

# Sources of Cardiomyocytes for Stem Cell Therapy: An Update

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**ABSTRACT:** The therapeutic use of stem cells for cardiac repair following myocardial infarction (MI) has generated a great deal of interest. Many types of stem/progenitor cells have been used in an attempt to regenerate the infarcted heart. We have reviewed the current state of progress using embryonic stem (ES) cells, hematopoietic stem and progenitor cells (HSCs/HPCs), mesenchymal stem and progenitor cells, resident and cardiac "stem" cells from other sources. Our view is that, even though the road ahead is long and tortuous, embryonic stem cells represent the most reliable font of CMC and the most promising type of cell to rely on for a future tool in heart failure treatment. (*Pediatr Res* 59: 79R–83R, 2006)

The proliferating and self-healing capacity of CMCs is limited. Thus, CMC loss after intrinsic or extrinsic damage can be partly if not at all replaced. For this reason, the field of cardiac stem cells is extremely exciting to work in. One of the major hurdles of this field is the definition of which is the ideal cardiac stem cell. In this review, we will review the literature on the major characteristics of cardiac stem cells described so far, trying to present the reader with a balanced view of the pros and cons of each cell type.

## EMBRYONIC CELLS

Three types of pluripotent stem cell lines have been established from mammalian embryos: ES, embryonic germ (EG) and embryonic carcinoma (EC) cells. ES cells are derived from the pre-implantation embryo, and like EG cells, can be cultivated *in vitro* in their undifferentiated state on feeder layers (mouse embryonic fibroblasts or SIM (Sandoz inbred Swiss mouse) thioguanine-resistant ouabain resistant cells), or addition of the differentiation inhibitor factor, LIF. The cells are capable of unlimited self-renewal with a relatively stable karyotype. Following re-implantation into a host blastocyst the cells have a pluripotent differentiation capacity, including the formation of gonad tissue, producing chimeras from which transgenic lineages with homologous recombination of specific genes can be selected. All three types of murine stem cells gave rise to CMCs. The most widely studied ES cells are

murine ES (mES) cells, due to the wide range of mouse homologous recombination techniques available.

Wobus *et al.* in the early nineties were the first to demonstrate the capability of mES cells to differentiate into beating CMCs. CMCs were observed in the initial aggregate called EBs, along with a wide range of other specialized cell types (1). EBs were formed by placing small drops of ES cells into plastic dishes lacking fibronectin, and then dissociated and re-plated in fibronectin precoated dishes. Spontaneously contracting CMCs were observed soon after plating (Figure 1). The terminally differentiated CMCs derived from ES cells have ultrastructural and functional characteristics similar to that of neonatal rodent myocytes (1). They also have cell to cell communication capacity due to the presence of functionally coupled gap junctions. They possess pharmacological and physiologic properties of specialized myocardial cells, including ventricular, atrial, pacemaker and Purkinje cells, and also have typical postnatal CMC electrophysiological characteristics. The CMCs have normal contractile sensitivity to calcium, and many features of excitation-contraction (E-C) coupling seen in isolated fetal or neonatal CMCs. Terminal, unlike early mES cell-derived CMCs, were shown to be responsive to beta-adrenergic stimulation (1).

The yield of CMCs differentiation rate from ESC can be increased after transfection with antibiotic selection marker driven by a cardiac specific promoter (2). ESCs-derived CMCs were able to functionally integrate into the myocardium of adult mice (2). *In vitro*, ESCs have also been used to generate myocardial tissue, not only myocytes. Zimmermann *et al.* mixed cardiac myocytes from neonatal rats with liquid collagen type I, matrigel, and serum-containing culture medium to form reconstituted engineered heart tissue (EHT) which demonstrated normal contractile function *in vitro* (3).

Human blastocysts have been used to produce human ES (hES) cell lines (4) and primordial germ cells have produced human embryonic germ (EG) cell lines (5). ES cells have been obtained from embryos frozen from *in vitro* fertilization procedures, resulting in only a few cell lines. Melton's group has

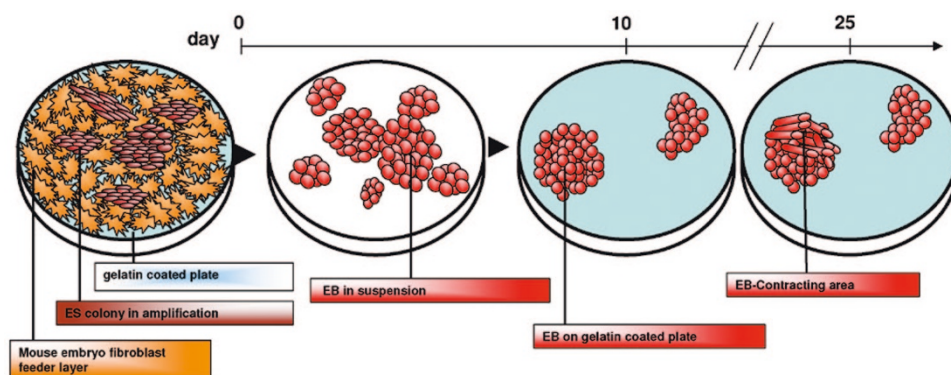
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**Abbreviations:**  $\beta$ -gal,  $\beta$ -galactosidase; BMSCs, bone marrow stem cells; CMC, cardiomyocytes; EBs, embryoid bodies; ES, embryonic stem; GFP, green fluorescent protein; HSCs/HPCs, hematopoietic stem and progenitor cells; LIF, leukemia inhibitory factor; MNCs, mononuclear cells; MSCs, mesenchymal cells; NOD/SCID, nonobese diabetic/severe combined immunodeficiency



**Figure 1.** Human ES propagation and *in vitro* differentiation to CMCs. hESC lines can be propagated continuously in the undifferentiated state when grown on top of the MEF feeder layer. When removed from these conditions and grown in suspension, they begin to generate three-dimensional differentiating cell aggregates termed embryoid bodies (EBs). Two weeks after plating on gelatin coated plate spontaneously contracting area appear within the EB.

described a simplified alternative procedure for generating 17 ES cell lines using frozen human blastocysts, and has produced a manual for their use throughout the scientific community (6).

Both mES cells and hES cells can grow in culture in an undifferentiated state on a feeder layer of mouse embryonic fibroblast (MEF) cells, although unlike mES cells hES cells grow in the absence of LIF. *In vitro* hES differentiation into CMCs is slower than that of mES cells (7). The main differences between mouse and human ESC are highlighted in Table 1. Recently, a coculture with a visceral endoderm-like cell line, END-2 and ascorbic acid in the medium improved CMC differentiation rate of hES (8), suggesting that it is very likely that cardiac differentiation rate will be improved in the near future.

The beating CMCs from contracting areas were similar to early-stage human CMCs in that they expressed cardiac-specific markers and also demonstrated electrical activity, calcium transients, and chronotropic response to adrenergic agents. The cells formed a functional syncytium with synchronous action, and possessed potential proliferation and pacemaker activity. Gepstein's group demonstrated that CMCs derived from hES cells could form structural and electromechanical connections with cultured rat CMCs. These cells were also able to pace swine hearts following transplantation into a swine model of atrioventricular block (9).

Several issues must be addressed before hES cells can be used for cardiac regenerative therapy. First, the CMC yield needs to be radically improved. The culture conditions need to be optimized including the correct cytokine mix to increase CMC differentiation from hES cells. Second, ES cell rejection following transplantation needs to be blocked. A decreased expression of the molecules of the MHC (MHC) would im-

prove immunologic tolerance, as ES cells express MHC I, with low or absent MHC II expression (10). Also, hES cells grown on mouse feeder layer cells express an immunogenic nonhuman sialic acid Neu5Gc which would need to be eliminated, preferably starting with fresh hESCs that have never been exposed to animal products using human serum with human feeder layers (11). In addition, an increase in the efficiency of CMC differentiation from hES cells is necessary to determine the extent of maturity of the CMCs in terms of E-C coupling.

#### HEMATOPOIETIC STEM AND PROGENITOR CELLS (HSCS/HPCS)

HSCs isolated from the mononuclear component of rat bone marrow (BM) have been shown to significantly regenerate the infarcted myocardium. The cells were isolated using negative (immunodepleting cells expressing markers of differentiated hematopoietic lineages, including CD34, CD45, CD20, CD45RO, and CD8) and positive (c-kit, the receptor for Stem Cell Factor, SCF) selection (12). Using male cells, which are identifiable by the presence of a Y chromosome, Orlic *et al.* demonstrated that injection of c-kit<sup>+</sup> lin<sup>-</sup> (BMSCs) into the rat myocardium generated smooth muscle, endothelial and cardiomyocytic cells, regenerating 60–70% of ischemia damaged tissue (12). Quaini *et al.* also confirmed the therapeutic potential of BMSCs in cardiac repair in humans, in a study that analyzed heart biopsies of female organ donors in male recipients several weeks following heart transplantation. The type of stem cell differentiation was determined using tissue-specific markers and the Y chromosome was used as a marker of cell origin (13). Markers of SM, endothelium or CMCs were expressed in more than 20% of the cells with a Y

**Table 1.** Differences between mouse and human ES

ES Source	Undifferentiated cell markers			Trans-stimuli able to induce ES-derived CMC			Condition needed to amplify ESs		Characteristics		
	SSEA1*	ALP†	Oct4‡	DMSO§	Retinoic acid	5AZA	Feeder layer	LIF¶	Doubling time	Maturity of derived CMCs	CMC yield Upscaling
Mouse	Y**	Y	Y	Y	Y	Y	N	Y	+++	++	Y
Human	N††	Y	Y	N	N	Y	Y	N	+	+	N

\* Stage specific Embryonic Antigen; † Alkaline Phosphatase; ‡ Octamer; § DiMethylSulfOxide; || 5-Aza-2'-deoxycytidine; ¶ LeukemiaInhibitory Factor; \*\* Yes; †† No.

chromosome, which would indicate BMSCs have the capacity to generate all types of cardiac cells. Several groups have attempted to reproduce these results with yields of CMC differentiation usually much lower than 1%. In another study, approximately 0.04% of CMCs originated from the host, with a median of 0.016%. Localization of Y chromosome positive CMCs was observed in areas of acute rejection, which would indicate an injury event is associated with such chimerism (14). A different study demonstrated 0.23+/-0.06% Y chromosome-positive CMCs in female recipients of sex-mismatched bone marrow transplantation (BMT) (15).

Results using genetically modified mice with the cre-lox recombination system have dramatically challenged the concept of differentiation of BM cells into CMCs, strongly indicating that fusion between cells, as opposed to differentiation, may account for the results from studies dealing with myocardium regeneration potential of BMSCs. CRE is a DNA recombinase which cleaves DNA at specific palindromic sequences, called LOX sites. Mice possessing the CRE gene driven by the ubiquitous cytomegalovirus (CMV) promoter were crossed with others having an allele in which the  $\beta$ -gal gene is arranged in a way that is activated following cleavage at a lox sites by CRE, causing cells to turn blue. Mice possessing the CRE-activatable LOX-containing  $\beta$ -gal gene were injected with BM cells from mice with the CRE gene. A low number of hepatocytes, CMCs and neurons turned blue, which would suggest -gal activation as a result of fusion of cells of different genetic origin, and not differentiation (16).

Two other groups used similar procedures to test the hypothesis of BM cell differentiation into CMCs. Mice with the  $\beta$ -gal gene driven by the cardiac specific  $\alpha$ -MHC promoter were used by Murry *et al.*, where cells turn blue upon activation of CMC differentiation (17). No blue cells were observed following MI in the  $\alpha$ -MHC  $\beta$ -gal mice, suggesting HSCs did not differentiate into CMCs. Balsam *et al.* used a model of parabiosis between a mouse with a WT genome and another expressing green fluorescent protein (GFP) under a universal promoter (18). An infarct was induced in the WT mouse thus producing stem cell chemo-attractants to determine whether GFP+ BM cells from the donor mouse would migrate to the peri-infarct area of the WT mouse (18). The only GFP-positive cells were of hematopoietic lineage and no GFP-CMCs were observed. Neither result supports the hypothesis of BMSC differentiation into CMCs.

A different study, demonstrated a purified population of hematopoietic stem and progenitor cells as well as unfractionated bone marrow cells engrafted within the infarcted myocardium. The engraftment was hematopoietic and transient, whereas a few bone marrow-derived CMCs were located outside the infarcted myocardium which were derived via cell fusion (19).

BM cells also contain endothelial progenitor cells, and the CD34+ fraction of BM mononuclear cells (BM-MNCs) can be induced, both *in vivo* and *in vitro*, to differentiate into endothelial cells. In addition, infusion of expanded endothelial (End) cells or BM-MNCs into murine models of acute ischemic damage improved myocardial perfusion and viability via angiogenesis (20). Furthermore, human cord blood CD34+

progenitor cells, in particular the KDR+/CD34+ fraction, were able to induce angiogenesis in ischemic damaged heart tissue of nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice, yet very few CMCs stained positive for human nuclei (21). *In vivo*, these cells have an anti-apoptotic effect on CMCs and they also synthesize growth factors. Therefore, the cardiac therapeutic potential of BM cells may be dependent on endothelial cell generation and growth factor synthesis. Following this observation, it was more recently shown that the number of circulating KDR+/CD34+ progenitor cells inversely correlate with cardiovascular events in man (22).

## MESENCHYMAL STEM AND PROGENITOR CELLS

The BM contains mesenchymal stem and progenitor cells (MSCs/MPCs) which are clonogenic nonhematopoietic stem/progenitor cells that have the ability to differentiate into multiple mesoderm-type cell lineages, *e.g.* endothelial-cells, osteoblasts and chondrocytes (23). In tissue culture, the cells adhere to plastic and have a finite lifespan expansion of 15–50 cell doublings. Multipotent adult progenitor cells (MAPCs), a relatively rare adherent stem-cell population have been isolated from BM cells grown for several months in growth medium containing specific growth factors, such as platelet-derived growth factor and epidermal growth factor (23). *In vitro*, they have the capacity to form classical endodermal, mesodermal, and ectodermally derived cell types such as neurons, hepatocytes and endothelial cells (23).

Makino *et al.* have used murine BM stromal cells to generate CMCs, *in vitro* (24). Immortalized stromal cells were treated with 5-azacytidine to induce spontaneously beating cells. Furthermore, human MSCs from adult BM transplanted into the adult murine heart differentiated into CMCs (25). Genetically modified rat MSCs that over-express the pro-survival gene Akt1, regenerated the infarcted myocardium and prevented remodeling (26).

Novel human BM-derived multipotent stem cells (hBMSCs) not previously described, were expanded *in vitro* and generated CMCs and cells of all three germ layers in co-culture conditions (27). Transplantation of hBMSCs into the myocardium following MI, resulted in engraftment of transplanted cells which co-localized with markers of CMC, EC, and smooth muscle cell (SMC), suggesting the hBMSCs differentiated into multiple cell lineages. The hBMSC-transplanted hearts demonstrated up-regulation of paracrine factors including anti-apoptotic factors, angiogenic cytokines, and proliferation of host ECs and CMCs (27).

## LOCAL CARDIAC STEM CELLS AND CELLS FROM OTHER SOURCES

Resident cardiac “stem” cells with the capacity to replicate and differentiate in CMCs have been described by several groups. A population of cells exists in BM, muscle, and skin which exclude Hoechst dye, giving a characteristic appearance in fluorescence-activated cell sorting (FACS) known as the side population (SP) (28). These cells have restricted ability to differentiate into striated skeletal myoblasts, similar to very

rare cells located in the myocardium (29). The presence of a population of cells with SP characteristics expressing stem cell antigen-1 (sca-1+) yet negative for c-kit, was demonstrated in the adult heart (30). These cells do not express cardiac specific genes under normal conditions, however, in the presence of the DNA demethylating agent 5'-azacytidine they are able to differentiate *via* a process that is partly dependent on a receptor for bone morphogenetic proteins (30).

Another resident population of cardiac stem cells exists which are similar to BMSCs (31). They are positive for c-kit (c-kit<sup>+</sup>), the receptor for SCF, and negative for blood lineage markers CD34, CD45, CD20, CD45RO, and CD8 (Lin<sup>-</sup>). *In vitro*, these cells have the capacity to propagate indefinitely, they are multipotent, and can be induced to differentiate into smooth muscle, endothelium and CMCs. Furthermore, they significantly contribute to the restoration of myocardium function following an infarction (31).

Undifferentiated adult cardiac stem cells that grow as self-adherent clusters known as "cardiospheres" have been isolated from human and murine hearts (32). The cells are clonogenic, express stem and endothelial progenitor cell antigens/markers, and have the ability to self-renew long-term. Following *in vitro* ectopic or orthotopic transplantation in SCID beige mice the cells were able to differentiate into myocytes and vascular cells.

It has also been shown that the anterior pharynx contains a subpopulation of cells that express the homeobox gene *islet-1* (*isl1*), that is lost during CMC terminal differentiation (33). These postnatal *isl1*<sup>+</sup> cells remain undifferentiated in the outflow tract, the atria, and the right ventricle, which develop from the "secondary heart field", of fully developed newborn rodent and human hearts (34). These cells express Nkx2-5 and GATA4, but not stem cell antigen 1 (Sca-1), CD31, or c-kit. The isolated progenitor cells have the ability to self-renew and differentiate into CMCs when cultivated on a cardiac mesenchymal feeder layer. In addition, *isl1*<sup>+</sup> cells co-cultured with neonatal myocytes can differentiate into a mature cardiac phenotype capable of myocytic marker expression (25%) with no indication of cell fusion, and exhibit intact Ca<sup>2+</sup>-cycling, and the generation of action potentials. This would suggest the cells are cardiomyoblasts, and using the cre-lox system it has been demonstrated that they are capable of both *in vitro* and *in vivo* differentiation. It is not yet known if these cells exist in adults.

Very recently, it was reported that in the stroma vascular fraction (SVF) of the adipose tissue there are pluripotent stem cells (34) able to generate CMCs. SVF-derived cells failed to express the skeletal marker MyoD and the smooth muscle actin. In contrast, these adipose tissue-derived cells expressed cardiac-specific transcription factors, the structural cardiac proteins, as well as late-stage cardiac specification proteins (35). However, it is not clear what is the differentiation rate.

CMCs were also obtained using a novel population of nonsatellite cells in adult murine skeletal muscle (36). These cells, called "Spoc" cells (skeletal-based precursors of CMCs) are CD34<sup>-</sup> and CD45<sup>-</sup> and are able to differentiate in co-culture systems in CMCs. Finally, a population of cardiac neural crest-derived cells was shown to be a multipotent stem

cells and differentiate into CMCs and typical neural crest-derived cells, including neurons, glia, and smooth muscle (37). Most probably, the list of potential CMC progenitor will increase in the near future.

## CONCLUSION

Although some studies suggest the potential use of adult/somatic stem cell for cardiac repair, other stringent data suggest that this potential is dependent on endothelial cell generation and growth factor synthesis rather than the formation of new CMCs.

Considering the limitations of adult stem cells and the evidence of beating CMCs generation from hESCs, our point of view is that hESCs are the more probable candidate for cell-therapy applications in cardiac repair. Infact, hESCs fulfill most properties of an ideal donor cell line.

One of the biggest hurdles for the utilization of hESCs for future myocardial regeneration strategies is the insufficient number of cardiomyocytes achieved by the currently available differentiation schemes. Therefore, strategies aimed at developing an efficient cardiomyocyte differentiation system are essential for the generation of large quantities of cells needed for successful therapeutic application. Differences between human and murine ESCs indicate that there is still some work to do for leveling the difference in yield between the ESC of the two species.

Other issues are to be solved for the clinical application of hES cells in cardiac diseases characterized by heart failure, among which immunologic compatibility, tissue engineering aspects and excitation-contraction differences between ESC-derived CMCs and adult CMCs. Moreover, for clinical applications, it is imperative to develop chemically defined culture media supplemented with recombinant cytokines and growth factors. Hopefully, in the next few years we will experience an acceleration of our knowledge in this exciting and important field.

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