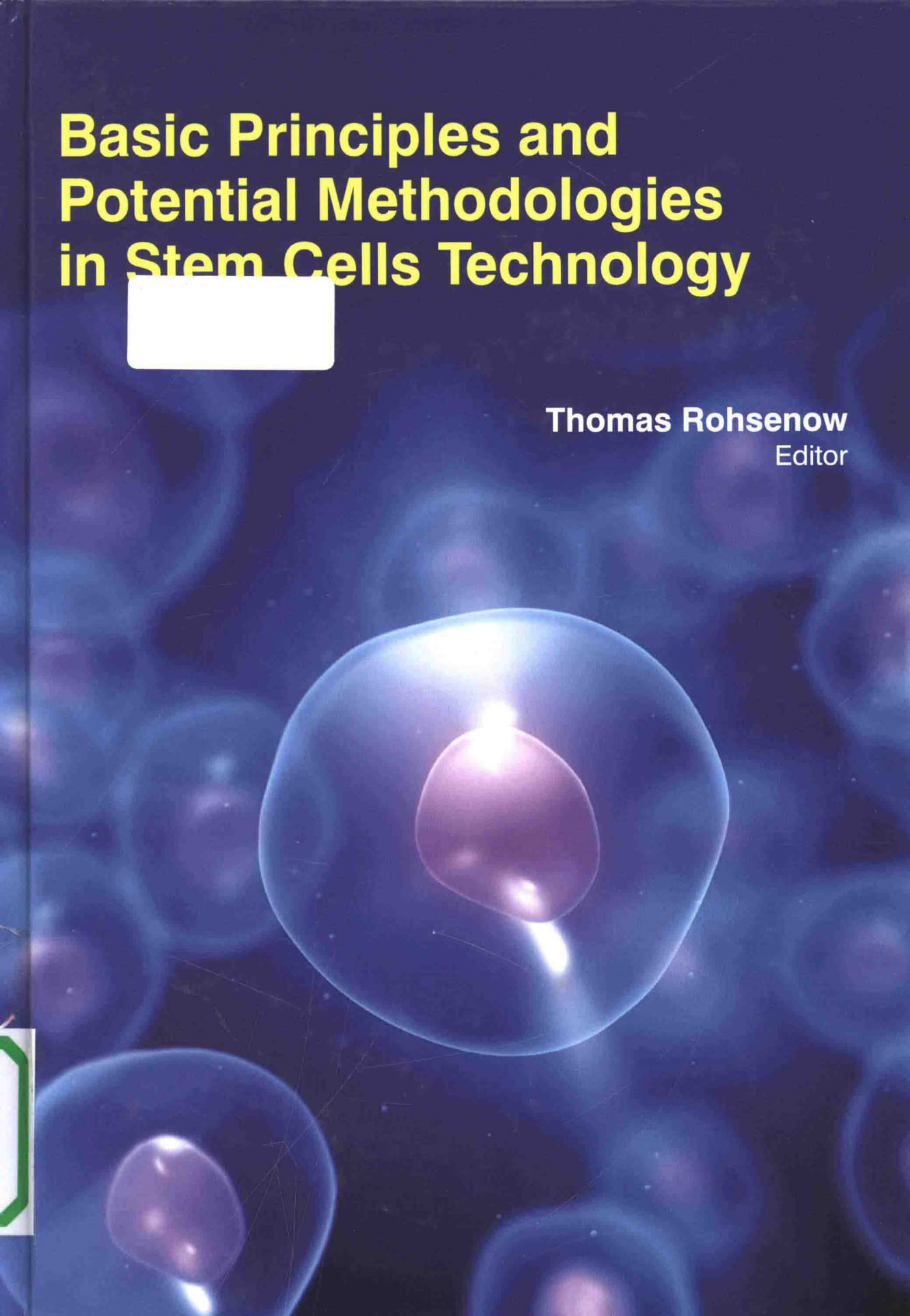
Basic Principles and Potential Methodologies in Stem Cells Technology

Thomas Rohsenow
Editor



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Preface

Stem cells are undifferentiated biological cells that can differentiate into specialized cells and can divide (through mitosis) to produce more stem cells. They are found in multicellular organisms. In mammals, there are two broad types of stem cells: embryonic stem cells, which are isolated from the inner cell mass of blastocysts, and adult stem cells, which are found in various tissues. In adult organisms, stem cells and progenitor cells act as a repair system for the body, replenishing adult tissues. In a developing embryo, stem cells can differentiate into all the specialized cells—ectoderm, endoderm and mesoderm —but also maintain the normal turnover of regenerative organs, such as blood, skin, or intestinal tissues. Stem cells can also be taken from umbilical cord blood just after birth. Of all stem cell types, autologous harvesting involves the least risk. By definition, autologous cells are obtained from one's own body, just as one may bank his or her own blood for elective surgical procedures.

Adult stem cells are frequently used in medical therapies, for example in bone marrow transplantation. Stem cells can now be artificially grown and transformed (differentiated) into specialized cell types with characteristics consistent with cells of various tissues such as muscles or nerves. In the first chapter we discussed, the successful establishment of human embryonic stem cells (hESCs) in culture has raised unprecedented public interest and expectation of treating intractable diseases such as diabetes, spinal cord injuries, neurodegenerative and cardiovascular diseases. Much of this enthusiasm was predicated on the unlimited self-renewal capacity of hESCs and their remarkable plasticity in differentiating into every cell type in our body. These features presented the tantalizing possibility of an unlimited cell source in regenerative medicine to generate any tissues to replace injured or diseased tissues. It is estimated that 2.1 million married couples or 5 million people in the United States are affected by infertility.1 Infertility is defined as failure to get pregnant after one year of unprotected intercourse. About 40% of infertility cases are due to a female factor and 40% due to a male factor. The remaining 20% are the result of a combination of male and female factors, or are of unknown causes. Issues of human infertility are extremely complex physiologically, psychologically, financially, legally and ethically. We discussed this in second chapter. We discussed in the chapter three, the application of embryonic stem (ES) cells to research and therapy has been a landmark development in science. Cell therapy

using ES cells depends on the progress of the stable culture conditions and differentiation induction methods. ES cells were first obtained directly from inner cell masses (ICMs) of blastocysts. These cells can self renew to produce stem cell itself and repopulate into many different tissues, including the somatic- and germ-cell lineages in chimeras. After developing the methods for establishing the ES (mES) cell lines of mice, ES cell lines of other species including primates and human were also established. In the forth chapter we discussed about stem cells, those elusive entities that have the capacity for producing, maintaining and reconstituting the integrity of a biological system, also demonstrate the potential to predict partial or life-threatening damage in response to drugs, environmental compounds and other agents. It is ironic however, that in the animal or human, prior to the manifestation of such potential biological damage most, if not all of the stem cells might have been eradicated. In the chapter five we discussed tissue engineering is a newly emerging biomedical technology and methodology which combines the disciplines of both the materials and life sciences to replace a diseased or damaged tissue or organ with a living, functional engineered substitute. The so-called triad in tissue engineering encompasses three basic components called scaffold, cell and signaling biomolecule. In the sixth chapter, we review studies that have examined the epigenetic instability of ES cells during generation and maintenance cultures, and discuss the candidate factors that may be responsible for this epigenetic instability. iPS cells promise a new paradigm in regenerative medicine. In the eighth chapter we discussed developing iPS technologies have the potential to generate patient specific stem cells, for use in generating any target phenotype within the human body for transplant.

In the research context as well, iPS cells have the potential to greatly advance existing disease models. Patient specific iPS cells could be used to create individualized disease models, potentially allowing for more specialized treatment of patients. Here in chapter seven we discuss a number of the technologies in development seeking to fulfill these promises, as well as their potential applications in both therapeutic and research settings. Acute ischemic injury and chronic cardiomyopathies lead to permanent loss of cardiac tissue, leading to heart failure. For pathologic situations, cell transplantation is thought to be an ideal therapeutic method for supplying de novo myocardium. Of the available cell sources for cardiac cell therapy, stem cells (e.g. pluripotent stem cells, bone-marrow derived stem cells, skeletal myoblasts and cardiac stem cells) are now being prioritized for basic research and clinical trials. The term stem cell includes a large class of cells defined by their ability to give rise to various mature progeny while maintaining the capacity to self-renew. Embryonic stem cells (ESCs) were first isolated from the inner mass of late blastocysts in mice by Sir Martin J. Evans and Matthew Kaufman and independently by Gail R. Martin. Later, it became possible to obtain ESCs from non-human primates and humans. In 1998, James Thomson and his team reported the first successful derivation of human ESC lines, thus extending the great potential of ESCs by providing the opportunity to develop stem cell-based therapies for human disease. This we discussed in chapter nine. In the tenth chapter we discussed about cancer stem cells. Cancer stem cells were proposed in 1994 by John Dick and coworkers as the cells that initiated leukemia [1]. It was thought that this leukemic cell was derived from the mutation of a hematopoietic

stem cell. Importantly, the term was used to distinguish a small subpopulation of leukemic cells that could initiate and maintain cancer from the rest of the leukemic cells that could not. Subsequently, it was also observed in other types of cancer that only a very small subpopulation of cancer cells had the ability to initiate cancer when transplanted into a new host. This subpopulation of cancer cells was considered as cancer stem cells.

Editor

Contents

Chapter 1

EMBRYONIC STEM CELLS FOR THERAPIES–CHALLENGES AND POSSIBILITIES

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INTRODUCTION

The successful establishment of human embryonic stem cells (hESCs) in culture (Thomson et al., 1998) has raised unprecedented public interest and expectation of treating intractable diseases such as diabetes, spinal cord injuries, neurodegenerative and cardiovascular diseases. Much of this enthusiasm was predicated on the unlimited self-renewal capacity of hESCs and their remarkable plasticity in differentiating into every cell type in our body. These features presented the tantalizing possibility of an unlimited cell source in regenerative medicine to generate any tissues to replace injured or diseased tissues. However, translating the potential of hESC into therapies has been challenging. Although translation of hESC has been severely impeded by social and political constraints placed on hESC research through ethical and religious concerns over the destruction of viable blastocysts during hESC isolation, the main challenges have been safety and technical issues.

CHALLENGES IN ESC THERAPY

Overcoming Tumor Formation

The two defining characteristics of ESCs are: 1) their pluripotency, or the potential to differentiate into all cell types in the adult body; and 2) their unlimited self-renewal capacity, or the ability to remain in an undifferentiated state and divide indefinitely. For mESCs, pluripotency is often demonstrated by the production of mESC-derived animals through germline transmission by chimeras resulting from injection of the cells into blastocysts or through tetraploid complementation. In hESCs, proof of pluripotency has been limited to formation of teratomas or teratocarcinomas, which are tumors composed of randomly distributed tissues from the three primordial germ layers in immunologically incompetent mice (Lensch et al., 2007). Karyotypically normal, low passage hESCs form benign teratomas that do not contain undifferentiated tissues and are less invasive (Blum et al., 2009;Reubinoff et al., 2000; Thomson et al., 1998) while high passage hESCs which have become karyotypically abnormal give rise to highly invasive, malignant teratocarcinomas (Herszfeld et al., 2006;Plaia et al., 2006; Werbowetski-Ogilvie et al., 2009; Yang et al., 2008).

Pluripotency coupled with unlimited self-renewal not only define ESCs, they are also the main appeal of ESC as the cell source for regenerative medicine but at the same time, pose significant challenges to the transplantation of differentiated ESCs to replace injured or diseased tissues. The propensity of ESC to differentiate into teratomas necessitates the need to eliminate any residual ESCs in the differentiated cell preparation. There have been many strategies to eliminate residual ESCs or enhance the purity of differentiated ESC preparations. The use of heterologous selectable gene markers such as antibiotic resistance gene or fluorescent protein markers (Klug et al., 1996; M. Li et al., 1998; Muller et al., 2000;Soria et al., 2000) is generally not a strategy of choice as this could introduce potentially deleterious gene mutations. Most of the strategies centered around the use of endogenous markers that are unique or highly expressed on ESCs and not on their differentiated progeny. For example, SSEA-4 and TRA-1-60 which are highly expressed on hESCs have shown

to be highly efficient in physically removing contaminating ESCs by magnetic or fluorescence-activated cell sorters (MACS or FACS) (Fong et al., 2009b). Another strategy exploit the flotation density of cell on discontinuous density gradients such as Puresperm- or Percoll-based gradients (Fong et al., 2009a). Using a relatively novel strategy, Choo et al. has raised antibodies against undifferentiated hESCs (Choo et al., 2008) and identified an antibody that was cytotoxic against hESCs by oncosis. This antibody was an IgM that recognizes podocalyxin-like protein-1(PODXL). hESCs that were treated with mAB 84 did not form teratoma when transplanted into SCID mice even after 18-24 weeks. Therefore, there are viable technologies to remove or reduce residual hESCs in differentiated hESC preparation and mitigate the risk of teratoma formation in patients receiving hESC-based cell therapy..

Overcoming Immunorejection

Like all tissue transplants, hESC-based cell therapy will have to circumvent host immune rejection to engraft in the recipients. One proposed strategy was to establish ESC repositories with lines expressing the combinations of HLA molecules that are compatible with HLA haplotypes present in the population (Nakajima et al., 2007; Taylor et al., 2005). Alternatively, the host's immune system could be manipulated to induce tolerance to foreign tissues by ablation of donor-reactive T cell in the thymus, generation of tolerogenic dendritic cells and induction of T_{reg} cells [reviewed in (Chidgey et al., 2008)]. However, with the development of induced pluripotent stem cell technology that makes the creation of "patient-specific" pluripotent cells containing the same genetic material as the recipient a highly viable and practical option, the issue of host rejection has become a non issue.

The quest to create "patient-specific" pluripotent cells began with therapeutic cloning or somatic cell nuclear transfer (SCNT) where the diploid nucleus of a somatic cell was injected into a haploid enucleated egg to be reprogrammed by soluble factors in the host cell. Upon stimulation, the re-programmed cell divides to form a blastocyst with an inner cell mass that has identical nuclear genetic composition as the nucleus donor. Although this approach has worked to generate ESCs from different animals such as mice,

rabbits, cats, sheep, cattle, pigs, goats [reviewed in (Wilmut et al., 2002)] and even primates (Byrne et al., 2007), no hESC has been generated through this approach as it remains a highly inefficient process and the use of human oocytes is ethically controversial (French et al., 2008;J. Li et al., 2009b). ESCs generated through SCNT are in principle, heterogeneous in their genetic composition as they contain nuclear DNA of the nucleus donor and mitochondrial DNA of the egg donor (Evans et al., 1999). This raises the possibility that SCNT-derived ESCs could be rejected by the innate immune system of the host with which the ESCs share the same nuclear but not mitochondrial genetic material (Ishikawa et al., 2010).

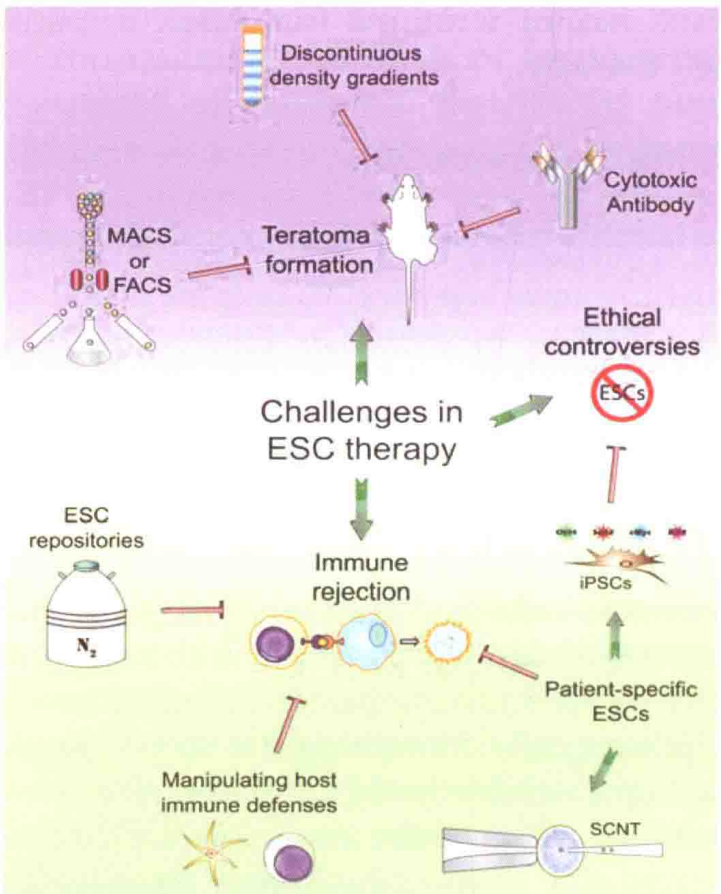

Figure 1. Mitigating tumor formation and immune rejection.

Two of the major challenges to the translation of ESCs into clinical applications are teratoma formation by residual undifferentiated ESCs in the cell preparation and immune rejection

of ESC-derived cells or tissues due to incompatible HLA profiles of ESC and recipient. To mitigate the risk of teratoma formation, several methods to remove residual hESCs have been developed using either physical or biological methods. Some of the physical separation methods are based on magnetic- or fluorescence-activated cell sorters (MACS or FACS) that sort against cells with ESC-associated surface markers, SSEA-4 and TRA-1-60 or on cellular density using discontinuous gradients of Percoll or PureSperm. Alternatively, residual ESCs can be destroyed using a cytotoxic antibody (mAb 84) specific for undifferentiated hESCs. To prevent immune rejection, one strategy proposed the establishment of ESC repositories to carry lines expressing HLA combinations compatible with all possible haplotypes in the population. Alternatively, donor cell tolerance can be induced by manipulating host immune defenses, such as eliminating donor-reactive T cells in the thymus, generating tolerogenic dendritic cells and inducing T_{reg} cells. An ideal approach would be to generate patient-specific ESCs. Some of early efforts include the use of somatic cell nuclear transfer (SCNT). More recently, induced pluripotent stem cell (iPSC) technology has enabled with great ease the generation of self pluripotent stem cells without the destruction of oocytes or embryos, hence bypassing ethical controversies.

The breakthrough in creating "patient-specific" pluripotent cells was achieved when Yamanaka demonstrated that the introduction of transcription factors which regulate ESC self-renewal, including Oct3/4 and Sox2 was sufficient to reprogram somatic cells into ES-like cells (Takahashi & Yamanaka, 2006). These induced pluripotent stem cells (iPSCs) are karyotypically normal with gene expression profiles highly similar to ESCs and can differentiate into cells of all three germ layers (Takahashi et al., 2007; Yu et al., 2007). Apart from being patient-specific, the major attraction of iPSCs lies in their derivation from somatic tissues and not from ethically contentious tissues such as human oocytes or embryos. However, retroviral and lentiviral vectors were required to express the transcription factors for reprogramming of the somatic cells and this carries a risk of insertional mutagenesis. To circumvent the need for viral vectors, non-viral genetic modification approaches were developed (Okita et al., 2008; Soldner et al., 2009; Woltjen et al., 2009). Recently iPSCs were obtained via a direct delivery of reprogramming factors into

cells using poly-arginine protein transduction domains (Zhou et al., 2009) or mRNA (Plews et al., 2010), thereby circumventing any form of genetic manipulation. These improvements have essentially abrogated the issue of host/donor cell immune compatibility and considerably enhanced the prospects of generating patient-specific iPSCs for regenerative medicine. However, a recent study demonstrated that some hiPSC derivatives exhibit limited expansion capability, increased apoptosis and early cellular senescence as compared to their hESC-derived counterparts, raising doubts about the clinical value of this reprogramming technology (Feng et al., 2010). Also, it remains to be determined if the progeny of these cells, which are genetically identical to the reprogrammed cell, will trigger any immune response when reintegrated into the donor.

ESC Differentiation

ESC owes its allure as the source of stem cells for regenerative medicine to two important potentials: 1) unlimited self-renewal potential and 2) the potential to differentiate into all the cell types in an adult. Unfortunately, the recent technological advances to circumvent the risks associated with transplantation of ESC-derived cells, namely teratoma formation and host immune rejection, were not matched by similar progress in differentiating hESCs into cells suitable for regenerative medicine. In contrast to adult stem cells where hundreds of clinical trials have been conducted to evaluate their clinical efficacy, the first testing of a hESC-based therapeutic candidate has only just been initiated. In Oct 2010, Geron Corp announced the enrollment of the first patient to test the safety of human embryonic stem cell (hESC)-derived oligodendrocyte progenitor cells, GRNOPC1, in treating spinal cord injury. With the progress made in reducing the risk of teratoma formation by residual ESC in differentiated ESC preparations and the generation of patient-specific iPSC, the major impediment to the development of hESC-based cell therapies remains the general lack of progress in developing protocols for efficient and reproducible differentiation of hESCs into clinically relevant cell types in sufficient quantity and purity suitable for transplantation studies in clinically relevant large animal models.

The pluripotent differentiation potential of hESCs has always been predicated on their ability to form teratomas in immune-compromised animals and embryoid bodies consisting of tissues from the three germ layers. This ability suggest that differentiation of ESC into the various cell types in the adult animal was not contingent on the presence of an embryonic microenvironment. Instead, it relies on a rather minimal environment that did not support the pluripotent and self-renewing state of the ESCs and bore little resemblance to the dynamically evolving microenvironment of a developing embryo. Nevertheless, much effort to direct differentiation of hESCs into potentially therapeutic cell types have focused on the recapitulation of the embryonic microenvironment based on a yet to be tested rationale that the embryonic microenvironment represents the optimal micro-environment for directed *in vitro*differentiation of ESC.

Recapitulating Embryonic Development to Induce Lineage Commitment

Embryogenesis is a highly dynamic complex process that is still being unraveled despite years of intensive research and much progress in elucidating the molecular and cellular processes involved in formation of an embryo. From a developmental perspective, the ESC represents cells that were frozen in the developmental state of a late-stage embryo just prior to differentiation and lineage commitment. The ability of ESC to re-enter the developmental process and differentiate when returned to the micro-environment of a blastocyst has provided compelling impetus to use the developing embryo to guide and direct *in vitro* differentiation of ESC to a specific cell type. Much effort has therefore been devoted to identifying the molecular cues that were involved in the differentiation of pluripotent cells in the blastocyst into specific terminally differentiated cells. The underlying rationale has always been that a temporal and spatial recapitulation of these cues *in vitro* will direct differentiation of ESC towards a specific cell type.

An early and critical phase of embryogenesis is gastrulation. During this process, the mono-layered blastula undergoes a series of transformation to form the tri-layered gastrula. The formation

of these three germ layers (endoderm, mesoderm and ectoderm) marks the first stage of cell fate determination. This is followed by organogenesis when tissues and organs are formed from further differentiation of the germ layers. The endoderm gives rise to the epithelia of the gut and respiratory system, and organs such as liver and pancreas; the mesoderm gives rise to muscles, the circulatory system, bone and connective tissues; and the ectoderm gives rise to the nervous system and the epidermis. Similarly, the initial step towards deriving functional cells and tissues from ESCs may involve germ layer induction *in vitro*.

The first visible sign of gastrulation is the formation of the symmetry-breaking structure called the primitive streak (PS). Epiblast cells, which are derived from the inner cell mass, ingress through the PS to form the mesoderm and definitive endoderm. The remaining epiblast cells that do not ingress form the ectoderm. Many molecular factors have been implicated in this process and they include members of the large transforming growth factor β (TGFβ) and Wnt signaling families (Conlon et al., 1994;Hogan, 1996; Schier, 2003; Yamaguchi, 2001). Painstaking research has revealed some of the temporal and spatial effects of these factors during embryogenesis and many of these factors exerted similar effects on the differentiation of ESC cells. As reviewed by Murry and Keller (Murry & Keller, 2008)], differentiation of ESCs into each of the three germ layers could be induced by the same factors known to induce them during gastrulation. For example, Wnt, Nodal or BMP4 which have been shown to be important in the formation of epiblast cells in the PS of a developing embryo (Kispert & Herrmann, 1994) could similarly induce the formation of PS-like cells from ESC (Kubo et al., 2004; Lindsley et al., 2006; Ng et al., 2005; Nostro et al., 2008). As in gastrulation, exposure of the PS-like cells to high levels of Nodal further differentiate these cells to a $Foxa2^{hi}$ cells that are comparable to cells in the anterior PS that forms the definitive endoderm (D›Amour et al., 2005; Kubo et al., 2004). In contrast, exposure to Wnt, low level of activin (which activates Nodal) and BMP4 causes the PS-like cells to differentiate into a $Flk\text{-}1^{+}$ posterior PS-equivalent population that forms the mesoderm (Nostro et al., 2008). Therefore, the three germ layers can be induced in ESCs by exposing the cells to factors known to be important in the formation of these three germ