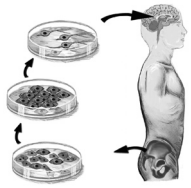

Regenerating the Heart Using Human Embryonic Stem Cells – from Cell to Bedside

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Key words: embryonic stem cells, myocardial regeneration, stem cell transplantation

Abstract

The adult human heart has limited regenerative capacity and, therefore, functional restoration of the damaged heart presents a great challenge. Despite the progress achieved in the pharmacological and surgical treatment of degenerative myocardial diseases, they are still considered a major cause of morbidity and mortality in the western world. Repopulation of the damaged heart with cardiomyocytes represents a novel conceptual therapeutic paradigm but is hampered by the lack of sources for human cardiomyocytes. The recent derivation of pluripotent human embryonic stem cell lines may provide a solution for this cell sourcing problem. This review will focus on the derivation of the hESC lines, their mechanism of self-renewal, and their differentiation to cardiomyocytes. The possible signals and cues involved in the commitment and early differentiation of cardiomyocytes in this model will be discussed as well as the molecular, structural and electrophysiologic characteristics of the generated hESC-derived cardiomyocytes. Finally, the hurdles and challenges toward fully harnessing the potential clinical applications of these unique cells will be described.

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Regeneration of body organs is a complex biological phenomenon that is limited only to some tissues and to a few species of vertebrates. The heart is one of the least regenerative organs and only a limited number of species (e.g., newts and zebrafish) are capable of renewing the heart. Since the human heart has a limited regenerative capacity, we can only venerate the ability of these organisms. Nonetheless, recent advances in the area of stem cell biology have provided scientists with potential tools to develop novel strategies for myocardial regeneration.

Stem cells can be derived from various sources including embryonic, fetal and adult tissues. All types of stem cells share a number of defining characteristics. First, they should be capable of self-renewal. Second, they must have the ability to differentiate into one or more mature cell types. Third, stem cells should be clonogenic, meaning that as single cells they are capable of spawning colonies of various differentiated cell types. In the last decade, various types of stem and progenitor cells aimed at regenerating the myocardium have been proposed. These include committed skeletal myoblasts, bone marrow-derived hematopoietic and mesenchymal stem cells, mouse and human embryonic stem cells, as well as several resident cardiac progenitor cells. In this review, we will focus on the ability of hESC to differentiate to cardiomyocytes, and discuss the opportunities and challenges they present for clinical translation.

Self-renewal and differentiation

The identification of embryonic stem cells from mouse blastocysts a quarter of a century ago represents a major advance in mammalian biology. The potential therapeutic applications and the unique opportunity to study early mammalian development exemplified by the murine embryonic stem cell model motivated researchers to establish similar human embryonic stem cell lines [1,2]. The establishment of hESC lines was achieved by explanting inner cell mass cells from the blastocyst into culture dishes of mitotically inactivated fibroblast feeder cells. The source for the blastocysts used for the creation of the hESC lines were supernumerary early-stage embryos derived by *in vitro* fertilization, originally for clinical purposes, and donated by individuals after informed consent. The hESC were grown in medium containing serum and plated on top of mouse embryonic fibroblast feeder layer cells. These conditions allow continuous undifferentiated propagation of the cells in culture [Figure 1]. However, from the perspective of therapeutic applications, a xeno-free and serum-independent culturing system is essential for ensuring high reproducibility and minimizing the risk for xenozoonoses. Hence, supporting systems based on human tissues were recently suggested as alternatives to the mouse embryonic fibroblast feeder cells [3]. Nevertheless, the secret of youth, the exact combination of factors that maintain the stemness of the cells, has not yet been fully elucidated.

In recent years, some of the key regulators mediating the ability of embryonic stem cells to self-renew have been described. The maintenance of the ESC self-renewal ability depends upon both extrinsic and intrinsic factors. The transcription factors oct-4, sox-2 and the recently identified nanog are the intrinsic factors

hESC = human embryonic stem cells

ESC = embryonic stem cells

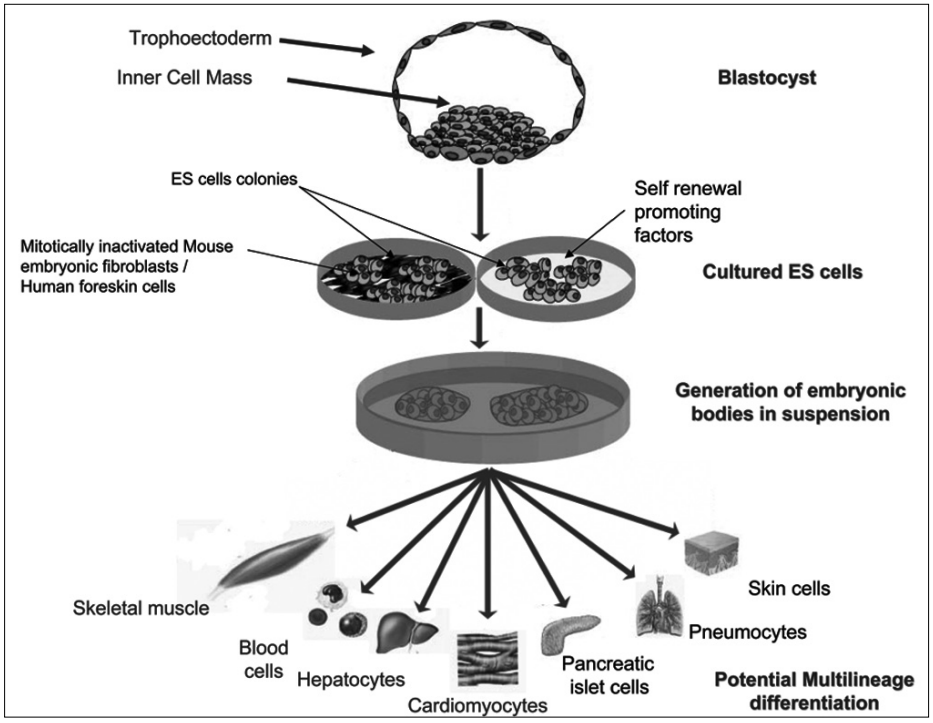

Figure 1. Early embryonic development and hESC isolation, propagation, and *in vitro* differentiation.
The hESC lines can be generated from human blastocysts. At this stage, the pre-implantation embryo is comprised of the trophoectoderm and the inner cell mass, which eventually will give rise to all tissue types in the embryo. The hESC lines were generated by isolating the inner cell mass cells by immunosurgery, and plating and propagating them on the mouse embryonic fibroblast feeder layer, human foreskin cells, or self-renewal promoting factors. When removed from these conditions and grown in suspension they begin to generate three-dimensional differentiating cell aggregates that are termed embryoid bodies. This *in vitro* differentiating system may be used to generate a plurality of tissue types, including skeletal muscles, hematopoietic cells, liver cells, cardiomyocytes, pancreatic islet cells, lung cells and keratinocytes.

needed for the maintenance of self-renewal in both the human and mouse models. However, the extrinsic factors mediating stemness of ESC differ between the mouse and the human models. The essential extrinsic signals in the mouse model include the bone morphogenic protein 4, which inhibits differentiation to neuroectoderm, and leukemia inhibitory factor, which inhibits differentiation toward endoderm and mesoderm [4]. In contrast, inhibition of BMP signaling via fibroblast growth factor-2 and noggin has been suggested as crucial extrinsic factors for the maintenance of hESC pluripotency [5]. Notwithstanding the recent advances, our understanding of the molecular mechanisms that govern hESC self-renewal is far from perfect and further studies are required to establish a reproducible, well-defined, animal and serum-free supporting system that may be upscaled and provide a safer alternative for future clinical applications of hESC.

When removed from the factors that maintain them in the undifferentiated state, hESC can differentiate, under appropriate conditions, to form cell derivatives of the three embryonic germ layers: endoderm, mesoderm and ectoderm. Since the initial report of the generation of the hESC lines, they have been shown to differentiate into cardiac tissue [6], neuronal tissue including dopaminergic cells [7], beta-islet pancreatic cells [8], hematopoietic progenitors [9], keratinocytes, bone tissue [10] and endothelial cells [11].

In vitro differentiation of hESC to cardiomyocytes

The generation of a cardiomyocyte-differentiating system from the clonal hESC line (H9.2) was originally described by Kehat et al. [6] from our laboratory. The induction of *in vitro* differentiation of hESC to cardiomyocytes requires their removal from the feeder layer and cultivation in suspension. The hESC then tend to generate three-dimensional differentiating cell aggregates termed embryoid bodies. Following 7–10 days in suspension, the embryoid bodies are cultured in adherent conditions and observed microscopically for the appearance of spontaneous contraction. Rhythmically contracting areas appear at 4–22 days after plating in ~8% of the embryoid bodies [6]. Recently, other groups reinforced our findings and generated hESC-derived cardiomyocytes using different clonal and non-clonal cell lines [12-14].

The hESC model may provide valuable information regarding the pathways involved in early cardiac lineage commitment, differentiation and maturation. The currently available cardiac differentiation system of the hESC is essentially spontaneous, however, and thus characterized by relatively low efficacy. One of the major obstacles for the utilization of these cells in future myocardial regeneration strategies is the insufficient number of cardiomyocytes achieved by the currently available differentiation scheme.

The development of a directed differentiation system is hampered by the relative lack of data regarding the inductive cues that lead to commitment and terminal differentiation of human cardiomyocytes. The heart arises from cells in the anterior lateral plate mesoderm of the early-stage embryo [15]. The anterior endoderm is in direct contact with the cardiac crescent and provides factors required for cardiac induction. The cardiogenic inductive role of the primitive visceral endoderm was also demonstrated to play a role in the cardiomyocyte differentiation of the hESC line. Co-culturing of a hESC line (hES2), which

BMP = bone morphogenic protein

does not regularly differentiate spontaneously to cardiomyocytes, with END-2 cells (a visceral endoderm-like cell line) provided the missing trigger for cardiac differentiation [13]. In terms of defined endogenous factors, various genetic and biochemical perturbations in several organisms have shown a key role for BMP members of the transforming growth factor-beta superfamily in specifying and/or maintaining the myocardial lineage. Along with the BMP-related proteins, additional factors such as fibroblast growth factors, TGF-β2, and members of the WNT/wingless signaling pathway [16] were also implicated in cardiac induction in the mESC system. In addition, a number of small molecules have also been reported to promote cardiogenesis in mESC. Xu and colleagues [14] demonstrated enhancement of cardiac differentiation in the hESC model by using 5-Aza-2'-deoxycytidene, but surprisingly not by dimethyl sulfoxide or retinoic acid [14]. However, even identification of the exogenous cardiac-promoting factors is not enough to generate a recipe for producing cardiomyocytes from hESC. In order to achieve such an ambitious goal, the exact concentration and timing for each factor should also be identified.

Structural maturation of hESC-CM

The temporal gene expression pattern of hESC-CM indicates that the cells are isolated at a relatively early stage of human cardiomyocyte development. Hence, it is not surprising that when grown in the absence of maturation-promoting factors, hESC-CM display a relatively immature cardiac phenotype. Respectively, light and electron microscopy studies revealed that during early stages of differentiation, hESC-CM had a large nucleus to cytoplasm ratio with disoriented myofibrils distributed throughout the cytoplasm in a random fashion. Nevertheless, at later stages of differentiation (~20–50 days) an ultrastructural maturation process was observed with the development of well-aligned sarcomeres accompanied by the development of the known sarcomeric striated pattern with recognizable A, I, and Z bands [6,17]. The period required for ultrastructural maturation of the hESC-CM is significantly longer than in the murine model, probably due to the differences in the gestation periods in both species.

Electrophysiologic characteristics of hESC-CM

The hESC-CM were shown to display functional properties, consistent with an early-stage, human, cardiac tissue phenotype. These include the presence of typical extracellular electrical activity, intracellular action potentials, and ionic currents. In addition, the hESC-CM displayed appropriate chronotropic responses to adrenergic and cholinergic stimuli, demonstrating the presence of functional receptors and signaling pathways. High resolution activation maps using a multi-electrode array electrical mapping system demonstrated the presence of functional electrical syncytium with spontaneous pacemaker activity and action potential propagation [18]. Immunostaining studies showed that this functional syncytium results from the formation of gap junctions between the cells. Moreover, the ability of the hESC-CM to integrate structurally and functionally with host cardiac tissue (a prerequisite for true systolic augmentation following myocardial cell transplantation) was also assessed in our laboratory. In these studies, the hESC-CM contracting areas were co-cultured with primary cultures of neonatal rat ventricular cardiomyocytes and were shown to integrate structurally and functionally with host cardiac tissue and to generate a single functional electrical syncytium [19].

Ion channel expression in hESC-CM

Similar to the mESC model, whole-cell patch clamp studies demonstrated that hESC-CM also display cardiac-specific action potential morphologies and ion currents. Additional work from our laboratory revealed the basis for the spontaneous automaticity in these cells, namely the absence of significant inward rectifier K^+ current coupled with a prominent Na^+ sensitive current and the presence of the hyperpolarization and cyclic nucleotide-activated ion channels pacemaker current [20]. The paucity of the inward current creates a high input resistance state that allows a small inward current to bring the membrane potential to threshold. Collectively, the mechanism of spontaneous activity of hESC-CM is fundamentally different from the murine model where the calcium channel $I_{Ca\text{-}L}$ plays a crucial role in action potential initiation. Clearly, future electrophysiologic and molecular characterization of the developmental cascade of cardiac ion channels using the hESC model will provide a unique insight into the development of excitability and the excitation-contraction coupling in early human cardiac tissue. This will help in identifying a threshold hESC-CM differentiation and maturation stage from which cells could be applicable for grafting while minimizing the risk for arrhythmia induction.

Regeneration of the functional myocardium

Heart failure is a growing epidemic in the western world due to the increasing aging of the population and the improved post-infarction survival rates. Heart diseases are the leading cause of death of Israeli citizens over age 65 (Ministry of Health, www.health.gov.il) and worldwide, with the failing heart accounting for ~7.2 million deaths annually (World Health Organization, www.who.int/whosis). Cell replacement therapy is emerging as an innovative therapeutic approach for the treatment of degenerative heart diseases. This therapeutic approach is based on the assumption that myocardial function may be improved by repopulating diseased areas with a new pool of functional cells. Based on this assumption a number of myogenic cells have been suggested for tissue grafting as mentioned above.

Skeletal myoblasts

The autologous origin and the high availability of adult skeletal muscle cells led to their application in recently conducted phase I and II clinical trials [21]. However, although previous reports suggest that these cells may have the ability to adopt cardiac-like phenotype following cardiac transplantation, it is now clear that

TGF-β2 = transforming growth factor-beta
mESC = murine embryonic stem cells
hESC-CM = hESC-derived cardiomyocytes

they do not possess such capabilities [22,23]. Importantly, of the first 22 patients reported in the literature to undergo skeletal myoblast transplantation, 10 experienced significant ventricular arrhythmias. Although a direct causal relationship is hard to prove in these initial non-controlled trials, given the expected high incidence of ventricular arrhythmias in this patient population, this potentially life-threatening side effect warrants further consideration.

Hematopoietic stem cells

Studies in animal models of ischemia as well as initial clinical trials suggested that delivery of hematopoietic stem cells may result in the eventual improvement of ventricular function. However, the initial assumption regarding the capability of bone marrow-derived hematopoietic stem cells to regenerate the heart by transdifferentiation to cardiomyocytes [24] was recently challenged by a number of studies that demonstrated cell fusion of the progenitor cells with the host cells [25,26]. Moreover, recent studies suggest that the hematopoietic stem cells continue to differentiate along the hematopoietic lineage [27,28], and the possible left ventricular functional improvement noted may not be related to transdifferentiation into the cardiac lineage, but rather, may result from indirect mechanisms such as their ability to enhance blood vessel formation. Despite the controversy related to the ability of bone marrow stem cells and especially hematopoietic stem cells to transdifferentiate to cardiomyocytes, increasingly more clinical trials are being conducted. Recently, Wollert et al. [29] reported from their clinical trial that intracoronary injection of unfractionated mononuclear cells resulted in 6% improvement when compared with controls. Initial clinical studies demonstrated the safety and feasibility of bone marrow stem cell injection to the heart with a possible improvement in cardiac performance. However, in our opinion, beyond the need for more randomized controlled clinical trials assessing cell transplantation efficacy, additional studies elucidating the mechanism by which the cells exert their positive effect on the infarcted myocardium are necessary.

Mesenchymal stem cells

The non-hematopoietic, stromal compartment of the bone marrow contains a promising multipotent stem cell population termed mesenchymal stem cells. These cells were suggested to differentiate to cardiomyocytes both *in vitro* and *in vivo* and their transplantation to the infarcted myocardium of rats and pigs resulted in improved cardiac function [30]. One possible advantage of mesenchymal stem cells is their ability to be delivered either in autologous procedures or using an allogeneic strategy; some reports suggest that they may be relatively immunoprivileged. These apparent attractive capabilities of the cells have led researchers to launch phase 1 clinical trials that have yet to be published.

Resident myocardial progenitors

The long-held belief that the adult mammalian heart has no intrinsic capacity for repair was recently brought into question when four types of resident myocardial progenitors were described. These include c-kit$^+$, sca-1$^+$, islet-1$^+$ and Hoechst dye effluxing side population cells. However, given the limited regenerative capacity of the adult heart, it is clear that the presence of the above-mentioned cells within the heart does not translate to a functionally significant cardiac differentiation following myocardial infarction. In addition, the description of four non-overlapping, discrete, progenitor cell populations in a non-regenerating organ may stem from the immature nature of this promising research area.

Embryonic stem cells

The derivation of the hESC lines offers a number of potential advantages over the currently available candidate donor cells. hESC are currently the only cell source that can potentially provide, *ex vivo*, an unlimited number of human cardiac cells for transplantation. Second, the ability of ESC to differentiate into a plurality of cell lineages may be utilized for transplantation of different cell types such as endothelial progenitor cells for induction of angiogenesis, and even specialized cardiomyocyte subtypes (pacemaking cells, atrial, ventricular, etc.) tailored for specific applications. Third, due to their clonal origin, the ESC-derived cardiomyocytes could lend themselves to extensive characterization and genetic manipulation to promote desirable characteristics such as resistance to ischemia and apoptosis, improved contractile function, and specific electrophysiologic properties. Fourth, the ESC-derived cells could also serve as a platform and a cellular vehicle for different gene therapy procedures aiming to manipulate the local myocardial environment by local secretion of growth-promoting factors, various drugs, and angiogenic growth factors. Last, the ability to generate potentially unlimited numbers of cardiomyocytes *ex vivo* from the hESC may also bring a unique value to tissue engineering approaches.

The availability of the mouse ESC model taught us important lessons about the potential myocardial regenerative applications of ESC. By using genetically selected mESC-derived cardiomyocytes (as detailed below), Klug and team [31] demonstrated that engraftment of the cells into the immunocompatible mouse heart resulted in the successful formation of stable intracardiac grafts. Interestingly, a study conducted by Puceat's group [32] indicated that undifferentiated mESC engrafted to immunocompetent mouse or rat heart survive, integrate and improve the myocardial function of the infarcted heart. Recently, Menard et al. [33] reported that cardiac committed mESC (following incubation with BMP-2) transplanted to the infarcted sheep heart differentiate to mature cardiomyocytes. Moreover, cell transplantation resulted in a significant improvement in cardiac function independent of whether the sheep were immunosuppressed or not [33]. These studies imply that mESC-derived cardiomyocytes may be immune-privileged and suggest that the host heart plays an instructive role in directing the differentiation of undifferentiated or cardiac-committed mESC and preventing the generation of teratomas. However, according to Laflamme and Murry [34], transplantation of undifferentiated mESC results in the formation of teratomas and these teratomas are rejected when non-syngeneic or immu-

notolerant rats are used. Similarly, in our hands, undifferentiated hESC transplanted to the uninjured myocardium in immunosuppressed rats formed teratoma-like structures and did not exhibit robust differentiation to cardiomyocytes (unpublished data).

Optimal functional improvement following cell grafting would require structural, electrophysiologic and mechanical coupling of donor cells to the existing network of host cardiomyocytes. In a recent study [19] we tested the ability of the hESC-CM to integrate structurally and functionally with host cardiac tissue both *in vitro* and *in vivo*. Initially, the ability of the hESC-CM to form electromechanical connections with primary cardiac cultures was assessed in a high resolution *in vitro* co-culturing system. Primary cultures were created from neonatal rat ventricular myocytes. The contracting areas within the embryoid bodies were then mechanically dissected and added to the co-cultures. Interestingly, within 24 hours post-grafting, we could detect synchronous contraction in the co-cultures that persisted for several weeks. Detailed studies of the hybrid cultures using the multi-electrode array mapping technique demonstrated tight electrophysiologic coupling between the two tissue types. The electrophysiologic assessment was reinforced by demonstrating the development of gap junctions between the human and rat cells as suggested by connexin 43 immunostainings.

To demonstrate the ability of the hESC-CM to survive, function and integrate also in the *in vivo* heart we assessed their ability to pace the heart and to function as a "biological pacemaker." In order to examine this possibility, the cells were transplanted to the posterolateral region of the left ventricle in a swine model of slow heart rate. Following cell grafting a new ectopic ventricular rhythm was detected in 11 of 13 animal studies, in 6 of which it was characterized by sustained and long-term activity. Pathologic studies validated the presence and integration of the grafted hESC-CM at the site of transplantation. Three-dimensional electrophysiologic mapping revealed that this ectopic ventricular rhythm originated from the area of cell transplantation. Recently, our findings were reinforced in a study conducted by Xue et al. [35] using a similar model of guinea pig heart block and demonstrating cell integration by optical mapping of the epicardial surface. Other studies demonstrated the ability of grafted hESC-CM to survive within the uninjured athymic rat heart for as long as 4 weeks without the generation of teratomas. Interestingly, the authors report that application of heat shock prior to transplantation results in a threefold increase in graft size.

From cell culture to bedside – challenges for clinical translation of hESC-CM [Figure 2]

Line characterization and development of good manufacturing practice culture conditions

For effective clinical translation of the enormous potential of hESC, characterization of the wide range of hESC lines representing the diverse human genetic pool is required. A meticulous characterization will also have to include epigenetic analysis of the various lines assessing for phenomena like autosomal imprinting and X-chromosome inactivation. Establishment of uniform criteria for hESC line characterization is required in order to give scientists a "common language" for comparing data between and within cell lines. Examination of the hESC for a minimal set of pluripotency markers, gene expression profile, and cytogenetic and epigenetic features may help in reducing the current heterogeneity between available hESC lines. Importantly, each cell line should also be evaluated for extended periods, assuring that no modifications occur during prolonged passaging. The essence of uniform characterization criteria is stressed in light of recent studies reporting karyotype abnormalities in hESC [36].

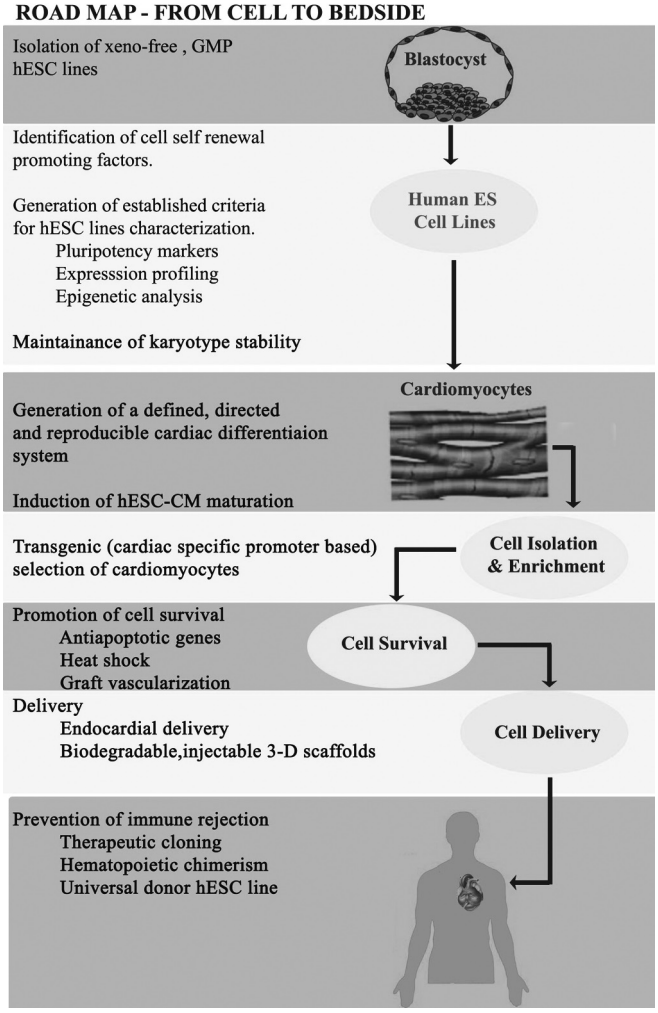

Figure 2. A road map – from cell to bedside. A proposed road map describing the challenges toward clinical translation of hESC-CM-based myocardial regeneration. A detailed list of the challenges is outlined on the left. (GMP = good manufacturing practice).

Selection and purification strategies

Although the development of directed differentiation systems is essential for increasing the cardiomyocyte yield from the hESC, it is unlikely that the degree of purity obtained would be sufficient for clinical purposes. Since the contracting areas derived from hESC comprise a mixed population of cells, selection strategies are crucial not only for increasing cardiomyocyte numbers but also for preventing the presence of other cell derivatives, as well

as ensuring the absence of pluripotent stem cells carrying the risk of teratomas. In addition, a similar strategy may aid in selecting specific cardiac cell types. An example for an elegant selection scheme to generate pure cardiomyocyte populations was the one suggested by Klug and team [31] in the mESC model. This scheme is based on genetically modifying the ESC by transfection with a fusion gene encoding for resistance to neomycin under the regulation of the cardiac specific α-myosin heavy chain promoter. Based on this approach, cardiomyocytes can be selected based on the resistance for neomycin.

Genetic and tissue engineering

Increasing the survival of engrafted cells is a critical issue since the hostile ischemic and scar environment may result in significant cell death. Since adequate vascularization of the graft tissue is critical to its survival, the potential ability to generate engineered hESC-CM resistant to apoptosis and ischemia as well as capable of promoting graft vascularization by secretion of angiogenic growth factors may bring an added value to these procedures. An alternative approach would be to generate vascularized cardiac tissue by combining both cardiomyocytes and endothelial cells in a similar manner to that produced by Levenberg [37] using skeletal myoblasts. Future studies should also determine the ideal nature of the graft and the optimal method for its delivery (e.g., direct injection of dissociated cells, or combined with scaffolding biomaterials). Additional factors to be determined include the ideal degree of differentiation of the transplanted cells and the appropriate delivery method (such as epicardial injections, transendocardial or via the coronary circulation).

Immune tolerance

A major obstacle for the utilization of hESC derivatives in regeneration of different tissue types is the prevention of their immune rejection. The hESC were shown to express relatively low levels of HLA class I molecules. This expression was only moderately increased after differentiation *in vitro* (to embryoid bodies) and *in vivo* (to teratoma cells), but was significantly augmented following interferon-gamma treatment. No expression of HLA class II molecules and the ligands for natural killer cell receptors was detected on the hESC or their differentiated products. The elegant human/mouse trimera model, enabling the assessment of the allo-immune response of the human immune system to transplanted hESC, was recently used to elucidate the immune co-stimulatory characteristics of hESC. Engrafted undifferentiated and differentiated hESC elicited only a minute immune response over the course of one month. These results imply that immunosuppressive regimes for future hESC-based therapeutics may be highly reduced in comparison to conventional organ transplantation [38]. Several strategies aimed at achieving immunologic tolerance were suggested. One of the most promising strategies is based on the generation of isogenic hESC lines tailored specifically for each patient. Recently, Hwang and co-workers [39] demonstrated the feasibility of this concept to derive hESC lines from an enucleated oocyte following somatic nuclear transfer.

Summary and future potential research directions

The derivation of the hESC lines and the resulting cardiomyocyte differentiation system may bring a unique value to several basic and applied research fields. Research based on the cells may help to elucidate the mechanisms involved in early human cardiac lineage commitment, differentiation and maturation. Moreover, this research may promote the discovery of novel growth and transcriptional factors using gene trapping techniques, functional genomics and proteomics, as well as providing a novel *in vitro* model for drug development and testing. Finally, the ability to generate, for the first time, human cardiac tissue provides an exciting and promising cell source for the emerging discipline of regenerative medicine and myocardial repair. Yet, despite the enormous potential of these technologies, as discussed above, several hurdles need to be overcome before this strategy can become a clinical reality.

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