Stem Cells and Their Potential in Cell-Based Cardiac Therapies

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Stem cells are potential agents for the treatment of myocardial infarcts among other heart diseases. Over the past decade, the scientific community has extensively used a wide variety of cells and examined their capacity to both regenerate the infarcted myocardium and improve functionally the diseased hearts. Some of the cells used include skeletal myoblasts, bone marrow–derived cells, adult cardiac resident stem cells, mesenchymal stem cells, and both mouse and human embryonic stem cells (*Nat Biotechnol* 2005;23:845-856). The reported cardiogenic capacity of the utilitized stem cells is assayed both in vitro through the use of differentiation paradigms and in vivo through transplantation into a variety of animal models of cardiac disease. The purpose of this review article is to summarize recent stem cell applications in cell-based cardiac therapies and their outcomes.

Stem cells have enormous potential for the treatment of a variety of human diseases. Stem cells as applied to regenerative medicine have a purpose of supporting and/or replacing existing host cells that may have ceased to function properly or have been lost. The heart is an organ that seems to lack any intrinsic regenerative capacity. A myocardial infarction is caused by an acute reduction of blood to the myocardium with the direct effect of diminished oxygen supply compared to the amount required by the myocardium. In humans this leads to myocardial necrosis and the loss of large numbers (>10^9 cells) of cardiomyocytes. Cardiac wall thinning and cardiac remodeling among other factors lead to a diminished capacity of the heart to pump blood effectively leading to eventual heart failure and ultimately death of the patient. The main purpose of stem cells as applied to the treatment of myocardial infarcts is to prevent heart failure by either rescuing the host myocardium or regenerating cardiac cells (Fig 1).

Since the initial conception of the idea that cells may be used as therapeutic agents for the grafting and regeneration of the myocardium, scientists have used a variety of cell types and examined their capacity to do so. In vitro studies examined the ability of stem cells to differentiate into cardiomyocytes and then proceeded to investigate the functional characteristics of these cells. In vivo studies examined the capacity of stem cells to graft into the host myocardium and then assayed the functional recovery of the diseased heart.

The main purpose of this article is to review recent reports on the use of stem cells for the examination of their in vitro cardiogenic potential and their in vivo capacity to graft and improve the functional properties of the infarcted heart. This article will encompass both adult (bone marrow cells, skeletal myoblasts, adult cardiac resident stem cells, mesenchymal stem cells [MSCs]) and embryonic stem cells as well as the variety of in vitro cardiac differentiation protocols and in vivo cardiac injury animal models.

Adult Stem Cells

Transplantation Studies

Evidence of the potential to regenerate the heart was initially determined through transplantation studies where the donor organ has a different genotype than the recipient, thus allowing the

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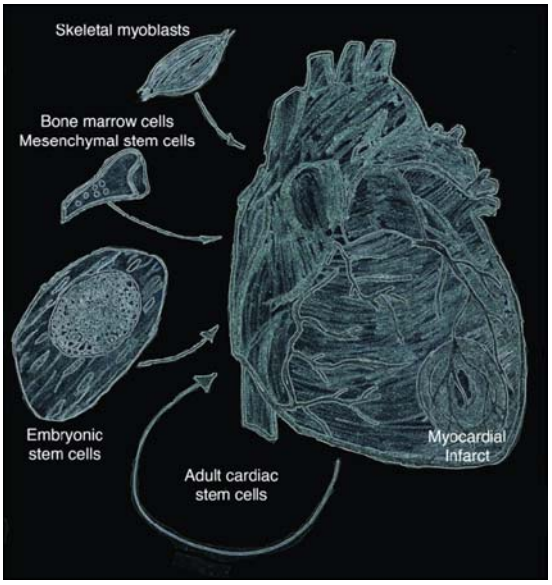


Fig 1. Stem cells as applied for the treatment of myocardial infarcts. A myocardial infarction is caused by a diminished blood supply to parts of the myocardium as a result of an occlusion to a coronary artery. This translates into low, or no oxygen supply to the cardiac muscle leading to wide apoptosis and necrosis of the cells. As a result of the infarct, the heart cannot pump blood effectively to the rest of the body, thus leading to heart failure and ultimately death of the patient. Stem cells have the capacity to differentiate into functional cells including cardiomyocytes and endothelial cells. The main purpose of stem cell therapies for the treatment of myocardial infarcts is the prevention and or regeneration of dying muscle. A variety of cell types have been used for such a treatment. These cells include skeletal myoblasts, whole bone marrow cells or sorted bone marrow–derived HSCs, bone marrow–derived MSCs, adult cardiac resident stem cells, and embryonic stem cells including their differentiated progeny.

detection of cells that engrafted in the heart posttransplantation. In the case where a male patient received a heart from a female donor, the presence in the heart of cells containing the Y chromosome would indicate the cellular engraftment from the male host. In one such study,[1] the male recipients had myocardial infarcts posttransplantation. It was determined that host-derived noninflammatory progenitor and endothelial cells were significantly increased compared with patients that did not sustain an infarct; however, a very small cell number (0.02%-0.07%) was determined to be male-derived cardiomyocytes. In a similar study,[2] 5 male patients were identified who had retained a female heart at least 9 months before death and necropsy. The authors of that study reported the number of Y chromosome cardiomyocytes to be 0.04% ± 0.016%, with most of these cells associated with areas of acute rejection and damaged myocardium. In addition, Muller et al[3] reported the detection of 0.16% ± 0.04% Y chromosome containing cardiomyocytes in the hearts of 8 male recipients of female hearts. Finally, in a study[4] where females had undergone sex-mismatched bone marrow transplantation, their hearts were reported to contain 0.23% ± 0.06% Y chromosome containing cardiomyocytes.

It is evident from the data described in the above studies that a cell type present in the recipient patient can indeed enter the host heart and contribute to the resident endothelial cell population and to a much lesser extent to cells of the myocardium. The progenitor cell that contributes to the donor heart may be a circulating cell, a bone marrow–derived MSC, or even an organ-specific progenitor cell that may transdifferentiate into the identified endothelial cells and cardiomyocytes. This example provides evidence that cardiac regeneration does indeed take place naturally even at a very low level.

Bone Marrow Cells

Bone marrow is one of the main cell sources that have been investigated extensively for their ability to regenerate the myocardium. In one of the first studies[5] that examined the regenerative capacity of bone marrow–derived cells, female dystrophic mice (mdx) received marrow from male wild-type mice. It was determined that both the skeletal and cardiac tissue of the recipient mice had muscle-specific cells that contained the Y chromosome. Bone marrow–derived hematopoietic stem cells were used in a later study[6] in lethally irradiated mice that also sustained ischemia through coronary artery occlusion after cell transplantation. The donor cells constitutively expressed lacZ, which allowed their easy identification. The authors reported identification of lacZ-expressing cardiomyocytes with a prevalence of around 0.02% in the peri-infract region. They also identified endothelial cells with a prevalence of around 3.3% in small vessels adjacent to the infarct. The initial data described in the above studies suggested that the bone marrow progenitor cells could be a good candidates for cell-based cardiac therapies.

In 2003, it was reported, however, by Alvarez-Dolado et al[7] that bone marrow–derived cells fuse in vivo with cardiac muscle without transdifferentiation occurring in the absence of a fusion event. Moreover, the frequency of these fusion events was determined to be very similar to the frequency of bone marrow–derived cardiomyocytes reported in previous human and mouse transplantation studies. This report immediately raised many concerns about the observations seen previously in both the transplantation studies and the bone marrow cell donor experiments, as the phenomenon of cell fusion was not taken into consideration in those cases.

Orlic et al[8] in 2001 examined the regenerative capacity of bone marrow–derived hematopoietic stem cells (HSCs). In their study, isolated HSCs that constitutively expressed green fluorescent protein (GFP) were injected in contracting myocardium wall surrounding an infarcted region produced by coronary artery ligation. They discovered that the newly formed myocardium occupied 68% of the infarcted portion of the ventricle 9 days after transplantation and that the developing tissue comprised proliferating myocytes and vascular structures. The transplanted cells were reported to coexpress GFP and cardiomyocyte-specific markers. At the functional level, the hearts of the transplanted animals exhibited significant recovery as assayed by echocardiography and hemodynamic parameters when compared with sham-operated animals. In 2004, three studies were published that directly contradicted the observations reported in the above study by Orlic et al. In the first study, HSCs were isolated from mice carrying a transgene that controlled the expression of lacZ or GFP through the cardiac α myosin heavy chain promoter.[9] Isolated HSCs that would express the α myosin heavy chain protein as a result of their transdifferentiation into cardiomyocytes would also express lacZ or GFP and thus would be easily detectable in the hearts of the recipient animals. The HSCs were injected in the myocardium of animals that had undergone coronary occlusion and had a myocardial infarct as a result of ischemia. The authors reported that they could find no evidence of cardiac-specific transgene expression and concluded that there was no significant cardiac differentiation of the HSCs after their transplantation. A study published at the same time also reported on the capacity of bone marrow isolated HSCs to regenerate the infarcted myocardium and differentiated into cardiomyocytes.[10] Hematopoietic stem cells isolated from transgenic mice expressing GFP were injected directly into the ischemic myocardium of wild-type mice. GFP^+ cells expressed mature hematopoietic markers CD45 and Gr-1 with no detectable expression of cardiac markers. They concluded that even in the microenvironment of the injured heart, the isolated HSCs adopt only traditional hematopoietic fates. Finally, in a study by Nygren et al,[11] it was reported that both unfractionated bone marrow cells and a purified population of HSCs only transiently engrafted in the infarcted myocardium. Low levels of donor bone marrow–derived cardiomyocytes were observed in the host myocardium but were found to be the result of cell fusion and not transdifferentiation. The observations described in the four studies lead to contradictory conclusions. Unlike the first study, which reported wide transdifferentiation of bone marrow–derived HSCs into cardiomyocytes in the injured myocardium, the other three studies reported only rare events that could be explained through cell fusion.

The group that published the original study[8] using bone marrow–derived HSCs repeated their experiments with $c\text{-}kit^+$ cells isolated from the bone marrow of male transgenic mice that constitutively expressed GFP.[12] They reproduced their original findings by demonstrating that the injected cells differentiated into cardiomyocytes and coronary vessels with no detectable differentiation into hematopoietic cells, no apparent detection of the "paracrine" effect, or cell fusion. In 2005, another group reported the isolation of a novel human bone marrow–derived multipotent stem cell with the capacity to self-renew without the loss of multipotency and the capacity to differentiate into cells of all three germ layers.[13] They reported that cardiac transplantation of these cells in a myocardial infarct model resulted in robust cellular engraftment of the cells. They concluded that transplanted human bone marrow–derived multipotent stem cells secreted factors that caused the proliferation and preservation of the host myocardium,

differentiated into cardiomyocytes and fused with the host cells.

The use of bone marrow–derived progenitor cells and their capacity to regenerate the infarcted myocardium is highly controversial to say the least as evidenced by the different observations and conclusions described in the studies above. This can only mean that further work is needed to determine unequivocally the true cardiac capacity of bone marrow–derived cells.

Bone marrow cells have been used in clinical trials in which the primary objective was the functional improvement of the infarcted hearts. In the BOOST (BOne marrOw transfer to enhance ST-elevation infarct regeneration), trial patients received percutaneous coronary interventions (PCI) and intracoronary transfer of autologous bone marrow cells.[14] They concluded that six months after transplantation, the transfer of cells enhanced the left ventricular systolic function in a myocardial segment adjacent to the infarcted area when compared with patients who only received the PCI intervention. The authors did not address the mechanisms by which such functional recovery occurred in their patients. In a similar clinical study reported by Chen et al,[15] patients who received intracoronary autologous bone marrow cell grafts exhibited improved wall function and a 14% increase in the ejection fraction when compared with the control patients who were injected with saline. Recently, three reports were published in *The New England Journal of Medicine* describing three different studies wherein they used bone marrow cells for treatment of myocardial infarcts. In the first study, patients with acute ST-elevation myocardial infarction of the anterior wall were treated with PCI and intracoronary injection of autologous bone marrow cells.[16] When compared with control patients (only PCI treatment), they exhibited an increase of 0.6% in left ventricular ejection fraction on single photon emission computed tomography, an increase of 0.6% on echocardiography, and a decrease of 3% on magnetic resonance imaging. The authors concluded that there were no significant effects of intracoronary injection of bone marrow cells on global ventricular function. In the second study, patients with acute myocardial infarctions received an intracoronary infusion of bone marrow cells or placebo medium 3 to 7 days after reperfusion therapy.[17] The authors reported a small but significant improvement in the global left ventricular ejection fraction (5.5% $\pm$ 7.3% vs 3.0% $\pm$ 6.5%) at 4 months posttherapy application. They also reported a reduction in the prespecified combined clinical end point of death, recurrence of myocardial infarction, and any revascularization procedure at 1 year postoperation. The third study examined the effect of transcoronary transplantation of either bone marrow cells or circulating blood cells in patients with healed myocardial infarction.[18] It was concluded that the transplantation is associated with moderate but significant improvement in the left ventricular ejection fraction three months after transplantation.

The described clinical studies are evidence that bone marrow cell therapies are rational but not sufficient for adequate functional recovery of the infarcted hearts. Further work needs to be done to increase the ability of bone marrow cells to improve cardiac function. Moreover, the mechanism through which the bone marrow cells act on cardiac function needs to be further examined.

Skeletal Muscle Cells

The skeletal muscle is able to regenerate itself after injury because it contains satellite cells or myoblasts, which retain the capacity to fuse with the surrounding muscle fiber and differentiate into functional skeletal muscle. Initially, it was hypothesized that cardiac muscle lacked a resident cell type equivalent to skeletal muscle satellite cells, as it was not able to regenerate after injury. Initial cell-based cardiac therapeutic studies used satellite cells isolated from skeletal muscle.[19-22] It was immediately evident that the satellite cells were differentiating into muscle cells and incorporating into the host myocardium; however, after further analysis, it was concluded that they were not becoming cardiomyocytes, rather they were differentiating into their skeletal muscle cell identity.[23,24] The cells were reported to lack the intercalated disk proteins N-cadherin and connexin 43; thus, they did not couple electrophysiologically with the host myocardium. Skeletal myoblasts have been used in a large number of experimental studies using both small and large animal models.[25] Most of these studies concluded that the grafted cells differentiated into skeletal muscle cells and allowed an improvement to both regional and

global left ventricular function.[26] In the most recent study, skeletal myoblasts were injected into the left ventricular wall of dogs that were subjected to continuous ventricular pacing.[27] At the end of the study, the authors detected improvement of the left ventricular ejection fraction along with increased wall thickness, significant reduction in fibrosis and apoptosis, and a significant increase in cell proliferation.

Skeletal myoblasts were the first cell candidate to be used in a clinical setting for the treatment of heart disease. The skeletal myoblast is thought to be an ideal cell for cell-based therapies because it can be grown in high numbers in vitro after initial biopsy, it is a progenitor cell able to only differentiate into multinucleated myotubes eliminating the risk of tumor formation, and it has a high resistance to ischemia-induced apoptosis.[28] Several phase I clinical studies have reported the use of skeletal myoblasts for the treatment of heart disease.[29-34] There seems to be an agreement in these studies concerning the functional recovery of the transplanted hearts as indicated by an improved ejection fraction and improved regional contractility of the myoblast implanted segments. However, there seems to be a gap in the degree to which recovery was observed in each of these studies. Further work should include standards by which the recovery can be measured to correctly understand the degree by which these myoblasts affect cardiac recovery.

Adult Cardiac Stem Cells

It was believed until recently that the heart was one of the only organs that did not possess a resident progenitor cell, which would have the capacity to regenerate sections of the healthy or injured myocardium. In 2003, several independent groups reported the discovery of such a cell type.

In one such study, a Lin^- $c\text{-}Kit^+$ cell was isolated from the adult rat heart, and it was reported to be self-renewing, clonogenic, and multipotent, being able to give rise to cardiomyocytes, smooth muscle cells, and endothelial cells.[35] The cells were injected into ischemic hearts and were reported to reconstitute up to 70% of the myocardium, forming both new vessels and cardiomyocytes. More importantly, the authors reported a significant increase in cardiac function of the animals that had received these cells. In a similar study, the same cell type was delivered to the coronary arteries of rats that had had a myocardial infarct, via a catheter positioned into the aortic root.[36] The cells were reported to induce myocardial regeneration through the decrease of the infarct size by 29% and also to improve the function of the injured heart.

At about the same time, a different type of adult cardiac stem cell was reported by Oh et al.[37] The cells, isolated from adult mouse hearts, were $Sca\text{-}1^+$ and initiated in vitro cardiac gene expression after treatment with the DNA demethylating agent 5-azacytidine. A mouse model of ischemia/reperfusion injury was used to assay the in vivo capacity of the $Sca\text{-}1^+$ cardiac stem cells to regenerate the infarcted myocardium. Transplanted cells initiated production of cardiac functional proteins and were identified in the mouse hearts 2 weeks after injection. Fusion between the donor cells and the host cardiomyocytes was evident in about 50% of the identified cells, whereas the other 50% had differentiated into cardiomyocytes.

Martin et al[38] reported on the identification of a resident cardiac population of adult stem cells with the unique expression of the Abcg2 transporter protein (side population cells). The $Abcg2^+$ cells were reported to differentiate into alpha actinin-positive cells when cocultured with adult cardiomyocytes; however, when cultured individually in methylcellulose, they gave rise to hematopoietic colonies. Finally, gene expression analysis revealed a unique transcriptional signature similar to that of endothelial and hematopoietic progenitor cells. Cell-based cardiac therapies using the $Abcg2^+$ cells are underway.

Islet-1, a LIM homeodomain transcription factor, is uniquely expressed in the adult heart by the fourth identified cardiac stem cell population. This marker is expressed by cardiac progenitor cells in the secondary cardiac field, a structure present during early development, which contributes to most cells in the heart.[39] During development, proliferating progenitor cells in the outflow tract, the right ventricle, and the atrium express isl1, without which they cannot contribute to the heart. $Isl1^+$ cells were identified in the postnatal rat, mouse, and human myocardium.[40]

A technique of conditional genetic marking was used to identify the isl1$^+$ population in a temporal/spatial manner. This allowed the authors to selectively isolate the isl1$^+$ cells at a particular developmental stage. When cocultured with isolated cardiac mesenchymal cells, the isl1$^+$ cells maintained isl1 expression and proliferated in culture without differentiating. The isolated cells could be induced to differentiate in culture into cardiomyocytes after exposure to 4-OH-TM or after coculture with neonatal cardiac myocytes. No cell-based cardiac therapy experiments have yet to be reported involving the isl1$^+$ cardiac stem cells.

The four adult cardiac stem cell populations reported isolated are Lin$^-$/c-Kit$^+$, Sca-1$^+$, Abcg2$^+$, and isl1$^+$ cells. When these cell types were examined for markers expressed by the rest of the stem cell populations, it was found that the c-Kit$^+$ cells did not express Sca-1, the Sca-1$^+$ cells did not express c-Kit, and the isl1$^+$ cells did not express c-Kit or Sca-1. The Abcg2$^+$ cells were reported to be approximately 50% Sca-1$^+$ and only about 2% c-Kit$^+$ (no data were given for isl1). The lack of any regenerative capacity of the myocardium led the scientific community until recently to believe that the adult heart contained no resident stem cells. These reports describe the isolation and characterization of adult cardiac stem cells that, however, seem to differ in their described gene expression patterns and differentiation potentials. If indeed these four described progenitor/stem cell populations reside in the myocardium, it would be informative to examine why under normal circumstances they do not regenerate the myocardium and how can we stimulate them to do so.

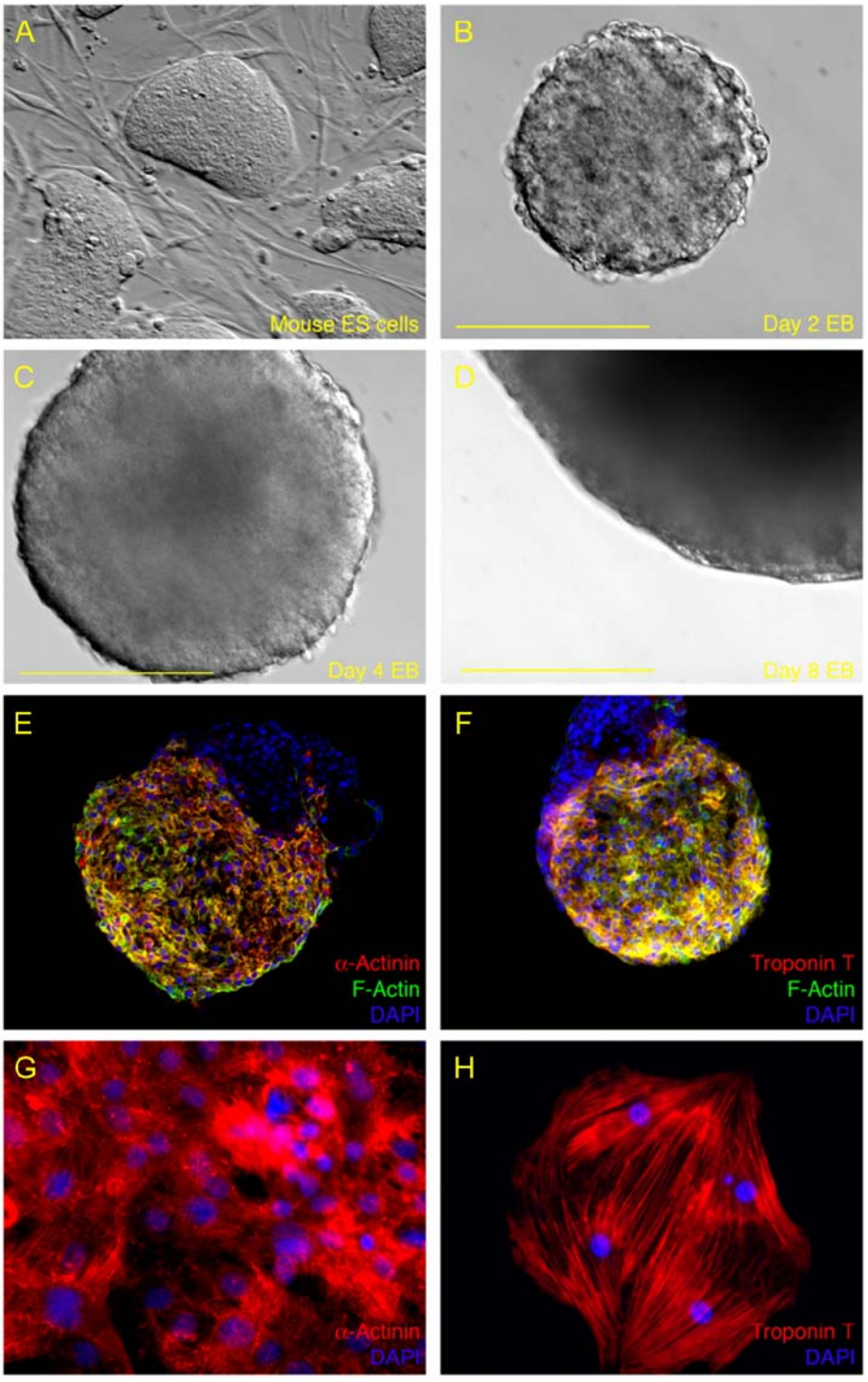
Mesenchymal Stem Cells

Mesenchymal stem cells are derived from the bone marrow stroma, which was originally believed to function as a structural framework for the hematopoietic cells that reside in bone marrow. Closer examination led to the discovery that these cells were expressing a variety of growth factors that supported hematopoiesis both in vivo and in vitro. Initial reports described the multipotential of MSCs to differentiate into a wide variety of mesenchymal tissues such as bone, cartilage muscle, bone marrow stroma, tendon, ligament, fat, and connective tissues.[41] Mesenchymal stem cells isolated from human bone marrow were also reported to be clonogenic in vitro and could be induced to differentiate into the adipocytic, chondrocytic, and osteocytic cell lineages.[42]

The in vitro capacity of mouse bone marrow–derived MSCs to differentiate into cardiomyocytes was first reported in 1999 by Makino et al.[43] The authors induced the cells to differentiate by treating them with 5-azacytidine: a global DNA demethylating agent that acts as a cytosine analog capable of altering expression of certain genes that may regulate differentiation. The morphology of about 30% of the treated cells changed within a week of treatment, and by the second week, the cells were spontaneously contracting and expressing cardiac specific proteins. Bittira et al[44] also reported the isolation of rat bone marrow–derived MSCs. LacZ labeled cells received either 5-azacytidine treatment or no treatment and were subsequently injected into the cryoinjured myocardium of isogenic rats. The authors reported that 4 to 8 weeks postinjection, the treated cells appeared myotube-like while expressing the cardiac marker troponin I-C. The data from both studies suggest that cell treatment with a DNA demethylating agent is necessary for the differentiation of MSCs into cardiomyocytes.

The effect of bone marrow–derived MSCs on cardiac function after myocardial infarction was also examined. A study reported by Shake et al[45] focused on the implantation of autologous MSCs in a swine myocardial infarct model. Labeled cells were administered 2 weeks postmyocardial infarction in the infarcted area through direct injection. The authors reported a significant attenuation in the degree of contractile dysfunction in the transplanted animals with reduced wall thinning in the infarcted region of the myocardium. In the second study, bone marrow MSCs were injected into the tail vein of rats that had had a myocardial infarct (MI).[46] The MSCs were reported to be recruited to the injured heart through the expression of the stromal cell derived factor 1, enhance angiogenesis, and improve cardiac function. The same authors also reported in another study that lacZ labeled rat bone marrow–derived MSCs were injected

into the tail vein. The cells were able to home to the injured area of the heart and were found at high concentrations in the peri-infarct region of the myocardium.[47] Toma et al[48] reported the injection of lacZ labeled human bone marrow–derived MSCs into the left ventricle of mice. One week postinjection, only a limited number of cells had survived; however, these cells were



reported to express cardiac-specific markers similarly to the host myocardium. Hattan et al[49] used a transgene that allowed expression of GFP under a ventricular-specific promoter. After MSC differentiation, GFP^+ cells were sorted and transplanted into the adult mouse myocardium with a reported long-term survival. In a most recent report, MSCs cultured in the presence of cardiogenic growth factors were injected into the myocardium of dogs that had had a myocardial infarct (coronary artery ligation) 8 weeks prior to the injections.[50] The authors report significant functional recovery of the transplanted hearts.

Bone marrow–derived MSCs are reported to have the ability to home to the areas of the heart that have sustained an injury as a result of a myocardial infarct. They have also been shown to express cardiac markers in the myocardium independent of 5-azacytidine treatment. One of their advantages is that they are an autologous cell source obviating the need for immunosuppression therapy. However, the time needed for MSCs to proliferate in culture to a sufficient cell number needed for the transplantation is far longer than the short amount of time the patient has postinfarction for the injection of these cells as shown by Bittira et al.[44] Furthermore, the capacity of these cells to regenerate the infarcted myocardium has not been proven other than the fact that the presence of the cells in the myocardium decreases the potential infarct size. Phase I studies with bone marrow–derived MSCs (Osiris Therapeutics) are currently under way at the Johns Hopkins medical institution.

Embryonic Stem Cells

Mouse Embryonic Stem Cells

Scientific discoveries that were initially reported in the early 1950s with the experimental production of teratomas and teratocarcinomas and the isolation of the embryonic carcinoma stem cells were essential stepping stones for the subsequent derivation of embryonic stem cells.[51] Embryonic carcinoma cell lines were derived from teratocarcinomas that formed when embryos were grafted at extrauterine sites. An attempt was then made to determine if it was possible to isolate cells directly from early embryos. The first reported mouse embryonic stem cell (mESC) lines were isolated from the inner cell mass of preimplantation mouse embryos.[52,53] The isolated mESCs were derived through plating on supporting feeder layers of division-incompetent mouse embryonic fibroblasts. It was further discovered that leukemia inhibitory factor (LIF; soluble factor) acts on established cultures of mESCs to maintain them in their undifferentiated state.[54,55] Removal of both the feeder layer of embryonic fibroblasts and LIF from the cell culture results in the differentiation of mESCs. Undifferentiated mESCs are characterized by the specific expression of markers, which are quickly silenced after the initial stages of differentiation. Stage-specific embryonic antigen 1, which was originally identified to be expressed by embryonal carcinoma cells,[56] is also highly expressed on the cell surface of mESCs. Transcription factors Pou5f1[57] and Nanog[58,59] are expressed specifically in the nuclei of mESCs. Finally, mESCs are characterized by high alkaline phosphatase activity.

Doetschman et al[60] reported the first evidence for the cardiogenic potential of mESCs in 1985. The cells, cultured in suspension in the absence of a feeder layer, formed 3-dimensional cystic bodies, termed embryoid bodies, which differentiated into cell types analogous of the visceral yolk sac, blood islands, and myocardium (Fig 2). Mouse embryonic stem cell–derived cardiomyocytes were determined to follow a stage-specific developmental myosin heavy chain transcription

Fig 2. Mouse embryonic stem cells and their cardiogenic potential. Colonies of undifferentiated mESCs are cultured on a supporting feeder layer of proliferation incompetent primary mouse embryonic fibroblasts (A). Embryoid bodies formed by differentiating mESCs at days 2, 4, and 8 of differentiation (bar, 500 μm) (B and D). A transgene introduced in mESCs allows the selection of mESC-derived cardiomyocytes under the control of the cardiac α myosin heavy chain promoter. Antibiotic selection is applied for a week after the first detection of spontaneously contracting areas in differentiating embryoid bodies. Cells in the contracting aggregates stain positive for the cardiac markers α-actinin and troponin T (×20) (E and F). Dissociated mESC-derived cardiomyocytes express stain positive for cardiac markers (×40) (G and H).

Table 1. Cell-Based Cardiac Therapies Using Embryonic Stem Cells

Authors	PMID	Cell Source	Animal Model	Cell Application	Observations
Klug et al	8690796	mESC-derived cardiomyocytes (aMHC promoter NeoR)	Adult dystrophic mice (*mdx*)	10,000 cells left ventricular wall	• Graft formation up to 7 weeks postengraftment
Min et al	1.2E+07	microdissected mESC-derived cardiomyocytes (hCMV promoter-GFP)	Male Wistar rats LAD ligation	30,000 cells 3 injections in infarcted and peri-infarcted area	• Transplantation significantly improved LV function and isometric contractility • Reduced infarct size
Min et al	1.3E+07	Microdissected mESC-derived cardiomyocytes (hCMV promoter-GFP)	Male Wistar rats LAD ligation	300,000 cells 3 injections in infarcted and peri-infarcted area	• Increased survival rate • Improved ventricular function • GFP cardiomyocytes in the myocardium • Increased number of blood vessels
Hodgson et al	1.5E+07	mESCs (α-actin promoter-ECFP)	Male Sprague-Dawley rats LAD ligation	300,000 cells peri-infarcted area 8 weeks postinfarction	• Significant LVEF improvement • Significant positive inotropic response • ECFP+ engrafted cardiomyocytes • No immunorejection or tumor formation
Min et al	1.7E+07	Microdissected mESC-derived cardiomyocytes (hCMV promoter-GFP)	Male Wistar rats LAD ligation	30,000,000 cells tail vein injections	• Significant increase in ventricular function, regional blood flow, and arteriole density • GFP+ grafts in infarcted area
Singla et al	1.6E+07	mESCs (CMV promoter-EGFP) (RNF4-lacZ)	C75BL/6 mice coronary artery ligation	30,000 cells infarct, peri-infarct, normal myocardium	• Engraftment of GFP+ or lacZ+ cells in the infarct region without evidence of tumor formation • Cells differentiated into cardiomyocytes, smooth muscle cells, and endothelial cells.

Nelson et al	1.7E+07	mESCs (aMHC-LacZ) (EF-LacZ)	C75BL/6 mice coronary artery ligation	50,000 cells inferior border of infarcted area	• mESC-derived tumors in the pericardial space (21% of the injected hearts) • Cell engraftment restricted in infarcted myocardium • Cardial functional improvement
Ke et al	1.6E+07	mESCs (CMV promoter-EGFP)	Mice (Friend leukemia virus) LAD ligation	50,000 cells seeded onto PGA scaffold patches	• Improved blood pressure and ventricular function • Significant higher survival rate • GFP+ cells engrafted in infarcted region
Kolossov et al	1.7E+07	mESC-derived cardiomyocytes (aMHC-Pac-IRES-EGFP)	SV129 mice cryolesion or LCA ligation	30,000-300,000 cells infarct and peri-infarct area	• Long-term engraftment with no teratoma formation • Improved cardiac function
kehat et al	1.5E+07	Microdissected hESC-derived cardiomyocytes	Pigs His-bundle ablation	40-150 contracting EBs. Posteriolateral wall of left ventricle	• hESC cardiomyocytes paced swine hearts that had complete atrioventricular block
Xue et al	1.6E+07	Microdissected hESC-derived cardiomyocytes (CAG promoter-GFP)	Adult guinea pigs	Contracting EBs anterior epicardium	• Functional hESC-derived pacemaker could be implanted in the left ventricle in vivo • Successful spread of membrane depolarization from the site of injection to the surrounding myocardium
Laflamme et al	1.6E+07	Percoll enriched hESC-derived cardiomyocytes	Male nude rats	5-10,000,000 cells left ventricular wall	• Grafted cells expressed cardiac specific markers • No connexin 43 detection • Rat and human specific angiogenesis • Proliferation of grafted cells

LV, Left ventricular; LAD, Left anterior descending; PMID, Pubmed ID number.

pattern similar to that seen during in vivo cardiogenesis.[61] It was also established that the derived cardiomyocytes were able to develop cell surface receptors, signal transduction mechanisms, and L-type Ca^{2+} channels similar to those found expressed by in vivo cardiomyocytes.[62] This information led to the conclusion that mESC-derived cardiomyocytes could be used as a model system to study cardiomyocytes and their response to toxicological agents. Patch-clamp analysis indicated that action potentials of early-stage differentiated cardiomyocytes represented those of pacemaker cells, whereas action potentials of terminally differentiated cells represented those of sinusnodal, atrial, and ventricular cardiomyocytes.[63] Westfall et al[64] examined cellular dimensions, sarcomere formation, and cell-cell contacts in differentiating cardiomyocytes. They concluded that mESC-derived cardiomyocytes exhibit cell morphology, sarcomere formation, and cell-cell junctions similar to those observed in cardiomyocytes developing in vivo. Through gene expression analysis of the mESC-derived cardiomyocytes, it was determined that the cells resemble their counterparts in the heart tube: an early stage of in vivo cardiogenesis.[65]

Currently, for an efficient cardiac differentiation of mESCs, the cells undergo a step of feeder layer subtraction and are resuspended in LIF-free culture medium at a very low density (5×10^4 cells/mL). Mouse embryonic stem cells are cultured in small drops (hanging droplets, $\approx 20\ \mu$L), which are formed on the lid of tissue culture dishes. The cells aggregate and form differentiating embryoid bodies in a hanging droplet microenvironment for 2 days. The formed embryoid bodies (EBs) are then transferred into ultralow attachment dishes where they further differentiate. Spontaneous contracting cells, evidence of the presence of newly formed cardiomyocytes, can be observed between days 7 and 8 of differentiation. The process of cardiogenesis in differentiating embryoid bodies is dependent on the culture conditions used. Many groups have reported on the capacity of either growth factors that are known to be active during in vivo development or small molecules to efficiently enhance cardiogenesis in cultures of differentiating mESCs. Molecules like bone morphogenic proteins, transforming growth factor β, fibroblast growth factor, nitric oxide, retinoic acid, and ascorbic acid have all been shown to increase the percentage of cardiomyocytes derived from these cultures.[66]

The possible applications of mESC-derived cardiomyocytes for cell-based cardiac therapies were evident early after their initial discovery. Two reports by Field and coworkers established that both, in a mouse and a canine animal model, fetal cardiomyocytes were able to form stable grafts that function in tandem with the host myocardium.[67,68] It was thus hypothesized that mESC-derived cardiomyocytes would be good cell candidates in cardiac engraftment studies, as previous reports had concluded that phenotypically, mESC-derived cardiomyocytes were embryonic-like. Klug et al[69] described the first such attempt: a transgene was stably introduced in undifferentiated mESCs allowing genetic selection (neomycin resistance) of only cells that expressed the cardiac α myosin heavy chain protein. The authors reported 99.6% cardiomyocyte purity after antibiotic selection. The capacity of the selected cardiomyocytes to form stable grafts with the host myocardium was tested by injecting the cells into the ventricular myocardium of adult *mdx* mice. They concluded that the selected cardiomyocytes were able to form stable intracardiac grafts having aligned with the host cardiomyocytes. Transgenes using either the Mlc-2v promoter or the Nkx2-5 promoter have also been used for the specific expression of selectable markers in ventricular cardiomyocytes or cardiac precursor cell populations, respectively.[70-72]

Embryonic stem and embryonic stem cell-derived cells have been used in a variety of studies for the treatment of myocardial infarcts (Table 1). Xiao and coworkers published two reports in which they describe the injection of microdissected GFP^+ mESC-derived cardiomyocytes ($\approx 3 \times 10^4$ to 3×10^5 cells) in the myocardium of rats that had myocardial ischemia through ligation of the left coronary artery.[73,74] The authors reported a significant survival rate of the animals that received the cells, along with improved ventricular function relative to the control group during the experimental period of 32 weeks. At the site of the infarct, they detected GFP^+ cells that also expressed cardiac markers, an increased overall level of cardiac proteins, and a greater number of blood cells. In a similar study, 3×10^5 undifferentiated mESCs (trans-

fected with a transgene that allowed the expression of a fluorescent marker under the control of the alpha actin promoter) were injected in the infarcted myocardium of rats that had had ligation of the left anterior descending artery 8 weeks before the transplantation.[75] The reported mESC-derived ECFP$^+$ cardiomyocytes that were detected in the myocardium were associated with normalized ventricular architecture, little scar formation, and a decrease in signs of myocardial necrosis. The authors saw no evidence of graft rejection, ectopy, sudden cardiac death, or tumor formation. Singla et al[76] reported injecting mESCs (3×10^4 cells) in the infarcted myocardium of mice with a ligated coronary artery. The authors reported that 2 weeks postinjection, the cells had engrafted in the infarcted region and differentiated into cardiomyocytes, vascular smooth muscle, and endothelial cells with no apparent tumor formation. Heart function was improved, and there was reduced heart remodeling. In another study, after initial seeding of GFP$^+$ mESCs on a biodegradable scaffold of polyglycolic-acid, the patch was transplanted on the surface of ischemic mouse myocardium.[77] The mice that received the patch with the cells were reported to have improved blood pressure and ventricular function, along with a higher survival rate. GFP$^+$ cells were detected in the infarcted area of the myocardium. A similar strategy was reported by Kofidis et al[78] (mESCs + Matrigel injection mixture) with reported prevention of ventricular wall thinning and improved fractional shortening and regional contractility. Finally, in a recent report by Min et al,[79] microdissected GFP$^+$ mESC-derived cardiomyocytes (3×10^7 cells) were injected intravenously in rats that had a myocardial infarction. Six weeks after injection, rats that received cells had increased ventricular function, regional blood flow, arteriole density, and the GFP$^+$ cells detected in the infarcted region expressed cardiac markers.[79]

Most studies described above used undifferentiated mESCs to treat myocardial infarcts. One of the main problems that may arise as a result of injecting the cells in their undifferentiated state is a risk of teratoma formation. A teratoma is a type of tumor, which is composed of cells derived from all three embryonic germ layers.[80] In some reported studies, the formation of teratomas worked against the therapeutic benefits of mESC transplantation.[81,82] Nelson et al[83] completed a study in which they examined the prevalence of teratoma formation as a result of mESC injections in the infarcted myocardium of mice. The authors reported restricted engraftment of the cells at the site of the infarct with significant increases in the ejection fraction, circumferential fiber shortening velocity, and peak mitral blood flow velocity. Most importantly, the authors found that 21% of the injected hearts had mESC-derived tumors in the pericardial space. The formation of tumors as a direct result of mESC injections seems to be a phenomenon not reported by all the groups that are following this therapeutic scheme. It is however a serious safety issue, which has to be resolved before any kind of similar therapies can be applied to humans. One suggested idea of eliminating this risk is to use ESC lines that would allow negative selection of any cells that remain undifferentiated after the transplantation process.

Immunoreactivity is also a problem associated with cell-based therapies in which mESCs or their differentiated progeny are used. One group examined the immunogenic characteristics of mESCs by injecting them in animal hearts that had sustained a myocardial infarct. Mouse embryonic stem cells (CGR8 mESC line) were injected in the hearts of immunocompetent rats that had sustained a myocardial infarct by left coronary artery ligation.[75,84] The authors reported significant cardiac recovery, with integration of mESC-derived cardiomyocytes in the infarcted rat myocardium. This observation may be evidence that embryonic stem cells and their differentiated cell derivatives are not immunogenic, a fact that would be greatly advantageous for cell-based cardiac therapies. This conclusion however is controversial because some groups have reported large-scale immunorejection of the transplanted cells.[66]

Finally, in a recently published study, Kolossov et al[85] injected either bone marrow cells, mESC-derived cardiomyocytes together with embryonic fibroblasts, or skeletal myoblasts in the hearts of mice that had a myocardial infarct. The authors reported detecting a long-term engraftment of the mESC-derived cardiomyocytes with no teratoma formation, accompanied by an enhancement in the cardiac function (significant enhancement of left

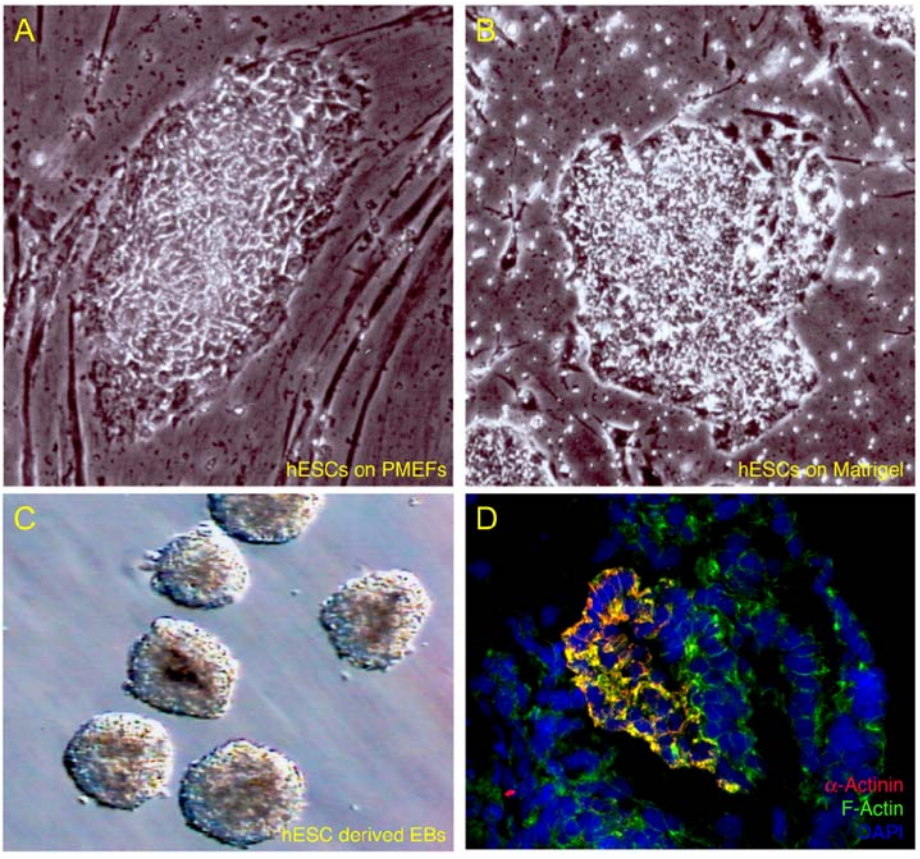


Fig 3. Human embryonic stem cells and their cardiogenic potential. Colony of undifferentiated hESCs cultured on a supporting feeder layer of primary mESCs (A). Before induction of differentiation, hESC colonies undergo feeder layer subtraction and are cultured on Matrigel-coated tissue culture plates (B). Embryoid bodies formed by differentiating hESCs (C). The hESC-derived cardiomyocytes among other cells present in an embryoid body stain positive for the cardiac marker α-actinin (D).

ventricular ejection fraction (LVEF), and reduction of end-diastolic volume). They also detected engraftment of the skeletal myoblasts in the infarcted region with a significant enhancement of the LVEF. On the other hand, the authors reported no contribution of the bone marrow cells to cardiac, endothelial, or smooth muscle tissue and no significant improvement of cardiac function.

From the reviewed reports it is apparent that mESCs or cell lineages derived from them have the capacity to engraft and regenerate the injured myocardium in a way that indeed improves cardiac function. Compared with other cell sources, these calls are unequivocally able to form cardiomyocytes that couple electrically with the host myocardium, as well as blood vessels and endothelial cells. However, mESCs are also associated with teratomas and immunorejection, which are serious issues that need to be addressed before using the cells in human cell-based therapies. Three recent reports describe the identification, isolation, and characterization of cardiac progenitor cells (CPCs) from mouse embryonic stem cells. Kattman et al describe the mESC-derivation of a *Brachyury*$^{+}$/*Flk1*$^{+}$ CPC population with the capacity to both proliferate and differentiate into cardiomyocytes, smooth muscle cells, and vascular endothelium cells.[95] In a similar report mESC-derived CPCs resembling progenitors of the secondary cardiac field (*Isl1*$^{+}$) also express *Nkx2-5/Flk1* and exhibit the same differentiation capacity.[96] Finally, Wu et al describe the isolation of a *Nkx2-5/Kit* CPC population with the capacity to differentiate into cardiomyocytes and smooth muscle.[97] The therapeutic potential of mESC-derived CPCs has yet

to be reported; however, utilization of the CPCs for cardiac cell-based therapies may be highly advantageous as the cells exhibit a capacity both for extended proliferation and differentiation into the cell types comprise the heart.

Human Embryonic Stem Cells

Thomson et al[86] reported the first successful isolation of human embryonic stem cells (hESCs) from human blastocysts in 1998, initiating a new era of stem cell biology. The isolated cells met all the criteria of embryonic stem cells: derivation from the pre- or peri-implantation embryos, prolonged undifferentiated proliferation under special conditions, and the capacity to form derivatives of all three germ layers. Scientists immediately started investigating the cardiogenic potential of hESCs (Fig 3). In 2001 Kehat et al[89] reported the successful differentiation of hESCs into cardiomyocytes, with 3 similar reports following soon after that on the same subject.[87-90] At the molecular level, it was determined that hESC-derived cardiomyocytes expressed markers characteristic of in vivo cardiomyocytes. Electrophysiological analysis demonstrated that most of the derived cells resembled human fetal ventricular myocytes. Moreover, electron microscopy revealed various degrees of myofibrillar organization consistent with early-stage cardiomyocytes. In cocultures, fetal cardiomyocytes were coupled through gap junctions with hESC-derived cardiomyocytes as demonstrated by real-time intracellular calcium measurements, Lucifer yellow injections, and connexin-43 expression. To isolate the cardiomyocytes, after hESC differentiation, cells are centrifuged through a Percoll gradient, which allows specific enrichment of the hESC-derived cardiomyocytes. Interestingly, the hESC-derived cardiomyocytes unlike their mouse counterparts are able to divide and proliferate in vivo in the presence of insulin growth factor 1.[91]

Three reports have been published to date that describe the use of the derived cardiomyocytes for in vivo cell-based studies (Table 1). In the first report, the authors completely blocked the resident pacemaking capability of the swine heart (immunosuppressed pigs) by ablating the His bundle.[92] They then injected bundles of contracting hESC-derived cardiomyocytes that had been mechanically dissected from embryoid bodies into the posterolateral region of the left ventricle. The authors reported that the hESC-derived cardiomyocytes were able to generate stable spontaneous pacemaking activity. They detected, however, sustained ectopic activity in only half of the animals, and that activity was interspersed with episodes of junctional escape rhythm. In the second report, the authors injected microdissected GFP expressing contracting areas of embryoid bodies into the left ventricular anterior wall of adult guinea pigs.[93] After cell injection, the pacemaking circuit of the hearts was diminished through cryoablation. The authors detected spontaneous action potentials in the hearts of the transplanted animals, which were consistent with epicardial wavefront propagation proceeding from the site of transplantation. Finally, according to the most recent report, 5 to 10×10^6 Percoll gradient enriched, heat-chock conditioned, hESC-derived cardiomyocytes were injected in the uninjured left ventricular wall of male nude rats.[94] In spite of the wide detection of noncardiac markers (cytokeratin and α-fetoprotein) immediately after cell injection, no such markers were detected 4 weeks later with only cardiac marker expressing markers grafted cells present at the site of injection. The grafted cells expressed high levels of the gap junction protein N-cadherin; however, no connexin 43 expression was detected. Both rat-specific and human-specific angiogenesis was observed in the grafts. Finally, BrdU staining along with immunocytochemical analysis specific for Ki-67 revealed a high degree of proliferative activity in the grafts.

Since the initial report of the derivation of cardiomyocytes from hESCs work from the scientific community has shown that these cells behave structurally and functionally as early-stage cardiomyocytes and they can integrate and couple in vivo with the host myocardium of animal models. There are, however, a few issues that need to be resolved before use of these cells in clinical studies. Mechanical microdissection of the cells only allows the derivation of a very small number of heterogeneous cell types that may not be sufficient in number or purity for any human cell-based cardiac therapies. Furthermore, Percoll gradient centrifugation only enriches for the hESC-derived cardiomyocytes, allowing the presence of other cell types, which may include undifferentiated hESCs, which can form teratomas at the injection site. The use of transgenesis

would be advantageous because it would allow the genetic selection of a pure population of hESC-derived cardiomyocytes be used. Moreover, the discovery of molecules that would enhance cardiogenesis during hESC differentiation would yield increased cardiomyocyte cell numbers, which when coupled with large-scale culture methods would allow the derivation of sufficient cardiomyocytes for cell-based cardiac therapies. Immunorejection is another major issue that must be resolved before any use of hESCs or their derivatives for therapeutic purposes. Specific solutions for this problem that have been suggested[94] include: (1) multidrug immunosuppression regiments; (2) cells that are isogenic with the recipient patient through either somatic cell nuclear transfer or somatic cell reprogramming; (3) expression by the hESCs of recipient specific major histocompatibility complex molecules; (4) establishment of hematopoietic chimeras and induction of immunologic tolerance.

Conclusions

The use of stem cells as regenerative agents for the treatment of myocardial infarcts is a relatively novel concept in the area cardiac therapies. Since its initial conception, about a decade ago, a large assortment of cell types have been used and their capacity of improve cardiac function examined. In vivo grafting of the cells into infarcted areas of the heart has proven their multipotential properties: preventing large-scale myocardial necrosis, differentiating into functional cardiomyocytes that couple with the host myocardium, growing new blood vessels, and secreting factors that support myocardial regeneration. Clinical application of cell-based cardiac therapies has been reported for some of the stem cell types reviewed in this article with some promising data involving cardiac functional recovery. Embryonic stem cells have vast multipotential differentiation abilities. With the recent isolation of hESCs, it is only a matter of time before the undifferentiated cells or their differentiated progeny are used for cardiac therapies in a clinical setting. Many issues must be resolved before use of any cell type in clinical studies including diminishing their immunogenic character, ensuring their cell purity, improving on cell survival along with long-term engraftment, and diminishing the possibilities of side effects like induction of cardiac arrhythmias or tumor formation. Regenerative medicine and stem cells seem to be the new future for the treatment of heart disease, and their role may ensure a good quality of life for the million of patients that have it.

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