins to regenerate, but some segments of the repaired muscle are eventually replaced by fibrosis starting

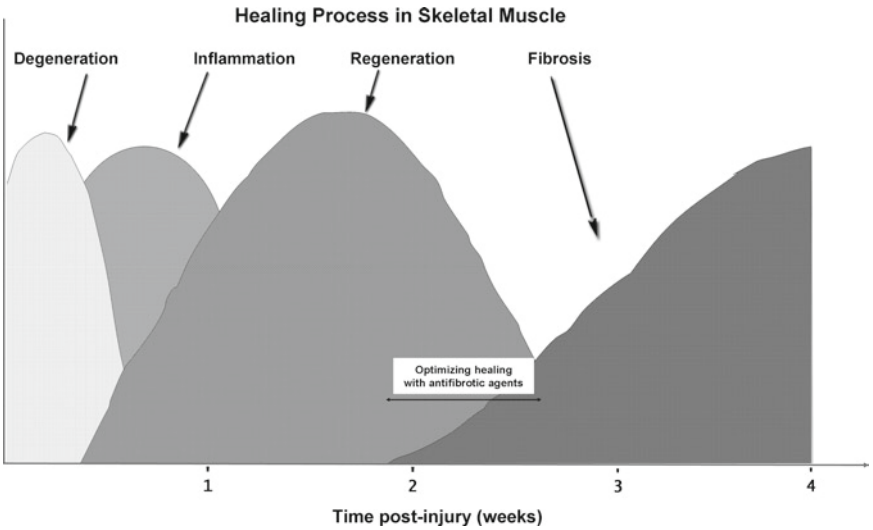

Fig. 3 The healing process in skeletal muscle comprises four phases. First is the initial degeneration after injury, followed closely by inflammation. The regeneration phase starts in the first week and peaks around week 2, which is the time that fibrosis begins forming in the muscle

about 2 weeks after the initial injury. After injury, regeneration of the muscle and scar tissue formation both occur [39], and an optimal outcome would be one that maximizes the regeneration and minimizes fibrosis by modulating these processes with gene and cell therapy and antifibrotic agents. The disorganized collagen in the fibrotic area is not always replaced by functional skeletal muscle tissue. Over time, this results in weakness and a tendency for reinjury in that location.

Myostatin is a regulator of muscle growth and is involved in fibrosis after injury in skeletal muscle. Myostatin increases the proliferation of fibroblasts and increases TGF-β1 secretion [40]. Decorin is a small, leucine-rich proteoglycan that upregulates the expression of follistatin, which is an antagonist of myostatin. In addition, treatment of cells with decorin counteracts the negative effects of myostatin and increases the differentiation of MDSCs and the upregulation of myogenic genes [40, 41]. The different interactions of these molecules are summarized in Fig. 4.

Another treatment, suramin, also has beneficial effects on skeletal muscle healing through both stimulation of regeneration and blocking fibrosis. Suramin also blocks the effects that myostatin has on cells that, when injected postinjury, improve muscle healing [42]. An additional therapy that has been found to be effective in reducing scar tissue formation and increasing regeneration is VEGF. Increasing levels of VEGF by transducing MDSCs with the gene leads to increased healing, while blocking VEGF action with soluble fms-like tyrosine kinase 1 (sFlt-1) leads to decreased repair [24].

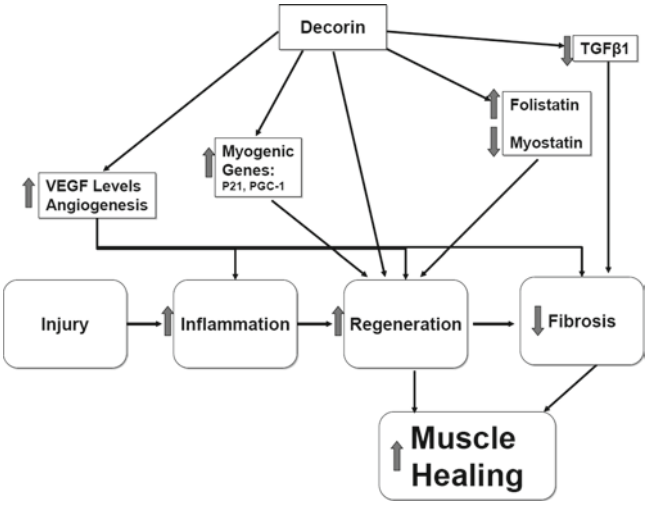

Fig. 4 The interactions between the antifibrotic agent decorin and other factors involved in the healing process. TGFB1, transforming growth factor beta 1; VEGF, vascular endothelial growth factor

8 Role of Muscle-Derived Stem Cells in Bone and Cartilage Regeneration

For bone healing applications, MDSCs have been examined for their ability to repair nonunion fractures in long bones, as well as for healing critical size skull defects, both of which are usually unable to heal without treatment. Since new bone formation with the appropriate mechanical properties is critical for bone repair, research has focused on the use of scaffolds, such as a fibrin gel seeded with cells, which can release paracrine factors to induce osteogenesis. Better bone healing is achieved when MDSCs were transduced to express bone morphogenetic protein 2 (BMP2), and results have shown that the healed nonunion injuries had the same mechanical properties as undamaged bone [43]. MDSCs can also be used for chondrogenesis. Increased chondrogenic defect repair was seen in MDSCs when transduced with BMP4 and sFlt-1: sFlt-1 is an antagonist of VEGF [44]. The transduced MDSCs prevented angiogenesis, which led to a decrease in cartilage resorption and consequently increased cartilage regeneration and repair.

9 Role of Muscle-Derived Stem Cells in Cardiac Muscle Regeneration After Infarction

Cellular cardiomyoplasty—the transplantation of exogenous cells into the cardiac muscle—is gaining recognition as an alternative method for cardiac repair [45, 46]. Using a myocardial infarction mouse model, we compared animals injected with myoblast to those injected with MDSCs. We found that MDSCs displayed better engraftment and induced more neoangiogenesis, less fibrosis, and, consequently, significantly better cardiac output as determined by echocardiography [22]. MDSCs also exhibited a greater ability to resist oxidative stress–induced apoptosis compared to myoblasts, which may partially explain the improved engraftment of MDSCs [47]. However, in a subsequent study [26], male-derived MDSCs injected into female hearts and tracked by Y chromosome probes showed that few donor-derived cells differentiated into myocytes that acquired a cardiac phenotype by fusion with host cardiomyocytes, and limited differentiation was observed. Furthermore, the grafts were found to lack Connexin-43, which indicated limited fusion and gap junction connections between donor and host cells. These findings indicate that MDSCs constitute an alternative to other myogenic cells for use in cardiac repair applications; however, further investigation of the behavior of these cells in a cardiac environment is greatly needed. The improvement in cardiac output by echocardiography indicated that the repair observed appears to be mainly due to a paracrine effect imparted by the implanted cells that promoted angiogenesis, reduced the spread of scar tissue, and allowed the host cells to participate in the repair process [26]. This was ascertained by using a gain- and loss-of-function approach in which MDSCs were transduced to express VEGF or sFlt1 (the VEGF antagonist mentioned earlier) [23]. When sFlt1 was present, cardiac repair

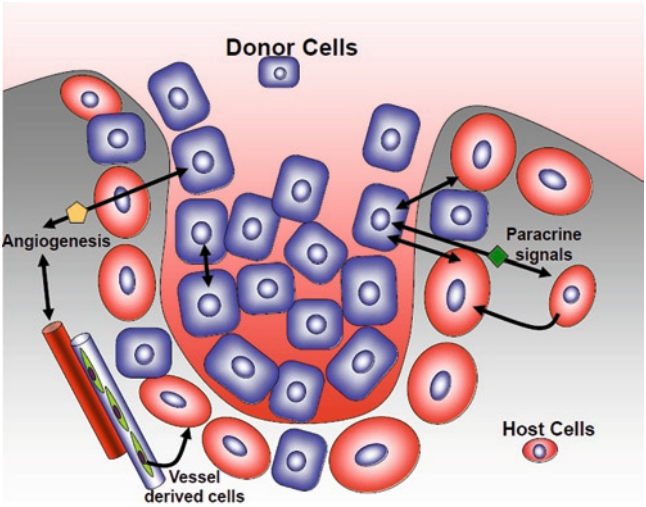

Fig. 5 Interactions between donor stem cells engrafted into a defect area and host tissue. Cells surviving the oxidatively stressful environment and promoting angiogenesis are likely as beneficial as the direct fusion of the donor cells into the resident tissue

was reduced, as was angiogenesis. Furthermore, MDSCs have been shown to secrete more VEGF than myoblasts, which probably contributed to the increased repair observed when MDSCs were transplanted with no genetic modification.

It is worth mentioning that in cardiomyoplasty experiments in which other cell populations are utilized, very little differentiation toward a cardiac phenotype has been seen. This supports the hypothesis that the beneficial effect seen with MDSCs was likely due to increased secretion of paracrine factors, including VEGF [27, 48–50]. Since the donor cells do not necessarily proliferate in large numbers or differentiate into the resident tissue in vivo to a significant extent, we believe that another measure of "stemness" is the ability of cells to have a paracrine effect on the host tissue and induce regeneration by affecting local immune responses and modulating a set of signals that shapes the local environmental niche. The capacity to differentiate into many lineages is valuable, but the ability to have an indirect contribution (where the cells promote angiogenesis, which in turn promotes a reduction in fibrosis and better remodeling) is to also highly important for cell therapeutic strategies (Fig. 5).

10 Factors That Could Influence Stem Cell Performance

Understanding basic biologic phenomena in muscle-derived stem cells is essential when designing cell therapy strategies. The effects of sex, age, genetic background, and so on are all likely important aspects of the healing process that have to be considered.

Stem cells have the ability to proliferate and self-renew far beyond the Hayflick limit, which constrains more-differentiated cells to a limited number of divisions before entering quiescence [51]. Aging can affect the microenvironment, the cells, or a combination of both. There is some debate as to how aging affects muscle progenitor cells, but recent research suggests that these cells may be able to escape the effects typically associated with aging [52]. Previous work has demonstrated that with increasing age, a reduction in potency and differentiation capacity is seen in a variety of stem cell types [53, 54]. Earlier work with MDSCs has shown that with up to 200 population doublings there is no decrease in the regenerative capacity of the cells [11]. The marker phenotype was also preserved over long-term expansion, and no difference was seen in the population doubling time [11]; however, expansion beyond 200 population doublings begins to result in altered cell behavior, including lower regeneration capacity and myogenic differentiation capacity [11]. As many of the potential cellular therapy patients are from an older patient population, understanding the effects of aging on the interaction between stem cells and the recipient tissue environment could improve cellular therapies.

Cell sex is another factor that may play a role in the MDSC's regenerative abilities. We investigated differences in skeletal muscle regeneration based on the sex of MDSCs and found that female cells are able to regenerate skeletal muscle to a greater extent than male cells even when transplanted into male animals [55]. Conversely, in cartilage and bone models, male MDSCs express more osteogenic markers and can create more bone and cartilage than female cells [28, 56]. In cardiac repair the data appear to be more ambiguous. Mesenchymal stem cells show a sex difference in their regeneration capacity, with female cells conferring better ventricular recovery and significantly more VEGF but less tumor necrosis factor alpha [57]. MDSCs appear to have no sex differences in their regeneration capacity in cardiac muscle [57, 58]. Transplantation of both male and female murine MDSCs attenuates functional deterioration after myocardial infaction, increases angiogenesis, and decreases scar tissue formation compared to control (phosphate-buffered saline injected) groups. Male and female MDSCs were also shown to exhibit similar survival rates under oxidative and inflammatory stresses both in vivo and in vitro and secrete comparable levels of VEGF [58].

11 Translational Clinical Applications Based on Muscle-Derived Stem Cells

Research performed on MDSCs in several models of injury in musculoskeletal and cardiac systems led to the use of the preplating technology to purify muscle cells from the slowly adhering fraction, by which MDSCs are enriched for use in translational application. Autologous human muscle–derived cells obtained by the preplate technique are being used in ongoing clinical trials in Canada to treat stress urinary incontinence [12]. Improvement was seen in more than half the patients of the phase I clinical trial, with no serious adverse effects reported, and plans are in

place to use the same method of isolating muscle-derived cells in a new clinical trial to treat patients who have suffered a myocardial infarction. We are optimistic that MDSCs will become a standard donor cell type for many regenerative medicine applications.

12 Future Directions

Work is underway to examine the mechanisms that have promoted the successful transplantation and regeneration capacity imparted by MDSCs, which includes the elucidation of the roles that angiogenesis and MDSC resistance to oxidative stress have in the regeneration process. Methods to improve cell transplantation efficiency, including gene therapy and other treatments to increase cell survival and resistance to oxidative stress, require further investigation. Furthermore, we suggest that the paracrine effects of stem cell transplantation are a major component for the successful transplantation and regeneration of tissues by stem cell therapy that need to be evaluated in regenerative medicine experimental designs. We have demonstrated that stem cells can ameliorate the conditions created in a variety of injury models by secreting growth factors that attract or signal a homing mechanism for other host cells to enter the injury site and contribute significantly to the regeneration process. All of the mechanisms contributing to successful stem cell transplantation and their regeneration capacity are not fully understood and will require further evaluation.

13 Conclusion

Stem cell transplantation is an innovative therapy for tissue regeneration and repair after an injury. The gold standard of cell therapy is autologous transplantation to circumvent the need for immunosuppression and thus avoid the concomitant adverse effects of lifelong use of immunosuppressive drugs. MDSCs are an excellent choice for cell therapy, as they exhibit stem cell characteristics, are easy to isolate, and can be used for autologous therapy. In addition, they have been shown to be superior to more-differentiated cells (i.e., myoblasts) for the repair of bone, cartilage, skeletal and cardiac muscle, and nerve [9, 13, 22, 31, 32], and this may be due to their higher capacity to survive the oxidative stress of the engraftment environment [47, 59, 60]. MDSCs have also been shown to be beneficial because of their capacity to improve angiogenesis and decrease scar formation and hence increase outcomes of functional recovery.

Continued research into cellular therapies for repair is imperative, as current treatment methods are insufficient and result in incomplete healing. In clinical translation, it is important to understand inherent differences between stem cell populations. Even within one group, MDSCs showed interesting sex- and

age-related differences in their regenerative capacity that was also tissue specific. Female-derived MDSCs were better at repairing skeletal muscle but were inferior to male-derived MDSCs in bone and cartilage repair protocols. In addition, examination of safe and effective methods of improving cell transplantation for all types of repair would move the field of regenerative medicine forward and aid in bringing translational therapies to patients.

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Regenerative Strategies for Cardiac Disease

Xiaojing Huang, James Oh, and Sean M. Wu

Abstract Current treatments for ischemic heart disease (IHD) are generally limited to palliative measures and do not halt or reverse the loss of cardiac muscle cells, the defining characteristic of the disease. Recent findings in the stem cell and developmental biology fields have suggested the possibility of generating new heart muscle using cells derived from a variety of sources. These include adult autologous stem cells found in bone marrow or skeletal muscle, autologous cardiac progenitor cells, embryonic stem cells, and induced pluripotent stem cells or other types of reprogrammed cells. Beating cardiomyocytes have been successfully obtained from a number of these different cell types in both murine and human models, but significant technical challenges remain before cell-based cardiac regeneration is a viable therapy. Nevertheless, a large research effort is underway to address these challenges, and the outlook for revolutionizing the treatment of IHD is optimistic.

Keywords Ischemic heart disease • Cardiomyocyte regeneration • Stem cell therapy • Cardiac progenitors

1 Introduction

Ischemic heart disease (IHD) is characterized by reduced blood flow to the heart muscle, or myocardium. It remains the leading single cause of death in the United States, despite medical and social developments that have reduced its fatality over the last 50 years [1]. The incidence of the disease continues to rise worldwide in

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both high- and low-income countries, creating debilitating burdens on their health care systems [2]. Established treatments for IHD include addressing risk factors that contribute to the disease, such as high cholesterol, hypertension, tobacco use, and diabetes, and improving impaired heart function directly by mechanical measures, such as biventricular pacemaker implantation, valve repair, bypass surgery, and heart transplant. Of these treatments, only transplant of a new heart resolves the underlying pathology of IHD, which is death of the heart muscle cells, or cardiomyocytes. Because the heart has limited capacity to regenerate [3, 4], events that induce cardiomyocyte death, such as acute myocardial infarction (MI), lead to permanently impaired heart function for which there are currently mainly palliative, but not curative, therapies. Cardiac transplant is the major exception, but its efficacy as a general treatment is limited by the small number of available donor organs. Consequently, the goal of cardiac regenerative medicine is to create robust, renewable sources of heart cells, particularly cardiomyocytes that can be used to restore lost heart function.

Broadly, there are two strategies for creating new, differentiated somatic cells. Cell-based methods begin with the isolation of a population of cells possessing properties that make them attractive for therapeutic use, such as pluripotency, immunocompatibility, or abundance. These cells can then either be introduced into the patient directly or first manipulated in vitro before transplant. Alternatively, a native cell population within a patient may be induced to expand and repair damaged tissue in vivo through the use of pharmaceutical agents, without any surgical interventions. Current research in cardiac regeneration has mainly focused on the cell transplantation approaches, with a wide variety of cell types being proposed or hypothesized to have regenerative potential for treating IHD. We discuss the properties and prospects of these different cell types in the following sections, as well as examine the standards for evaluating their effectiveness in enabling true cardiac regeneration.

2 Autologous Noncardiac Stem Cells and Progenitor Cells

Certain types of tissue in the adult human body, including liver, skin, blood, and skeletal muscle, are able to repair themselves after sustaining damage. These repair processes rely on populations of undifferentiated stem cells or progenitor cells that reside in the tissue and are activated to divide and differentiate following injury [5–8]. Whether the adult mammalian heart possesses a similar pool of cells is a subject of debate, although recent studies suggest that it does have a limited capacity for regeneration [9–12]. If a population of cardiac progenitor cells does exist, however, its contribution to native cardiac repair is clearly limited, given the persistence of tissue damage in the heart following MI [13]. Consequently, early experimental efforts in cardiac regeneration have focused on the larger, better-identified populations of noncardiac stem cells and progenitor cells, particularly those related to skeletal muscle and blood.

The cardiac regenerative potential of the skeletal muscle progenitor pool, alternatively designated as satellite cells and/or myoblasts, was first examined in animal models in the early 1990s [14], with demonstrated improvement in the function of infarcted myocardium upon transplant of such cells into the heart [15]. In their native in vivo environment, myoblasts differentiate into skeletal muscle cells and integrate into existing muscle by cell fusion, but they can be manipulated to follow a cardiomyogenic pathway in vitro. Mouse myoblasts have been shown to transform into cardiomyocyte-like cells when cocultured with beating rat cardiomyocytes [16], and a subpopulation of these cells has been found to spontaneously differentiate into beating cells with cardiomyocyte features [17]. However, these results have not been replicated for human myoblasts either in vitro or in vivo, and while clinical trials in heart attack patients have shown modest benefit from the injection of autologous myoblasts into the injury site [18–20], the emerging consensus is that any observed improvement in heart function is due to paracrine effects mediated by the injected cells rather than neocardiomyogenesis [21]. The evidence for this hypothesis comes from the observation of functional improvement without significant concomitant engraftment of the injected cells, a result that also suggests that current transplant protocols are not conducive to the cells' long-term survival [22]. Finally, injected myoblasts do not integrate into the heart's conduction system and hence do not beat in synchrony with rest of the myocardium [23]. This phenomenon has given rise to malignant ventricular arrhythmias in a number of patients, who subsequently required implanted defibrillators to prevent sudden cardiac death [24].

The other major population of autologous adult progenitor cells purported to have cardiac regenerative potential consists of the bone marrow–derived cells (BMCs) [13, 22]. BMCs include the blood progenitors, or hematopoietic stem cells, and the mesenchymal stem cells, which differentiate into connective tissue such as bone, cartilage, and adipose tissue. BMCs are characterized by their free circulation in the vascular system and their contribution to diverse tissues in the body, including those of the heart. This latter property is most evident following heart and bone marrow transplants, in which host-derived endothelial [25] and smooth muscle cells [26] are found in the vasculature of the transplanted heart and donor-derived endothelial cells are found integrated into the host coronary vasculature [27], respectively. These host–donor chimerisms suggest that BMCs play a role in the repair of an injured heart by promoting angiogenesis, a hypothesis that is supported by more direct cell-lineage-tracing experiments in animals [28]. The promotion of vascularization, along with as-yet-uncharacterized paracrine effects [13, 22], has also been proposed to be the mechanism of benefit underlying the modest positive results of human clinical trials in which autologous BMCs were injected into infarcted hearts [22, 29, 30]. Although some studies have reported the cardiomyogenic differentiation capacity of BMCs [25, 27, 31], these findings remain controversial and are poorly reproducible. In any case, the number of new cardiomyocytes reported in these studies is extremely low and is unlikely to reconstitute the estimated one billion cardiomyocytes that are loss during MI [22].

Skeletal myoblasts and BMCs have been the preferred cell sources for clinical trials of cell-based cardiac regeneration for a number of reasons, including, principally, their autologous origin, which eliminates the need for immunosuppressive therapy, and their ability to expand ex vivo to sufficient numbers for transplantation [22]. In addition, they are known to contribute to the repair processes of tissues that are closely related to myocardium, either by physiologic characteristics, as is the case with skeletal muscle, or by proximity, as is the case with coronary-vascular endothelium and smooth muscle. However, from a developmental perspective, skeletal muscle and bone marrow represent sufficiently distant lineages from myocardium that conversion of the former into the latter should not be expected to occur readily in vivo, even in the altered environment of the ischemic heart [32]. This hypothesis is corroborated by the failure to observe any substantial formation of new myocardium following injections of skeletal myoblasts and BMCs. Consequently, the focus of cardiac regenerative medicine has recently turned to cell types that have been unequivocally demonstrated to differentiate into cardiomyocytes, as well as to genetic and biochemical manipulations that can induce or enhance conversion of noncardiac cells into cardiomyocytes prior to transplant.

3 Embryonic Stem Cells

Unlike the autologous stem cells and progenitor cells, embryonic stem cells (ESCs) have the ability to spontaneously and unambiguously generate all cell types in an organism [33]. Studies of both mouse [34] and human [35] ESCs have shown that under permissive culture conditions, these cells develop into aggregates called embryoid bodies that include clusters of differentiated, beating cardiomyocytes. These clusters recapitulate developing embryonic myocardium, as assessed by the cells' molecular markers, morphology, electrophysiologic properties, and contractility [36]. Because of their remarkable similarity to native cardiac muscle, ESC-derived cardiomyocytes represent a promising population of cells for transplant, but a number of significant challenges remain before they can be used clinically for heart regeneration.

Chief of these challenges is the tendency of ESCs to form teratomas, or benign tumors that contain cells derived from all three germ layers [37]. As the presence of teratomas in the heart could potentially be highly arrythmogenic and significantly impair contractility, extreme caution must be taken to prevent their formation when ESC-derived cells are introduced into a patient. One study suggests that undifferentiated ESCs can be directly transplanted into the heart without causing teratoma formation [38], but the consensus among most investigators is that ESCs must first be differentiated into cardiomyocytes, then purified to eliminate any residual undifferentiated, teratoma-producing cells. This purification step is technically challenging because in most differentiation experiments, the number of cardiac cells produced represents at best a few percent of the total cell population [39]. Efforts are being made to develop directed differentiation protocols consisting

of chemical or protein factor treatments that induce ESCs to preferentially commit to a cardiac lineage. While no consensus has emerged regarding the optimal protocol, a number of chemical and protein candidates have been identified to promote cardiomyogenic differentiation of ESCs, including retinoic acid [40], ascorbic acid [41], bone-morphogenic proteins [42], and members of the Wnt signaling pathway [43]. ESCs can also be guided to yield only the cardiac fraction by using transgenic methods, in which selectable markers such as drug resistance are attached to cardiac promoter sites and transfected into ESCs [39]. Only those cells committing to the cardiac lineage survive the selection, and the resulting product is thus a highly pure population (greater than 99%) of cardiomyocytes.

Additional challenges facing ESC-based therapies stem from the nature of the starting material. ESCs are allogeneic sources and are thus subject to immune rejection unless administered in conjunction with immunosuppressive therapies [37]. The creation of patient-matched ESCs through somatic cell nuclear transfer, or what is popularly termed therapeutic cloning, is theoretically possible, although the technique has yet to work in humans and is subject to considerable ethical concerns [44]. Ethical considerations also restrict the current number of available ESC lines, and any attempt at large-scale manufacture of ESC-derived cardiomyocytes would have to contend with this limited supply. A potential solution to the problem may lie in the recent discovery of purported mouse female germline stem cells that differentiate into the oocytes necessary to make ESCs [45]. If a similar population is found in humans, then the possibility exists that oocytes may be mass produced in vitro without involving egg donors.

Apart from the technical and ethical challenges of working with ESCs, investigations into the cardiac regenerative potential of these cells have shown mixed results. Human ESC-derived cardiomyocytes have shown sustained proliferation both in vitro [46] and in vivo [47], suggesting that a sufficient number of cells may be obtained for successful therapy without requiring a large number of starting ESCs [22]. When these cells were injected into uninjured hearts of immunosuppressed rats, they integrated both electrically and mechanically with the existing myocardium, and the initial graft size expanded as much as sevenfold over a month-long period [47]. However, injection of the cells into infarcted rodent hearts required simultaneous treatment with a cocktail of survival-promoting proteins and compounds in order to obtain similar graft expansion [48]. Moreover, while the transplanted cardiomyocytes improved the function of infarcted hearts at 4 weeks following injection [48], the improvement was not sustained to the 12-week follow-up [49]. These results indicate that cardiac regeneration in the postinjury environment is a highly complex problem requiring more than just engraftment of new cardiomyocytes.

4 Patient-Derived Induced Pluripotent Stem Cells

Induced pluripotent stem (iPS) cells are a recent innovation [50] that have the potential to circumvent some of the technical and ethical issues facing ESCs. iPS cells can be derived from the patient's somatic cells without the use of embryos,

and, like ESCs, they can differentiate into cells of all lineages in the body [50]. Thus, the problems of immune rejection and limited starting material are largely irrelevant for iPS cells, although the concern of teratoma formation still applies [50]. Both mouse [51] and human [52] iPS cells have been shown to generate embryoid bodies with beating cardiac clusters similar to those obtained from ESCs.

Despite their similarity to ESCs, iPS cells possess several distinct characteristics that present special challenges to their use in cardiac regeneration. To begin, they are not an entirely well-defined cell population, because the reprogramming process that returns differentiated cells to a pluripotent state remains poorly understood [53]. Consequently, it is difficult to determine a small, convenient set of properties that sufficiently distinguishes cells that have attained true pluripotency from those that are incompletely reprogrammed. Incompletely reprogrammed cells compound the problem of purifying out the desired differentiated cell type, because they can generate precursor cells that later fail to mature to the desired phenotype, which causes problems for purifying out the desired differentiated cell type. Purification schemes based on the selection of early precursor markers would then fail to generate a cell population with true regenerative potential. Preliminary data indicate that this scenario merits serious attention because mouse iPS cell–derived cardiomyocytes initially develop in embryoid bodies in a manner similar to that of ESC-derived cardiomyocytes, but they fail to fully mature and eventually degenerate without attaining an adult cardiomyocyte phenotype (S. M. Wu, unpublished data).

In addition to teratoma formation, iPS cells are also subject to other safety concerns, which arise mainly because of the reagents used in the reprogramming process. The initial derivation of iPS cells [50] relied on retroviruses carrying genes for a set of transcription factors that have been implicated in oncogenic pathways [54]. Continued expression of these genes in differentiated derivatives of iPS cells could lead to aberrant cell behavior that poses a danger to patients. In addition, the viruses may integrate into the host genome in a manner that induces oncogene expression, again leading to possible aberrant behavior in iPS cell derivatives. Much effort is being devoted to finding alternative methods of reprogramming, such as using non-integrating adenoviruses to deliver the transcription factors [55], excisable constructs that can be removed from the genome once reprogramming is complete [56], and chemical or protein factors [57]. These methods generally suffer from very low yields, and characterization of the products remains incomplete, but improved understanding of the reprogramming process should lead to optimized protocols that generate safe iPS cells suitable for regenerative applications.

5 Direct Reprogramming of Other Cell Types to the Cardiac Phenotype

An alternative paradigm to converting differentiated cells to iPS cells is to reprogram them directly to the desired phenotype, without first achieving a pluripotent intermediate. This process, also known as lineage reprogramming or

transdifferentiation, has natural analogs in newt limb, eye, and heart repair, in which terminally differentiated cells dedifferentiate into progenitors, which then develop into the necessary cells [58]. Although similar processes have not been shown to occur spontaneously in mammals, particularly with regard to the conversion of other cell lineages to cardiomyocytes, lineage reprogramming can be experimentally induced in some cell types by ectopically overexpressing certain key regulatory genes, a method similar to the current process for generating iPS cells. For example, MyoD, a transcription factor that is central to the specification of skeletal muscle during embryonic development, converts mouse embryonic fibroblasts to contracting muscle cells [59], while C/EBP, a ubiquitous bZip transcription factor, converts mouse B lymphocytes into macrophages [60]. Although these examples require only a single factor for full conversion, the majority of transdifferentiation protocols are likely to be much more complex, necessitating treatment of the starting cells with a number of different factors, whose expression may also need to be controlled temporally or spatially. Moreover, the specific set of factors and conditions may vary depending on the identity of the starting cell population [32], in contrast to the situation for generating iPS cells, where the same set of four factors has thus far been able to reprogram nearly every cell type to a pluripotent state [50].

It has been hypothesized that the set of factors most likely to be successful in facilitating a particular transdifferentiation can be gleaned from the set of gene regulatory elements that are active during the embryonic development of the target cell type [32]. Working on the basis of this idea, Melton and colleagues identified a set of nine transcription factors that were specific and necessary for normal pancreatic β cell development, as assayed by in situ hybridization and knockout assays. They delivered these factors using adenoviruses into adult mouse pancreatic exocrine cells and successfully transdifferentiated them in vivo into the insulin-secreting β endocrine cells [61]. Further experiments showed that the set of nine factors could be reduced to three, and treatment of type I–like diabetic mice with this set led to improvement in the animals' blood sugar regulation. This study represents the first successful example of in vivo lineage reprogramming in animals where the desired cell population is regenerated and the disease phenotype is rescued.

Restoring heart function is a fundamentally different problem from restoring pancreatic endocrine function, as the former involves electrical, mechanical, and signaling requirements that are missing or different from those of the latter. However, the general approach that adopts cues taken from the developmental biology of the organ of interest can still be instructive. The regulatory elements controlling embryonic heart development are well known at this point (Fig. 1 and Fig. 2), although the temporal and spatial patterns of their activity and their downstream targets are less understood [62]. Beyond the selection of which genes to test for reprogramming potential, protocols for transdifferentiation to the cardiac lineage must also consider the choice of starting cell population. Ideally, the population should be abundant so as to facilitate autologous derivation, but it may also be necessary to consider proximity to the target tissue, either in location or developmental lineage.

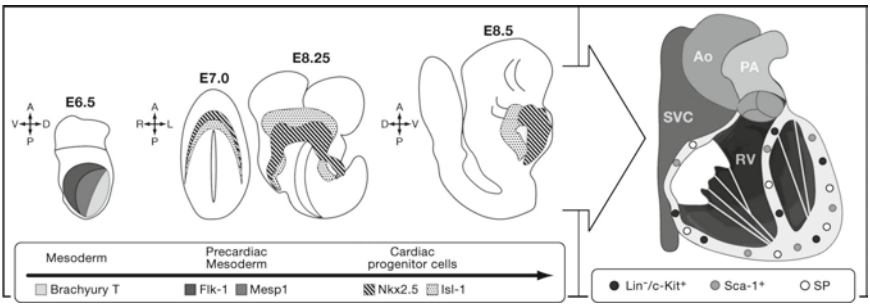

Fig. 1 Origins of cardiac progenitor cells in the developing heart. Cells from the developing mesoderm of the mouse embryo are marked by the expression of Brachyury T at E6.5 of embryonic development (*light gray*). As they transition into precardiac mesoderm, they start to express Mesp1 (*medium gray*) and Flk-1 (*dark gray*) and commit irreversibly to become cardiac progenitor cells by expressing Nkx2.5 (*striped*) or Isl-1 (*dotted*) at E7.0. As the heart progresses through embryonic and postnatal development, it acquires a four-chambered identity and is functionally integrated with the systemic vasculature. Within the adult heart reside several different populations of cardiac stem cells, including side population (SP) cells and cardiospheres (not shown) and cells that express c-Kit, Sca-1, or Isl-1 (not shown). Ao, aorta; SVC, superior vena cava; PA, pulmonary artery; RV, right ventricle. (Adapted from ref. [72].)

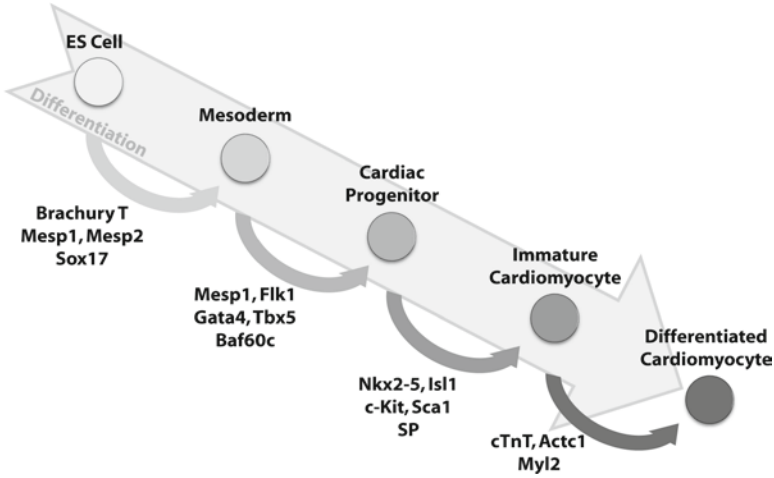

Fig. 2 Factors involved in the differentiation of embryonic stem (ES) cell to cardiomyocyte. Specific transcription factors and signaling proteins have been identified to be necessary for cardiac differentiation in in vivo and ex vivo assays [62]. As an ES cell matures into a cardiac progenitor and ultimately differentiated cardiomyocyte, specific sets of genes are combinatorially upregulated at various stages of differentiation. Expression levels of these gene sets are used to identify maturity

Selecting a population that is physically close to the myocardium may permit in vivo transdifferentiation, while selecting one that is developmentally related may give a higher probability of successful reprogramming. Skeletal myoblasts fulfill this last criterion to a certain degree, as well as the criterion of abundance. A recent study has shown that noncardiac embryonic mesoderm, from which skeletal myoblasts are eventually derived, can be differentiated into cardiomyocytes upon transfection with constructs encoding a cardiac-enriched chromatin-remodeling complex and three cardiac-specific transcription factors [63]. This result, as well as current knowledge of the embryonic cardiac development program, provides a starting point for attempts to transdifferentiate adult somatic cells from variety of mesoderm lineages into cardiomyocytes.

6 Expansion of Native Cardiomyocyte, Cardiac Stem Cell, or Cardiac Progenitor Cell Populations

A recent study using a novel carbon-14 dating technique has provided the strongest evidence yet of cardiomyocyte turnover in postnatal human hearts [64]. It is not clear whether this turnover arises from the division of existing cardiomyocytes, from the expansion and differentiation of resident cardiac stem cells (CSCs) or progenitors, or both. Results from pulse-chase type experiments in mice suggest that the cardiomyocyte pool is replenished after injury by stem cell expansion and differentiation [65]. As mentioned previously, the low turnover of cardiomyocytes in humans, regardless of its underlying mechanism, is insufficient to restore adequate function following trauma to the heart. However, the fact that even a low rate of renewal seems to be present natively is encouraging for regenerative efforts, because therapeutic enhancement of this rate represents the most likely method of generating cardiomyocytes that are indistinguishable from the resident population. Although theoretically possible, the derivation of cardiomyocytes from pluripotent or noncardiac precursors, as described earlier, involves a large number of experimental manipulations, during which deleterious aberrations can be introduced into the cells and their DNA [66]. In contrast, expansion of the native pools of cardiomyocytes or cardiomyogenic progenitors, a process that now appears to occur naturally, is likely to require fewer treatment steps. For example, introducing a single protein, periostin, into rat myocardium following infarction has been shown to increase the rate of cardiomyocyte cell division, with a consequent improvement in heart function at a 12-week follow-up [67].

Although expanding the population of native cardiac cells is an attractive regenerative strategy, significant challenges remain, especially with regard to exploiting the elusive population of CSCs. No single cell surface marker has been identified that allows for specific isolation of the CSC population [22], while at least five different combinations of multiple markers have been proposed [68] (Fig. 1).

The cells isolated based on these marker combinations have been shown to differentiate into beating cardiomyocytes and other cardiac cell types in vivo [9], in vitro [69], or both [70], with some experiments also showing improvement in postinjury cardiac function following injection of the purported CSCs into the heart [71]. Despite these positive results indicating that CSCs exist and can contribute to the observed cell renewal of the heart, they remain unsuitable for therapeutic use until they can be better characterized. It is unknown, for instance, whether the different combinations of surface markers designate different populations or the same population at different stages of differentiation [13] and whether these differences matter in terms of the regenerative potential of the cells. Another important issue to consider is whether the apparent multipotency of CSCs is an artifact of in vitro culture or whether these cells exist in vivo as true progenitors [13]. If the latter happens to be true, then drug treatments intended to expand and differentiate CSCs may be administered in vivo, whereas if the cells acquire their multipotency as a result of being cultured, then the course of therapy would involve extraction of the cells from patients, in vitro expansion and treatment, followed finally by injection of the products back into patients.

7 Conclusion: Standards of Evaluation and Prospects for the Future

This chapter has focused on cell-based methods for regenerating cardiomyocytes that are lost due to IHD or other injury, with the end goal of restoring compromised heart function. For a particular treatment to be considered successful, it is necessary that gross measures of heart function improve following treatment and that the effect is sustained over the long term (e.g., greater than 12 months). In addition, at the cellular level, the newly generated cardiomyocytes must engraft onto the patient's existing myocardium, restore the contractile force of each heartbeat, and respond appropriately to neural and hormonal signaling. Results from early clinical trials of injected skeletal myoblasts and BMCs show that minor improvement in heart function can be obtained without cardiomyocyte regeneration; however, these results are generally not regarded as successful examples of true regenerative medicine. Nevertheless, the observed improvements are instructive of the complex environment of an injured heart, because the injected cells may confer their benefit by ameliorating the effects of the attendant hypoxia and inflammation [13,22]. It has been hypothesized that the severity of these physiologic reactions following cardiac injury hampers the body's inherent repair mechanisms [13], and thus they must be successfully managed in therapeutic repair efforts as well. It may be the case that regenerating lost cardiomyocytes is necessary but not sufficient for restoration of heart function following injury, as concurrent activation or inhibition of certain hypoxic and inflammatory responses may also be needed.

Presently, all of the aforementioned cell-based methods for cardiac regeneration are subject to intense research. The most promising of these from a current clinical and commercial prospective is the directed differentiation of ESCs,

because the pathway from these cells to cardiomyocytes is the best-characterized and best-understood of all the cell types discussed here, as well as the one most amenable to efficient scale-up. iPS cell–based approaches, direct reprogramming, and expansion of native CSCs are also highly promising methods, as they are patient-specific therapies (Fig. 3) that may represent viable alternatives to the use of ESCs. However, as personalized therapies, they are also likely to be subject to cost concerns later in their development. Nevertheless, these approaches represent an interesting and exciting intersection between two sweeping trends in science and biomedicine today, namely regenerative medicine and personalized medicine. As the health profile worldwide continues to change toward greater incidence of metabolic- and age-related diseases, research on all approaches to regenerative therapies should benefit the increasing number of people suffering from heart failure, the one such disease that has become the epidemic of our generation.

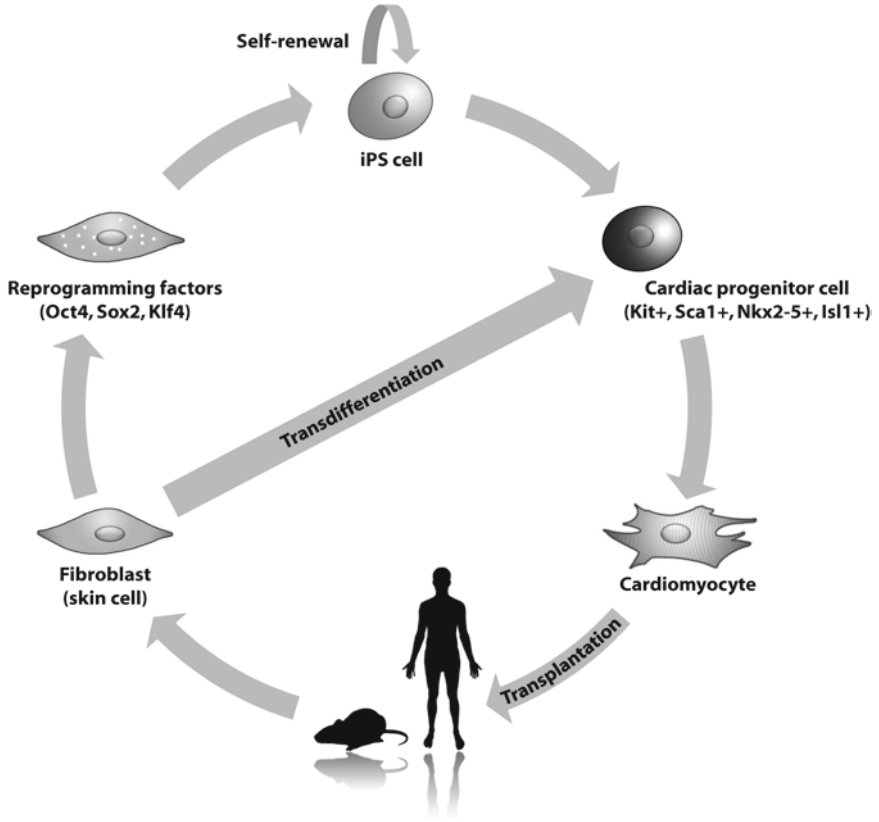

Fig. 3 Induced pluripotent stem (iPS) cells and cardiac transdifferentiation for transplantation therapy. Fibroblasts isolated from the donor may follow two paths in this reprogramming scheme. They can be reprogrammed by the overexpression of reprogramming factors to become embryonic stem cell–like induced pluripotent stem cells, which can be guided to differentiate into cardiomyocytes for autologous transplantation. Alternatively, fibroblasts can theoretically be directly reprogrammed to transdifferentiate from fibroblast to cardiac progenitor cell, eliminating the need of converting the donor cells back to complete pluripotency

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Collecting, Processing, Banking, and Using Cord Blood Stem Cells for Regenerative Medicine

David T. Harris

Abstract It is estimated that almost one in three individuals in the United States might benefit from regenerative medicine. Embryonic stem (ES) cell therapies have been most often touted as the optimal stem cell source for such applications due to their ability to become any tissue in the body that might need therapy. Unfortunately, ES cell applications are currently limited by ethical, political, biologic, and regulatory hurdles. Thus, for the foreseeable future, translation of regenerative medicine to the clinic will depend upon the development of non–ES cell therapies. Over the last decade the collection and banking of cord blood has become the focus of many medical centers in the United States, as cord blood provides a virtually unlimited source of ethnically diverse stem cell donors for a variety of clinical transplant applications. In addition, the recent use of cord blood in regenerative medicine clinical trials has demonstrated therapeutic flexibility of cord blood and has further spurred the collection and banking of these stem cells. This chapter reviews the latest developments in cord blood collection, processing, and banking, as well as the recent use of cord blood stem cells in transplant and regenerative medicine.

Keywords Cord blood • Stem cells • Transplantation • Regenerative medicine

1 Introduction

It is estimated that as many as one in three individuals in the United States, or 128 million people, could benefit over their lifetime from the applications of regenerative medicine, including therapies for cardiovascular disease and endocrine and

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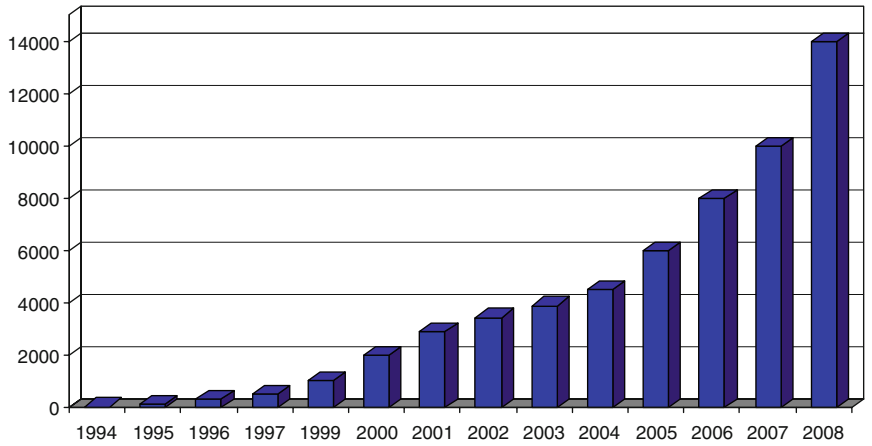

Fig. 1 Growth of use of cord blood in stem cell transplantation. Increase in cumulative numbers of cord blood transplants performed between 1994 and 2008.

orthopedic treatments (http://www.dhhs.gov/reference/newfuture.shtml). Previously incurable diseases, such as type I diabetes, myocardial infarction, stroke, and spinal cord injury, could be ameliorated with advances in regenerative medicine. However, translation of these therapies into reality depends upon the identification of stem cell sources that are medically and economically feasible. Political and ethical controversy surrounding the use of embryonic stem (ES) cells, as well as significant biologic and regulatory limitations to their utilization, has spurred a growing interest in the potential of other, non-ES sources, particularly umbilical cord blood (CB). Over the last 15 years CB has been well established as a substitute for bone marrow in stem cell transplantation (Fig. 1), while recent use of CB in regenerative medicine applications during the last few years has expanded its clinical applications.

Blood and marrow transplantation (BMT) is an often-used means of therapy for a variety of diseases (Table 1), including chemotherapy-resistant malignancies and genetic blood disorders. If a family member cannot provide a bone marrow donor, then a search must be performed for an HLA-matched, unrelated volunteer stem cell donor. The difficulty of finding suitably HLA-matched donors for patients needing stem cell transplant (especially minority patients) has left many patients without treatment [1, 2]. Furthermore, graft versus host disease (GVHD) has been reported to occur in more than 50% of unrelated BMTs [3], with approximately half being severe and life threatening. Due to the problems of a lack of suitable donors and the high incidence of GVHD, researchers began to utilize CB stem cells in the late 1980s [4–10]. Cord blood offers a number of advantages, including a lower incidence of GVHD and less strict HLA-matching requirements, which have increased its availability to transplant patients [10,11]. Furthermore, using related samples instead of unrelated donor samples, when available, has doubled survival rates for patients [10,12]. Over the last 10 years more than 14,000 CB transplants have been conducted worldwide, demonstrating that CB is an excellent

Table 1 Diseases amenable to treatment with cord blood stem cell transplant

Blood cancers	Other cancers	Bone marrow failure disorders	Blood disorders	Inherited metabolic disorders	Inherited immune disorders	Other disorders
Acute biphenotypic leukemia	Brain tumors	Amegakaryocytosis	Beta-thalassemia major	Adrenoleukodystrophy	Chronic granulomatous disease	Congenital erythropoietic porphyria (Gunther disease)
Acute lymphocytic leukemia (ALL)	Ewing sarcoma	Aplastic anemia (severe)	Sickle cell disease	Fucosidosis	Congenital neutropenia	Osteopetrosis
Acute myelogenous leukemia (AML)	Neuroblastoma	Blackfan Diamond anemia	Familial erythrophagocytic lymphohistiocytosis	Gaucher disease	Leukocyte adhesion deficiency	Chronic active Epstein-Barr
Acute undifferentiated leukemia	Ovarian cancer	Congenital cytopenia	Hemophagocytosis	Hunter syndrome (MPS-II)	Adenosine deaminase deficiency	Evans syndrome
Adult T cell leukemia/ lymphoma	Renal cell carcinoma	Congenital dyserythropoietic anemia	Langerhans cell histiocytosis (histiocytosis X)	Hurler syndrome (MPS-IH)	Bare lymphocyte syndrome	Multiple sclerosis
Chronic lymphocytic leukemia (CLL)	Rhabdomyosarcoma	Dyskeratosis congenita	Acute myelofibrosis	Krabbe disease	Chediak–Higashi syndrome	Rheumatoid arthritis
Chronic myelogenous leukemia (CML)	Small-cell lung cancer	Fanconi anemia	Agnogenic myeloid metaplasia (myelofibrosis)	Lesch–Nyhan syndrome	Kostmann syndrome	Systemic lupus erythematosus
Hodgkin lymphoma	Testicular cancer	Paroxysmal nocturnal hemoglobinuria (PNH)	Amyloidosis	Mannosidosis	Omenn syndrome	Thymic dysplasia

(continued)

Table 1 (continued)

Blood cancers	Other cancers	Bone marrow failure disorders	Blood disorders	Inherited metabolic disorders	Inherited immune disorders	Other disorders
Juvenile chronic myelogenous leukemia (JCML)	Thymoma (thymic carcinoma)	Pure red cell aplasia	Chronic myelomonocytic leukemia (CMML)	Maroteaux–Lamy syndrome (MPS-VI)	Purine nucleoside phosphorylase deficiency	
Juvenile myelomonocytic leukemia (JMML)			Essential thrombocythemia	Metachromatic leukodystrophy	Reticular dysgenesis	
Multiple myeloma			Polycythemia vera	Mucolipidosis II (I-cell disease)	Wiskott–Aldrich syndrome	
Myeloid/natural killer (NK) cell precursor acute leukemia			Refractory anemias (RA)	Neuronal ceroid lipofuscinosis (Batten disease)	X-Linked lymphoproliferative disorder	
Non-Hodgkin lymphoma				Niemann–Pick disease	DiGeorge syndrome	
Prolymphocytic leukemia				Sandhoff disease		
Plasma cell leukemia				Sanfilippo syndrome (MPS-III)		
Waldenstrom macroglobulinemia				Scheie syndrome (MPS-IS)		
				Sly syndrome (MPS-VII)		
				Tay-Sachs disease		
				Wolman disease		

alternative to bone marrow [13]. In addition to its use in hematopoietic stem cell transplantation, CB has recently been used in a variety of regenerative medicine applications. Work done by McGuckin et al. [14,15], Rogers et al. [16], Kucia et al. [17], and Harris et al. [18,19] has shown that CB contains a mixture of pluripotent stem cells capable of giving rise to cells derived from the endodermal, mesodermal, and ectodermal lineages. Thus, CB appears to be a practical substitute for embryonic stem cells and readily available for use in tissue engineering and regenerative medicine. Recently clinical trials have begun using CB stem cells to treat type I diabetes, cerebral palsy, and peripheral vascular disease, among others.

In order for CB to attain its maximal clinical potential it is necessary to collect, process, and bank these stem cells in a reproducible and economical manner. Our laboratory has had extensive experience in these endeavors since 1989 [20–24]. We have established methodologies needed for efficient and reproducible CB collection, processing, and banking for clinical use [25–29]. In June 1992, our laboratory established the first CB bank in the world, which became the Cord Blood Registry (Cbr Systems, San Bruno, CA) in 1996. Currently, the Cord Blood Registry has more than 260,000 CB samples banked (http://www.cordblood.com). In this chapter we review the latest developments in CB collection, processing, and banking, as well as the recent use of CB stem cells in transplant and regenerative medicine.

2 Collection and Processing of Cord Blood Samples

All donors must sign informed consents prior to collection of the CB sample. Maternal blood draws are conducted to test for potential infectious disease contamination (i.e., reactivity for HIV, hepatitis B and C, etc.) of the CB as is typically done with blood donors. Furthermore, the collected CB samples are tested for microbial sterility using an automated system (bioMérieux, Hazelwood, MO) [30]. In all instances the CB samples are harvested by the patient's caregiver (physician or midwife), and in the majority of cases the collections are made after delivery of the infant, prior to expulsion of the placenta. Prior to collection the cord is wiped with alcohol and/or Betadine to ensure sterility of the collection [29].

In practice there are a variety of methods used to collect CB, although small gravity bags (approximately 400 cc) are utilized most commonly. Routinely, collections are completed within 5 minutes (prior to placental expulsion) through a simple venipuncture of the umbilical cord. Experienced collectors are able to collect comparable samples using bags or syringes, and many collectors have expressed the opinion that bag collections are simpler to perform. Most CB banks provide sterilized collection methods for use during surgical deliveries. In addition, most banks provide the syringe and gravity bag collection methods preloaded with anticoagulant, with the kit containing all necessary shipping materials for delivery to the processing facility. The kit containing the CB is generally shipped overnight to a processing facility to be received within 28–34 hours to ensure optimal processing outcomes. In accordance with regulatory requirements, a maternal

blood sample is taken for the infectious disease marker testing near the time of CB collection.

CB collections are routinely red blood cell (RBC) reduced prior to cryopreservation in order to reduce storage volumes, minimize certain transplant complications due to excess cryopreservent and red cell lysis [31–33], and maximize cell recoveries upon thawing. Methods to accomplish this goal have included Hespan (Abbott Laboratories, North Chicago, IL) sedimentation to obtain a modified buffy coat, density gradient centrifugation to obtain enriched mononuclear cells (MNCs), and two automated processes, Sepax (Biosafe, Eysins, Switzerland) and AutoXpress Platform (AXP) (ThermoGenesis Corp., Rancho Cordova, CA), that also result in a buffy coat product [11, 29, 34, 35]. The Hespan, Sepax, and AXP processing methods result in CB products containing the nucleated cell population found in the original collection (MNCs, neutrophils, some nucleated RBCs), while the Ficoll method enriches for the stem cell–containing MNC subpopulation (generally greater than 85% MNCs, with a few contaminating neutrophils and nucleated RBCs). Cell counts obtained in the final Ficoll product are generally half the cell counts found in the other processes for this reason, although the stem cell recoveries are similar.

3 Cryopreservation and Banking and Banking of Cord Blood Samples

The majority of CB banks cryopreserve samples using automated, microprocessor-controlled cell freezers, which provide both reproducibility and documentation for regulatory purposes. In preparation for cryopreservation, CB cells are resuspended in autologous plasma containing the cryoprotectant dimethyl sulfoxide. Autologous plasma is used for cryopreservation to avoid exposure to nonself and/or animal proteins (and the inherent infectious disease risks associated with such use). Temperatures are then slowly reduced to -180°C, and the cells are stored in a specially constructed liquid nitrogen freezer or Dewar (MVE, Inc., model 1830, Pacific Science, Laguna Beach, CA), which allows for vapor storage at liquid nitrogen temperatures [36]. Vapor-phase storage prevents cross-sample contamination. Certain viruses, such as hepatitis and papilloma viruses, are known to survive exposure to liquid nitrogen and have been shown to cross-contaminate bags of bone marrow during liquid nitrogen storage [37]. Cord blood samples are generally frozen in multiple aliquots using either multiple cryovials or multicompartment bags. Multiple aliquots allow for future use of the stem cells in cell expansion, in gene therapy, or for regenerative medicine uses that may only require a fraction of the frozen sample. Thus, it is not necessary to thaw the entire sample unless absolutely needed, avoiding the damaging effects of repeated episodes of freezing/thawing. Multiple aliquots also allow for repeated testing of the sample if needed to resolve any issues of misidentification or sample potency. Each storage Dewar must be monitored continuously for liquid nitrogen levels and Dewar temperature, and multiple back-up and fail-safe systems must be put in place, including the immediate availability of spare Dewars, in order to meet regulatory requirements.

4 Cord Blood Stem Cells and Clinical Applications

Over the last decade scientists have demonstrated that CB stem cells not only can differentiate into many cell types in vitro, but also can improve function in a variety of animal models of disease. The presence of multiple primitive and pluripotent stem cell populations in CB helps to explain the mechanisms for the effects behind these observations in addition to cellular reparative factors shown to be secreted by the stem cells. This research has paved the way for several clinical trials in human patients. The body of data is extensive for many disease states (e.g., brain injury, cerebral palsy, type I diabetes, heart disease, and stroke), including the following salient examples. It must be noted that use of stem cells for regenerative medicine differs significantly from use of stem cells in typical stem cell transplant settings for hematologic diseases and generally will require the use of autologous stem cells for implementation (Fig. 2).

5 Cardiovascular Disease

Cardiovascular disease is the leading cause of morbidity and mortality for both men and women in the United States. Approximately one million people die of cardiovascular disease annually despite medical intervention. Coronary artery disease accounts for approximately half of these deaths. As heart cells have a limited capacity to regenerate after myocardial infarction (MI), application of

Traditional	Regenerative
▪ Current clinical use	▪ In preclinical research & clinical trials phases
▪ Stem cells regenerate the blood and immune system	▪ Stem cells regenerate or repair damaged cells other than blood and immune cells
▪ Typically requires immune suppression or ablation (destruction)	▪ Autologous cells are used in clinical trials
▪ Allogeneic or autologous cells are used	▪ Generally being researched for conditions not immediately life threatening
▪ Generally used for treatment of immediately life threatening conditions	

Fig. 2 Comparison of traditional stem cell transplantation with regenerative medicine stem cell applications

exogenous stem cells seems a logical alternative for therapy. Recently, numerous preclinical and clinical studies have examined the use of adult hematopoietic stem cell sources [38]. To date, only nonembryonic stem cells have been examined in cardiac clinical trials due to political, ethical, and biologic constraints. In addition, there have been no clinical trials using CB stem cells for cardiovascular disease. The lack of clinical trials has been due to the relative youth of the CB banking industry and the older age of cardiovascular disease onset, limiting access to autologous cord blood. However, although no clinical trials utilizing CB stem cells for heart failure have been conducted, a number of preclinical animal studies have been performed [19, 39–44]. Several common observations have been noted in these studies regardless of the protocols used, including selective migration of the CB stem cells to the injured cardiac tissue, increased capillary density at the site of injury, decreased infarct size, improved heart function, and a general lack of myogenesis. These observations are thought to be due to the production of angiogenic factors and induction of angiogenesis/vasculogenesis [19, 45, 46]. In fact, studies in myocardial infracted rats showed that $CD34^+$ CB stem cells induced blood vessel formation, reduced infarct size, and restored heart function [19, 38]. These effects are believed to be due to the release of angiogenic and growth factors (e.g., VEGF, EGF and angiopoietin-1 and 2) induced by hypoxia, as shown by gene array analyses. Of interest, as a prelude to human clinical trials for MI, it has been shown that it is possible to isolate therapeutic cells from CB using a clinical-grade apparatus, making the transition from bench to beside a bit more facile. Finally, work from numerous groups seems to indicate that more than one population of pluripotent cells contained in CB are capable of mediating this effect, as shown by the ability of $CD34^+$, $CD133^+$,and $CD45^-$ cells to induce cardiac repair after MI [19, 39, 43, 44, 47]. Even more important, the number and potency of these cells found in CB seem sufficient for adult human applications, as shown by work performed in a porcine model [43].

Aside from its application to MI, CB stem cells, via their angiogenic capability, also appear to be useful for the treatment of various ischemic diseases. Many investigators have demonstrated that not only does CB contain cells displaying the phenotypic characteristics of endothelial precursors that are responsible for blood vessel formation, but these cells also are capable of differentiating into endothelial cells and becoming blood vessels [48–52]. These bioengineered blood vessels appeared similar to native blood vessels in terms of their (three layered) tissue organization as well as expression of matrix components [48, 53]. Furthermore, when placed in animal models CB stem cells were able to significantly reverse the effects of ischemia in several model systems [49–51]. In models of hind limb ischemia, transplantation of CB stem cells or endothelial cells derived from CB stem cells appeared to be able to reverse surgery-induced ischemia, resulting in limb salvage [54–57]. These observations have led to the recent announcement of clinical trials using CB stem cells at the Indiana University Medical School and the University of Toronto for patients with peripheral vascular disease (for more information, please see the respective websites of the institutions).

6 Diabetes

Type I diabetes (TIDM) can be expected to be found in 1 in every 300 births. Of the 20.8 million individuals with diabetes in the United States, approximately 5%–10% display the type I diabetic phenotype. Thus, approximately two million individuals in the United States currently have TIDM. TIDM is due to destruction by the immune system of the β cells in the pancreatic islets responsible for insulin production. The end result is uncontrolled blood glucose. Diabetic complications include cardiomyopathy and coronary artery disease, peripheral vascular disease, and neurologic complications. In an effort to treat TIDM, surgical procedures have been developed to transplant islets across histocompatibility barriers, with limited success due to immune rejection and the lack of donors. Thus, novel approaches are desperately needed to address this serious health issue. Investigators have tried to address the issue of TIDM through the use of stem cells and regenerative medicine [58]. Currently, autologous CB mononuclear (stem) cells are being evaluated in a clinical trial of TIDM in children. Twenty-three children have been treated, and preliminary analyses of the first 8 patients showed evidence that the treatment is safe and provides some slowing to the decline of endogenous insulin production [59]. The rationale for the clinical trial was established in animal studies that showed CB treatment lowered blood glucose levels, reduced insulitis, and increased lifespan compared to control diabetic animals [60, 61]. Similar stem cell trials are being proposed at other centers as well.

Although the mechanisms of action behind CB stem cell therapy for TIDM are not known, it is postulated that once in vivo, the infused CB stem cells may differentiate into insulin-producing β cells and act as nurse cells to foster proliferation of new islets from remaining viable tissue, and that regulatory T cells may facilitate direct or bystander suppression of effector T cells, allowing for restoration of immune tolerance [59] (Fig. 3). In fact, recent results have indicated that in vitro CB stem cells can indeed be driven to become insulin-secreting islet cells, as indicated by the production of C-peptide, an offshoot of the de novo secretion of insulin [62, 63]. In both instances, the islet cell differentiation was attributed to the presence of the ES-like stem cells found in CB.

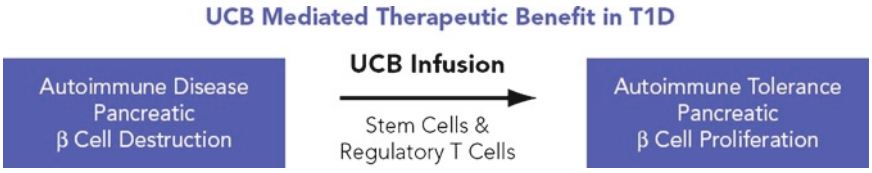

Fig. 3 Potential mechanism of action for cord blood infusions in type I diabetes

7 Neurologic Damage

Neurologic repair has a much more complex etiology than many other conditions being evaluated for CB-based stem cell therapy. Nonetheless, in animal models of traumatic brain injury, stroke, amyotrophic lateral sclerosis, Parkinson disease, cerebral palsy, and spinal cord injury, CB stem cell infusion has resulted in observable behavioral improvement compared to control animals [14, 40, 64–82].

Several investigators have demonstrated that it is possible to derive neurologic-like cells utilizing CB stem cells in vitro. McGuckin et al. and Rogers et al. demonstrated that ES-like CB stem cells could be expanded in culture, and that the cells expressed neuronal cell morphology as well as neuronal markers (GFAP, nestin, musashi-1, and nectin) [14,16]. These neuronal-like cells also released glial-derived neurotrophic factor into the cultures. Jang et al. also showed that CB $CD133^+$ stem cells exposed to retinoic acid differentiated into neuronal (astrocytes and oligodendrocytes) and glial cells with neuronal markers (including tubulin βIII, neuron-specific enolase, NeuN, microtubule-associated protein-2, and the astrocyte-specific marker glial fibrillary acidic protein) [81]. Furthermore, nonhematopoietic stem cells found in CB (most likely mesenchymal stem cells [MSCs]) also could become neural-like cells in culture capable of becoming astrocytes and oligodendrocytes [80]. The utility of these progenitor cell populations for use in cell-based treatments of brain injuries and neurologic diseases has recently been reviewed by Chen et al. [83]. In confirmation of these reports, work from Harris et al. has also shown that $CD133^+$ and Lin^- populations isolated from CB could become glial cells, astrocytes, and oligodendrocytes in vitro [82].

8 Stroke

Cerebrovascular diseases remain the third leading cause of death in the United States, not including the multitudes of individuals who survive only to suffer debilitating lifelong injuries. According to the American Heart Association, cerebral ischemia is by far the most prevalent cause of stroke (87%). Approximately 700,000 people in the United States are affected by stroke annually; 1 in 16 Americans who suffer a stroke will die from it. The brain is extremely sensitive to hypoxia, and some degree of tissue death is likely from stroke. At a relatively young age the brain loses most of its plasticity, so any significant tissue death can be profoundly devastating. Of interest, in young children the brain is very plastic, and very large portions of the brain can be removed (such as removal of tumors or hemispherectomy for severe seizures) with relatively low to no noticeable long-term neurologic damage. These facts suggest that younger and more naïve cells, which could be generated by differentiation from CB, might have a greater capacity to regenerate the injured brain.

Nowhere has the potential significance of CB stem cell therapy for the treatment of neurological disease been greater than in the area of stroke therapy. As early as

2001, it was demonstrated that the infusion of CB stem cells into rats with the commonly used medial carotid artery occlusion (MCAO) model of stroke could ameliorate many of the physical and behavioral deficits associated with this disease [78]. Studies demonstrated that direct injection of the stem cells into the brain was not required, and in fact, beneficial effects could be observed even if the stem cells did not actually make their way into the target organ (probably via the release of growth and repair factors triggered by anoxia) [75, 76, 79]. The beneficial effects seemed to be dose-dependent and could reduce the size of the infarcted tissue [77]. Once again, it appeared that multiple progenitor populations may be capable of mediating these effects [74]. It is significant that, unlike current pharmacologic interventions that require treatment in the first few hours after stroke, CB stem cell therapies were still effective up to 48 hours after the thrombotic event [73]. In fact, administration of CB stem cells immediately after the ischemic event may be detrimental, in that the inflammatory milieu may be toxic to the administered stem cells.

In fact, the majority of reported studies have shown that CB administration in stroke resulted in some degree of therapeutic benefit with no adverse effects [66, 72–74, 77, 84–86]. Neuroprotective effects as well as functional/behavioral improvements of CB therapies have been widely reported [72–74, 84–87]. Neurologic improvement was accompanied by decreased inflammatory cytokines [72], neuron rescue, reduced ischemic volume [73, 74, 85], and lowered parenchymal levels of granulocyte and monocyte infiltration and astrocytic and microglial activation [73]. Thus, the mechanisms behind the observed beneficial effects afforded by CB therapies included reduced inflammation [85], apoptotic protection [74], and a combination of trophic actions and nerve fiber reorganization [74]. This later postulation is particularly encouraging, as it implies that CB therapy can mediate both direct restorative effects to the brain and trophic neuroprotection. Many of the studies lend support to this trophic role, in that several investigators reported neural protection with little to no detection of CB cells engrafted in the brain [74, 84, 86]. The level of engraftment in the brain appeared to be a function of the route of CB administration. When CB was administered intravenously, little or no CB migration to the brain was found [84, 86–88]. However, when CB was given intraperitoneally, there was evidence of neural restorative effects [88]. Two studies investigated the optimal delivery time of cells.

Early studies have also shown benefit in animal models of hemorrhagic (as opposed to embolic) stroke [72]. For additional information see the recent review on cell therapies for stroke by Bliss et al. [69].

As the transfusion of CB stem cells in animals with stroke has shown significant effects, it should not be surprising that similar therapies may be effective in treating other forms of neurologic damage. Along those lines, Lu et al. demonstrated that intravenous administration of CB mononuclear cells could be used to treat traumatic brain injury in a rat model [68]. In this model the CB cells were observed to enter the brain, selectively migrate to the damaged region of the brain, express neural markers, and reduce the neurologic damage. Similarly, CB stem cell transplant could also alleviate symptoms of newborn cerebral palsy in a rat model, with improved neurologic effects [66]. These observations have been turned into clinical

efforts. The Cord Blood Registry has released over 50 CB stem cell samples for autologous use in the treatment of cerebral palsy and anoxic and traumatic brain injury (http://www.cordblood.com). Early, albeit anecdotal, reports have indicated beneficial effects from the CB mononuclear cell infusions. Several investigators have begun planning clinical trials to treat children with traumatic brain injury utilizing autologous CB stem cell infusions.

The finding that CB stem cells have the ability to become different types of nervous cells and restore neurologic function extends to other areas of neurologic damage, including spinal cord injury. Spinal cord–injured rats infused with CB stem cells showed significant improvements 5 days posttreatment compared to untreated animals [78]. The CB stem cells were observed at the site of injured nervous tissue but not at uninjured regions of the spinal cord [78]. This finding is supported by studies showing that CB stem cells transplanted into spinal cord–injured animals differentiated into various neural cells, thereby improving axonal regeneration and motor function [70]. It is significant that in a 2005 case report of clinical use of CB stem cells to treat a patient with a spinal cord injury, it was stated that transplantation of CB cells improved her sensory perception and mobility in the hip and thigh regions [67]. Both computed tomography and magnetic resonance imaging studies revealed regeneration of the spinal cord at the injury site. Since the CB stem cells were allogeneic in origin, it will be significant to determine whether immune rejection or other immune-mediated problems occur that might jeopardize the early improvement.

Other investigators have shown that CB stem cells have potential for developing effective therapies for Parkinson disease [64, 65, 89, 90]. In both animal models, CB stem cell infusion delayed symptom onset and progression, as well as prolonged survival. Finally, two recent preclinical animal studies are of significant interest. In the first study, Bachstetter et al. demonstrated that intravenous injections of CB mononuclear cells could stimulate neurogenesis in the brains of aged rats, as evidenced by a variety of histologic analyses [91]. The mechanism of action was postulated to be a rejuvenation of the aged brain microenvironment mediated via a decrease in inflammation (i.e., cytokines) and a decrease in activated microglia. It will be of interest to learn whether such therapy is correlated with improvements in cognitive function. In the second study, Nikolic et al. demonstrated that intravenous administration of CB stem cells could modify the progression of an Alzheimer disease animal model [92]. That is, a marked reduction in beta-amyloid plaques and associated astrocytosis was observed following multiple low-dose infusions of CB stem cells in the Tg2576 AD mouse model.

9 Orthopedic Applications

Although many researchers have investigated the use of bone marrow MSCs for the repair of bone and cartilage, the potential of CB stem cells has been only recently examined. Orthopedic applications could present a tremendous opportunity for the use of CB stem cells to progress rapidly to the clinic, and additional studies should

be expected in the near future. It is estimated that more than one million individuals in the United States annually suffer from articular joint injuries involving cartilage, ligaments, and/or tendons, as well as difficult-to-heal bone fractures [93]. As CB contains both ES-like stem cells as well as MSC, one should not be surprised that it is capable of differentiating into both bone and cartilage. Cord blood stem cells have been shown to be capable of becoming bone in vitro when subjected to shockwave induction [94]. In fact, when these cells were placed into animals with fractured femurs there was significant bone healing. Work from our laboratory and that of J. Szivek (unpublished data) has also examined the ability of CB stem cells to become cartilage in comparison to tissues derived from bone marrow MSC and adipose stem cells, with early encouraging results [95].

10 Epithelial Tissue Applications

Cord blood contains stem cells capable of giving rise to epithelial tissue, making it amenable for use in regenerative medicine applications for the eye (cornea), skin (wound healing), and other such tissues (e.g., gut and lung). In terms of the eye, the cornea appears to be suitable for routine clinical applications. The outer layer of the eye is made up of the central cornea, the limbus, and the sclera. The cornea epithelium is a rapidly self-renewing tissue, implicated as having its own source of stem cells (the limbus) specialized for this purpose. Corneal epithelial stem cell deficiency is an important cause of visual disability, resulting from alkali injury, Stevens-Johnson syndrome, ocular cicatricial pemphigoid, aniridia, chronic rosacea keratoconjunctivitis, and iatrogenic causes. Without a normal corneal epithelium, a clear image cannot be focused on the retina. Autologous corneal epithelial stem cell grafts have been successful for patients with unilateral disease. However, harvesting cells from the functional eye places the healthy eye at risk for vision loss. In addition, under bilateral conditions, autologous grafts are not available. The best current solution for bilateral disease is a corneal epithelial stem cell allograft. Allografts require chronic antirejection therapy, with possible systemic side effects. In addition, the average survival of allografted corneal stem cells is 2 years. Severe corneal wounds requiring intervention are not uncommon. In fact, corneal wounds make up 37% of all visual disabilities and almost one fourth of all medical visits for ocular problems in North America [96, 97].

Work from the group of Nichols et al. has used CB $CD34^+$ stem cells as a viable therapeutic modality for ocular surface disease, as differentiated human CB stem cells represent an unlimited source of tissue for ocular surface reconstruction [18, 98, 99]. Although there is no extensive published literature regarding the use of CB stem cells for ocular surface reconstruction, preliminary laboratory and animal data are supportive of this hypothesis. Histology and immunohistochemistry of differentiated CB stem cells revealed a resultant cell sheet that was morphologically indistinguishable from corneal epithelial cells. Cord blood stem cells were capable of expressing the corneal epithelial-specific cytokeratin k3. When

New Zealand White rabbits were transplanted with the cell sheets, it was able to reconstitute the cornea, forming an optically clear surface. It is significant that this work once again implicated the existence of multiple progenitor cell populations in CB capable of becoming corneal epithelium. Other investigators have demonstrated that MSC stem cells are also capable of reconstituting the cornea in a rat model. In a rat model of chemically induced loss of the cornea, human bone marrow–derived MSC could replace it and appeared to work in a way similar to limbal stem cells [100]. As CB also contains MSCs, this observation may partially explain the mechanism of action of CB stem cells in the previous application.

Since CB stem cells could become corneal epithelial cells, it should not be unexpected that these cells could also differentiate into skin epithelial cells and thus be useful in facilitating wound repair (e.g., for diabetic ulcers). Work from our laboratory (unpublished data) and that of D. Ablin (personal communication) has begun to investigate this premise, knowing that previous studies have demonstrated a bone marrow stem cell contribution to wound healing in mice, presumably due to MSC stem cells [101]. In agreement with this hypothesis, in 2004 there was an initial report of the use of allogeneic CB $CD34^+$ progenitor cells in two patients to promote skin wound/lesion repair [102]. In this instance the progenitor cells were admixed with an autologous fibrin matrix, and 3×10^4 cells were injected in a volume of 3 mL into the margins of the lesions. At 3- and 7-month follow-up there was no sign of GVHD, and, most important, there was evidence of significant healing in both patients.

11 Conclusions

As described in this chapter, the collection, processing, and banking of CB for immediate or future clinical use can be reproducibly performed with the proper methodology. It is important to note that all procedures used in the CB banking endeavor described here have met and passed regulatory scrutiny (including AABB accreditation). Regulatory compliance is essential in providing the assurance to clients and the transplant physician that each sample is banked under optimal conditions and will continue to be in optimal condition years later, if needed. Although many individuals elect to bank CB for its potential use in the treatment of cancer and genetic blood disorders, more and more, clients are banking upon uses that are only now coming to light, such as regenerative medicine.

The recent body of stem cell research shows that CB has rapidly moved past its traditional hematologic applications into the field of regenerative medicine. This evolution in CB utility has been demonstrated by the discovery of CB pluripotency and the identification of primitive, plastic stem cells within CB, in addition to the cells' ability to secrete reparative factors. However, the true test of therapeutic potential is the ability of these cells to engraft in a living organism and produce measurable improvement in disease states. Cord blood has shown this ability in several therapeutic areas.

Already, in the examples of type I diabetes and neurologic (cerebral palsy and brain injury) applications, CB has transitioned from the laboratory to the clinic, as patients are currently being treated in clinical studies. Clinical trials are beginning for patients with peripheral vascular disease, nonhealing wounds, and spinal cord injury. Other trials will likely follow, including therapies for stroke, the eye, liver, and joints. Farther into the future we would expect to see treatments for other forms of cardiovascular disease, as well as Parkinson disease. However, the key to these advances lies in the ability of CB stem cells to be used in many instances under the practice of medicine (rather than under an investigational new device or IND) since it appears in many systems that it is possible to merely infuse the stem cells directly without timely and costly in vitro culture and differentiation steps.

Regenerative medicine offers the hope of remedial therapy, if not a cure, for many degenerative diseases, including those of neurologic origin. However, in order to make this possibility a reality, one must have available a source of stem cells derived from the patient, the stem cells must be available in large numbers, and the process must be economical. CB is the best source of these stem cells, as it can be used to derive tissues from all three (mesodermal, endodermal, and ectodermal) germ lineages. CB contains a mixture of different types of stem cells in numbers not seen in any other location, including embryonic-like stem cells, hematopoietic stem cells, endothelial stem cells, epithelial stem cells, and MSCs. Therefore, CB collection, processing, and banking will play a significant role in the advance of regenerative medicine now and for the long-term future.

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