Stem Cells and Cancer Stem Cells 1
Therapeutic Applications in Disease and Injury

M.A. Hayat
Editor

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Therapeutic Applications in Disease and Injury

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"Although touched by technology, surgical pathology always has been, and remains, an art. Surgical pathologists, like all artists, depict in their artwork (surgical pathology reports) their interactions with nature: emotions, observations, and knowledge are all integrated. The resulting artwork is a poor record of complex phenomena."

Richard J. Reed MD

Preface

It is recognized that scientific journals and books not only provide current information but also facilitate exchange of information, resulting in rapid progress in the medical field. In this endeavor, the main role of scientific books is to present current information in more detail after careful additional evaluation of the investigational results, especially those of new or relatively new therapeutic methods and their potential toxic side-effects.

Although subjects of diagnosis, cancer recurrence, resistance to chemotherapy, assessment of treatment effectiveness, including cell therapy and side-effects of a treatment are scattered in a vast number of journals and books, there is need of combining these subjects in single volumes. An attempt will be made to accomplish this goal in the projected seven-volume series of Handbooks.

In the era of cost-effectiveness, my opinion may be minority perspective, but it needs to be recognized that the potential for false-positive or false-negative interpretation on the basis of a single laboratory test in clinical pathology does exist. Interobservor or intraobservor variability in the interpretation of results in pathology is not uncommon. Interpretative differences often are related to the relative importance of the criteria being used.

Generally, no test always performs perfectly. Although there is no perfect remedy to this problem, standardized classifications with written definitions and guidelines will help. Standardization of methods to achieve objectivity is imperative in this effort. The validity of a test should be based on the careful, objective interpretation of the tomographic images, photomicrographs, and other tests. The interpretation of the results should be explicit rather than implicit. To achieve accurate diagnosis and correct prognosis, the use of molecular criteria and targeted medicine is important. Equally important are the translation of molecular genetics into clinical practice and evidence-based therapy. Translation of medicine from the laboratory to clinical application needs to be carefully expedited. Indeed, molecular medicine has arrived.

Although current cancer treatment methods have had an important impact on cancer-related morbidity and mortality, the cure rates are modest. On the other hand, cell-based therapy has the potential to treat human conditions not treatable with available pharmaceutical agents, radiation, surgery, chemotherapy or hormonal therapy. Stem cells present important opportunity to elucidate manifold aspects of molecular biology and potential therapeutic strategies, especially in the areas of cancer and tissue/organ injuries. In other words, stem cell field has tremendous potential in deciphering the molecular pathways involved in human diseases. Some stem cell therapies already are being clinically used routinely; for example in leukemic therapy.

Human stem cells also have the potential for application in regenerative medicine, tissue engineering, and in vitro applications in drug discovery and toxicity testing. Stem cells represent populations of primal cells found in all multicellular organisms, which have the capacity to form a variety of different cell types.

A brief statement on the difference between tissue specific stem cells and embryonic stem cells is in order. Tissue specific stem cells (adult or somatic stem cell) can be isolated from a range of organs and tissues from fetal or adult organisms. These cells have a limited life span, each explicative senescences during in vitro to propagation and are multipotent, and thus can be differentiated into a limited number of specialized cells. Embryonic stem cells, on the other hand, are isolated from the inner cell mass of a fertilized egg that has been cultured in vitro to match the blastocyte stage (5–7 days post-fertilization). These cells possess infinite capacity to proliferate in vitro provided maintained in an appropriate condition. The advantage of these cells is that they are pluripotent and can give rise to any fetal or adult cell type.

This is volume 1 of the seven-volume series, *Stem Cells and Cancer Stem Cells: Therapeutic Applications in Disease and Injury*. Support and development of the stem cell field, especially the application of human pluripotent stem calls (embryonic cells), mesenchymal stem cells, and hematopoietic stem cells in cancer therapy and tissue/organ regeneration, are discussed. Role of neural cancer stem cells in brain tumors, including their role in brain tumor therapy and the role of CD133 stem cell antigen in glioma patients, is explained. Therapeutic role of bone marrow-derived stem cells in myocardial infarction and the use of mesenchymal stem cells in orthopedics are explained. Transplantation of umbilical cord hematopoietic stem cells and allogenic hematopoietic stem cell transplantation followed by graft-versus-host disease are presented.

The contents of the book are divided into four sections, Introduction, Neural Stem Cells, Gliomas, and Transplantation, for the convenience of the readers. Vast applications of stem cells, cancer stem cells, mesenchymal stem cells, and pluripotent human stem cells are discussed. Role of cancer stem cells specifically in glioblastoma and medulloblastoma is explained. Interferon treatment for glioma-initiating cells is discussed. Transplantation of embryonic stem cells to reduce brain lesions is included. Complex role of stem cells in angiogenesis is detailed. Targeting of cancer stem cells is also included. Insights on the understanding of molecular pathways involved in tumor biology are explained, which lead to the development of effective drugs. Information on pathways (e.g., hedgehog) facilitates targeted therapies in cancer.

By bringing together a large number of experts (oncologists, neurosurgeons, physicians, research scientists, and pathologists) in various aspects of this medical field, it is my hope that substantial progress will be made against terrible human disease and injury. It is difficult for a single author to discuss effectively the complexity of diagnosis, therapy, including tissue regeneration. Another advantage of involving more than one author is to present different points of view on a specific controversial aspect of cancer cure and tissue regeneration. I hope these goals will be fulfilled in this and other volumes of the series. This volume was written by 45 contributors representing 14 countries. I am grateful to them for their promptness in accepting my suggestions. Their practical experience highlights their writings, which should build and further the endeavors of the readers in this important area of disease. I respect and appreciate the hard work and exceptional insight into the nature of cancer provided by these contributors. The contents of the volume are divided into four subheadings:

Introduction, Neuronal Stem Cells, Gliomas, and Transplantation for the convenience of the reader.

It is my hope that subsequent volumes of the series will join this volume in assisting in the more complete understanding. There exists a tremendous, urgent demand by the public and the scientific community to address to cancer diagnosis, treatment, cure, and hopefully prevention. In the light of existing cancer calamity, government funding must give priority to eradicating deadly malignancies over military superiority.

I am thankful to Dr. Dawood Farahi and Dr. Kristie Reilly for recognizing the importance of medical research and publishing through an institution of higher education.

Union, New Jersey M.A. Hayat
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Part I
Introduction

Chapter 1

Pluripotent Human Stem Cells: An Overview

Gina Y. Chen and Jiang F. Zhong

Abstract For the past few years, cell therapy with pluripotent stem cells has been central to the prospect of regenerative medicine. From the traditional human embryonic stem cell (HSC) to the more recent induced pluripotent stem cells (iPSC), the main objective across all is to make use of the property of cell pluripotency to generate target cell types for therapeutic purposes. This chapter reviews some aspects of pluripotency in human cells, including its characteristics and regulatory factors, as well as various cell-reprogramming and single-cell analysis techniques developed for the manipulation of pluripotency in human cells and the clinical aspects of current methods.

Keywords Stem cells · iPSC · Pluripotency · ESC · SCNT · Trans-differentiation

Introduction

Stem cells are found in most multi-cellular organisms. They are characterized by the ability to renew themselves through cell division to maintain a stable population, and differentiate into a wide range of specialized cell types. There are two broad types of stem cells naturally found in humans: embryonic stem cells (ESCs) and adult stem cells (ASCs).

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Embryonic Stem Cells

Embryonic stem cells are cells isolated from the inner cell mass of a 3–5-day-old embryo, known as a blastocyst. They are pluripotent, having the potential to give rise to any of the three embryonic germ layers (endoderm, mesoderm, and ectoderm), and therefore, any of the adult cell types. In culture, they can proliferate indefinitely and still maintain the undifferentiated state. Human embryonic stem cells (hESCs) were first generated using embryos from in vitro fertilization by Thomson et al. (1998) from the inner cell mass of human blastocyst. With the appropriate extrinsic environment in laboratories, hESC can undergo extensive proliferation for long periods in culture without differentiation, and thereby keeping their developmental potential.

Much hope in embryonic stem cell research lands on the possibility to provide various therapeutic applications. Pluripotent cells, if obtained from specific patients, can serve as a tool for disease modeling, or be subsequently differentiated into specific cell types for replacement therapy in patients with tissue loss due to injuries or degenerative diseases. However, some limitations have restricted the use of embryonic stem cells in regenerative medicine. The possibility of teratoma formation remains one of the major concerns for stem cell therapy. Teratomas are benign tumors that contain differentiated cells of all three germ layers, giving rise to organ-like structures. Because of the intrinsic pluripotency property found in hESC and the difficulty in obtaining pure cell cultures, the undifferentiated hESC can form all cell types and have the possibility of forming teratoma. Patients undergoing hESC therapies are also

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subject to immune rejection complications due to the use of hESC-derived cells with different major histocompatibility complexes (MHCs). Harvesting hESCs from human embryos also entails ethical controversies. Despite their limited potential in regenerative medicine, they are still a powerful tool for developmental studies. Embryonic stem cells have made it possible for researchers to understand many aspects of the self-renewal mechanism and cell lineage regulation. They are the best characterized stem cells for research purposes.

Adult Stem Cells

Another type of stem cell, adult stem cell (ASC), can be found in many tissues or organs. Some examples are hematopoietic stem cells (HSCs), mesenchymal stem cells, and neural stem cells. They act as a repair system, dividing to replace damaged or worn-out cells throughout life. For instance, hematopoietic stem cells (HSC) found in bone marrow give rise to all blood cell types. They constantly divide to replace dying blood cells. In addition, they also self-renew to maintain a constant HSC population, and are subjected to regulations through the bone marrow microenvironments as demonstrated by our group with mouse model (Zhong et al., 2002).

ASCs are distinguished from hESC in many aspects besides where they are originated from. While hESCs are pluripotent, ASCs are mostly classified as multipotent because they are often found to differentiate into only particular cell lineages. Traditionally, these cells are considered to be merely capable of differentiating into limited cell types, primarily those found in the organs from which they originate. However, recently more evidence suggests that ASCs may have greater plasticity than previously thought.

The clinical use of ASC is most commonly found in bone marrow transplant of patients diagnosed with leukemia and various blood diseases. In this procedure, the patient's own hematopoietic systems are first destroyed using radiation or chemotherapy. The matching donor's bone marrow stem cells are then infused into the patient's blood and migrate towards bone marrow, in which they differentiate into all types of blood cells for regeneration of a healthy hematopoietic system. ASCs have potentially wider range of use in cell replacement therapy if the differentiation can be controlled in vitro. The advantage of using ASCs is that they are patient-derived and do not mount an immune response if autologous cells are used. In addition, ASCs often will not generate teratomas. They are also ethically more acceptable in that the use and destruction of human embryos is avoided.

Characterization of Pluripotency

One of the major characteristics of stem cells is their pluripotency. Pluripotency refers to the ability of a stem cell to give rise to all cell types found in an organism. It is worth noting that pluripotent stem cells alone cannot develop into functional organisms due to the inability to generate extraembryonic tissues. In humans, cells isolated from the inner cell mass of a blastocyst retain the capacity to differentiate into any of the three germ layers. Once isolated, however, they cannot each form a human embryo.

Pluripotency is best defined functionally and characterized by the developmental potential. For this reason, the most definitive way to assess pluripotency is to perform a functional assay to test the ability of a cell to give rise to all kinds of tissues. The most extensive functional test is the formation of chimeric animals. In such experiments, genetically marked cells (e.g., GFP tagged cells or cells carrying specific DNA sequences) are injected into embryos to generate full-term chimeric animals. The contribution of the injected cells in the chimeric animals serves to indicate the cell types that can be differentiated from the injected cells. Another common functional test is injecting the cells into an immune-suppressed animal, and subsequently observing the formation of teratomas. Teratoma formation is an indication that the cells have the potential to form all cell types (the three germ layers) and are indeed pluripotent. The in vitro approach to pluripotency evaluation is the formation of embryoid bodies. Embryoid bodies are an aggregate of cells derived from embryonic stem cells. They contain cell types from all three germ layers, and are thus served as a tool for pluripotency screening.

Alternative from the expensive and labor consuming functional assays, pluripotency biomarkers can assess pluripotency to certain degrees. In conjunction

with the functional assays, molecular analysis of biomarker genes provides additional screening cellular pluripotency. The expression of pluripotency related genes are often used to roughly sieve out the non-pluripotent cell populations. However, this method does not exclusively discriminate between pluripotent and nonpluripotent cells. Therefore, pluripotency marker gene alone is not yet a reliable tool to verify cell pluripotency.

Maintenance of Pluripotency

The properties of self-renewal and pluripotency are governed by an intricate set of extrinsic cues as well as intrinsic gene regulatory mechanisms. Intrinsic regulation comes mainly from the expression of transcription factors and methylation of DNA. Inside a stem cell, transcription factors of OCT4, SOX2 and NANOG, which together compose the core transcriptional regulatory circuitry, are essential in maintaining the undifferentiated state and self-renewal. These transcription factors co-occupy the promoters of their target genes, including the genes that code for the transcription factors themselves. The result is a self-regulatory and feed-forward loop of pluripotency. Yu et al. (2007) successfully reprogrammed human somatic cells to embryonic stem cell-like stage using OCT4, SOX2, and NANOG, together with 1 other factor LIN28, demonstrating the power of these intrinsic factors. At the same time, Takahashi et al. (2007) also demonstrated the reprogramming of human fibroblasts into pluripotent state with OCT4, SOX2, KLF4, and c-Myc.

Besides the intrinsic gene regulatory network, the extrinsic regulation also plays an important role in the maintenance of pluripotency. The extrinsic regulation is primarily associated with the microenvironment surrounding the cells. It is the balance of intrinsic and extrinsic factors that results in the pluripotency status of a cell. While the intracellular transcription factors in stem cells ensure the maintenance of pluripotency, signals from outside the cells can act in reverse to trigger differentiation. It was proposed by Schofield (1978) that extrinsic stimuli, such as soluble growth factors, extracellular matrix, and signals sent from neighboring cells, are important determinants of cell fate. Studying these extrinsic factors, together with their downstream intracellular signal transduction pathways, has led to the understanding of how to control and direct the differentiation of stem cells.

Growth factors comprise a major part of extrinsic determinants, and are local regulators that stimulate nearby cells to grow and divide. Various growth factors have shown to facilitate or suppress differentiation. It was shown by Xu et al. (2002) that hESCs are highly subject to differentiation in the presence of bone morphogenetic proteins (BMPs), a group of transforming growth factors capable of inducing bone and cartilage formation. BMP4-treated hESCs undergo differentiation and become trophoblasts, which contribute to a large portion of placenta in human embryogenesis. Interestingly, BMP has opposite effect in mouse in that it maintains pluripotency in the mouse ESCs. Such phenomena supports the hypothesis that pluripotency is a result of a balance between intrinsic and extrinsic factors.

While some growth factors promote differentiation, others can maintain the undifferentiated state of hESC. These signaling molecules include transforming growth factor-β (TGFβ) superfamily (Vallier et al., 2005), fibroblast growth factors (Amit et al., 2000), and insulin-like growth factor (Wang et al., 2007). The TGFβ superfamily members include TGF-β protein, activin and nodal, and growth differentiation factors (GDF). Many of these act to offset the differentiation signals from other signaling pathways. For instance, it was reported by Xu et al. (2008) that TGFβ and activin can counteract the induction of differentiated cells from BMP4 signaling by promoting the expression of the transcription factor NANOG. Levine and Brivanlou (2006) also reported that growth differentiation factors help maintain pluripotency by inhibiting BMP-induced differentiation. Blockage of signaling pathways, such as FGFs (Amit et al., 2000) and IGFs (Wang et al., 2007), results in differentiation of hESCs.

Besides growth factors, extracellular matrix is required to keep hESCs in their undifferentiated state. hESCs cultured on plastic without extracellular matrix components are subject to differentiation and programmed cell death. Commonly used extracellular matrix components are feeder cells such as mouse embryonic fibroblast (MEF) and human fibroblast. Furthermore, it was proven by Levine et al. (2009) that human fibroblast can produce differentiation-inhibiting factors or pluripotency-promoting factors. The feeder cells can also be replaced with synthetic extracellular matrix proteins with similar effects.

Pluripotency Spectrum

The balance of intrinsic and extrinsic factors often results in different degrees of pluripotency. Pluripotency is a dynamic functional status. Stem cells with higher degree of pluripotency have a stronger self-renewal capacity and can differentiate into more cell types than those of lower degree of pluripotency. This phenomenon has been well studied in the hematopoietic system. A given population of cells can be classified into a pluripotency hierarchy, with the cells at the top having greatest ability to self-renew and differentiate into more hematopoietic cell types. The existence of this hierarchy system suggests the plasticity of pluripotent stem cells and the heterogeneity of these cell populations.

Recent studies suggest that hESCs also exhibit high levels of heterogeneity of pluripotency. Subpopulations can be isolated by cell-surface markers based on the expression of different surface glycolipids and glycoproteins. Using flow cytometry, it was shown by Enver et al. (2005) that a subpopulation of hESCs express surface protein SSEA-3 while others do not. In examining the expression of pluripotency marker GCTM2 and CD9, the expression levels found in different hESC subpopulations display a continuum of spectrum (Hough et al., 2009). A study of single-cell analysis conducted by Zhong et al. (2008) also reveals that individual hESCs possess different transcription profiles. The single-cell assessment of commonly expressed genes, such as B2M, Nodal, and Fzd4, shows that not all cells express all three genes. The expression level of each gene also fluctuates among individual cells. This analysis proves the existence of different degrees of pluripotency among the apparently pluripotent hESC.

Manipulation of Pluripotency

The two major methods for manipulating pluripotency are induction of pluripotent stem cell (iPSC) technology and somatic cell nuclear transfer (SCNT). Both methods can generate pluripotent stem cells which resemble hESCs. Although these methods cannot be verified with chimeric human embryos, chimeric mice were produced to demonstrate that stem cells obtained from both technologies can develop into whole mice. The central concept shared by both methods is related to the importance of intrinsic determinants of pluripotency, the factors associated with the maintenance of the defining characteristics of pluripotent stem cells.

Somatic Cell Nuclear Transfer

Somatic cell nuclear transfer (SCNT) is a technique that uses an egg cell and the nucleus of a somatic cell to obtain reprogrammed pluripotent stem cells. In SCNT, the nucleus of the egg cell is removed, and the donor nucleus is inserted into the egg cell. The somatic cell nucleus is reprogrammed during the process by unknown factors in the egg. After a brief electric shock, the resulting new egg divides to form blastocyst, with the genetic materials almost identical to the donor. SCNT can be used for reproductive as well as therapeutic cloning. Wilmut et al. (1997) successfully cloned Dolly the sheep using this method. Since then, researchers have been motivated to utilize the nuclear transfer method clinically to generate patient-specific embryonic stem cells. These cells isolated from the blastocyst stage can then be used for studying disease or potentially for transplanting back to the donor after linage-specific in vitro differentiation for cell replacement therapy.

Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) are adult somatic cells epigenetically reprogrammed to acquire stem cell-like properties by forced expression of certain transcription factors, so called reprogramming factors (RFs). The RFs are either factors that maintain the continuous proliferation and suppress differentiation, or tumor-related factors. Unlike SCNT, iPSC technology directly reprograms the DNA of somatic cells into a pluripotent state. This method was first demonstrated by Takahashi and Yamanaka (2006) using the forced expression of only 4 transcription factors, Oct3/4, Sox2, c-Myc, and Klf4, on mouse fibroblasts. Takahashi et al. (2007) successfully produced iPSC using human fibroblasts with the same 4 factors. At the same time, Yu et al. (2007) also successfully reprogrammed human somatic cells to an hESC-like state using OCT4, SOX2, NANOG,

and LIN28. The resulting iPSCs were found to resemble hESCs in morphology, self-renewal ability, cell-surface-marker expression, epigenetic status, germ-line competence, embryoid body formation, and teratoma formation. Chimeric animals were used to demonstrate the pluripotency of iPSCs in mice.

During the initial stage of iPSC discovery, the reprogramming was found to be incomplete because the resulting iPSC colonies had gene expression and DNA methylation patterns slightly different from ESCs. They were also not germ-line competent, which is a characteristic marked by ESCs. Wernig et al. (2007) modified the reprogramming protocols and showed that reactivation of OCT4 gene is necessary for the generation of germ-line competence. Since then, methods have been reported that employ different combinations of RFs to successfully induce pluripotency. A variety of cell types can be used for reprogramming. Some of them include embryonic and adult fibroblasts, hepatocytes, keratinocytes, stomach epithelial cells, and pancreatic β cells. The reprogramming efficiency is controlled by the protocols used and the selection of the starting somatic cell types. It was reported by Aasen et al. (2008) that the generation of iPSC from human keratinocytes are found to be more rapid and 100 times more efficient than human fibroblasts.

Molecular Mechanism of Pluripotency

The iPSC technology allows for investigating the molecular mechanism of pluripotency changes. The molecular mechanism of reprogramming, the stepwise gene regulation during reprogramming, can be examined by comparing the molecular profiles of cells at various intermediate stages of reprogramming. However, the low efficiency of the present iPSC technology remains a major hurdle for isolation of pure cell populations of different stages. Traditional gene expression profiling approaches measure the average mRNA or protein levels for a population of cells. Due to heterogeneity of cell types and cell cycle phase, interpreting these bulk-scale data is challenging. In addition, isolating a pure population of cells at a particular developmental stage of reprogramming is difficult. Although stable cell lines of partially reprogrammed cells can be obtained, these cell lines may be very different from its parental cells because cell development is a dynamic event. Studies of partially reprogrammed cell lines may not reveal the true characteristic of intermediate status of reprogramming. Therefore, studying intermediate reprogramming stages using the single-cell approach is an efficient way to investigate reprogramming mechanism.

Our laboratory developed multiple microfluidic tools to perform reliable and large scale single-cell analysis for such studies. With these novel single-cell analysis tools, the need of isolating a large number of synchronized cells from intermediate reprogramming stages is circumvented. These devices can manipulate several nanoliters of reagents for biochemical reactions (Fig. 1.1). They can also simultaneously extract total mRNA from thousands of individual cells, and convert mRNA to cDNA with a 5-fold higher efficiency than that of bulk assays. With these microfluidic devices, individual cells from entire hESC or iPSC colonies consisting of cells in a continuous spectrum of pluripotency can be profiled. The information obtained can be used to construct a high resolution map of gene regulation. These maps are dynamic records of the stepwise reprogramming event.

Minimizing material loss is a major advantage of these microfluidic devices. Uemura (1980) and Brady (2000) reported that a single mammalian cell contains 20–40 pg of total RNA, and only 0.5–1.0 pg of mRNA, which is equivalent to 10^5–10^6 mRNA molecules. The small amount of materials presents a challenge for single-cell mRNA profiling using current techniques, which are designed for biochemical reactions at the micro-liter scale. The relatively huge dead volumes of micro-pipette and micro-centrifuge tubes cause significant material loss in single-cell analysis, and thus a nanoliter scale reactor is needed for reliable single-cell analysis. In order to produce consecutive gene expression profiles, a large number of cells also must be profiled for a particular experiment to cover all intermediate reprogramming stages. Our microfluidic devices which perform reactions in 10-nl scale can meet the requirements for a large scale single-cell analysis (Fig. 1.2).

After obtaining sufficient single-cell transcriptome profiles, the gene regulation of reprogramming can be inferred with a bioinformatics approach. Regulatory relationships among genes are often masked in mRNA extracted from a cell population because cell differentiation is a continuous event, and bio-markers for distinguishing cells in close differentiation/maturation