Derivation and Potential Applications of Human Embryonic Stem Cells

Lior Gepstein

Abstract—Embryonic stem cells are pluripotent cell lines that are derived from the blastocyst-stage early mammalian embryo. These unique cells are characterized by their capacity for prolonged undifferentiated proliferation in culture while maintaining the potential to differentiate into derivatives of all three germ layers. During in vitro differentiation, embryonic stem cells can develop into specialized somatic cells, including cardiomyocytes, and have been shown to recapitulate many processes of early embryonic development. The present review describes the derivation and unique properties of the recently described human embryonic stem cells as well as the properties of cardiomyocytes derived using this unique differentiating system. The possible applications of this system in several cardiac research areas, including developmental biology, functional genomics, pharmacological testing, cell therapy, and tissue engineering, are discussed. Because of their combined ability to proliferate indefinitely and to differentiate to mature tissue types, human embryonic stem cells can potentially provide an unlimited supply of cardiomyocytes for cell therapy procedures aiming to regenerate functional myocardium. However, many obstacles must still be overcome on the way to successful clinical utilization of these cells. (Circ Res. 2002;91:866-876.)

Key Words: stem cells • cardiomyocyte differentiation • cell therapy • tissue engineering • myocardial regeneration

One of the most promising areas in basic research today involves the use of stem cells. These unique cells have the capability to transform and replenish the different tissue types that make up the body, and they also represent the fundamental building blocks of human development. In general, stem cells can be divided into two broad categories: adult (somatic) stem cells and embryonic stem (ES) cells. The recent derivation of human ES cell lines from human blastocysts1,2 and human embryonic germ (EG) cell lines from primordial germ cells3 has aroused intense public and scientific discussion. This interest stems in part from the controversy surrounding the origin of these lines but, more importantly, from the widespread conviction that their availability will have a major impact on several scientific areas. The focus of the present review will be to describe the derivation and unique properties of human ES cells. Particular emphasis will be placed on describing the potential research and clinical applications of this new technology in the field of cardiac research. These applications will be discussed in the context of the extensive experience gathered over the years...
from the analogous murine ES cell model and through a
description of the steps required to fully harness the research
and clinical potential of these cells. The ethical and legal
issues associated with human ES cell research will not be
discussed in the present review but have received thoughtful
coverage elsewhere.4–6

Derivation of ES Cells

All stem cells are defined as having two basic properties:
prolonged self-renewal and the potential to differentiate into
one or more specialized cell type. In adults, a highly regulated
process of stem cell self-renewal and differentiation sustains
tissues with high cell turnover. In recent years, this type of
adult stem cell was also described in tissues that were
originally thought to have relatively limited regenerative
capacity, such as the brain and pancreas.7,8 Although adult
stem cells have been found to be more versatile than
originally believed,9 they typically can differentiate to a
relatively limited number of cell types.

In contrast, cells in the early mammalian embryo have the
potential to contribute to all tissue types in the body, a
property that is termed pluripotency. After fertilization, at the
blastocyst stage, a hollow sphere of cells is formed that
contains an outer cell layer and an inner cluster of cells
tumed the inner cell mass (ICM, Figure 1). Whereas the outer
cells become the trophectoderm and subsequently give rise to
the placenta and other supporting tissues, the ICM cells will
ultimately create all tissues in the body, as well as nontro-
phoblast structures that support the embryo and are therefore
truly pluripotent.10

The term ES cells originated from the isolation in 1981 of
pluripotent stem cell cultures from mouse blastocysts by
Evans and Kaufman11 and independently by Martin.12 The ES
cells were discovered to be capable of self-renewal and
prolonged undifferentiated proliferation in culture. However,
because of their origin in the early embryo, ES cells differed
from other stem cells in their ability to retain the potential
to generate derivatives of all three germ layers. Interestingly,
although these cells could proliferate indefinitely in the
undifferentiated state in cultures, the embryonic cells from
which they were derived (the ICM cells), in vivo, are
normally short-lived and soon disappear from the developing
embryo as they give rise to specialized progenitor cells of the
three germ layers.

Given the large impact that the development of the murine
ES cell lines has had on several research fields in the last 20
years, it is not surprising that significant efforts have been
invested in the derivation of human pluripotent stem cell
lines. To date, three pluripotent cell types have been estab-
lished from human tissue: human embryonic carcinoma (EC)
cells,13,14 human EG cells,3 and human ES cells.1,2

Human EC cell lines and their murine counterparts were the
first pluripotent stem cell lines to be established. They
were derived from the undifferentiated stem cell component
of germ cell tumors. The EC clones could be expanded
continuously in culture but could also differentiate to produce
derivatives of all three germ layers either in vitro or through
teratocarcinoma formation. Compared with human ES cells,
however, these cells seem to have less differentiating capac-
ity, and they are usually aneuploid and, therefore, not suitable
for clinical applications.15 Pluripotent human and mouse EG
lines were derived from primordial germ cells in the genital
ridges of the developing embryos, typically at 5 to 9 weeks
after fertilization in humans,3 and were also shown to be
pluripotent.

The origin of human ES cells, in contrast to EC and EG
cells, is from the preimplantation embryo. These cell lines
were derived from the ICM cells of human blastocysts, which
were derived from the ICM cells of human blastocysts,
produced by in vitro fertilization for clinical purposes and
donated by individuals after informed consent. The human ES
cell lines were created in a manner similar to that of
mouse11,12 and rhesus16 ES cells. In this process, the outer
trophectoderm layer of the blastocyst was selectively re-
moved using specific antibodies (immunosurgery), and the
ICM cells were isolated and plated on a mitotically inacti-
Vated mouse embryonic fibroblast (MEF) feeder layer (Figure

Figure 1. Early embryonic mammalian development and deriva-
tion of the ES cell lines. At the blastocyst stage (5 days after
fertilization), the embryo is composed of the trophectoderm and
the ICM, which will eventually will give rise to all tissue types in the
embryo. The ES cell lines were generated from the ICM. These
cells were isolated by immunosurgery and plated on the MEF
feeder layer. The resulting colonies were propagated and
expanded. ES cells can be propagated in the undifferentiated
state while being cultured on top of the MEF feeder layer. When
they are removed from the feeder layer and cultivated in sus-
pension as 3D cell aggregates (EBs), the ES cells differentiate
into specialized cells, including neuronal, hematopoietic, skeletal
muscle, smooth muscle, and cardiac tissue.
1). Cells from the periphery of the colonies that formed were mechanically isolated and replated in the same fashion until homogenous colonies appeared. These colonies were selected, passaged, and expanded for the creation of the ES cell lines.

The human ES cells were demonstrated to fulfill all the criteria defining embryonic stem cells, namely, derivation from the preimplantation embryo, prolonged undifferentiated proliferation in culture under special conditions, and the capacity to form derivatives of all three germ layers.\(^1\) In addition, the human ES lines have also been shown to keep a stable diploid karyotype and to continuously express a high level of telomerase activity during long-term propagation in culture.\(^1,17\)

The pluripotency of ES cells can be established traditionally using three different approaches. Mouse ES cells can be retransferred into early mouse embryos, where they eventually give rise to all somatic cells of the chimeric embryo, including the germ cells.\(^18\) Such a test cannot be applied to human ES cells for obvious ethical reasons. The second approach relates to the demonstration that ES cells can differentiate to generate derivatives of all three germ layers in vivo. When human ES cells were injected into immunodeficient mice, they formed benign teratomas containing advanced differentiated tissue types representing all three germ layers.\(^1,17\) The third approach establishes ES pluripotency during in vitro differentiation. Both mouse and human ES cells, when removed from the MEF feeder layer and allowed to differentiate, could form 3D cell aggregates, termed embryoid bodies (EBs), that contained tissue derivatives of endodermal, ectodermal, and mesodermal origin.\(^19,20\)

The originally described human ES cell lines were not clonally derived from a single cell; therefore, their pluripotency could theoretically be attributed to a population of cells. In further studies, Amit et al\(^17\) produced single-cell clones from the human ES cell lines. This was accomplished by plating individual cells derived from the parental line under direct observation on individual wells containing the MEF feeder. Although the cloning efficiency of the human ES cells was relatively poor, a severalfold increase was observed when serum-free medium supplemented with basic fibroblast growth factor (FGF) was used. The human ES cell-derived clones retained all the properties of the parental line, including prolonged undifferentiated proliferation with a stable karyotype, expression of high levels of telomerase, and the ability to generate teratomas after in vivo transplantation to immunodeficient mice.

**In Vitro Differentiation**

One of the most fascinating and important aspects of ES cell lines is their ability to differentiate in vitro, via precursor cells, into terminally differentiated somatic cells of all tissue types. The mouse ES cells were shown to differentiate in vitro into a variety of cell types, including cardiomyocytes,\(^19\) skeletal and smooth muscle cells,\(^21,22\) neuronal and glial cells,\(^23,24\) hematopoietic progenitor cells,\(^19\) adipocytes,\(^25\) endothelial cells,\(^26\) pancreatic islet cells,\(^27\) and several other tissue types.\(^28\) Ever since the initial report of derivation of the human ES cell lines, a variety of studies have also established in vitro spontaneous and directed differentiation systems to several lineages, including cardiac tissue,\(^29\) neuronal tissue,\(^30,31\) β islet pancreatic cells,\(^32\) hematopoietic progenitors,\(^33\) and endothelial cells.\(^34\)

The most common method used for in vitro differentiation is to remove the ES cells from the feeder layer and to cultivate them into 3D cell aggregates, termed EBs (Figure 1). This aggregation step is required for the differentiation of most cell lineages (except neurogenesis), and it is believed that the cell interactions within the 3D structure are important for differentiation. Different protocols have been used in the murine ES cells for such cultivation, including the “mass culture” technique,\(^19\) cultivation in methylcellulose, or the “hanging drops” technique.\(^35\) In the latter technique, a definite number of mouse ES cells (usually 400) are cultivated in hanging drops for 2 days.\(^36\) The EBs are then collected and further cultivated for an additional 5 days in suspension and then plated on coated culture dishes for further differentiation. Among other cell types, cardiomyocyte tissue could be identified in the outgrowth of the plated murine EBs as clusters of spontaneously beating areas.\(^36\)

Similar to the mouse model, human ES cells, when removed from the MEF feeder layer and cultivated in suspension, begin to differentiate by forming EBs, some of which develop to cystic EBs.\(^37\) Although these EBs were found to be somewhat less organized than the mouse-derived EBs, they characteristically displayed regional expression of embryonic markers specific to different lineages of ectodermal, mesodermal, and endodermal origin.\(^20\)

More recently, we have used methodologies slightly different from those reported in the mouse model to generate a reproducible spontaneous cardiomyocyte-differentiating system from human ES cells.\(^29\) Human ES cells were dissociated into small clumps of 3 to 20 cells and grown in suspension for 7 to 10 days, where they formed EBs. The EBs were then plated on gelatin-coated culture dishes and observed microscopically for the appearance of spontaneous contraction. Rhythmically contracting areas appeared at 4 to 22 days after plating in 8.1% of the EBs.

Several lines of evidence confirmed the cardiomyocyte phenotype of these contracting areas (Figure 2). Cells isolated from the beating areas expressed the cardiac transcription factors GATA4 and Nkx2.5 and cardiac-specific genes, such as cardiac troponin I and T, atrial natriuretic peptide (ANP), and atrial and ventricular myosin light chains (MLCs). Immunostaining studies demonstrated the presence of the cardiac-specific sarcomeric proteins myosin heavy chain, α-actinin, desmin, and cardiac troponin I as well as ANP (Figure 2A). Electron microscopy revealed varying degrees of myofibrillar organization, consistent with the ultrastructural properties reported for early-stage cardiomyocyte (Figure 2B).

The human ES-derived cells also exhibited a functional phenotype of early-stage human cardiomyocytes, including electrical activity, calcium transients, and chronotropic response to adrenergic agents (Figures 2C and 2D). More recently, we have demonstrated that this system is not limited to the differentiation of isolated cardiomyocyte cells but rather that a functional cardiomyocyte syncytium is generated.
with synchronous action potential propagation and pacemaker activity\(^\text{38}\) (Figure 2E).

**Differences Between Human and Murine Models**

Although experience with human ES cells is relatively limited, important differences can already be noted when these cells are compared with the murine model.\(^\text{39}\) Human ES cells form relatively flat and compact colonies that can be easily dissociated into single cells with conventional mechanical and enzymatic techniques. The population-doubling time of human ES cells (36 hours) is significantly longer than that of mouse ES cells (12 hours). In addition, the two sources differ in some of their antigenic phenotypes. For example, human ES cells express stage-specific embryonic antigen (SSEA)-3 and SSEA-4, which are not expressed in mouse ES cells but lack SSEA-1, which is expressed in the latter model.\(^\text{1}\)

One of the most important differences between the two models lies in their in vitro culturing requirements. In contrast to the mouse model in which the undifferentiated propagation of ES cells can be achieved by the administration of leukemia inhibitory factor, the human ES cells require the presence of a feeder layer. This is achieved by propagating the cells on top of the MEF feeder layer in the presence of serum or serum replacement supplemented with basic FGF. More recently, the undifferentiated proliferation of the human ES cells was also described in feeder-free settings using conditioned media from the MEF feeder layer\(^\text{40}\) and also by maintaining the ES cells on a human feeder cell layer.\(^\text{41}\)

In addition to the differences observed between the human and mouse ES cell lines, there are also important variations in the in vitro cardiomyocyte differentiation properties of the lines. Compared with murine cells, human ES cells differentiate in vitro into cardiomyocytes at a slower rate. Furthermore, whereas transmission electron microscopy revealed a
reproducible progressive process of ultrastructural development from an irregular myofilament distribution to a more mature sarcomeric organization during <2 weeks in the mouse model. This process was more heterogeneous, lasted longer, and did not reach the same level of maturity in the human model. These findings are not surprising considering that there are differences in the length of the gestational periods and that the in vivo formation of the human heart takes place during the first 35 days, in contrast with 12 days in the mouse. These differences may represent differences between the species, differences between the cell lines, or differences in the in vitro culturing techniques.

Implication for Basic Research and Clinical Applications

Because of the outstanding potential demonstrated by mouse ES lines, the introduction of human ES cell lines may hold important research and clinical applications. Specifically, important topics related to cardiac research may include the following: (1) investigation of the mechanisms involved in early human cardiac lineage commitment, differentiation, and maturation; (2) derivation of a unique in vitro model to study human cardiac tissue; (3) functional genomics, drug and growth factor discovery, drug testing, and reproductive toxicology; and (4) development of cell-based therapies and tissue-engineering strategies.

In contrast to the fairly well-characterized process of the morphogenetic transformation of the primitive heart into the 4-chambered structure, the inductive clues that lead to specification and terminal differentiation of cardiomyocytes are somewhat less known. Although organogenesis or significant tissue organization does not occur within the EB model, valuable information can be gathered regarding the process involved in lineage commitment and differentiation. In fact, in vitro differentiation within the EB model system may provide a number of advantages over comparable approaches in the whole embryo. First, it provides access to the population of early precursor cells that are difficult if not impossible to identify in vivo. Second, it could allow the study of targeted mutations of genes that may be lethal in vivo but can be studied in vitro. These advantages are even more important for human embryology because of the limited access to early-stage human tissue.

Data derived from amphibian and chick embryos and, more recently, from mouse embryos have suggested that signals emanating from the primitive endoderm in the early embryo may be involved in the processes of cardiomyocyte induction of the precursor cells in the adjacent anterior mesoderm. Recent studies have also identified some of the growth factors that regulate cardiomyogenic induction. These include molecules that promote cardiomyogenesis (bone morphogenetic proteins [BMPs], FGFs, and inhibitors of the Wnt family of morphogens) as well as factors that inhibit the process (Wnt family of morphogens, noggin, and chordin).

Evidence exists that the above-mentioned findings may also be relevant to ES cell differentiation. Coculturing experiments of undifferentiated P19 EC cells, mouse ES cells, and human ES cells with END-2, an endoderm-like cell line, promoted their differentiation into immature cardiomyocytes. Similarly, mouse EBs depleted of primitive endoderm or parietal endoderm did not develop beating cardiomyocytes. Several lines of evidence also indicate a role for extracellular matrix proteins in the regulation of ES cell cardiac differentiation. For example, studies on β1-integrin-deficient mouse ES cells demonstrated impaired cardiac differentiation with delayed expression of cardiac-specific genes and action potentials and impaired sarcomeric organization. Interestingly, these studies also implicate an important role for Wnt-1 and -4 pathways on cardiogenesis, in a manner similar to that of the in vivo findings.

The in vitro formation of cardiomyocytes within the EB also provides a unique tool for the investigation of early cardiomyogenesis. Detailed ultrastructural, immunohistochemical, molecular, and electrophysiological studies have shown that the developmental stages of the mouse ES cell–derived cardiomyocytes in vitro recapitulate those of the in vivo murine heart. During in vitro cardiomyocyte differentiation, a developmentally controlled expression of cardiac-specific genes was noted. The mesoderm-specific genes, such as BMP-4, were expressed initially, followed by gene expression of cardiac-specific transcription factors (Nkx2.5) and structural cardiac proteins, with the chamber-specific genes, such as MLC-2V, expressed last.

The formation of cardiomyocytes within the EB may also provide a unique in vitro tool for investigation of the physiological processes involved in early cardiomyocyte differentiation and maturation. The advent of the murine ES cell model has thus provided important insights into the early stages of development of excitability and electromechanical coupling, including patterns of gene expression, myofibrillogenesis, ion channel development and function, calcium handling, receptor development, and the signal machinery involved in these processes. Moreover, because of the relative ease in genetically manipulating the ES cells, reporter gene constructs may be used in conjunction with early cardiac-specific promoters to identify and study early-stage cardiac precursor cells before the initiation of spontaneous contraction.

Detailed electrophysiological studies of cardiomyocytes within the developing murine EBs have revealed a developmental cascade of ion channel expression and modulation. The noncontracting precursor cells already displayed voltage-dependent L-type Ca2+ channels at very low densities. Cardiomyocytes of a very early differentiating stage had an action potential that was generated by only two main types of ion channels, the L-type Ca2+ channel and the transient K+ channel. Additional ion channels, such as the voltage-dependent Na+ channel, delayed outward rectifying K+ channel, inward rectifying K+ channel, muscarinic activated K+ channel, and pacemaker channel, were demonstrated, only in more differentiated cardiomyocytes. The increased number of different ion channels caused a diversification of cardiac phenotypes, with cardiomyocytes in late-stage EBs displaying a ventricle-like, atrium-like, and Purkinje-like phenotype.

Another important property of the human ES cell–differentiating system is the ability to reproducibly provide differentiated nontransformed cardiomyocytes for long term in...
vitro assessment of cardiac tissue. Although the heart has been thoroughly investigated in its intact form, only a small number of in vitro models are currently available for the study of its structural and functional properties during normal physiological and pathological states. These models include a number of primary cultures, which may be limited by their relatively short-term availability and by the lack of a similar human model. The ability of human ES cells to provide in vitro cardiomyocyte tissue for long-term assessment may also prove invaluable for drug discovery, drug screening, and toxicity testing. Furthermore, by use of the differentiation of the murine ES cells to cardiomyocytes, a standardized in vitro model (the so-called embryonic stem cell test) has already been derived to analyze the embryotoxic effects of chemical compounds.

Another possible important application of the human ES cell–differentiating system is to determine the function of novel genes during differentiation and also in terminally differentiated cells. Because the cardiomyocytes in this model can be derived from a clonal precursor, genetic manipulation may be performed with relative ease. This theoretically may provide a unique system to assess the function of human genes through “loss of function” studies or “gain of function” approaches.

**Developing Transplantation Strategies for Myocardial Regeneration**

The most exciting and frequently discussed application of ES cells is in cell replacement therapy, involving the replacement of diseased, absent, or malfunctioning tissue. The adult heart has a limited regenerative capacity; therefore, any significant cell loss, such as that which occurs during a large myocardial infarction, is mostly irreversible and may lead to progressive deterioration in ventricular function and to the development of heart failure. Congestive heart failure is currently a growing epidemic affecting >5 million Americans, with 400 000 new cases each year. Despite advances in pharmacological, interventional, and surgical therapeutic measures, the prognosis for patients with this disease remains poor. With a chronic lack of donors limiting the number of patients who can benefit from heart transplantsations, development of new therapeutic paradigms for heart failure has become imperative.

One of the most attractive therapeutic approaches for the treatment of heart failure may be the development of myocardial regeneration strategies aiming to replace the dysfunctional myocardium with new contractile tissue. A number of myogenic cells have been suggested as potential sources for tissue grafting, including skeletal myoblasts, fetal cardiomyocytes, smooth muscle cells, murine embryonic stem cells, and bone marrow–derived stromal cells and hematopoietic stem cells. Recent animal studies have shown that cells derived from all sources may survive, differentiate, and even improve cardiac function after cell grafting.

Although a number of myocyte preparations have been used in the aforementioned studies, the inherent electrophysiological, structural, and contractile properties of cardiomyocytes strongly suggest that they may be the ideal donor cell type. Consequently, much effort has been spent over the years in assessing the ability of fetal, neonatal, or adult cardiomyocytes to survive and improve myocardial performance in animal models. In the pioneering work of Soonpaa et al., fetal cardiomyocytes transplanted into mice hearts were demonstrated to survive, align with host cells, and form cell-to-cell contacts with host myocardium. The same group also reported on the formation of stable fetal grafts in the myocardium of dystrophic mice and dogs.

Other studies have demonstrated that fetal or neonatal cardiomyocytes can also be engrafted into infarcted or cryoinjured hearts. An interesting report also suggests that early-stage cardiomyocytes (fetal and neonatal cells) may serve as better candidates than adult cardiomyocytes because of their superior survival rate after transplantation in healthy, injured, and scarred rat hearts. More recently, it was demonstrated in a rat model of chronic infarction that these cells could survive and improve cardiac function for up to 6 months. Cardiomyocyte cell transplantation has been associated with smaller infarcts and has been shown to prevent cardiac dilatation and remodeling after myocardial infarction and to improve ventricular function. The mechanism underlying this functional improvement is not clear. A number of potential mechanisms have been proposed, including direct contribution to contractility by the transplanted myocytes, attenuation of the remodeling process by changing the elastic properties of the scar, and improvement in the function of viable myocardial tissue by the induction of angiogenesis.

Despite these encouraging results, the clinical utility of fetal cardiomyocyte transplantation is significantly hampered by the inability to obtain human fetal cardiomyocytes in sufficient numbers for practical and ethical reasons. In addition, fetal and neonatal cells are prone to immune rejection after transplantation, and they have limited proliferative potential in vitro and are relatively sensitive to ischemic injury. Consequently, significant cardiomyocyte cell death has been reported after cell transplantation, limiting the amount of new myocardium that can be formed.

The derivation of human pluripotent ES cell lines offers a number of potential advantages for cell therapy procedures. Because of their in vitro ability to be propagated in mass and to differentiate into cardiomyocytes with the characteristic electrophysiological, structural, and contractile properties, ES cells can potentially provide an unlimited number of cells for transplantation. Furthermore, specialized subtypes of cardiomyocytes with different phenotypes (eg, atrial, ventricular) can be differentiated in vitro from ES cells and can be tailored to specific procedures. The ES cell cultures could lend themselves to extensive characterization and genetic engineering to promote desirable characteristics, such as resistance to ischemia and apoptosis and improved contractile function.

Despite the exciting advances in human ES research, much basic work is still required, and several obstacles remain to be overcome before this technology can enter any serious clinical practice. Specifically, a number of important milestones need to be achieved: (1) Strategies need to be developed for directing in vitro differentiation to a specific lineage. (2) Selection protocols must be devised to generate a pure
population of cells of a specific lineage (cardiac) or even of a specific cell type (eg, ventricular, atrial). (3) Culturing techniques need to be upscaled for mass production, yielding clinically relevant quantities of cardiomyocytes. (4) A transplantation technique should be developed, and important issues regarding the in vivo properties of the graft should be resolved. (5) Antirejection regimens need to be developed.

Directed Differentiation and Cardiomyocyte Enrichment Strategies

Although both human and mouse ES cells have been demonstrated to differentiate spontaneously in vitro to cardiomyocyte tissue using the EB system, the cues involved in this process are still largely unknown. Moreover, cardiomyocyte cells account for only a minority of the cells within the EBs, and spontaneously contracting areas are not observed in all EBs, even less so in the human model.

Strategies for directed differentiation may follow development in the understanding of the biological processes involved in early cardiomyocyte differentiation or may follow a trial-and-error approach using different growth factors, transcription factors, feeder layers, and physical perturbations. Some evidence suggests a possible role for a number of soluble factors in promoting cardiomyocyte differentiation. These include members of the transforming growth factor-β family, retinoic acid, leukemia inhibitory factor, and other factors when provided at the appropriate timing and dosage. However, the extent to which these different factors may actually promote cardiogenesis is still unknown. A similar strategy may be applied to promote differentiation of a specific sublineage phenotype. Recent studies have identified a number of transcriptional factors and growth factors that may play a role in the specification of atrial, ventricular, and conduction system cells.

Evidence also exists indicating that undifferentiated human ES cells and their more differentiated derivatives express receptors for various growth factors. Hence, supplementation of the human ES culture medium with 8 different growth factors altered the expression profile of an array of tissue-restricted genes. Although some of these growth factors were shown to increase differentiation to mesoderm, ectoderm, or endoderm derivatives, none of the growth factors directed differentiation exclusively to one cell type.

Cell-Selection Strategies

Although differentiation toward a specific lineage may be enhanced somewhat by the addition of growth factors, the degree of purity that will be achieved will probably be insufficient for clinical purposes. Besides the obvious need for larger number of cardiomyocytes, a selection process may be required to avoid the presence of other cell derivatives and to ensure the absence of remaining pluripotent stem cells in the graft. The latter may be crucial to prevent the possible generation of ES cell–related tumors, such as teratomas. A similar strategy may also be necessary to select for the specific cardiac cell types (eg, atrial, ventricular, or pacemaking cells) required for different cell therapy strategies.

Given the heterogeneous cell mixture within the EB, derivation of a relatively homogenous cell population will ultimately depend on the selection of a specific cell type from the mixed population of cells. An elegant and relatively simple approach to generate pure cardiomyocyte cultures was first demonstrated by Field’s group (Klug et al⁷³) in the murine ES model. This approach is based on the use of a tissue-specific promoter to drive a selectable marker, such as an antibiotic resistance gene. Using this approach, Klug et al generated, by stable transfection, an ES cell line carrying a fusion gene composed of the α-cardiac myosin heavy chain (α-MHC) promoter and a cDNA encoding aminoglycoside phosphotransferase (neo⁵). The ES cells were then allowed to differentiate in vitro and were subjected to selection with the appropriate antibiotic (G418). Using the selection process during in vitro differentiation, the authors have shown that >99% pure cardiomyocyte cultures could be generated. The selected cardiomyocytes were further demonstrated to form stable grafts after transplantation into adult dystrophic mice hearts.

Interestingly, a similar enrichment strategy has also been successfully attempted in murine ES cells to produce purified cultures of neurons⁸² and insulin-secreting pancreatic β cells⁷² using the appropriate promoters.

An alternative approach could involve the introduction of a gene construct containing a tissue-specific promoter/enhancer controlling the expression of a green fluorescence protein gene. Selection of the desired cells can then be performed using fluorescence-activated cell sorting. Using this approach, Muller et al⁹³ transfected murine ES cells with a construct encoding a cytomegalovirus enhancer and a ventricle-specific (MLC-2V) promoter driving the green fluorescence protein product. The use of Percoll gradient centrifugation and subsequent fluorescence-activated cell sorting yielded 97% pure cardiomyocyte fractions.

Both of the above-mentioned approaches would require the development of efficient gene transfer methodologies for human ES cells. In a recent report, Eiges et al⁹⁴ reported on the establishment of human ES cell–transfected clones carrying a marker for undifferentiated cells. In that report, various transfection methodologies were used, and their efficiency in introducing DNA into human ES cells was compared.

Upscaling Methodologies and Strategies for Prevention of Immune Rejection

In addition to the conceptual milestones that remain to be achieved for the development of any clinical application, several technical obstacles also need to be overcome. These include the development of mass production methods to yield a clinically relevant number of cells, replacement of animal-related products used during human ES culturing, quality-control testing, and development of methods for storing and shipping the cells, such as cryopreservation.

A major barrier for the possible use of human ES cells in cell transplantation strategies is the generation of sufficient numbers of cardiomyocytes. It is estimated that a typical myocardial infarction that induces heart failure kills ≈1 billion cardiomyocytes; (≈20 million myocytes/g)×(≈200 g/left ventricle)×(≈25% of left ventricle infarcted). Generation of this number of cells can be achieved by increasing the initial number of ES cells used for cardiomyocyte differen-
Another major consideration and significant obstacle for the possible clinical utilization of the ES cells is the expected immune-mediated rejection of the ES cell–derived grafts. Although a detailed discussion of this issue is beyond the scope of the present review, we will briefly discuss some of the strategies suggested to deal with this problem.

The first question to be contended with is as follows: Precisely how immunogenic are tissues derived from ES cells? In a recent report, human ES cells were shown to express low levels of MHC class I proteins. This expression was only moderately increased during in vitro and in vivo differentiation but was significantly augmented after treatment of the cells with interferon-γ. In contrast, MHC class II antigens and the ligands for NK cell receptors were not expressed on the surface of the ES cells or their derivatives. The absence of MHC class II antigens on human ES cells and their cardiomyocyte derivatives may be important because cells expressing these proteins, such as B cells, macrophages, dendritic cells, and endothelial cells, are believed to be highly immunogenic. This may provide an inherent immune advantage to human ES cell–derived grafts, which could possibly require milder immunosuppressive regimens. In addition, strategies aimed at reducing the mass of alloreactive T cells are being developed, and these and other novel therapies with particular relevance to the anticipated immune response.98,99

Other approaches for reducing graft rejection may be the generation of a panel of ES cell lines to allow matching of the major histocompatibility determinants and genetically modifying the ES cells to reduce their immunogenicity. The latter can be achieved by engineering a “universal cell donor” by knocking out the major histocompatibility complexes97 or by inserting or deleting other genes that can modulate the immune response.98,99

The concept of hematopoietic chimerism was demonstrated in bone marrow–transplanted patients receiving a solid organ transplant from the same donor, in which no immune suppression was required to prevent rejection.100 The same concept theoretically can be applied also to ES cell–derived transplantation using the same ES cell line to derive both lymphohematopoietic stem cells and cardiomyocyte tissue. Tolerance can then be induced by initially achieving hematopoietic chimerism followed by cardiomyocyte cell transplantation.

Derivation of a human ES cell line specifically for each patient with the use of somatic nuclear transfer technology is another approach that has been suggested to overcome tissue rejection. Although the feasibility of this scenario has already been demonstrated in a bovine population101 in which immunocompatible tissue grafts were generated, a similar therapeutic cloning strategy in humans would be exceptionally controversial. In addition, the poor availability of human oocytes and the technical difficulties involved in the process of the creation of each ES cell line makes this approach unlikely to become a routine clinical procedure.

In Vivo Transplantation

Although changes in the remodeling processes, prevention of ventricular dilatation, and improvement in the ventricular diastolic properties have been reported after the transplantation of a variety of cell types into the infarcted region, systolic augmentation would depend on the functional integration between graft and host cardiomyocytes. This would require the long-term survival of the grafted cells, the presence of a critical tissue mass, the appropriate alignment of the donor cells, and their structural and functional integration with the host tissue.

Several questions and issues remain to be addressed in this area.66 First, the size of the cell graft may have important applications for its ultimate success in improving the ventricular mechanical function. Cell death occurring after engraftment is believed to have a major negative impact on graft size.83,84 Hence, cell survival after transplantation may depend on adequate vascularization of the graft, which may require additional revascularization procedures or induction of angiogenesis. In that respect, the ability to genetically manipulate the ES cell derivatives may be used to generate cell grafts that are more resistant to ischemia or apoptosis, that display larger proliferative capacity, that can secrete angiogenic growth factors, or that may be coupled with ES cell–derived endothelial progenitor cells. Additional factors that remain to be determined in future studies include the ideal nature of the graft (individual cells, small cell clumps, or combined with scaffolding biomaterials), the appropriate delivery method (epicardial, endocardial, or the coronary circulation), and the timing of cell delivery relative to the timing of the infarct.66

The ability to generate potentially unlimited numbers of cardiomyocytes ex vivo from human ES cells may bring a unique value also to tissue-engineering approaches. This new discipline combines functional cells with 3D polymeric scaffolds to create tissue substitutes.102 These biodegradable scaffolds can be engineered to provide adequate biomechanical support for the cell graft, to control graft shape and size, and to promote angiogenesis. Recently, scaffolds that may allow mechanical contraction have also been described.103

An important aspect related to the possible utilization of ES cell–derived cardiomyocytes for cell transplantation strategies relates to the safety of these procedures. A major safety concern is the possible development of ES cell–related tumors. As described above, injection of undifferentiated ES cells into immunodeficient mice has resulted in the generation of teratomas.1 Hence, as ES cells are coaxied to differentiate in vitro to terminally differentiated cardiomyocytes, efforts need to be made to ensure that the cell graft is depleted of any undifferentiated stem cells before transplantation. In that respect, studies involving in vivo transplantation of differentiated ES cell derivatives have failed to show any evidence of teratoma or the presence of any other ES cell–derived tumor.30,31,73

A second major concern relates to the development of cardiac arrhythmias after cell transplantation. Besides the
known increased risk for malignant ventricular arrhythmias in patients with reduced ventricular function, cell transplantation may modify the electrophysiological properties of the scar and theoretically may change (increase or decrease) the propensity for development of arrhythmias.

**Summary**

During the last 20 years, mouse ES cells have revolutionized many areas of modern biology. The derivation in 1998 of human ES cell lines was accompanied by significant enthusiasm but also by major public debate. Although research using human ES cells is still at its “embryonic” stage, important research accomplishments have already been achieved. These include the derivation of a number of ES cell lines and clones, verification of the pluripotent nature of human ES cell lines both in vivo and in vitro by creation of teratomas and EBs, respectively, and the establishment of in vitro differentiating systems to generate variable tissue types, including cardiomyocytes.

In addition, basic methodologies for large-scale cultures have been developed, initial functional and morphological studies of ES cell–derived cardiomyocytes have been performed, and the initial groundwork for genetic manipulation of these lines has been established. On the basis of these initial achievements, together with the extensive experience gathered from the murine model, we can predict that human ES cells may be useful in the future in a variety of cardiovascular research areas, including human developmental biology, functional genomics, pharmacological testing, tissue engineering, and gene- and cell-based therapies. Nevertheless, important knowledge is still lacking in many of these areas, much basic work is required, and several obstacles need to be overcome before any clinical breakthroughs can be expected.

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**References**

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Lior Gepstein

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