Strategies for Developing Therapeutic Application of Human Embryonic Stem Cells

The ongoing debate on human embryonic stem cells (hESC) is fuelled by ethical concerns but also by the legitimate hope that hESC could one day be used for the cure of presently untreatable human diseases. Here we discuss current approaches to and constraints upon hESC differentiation and describe their potential application in clinical medicine.

The first viable human embryonic stem cells (hESC) were generated in Madison, Wisconsin, by Thompson’s group in 1998 (99). In contrast, murine ESC were isolated and initially described in the 1980s (27, 83) and have been used in many different models ever since, paving the way for current studies on hESC. The promise generated by the possible application of hESC to disease treatment has led to a tremendous interest in stem cell research overall. However, success in the cloning of large animals has raised concerns that stem cell research could eventually result in human cloning (19, 106).

Current methods for ESC derivation are still very elaborate and require specialized labs for the successful derivation of ESC. Two such approaches for the derivation of hESC have been described. A number of hESC lines have been derived by immunosurgery from the inner cell mass of the blastocyst-stage preimplantation embryos produced by in vitro fertilization (99). An alternative to the use of embryos derived after egg fertilization is nuclear transfer (NT), a technique whereby the nucleus of a somatic cell is transferred into an enucleated oocyte. This procedure has already been successfully performed in sheep and bovine cells (19, 106). These results demonstrate a potentially less controversial and ethically more acceptable method of ESC derivation in humans. It appears therefore that stem cell research could eventually result in human cloning (19, 106).

Recent studies have indicated the expression of an immunogenic nonhuman sialic acid N-glycolyneuraminic acid (Neu5Gc) by hESC cultivated in bovine serum (64). As a consequence, if any terminally differentiated cells derived from these hESC were to be transplanted into patients for therapeutic purposes, they would likely be rejected. This critical report emphasizes an urgent need for the development of methods and tools that are unique for hESC to harness their unique properties for future clinical trials without increasing their immunogenicity.
ESC, with a population-doubling period of ~36 h. The hESCs exhibit high levels of telomerase activity even after >300 population doublings and passages for >1 yr in culture (2, 99). hESC express the stage-specific antigens SSEA-3 and SSEA-4; the glycoproteins tumor recognition antigen (TRA)-1–60, TRA-1–81, and TRA-2–54; GCTM-2; trophoblast giant (TG) 343 and 30; CD9 and 133; Oct 4; Nanog; Sox-2; Tfdg1; LeftyA; Rex-1; Stellar; Dazl; Nanos 1; Pum1 and 2; Pumilio 1 and 2; Gd3; Thy-1 cell-surface antigen; and alkaline phosphatase (6, 18, 20, 30, 34, 44, 68, 81, 99). A wide variety of transcriptional factors have been shown to play a crucial role in maintaining the undifferentiated as well as the differentiated state in hESC. The self-renewal of hESC is critically dependent upon a stoichiometric balance among various signaling molecules, and any imbalance among these can cause loss of ESC identity.

Abeyta and colleagues (1) have compared the gene-expression profiles of three hESC lines: HSF-1, HSF-6, and H9. The karyotype of the HSF-6 and H9 lines is 46XX and that of HSF-1 is 46XY. To assess variation among these hESC lines, their ability to contribute to the three germ layers was examined using gene-expression markers indicative of differentiation to endoderm [α-fetoprotein (AFP)], mesoderm (VEGFR2), and ectoderm (NCAM1). The expression patterns of the AFP, keratinocyte differentiation factor, and NCAM1 were found to differ markedly among these three hESC lines. Whether the observed variability is due to karyotypic differences, is a result of random fluctuation in the differentiation process, or is a reflection of variation inherent to each line needs to be investigated further. In addition, all three hESC lines were analyzed for the expression of various important adult stem cell genes. It was reported that 52% of the genes examined were expressed in all three independently derived hESC lines (1). Therefore, a comparative analysis of the transcriptional profiles of the currently available hESCs has the potential to uncover a common set of molecular markers as well as unique expression signatures. If it turns out that every hESC possesses a unique gene-expression signature, it would be a cause of great concern since the application of genetically and epigenetically unstable hESC lines in transplantation could prove detrimental. It is possible that differences in gene expression by hESCs may be an effect of different culture conditions, different passage levels, as well as different gender, which might necessitate standardization of optimal protocols.

**Differentiation Potential of ESC into functional tissues**

Despite the well-recognized pluripotency of hESC, it has proven rather challenging to direct the differentiation of hESC into specific lineages. However, an ESC-based strategy could permit the generation of an unlimited supply of desirable, fully functional cell types from an abundant, renewable, and readily-accessible source. The differentiation of hESC into specific lineages could provide a tool for studying the molecular mechanisms underlying lineage specification, early embryonic developmental pathways, and the pathological basis of genetic disorders, as well as provide a source of transplantable cells and tissue (Figure 1). Here we will focus on the representative differentiation of the pluripotent hESC into pancreatic islets, cardiomyocytes, and neurons, respectively.

**Differentiation of ESC into insulin-producing cells**

Type 1 diabetes affects 4–5% of the total world population, representing a patient population that could benefit immensely from cell-replacement therapy. There are >18 million patients in the United States alone with diabetes mellitus, in addition to 17 and 40 million people who have insulin resistance, impaired glucose tolerance, or metabolic syndrome. The number of patients with diabetes worldwide is predicted to exceed 350 million by 2010. Type 1 diabetes results from the autoimmune destruction of β-cells in pancreatic islets and can be reversed by pancreatic or islet cell transplantation. However, the major limitation to the wider application of this potentially curative therapy is an inadequate source and supply of islets from cadavers. ESC-based therapy for type 1 diabetes represents one of the most compelling areas in regenerative medicine.

The normal human adult pancreas contains ~250,000–1,000,000 distinct microanatomic structures known as the islets of Langerhans. In adults, 70–80% of the islet consists of insulin-producing β-cells, 15–20% either glucagon-producing α-cells or pancreatic polypeptide-producing PP cells, and 5% somatostatin-producing δ-cells. Insulin secretion from pancreatic islet β-cells is a tightly regulated process, under the precise control of blood glucose concentrations, neural inputs, and circulating hormones. The pancreatic β-cells have an amazing capacity for storing insulin such that ~10% of cellular protein consists of insulin. On an average, a single β-cell contains ~13,000 granules and 20 pg of insulin, whereas the adult human pancreas contains ~200 U or 8 mg of insulin.

A detailed understanding of the molecular mechanisms by which glucose and other nutrients trigger insulin secretion will probably be essential to allow for the development of novel therapies including derivation of surrogate β-cells from ESC as a treatment form for type 1 diabetes. Cre-loxP lineage-tracing studies as well as knockout mice have revealed valuable information about the roles of a wide variety of transcription factors involved in pancreatic development (24, 26, 38, 42). As depicted in Figure 2, ESC differentiation into insulin-producing cells requires a series of steps via embryoid body formation into fully devel-
There are several recent reports demonstrating the generation of insulin-secreting pancreatic β-like cells following multistep differentiation of endoderm-derived mouse as well as hESC. For example, Soria and colleagues (93) have developed a cell-trapping system for the differentiation of the mouse ESC into insulin-secreting cells. Transplantation of these insulin-producing cells into the spleen of diabetic mice resulted in the correction of hyperglycemia within 1 wk and restoration of body weight in 4 wk. Employing a similar cell-trapping strategy and a three-step differentiation protocol, their group reported a further improvement in the yields of insulin-expressing cells (59). In contrast, Miyazaki and co-workers (69) have used a Tet-Off system for the regulated expression of Pdx1 levels to enhance insulin production after derivation of islet-like cells. Although Pdx1 expression increased the levels of insulin 2 gene expression, the amount of insulin secreted into the medium was still much lower than that from the adult pancreatic β-cells. However, transplantation of these differentiated cell clusters into streptozotocin-induced diabetic mice failed to lower their blood glucose levels. Interestingly, Lumelsky and colleagues (61) have used a five-step protocol to differentiate mouse ESC into insulin-secreting structures similar to pancreatic islets. Their experimental strategy involves generation and enrichment of nestin-positive cells from embryoid bodies, by employing serum-free medium containing insulin, transferrin, selenium, and fibronectin (ITSFn). These nestin-positive cells were then expanded and further cultivated in media containing B27 supplement and nicotinamide to improve the yield of pancreatic endocrine cells. These differentiated cells expressed low levels of insulin and other pancreatic endocrine hormones, and they self-assembled into three-dimensional clusters that were similar in morphology to normal pancreatic islets. However, transplantation of these insulin-producing cells into diabetic mice failed to correct the diabetic phenotype.

In yet another study, Segev and co-workers (86) demonstrated further differentiation and increased synthesis and secretion of insulin by insulin-releasing
cell clusters by modifying Lumelsky’s protocol (61). Using a different approach, Hori and co-workers (37) have reported improved insulin content and glucose-dependent insulin release following mouse ESC differentiation in the presence of phosphatidylinositol 3-kinase inhibitor LY-294002. Insulin levels in the final population were >50-fold higher, and even when allowance was made for the differences in the percentage of insulin-positive cells produced by the two methods, insulin-positive cells made in the presence of LY-294002 contained about fivefold more insulin and ~10% of the amount found in a normal β-cell. Transplantation of the cell aggregates increased the circulating insulin levels, improved glycemic control, and completely rescued survival in mice with diabetes. The graft removal resulted in rapid relapse and death.

On the other hand, Blyszczuk and co-workers (15) have shown that constitutive expression of Pax4 and to a lesser extent that of Pdx1 modified the differentiation of mouse ESC and promoted the development of insulin-producing cells. Transplantation of these cells into streptozotocin-induced diabetic mice resulted in the normalization of blood glucose levels. More recently, Blyszczuk and co-workers (14) have developed a new strategy for the differentiation of ESC into insulin-producing cells via a multistep process without selection and induction of nestin-positive cells. The differentiated cells released insulin in response to glucose, showed β-cell-specific ion-channel activity, and were able to rescue diabetes in an animal model.

Surprisingly, Ku and co-workers (55) have reported that ESC-derived embryoid bodies exposed to a high concentration of monothioglycerol and serum leading to codevelopment of Flk-1-expressing cells and subsequently cultured in the presence of the β-cell specific and differentiation factors activin βB, nicotinamide, and exendin-4 added at later stages of differentiation further increased insulin-positive cells. Since pancreatic epithelium requires contact from blood vessels to differentiate into insulin-expressing cells (57), it is possible that the codevelopment of Flk-1-expressing cells might play a critical role in the β-cell differentiation process. Exendin-4, a long-acting GLP-1 receptor agonist, has been shown to be capable of stimulating both the differentiation of β-cells from ductal progenitor cells as well as proliferation of β-cells (95, 110, 115). Using a five-step differentiation protocol and GLP-1 or exendin-4, Bai and colleagues (7) have reported a significant increase in insulin content and C peptide secretion by insulin-producing cells derived from mouse ESC.

Recently, Shi and co-workers (89) have developed a three-step differentiation protocol based on the combination induction by activin A, all trans-retinoic acid, and other factors to induce murine ESC to differentiate into insulin-producing cells. Transplantation of the insulin-producing cells was sufficient to normalize blood glucose levels in diabetic mice. However, in these studies, tumor formation was observed in the kidney of some of the mice transplanted with the induced cells. These findings imply that we need an additional step to purify and enrich insulin-producing cells before transplantation so as to minimize or eliminate altogether the danger of tumor formation.

The differentiation of mouse ESC into insulin-producing cells can be further enhanced by the delivery of protein transduction domain (PTD) fused with key transcription factors involved in pancreatic development to ESC and progenitor cells. This novel approach has already shown promising results and has the potential for maximizing the efficacy of ESC differentiation protocols into various types of lineages. Dominguez-Bendala and coworkers (23) have used TAT/ngn3 fusion protein transduction to stimulate pancreatic endocrine differentiation in cultured mouse pancreatic explants. Further, Kwon and colleagues (56) have demonstrated that the transduction of hESC with TAT-PDX1 fusion protein led to activation of downstream target genes and induced insulin production in hESC. These studies provide an excellent opportunity for the development of improved ESC differentiation protocols as well as for exploring the molecular mechanisms underlying pancreatic differentiation.

Although cells containing insulin and various other β-cell markers could be generated from ESC, one of the current controversies is that the insulin staining could be artifactual, reflecting insulin uptake by apop-
Cardiomyocyte differentiation of mouse ESC involves a complex interplay of defined endogenous factors, resulting in the activation of multiple transcription factors that act through intracellular signal-transduction pathways, and by a plex process of extracellular signaling, by multiple bodies. In this direction, the most successful approach so far has been genetic selection using a selection marker driven by a cardiac-specific promoter (51). In this approach, a cardiac-specific fluorescent protein marker is used for enrichment of ESC-derived cardiomyocytes (36, 53, 66). In this approach, a cardiac-specific fluorescent protein marker is used for enrichment of ESC-derived cardiomyocytes (36, 53, 66).

In contrast, the potential differentiation of ESC into cardiomyocytes could lead to a ready source of cells for use in repairing damaged cardiac tissue. Cardiomyocyte differentiation is governed by a complex process of extracellular signaling, by multiple extracellular signal-transduction pathways, and by activation of multiple transcription factors that activate a repertoire of cardiac-specific genes. Cardiomyocyte differentiation of mouse ESC involves a complex interplay of defined endogenous factors, including transforming growth factor-β1 (9), bone morphogenic proteins (9, 47) or their endogenous antagonists (111), fibroblast growth factors (22), nitric oxide (45), and various members of the Wnt/wingless signaling family (98). The induction of mouse ESC into cardiomyocytes has been reported using retinoic acid (98, 107, 112), ascorbic acid (97), dynorphin B (103), 5-azacytidine (28a), and oxytocin (4, 65, 74, 77, 92).

These methods consistently result in the formation of frequently pulsating areas in coordinated, node-like structures 4–22 days after plating, as shown in FIGURE 3. However, the current strategies may not be efficient enough to produce a sufficiently pure population of cardiomyocytes free of nondifferentiated cells for human therapeutic applications.

During in vitro differentiation, cardiomyocytes within the embryoid bodies express cardiac-specific genes, proteins, ion channels, receptors, and signal-transduction pathways in a developmental pattern that closely resembles the developmental pattern of early in vivo cardiomyogenesis (16, 35, 67). The mouse ESC-derived cardiomyocytes express cardiac-restricted genes in a temporally regulated fashion, with the mesodermal genes such as BMP-4 expressed initially followed by the expression of cardiac-restricted transcription factors Nkx2.5 and GATA4. This in turn is followed by the expression of the cardiac-specific structural proteins such as atrial natriuretic factor (ANF), myosin heavy chains (α-MHC and β-MHC), and phospholamban, with the chamber-specific genes such as myosin light chain-2v (MLC-2v) expressed last (16). Studies have suggested that the early differentiated cardiomyocytes are typical of primary myocardium and subsequently differentiate to atrial, ventricle, Purkinje, and pacemaker-like cardiomyocytes (35, 63). Compared with their mouse counterparts, hESC-derived cardiomyocytes show the expected molecular, structural, electrophysiological, and contractile properties of nascent myocardium (33, 48, 70, 109). The precise culture conditions and the particular hESC used are the major factors influencing the efficiency of spontaneous cardiogenesis (48, 70, 109).

Given the undeniably inefficient cardiogenesis within the embryoid bodies, it might be worthwhile to devise strategies for enriching ESC-derived cardiomyocytes from spontaneously differentiating embryoid bodies. In this direction, the most successful approach so far has been genetic selection using a selection marker driven by a cardiac-specific promoter (51). In this approach, a cardiac-specific α-cardiac myosin heavy chain promoter was used to drive the expression of aminoglycoside phosphotransferase, thereby conferring neomycin resistance selectively to the cardiomyocytes in the mixed culture. Following neomycin selection for 8 days, 99.6% of the cells were myosin-positive cardiomyocytes. Using an analogous approach, various other groups have demonstrated that α-cardiac myosin heavy chain, Nkx2.5, or myosin light chain-2v promoter-driven expression of fluorescent protein markers followed by fluorescence-activated cell sorting can be used for enrichment of ESC-derived cardiomyocytes (36, 53, 66). In general, the derivation of cardiomyocytes from ESC is less difficult than the derivation of insulin-producing cells. The obstacle to using this technique is the subsequent purification of cardiomyocytes from nondiff-
ferentiated cells. However, clearly this would be a much preferred approach compared with the use of bone marrow stem cells that are being infused in patients with infarction, as some of these studies have led to arrhythmia (49, 54, 84). Thus the use of predifferentiated cardiomyocytes would be ideal, although data showing improvement of cardiac function after treatment with ES-derived cardiomyocytes are still elusive.

**Neuronal differentiation of ESC**

Neurodegenerative diseases and spinal cord injury represent another set of diseases ideal for ESC-based therapy. Various groups have reported success in differentiating murine as well as hESC into specific neuronal subtypes (17, 50, 80, 85, 104, 114). One of the most compelling approaches for directed neuronal differentiation has come from the laboratories of McKay (50) and Jessell (104). Both of these groups have utilized factors that program neuronal development during embryogenesis and recapitulated the timing and sequence of exposure to direct neuronal patterning during in vitro ESC differentiation. McKay’s group has used the morphogen sonic hedgehog and FGF8 to drive commitment of ESC to ventral midbrain fates and ultimately to tyrosine hydroxylase-positive dopaminergic neurons. Transplantation of these dopaminergic neurons was shown to be functional in a rodent model of Parkinson disease (50). On the other hand, Jessell and colleagues used retinoic acid to program ectodermal commitment followed by exposure to sonic hedgehog to induce the formation of spinal motor neurons that successfully engrafted the embryonic spinal cord of the chick, extended axons, and formed synapses with target muscles (104). Interestingly, Isacson’s group (13) has demonstrated an improvement in a rodent model of Parkinson disease following transplantation of undifferentiated ESC into the striatum, thereby suggesting that the local microenvironment may play a significant role in inducing proper development of dopaminergic neurons.

The differentiation of hESC into highly enriched cultures of neuronal progenitors has been achieved using a two-step protocol (10), involving the use of bone morphogenetic protein antagonist noggin and various mitogens. The vast majority of cells within the neurospheres expressed neural progenitor markers. Successful differentiation of the hESC-derived neural progenitors into midbrain dopaminergic neurons requires the induction of genes that are expressed by the neural progenitors during the development of midbrain in vivo, e.g., Pax2 and Pax5 (108). In this study, the progenitors were grafted into the striatum of Parkinsonian rats, where the grafted cells differentiated into dopaminergic neurons and the transplanted rats exhibited a significant partial correction of D-amphetamine- and apomorphine-induced rotational behavior. Most recently, Li and coworkers (60) have differentiated hESC lines H1 and H9 into neuroepithelial cells using a chemically defined adherent colony culture system. Only the early, not the late, neuroectodermal cells were efficiently posteriorized by retinoic acid and in the presence of sonic hedgehog, differentiated into spinal motoneurons.

Functional motoneurons generated from hESC may be useful for screening pharmaceuticals targeting motoneuron-related disorders such as amyotrophic lateral sclerosis and may provide replacement motoneurons for applications in patients with motoneuron diseases or spinal cord injury. Nistor and colleagues (72) have developed a method for obtaining oligodendrocytes and their progenitors from hESC. Transplantation into the shiverer model of dysmyelination resulted in integration, differentiation into oligodendrocytes, and compact myelin forma-
tion. The neural progenitors derived from hESC differ depending on the inducing agents employed. Retinoic acid treatment of hESC results in the generation of neural cells at various developmental stages (17, 85), similar to spontaneous differentiation using high-density cultures, which also yields similar results (80). In contrast, FGF induction causes a synchronized differentiation of hESC to neuroepithelial cells (114). However, the neurons differentiated from hESC using FGF induction remain physiologically inactive for 3 wk in differentiation cultures. Whether the hESC-derived neural cells incorporate into the adult brain and contribute to neural function is a critical factor for the success of the potential future therapeutic application of hESC in restoring neurological function.

Challenges and Future Directions

Although the potential use of hESC-derived cells for the treatment of diseases is exciting, there are considerable issues that must be overcome before this type of therapy becomes clinically feasible. For example, there is a need for establishing strategies for optimal regulated gene expression and cell-fate determination. A major concern is the possible formation of teratomas (99, 100). As these tumors are nonmetastatic, they do not rapidly kill the host. The tumor growth in immunodeficient animals requires the presence of a stem cell population remaining in partially undifferentiated cultures. If the ESCs fully differentiate into terminally differentiated cells, the possibility of uncontrolled tumor growth can be essentially eliminated. Nonetheless, it will be very difficult to completely remove nondifferentiated hESC from differentiated cultures. One of the strategies to overcome this problem would be to genetically engineer a safety mechanism that would selectively eliminate the transformed ESC. The ESCs derived from nonhuman primates could provide a model for developing novel strategies for countering the immune system and for demonstrating the safety and therapeutic efficacy of ESC-based therapies. Strategies such as transplantation tolerance induction, knockout, or deleting foreign MHC genes; knockin of the desired MHC genes to make them look like self; expression of immunosuppressive molecules like Fas-ligand; and siRNA-mediated inhibition of important immune-stimulating proteins like B7 or CD40 ligand invariably represent approaches to the immunological barrier to ESC transplantation, especially given the fact that hESC can indeed be induced to form hematopoietic stem cells in vitro (46). Finally, to assist in moving the field forward, there is a need for more support at national level so that more groups can move into this exciting area. Although more data are now available in the mouse model, data on hESC are still very limited. A greater understanding of the differentiation of hESC and their gene regulation will accelerate new discoveries and their application in disease treatment. However, despite the current challenges, there is optimism and hope that in the future hESCs might form the basis for novel therapies to treat human diseases and that the derivation of new hESC lines, including those with congenital diseases, will move forward.

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References

The image contains a page from a scientific journal article. The text is in English and discusses various topics related to stem cell differentiation and regeneration. The page includes references to research studies published in journals such as Circ Res, Proc Natl Acad Sci USA, and Nat Med. The text refers to studies on human embryonic stem cells, pancreatic development, and regeneration of ischemic cells. The page also includes a reference to the website www.physiologyonline.org.


