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Pluripotent Stem Cell Derived Cardiomyocytes for Cardiac Repair

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Opinion Statement

The adult mammalian heart has limited capacity for generation, so a major injury such as a myocardial infarction results in the permanent loss of up to one billion cardiomyocytes. The field of cardiac cell therapy aims to replace these lost contractile units with de novo cardiomyocytes to restore lost systolic function and prevent progression to heart failure. Arguably the ideal cell for this application is the human cardiomyocyte itself, which can electromechanically couple with host myocardium and contribute active systolic force. Pluripotent stem cells from both human embryonic or induced pluripotent lineages are attractive sources for cardiomyocytes, and preclinical investigation of these cells is in progress. Recent work has focused on efficient generation and purification of cardiomyocytes, tissue engineering efforts, and examining the consequences of cell transplantation from mechanical, vascular, and electrical standpoints. Here we discuss historical and contemporary aspects of pluripotent stem cell-based cardiac cell therapy, with an emphasis on recent preclinical studies with translational goals.

Keywords

embryonic stem cells; pluripotent stem cells; cardiomyocyte; myocardial infarction; regenerative medicine; cardiac cell therapy

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Conflict of Interest

Dr. Scott D. Lundy, Dr. Jay A. Gantz, Dr. Chelsea M. Pagan, Dr. Dominic Filice, and Dr. Michael A. Laflamme each declare no potential conflicts of interest.

Compliance with Ethics Guidelines

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Introduction

Despite significant improvements in medical management, heart disease still claims one life every 40 seconds in the United States and is now the number one cause of mortality worldwide[1]. The adult heart lacks robust intrinsic regenerative capabilities, and any major injury to the myocardium results in the replacement of muscle with non-functional scar tissue, reduced contractile performance, and the initiation of a vicious cycle of adverse remodeling. Motivated by this challenge, a number of investigators began experimenting with exogenous cell transplantation in the early 1990s with the goal of remuscularizing the infarcted heart [2,3]. Early proof-of-concept experiments with neonatal and fetal cardiomyocytes showed that stable grafts of new myocardium could be formed in injured hearts [4,5], but these cell sources have obvious ethical and practical limitations that prevent clinical use. Skeletal myoblasts were next investigated as an alternative source of striated muscle tissue [6,7], but subsequent work raised concerns about arrhythmias [8–10] and efficacy [11] with this cell source. More recently, a variety of bone marrow-derived cell types have been explored in preclinical and clinical studies, but these cells do not generate significant numbers of de novo cardiomyocytes, and any beneficial contractile effects likely result from indirect mechanisms such as paracrine signaling [12,13].

If the ultimate goal of cardiac cell therapy is to regenerate human myocardium, then no cell type is better suited to the task than the human cardiomyocyte itself, as it is capable of electrically integrating with host muscle and generating systolic force. However, the initial practical challenge was to identify a suitable source for obtaining large quantities of phenotypically unambiguous cardiomyocytes. The isolation of human embryonic stem cells (hESCs) in 1998 [14] and human induced pluripotent stem cells (hiPSCs) a decade later [15] represented potential solutions to this problem. Here, we review the current status of cardiac repair using pluripotent stem cell-derived cardiomyocytes (PSC-CM), with an emphasis on recently published work that is aimed at moving this emerging technology towards clinical translation.

Sources of Pluripotent Stem Cells

Pluripotent hESCs are derived from preimplantation-stage human blastocysts donated after in vitro fertilization efforts [14]. In addition to political and ethical concerns, these cells also pose a practical challenge for therapeutic applications because their differentiated progeny will evoke an immune response from allogeneic recipients [16,17]. Some of these limitations are avoided at least in principle by the use of hiPSCs, which are derived by reprogramming somatic cells (e.g. dermal fibroblasts) to a pluripotent state [15]. While this reprogramming was initially accomplished by the forced over-expression of key stem cell-related transcription factors delivered via integrating viral vectors, a number of virus-free alternative approaches have since been developed including reprogramming via recombinant proteins [18], mRNA [19], non-integrating episomal vectors [20], and even small molecules [21].

Significant attention has been focused on comparing hESCs, hiPSCs, and their differentiated progeny. The Loring group has extensively investigated the genotype of hESC and hiPSC

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lines and found that both demonstrate similar degrees of genomic instability, underscoring the need for thorough characterization and validation before clinical application [22,23]. Toivonen and colleagues compared multiple hESC and hiPSC lines for transgene persistence and their ability to differentiate into cardiomyocytes and other lineages [24], and they reported differences in both differentiation potential and transgene silencing. Subsequent work by Sepac and colleagues found similar differences across several hESC and hiPSC lines in terms of cardiomyogenic potential [25]. While other studies have also highlighted potential epigenetic differences [26,27], it is nonetheless reassuring that differentiated hESC- and hiPSC-CMs seem to share a nearly identical functional phenotype [28,29].

Parthenogenetic stem cells are another pluripotent stem cell type that may avoid many of the ethical objections associated with hESCs, with the added potential benefit of reduced immune rejection since they display the HLA profile of only a single donor [30]. Although to our knowledge no one has characterized cardiomyocytes from human parthenogenetic stem cells, Didié and colleagues recently showed that cardiomyocytes from mouse parthenogenetic stem cells have a phenotype very similar to their counterparts from mouse ESCs and iPSCs [31]. These authors also showed that cardiomyocytes derived from mouse parthenogenetic stem cells integrated electrically with host myocardium and improved contractile function following transplantation in a mouse infarct model [31].

Generation and Enrichment of Cardiomyocytes from Pluripotent Stem Cells

hESC-CMs were first isolated from serum-stimulated embryoid bodies (EBs) [32,33] and later derived by co-culture with mouse endodermal cells [34]. Early differentiation protocols were inefficient with cardiomyocyte yields below 1%, but more recent directed cardiac differentiation protocols have resulted in dramatically improved cardiac purities (~30–70% cardiomyocytes) [35,36]. Additional refinements to these protocols include the addition of extracellular matrix proteins [37], modulation of Wnt signaling [38], and optimization of activin-nodal signaling [39]. These advancements now permit the reliable production of large quantities of relatively pure cardiomyocytes and give reason for optimism that the scaled production of clinical-grade cells for cardiac repair will be possible in the near future.

If an even higher degree of cardiomyocyte purity is required, subsequent enrichment steps are possible. Arguably the most successful strategy has been genetic selection using a cardiac-specific promoter that drives expression of a fluorescent protein and/or antibiotic resistance. Early proof of concept for genetic selection came from work by Field and colleagues using mouse ESC-CMs [40,41], and other groups have more recently extended this approach to human cells [42–44]. hESC-CMs have also been enriched based on sorting for surface markers (e.g. SIRPA [45], VCAM1 [46] and EMILIN2 [47]), via light-scattering properties via Raman micro-spectroscopy [48], and by differences in metabolic status[49]. While the latter approaches avoid the need for genetic modification, they are currently limited by cell yield and throughput. It remains to be determined what threshold of purity will be deemed acceptable for future clinical therapies.

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The Phenotype of Stem Cell-Derived Cardiomyocytes

By light microscopy, early human PSC-CMs appear as small, nondescript, mononucleated cells of variable size and shape [29,32–34]. They express sarcomeric proteins including α-actinin, cardiac troponins I and T, α- and β-myosin heavy chains, atrial- and ventricular-myosin light chains, desmin, and tropomyosin [29,33,34,50,51]. Ultrastructurally, PSC-CMs show poorly organized sarcomeres and intercalated discs [28,50]. Interestingly, PSC-CMs show robust proliferative activity characteristic of early chamber myocardium, with cell cycle activity slowly tapering off over several weeks of in vitro maturation [52,53].

PSC-CMs have significant automaticity and exhibit action potentials (APs) that have been classified as either nodal- or ventricular-like [34,54]. In both subtypes, early hESC-CMs show immature AP properties (i.e. a more rapid spontaneous rate, a slow AP upstroke, and a depolarized maximum diastolic potential), but these parameters improve somewhat with prolonged duration in culture [54,55]. In voltage-clamp studies, hESC-CMs exhibit most of the major cardiac ion currents, including fast sodium, L- and T-type Ca^{2+}, pacemaker, and transient outward and inward rectifier K^+ currents [34,55–57]. Depolarization in these cells is dominated by Na^+ influx via the $Na_V1.5$ channel, and this current is at least partially responsible for their spontaneous electrical activity [57]. As in adult cardiomyocytes, depolarization activates L-type Ca^{2+} channels in hESC-CMs, which results in a Ca^{2+} influx that is amplified by release from sarcoplasmic reticulum stores [56,58,59]. Recent data from our group indicates that this calcium-induced-calcium release process operates via a tight “local control” mechanism, similar to that of adult myocardium [56].

The first measurements of hESC-CM contractility were performed by Binah and colleagues using videomicroscopic contraction analysis of EBs [60,61]. While the use of EBs has some limitations (including low cardiomyocyte purity and heterogeneity in size and shape), these investigators showed that hESC-CMs had relatively immature contractile properties, including a negative force-frequency relationship. Our own group later used videomicroscopy to quantify both the magnitude and kinetics of spontaneous contractions of individual hESC- and hiPSC-CMs, and we found that these cardiomyocytes had contraction amplitudes of ~5% after ~25 days of in vitro maturation [28]. Hazeltine and colleagues used traction force microscopy to show that force production by hESC-CMs increased with culture on stiffer substrates [62]. Other groups have used atomic force microscopy to directly measure contractile force generation by PSC-CMs and reported values of ~0.2nN per cell [63,64]. By any measure, however, the forces generated by immature PSC-CMs appear miniscule: approximately10- to 100-fold lower than those of mature adult cardiomyocytes. This remains a major challenge for the field, and much additional work will be required to identify scalable methods for enhancing the mechanical properties of PSC-CMs.

Cardiac Tissue Engineering

Motivated in part by this increasing recognition of the immature functional properties of PSC-CMs under two-dimensional monoculture conditions, a number of groups have explored cardiac tissue engineering as a means of imposing both maturation and

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multicellular organization [65–67]. In pioneering experiments, Zimmermann and Eschenhagen first seeded primary rat cardiomyocytes in hydrogels [68], and several groups have subsequently applied this general approach to PSC-CMs and fibrin-based hydrogels [69–72]. This technique allows constructs to be prepared in almost any pattern, including as an injectable delivery vehicle [73]. Working independently, both the Murry and Martin groups showed that static stretch induces an increase in cardiomyocyte alignment and maturation within collagen-based hydrogel constructs [70,74]. In an interesting variation on this approach, the Radisic group exposed their constructs, cylindrical-shaped “biowires” of hPSC-CMs and non-cardiac supportive cells, to electrical field stimulation [75], and they found this promoted a more mature structural and electrophysiological phenotype. Recently, Bursac and colleagues seeded hESC-CMs into fibrin-based hydrogels over a PDMS template to form relatively large three-dimensional patches, and the resultant constructs exhibited force generation and conduction velocity measures that far exceed those previously reported [72].

Engineered tissue constructs can also be created by seeding PSC-CMs into three-dimensional scaffolds specifically designed for the inclusion of supporting non-cardiac cell types. The Gepstein group was among the first to compare outcomes with hESC-CMs alone versus a “tri-cell” mixture of hESC-CMs, endothelial cells and fibroblasts, by seeding both cell preparations into a poly-L-lactic acid (PLLA) scaffold [76]. Both expressed cardiac markers, but scaffolds seeded with the tri-cell combination showed far greater vascular organization in vitro. Qualitatively similar results were observed in vivo following engraftment in healthy rat hearts [77].

Two alternatives to the preceding artificial scaffold-based strategies are the use of decellularized native heart tissue [78] and scaffold-free tissue constructs [79–81]. Lu and colleagues seeded multipotent cardiovascular progenitors from hiPSCs into decellularized mouse hearts and found that the resultant constructs exhibited spontaneous contractile activity and responded appropriately to chemical agonists [78]. In pursuing the scaffold-free approach, the Murry group showed that hESC-CMs and supportive non-myocyte cell types will spontaneously aggregate to form viable cardiac patches [79–81]. It remains to be seen if either of these approaches are scalable for human applications.

Taken collectively, these studies suggest a potential role for tissue engineering in future therapeutic applications, but the identity of the most appropriate construct for achieving cardiac repair remains uncertain.

In Vivo Studies

Small Animal Models

Early in vivo proof-of-concept for the use of PSCs in cardiac repair came from the Field group, who showed that genetically-selected mESC-CMs formed stable myocardial grafts following transplantation in the hearts of dystrophic mice [82]. Xiao and colleagues later extended this work by microdissecting mESC-CMs from spontaneously beating EBs and transplanting them into a rat infarct model [83]. Somewhat surprisingly, in the absence of immunosuppression, this xenogeneic cell transplantation resulted in the formation of graft

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myocardium with beneficial effects on LV dimensions, fractional shortening and hemodynamics that were sustained for up to 32 weeks [83].

Transplantation studies with hESC-CMs followed a similar trajectory, with work first commencing in uninjured rodent hearts [84,85] and then gradually transitioning to rodent infarct models [35,86–90]. Our group transplanted enriched populations of hESC-CMs (~80% cardiac) into infarcted athymic rat hearts and saw partial remuscularization of the infarct zone [35]. Compared to infarcted controls receiving either non-cardiac hESC derivatives or vehicle, infarcted recipients of hESC-CMs showed better preserved LV dimensions, fractional shortening and regional wall motion. Interestingly, Mummery and colleagues found that while hESC-CMs formed nascent myocardium and improved contractile function in the infarcted hearts of immunodeficient mice, these beneficial effects appeared to be transient in nature [87,89]. While there were major experimental differences between the latter studies and our own (including the formation of qualitatively smaller grafts and the use of a mouse model in which hESC-CMs were unlikely to couple electrically), the Mummery group's findings nonetheless underscore the need for future studies with a greater duration of follow-up.

All of the preceding studies examined outcomes following hESC-CM transplantation in acute or subacute infarct models. More recently, Fernandes and colleagues transplanted hESC-CMs into chronically infarcted rat hearts, which perhaps represent a more relevant model for the end-stage patient in which such novel cell therapies would be first applied [88]. Interestingly, although hESC-CMs formed large myocardial grafts in chronically injured hearts, their engraftment was not accompanied by beneficial effects on contractile function. This study raises concerns that hESC-CMs cannot efficiently integrate and provide new force-generating units in chronically injured hearts or that they may be unable to reverse deleterious remodeling in hearts with already established failure.

Recent studies have also begun to focus on the application of iPSCs in rodent models of cardiac injury. Proof-of-concept experiments with murine iPSC-CMs have shown the ability of these cells to survive and form nascent myocardium [91,92]. Recently, the Gepstein group transplanted hiPSC-CMs from spontaneously beating EBs into uninjured rat hearts [93], and, while their study had a relatively short duration of follow-up (7–10 days), they did find histological evidence of engraftment and structural features of host-graft coupling (i.e. immunostaining for gap junctions between graft and host myocytes). Another recent study of interest by Carpenter and colleagues involved the transplantation of multipotent cardiovascular progenitors from hiPSCs in a rat infarct model [94]. Although the effect did not reach statistical significance, the cell-treated hearts showed a trend toward less deterioration in ejection fraction at 10 weeks post-infarction than untreated controls. The grafts in this study seemed modest in size, inviting speculation as to whether better graft survival post-transplantation might yield greater improvements in cardiac function.

Large Animal Models

The preceding experience in rodent infarct models set the stage for transplantation studies with hPSC-CMs in more relevant large animal preclinical models. Surprisingly, the first large animal study was performed nearly a decade ago when the Gepstein group transplanted

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hESC-CMs microdissected from beating EBs into the left ventricle of swine with complete atrioventricular block [50]. They detected ectopic pacemaking activity in the hearts of hESC-CM recipients and localized this signal to the site of cell transplantation by electroanatomic mapping. Although their study involved a relatively crude cell preparation and a very short duration of follow-up, it nonetheless remains a landmark study in the field.

The next relevant large animal study was reported in 2010 by the Menasché and Pucéat groups, who transplanted multipotent cardiovascular progenitors derived from rhesus ESCs in a primate infarct model [95]. These investigators found that while the recipients of undifferentiated ESCs grew teratomas, those receiving cardiovascular progenitors showed remuscularization of up to 20% of the infarct area. Unfortunately, their study did not include any functional endpoints, leaving open the question as to whether the partial remuscularization of the infarct results in a meaningful restoration of lost cardiac function in primates.

More recently, the Sawa group incorporated hiPSC-CMs into bioengineered sheets that were then applied to the epicardial surface of infarcted pig hearts [96]. At 8 weeks post-transplantation (12 weeks post-infarction), the recipient hearts had very few surviving graft cells, but they nonetheless showed better preserved ejection and left ventricular dimensions than untreated controls. More recently, this same group has shown that graft cell survival can be somewhat improved by delivering hiPSC-CM sheets with a pedicle omentum flap [97].

Remaining Hurdles to Translation

While this progress in animal models gives reason for hope, a number of major hurdles must still be overcome if PSC-based cardiovascular therapies are to reach clinical application. Some of these challenges and their potential solutions have been discussed above. For example, to avoid teratomas, it is likely that we will need highly purified preparations of cardiomyocytes (or cardiomyocytes with the appropriate supportive cell types). With improved directed differentiation protocols and enrichment strategies, such cell production now seems feasible. Another major issue is the immature phenotype of the PSC-CMs generated by existing protocols. While this issue will clearly require much additional work, a number of potential solutions have been previously mentioned, including prolonged duration in culture [28], electromechanical conditioning [75], and/or tissue engineering approaches [72]. In the following sections, we highlight four other remaining barriers to PSC-CM-based therapies: electromechanical integration and the risk of arrhythmias, graft cell death, graft vascularization, and immune rejection.

Electromechanical Integration and Arrhythmia Risk

Early work by Gepstein and others showed that hESC-CM grafts were capable of electromechanical integration in uninjured hearts [50,98], but their ability to couple with host myocardium following transplantation in injured hearts remained uncertain until quite recently. To address this question directly, our group generated transgenic hESC-CMs that stably express the fluorescent calcium indicator GCaMP3 [99]. GCaMP3+ hESC-CMs exhibit fluorescent transients with each contractile cycle, providing a convenient readout of

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graft activation that can then be correlated with the host electrocardiogram. Using this approach, we found that hESC-CM grafts in uninjured guinea pig hearts always activated synchronously with host myocardium. Outcomes following transplantation in injured hearts, however, were mixed, with only ~60% of injured hearts containing coupled hESC-CM graft regions. Equally concerning was the finding that even well-coupled graft regions in injured hearts typically showed relatively slow conduction velocities, a situation likely to favor pro-arrhythmic reentrant phenomena. In summary, while we found direct evidence that hESC-CMs could form functionally integrated myocardium in injured hearts, more work will be required to improve their integration and maximize the functional benefits.

A closely related issue is the risk of graft-related arrhythmias, the incidence of which could be conceivably either increased or decreased by improvements in graft electromechanical integration. Indeed, PSC-CM grafts could plausibly contribute to all three fundamental arrhythmia mechanisms: automaticity, triggered activity, and reentry. First, as immature cardiomyocytes, PSC-CMs exhibit some degree of automaticity, although this diminishes somewhat with duration in culture [28]. Second, some reports suggest that these myocytes are particularly prone to exhibiting early- and after-depolarizations and triggered activity that is thought to underlie many episodes of ventricular tachycardia [100]. Finally, PSC-CM transplantation may promote reentrant phenomenon by slow propagation through irregularly-shaped islands of graft myocardium isolated by scar tissue. While work in small animals suggested that hESC-CM transplantation might actually exert an arrhythmia-suppressive effect in injured hearts [99], more recent preliminary studies in larger animal models with slower heart rates suggest that these cells may instead promote arrhythmias [101]. In our opinion, if such pro-arrhythmic effects are significant, this may prove the most challenging hurdle to the successful development of PSC-based cardiovascular therapies.

Cell death

It is known that the vast majority of implanted cardiomyocytes die shortly after intra-cardiac transplantation as a consequence of anoikis, ischemia and inflammation [102,103]. While this initial wave of cell death is somewhat compensated for by the subsequent proliferative activity of PSC-CMs in vivo [84], it is nonetheless an inviting target for improving graft outcomes. Our group has identified a number of interventions that each help attenuate graft cell death to a degree, including transient heat shock of the cells pre-transplantation [84], delivery in the presence of a cocktail of pro-survival factors [35] and treatment with carbamylated erythropoietin [103]. Additionally, the Wu lab has described elegant, longitudinal and non-invasive imaging techniques that will likely prove useful in testing other methods of enhancing graft cell survival [104,105]. It is likely that the ideal strategy for improving cell survival and integration will involve a multifaceted approach.

Immune rejection

Another obvious cause of graft cell death is immune rejection. While undifferentiated PSCs have some degree of immune privilege [106], their differentiated progeny including cardiomyocytes are immunogenic and clearly evoke an immune response in allogeneic recipients [16,107]. A number of exotic solutions to this problem have been proposed including the creation of isogeneic PSCs by somatic cell nuclear transfer [108], the creation

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of “universal donor” PSC lines via HLA engineering [109,110], and the induction of tolerance via bone marrow microchimerism [111,112]. Because iPSCs can be genetically matched to their recipient, they represent another theoretical solution to this problem, although the creation of “customized” autologous iPSC therapies would likely present a new set of practical hurdles in terms of scalability, economics, and regulatory burden. In our opinion, initial PSC-based therapies will require pharmacological immunosuppression, although we speculate that stem cell banking and the exclusion of antigen-presenting cell types (e.g. endothelial cells) from grafts may allow the use of a less aggressive immunosuppression regimen than is required for conventional solid organ transplants. The Wu group is using the aforementioned longitudinal imaging techniques to help define optimal immunosuppressive strategies for PSC-CMs [113,114].

Graft vascularization

A robust vascular supply is critical for PSC-CM grafts to thrive in the heart, but surprisingly the vascular consequences of PSC-CM transplantation have not been extensively investigated. In our own hESC-CM transplantation studies in intact and injured rodent hearts, we demonstrated the formation of new microvessels within the graft myocardium [35,84,99]. These neovessels were largely of host origin, but we did find chimeric vessels when less pure populations of cardiomyocytes were injected. Importantly, while the density of new capillaries within the graft tissue approached that in the surviving distant host myocardium, vessel densities in the border zone and scar tissue outside of the graft were unchanged relative to those in untreated controls [35,84]. Working independently, both the Murry and Gepstein groups have compared outcomes following the implantation of bioengineered constructs seeded with hESC-CMs alone versus those seeded with a mixture of hESC-CMs, endothelial cells, and fibroblasts [77,79]. Not surprisingly, the latter preparation produced more graft-derived vessels, but both were perfused by vessels that were connected to the host vasculature, as evidenced by the presence of either intraluminal erythrocytes or fluorescent microspheres. It remains to be demonstrated how the function of these vessels will compare to those obtained following the injection of cell suspensions alone.

In recent work, our group imaged engrafted hearts by micro-CT angiography to look for effects on larger vessels that cannot be readily examined by histology [115]. Interestingly, skeletal myoblast grafts evoked significant remodeling of larger conducting vessels in myocardium distant to the graft, an effect that might enhance perfusion of both host and graft tissue. It remains to be seen whether PSC-CM grafts can mediate a similar response.

Closing Perspectives

While the development of cardiovascular therapies based on PSCs certainly lags that of certain adult stem cell types (some of which have reached human trials), substantial progress has been made in recent years. PSC-CMs can now be made in large quantities at high purities using reagents that comply with “Good Manufacturing Practice” standards [116]. The phenotype of PSC-CMs has been extensively characterized in vitro, and promising new strategies for promoting their maturation are currently under development. Preclinical studies in small animal models have shown convincingly that these cells survive in both

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healthy and infarcted myocardium; and, in most studies, they also exert modest beneficial effects on cardiac function. This work has provided compelling rationale for large animal studies, which are now well underway. Despite these advances, there are still critical gaps in understanding that should be addressed before clinical trials can commence. For example, while recent studies have shown that hESC-CM grafts are indeed capable of coupling with host myocardium and contributing new force-generating units, it is still uncertain whether this mechanism accounts for the observed beneficial effects on contractile function. Finally, valid concerns regarding the risk of arrhythmias must be addressed. In summary, while the long-term prospects for PSC-based cardiac repair are favorable, much work remains to be done.

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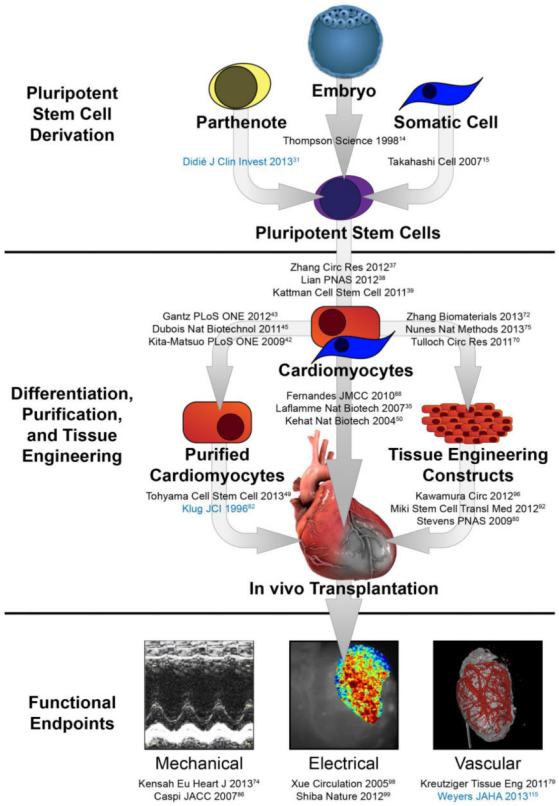

Figure 1. Schematic representing the generation, transplantation, and functional endpoints associated with stem cell-derived cardiomyocyte therapy

Critical manuscripts in each area are listed, with rodent cell work denoted in blue and human cell work denoted in black.