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Cardiac Applications for Human Pluripotent Stem Cells

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Abstract

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) can self-renew indefinitely, while maintaining the capacity to differentiate into useful somatic cell types, including cardiomyocytes. As such, these stem cell types represent an essentially inexhaustible source of committed human cardiomyocytes of potential use in cell-based cardiac therapies, high-throughput screening and safety testing of new drugs, and modeling human heart development. These stem cell-derived cardiomyocytes have an unambiguous cardiac phenotype and proliferate robustly both in vitro and in vivo. Recent transplantation studies in preclinical models have provided exciting proof-of-principle for their use in infarct repair and in the formation of a “biological pacemaker”. While these successes give reason for cautious optimism, major challenges remain to the successful application of hESCs (or hiPSCs) to cardiac repair, including the need for preparations of high cardiac purity, improved methods of delivery, and approaches to overcome immune rejection and other causes of graft cell death. In this review, we describe the phenotype of hESC- and hiPSC-derived cardiomyocytes, the state of preclinical transplantation studies with these cells, and potential approaches to overcome the aforementioned hurdles.

INTRODUCTION

Heart disease is among the most important therapeutic targets for the emerging field of regenerative medicine. Most of the pathological processes that commonly initiate irreversible heart failure (e.g. ischemic heart disease, cardiomyopathies) either result from or are exacerbated by a loss of cardiomyocytes, while many other cardiac diseases might be effectively treated by the replacement of specialized cardiac structures (e.g. congenital heart disease, “sick sinus” syndrome). Hence, one of the primary goals of cardiovascular regenerative medicine is to use stem cells to generate large quantities of human cardiomyocytes for such applications. A large number of stem and progenitor cell types have been reported initially to differentiate into cardiomyocytes either in culture or following intra-cardiac transplantation, including skeletal myoblasts [1-3], hematopoietic stem cells [4], and mesenchymal stem cells [5-8]. Unfortunately, many of these were subsequently shown to have limited or no cardiomyogenic potential in follow-up studies (for a comprehensive review, see refs [9,10]). At present, the two human stem cell sources that are generally regarded as having the greatest cardiomyogenic potential are resident cardiac stem cells and pluripotent stem cells (ESCs and the related induced pluripotent stem cells (iPSCs)).

While still at an early phase of preclinical development, human ESCs and iPSCs have a number of attractive qualities for cell-based cardiac repair and related applications. First and foremost, as reviewed in detail below, both of these pluripotent stem cell types have unquestioned

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cardiomyogenic potential [11-13], which places them in stark contrast to many adult stem cell types for which this capacity is controversial. Second, hESCs and hiPSCs and their cardiac progeny are readily expandable, an important consideration given that a typical human infarct involves the loss of more than one billion cardiomyocytes [14]. Undifferentiated hESCs and hiPSCs retain their phenotype through many more than a hundred population doublings, and differentiated pluripotent stem cell-derived cardiomyocytes proliferate robustly both in vitro [15-17] and in vivo following intra-cardiac transplantation [18]. (See Figure 1.) Finally, while the isolation of other candidate stem cell populations has sometimes proven difficult to reproduce, hESCs and hiPSCs have been derived by many laboratories using well-established protocols. The techniques used to generate hiPSCs are particularly straightforward, as demonstrated by their successful derivation by multiple independent laboratories despite having only been relatively recently described. This is an often overlooked issue—if a candidate cell for therapy cannot be reliably obtained for preclinical studies by sophisticated research labs, it is difficult to imagine its successful use in widespread clinical application.

In this review, we describe the phenotype of hESC- and iPSC-derived cardiomyocytes, as well as approaches to their derivation and maturation. We also consider their prospects for diverse applications, including infarct repair, in vitro toxicology screens, cardiac pacemaking, and suppression of arrhythmias. Finally, we outline potential approaches to overcome the major challenges remaining to the successful application of these cells, including the need for preparations of high cardiac purity and strategies to overcome immune rejection and other causes of graft cell death.

HUMAN PLURIPOTENT STEM CELLS: DERIVATION, PHENOTYPE, AND MAINTENANCE IN THE UNDIFFERENTIATED STATE

Embryonic stem cells

Murine ESCs were first isolated in 1981 [19,20], and their human counterparts in 1998 [21]. In both cases, ESCs are derived from a specialized population of cells present in mammalian blastocysts, the inner cell mass, which ultimately gives rise to all tissue types of the developing embryo. All currently available hESC lines were derived from pre-implantation stage human blastocysts that were no longer intended for clinical use, and so were donated after informed consent rather than discarded. While beyond the scope of this review, ethical and legal concerns regarding hESC research have continued, resulting in efforts to develop alternative, nondestructive approaches to derive hESCs, for example, using blastomere “biopsies” from morula-stage embryos [22]. Reprogramming somatic cells into iPSCs (discussed further below) represents another approach to obtaining cells with ESC-like properties that does not involve the destruction of human embryos.

Undifferentiated hESCs have two defining properties: First, hESCs can be propagated indefinitely as a stable, self-renewing population. Second, they have a pluripotent phenotype, meaning they can differentiate into cell types belonging to all three embryonic germ layers, as evidenced by their capacity to form benign teratomas following transplantation into immunodeficient animals [19-21]. Given appropriate culture conditions, ESCs have been induced to differentiate into a variety of somatic cell types, including multiple cell types relevant to the cardiovascular system (e.g. endothelial cells, smooth muscle cells, and cardiomyocytes). Thus, there is tremendous interest in the use of hESCs as a model of human heart development, a tool for drug discovery and arrhythmia safety screening, and a source of human cells for cardiac repair.

Several culture systems have been developed to maintain and expand hESCs in the pluripotent state. Historically, the most commonly employed approach has been to culture hESCs directly

on a feeder layer of mouse embryonic fibroblasts (MEFs) or human feeder cell alternatives [23-26], which release an as yet incompletely characterized set of factors that support the proliferation and self-renewal of undifferentiated hESCs [27-29]. However, because direct co-culture is undesirable for cell transplantation applications, our group has generally preferred to use a feeder-free, conditioned medium system [30]. More recently, several defined media formulations involving specific growth factors (usually including fibroblast growth factor-2 (FGF-2)) have been developed [28,31-35], reflecting our improved understanding of hESC self-renewal. Continued progress toward the identification of reliable and inexpensive defined medium alternatives for hESC culture will be required if the field is to transition from the benchtop to large-scale production and fulfill the good manufacturing practice requirements needed for clinical applications.

Induced pluripotent stem cells

The cloning of Dolly proved that differentiation can be reversed and that mammalian oocytes contain trans-activating factors that are sufficient to “reprogram” the epigenetic status of a differentiated nucleus back to the pluripotent state [36]. To screen for factors capable of mediating a similar reprogramming in cultured somatic cells, the Yamanaka group transduced mouse fibroblasts with a panel of retroviral vectors, each encoding for a candidate reprogramming gene, and eventually identified Oct4, Sox2, Klf4, and c-myc as the minimal set of factors sufficient to convert the fibroblasts to an ESC-like phenotype [37]. The pluripotency of the resultant murine iPSCs was rigorously confirmed by multiple techniques, including in vitro differentiation into cell types from all three embryonic germ layers, teratoma formation after transplantation in mice, and contribution to chimeric mice following injection into blastocysts [37-40]. A number of groups subsequently reported the independent generation of human iPSCs by reprogramming human somatic cells with overexpression of either Oct4, Sox2, Klf4, and c-Myc or Oct4, Sox2, Nanog, and LIN28 [41-44]. Human iPSCs exhibit a phenotype very similar to hESCs: they express typical hESC markers, show trilineage differentiation in vitro, and form teratomas after transplantation in immunodeficient mice. As discussed further below, hiPSCs appear to have a cardiac potential comparable to hESCs, and hiPSCs can be differentiated into cardiomyocytes using similar techniques [41].

Human iPSCs are quite new, and so it is difficult to assess their prospects for use in regenerative medicine. On the one hand, hiPSCs have two major potential advantages over hESCs: 1) ethical objections to the destruction of human embryos are avoided, and 2) hiPSCs could, in principle, be used in transplantation medicine in an autologous fashion. On the other hand, there are important safety concerns regarding the derivation of hiPSCs that must be overcome if these cells are to reach mainstream clinical application. The most obvious of these concerns is that the genome-integrating retroviral vectors presently used to introduce the reprogramming factors may result in neoplastic transformation. Fortunately, recent data gives reason for optimism that safer methods of reprogramming without integrating viruses are possible, e.g. gene delivery via non-integrating adenoviral vectors [45] or plasmid transfection [46] or substitution of small molecules for the genetic factors [47,48]. Another less understood safety issue is the functionality and phenotypic stability of the “redifferentiated” cells derived from hiPSCs. For example, our group has found that hiPSC-derived cardiomyocytes (hiPSCCMs) have an immunophenotype and functional properties comparable to hESC-CMs (data not shown), but we cannot, as yet, exclude subtle but potentially important differences in their phenotype, proliferative capacity, viability, or ultimate maturation potential. Given their novel origin, such characterization is essential before hiPSC-CMs (or other hiPSC derivatives) reach clinical application.

CARDIOMYOCYTES FROM HUMAN PLURIPOTENT STEM CELLS

In vitro phenotype of hESC-CMs

Multiple laboratories, including our own, have reported the successful derivation of cardiomyocytes from hESCs, using either embryoid body (EB) differentiation [11,16] or the guided differentiation approaches [12,49] discussed in more detail below. Regardless of the method of cardiac induction, the resultant hESC-CMs show an unambiguous cardiac phenotype. As demonstrated by RT-PCR and immunohistochemical studies, hESC-CMs express early cardiac-specific transcription factors (e.g. Nkx2.5, GATA4, myocyte enhancer factor 2C (MEF2c), Tbx-5, and Tbx-20 [11,16,17,50-52]), sarcomeric proteins (e.g. α -actinin, cardiac troponins I and T, sarcomere myosin heavy chain (MHC), atrial- and ventricular-myosin light chains (MLC2v and MLC2a), desmin, and tropomyosin [11,12,16,51-53]), gap junction proteins [11,12,16,54,55], as well as other cardiac and muscle-specific proteins (e.g. atrial natriuretic peptide (ANP), creatine kinase-MB and myoglobin [11,16]). At the ultrastructural level, hESC-CMs show clearly identifiable sarcomeres and intercalated discs [13,16,47].

Cultured hESC-CMs also exhibit the expected functional properties of primitive cardiomyocytes. As in mature cardiomyocytes, the trigger for contraction in hESC-CMs is a rise in intracellular calcium, and these cells do exhibit calcium transients [12,56,57]. Consistent with previous findings in murine ESC-derived cardiomyocytes, hESC-CMs show unambiguous cardiac-type action potentials (AP), but with comparatively immature AP parameters (e.g. automaticity, a slower AP upstroke, a relatively depolarized maximum diastolic potential) that gradually transition toward somewhat more mature values with increasing duration in culture [53,58]. Interestingly, despite their apparent electrophysiological immaturity, hESC-CMs are comprised of an admixture of myocytes with nodal- and “working-” (i.e. ventricular- and atrial- chamber) like AP phenotypes [12,53]. In voltage-clamp studies, hESC-CMs exhibit expected ionic currents, including fast sodium current, L-type calcium current, pacemaker currents, as well as transient outward and inward rectifier potassium currents [12,58,59]. Finally, hESC-CMs show dose-dependent inotropic and chronotropic responses to isoproterenol, showing they have intact β -adrenergic signaling [11,16,53,60].

In vitro phenotype of iPSC-CMs

At present, there is considerably more data available regarding the phenotype of murine iPSC-CMs. The Martin group has described the phenotype of cardiomyocytes generated from a miPSC line via embryoid body differentiation [61], while the Yamashita group obtained cardiomyocytes from three other miPSCs lines by co-culturing mesodermal miPSC derivatives with OP9 mouse stromal cells [62]. In both cases, the resultant miPSC-CMs showed an unambiguous cardiac phenotype comparable to that of mESC-CMs. miPSC-CMs expressed expected cardiac markers including α -myosin heavy chain, cardiac troponin T, and ANP [61, 62]. They also exhibited spontaneous contractile activity that could be modulated by isoproterenol or carbachol, indicating intact neurohumoral signaling [61]. Finally, miPSC-CMs exhibited both spontaneous and caffeine-induced calcium transients, indicating functional sarcoplasmic reticulum calcium stores [61]. Of some concern, the Yamashita group reported that prolonged in vitro culture of miPSC-CMs resulted in the occasional re-expression of reprogramming factors, including the proto-oncogene c-Myc [62], a finding that emphasizes the need for improved methods of reprogramming.

To our knowledge, the only published characterization of hiPSC-CMs to date appears in the original report by the Yamanaka group describing the isolation of hiPSCs, and their description of the cardiac phenotype of the resultant hiPSC-CMs was quite limited. To induce

cardiogenesis in hiPSCs, these authors used a directed differentiation protocol previously developed by our group for hESCs (see Figure 2B and below) and showed that the differentiating cultures exhibited spontaneously contracting foci and expressed cardiac markers (e.g. Nkx2.5, MEF2c, and β -myosin heavy chain) by RT-PCR. In preliminary studies, our own group has found that this protocol induces cardiogenesis in at least two other hiPSC lines and that the resultant cardiomyocytes show an immunocytochemical, electrophysiological, and contractile phenotype indistinguishable from hESC-CMs (data not shown).

APPROACHES TO DERIVING CARDIOMYOCYTES FROM HUMAN PLURIPOTENT STEM CELLS

“Guided” cardiomyogenic differentiation

Historically, the most common method by which cardiomyocytes have been derived from murine and human ESCs has involved their differentiation via spontaneously forming three dimensional aggregates, so-called embryoid bodies (EBs) [11,63]. In this approach, ESCs are dispersed, removed from the conditions needed to maintain pluripotency, and are placed in suspension cultures, typically in the presence of a high percentage of fetal calf serum. The resultant EBs, which include an admixture of differentiating cell types, are then re-plated onto two-dimensional substrates. Several days later, a subset of EB outgrowths will show cardiac differentiation, as indicated by the appearance of rhythmically contracting foci. Unfortunately, the cardiac purity of these cell preparations is typically quite low. In 2001, Kehat et al. reported that less than 10% of human EBs showed spontaneous beating activity [11], a finding consistent with our own group's experience that cardiomyocytes typically comprise <1% of total hEB-derived cells [18,49].

For hESC-CMs to reach serious consideration for clinical applications, preparations of a much higher degree of cardiac purity will be required, and these should be obtained using well-defined and animal product-free culture conditions. Fortunately, information gleaned from models of cardiac development has led to protocols with improved cardio-inductive efficiency. Figure 2 depicts the three guided cardiac differentiation protocols that have resulted in the highest degree of cardiac purity to-date.

The first approach, developed by the Mummery group, was inspired by developmental studies indicating the critical role of anterior endoderm in the cardiac induction of adjacent mesodermal structures [64-66]. The Mummery group had previously shown that undifferentiated murine P19 embryonal carcinoma cells undergo cardiogenesis when grown in medium conditioned by END-2 cells, a visceral endoderm-like derivative of P19 cells [67]. To validate this approach with hESCs, they co-cultured END-2 cells with the hES2 hESC line, a line which does not spontaneously form EBs [12]. (See Figure 2A.) A subset of the hES2 hESCs differentiated into definitive cardiomyocytes, as confirmed by immunocytochemical, electrophysiological, and calcium imaging studies. These investigators were able to further enhance the efficiency of cardiogenesis from hESCs/ END-2 co-cultures by either eliminating serum from the medium used during co-culture [68] and/or adding a small molecule inhibitor of p38 MAP kinase [69]. These modifications to their protocol reportedly resulted in preparations of ~20-25% cardiomyocytes.

At least a portion of the cardio-inductive activity of anterior endoderm is mediated by growth factors belonging to the transforming growth factor- β (TGF β) superfamily [70,71], and our group recently reported a guided cardiac differentiation protocol involving two TGF β family members, activin A and BMP 4 [49]. (See Figure 2B.) In this approach, hESCs are maintained throughout in monolayer culture. After growth to confluency in either MEF-conditioned

medium [30] or other defined medium substitutes for maintaining pluripotency, the cells are sequentially treated with activin A and BMP4 in serum-free medium (Fig. 2B). The growth factors are then removed, and the cells are maintained in serum-free medium for an additional 2-3 weeks in the absence of exogenous growth factors. Spontaneously contracting areas are usually observed approximately 10 days post-induction with activin A, and enzymatically dispersed preparations at three weeks post-induction typically consist of > 30% cardiomyocytes. As noted above, this cardiac induction protocol appears to work comparably well with hiPSCs [41].

A third protocol, developed by the Keller group, involves TGF β family molecules but also exploits the important roles of canonical Wnt signaling in cardiogenesis. Canonical Wnt signaling exerts stage-dependent effects on cardiac differentiation: it is required for mesoderm induction but must be inhibited later for the induction of pre-cardiac mesoderm [72,73]. Based on this information, the Keller group developed a guided differentiation protocol for hESC-CMs, involving induction of a primitive streak-like population and mesoderm with activin A, BMP4, and bFGF, followed by cardiac specification with the Wnt inhibitor, dickkopf homolog 1 (DKK1) [74]. This protocol reportedly generates populations consisting of ~40-50% cardiomyocytes.

The Keller group's guided differentiation protocol was further enhanced by sorting the differentiating cultures for an early cardiovascular progenitor based on expression of the Flk-1 tyrosine receptor kinase (otherwise known as vascular endothelial growth factor receptor 2 or KDR). In previous studies in the mESC differentiation system, these authors had shown that early differentiating mEBs include two KDR+ populations: an early hemangioblast population (i.e. hematopoietic and endothelial progenitors) and a later multipotent cardiovascular progenitor population [75]. These temporally distinct populations are also present in hESC derivatives generated using the Keller induction protocol, and, at 5-6 days post-induction, they can be distinguished based on their differential expression of KDR and the stem cell marker c-Kit [74]. In particular, if KDR^{high}/c-Kit+ cardiovascular progenitors are selected by fluorescence-activated cell sorting (FACS) at this timepoint and then replated in monolayer cultures, they subsequently differentiate into highly enriched preparations of cardiomyocytes, endothelial cells, and smooth muscle cells. (See Figure 2C.)

Purification of stem cell derived cardiomyocytes

While the field has made tremendous progress in the development of guided differentiation protocols that are efficiently cardiogenic, none of the currently available protocols result in homogeneous preparations of cardiomyocytes. Fortunately, there are multiple approaches by which such preparations can be further purified. The most straightforward approach is mechanical dissection of the spontaneously beating areas within hEB outgrowth cultures. While microdissected preparations from even relatively low-purity hEB-derived cells can include up to 70% cardiomyocytes, it is difficult to imagine scaling up this extremely laborious approach to obtain the cell numbers required for clinical applications [76]. While also fairly laborious, a more scaleable method is Percoll gradient centrifugation, which takes advantage of the unique buoyant properties of cardiomyocytes. In our hands, Percoll centrifugation results in a three- to seven-fold enrichment of hESC-CMs when applied to enzymatically dispersed cells from hEBs [16,18] or cultures "guided" by treatment with activin A and BMP4 [49].

To-date, the highest levels of cardiac purity have been obtained using genetic selection. In this strategy, undifferentiated hESCs are genetically modified to carry either a fluorescent marker or mammalian selection gene (e.g. antibiotic resistance) under the control of a cardiac-specific promoter. The transgenic cells are then induced to differentiate and then selected based on activation of the cardiac-specific promoter. While the major disadvantage of this approach is that it entails the usual risks of genetic modification (e.g. insertional oncogenesis), it is capable

of an impressive degree of cardiac enrichment. This was first demonstrated over a decade ago in a pioneering study by the Field group [77]. These authors stably transfected undifferentiated murine ESCs with a transgene in which expression of the aminoglycoside phosphotransferase gene (i.e. neomycin resistance, which confers resistance to the antibiotic G418 in mammalian cells) was driven by cardiac-specific α -myosin heavy chain. The transgenic mESCs were differentiated using the EB system and then were selected based on G418 resistance, resulting in preparations of >99.6% purity from mESC-CMs. This selection strategy was subsequently adapted to generate and purify mESC-derived cardiomyocytes on a large scale [78,79]. Genetic selection based on activation of either the human α -MHC [80] and MLC2v promoters [81] has been shown to generate preparations of >90% hESC-CMs.

CARDIAC TISSUE ENGINEERING WITH PLURIPOTENT STEM CELLS

The technological advances described in the preceding sections provide for a theoretically unlimited supply of functional, proliferative, and, in the case of hiPSCs, perhaps autologous human cardiomyocytes. With these cells now in hand, attention must turn to how they might be employed clinically. To date, preclinical transplantation studies with hESC-CMs have followed the approach used with other candidate cell types for cardiac repair, namely the direct injection of suspensions of dispersed cells into infarcted heart. The hope is that the injected cells will repopulate the infarct zone, integrate with the host myocardium, and thereby provide additional force generation. As described further below, while transplanted hESC-CMs have been shown to engraft in preclinical models and favorably influence mechanical function, the resultant intra-cardiac implants have shown limited myocardial organization and uncertain host-graft electromechanical integration. Indeed, hESC-CM grafts are typically comprised of small, irregularly contoured islands of primitive cardiomyocytes with inconspicuous sarcomeric organization and alignment, generally isolated from the host myocardium by an insulating zone of fibrosis. By contrast, adult ventricular myocardium is an extremely complex tissue type that includes highly aligned and interconnected “working” cardiomyocytes, specialized fast-conducting (Purkinje) fibers, stromal cells, and an intimately positioned vascular network, all held together within a carefully structured extracellular matrix. It is doubtful that the simple injection of dispersed parenchymal cells alone will result in nascent tissue recapitulating this organization and complexity, particularly in an organ known for negative remodeling, scar formation, and limited (if any) endogenous regeneration.

For this reason, the field has increasingly looked to tissue engineering strategies to provide a hierarchical organization for the cells. In general, the concept is to combine the appropriate cell types with a scaffold or other support structure meant to sustain the development of functional tissue. Whether in vitro or in vivo, the cells in such environs may be provided nutrients, perfusion, mechanical stability, mechanical and/or electrical stimulation, and soluble or bound factors to promote viability, proliferation, or maturation. Historically, cardiac tissue engineering efforts have tended to use easy-to-obtain primary rodent cardiomyocytes (e.g. rat neonatal cardiomyocytes), but large numbers of human cardiomyocytes will be required for the development and implementation of human tissue constructs. For this, the cardiomyogenic differentiation of human pluripotent stem cells is obviously an enabling technology, and methods for the large scale production of such cells are under development [82].

Tissue engineering approaches

A wide range of cardiac tissue engineering efforts have been explored to date, ranging from engineered hydrogels used as a carrier and adjuvant to injected cell suspensions, to fabricated polymer scaffolds upon which cells are cultured in vitro, to decellularized whole organs which are later re-cellularized. A few selected examples are highlighted here, while several other recent comprehensive reviews of this topic can be found in references [83-88].

Several groups have developed gel-based carrier and scaffold systems, which may serve a direct scaffolding role during in vitro growth and may improve retention upon injection. For example, Davis and coworkers have engineered a self-assembling peptide hydrogel, which is designed to both provide a suitable microenvironment for cardiomyocytes and promote the ingrowth of host vascular cells [89]. The Zimmerman and Eschenhagen groups have combined rat neonatal cardiomyocytes with collagen I and matrigel to create sizeable, spontaneously contracting tissue constructs in the three dimensional form of bands and pouches [90]. When transplanted onto the epicardial surface of the rat heart, this neotissue successfully integrates with the host.

Others have proposed more elaborate solid scaffolds built from synthetic (e.g. poly-glycerol sebacate, poly-lactide-co-glycolic acid (PLGA), polyetherurethane, others) or naturally derived (e.g. collagen, fibrin) degradable polymers. For example, Freed and coworkers recently used laser microfabrication techniques to build a structured three-dimensional porous scaffold made from bio-erodable polyglycerol sebacate, which is meant to closely mimic the anisotropic properties of myocardium [91]. Cardiomyocytes cultured on these scaffolds exhibited axial alignment and enhanced electrophysiologic properties. In one of the first engineering efforts to specifically make use of hESC-derivatives, the Gepstein group used PLGA scaffolds with hESC-CMs as well as hESC-derived endothelial cells and fibroblasts to construct vascularized muscle tissue [92]. Finally, in perhaps the ultimate deference to the native structure of the heart, the Taylor lab used detergent perfusion techniques to decellularize a whole heart. They then repopulated the decellularized matrix structure with cardiomyocytes and endothelial cells to yield a organ-shaped construct with modest pumping capacity [93].

While scaffolds may play an important role in imparting structural organization early in tissue development, there is some consensus that the polymeric support structure should be cleared fairly rapidly and replaced by matrix produced by the new tissue. Consistent with this notion, Okano and co-workers have minimized the role of the scaffold by forming sheets of confluent neonatal cardiomyocytes in conventional two-dimensional cultures. They then detach these intact cardiomyocyte sheets by modulating the hydrophobicity of the tissue culture substrate through the use of a temperature-sensitive coating (reviewed in [94]). These centimeter-sized sheets can then be layered together to form thicker constructs that exhibit characteristic cardiac structures (e.g. sarcomeres, gap junctions) and contractile activity. When implanted subcutaneously, these tissue constructs become vascularized and exhibit sustained contractile activity. Moreover, when implanted on the epicardial surface of the infarcted rat heart, functional gap junctions were demonstrated between host and graft tissue [95]. Finally, Stevens and colleagues have pursued a very minimalist approach that relies only on externally applied physical forces and the self-aggregating properties of hESC-CMs themselves [96]. In particular, by applying carefully tailored rotational forces in culture, they developed flat, circular “patches” of human myocardial tissue in the absence of a scaffold.

Other cell types from human pluripotent sources

While cardiomyocytes make up 70% of the mass of the heart, they represent only 30% of total cell number, with the balance being comprised of non-cardiac cell types including fibroblasts, vascular smooth muscle, and endothelial cells. Hence, successful cardiac tissue engineering efforts will likely require the inclusion of critical non-cardiac cell types. While these non-cardiac cells could in principle be obtained from other adult stem cell sources (many of which are poorly cardiomyogenic but efficiently differentiate into stromal or vascular cell types [9]), they can also be readily derived from human pluripotent stem cells [97-100]. Indeed, the multipotential $KDR^{high}/c\text{-Kit}^{+}$ cardiovascular progenitors isolated by from ESCs by the Keller group represents an attractive “one-stop” source for large quantities of cardiomyocytes, smooth muscle cells, fibroblasts, and endothelial cells [74,101].

USE OF STEM CELL DERIVED CARDIOMYOCYTES IN INFARCT REPAIR

Proof of principle for the use of cell-based therapies in infarct repair was provided by early preclinical studies in which terminally differentiated cardiomyocytes from fetal and neonatal sources were transplanted into infarcted rodent hearts and were shown to help preserve left ventricular function [102-106]. The transplanted cardiomyocytes retained their cardiac phenotype (including gap junction expression), and they were later shown by intravital imaging studies to be electrically coupled with host myocardium [107-109] hearts.

Recognizing that mESCs represented an essentially inexhaustible supply of committed, albeit immature cardiomyocytes, a number of laboratories investigated the capacity of mESC-CMs to mediate similar cardiac repair. Using the genetically selected cell preparation described above, the Field group was the first to demonstrate that mESC-CMs could form stable intracardiac grafts in uninjured rodent hearts [77]. The Xiao group subsequently extended this work to show that mESC-CM grafts would survive and favorably influence cardiac function in infarcted hearts [110,111], a finding that has been independently confirmed by others [112-116].

Initial transplantation studies with hESC-CMs were focused on uninjured hearts and demonstrated the capacity of these cells to form intra-cardiac implants of proliferating human myocardium in rats [18,54] and swine [117]. More recent work has shown that hESC-CMs can engraft in infarcted hearts and that their transplantation mediates beneficial effects on contractile function, at least at early timepoints. (Please see Table 1 for a comprehensive list of preclinical studies examining hESC-CM transplantation in infarct models.). Our own group demonstrated the capacity of these cells to partially remuscularize the infarct zone and preserve regional and global cardiac function in a nude rat infarct model. For this study, we generated a highly purified preparation of human cardiomyocytes (mean of 83% cardiomyocytes), using the directed differentiation protocol described above (see Figure 2B), followed by Percoll gradient enrichment [49]. When these cells were transplanted into recently infarcted rats in the presence of a pro-survival cocktail (PSC) including anti-oncotic and anti-apoptotic factors, significant amounts of human myocardium were formed (up to 11% of the infarct volume) in all recipient heart. No teratomas were observed and, in fact, greater >99% of the surviving graft cells were cardiomyocytes, as demonstrated by combining immunohistochemistry for cardiac markers and in situ hybridization with a human-specific pan-centromeric probe. Cardiac function was assessed at 4 weeks post-transplantation by echocardiography and magnetic resonance imaging (MRI). Recipients of hESC-CMs in PSC showed preserved left ventricular dimensions, regional wall thickening, and global contractile function, relative to controls receiving vehicle, PSC alone, or non-cardiac hESC derivatives in PSC [18].

Similarly encouraging results were reported by the Gepstein group, which injected either undifferentiated hESCs, hESC-CMs (microdissected from spontaneously beating EBs), non-cardiac hESC derivatives, or saline vehicle into infarcted rat hearts [76]. As would be expected, the transplantation of undifferentiated hESCs resulted in the formation of teratomas by four weeks post-transplantation. By contrast, animals receiving microdissected hESC-CMs did not show teratomas, but instead contained small intra-cardiac implants of human myocardium (although the extent of remuscularization was not assessed). More importantly, the recipients of hESC-CMs demonstrated greater preservation of left ventricular dimensions and global cardiac function than did controls, as measured by echocardiography at 4 and 8 weeks post-transplantation.

A cautionary note was suggested by the Mummery group, based on their hESC-CM transplantation studies in a murine acute infarct model [118,119]. For this work, the authors generated hESC-CMs using their END-2 co-culture system (see Figure 2A). They transplanted

these cells into acutely infarcted immunodeficient (NOD-SCID) mice and then followed the animals using histological endpoints and MRI through a 12-week timepoint. Although they observed modest improvements in cardiac function in recipients of hESC-CMs at 4 weeks, these beneficial effects proved transient, and no significant differences were found between hESC-CM recipients and controls at 12 weeks.

Based on the findings of the latter study, which includes by far the longest duration of follow-up after hESC-CM transplantation, Mummery and colleagues have urged caution in drawing conclusions from cell transplantation studies that rely solely upon earlier timepoints, including our own [49]. While wholeheartedly agreeing with the value of including later timepoints in future preclinical studies, we note that this can be challenging in a xenotransplant context. In our experience, the residual immunity of even immunodeficient animals generally “catches up” with and rejects the human xenograft over time. (That said, Mummery and colleagues concluded that a reduction in graft size from 4 to 12 weeks was not responsible for the loss of late-term functional improvement in their study. They assessed graft size using a semi-quantitative grading system, and, while there was a modest declining trend in graft size from 4 to 12 weeks, this change was not statistically significant.) There are other important differences between the Mummery group’s work and our own that preclude a direct comparison: species (mouse versus rat), timing of injection (they injected cells at the time of infarction, while we did so 4-5 days post-infarction), use of prosurvival adjuvants (which we found to be absolutely required for long-term engraftment), and the methodology used to assess graft size (they used a semi-quantitative histological score, while we used histomorphometry to express graft size as a fraction of infarct and left ventricular volume). The disparate methods used to measure graft size make it particularly challenging to compare this important parameter between studies and represents an area where standardization in the field would be helpful. The field would also benefit from the development of improved model systems in which to conduct long-term studies with pluripotent stem cell-derivatives (e.g. hosts with chimeric matched human-animal bone marrow or iPSCs from relevant preclinical models).

Overall, despite these important differences in experimental design and interpretation, several encouraging conclusions can be drawn based on hESC-CM transplantation experience in rodent models. First, as illustrated by Table 1, multiple groups have now independently demonstrated the successful engraftment of hESC-CMs within infarcted hearts. Second, while the transplantation of cell preparations of low cardiac purity can give rise to teratoma-like graft elements [49,76,120], hESC derivatives do not form a teratoma when sufficiently enriched for cardiomyocytes. Third and perhaps most importantly, all studies to date have found that hESC-CM transplantation exerts at least transient beneficial effects on left ventricular remodeling and function, effects that do not appear to be mediated by non-cardiac hESC derivatives [49, 76,119]. Clearly, transplantation studies in a larger, more relevant preclinical model are warranted.

Finally, some commentary is warranted regarding the mechanism(s) of the beneficial functional effects of hESC-CM transplantation. One possibility is that the nascent human myocardium becomes electromechanically integrated with the host and thereby contributes new force generating units. Consistent with this hypothesis, hESC-CMs form electromechanical junctions and beat synchronously with rat cardiomyocytes in vitro [117, 121], and, as discussed further below, they can function as an ectopic pacemaker following transplantation [117,121]. Moreover, we have shown that systolic wall thickening in the infarct region is enhanced 2.5-fold in hearts receiving hESC-CMs relative to controls [49]. However, these observations represent only indirect evidence for host-graft coupling. It is entirely possible that the beneficial effects of hESC-CM grafting result from secondary, so-called “paracrine” mechanisms of actions, e.g. by favorably influencing post-infarct remodeling or

by promoting collateral formation, etc. [122]. Our group is currently pursuing intravital imaging studies to directly address this uncertainty.

OTHER CARDIAC APPLICATIONS FOR HUMAN PLURIPOTENT STEM CELLS

While much attention has been understandably focused on the use of hESC- and hiPSC-CMs for ventricular infarct repair, we expect these cells will prove useful for other in vitro and in vivo applications. Indeed, because many of these applications require smaller numbers of cells and avoid the challenges of cell transplantation within the hostile environment of the infarct zone, we predict that hESC- and hiPSC-CMs will reach mainstream use in these areas much earlier than with infarct repair.

Before considering some of the other potential clinical uses of hESC- and hiPSCs, some commentary is warranted regarding their utility as high-throughput in vitro models of cardiac development and disease. Non-human model systems have proven to be invaluable tools for studying generally well-conserved events and signaling effectors during cardiac development, but species differences are inevitable. Differentiating hESCs and hiPSCs allow one to test lessons from these non-human models in a human context and to identify aspects of heart development that may be idiosyncratic to humans. hiPSCs also represent a unique resource with which to generate multiple somatic cell types from individuals with hereditary diseases. For example, somatic cells from patients with congenital heart disease, channelopathies, or hereditary cardiomyopathies can be reprogrammed into hiPSCs, which may then be redifferentiated into cardiomyocytes for in vitro disease investigation and/or drug screening studies.

Drug screening and safety studies

The most frequent reason that drugs are removed from the market is adverse cardiac side effects [123,124], and so regulatory agencies and the pharmaceutical industry have developed several high-throughput in vitro systems to screen compounds for pro-arrhythmic or other cardiotoxic effects. Commonly used in vitro screening platforms include primary ventricular cardiomyocytes or Purkinje fibers, isolated heart models, and mammalian cell lines with stable, heterologous expression of relevant cardiac ion channels (e.g. HEK cells overexpressing the hERG K⁺ channel) [125,126]. While not yet in mainstream use in drug screening studies, cardiomyocytes from hESCs or hiPSCs have a number of potential advantages over these existing systems. First, hESC- and hiPSC-CMs are *human* cells and so avoid problems related to interspecies variability (e.g. interspecies differences in ion channel expression). Human iPSC-CMs may be particularly relevant in providing disease-specific screening models or even predicting effects in individual patients, thereby enabling personalized medicine. Second, these stem cell derived preparations can be expanded in either the undifferentiated or differentiated state, while primary adult cardiomyocytes are essentially post-mitotic and so must be repeatedly isolated from experimental animals using complicated enzymatic dispersion techniques. Isolated heart preparations are similarly low-throughput and are challenging to standardize between laboratories. Third, while drug screening in heterologous expression systems is certainly high-throughput, these systems do not represent a “natural” cardiac context and so may lack important accessory proteins or secondary targets that may mediate relevant adverse effects.

The most important rationale for in vitro drug safety screening systems is to identify compounds that prolong the QT interval (the duration of time between the Q-wave and completion of the T-wave components of the electrocardiogram), a phenomenon associated with a potentially fatal ventricular arrhythmia called ‘torsade de pointes’. Numerous otherwise useful drugs have been removed from the market or restricted in their use because of QT-prolonging effects, including various antihistamines, antimicrobial agents, antipsychotics, and

anti-arrhythmic drugs. Most drugs of these prolong the QT interval by inhibiting the rapidly activating delayed rectifier potassium current (I_{Kr}), which is conveyed by the human ether-à-go-go-related gene-encoded voltage-dependent potassium channel (hERG K^+ channel). Importantly, hESC-CMs do express hERG and exhibit an I_{Kr} current that is sensitive to canonical long QT-inducing inhibitors including E4031, sotalol, and cisapride [58,127]. While patch-clamp studies to detect effects on I_{Kr} may be somewhat more technically challenging in hESC-CMs than with heterologous expression systems, hESC-CMs again offer potential advantages. First, hESC-CMs are more likely than heterologous cell types to express important accessory subunits that modify the I_{Kr} current, and hESC-CMs express other ionic currents (e.g. I_{Na} [59]) that influence repolarization and have also been implicated in drug-induced long QT syndrome [128]. Second, some QT-prolonging drugs (e.g. arsenic [129] and pentamidine [130]) appear to work by reducing cell surface expression of the hERG channel, rather than acute block. Stem cell derived cardiomyocytes are likely to be a more relevant model in which to look for such effects on endogenous channel expression. Finally, there are patients with polymorphisms in ion channel genes that place them at higher risk for drug-induced long QT syndrome [131]. If iPSCs can be derived from such individuals, these mutant iPSC-CMs would likely be a far more sensitive screening tool.

Currently available hESC- and hiPSC-CM preparations have two limitations with regard to their use in drug screening and safety studies. First, these are *embryonic* cardiomyocytes, and so it is important to be mindful of the implications of the significant phenotypic differences between these cells and adult cardiomyocytes. Second, as discussed above, hESC-CMs include distinct nodal and working cardiac subtypes, and so genetic selection using available subtype-specific promoters may be required to obtain more homogenous ventricular preparations [81, 132].

Stem cell derived biological pacemakers

Three million people worldwide currently have a man-made implanted pacemaker for cardiac rhythm disturbances, and up to 600,000 patients receive new ones each year [133]. These devices successfully treat a broad range of electrophysiological abnormalities, but they have shortcomings including increased susceptibility to infection, finite battery life, significant patient discomfort related to the permanent implantation of a foreign device, and lack of intrinsic responsiveness to neural and hormonal regulation. These liabilities have led to recent interest in cell-based therapies as an alternate strategy, that is, the formation of a “biological pacemaker” via delivery of cells with pacemaking properties [117,134,135].

Exciting proof-of-principle for the use of hESC-CMs in this application was provided by a pair of recent studies by the Gepstein and Li groups [117,121]. The Gepstein group microdissected spontaneously beating foci from hEBs and then demonstrated the capacity of these cells to form gap junctions with and pace co-cultured rat neonatal cardiomyocytes in vitro [117]. They next moved to in vivo transplantation experiments using a swine model of complete heart block [117]. In brief, complete heart block was induced by radio frequency ablation of the bundle of His, and an electronic pacemaker was implanted to prevent bradycardia. A subset of the animals then received an injection of 40-150 beating EBs into the posterolateral left ventricular wall. The EB recipients later showed an ectopic ventricular rhythm with a rate of ~60 beats per min and an ECG morphology distinct from either the junctional or paced rhythms. More definitive proof that the EB implants were functioning as an ectopic pacemaker was provided by three-dimensional electroanatomic mapping studies, performed at 1-3 weeks post-transplantation, in which the origin of ventricular activation was mapped to the posterolateral injection site. No such ectopic activity was observed in control animals receiving an injection of non-myocyte hESC derivatives.

The Li group used an ex vivo guinea pig preparation to demonstrate the pacemaking capacity of transplanted hESC-CMs [121]. In brief, these authors microdissected spontaneously beating embryoid bodies, which were marked with GFP via lentiviral vector and then transplanted into the anterior left ventricular wall of the guinea pig heart. At 2 to 3 days post-transplantation, the heart was explanted and mounted on a Langendorf apparatus for optical voltage mapping. After cryoablation of the host AV node, control animals showed complete electrical silence, while recipients of the embryoid bodies showed waves of epicardial activation originating from the injection site, as confirmed by the presence of GFP+ cells.

These two studies are useful because they confirm the capacity of transplanted hESC-CMs to couple with host myocardium and function, at least transiently, as an ectopic pacemaker. However, enthusiasm should be tempered by the relatively early timepoints examined. While early embryonic cardiomyocytes all show some degree of automaticity, this property dissipates with maturation into working ventricular cardiomyocytes. Therefore, to form implants with sustained pacemaking activity, it may prove necessary to employ preparations of enriched pacemaker/nodal-type cells or to genetically modify the cells to express pacemaker ion channels [134,136].

Electrical stabilization of the post-infarcted heart

In an intriguing recent report, the Fleischmann group showed that the transplantation of primary embryonic cardiomyocytes suppresses post-infarct arrhythmias in a mouse model [137]. This exciting finding begs the question whether hESC- or hiPSC-CMs, which are committed cardiomyocytes with a similar phenotype, might also mediate such anti-arrhythmic effects. If so, this would represent a tremendously useful application for pluripotent stem cell derivatives, as ventricular arrhythmias are a leading cause of death in patients following a myocardial infarction [138-140].

In some respects, such an effect is counterintuitive because hESC-CMs grafts can plausibly contribute to all three fundamental arrhythmia mechanisms: automaticity, triggered activity, and reentry. First, all hESC-CMs exhibit some degree of automaticity, and, as discussed, transplanted hESC-CMs can function as an ectopic pacemaker [117,121]. Second, murine ESC-CMs seem to be particularly prone to exhibiting early- and after-depolarizations [141], triggered activity that is thought to underlie many episodes of ventricular tachycardia. Third, hESC-CM transplantation may promote reentrant phenomena by introducing electrical heterogeneity. As discussed above, hESC-CMs themselves are electrically heterogeneous, and they form irregularly shaped grafts that are generally isolated by scar tissue [18,49]. hESC-CMs also tend to be depolarized relative to adult ventricular myocardium.

However, while there is much to suggest that pluripotent cell-derived CMs might be proarrhythmic, the result of the Fleischmann group's study underscores the need to test such assumptions directly. Using a mouse cryoinjury model in which ventricular tachycardia was inducible in 96% of control mice, these authors demonstrated a robust protective effect with the transplantation of either primary murine embryonic cardiomyocytes or connexin-43 overexpressing skeletal myoblasts (reducing inducibility to 36% and 38% of recipients, respectively). Of note, this suppressive effect was not observed in recipients of wildtype (i.e. connexin-43 null) skeletal myoblasts, and it did not correlate with preservation of mechanical function. Ex vivo optical mapping studies revealed a decreased incidence of conduction block and ectopic activity in the border zone in hearts receiving cardiomyocytes relative to controls. While the precise mechanisms underlying this electrical stabilization remain undetermined, studies to determine whether hESC- or hiPSC-CMs are capable of mediating similar effects are clearly warranted.

REMAINING HURDLES TO THE DEVELOPMENT OF CARDIAC THERAPIES

There is certainly unmet clinical need for which stem cell based cardiac therapies may be extremely useful, but it is important to keep in mind that contemporary medicine has already developed fairly successful approaches to each of the cardiovascular diseases discussed in the preceding sections. Many patients do quite well following a modest myocardial infarct under conventional medical management, and the device industry has developed remarkably sophisticated electronic pacemakers. Therefore, to successfully compete with existing therapies, stem cell based approaches must be developed with the goal of maximizing efficacy and safety, especially in the case of novel stem cell populations such as hESCs and hiPSCs. Well-publicized bad outcomes in the gene therapy field underscore the dangers inherent in moving promising but underdeveloped new therapies to the clinic.

Table 2 lists the most important of the remaining hurdles to the development of safe, efficacious cardiac therapies based on hESCs and/or hiPSCs. In brief, the field must do the following: 1) generate purified cell preparations that eliminate the risk of teratoma formation, 2) demonstrate that the incidence of arrhythmias is unchanged or reduced in cell recipients, 3) minimize graft cell death following transplantation, and 4) avoid immune rejection of the graft. While potential approaches to the first three issues have been considering at length in preceding sections, some discussion is warranted regarding the issue of immune rejection.

The immunogenicity of ESCs increases with differentiation, and cardiac transplants of differentiated ESC-derivatives are recognized and rejected by allogeneic recipients [142, 143]. Thus, while hESC-CMs would likely be less immunogenic than whole organ allografts, their transplantation would likely necessitate some degree of pharmacological immunosuppression. Obviously, this would only be appropriate in patients for whom the benefits of cell therapy outweigh the intrinsic risks of immunosuppression. Fortunately, there are a number of other strategies by which the immune rejection of hESC derivatives might be avoided, including nuclear reprogramming to generate an “autologous” hESC cell source, genetic manipulation of major histocompatibility genes to produce a “universal” donor hESC line, and induction of immune tolerance via the transplantation of hESC-derived hematopoietic precursors and establishment of bone marrow chimerism [144]. Finally, hiPSCs represent a potential autologous source of cells with an hESC-like phenotype and thus would be particularly useful for clinical applications if safer, non-viral approaches to somatic cell reprogramming can be developed.

There are many other parameters for which we have incomplete information, including the optimal timing for cell transplantation post-infarct, the appropriate level of maturation of the input cell preparation (or engineered tissue construct), and which mechanism of delivery (e.g. catheter-based injection or direct implantation by the surgeon) will best facilitate successful engraftment. Of course, these issues are common to all cell-based therapies and can likely be addressed by continuing work in pre-clinical models.

SUMMARY

Adult mammalian cardiomyocytes are essentially post-mitotic, and so the capacity of hESCs to generate large quantities of proliferating *human* cardiomyocytes for research and regenerative medicine applications has engendered considerable excitement in the field. Cardiomyocytes from hESCs have already proven useful as a model of human heart development, while cardiomyocytes from disease-specific hiPSC lines are expected to yield important new insights into the pathogenesis and treatment of heritable cardiac disorders. Both hESC- and hiPSC-CMs have an unambiguous cardiac phenotype, and recent improvements in the guided differentiation and/or selection of these cardiomyocytes have improved their prospects for use in larger-scale applications. Certainly, substantial hurdles

remain to the successful development of hESC- and hiPSC-CMs as research tools and a clinically useful cell-based therapy. Nonetheless, we envision the use of these cells in the near-term as part of high-throughput screens for drug development and safety testing. Longer-term, these cells have considerable promise for use in both infarct repair and biological pacemaker formation, and recent preclinical studies provide exciting proof-of-principle for such applications.

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ABBREVIATIONS

ANP	atrial natriuretic peptide
AP	action potential
BMP	bone morphogenetic protein
DKK1	dickkopf homolog-1
EB	embryoid body
EC	endothelial cell
EF	ejection fraction
END2-CM	medium conditioned by the endodermal cell line END2
ESC	embryonic stem cell
FACS	fluorescence-activated cell sorting
FS	fractional shortening
GATA4	GATA binding protein 4
hERG	the human <i>ether-à-go-go</i> related gene
hESC-CM	human embryonic stem cell-derived cardiomyocyte
hiPSC	human induced pluripotent stem cell
hiPSC-CM	human induced pluripotent stem cell-derived cardiomyocyte
KDR	kinase insert domain receptor
Klf4	Kruppel-like factor 4
MEFs	mouse embryonic fibroblasts
MAP	mitogen-activated protein
Mef2c	myocyte enhancer factor 2C
MHC	myosin heavy chain
MLC	myosin light chain
MRI	magnetic resonance imaging
Nkx2.5	NK2 transcription factor related, locus 5
NOD-SCID	non-obese diabetic severe combined immunodeficiency
Oct4	Octamer-4

PLGA	poly-lactide-co-glycolic acid
RT-PCT	reverse transcriptase-polymerase chain reaction
SMC	smooth muscle cell
Sox2	sex determining region Y-box 2
TGFβ	transforming growth factor β

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Figure 1. Cardiomyocytes from human pluripotent stem cells

Two human pluripotent stem cell types show efficient cardiogenesis: embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs). hESCs are derived from the inner cell mass of pre-implantation stage blastocysts remaining after in vitro fertilization efforts. hiPSCs show a very similar phenotype but are derived by reprogramming skin fibroblasts or other somatic cell types via the over-expression of stem cell related genes. In both cases, the stem cell populations can be expanded in the undifferentiated state or differentiated into cardiomyocytes via the formation of embryoid bodies or by treatment with inducing factors. These stem cell derived cardiomyocytes include “working” (i.e. ventricular and atrial chamber-like) and nodal type cells.

Figure 2. Approaches to generating enriched preparations of hESC-derived cardiomyocytes

A. In this protocol developed by the Mummery group [69], hESC derivatives are guided into the cardiac lineage via treatment with medium conditioned by endodermal END-2 cells and a p38 MAP kinase inhibitor. B. Our group reported this protocol [49], which involves serial treatment of hESCs with the growth factors activin A and bone morphogenic protein-4 (BMP4). In this procedure, hESC derivatives are grown in monolayer culture in the absence of serum. C. In this protocol developed by Keller group [74], hESCs are first guided into the mesodermal lineage by treatment with activin A, BMP4, and basic fibroblast growth factor (bFGF), followed by cardiac induction by treatment with the Wnt inhibitor, dickkopf homolog 1 (DKK1), vascular endothelial growth factor (VEGF), and bFGF.

Preclinical studies examining the transplantation of hESCs or hESC-derived cardiomyocytes in rodent infarct models.

Table 1

Investigator	Cardiomyocyte Purity	Cell Host	Dose Species	Duration of Follow up	Functional Effects	Comments
Caspi et al. [76]	71%	1.5 × 10 ⁶	Rat	≤9 weeks	Preserved LV dimensions and FS	Used microdissected EBs
Dai et al. [54]	13%	2 × 10 ⁶	Rat	4 weeks	ND	Histological endpoints only.
Kofidis et al. [145]	15%	1 × 10 ⁶	Mouse	3 weeks	Preserved EF and wall thickening	Cardiac engraftment was enhanced by treatment with allopurinol/uricase and ibuprofen
Laflamme et al. [49]	>80%	5 × 10 ⁶	Rat	4 weeks	Preserved LV dimensions, FS, and wall thickening	Cardiac engraftment required delivery in a pro-survival cocktail
Leor et al. [120]	ND	0.5-1 × 10 ⁶	Rat	2-4 weeks	Preserved FS and wall thickening	Observed functional benefits in the absence of cardiac graft survival. Teratomas were found in 1 of 7 hESC-CM recipients.
Tomesco et al. [146]	ND	3 × 10 ⁶	Rat	8 weeks	ND	Transplanted BMP-2 treated hESCs. Histological endpoints only.
van Laake et al. [118,119]	20-25%	1-3 × 10 ⁶	Mouse	≤12 weeks	Preserved EF at 4 weeks, but no beneficial effects at 12 week-time point	
Yang et al. [74]	40-50%	1 × 10 ⁵	Mouse	2-11 weeks	Preserved EF	Transplanted KDR ^{low} /c-Kit ^{neg} hESC-derivatives. Observed cardiac, endothelial, and SMC differentiation.

Abbreviations: BMP—bone morphogenic protein, EBs—embryoid bodies, EF—ejection fraction, FS—fractional shortening, hESC-CM—human embryonic stem cell derived cardiomyocytes, KDR—kinase insert domain receptor, LV—left ventricle, ND—not determined, SMC—smooth muscle cell.

Table 2

Major hurdles to the development of cardiac therapies based on human pluripotent stem cells and selected approaches to potentially overcome them.

Issue	Potential Approaches
Risk of teratoma formation	<ul style="list-style-type: none"> Guided cardiac differentiation Enrichment of cardiomyocytes based on cell surface markers or unique physical properties Genetic selection of cardiomyocytes using cell type-specific promoters
Risk of arrhythmias	<ul style="list-style-type: none"> Elimination of pacemaker/nodal cardiomyocytes Use of in vitro conditioning and/or tissue engineering approaches to optimize the maturation, organization, and electromechanical integration of the stem cell derived graft
Retention and acute graft cell death	<ul style="list-style-type: none"> Delivery in pro-survival factors Use of in vitro conditioning and/or tissue engineering approaches Genetic modification of the graft cells
Ensuring longer-term graft survival and host-graft integration	<ul style="list-style-type: none"> Co-delivery of factors to promote host vascular ingrowth into the graft Co-deliver of vascular progenitors Tissue engineering approaches (e.g. implantation in constructs with a pre-formed vasculature)
Immune rejection of the graft cells	<ul style="list-style-type: none"> Pharmacological immunosuppression Generation of isogeneic stem cell sources via either reprogramming (i.e. patient-specific iPSCs) or somatic cell nuclear transfer ("therapeutic cloning") Banking of MHC-typed stem cell preparations Induction of graft tolerance in the host by using stem cells to form hematopoietic chimerism Generation of a universal donor hESC line via genetic manipulation of MHC genes, etc.

Abbreviations: hESC—human embryonic stem cell, iPSC—induced pluripotent stem cell, MHC—major histocompatibility gene.