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Forum Review Article

Embryonic Stem Cells in Cardiac Repair and Regeneration

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Abstract

Cell transplantation is a subject of fast-growing research with a potential of a therapeutic approach for the treatment of heart diseases. Clinical applications require preparation of large number of donor cells. Stem cell studies published to date demonstrate that scientists have not reached the general consensus to use an optimal cell type for better cardiac repair and regeneration. We used embryonic stem (ES) cells and their released factors for cardiac repair and regeneration. The major concern of cardiac regeneration with stem cells includes engraftment, differentiation, and teratoma formation after ES cell transplantation. Our current knowledge of ES cell transplantation in the heart is very limited. This review discusses the use of various growth factors to enhance ES cells engraftment and differentiation, as well as the issue of teratoma formation. *Antioxid. Redox Signal.* 11, 1857–1863.

Introduction

Coronary artery disease and congestive heart failure remain significant health problem despite advances in early diagnosis and therapeutic interventions (17, 19–21). Acute myocardial infarction (MI) leads to cardiac remodeling, which is associated with coagulative necrosis of the myocardium (myofibrillar hypereosinophilia and loss of nuclei), myocyte cell loss *via* apoptosis, and infiltration of inflammatory cells such as neutrophils (17, 19–21).

The development, progression, and pathogenesis of heart failure is complex and multifactorial. Remodeling begins within hours of the MI and is initiated by migration of inflammatory cells, mainly macrophages and neutrophils, as well as fibroblasts, which produce tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β1 (17, 21, 59, 60). These cytokines stimulates mast cells and cardiac fibroblast proliferation (17, 21, 59, 60). The cross-talk of released factors and stimulated cells accelerates remodeling of the extracellular matrix (ECM), leading to a net accumulation of ECM. Cardiac remodeling is evidenced with enhanced collagen synthesis after MI and also is associated with concurrent ECM degradation through activation of matrix metalloproteinases (MMPs) (17, 21, 59, 60).

Existing treatment strategies for acute MI and subsequent heart failure include drugs (diuretics. Angiotensin-converting enzyme inhibitors, aldosterone antagonists, and β-blockers), ventricular-assist devices, implantable artificial hearts (still experimental), and ultimately, heart transplantation (2, 20, 21). However, recent stem cell research progress points to the potential of cell therapy as a future treatment strategy for heart failure. The most appropriate donor cell types, techniques, and patient populations for cell therapy remain uncertain. To avoid confusion between stem cells and progenitor cells, the definition of what constitutes a stem cell is as follows:

"A stem cell is clonogenic, capable of unlimited self-renewal by symmetric division, while maintaining a stable diploid karyotype. It is also capable of asymmetric division, one daughter resembling its mother, and one daughter giving rise to multiple types of differentiated cells."

In contrast, the progenitor cells that have been identified in adult organs thus far do not meet all of these criteria. The major aim of this review is to provide state-of-the-art knowledge on the cardiac repair and regeneration after embryonic stem (ES) cell transplantation.

Stem Cell Therapy

Over the past decade, cell transplantation to treat heart disease has been studied in a variety of animal models (1, 13, 26, 47). Demonstration of successful engraftment in injured myocardium has made cell transplantation, as a means to replace dysfunctional myocardium and treat heart failure, a realistic possibility. One rationale underlying this approach is that regeneration of injured myocardium with new heart-cell

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types will improve the mechanical properties of the infarcted region (13). Cell types used include multipotent progenitor cells, skeletal myoblasts, smooth muscle cells, fetal and embryonic cardiomyocytes, bone marrow stromal and hematopoietic stem cells, and mouse ES cells (1, 13, 26, 47). Most cell-transplantation studies have shown significant improvement in cardiac function despite often limited regeneration [*i.e.*, regeneration that is insufficient to compensate for cell loss due to apoptosis or necrosis or both (1, 13, 26, 47)]. The amount of regeneration has varied significantly depending in part on the number and type of cell transplantation.

Transplantation of purified Lin_c-kit^{pos} BMS cells in a mouse MI model resulted in partial regeneration of myocardium including endothelial cells, smooth muscle cells, and cardiomyocytes (41, 47). Results of these studies have spurred clinical testing of the potential use of adult stem cells for therapy (12, 47). The randomized clinical trials suggest that transplantation of autologous BMS cells into the myocardium of patients with a first ST-segment–elevation MI improves LV ejection fraction (EF) at 6 months' follow-up (4, 31, 44, 61); however, no significant difference was observed in these patients at 18 months (32). The recent clinical trial suggests that transplanted BM-derived progenitor cells in acute myocardial infarction (AMI) patients improve cardiac function at 4-month follow-up (45). In contrast, mononuclear BM cells transplanted in the AMI patients demonstrate no significant improvement in cardiac function at 6-month follow-up (29). However, these results are confounded by the fact that cell-transplantation studies in patients have usually been performed in association with surgical or percutaneous revascularization.

Skeletal myoblasts also are a major cell type studied to regenerate infarcted myocardium (14). This requires isolation and purification of skeletal myoblasts from skeletal muscle. Autologous skeletal muscle cell transplantation has also been shown to improve cardiac function in snake cardiotoxin-induced lesions in the sheep and cryoinjury-induced scars in rats (14). These studies have encouraged testing of adult stem cells in clinical medicine. The first clinical trial using autologous skeletal muscle cells demonstrated encouraging results after 5 months of follow-up, with respect to improved heart function (14). Autologous skeletal myoblasts transplanted in patients with ischemic cardiomyopathy show cell survival and formation of stable viable grafts in heavily scarred human myocardial tissue (14). Moreover, bone marrow stem cell applications remain controversial because their plasticity for heart regeneration has been seriously challenged (36, 61). Similarly, skeletal muscle progenitor cells clinical trials were stopped because of formation of arrhythmia after cell transplantation (14).

Embryonic Stem Cells and Cardiac Regeneration

ES cells are immortal, pluripotent cells derived and propagated from the inner cell mass (ICM) of the preimplantation blastocysts by using embryo-free techniques (50). They are unique because they propagate without differentiation in cell culture while maintaining the potential to differentiate into all three embryonic germ layers: ectoderm, endoderm, and mesoderm (50). The successful isolation and propagation of human ES cells has led to a great deal of interest in understanding their cellular and molecular biology in anticipation of their potential as a powerful new tool for regenerative medicine (57). By contrast, adult stem cells have limited plasticity and exhibit senescence when maintained in culture. Embryoid bodies (EBs) are aggregates of ES cells, most commonly developed by the hanging-drop method (Fig. 1A). As discussed later, we have shown that EB cells have the potential to form all three major heart-cell types *in vitro* (Fig. 1A) (50). However, the differentiation potential of ES cells to become cardiomyocytes (5–15%) has been limited in cell culture (47, 51). Accordingly, many investigators have used different growth factors/cytokines to enhance differentiation of cardiomyocytes from ES cells, as recently reviewed (42). We demonstrated that exposure of ES cells to TGF-β2, but not to TGF-β1 or TGF-β3, significantly increased spontaneous and rhythmically beating EBs (50%) found to be cardiomyocytes (51). Exposure to bone morphogenic proteins (BMP2) and fibroblast growth factor 2 (FGF2) has also been shown to enhance the differentiation of ES cells into beating cardiac myocytes (22). Consistent with this, clusters of beating cardiac myocytes compose more than 90% of colonies in EBs derived from FGFR$^{+/-}$ ES cells compared with 10% in EBs derived from FGFR$^{+/-}$ ES cells (6).

Transplantation of undifferentiated ES cells into the injured heart results in stable engraftment and differentiation of cardiomyocytes (16, 47). We have found that undifferentiated ES cells labeled with GFP/β-gal can be transplanted successfully into infarcted mouse hearts (47). We also demonstrated that transplanted cells can engraft and differentiate into all the major heart cells (cardiomyocytes, endothelium, and vascular smooth muscle) after 2 weeks (Fig. 1B) (47). Transplanted mouse ES cells 12 weeks after infarction exhibit differentiation into cardiomyocytes (16). Many studies have shown that transplanted mouse ES cells in the infarcted heart improve heart function (3, 16, 47, 53). Moreover, mouse ES cells transplanted into the infarcted sheep heart differentiate into cardiomyocytes and improve function (30). However, the actual amount of engraftment and differentiation into cardiomyocytes or other major heart cells has been limited.

In an attempt to enhance engraftment and regeneration, many investigators have used growth factors to induce differentiation of ES cells *in vitro*. However, it remains to be seen whether growth factor–primed ES cells used for cell transplantation will enhance cardiac differentiation and regeneration *in vivo*. Recent studies have shown that recombinant mouse IGF-1 added to a suspension of undifferentiated mouse ES cells subsequently transplanted into infarcted mouse hearts promotes cardiac myocyte differentiation and improved heart function compared with transplanting ES cells without IGF-1 stimulation (23). TGF-β added to suspensions of ES cells before transplanting them into post-MI mouse hearts was demonstrated to be the most effective agent with respect to eliciting an increase in the donor graft cells to the infarcted heart. Moreover, expression of connexin-43, a gap junction protein, suggesting coupling of the newly differentiated cells and improved heart function (24).

Donor Stem Cells Apoptosis After Transplantation

Cell therapy has demonstrated successful engraftment and differentiation after transplantation in the infarcted heart (Fig. 1A) (3, 12, 41, 47). However, results of recent cell-transplant studies suggest that cell survival *versus* donor cell death after transplantation is a major limiting factor for engraftment and differentiation (63). For example, skeletal muscle cells,

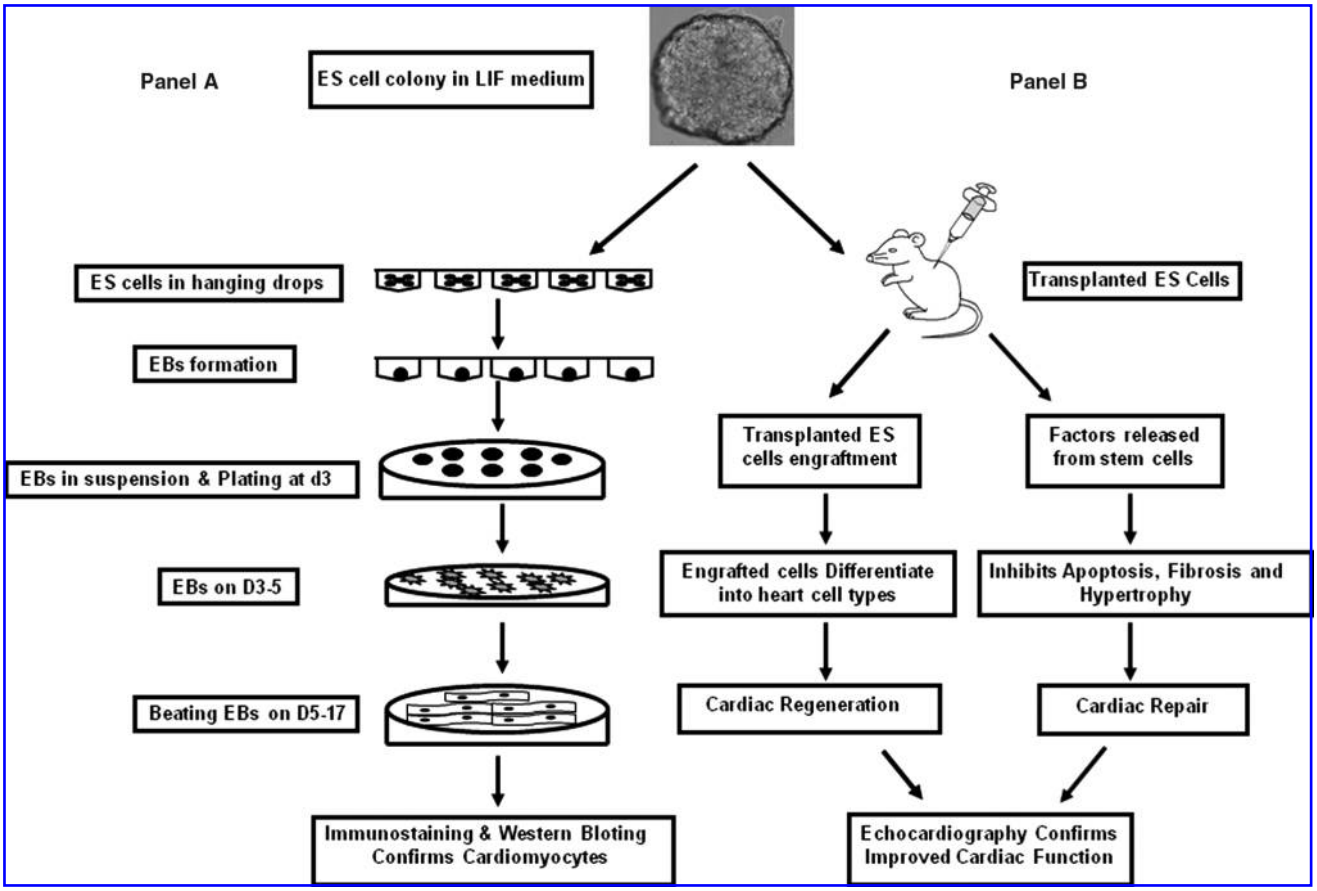

FIG. 1. *Left panel* (**A**) **demonstrates a general scheme outlining differentiation of cardiac myocytes from the mouse ES-EB *in vitro*.** *Right panel* (**B**) shows that transplanted ES cells in the infarcted mouse heart can engraft and differentiate into heart-cell types, as well as inhibit adverse cardiac remodeling and improve heart function.

neonatal cardiomyocytes, smooth muscle cells, and bone marrow stem cells survival after transplantation in the infarcted heart varies from 4 to 28% (15, 35, 54, 63). The cell-survival percentage depends on the number of cells transplanted, cell type, and method of transplantation (15, 35, 54, 63). Our unpublished data demonstrate that ES cell survival was $\sim$70 cells/section (detected with oct4^{+ve} marker) at day 1 after transplantation (3×10^4 ES cells *via* direct injection) in the infarcted mouse heart. The number of oct4^{+ve} cells was almost undetectable at days 14 and 28. A majority of donor cells die because of apoptosis or necrosis. For example, apoptosis has been shown after transplantation of dopaminergic neurons for Parkinson disease, islet cells for diabetes mellitus, and skeletal myoblasts for muscular dystrophy (7, 11, 38, 46, 52). Similarly, transplantation of neonatal or adult cardiomyocytes into injured myocardium is associated with significant apoptosis, necrosis, or both (35).

Strategies to Decrease Donor Cell Apoptosis After Transplantation

Cell survival after transplantation requires adaptation to diverse ischemic cardiac microenvironments. Various different strategies such as antiapoptotic gene transfection (9, 28, 37), pharmacologic and hypoxic preconditioning (5, 39, 58), and heat-shock protein (18, 55) treatment have been successfully used to enhance cell survival. Cardiomyocytes transfected with the antiapoptotic gene Akt have less donor-cell death after trans plantation (9, 10). Similarly, mesenchymal stem cells (MSCs) transfected with the Akt gene have better survival compared with that of wild-type MSC cells (9, 10). Another antiapoptotic gene, Bcl2, transfected in smooth muscle cells and MSCs enhances cell survival and engraftment.

Moreover, heme oxygenase-1 (HO-1, an inducible enzyme that releases antioxidative stress and cell-death factors), transfected myogenic precursor cells protects cell death from staurosporin-induced apoptosis in the cell-culture model (27). Vascular endothelial growth factor (VEGF)-transfected ES cells attenuate apoptosis in the cell-culture model (62). Beating cardiac EBs derived from VEGF-transfected ES cells after transplantation in the infarcted mouse heart demonstrate significant enhanced cell survival and cardiac function (62). Similarly, cyclosporin A pretreatment protects MSCs from hypoxia/reoxygenation-induced apoptosis (5).

Transplanted Stem Cells Release Factors That Inhibit Apoptosis and Cardiac Remodeling

Recent studies suggest that stem cells transplantation in the infarcted heart significantly improve cardiac function despite limited cell survival and engraftment. New emerging evidence suggests that surviving (10 to 20%) or dying cells (80–90%)

release autocrine or paracrine factors after transplantation. Factors released include antiapoptotic, antifibrotic, and cell-survival growth factors that may contribute in the attenuation of native heart cell apoptosis, fibrosis, and hypertrophy (Fig. 1B). We reported that ES cell transplantation in the infarcted heart decreases post-MI apoptosis, hypertrophy, and fibrosis (Fig. 1B) (48). Whether the decrease in apoptosis and fibrosis results from released autocrine or paracrine factors or is a direct consequence of regeneration remains unknown. We recently prepared ES cells–conditioned medium from surviving and dying cells after treatment with H_2O_2 (49). Our factor-analysis data indicate that the ES-CM contains antiapoptotic and antifibrotic factors suggesting that inhibiting apoptosis and fibrosis observed in the previous study could be the consequence of factors released after ES cells transplantation (49). Furthermore, our H_2O_2-induced apoptosis in H9c2 cells was significantly inhibited with the ES-CM, giving direct evidence of inhibition of apoptosis with the released factors (49).

A group showed that the CM from Akt-MSC demonstrates inhibition of apoptosis in cardiomyocytes (9, 10). Furthermore, they showed that several genes coding for VEGF, FGF-2, hepatocyte growth factor (HGF), insulin growth factor (IGF-1), and thymosin β4 (TB4) were upregulated and might be potential mediators of antiapoptotic effects observed with the CM (9, 10). Similarly, mesoangioblast and BM progenitor cells release basic fibroblast growth factor, insulin, and hepatocyte growth factors in their conditioned medium. Transplanted mesoangioblast and BM progenitor cells in the infarcted mouse heart significantly inhibit cardiac myocyte apoptosis, increase capillary density, and improve cardiac function. These data demonstrate that factors released from MSCs and ES cells are different, may have different mechanisms of inhibition of apoptosis, and require further investigation.

Transplanted Undifferentiated ES Cells and Teratoma Formation

It is well established that mammalian ES cells can form complex teratomas when engrafted into an immune-deficient host, a characteristic of ES cells resulting from their pluripotency (43, 57). Teratomas resulting from transplanted ES cells include highly organized and differentiated cell types representative of all three germ layers (8, 43, 50, 57). The cell types identified by using cell-specific markers include keratinized cells, hair follicles, muscle cells, cardiomyocytes, epithelial cells, neural ganglia, and pigmented cells (8, 43, 50, 57). Teratoma formation is thus a major potential limitation in the therapeutic use of ES cells.

Given the prospect of ES cells being able to differentiate into all three germ layers, it is critical to examine such potential after *in vivo* transplantation. After transplantation, engrafted cells are exposed to various combinations of cytokine/growth factor signals in their own local microenvironment and experience direct cellular interactions with adjacent host tissues. Results from recent studies suggest that local microenvironmental cues from the host tissue encompassing the transplanted cells influence the ability of the grafted cells to differentiate (25, 34, 47). This has led to the concept that organ-specific cytokine/growth factors can direct ES cells into organ-specific cell-type differentiation. For example, undifferentiated ES cells (1×10^5 cells) transplanted into the CCl4-treated mice will differentiate into hepatic cells when they are exposed to local liver-specific cytokines/growth factors, with no evidence of teratoma formation (25, 34, 47). Based on this concept of tissue-specific microenvironments, many investigators have transplanted undifferentiated ES cells into different organs and observed tissue-specific differentiation without teratoma formation (25, 34, 47).

In contrast, some investigators have reported that teratomas do form after ES cell transplantation (40, 56). In reviewing these results, it is important to consider the dose or number of ES cells, the microenvironment to which the ES cells are exposed after transplantation, and interactions between the two. We propose that two potential microenvironments exist after ES cell transplantation: one dominated by local cardiac cells and the other dominated by the transplanted ES cells themselves. We hypothesize that low numbers of transplanted ES cells are exposed to a predominantly cardiac microenviron-

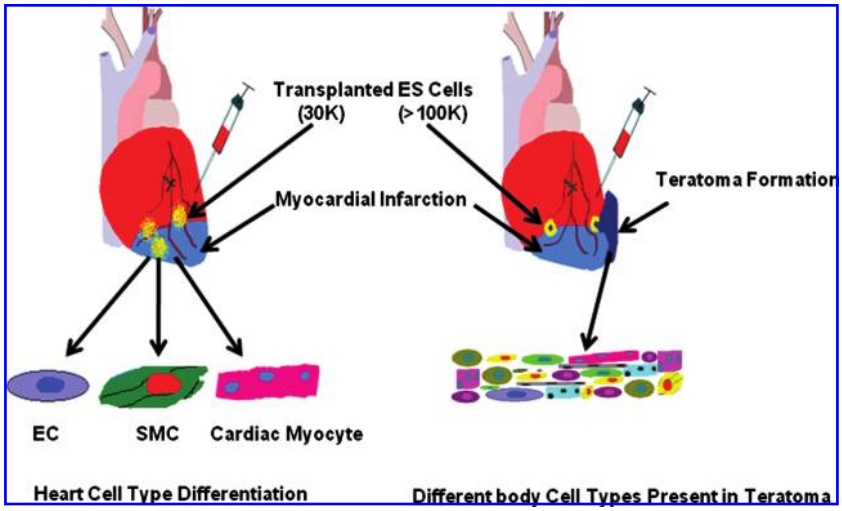

FIG. 2. ***Left panel*** **demonstrates that transplanted undifferentiated ES cells (*yellow*, 30K) in the peri-infarcted (*red* and *blue*) and infarcted (*blue*) mouse heart can easily spread out in the cardiac microenvironment (*dispersed small yellow dots*) and differentiate into heart-cell types (ECs, endothelial cells; SMCs, smooth muscle cells; and cardiac myocytes).** In contrast, the *right panel* demonstrates that transplanted ES cells (>100K) may localize to the needle-track area of injection (*thick yellow dot*) and be exposed to the internal ES cell microenvironment (*dark blue area inside the yellow thick dot*) and later develop a teratoma (*dark blue area*). The teratoma contains most of differentiated body-cell types (different colors represent various cell types). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

ment and therefore differentiate into cardiac cell types, whereas high numbers of transplanted ES cells retain an ES-cell microenvironment and have a higher potential to form teratomas (Fig. 2). In our hands and those of others, transplantation of up to 300,000 undifferentiated ES cells into the mouse heart by direct injection is safe and effective for cardiac regeneration and thus far has not resulted in formation of teratomas during a follow-up period of 12 weeks (16). We have also injected a total of 30,000 cells at three different sites in the infarcted mouse heart and have seen no evidence of teratoma formation (Fig. 2) (47). Behfar *et al*. (3) reported that 300,000 undifferentiated ES cells transplanted into the rat heart by using three separate injections does not induce teratoma. Similarly, a number of other investigators have transplanted undifferentiated ES cells and have not noted teratoma formation (23, 24, 33).

Very recently, Nussbaum *et al*., in *The FASEB Journal,* reported systematic evaluation of teratoma formation in the heart after transplantation of varying doses of ES cells (40). Their study suggests that transplantation of fewer than 100,000 undifferentiated ES cells into the mouse heart does not cause teratomas. However, they reported that teratomas do form if >100,000 ES cells are injected into normal or injured hearts (Fig. 2) (40). Importantly, they were unable to detect grafting of undifferentiated cells with a dose of 50,000 cells. The most likely explanation for their in ability to detect undifferentiated ES cells may be that the comparatively low number of transplanted ES cells were exposed to a microenvironment of cardiac cytokines and growth factors and differentiated into cardiac-specific cell lineages, as reported by us (47). Notably, Nussbaum *et al*. (40, 47) injected 100,000 ES cells in a single injection with 5–10 μl of cell-culture medium and compared this with 30,000 cells injected at three different sites in the mouse heart. We hypothesize that large doses of ES cells injected at a single site in small volumes of cell-culture medium will afford an ES cell microenvironment and lead to formation of ES cell clumps and teratoma formation. In contrast, with a low dose of ES cells exposed to a cardiac microenvironment, cardiac differentiation is induced, but no teratomas. This may explain the teratoma formation observed in such studies.

Recently, Menard *et al*. (30) transplanted 30 million undifferentiated ES cells primed with BMP2 (considered to be a cardiac-specific lineage growth factor for ES cells) and demonstrated engraftment and cardiomyocyte differentiation in the infarcted sheep heart, with no evidence of teratoma formation at 25 different sites. Thus, with BMP2 priming, little or no threat of teratoma formation occurred.

Future Strategies

After reviewing these recent studies, it would seem reasonable to consider ES cells as being potentially dangerous because of the threat of teratoma formation. An insight resulting from these studies is that future efforts should be directed toward purification of various tissue-specific cell types from ES cells for further therapeutic applications. Many laboratories recently described the isolation and purification of cardiac-specific progenitor cells from ES cells. Moreover, future efforts should be directed toward priming ES cells to direct them better toward differentiation into cardiac- or tissue-specific cell types, as needed. The use of antiapoptotic strategies to enhance engraftment and cell differentiation is another future direction. The use of released factors from ES cells could also enhance cardiac repair and regeneration. Last, an urgent need exists to examine the potential use of these cells in large-animal models.

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Abbreviations

AMI, acute myocardial infarction; BM, bone marrow; BMP2, bone morphogenic protein; BMS, bone marrow stem cell; CM, conditioned medium; EBs, embryoid bodies; EC, endothelial cell; ECM, extracellular matrix; EF, ejection fraction; ES, embryonic stem; FGF_2, fibroblast growth factor 2; GFP, green fluorescence protein; HGF, hepatocyte growth factor; ICM, inner cell mass; IGF1, insulin-like growth factor; MI, myocardial infarction; MMP, matrix metalloproteinase; MSCs, mesenchymal stem cells; SMCs, smooth muscle cells; TB4, thymosin beta 4; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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