

This Review is part of a thematic series on **Stem Cells**, which includes the following articles:

Differentiation of Pluripotent Embryonic Stem Cells Into Cardiomyocytes

Derivation and Potential Applications of Human Embryonic Stem Cells

Stem Cells for Myocardial Regeneration

Myocyte Death, Growth, and Regeneration in Cardiac Hypertrophy and Failure

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Mesenchymal Stem Cells and Their Potential as Cardiac Therapeutics

Therapeutics and Use of Stem Cells

Toren Finkel, Roberto Bolli, Editors

Mesenchymal Stem Cells and Their Potential as Cardiac Therapeutics

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Abstract—Mesenchymal stem cells (MSCs) represent a stem cell population present in adult tissues that can be isolated, expanded in culture, and characterized in vitro and in vivo. MSCs differentiate readily into chondrocytes, adipocytes, osteocytes, and they can support hematopoietic stem cells or embryonic stem cells in culture. Evidence suggests MSCs can also express phenotypic characteristics of endothelial, neural, smooth muscle, skeletal myoblasts, and cardiac myocyte cells. When introduced into the infarcted heart, MSCs prevent deleterious remodeling and improve recovery, although further understanding of MSC differentiation in the cardiac scar tissue is still needed. MSCs have been injected directly into the infarct, or they have been administered intravenously and seen to home to the site of injury. Examination of the interaction of allogeneic MSCs with cells of the immune system indicates little rejection by T cells. Persistence of allogeneic MSCs in vivo suggests their potential “off the shelf” therapeutic use for multiple recipients. Clinical use of cultured human MSCs (hMSCs) has begun for cancer patients, and recipients have received autologous or allogeneic MSCs. Research continues to support the desirable traits of MSCs for development of cellular therapeutics for many tissues, including the cardiovascular system. In summary, hMSCs isolated from adult bone marrow provide an excellent model for development of stem cell therapeutics, and their potential use in the cardiovascular system is currently under investigation in the laboratory and clinical settings. (*Circ Res.* 2004;95:9-20.)

Key Words: cardiomyoplasty ■ mesenchymal ■ cell therapy ■ stem cell

The last 5 years have witnessed an explosion of new reports on human stem cells isolated from a variety of sources including embryonic, fetal, and adult tissues. The recognition that stem cells are found in many adult tissues and that these cells may lend themselves to tissue repair or regeneration has been particularly exciting. But where do stem cells originate, how do they maintain themselves, how is their fate determined, and can stem cells move from one tissue to another to provide regenerative cells? These ques-

tions quickly give rise to other questions centered around whether we can manipulate stem cells: Can they be isolated for study or only maintain their stem cell abilities in their in vivo niches? Can they be culture expanded or does this change their identity, characteristics, and abilities? Can they be delivered from exogenous sources to repair tissue, or can their numbers be boosted in situ? How may they be used to treat damaged and diseased tissues? In this review, we focused on the human mesenchymal stem cells (hMSCs)

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isolated from adult bone marrow and used by many investigators. Plating studies indicate MSCs are present as a rare population of cells in bone marrow, representing perhaps 0.001% to 0.01% of the nucleated cells, \approx 10-fold less abundant than hematopoietic stem cells (HSCs), but MSCs can be readily grown in culture. Although the current knowledge of MSCs will further evolve, it is possible to move forward and use these isolated multipotential cells to ask critical questions that could not be addressed previously. MSCs offer a very useful compromise among the many different stem cells now described in terms of the ease of accessibility, handling, and multilineage potential, and their investigation illustrates how much has been learned and how much there is to understand still. Importantly, cultured MSCs have already been infused in humans for safety and early clinical testing for support of bone marrow transplantation, treatment for osteogenesis imperfecta, and glycogen storage disease, fields where there are not many therapeutic options.^{1–5a}

Enthusiasm for cell therapy for the injured heart has already reached the clinical setting, with physicians in several countries involved in clinical trials using several cell populations. Results from these early reports suggest new therapies may come from the use of progenitor cells such as myoblasts⁶ or the stem cells found in bone marrow.^{7,8} Recognizing there are stem/progenitor cells in marrow including the MSC, the HSC, as well as those of the endothelial system,⁹ it will be necessary to sort out the relative contributions of different cell types. Purified MSCs have tested well in animal cardiac models and are now being readied to head into the clinic.

There has long been evidence for a cellular basis for tissue damage repair, and there has been debate over whether the cells involved in tissue repair were of local origin or arrive by way of circulation. With the search for cells that would allow for survival after radiation exposure during the 1940s and 1950s, it became apparent that an HSC function could be found in the spleen and bone marrow.^{10,11} HSCs were elusive, difficult to isolate and grow in culture (and this remains true today). During this time period, studies also suggested that bone marrow, when transplanted to an ectopic site, could form new bone tissue.¹² Recognition that there might be particular isolatable cells present in bone marrow capable of becoming osteoblasts and responsible for ectopic bone formation was provided by Friedenstein et al using a guinea pig model.¹³

Research on bone-forming cells of marrow was continued by many investigators.^{14,15} There was also recognition of a bone marrow connection between bone-forming and adipose-forming cells.¹⁶ During this period, bone marrow stroma continued to be seen as providing the essential microenvironment for HSC survival, and marrow cell studies of stromal function were performed.^{17,18} The appreciation that there may be an MSC capable of differentiating to many or all mesenchymal lineages developed in part as a result of understanding lineage diagrams drawn for HSCs.^{14,19} Isolation of the hMSC was undertaken by Caplan in the late 1980s and involved a rethinking of the isolation procedures and means to assay for the presence of the rare MSCs in small aspirates

of bone marrow.¹⁹ An important contribution was the use of an osteoconductive ceramic matrix for implantation studies that would faithfully reveal the cell osteochondral potential of the human cells cultured *ex vivo* when implanted in immunocompromised mice. This *in vivo* assay allowed them to select appropriate culture conditions for isolation and expansion of the hMSCs from bone marrow.²³ Of particular importance was attention to the lot of FBS selected for MSC growth.²⁰ Because we do not understand the full growth requirements of hMSCs (or other stem cells), a serum-free medium with defined growth factor composition has not been fully developed yet.

MSCs or Mesenchymal Progenitor Cells

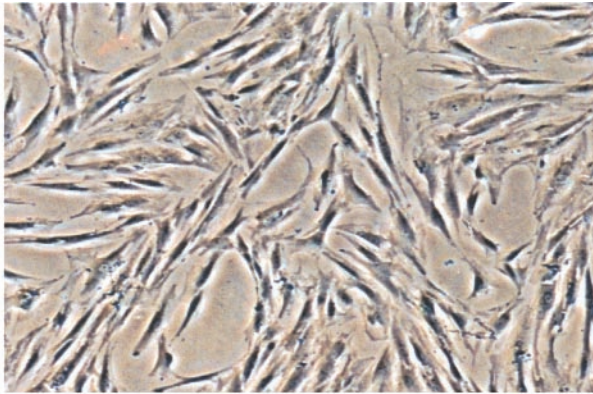
The appropriately selected cell from adult tissue, which can be greatly and efficiently expanded in culture and can differentiate to several specific mesenchymal cell lineages, is a proper stem cell, an MSC. The hMSC from bone marrow can be cloned and expanded *in vitro* \geq 1 million-fold and retain the ability to differentiate to several mesenchymal lineages.^{21–23} Researchers have not yet found conditions that allow continuous, indefinite hMSC growth, yet it is possible to produce billions of MSCs *in vitro* for cellular therapy from a modest bone marrow aspirate drawn through the skin. MSCs need to be expanded *ex vivo* because they apparently are very contact inhibited, and there is little evidence of *in vivo* expansion as MSCs labeled with membrane dyes, that would be diluted and undetected from dividing cells after \approx 3 divisions, are found months later even in repairing tissue (see Figure 5).

The current work with embryonic stem (ES) cells and neural stem cells is hindered by difficulties in producing enough cells in a reproducible manner to contemplate therapeutic development. The hMSCs, with their attributes of (1) ease of isolation, (2) high expansion potential, (3) genetic stability, (4) reproducible attributes from isolate to isolate, (5) reproducible characteristics in widely dispersed laboratories, (6) compatibility with tissue engineering principles, and (7) potential to enhance repair in many vital tissues, may be the current preferred stem cell model for cellular therapeutic development.^{21–23,30,41–43,45,49}

Whether MSCs are capable of transdifferentiation to ectodermal lineages awaits full evidence, but results from RT-PCR and microarray analysis show that MSCs express genes normally thought to be expressed in neurogenic, ectoderm-derived tissue. Several studies have demonstrated the expression of neural genes by MSCs and improved results from MSC implantation at the sites of spinal or intracranial injury.^{24–27} Whether such improvements come from MSCs producing growth factors and cytokines, MSC differentiation, or MSCs interacting with other cell types at the site of injury is still under investigation.

Sources and Characteristics of hMSCs

The fibroblastic cells from bone marrow are sometimes generically termed bone marrow stromal cells, but not all stromal cells from bone marrow are multipotent.^{21,28,29} Researchers have described a variety of cells from bone marrow and given them different names, and at times, a particular



hMSC Surface Markers

Positive

CD13, CD29, CD44, CD49a, b, c, e, f, CD51, CD54, CD58, CD71, CD73, CD90, CD102, CD105, CD106, CDw119, CD120a, CD120b, CD123, CD124, CD126, CD127, CD140a, CD166, P75, TGFb1R, TGFbIIIR, HLA- A,B,C, SSEA-3, SSEA-4, D7

Negative

CD3, CD4, CD6, CD9, CD10, CD11a, CD14, CD15, CD18, CD21, CD25, CD31, CD34, CD36, CD38, CD45*, CD49d, CD50, CD62E,L,S, CD80, CD86, CD95, CD117, CD133, SSEA-1

laboratory has changed names to refer to the same cells, leading to some nomenclature confusion. Most surface markers have been found inadequate as a means to identify stem cells because the putative marker(s) may also be found on nonstem cells, or a particular marker may only be expressed on a stem cell at a certain stage or under certain conditions, such as with CD34 on HSCs. Nevertheless, surface markers or other attributes are useful in characterizing the stem cell as isolated or cultured, and as a means to begin to understand its potential interactions with neighboring cells and the cell environment. A number of surface molecules on hMSCs are given in Figure 1 and, although extensive, are incomplete. Some variation in the surface molecules on hMSCs has been seen from laboratory to laboratory, and further work is necessary to understand whether these differences represent separate stem cell populations, different culture techniques, or means of analysis. MSCs from different species, studied by somewhat different methods, and in labs worldwide, have remarkably reproducible attributes.^{21–23,42–45}

Adult bone marrow MSCs are thought to be largely quiescent, and the extent to which they proliferate in vivo is unknown. The initial expansion process is a result of those colony-forming units (the single cells that continue to divide to form clonal cell colonies). Although perhaps quiescent, bone marrow MSCs can divide rapidly once cell division begins, and this is seen best at very dilute plating density. Although differences in cell morphology and characteristics may initially exist, the MSC population becomes very homogeneous with time in culture and remains so for many passages. Cytogenetic testing of late passage–cultured hMSCs has not detected chromosomal abnormalities, and telomerase activity was also evident in these cultured late-passage hMSCs.²⁵

Figure 1. hMSCs in culture appear fibroblastic and homogeneous in size and morphology by second passage. Flow cytometry applying fluoresceinated antibodies was used to determine surface molecules present in the expanded cell population, and both positive and negative surface molecules are listed.

Subpopulations of hMSCs

MSCs are not defined by their source, and even within a tissue such as bone marrow, there may be microenvironments wherein closely related or identical cells may express different surface molecules. For example, those MSCs isolated directly from bone marrow and tested for surface molecule expression will differ in some aspects from MSCs cultured for weeks in vitro. This includes CD45, which is considered a marker of hematopoietic lineages, yet it is identified on some MSCs from bone marrow at low levels and is quickly lost in culture.³⁰ Similarly, culture-expanded MSCs with slightly different characteristics may be derived depending on the culture methods used or the analysis methods. The Table compares the detected surface molecules on multipotential MSC-like cells from several labs, cultured in somewhat different conditions. Many of these isolated cells behaved similarly in in vitro differentiation experiments.

Prockop et al^{31,32} have used the low-density plating methods of Friedenstein and isolated and studied the population of rapidly dividing cells from human bone marrow, termed recycling stem (RS) cells, as a subpopulation of MSCs. RS cells were termed RS-1 for the small agranular, rapidly dividing cells, and RS-2 for small, granular cells, whereas the more typical fibroblastic MSCs were considered mature MSCs in the cultures. RS cells divided very quickly when plated at low density and may yield 10^9 cells in 6 weeks.³³ Most recently, Prockop et al identified the Wnt inhibitor dickkopf-1 as an effector molecule for hMSC proliferation in vitro.³⁴

Exciting recent findings have come from a series of articles from Verfaillie et al, who described marrow progenitor cells or multipotent adult progenitor cells (MAPCs).^{35–38} The cultured cells have many of the attributes of MSCs, but they

Surface Antigen Comparison Among MSC-like Cells

Surface Antigen	MSCs ²¹	MAPCs ³⁸	RS-1 ³³	PLAs ⁴³	APCs ⁴²
CD9					+
CD10		-	-		+
CD11a,b	-		-		-
CD13	+	+		+	+
CD14	-		-	-	-
CD18 integrin β 2	-				-
CD29	+			+	+
CD31 PECAM	-	-	+/-	-	
CD34	-	-	-	-	+
CD44	+		+	+	+
CD45	-*	-	-	-	-
CD49b integrin α 2	+	+			
CD49d integrin α 4	-			+	+
CD49e integrin α 5	+		+		+
CD50 ICAM3	-	-			-
CD54 ICAM1	+				+
CD56 NCAM				-	-
CD62E E-selectin	-	-		-	-
CD71 transferrin rec	+		+	+	
CD73 SH-3	+			+	
CD90 Thy-1	+	+	+/-	+	
CD105 endoglin, SH-2	+			+	+
CD106 VCAM	+	-		-	+
CD117	-	-			
CD133	-	(+)	-	-	
CD166 ALCAM	+				+
Others					
β 2 microglobulin	+	+			
Nestin	+			+	
p75	+			+	
HLA ABC	+	-	+/-		+
HLA DR	- induc	-	-		-
SSEA-4	+	+			
TRK (A, B, C)	+		+		
Differentiation in vitro					
Osteo	+	+	+	+	+
Adipo	+	+	+	+	+
Chondro	+	+	+	+	
Neural	(+)			(+)	
Stromal	+	+			
Myoblast Sk	(+)	+		+	
Endothelial	(+)	+			

(+) indicates detection varied; *positive upon isolation.³⁰

APCs indicates adipose progenitor cells; PLAs, processed liposuction aspirates; PECAM, platelet endothelial cell adhesion molecule; ALCAM, activated leukocyte cell adhesion molecule; ICAM, intercellular adhesion molecule; NCAM, neural cell adhesion molecule; VCAM, vascular cell adhesion molecule; HLA, human leukocyte antigen.

are reported to expand indefinitely and have lineage potentials that include extended ectodermal and endodermal cell types, making them more like ES cells. Additionally, they

lack major histocompatibility complex (MHC) class I and class II on their surface, so presumably, they may be used allogeneically, although the lack of MHC I molecules may make them vulnerable to elimination by natural killer cells. Much of the published data are of cells of mouse origin, and it will be important to verify that the human MAPCs are as potent in these respects as the mouse MAPCs.^{39,40}

It is possible to isolate MSCs from different mesenchymal tissues, and recently, it was shown that fibroblastic cells from adipose differentiate to mesenchymal lineages, and their characteristics and behavior are virtually indistinguishable from bone marrow-derived MSCs.⁴¹⁻⁴⁴ These cells have been termed adipose stromal cells, adipose progenitor cells, or processed liposuction aspirates.

Stem cells need to be functionally defined. Although there may be characteristics or surface markers that lead one to suspect a cell is a potential stem cell, the ability to produce daughter cells as well as differentiate to multiple phenotypes must be assayed. Small differences in surface markers may not be sufficient to distinguish types or subpopulations of stem cells. Functional comparison among isolated MSC populations is a better approach. In an effort to compare MSCs, adipose progenitor cells, and MAPCs prepared by protocols from different laboratories, Lodie et al performed an evaluation of their cellular characteristics and differentiation potential.⁴⁵ These authors found the cells produced by the different methods to be virtually indistinguishable. However, it is possible that a particular source of multipotential cells or a particular culture method may prove superior to other methods when cells are tested in vivo for the ability to repair a particular damaged tissue, and therefore, it is still useful to examine sources of stem cells and culture conditions.

There have been recognized differences between stem cells found in distinct species such as different in vitro requirements to culture HSCs from mice and man.^{46,47} Similarly, MSCs can be isolated by a number of methods from different tissue sources, but their stem cell nature cannot be assured; it must be assayed. Recently, Simmons et al^{18,48} reported the characterization of a subpopulation of marrow stromal cells selected by an antibody to an unknown surface marker, STRO-1. The selected cells from bone marrow have attributes of MSCs.⁴⁹

Immune Responses and MSCs

Several laboratories have begun to characterize the interactions between MSCs and cells of the immune system. hMSCs have a number of surface molecules that would predict interaction with T cells. MHC I, Thy-1 (CD90), vascular cell adhesion molecule (CD106, VCAM), intercellular adhesion molecule 1 (ICAM-1) and ICAM-2, activated leukocyte cell adhesion molecule (CD166, ALCAM), lymphocyte functional antigen-3, and integrins α 1 to α 3, α 5, α 6, β 1, β 3, and β 4 (CD49 a, b, c, e, and f) are surface molecules expressed on hMSCs that have cognate ligands on T cells.^{21,28,50,51} Additionally, although not expressed at appreciable levels on hMSCs, MHC II expression is upregulated by incubating the cells with interferon- γ . However, the hMSCs lack the B7 costimulatory molecules CD80 and CD86, and these are not expressed after interferon treatment.^{52,53}

Intuitively, one might expect allogeneic MSCs (allo-MSCs) would stimulate T cell proliferation and that donor MSCs would be recognized by responder T cells and rejected by a recipient host. However, experimental evidence indicates this may not be the case. MSCs have been shown to inhibit T cell proliferation in several laboratories.^{52–55}

The interaction of T cells with other cell types can be studied *in vitro*. The mixed lymphocyte reaction (MLR) or exposure of peripheral blood mononuclear cells or isolated T cells to cells from another individual usually evokes a T-cell proliferative response that is measured conveniently by incorporation of [³H]-thymidine into DNA during a several-day period. However, MSCs cultured with responder T cells do not generally cause T cell proliferation, and they usually reduce the response of responder T cells to other stimulator cells or nonspecific activators such as phytohemagglutinin. The lack of response is not because of T cell apoptosis or other deleterious effects because the T cells can be recovered and respond to other stimulators in the absence of MSCs. Tse et al described similar experiments including stimulating T cells with anti-CD28 and anti-CD3 antibodies before addition of MSCs.⁵³ The MSCs can inhibit the T cell proliferative response through a Transwell membrane insert, suggesting a soluble component is involved. Di Nicola et al⁵⁴ have shown that antibodies to hepatocyte growth factor and transforming growth factor β (TGF- β) restored the proliferative response, suggesting these factors are at least partially responsible. Le Blanc et al confirmed the MLR experiments and also demonstrated the dose-dependent inhibition of T cell proliferation, independent of the source of cultured MSCs.⁵⁵ Related studies in mice found MSCs inhibited the response of memory T cells to their antigen.⁵⁶

It has been suggested that once stem cells begin to differentiate, they will express MHC II molecules and be rejected by T cells. However, Le Blanc et al caused MSCs to differentiate *in vitro* and then tested their interaction with T cells and found no increased antigenicity.⁵⁷ Although not all published, numerous animal studies have found the persistence of allo-MSCs *in vivo*, some of which are highlighted below.

Overall, MSCs appear to prevent maturation of T cells to their cognate antigen or other antigens by a direct cell–cell effect and a soluble factor or factors. Then it is possible that MSCs in close proximity can play a role in modulating the immune response of several effector immune cells (S. Aggarwal and M.F. Pittenger, submitted for publication). MSCs may serve a role to preserve progenitor or immature lymphocytes in the central bone marrow space while allowing for expansion elsewhere in the periphery as needed. These concepts will need further study *in vivo* and *in vitro* before we understand the direct and indirect effects MSCs can have on the immune response.

Several studies have used allo-MSCs *in vivo*, and experience suggests the allo-MSCs are not rejected and may have positive effects on engraftment of third-party cells or tissue.⁵⁸ Bartholomew et al tested the ability of MSCs to enhance skin engraftment in an allogeneic baboon model. In another study, MSCs were found after 9 months in multiple tissues in an irradiated baboon model of bone marrow transplantation, whether allogeneic or autologous baboon MSCs were used.⁵⁹

Stem cell–based therapies could be very expensive to fully develop if they were based on autologous cells because it would be necessary to obtain a biopsy from the patient, expand the cells in culture, perform the required testing, and store retains before administering the therapy. Also, it is not clear whether each donor will produce sufficient stem cells of required potency at the needed time. An alternative approach is use of allogeneic stem cells. The advantages of allo-MSCs are many: the donor can be chosen ahead, qualified, and tested for absence of different disease organisms, the MSCs ready in advance so they are immediately available when needed by a patient.

Collectively, current studies on the interactions between MSCs and T cells support the potential use of allo-MSCs in cell therapy. Along with studies of allo-MSCs introduced into infarcted heart models and encouraging early clinical results, the future use of allo-MSCs has significant clinical potential. Hurdles exist, but many have already been overcome.

Cellular Cardiomyoplasty

Loss of cardiomyocytes after myocardial infarction (MI), combined with the absence of endogenous repair mechanisms, is a causative factor in progression to heart failure. Pathologic ventricular remodeling ensues as damaged myocardium is replaced by a fibrous scar composed of extracellular matrix produced by nonmyocytic cells, resulting in ventricular function loss.

In an effort to replace cardiomyocytes lost after ischemia, cellular transplantation has been investigated as a potential therapy for MI.⁶⁰ Termed cellular cardiomyoplasty, the approach has gone from an interesting research novelty to clinical reality. Adult cardiomyocytes have essentially no regenerative capacity, and although a cardiac stem cell has been described recently,⁶¹ its physiologic role in repair after infarction appears minimal and functionally inadequate. Therefore, implantation of exogenous cells may allow for meaningful replacement of damaged cardiac cells. Various types of isolated cells have been delivered to the heart, including fetal cardiomyocytes,^{62–64} skeletal myoblasts,^{65–67} and more recently, progenitor or stem cells such as those of the endothelial⁹ or mesenchymal lineages.^{68–71} Studies evaluating these cell types have documented repeatedly that exogenous cells can engraft in adult myocardium and, in some cases, have a measurable functional impact on damaged myocardium. Additionally, these studies have addressed fundamental questions that must be considered when evaluating MSCs for cardiac cell therapy. For example, issues involving delivery strategies and the spatial placement of the cell graft, the ideal temporal relationships between cell delivery and cardiac injury, and the electromechanical properties of cell grafts have been addressed with fetal cardiomyocyte and skeletal myoblast grafts. Research with fetal cardiomyocyte grafts has been curtailed because of the improbable likelihood of procuring sufficient amounts of fetal cardiomyocytes thought to be necessary for the damaged adult heart.

In the early 1990s, Chiu et al⁷² demonstrated the ability of skeletal myoblasts to be isolated, expanded in culture, and successfully delivered to injured myocardium. Since this pioneering work, skeletal myoblasts have been examined as a

cellular therapy in animal models of cardiac injury and are currently being tested in clinical trials in humans.⁶ Skeletal myoblasts can be isolated from fetal, neonatal, and adult muscle and expanded in tissue culture.⁷³ However, myoblasts are committed progenitor cells and have been shown to differentiate to mature skeletal myocytes, not cardiomyocytes, when implanted into normal and injured myocardium.^{60,63,65} That is, there is not current evidence of skeletal myocytes transdifferentiating to a cardiomyocyte phenotype after implantation in the heart.⁶⁵ Inherent differences in action potential generation and refractory period between these fiber types, alterations in cardiac conduction, and potential arrhythmogenesis may limit the long-term clinical utility of skeletal myoblasts.

MSCs for Cardiomyoplasty

Interest in MSCs, in general, and as a cardiac therapy, in particular, has increased exponentially during the past 5 years. Compared with other cells types considered for cardiomyoplasty, MSCs appear to possess unique properties that may allow for convenient and highly effective cell therapy. MSCs can be used allogeneically, delivered systemically, and differentiate into a cardiomyocyte-like phenotype when implanted in healthy myocardium.^{28,69} Furthermore, MSCs can be readily transduced by a variety of vectors and maintain transgene expression after *in vivo* differentiation.^{69,74} Transgene expression by MSCs may be used ultimately to augment cell engraftment or the extent of differentiation. Recent data from Mangi et al⁷⁵ support this possibility. This group used a retroviral vector to overexpress the prosurvival gene Akt in MSCs before implantation in infarcted rat myocardium. Akt protein overexpression was reported to greatly enhance MSC survival and prevent pathologic remodeling after infarction, with impressive improvement in cardiac output.

The first use of bone marrow cells for cardiomyoplasty was reported in 1999 by the laboratories of Weisel and Lee at the University of Toronto.⁷⁰ In this study, autologous bone marrow cells were implanted in the left ventricle (LV) of rats by direct injection 3 weeks after cryoinjury. Transplanted marrow cells could be identified in all animals 8 weeks after injury and were found to express muscle-specific proteins not present before implantation. Furthermore, improved systolic and diastolic functions were reported in animals that received cells pretreated with the DNA-demethylating agent 5-azacytidine, which has been reported to enhance myogenic differentiation of pluripotent cells. It should be noted that bone marrow, as opposed to a purified MSC population, was used in this study. Development of a causative relationship between the observed functional improvements and a particular cell population is problematic because of the fact that in addition to being a major repository for MSCs, whole marrow contains a number of cell types.

As opposed to the muscle precursor cells, allo-MSCs have the ability to be used immediately after acute injury.⁷⁶ The ability to treat MI patients with allo-MSCs in an emergent setting at the time of coronary reperfusion may constitute a distinct clinical advantage over autologous cellular cardiomyoplasty. Furthermore, MSCs appear to have the ability to

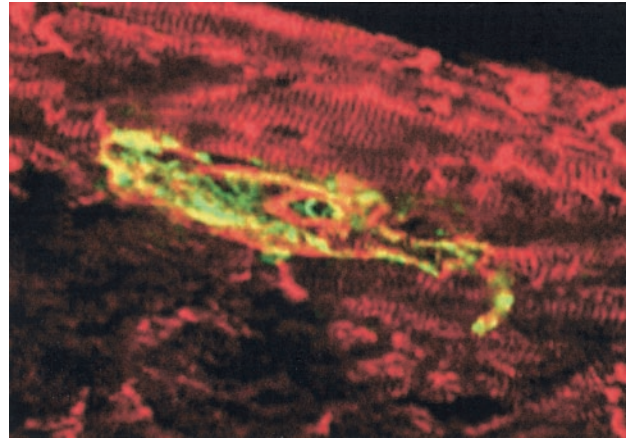


Figure 2. hMSC present in the ventricle wall of an adult mouse heart. The hMSC is identified with an antibody to transduced gene product β -gal (green), and the heart section has been counterstained with an antibody to desmin (red).

home to the site of myocardial injury when administered intravenously after acute infarction (see below).

Pathologic Remodeling Inhibition After Infarction

Our laboratory and others have demonstrated that after injection into infarcted myocardium, engrafted MSCs differentiate toward a myogenic lineage, as evidenced by expression of muscle-specific proteins including α -actinin, troponin-T, tropomyosin, myosin heavy chain-MHC, phospholamban, and other muscle-specific proteins (Figure 2).⁷⁷ Additionally, the presence of connexin-43, a protein responsible for intracellular connection and electrical coupling between cells, further suggests cardiomyocyte differentiation. However, complete myogenic differentiation with mature sarcomeric organization, intercalated discs, etc, has not been observed after implantation of MSCs in infarcted myocardium. This is in contrast to our experience regarding hMSC injection into viable, adult, noninfarcted murine myocardium, where evidence of sarcomeric organization and extensive cardiomyogenic differentiation has been observed.⁶⁹ The lack of extensive myocytic differentiation in infarcted heart likely speaks to the importance of the local extracellular milieu in driving differentiation of MSCs.

However, even without appropriate differentiation within infarcted tissue, MSC cardiomyoplasty has been associated with a number of significant functional improvements in the postinfarcted heart. We described previously the benefits of engrafted MSCs including prevention of pathologic wall thinning (30% improvement in end-diastolic wall thickness at 12 weeks) and improved postinfarction hemodynamics (Figure 3).⁷⁷ Significantly lower end-diastolic pressure in MSC-treated animals (\approx 50% reduction in left ventricular end-diastolic pressure at 6 months) suggests improved diastolic relaxation and decreased wall stress that are likely attributable to favorable ventricular remodeling.⁷⁸ Absence of improvement in systolic function may be considered somewhat expected in light of the absence of sarcomeric organization in MSCs implanted in the infarct. Together, these data suggest that the implantation of MSCs in damaged myocardium



Figure 3. Unretouched photographs of the LV (cross-sections) at the level of MSC injection. These data provide evidence supporting the hypothesis that cellular cardiomyoplasty may limit LV wall thinning. Control hearts (top) had significant scarring, wall thinning, compensatory hypertrophy in adjacent myocardium, and loss of normal ventricle chamber geometry (dilation). In contrast, hearts of MSC-treated animals (bottom) had preserved wall thickness and LV geometry, despite equally large infarctions. Reprinted from Shake et al,⁷⁷ with permission from the *Society of Thoracic Surgeons*.

results in a more compliant, less stiff ventricle with improved diastolic-filling properties. How implantation of MSCs improves diastolic function mechanistically is not yet clear, but early evidence suggests that improved pathological remodeling, specifically alterations in tissue cellularity and extracellular matrix remodeling, may be involved. Although patients with isolated left ventricular diastolic dysfunction have lower mortality compared with those with systolic dysfunction, significant morbidities are common, including symptoms of recurrent chest pain and congestive failure, and diastolic benefits of MSC cardiomyoplasty may play an important role in ischemic heart disease treatment.

Allogeneic Use of MSCs in Cellular Cardiomyoplasty

As described previously, MSCs from human and other species have a cell surface phenotype that is of low immunogenicity.^{21,50,79} In 2000, data from several research groups demonstrated long-term allo-MS-C engraftment in a variety of noncardiac tissues in the absence of immunosuppression.^{52,58,80} On the basis of these observations, our studies began to investigate the ability of allo-MS-Cs to engraft in infarcted myocardium. Initially in rats, and later in swine, allo-MS-Cs were found to readily engraft in necrotic myocardium and favorably alter ventricular function after infarction. Furthermore, allo-MS-C engraftment in the myocardium occurs without evidence of immunologic rejection or lymphocytic infiltration, even in the absence of immunosuppressive therapy. These results using allo-MS-Cs further emphasize some of the apparent advantages of these cells over other cell populations for cellular cardiomyoplasty. These MSC characteristics may allow for enhanced clinical applicability because allo-MS-Cs can be readily available and administered with immunologic acceptance by the recipient.

The immunologically privileged status of MSCs may even extend to the xenogeneic setting. In a recent study from Saito et al,⁸¹ MSCs obtained from C57BL/6 mice were injected intravenously into immunocompetent adult Lewis rats. Labeled mouse cells engrafted into the bone marrow for at least

12 weeks without immunosuppression. When these animals were later subjected to MIs, murine MSCs could be identified in the region of necrosis, and these cells expressed muscle-specific proteins not present before coronary ligation. Xenogeneic MSC engraftment was not associated with immunologic activation or rejection, but rather it appeared to form a stable cardiac chimera.

MSC Role in Angiogenesis

Because cardiac function depends critically on myocardial perfusion, restoration of cardiac function after MI must require not only replacement of lost cardiomyocytes but also revascularization of the injured region. Also, there is evidence from ischemic hind-limb models that circulating stem cells are incorporated into newly formed blood vessels and contribute to increased capillary density and enhanced tissue perfusion.^{82,83}

Recent studies have also shown that stem and progenitor cells isolated from adult tissues have the potential for enhancing neovascularization and regenerating infarcted myocardium.^{9,70,82,84–88} Myocardial perfusion is characterized by close matching of blood flow to myocardial demand. Therefore, it will be important to examine not only maximal blood flow (indicative of total vascularity) but also coronary flow responsiveness to changes in myocardial demand and the distribution of myocardial perfusion.

Experiments to evaluate the effect of MSC therapy on myocardial perfusion are ongoing. However, evidence for MSC involvement in vascular repair or regeneration can be found in Figure 4. The image was obtained from a pig 8 weeks after catheter-based endocardial delivery of allo-MS-Cs. The hematoxylin/eosin (H&E) images in the top panels clearly illustrate blood vessel presence within a region of generalized myocardial necrosis. In the confocal images of serial sections (bottom panels), 4',6-diamidino-2-phenylindole (DAPI)-labeled cells can be seen throughout the section. However, localization of implanted MSCs can be readily identified surrounding and associated with these blood vessels. DAPI-labeled MSCs (blue) were localized within and intimately associated with the smooth muscle layer of the vessel. In addition to smooth muscle actin, implanted porcine MSCs have been demonstrated to express the von Willebrand factor, vascular endothelial growth factor (VEGF), and other proteins indicative of angiogenesis. These proteins are not found in cultured MSCs, but rather are expressed only after several weeks in the cardiac environment. Recent data from Kinnaird et al⁸⁸ suggest that the mechanism of MSC-mediated improvements in perfusion may reside in their ability to secrete a variety of angiogenic cytokines (fibroblast growth factor, VEGF, matrix metalloproteinases, platelet-derived growth factor, TGF- β , interleukin 1, angiopoietin, and others), many of which are upregulated with hypoxia.

MSC Cardiac Homing to Infarction Sites

One of the most interesting characteristics of MSCs is their ability to home to sites of tissue damage or inflammation.⁸⁹ Although the cytostatic factors responsible for injury-specific MSC migration and its physiologic consequences have yet to

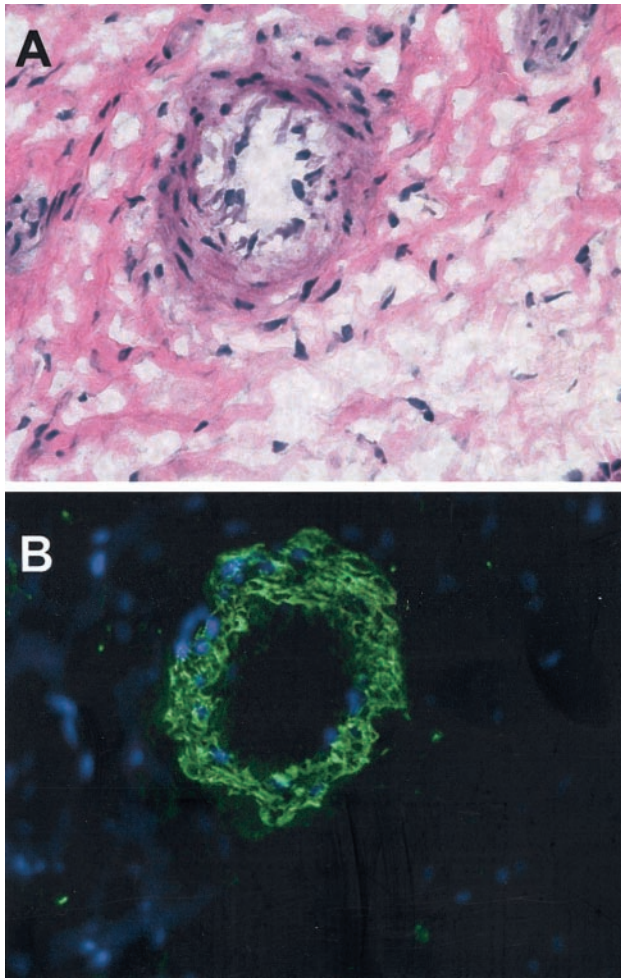


Figure 4. Immunohistochemical staining suggestive of MSC participation in postinfarction angiogenesis. A is an H&E-stained section of myocardium immediately adjacent to infarction (bottom) and illustrates a developing arteriole. A serial section viewed under confocal microscopy is shown in B. MSCs were labeled with DAPI before their intravenous injection 10 minutes after ischemia/reperfusion in a rat. DAPI-positive cells (blue) home to infarcted tissue and can be seen throughout the field, but only those integrated with the developing vessel are positive for smooth muscle actin (green).

be fully elucidated, it is postulated that MSCs migrate to participate in wound repair. This extraordinary ability of MSCs to home to sites of acute tissue injury has been demonstrated in the settings of bone fracture^{74,89,90} and cerebral ischemia,⁹¹ as well as the infarcted heart.^{81,92}

Saito et al⁸¹ were the first to demonstrate that MSCs administered intravenously engraft within regions of MI. In this study, mouse MSCs were transduced with a LacZ reporter gene and injected intravenously to rats (in the absence of immunosuppression). In healthy (noninjured) animals, intravenous MSCs preferentially engraft in the marrow cavity. However, when these rats were subjected to a cycle of ischemia/reperfusion, significant numbers of labeled xenogeneic MSCs could be identified in the circulation and, subsequently, the infarcted region of the heart. The majority of engrafting MSCs were found to be positive for cardiomyocyte-specific proteins, whereas a distinct subpopulation was determined to participate in angiogenesis.

Our laboratory has used a similar model in an attempt to characterize MSC movements after intravenous administration. MSCs obtained from the ACI rat strain were transduced with a retroviral vector encoding for β -galactosidase (β -gal) expression. These labeled MSCs were identified within the bone marrow of Fisher rats 10 days after intravenous injection, and MSCs were not detected in the heart or lungs at that time. When rats were submitted subsequently to a 45-minute cycle of left anterior descending artery occlusion and reperfusion, a significant number of β -gal-positive MSCs were detected within the infarction region (Figure 5A). This engraftment was highly specific in that labeled MSCs were not detected in noncardiac tissues or the viable myocardium of the right ventricle, atria, or LV posterior wall.⁹³

Although these data shed some light on the physiology of MSCs in wound repair, the question remained whether MSCs administered intravenously after infarction could similarly home to and possibly aid in repair of damaged myocardium. When administered during the acute reperfusion period, this in fact appears to be the case.^{93,94} In a rat infarct model, administration of 5 million MSCs intravenously after 10 minutes of reperfusion resulted in significant and sustained cardiac engraftment. However, the “window” of MSC homing is limited. If MSC administration was delayed until 2 weeks after infarction, no significant cardiac engraftment was observed, with most cells returning to the bone marrow (Figure 5B). The remarkable specificity with which MSCs home to regions of injury after systemic delivery with essentially no engraftment in normal tissue is illustrated in Figure 6. Although the factors responsible for MSC migration have not yet been defined clearly, the transient nature of the phenomenon is consistent with the involvement of an inflammatory mediator similar to those responsible for macrophage and neutrophil infiltration in injured tissue. Further complexity is added by a recent report suggesting that expansion of murine MSCs in culture may diminish the efficacy of injury-induced homing.⁹⁵ The degree to which species differences may account for these varying results requires further investigation.

Tracking and Quantification of Engrafted MSCs

One of the problems inherent to the study of cellular cardiomyoplasty, particularly with intravenous administration, is the difficulty in tracking the movement of implanted cells. Recently, Barbash et al⁹⁶ used γ camera imaging of ^{99m}Tc-labeled MSCs to determine their distribution after intravenous or intraventricular injection in a rat MI model. They reported that 4 hours after intravenous injection, a significant number of MSCs were trapped in the lungs, with lesser numbers in the heart and other organs. Cardiac engraftment was augmented significantly (and pulmonary plugging attenuated) with intraventricular administration. MRI of iron-labeled cells is another technique that has been used recently to track MSC distribution in vivo.^{97,98} Hill et al⁹⁸ demonstrated that iron labeling does not alter MSC multipotentiality and that imaging in a standard 1.5-T magnet provides excellent spatial resolution in the beating heart. Furthermore,

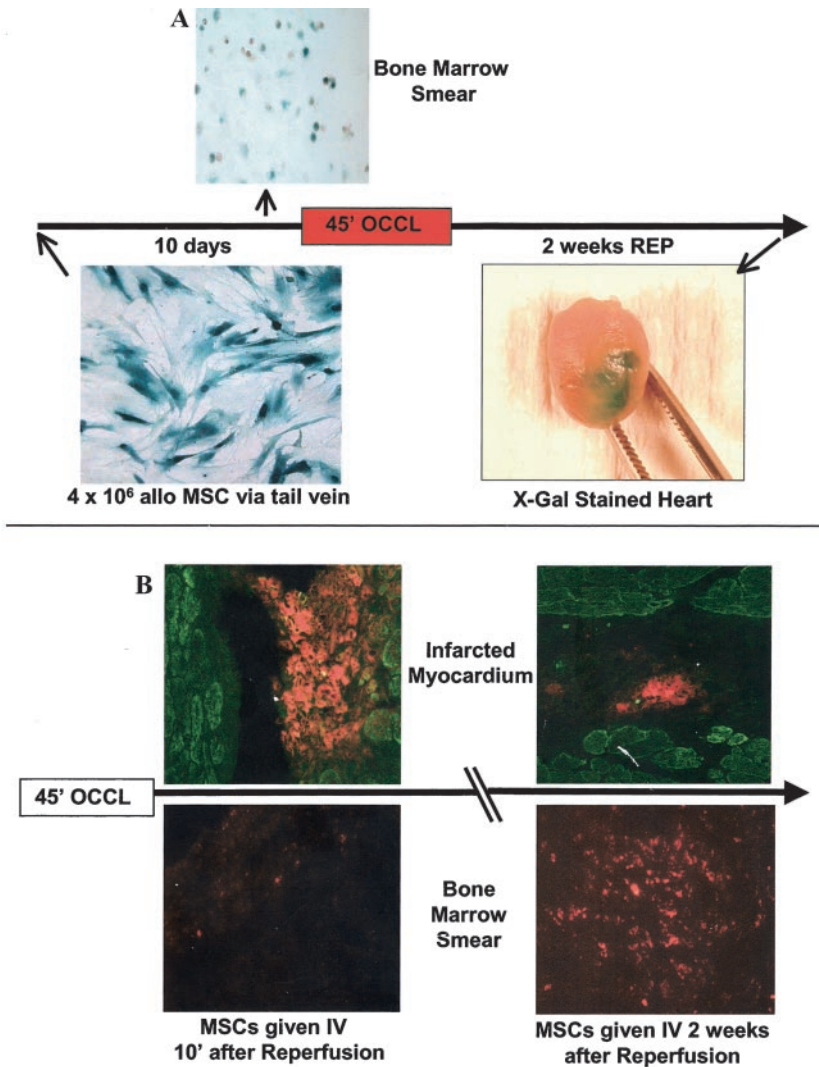


Figure 5. A, Rat MSCs were transduced in vitro with the β -gal reporter gene to follow their distribution after intravenous delivery. Left, Nearly 100% of the transduced MSCs stained positive for β -gal activity before injection; 3.9×10^6 MSCs were injected into the tail vein of healthy Fisher rats. β -Gal-positive cells were only detected in the bone marrow 10 days after MSC delivery. When these animals were subjected to 45 minutes of ischemia and 2 weeks of reperfusion, labeled MSCs migrated to the site of infarction. Labeled cells were not detected in noncardiac tissues at 2 weeks after infarction. B, To determine whether MSCs could home to sites of infarction when administered systemically at reperfusion, 2×10^6 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI)-labeled rat MSCs were delivered intravenously either 10 minutes (left) or 14 days (right) after reperfusion. In both groups, animals were euthanized 14 days after cell injection, and the hearts (top) and bone marrow (bottom) were examined for the presence of DiI-positive MSCs. Qualitative assessment suggests that MSC delivery at 10 minutes after reperfusion results in a greater degree of MSC engraftment in the infarcted myocardium than delivery at 14 days. Conversely, MSC engraftment in the bone marrow is increased with delayed administration.

the iron label can be conjugated to a fluorescent particle to aid in histologic MSC identification.

MSC localization in vivo with either MRI or γ imaging is of tremendous value to those attempting to demonstrate the biodistribution or persistence of MSC cardiomyoplasty. However, the inability of the techniques to accurately quantify the number of MSCs is troubling to those seeking to optimize the therapeutic use of MSCs. When attempting to calculate dose/response relationships in such studies, it is the number of cells engrafting that is of concern, as opposed to the

number administered. Accurate cell engraftment quantification has been elusive regardless of cell type examined. Some investigators have attempted to generate indexes of engraftment by simply counting the number of labeled cells in a representative sampling of high-powered fields. This methodology is hindered by the possibility of counting multiple cell layers or fractions of cells and, therefore, is not exact. We have attempted to quantify MSC engraftment using RT-PCR amplification of transgene expression. Although theoretically sound, in our experience, PCR-based quantification of trans-

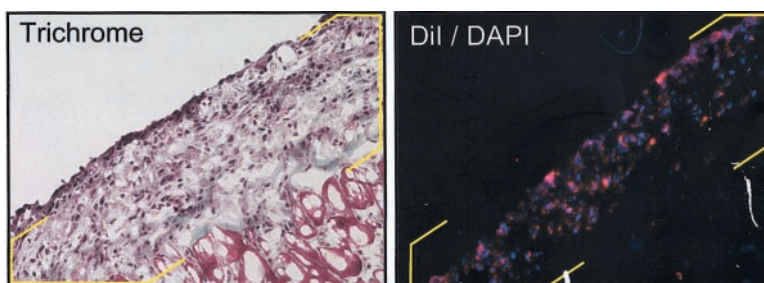


Figure 6. Histologic analysis was used to determine the specificity with which MSCs home to regions of infarction after intravenous delivery. Rat MSCs were labeled with DAPI (blue fluorescence) and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI; red fluorescence) in culture immediately before injection. Left, Infarcted tissue is identified in trichrome-stained samples by blue-green coloration (yellow brackets). Note region of viable myocardium in bottom right portion of image. Right, Fluorescently labeled MSCs were clearly identified in serial tissue sections using

confocal microscopy. Comparison of fluorescent images to trichrome-stained sections demonstrated a high degree of correlation between the region of MSC engraftment and the region of necrosis. Essentially, no cells were identified in regions of viable myocardium or noncardiac tissues.

duced MSCs lacks the sensitivity required for accurate tissue quantification when the number of engrafted cells is low.

A method found to provide a more accurate quantification of MSC engraftment is radiolabeling of MSCs with tritiated thymidine ($[^3\text{H}]$ -thymidine). After implantation, the degree of radioactivity in a tissue can be correlated to MSC engraftment in a linear fashion. Although PCR techniques would allow for detection of 50 000 cells in a rat heart, tritium labeling has a 10-fold lower threshold of detection (≈ 5000 cells), greatly improving accuracy of measurements as well as our ability to detect modest numbers of cells in the heart.

Using these types of techniques has allowed investigators to quantify the degree of MSC engraftment in the heart after delivery. Although the exact numbers vary, it can be said that in all cases, the extent of engraftment is currently modest. Some experiments have indicated that $<3\%$ of MSCs administered by direct injection persist after 2 weeks. Administration of higher numbers of cells results in only modest augmentation of long-term engraftment.

Summary

Adult stem cells are found in many tissues and participate in adult growth as well as damaged tissue repair and regeneration. The cellular and tissue environment in the adult is likely very different from the early embryo conditions that produce ES cells. Bone marrow provides an accessible and renewable source of adult MSCs that can be expanded greatly in culture and characterized. Culture-expanded and characterized MSCs have been tested for their ability to differentiate into several lineages in vitro and also tested in animal models for their ability to enhance tissue repair and undergo in vivo differentiation. One of the greatest attributes of MSCs is their potential to supply growth factors and cytokines to repairing tissue. MSCs do not appear to be rejected by the immune system, allowing for large-scale production, appropriate characterization and testing, and the subsequent ready availability of allogeneic tissue repair-enhancing cellular therapeutics. This provides for the further development of this new field and paves the way for the use of yet other stem cells. The potential to use MSCs to repair damaged cardiovascular tissue is very promising and moving forward quickly. The current results from many labs and early cardiac clinical studies suggest important therapeutic approaches will be forthcoming through MSC use. Perhaps most important, understanding adult stem cells such as MSCs will provide us with greater insight into human biology.

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