Cardiovascular disease is the leading cause of mortality and morbidity in the United States and worldwide.précardiomyocardial infarction (MI) and its fatal sequelae, heart failure, and sudden cardiac death, cardiovascular diseases carry an enormous psychological and financial burden to patients, their families, and society. Over the past half a century, conventional medicine and surgery have offered many breakthroughs, resulting in a dramatic decline in CV mortality. Despite the major advances, medical or surgical treatment of chronic heart disease yields only temporary delay in a progressive disease process, with the only definite cure remaining heart transplantation.

The use of mesenchymal stem cells (MSCs) for therapy of cardiac disease has been a rapidly evolving field. MSCs have been shown to differentiate into mesoderm- and nonmesoderm-derived tissues, their immunomodulatory effects, their availability, and their key role in maintaining and replenishing endogenous stem cell niches have rendered them one of the most heavily investigated and clinically tested type of stem cell. Accumulating data from preclinical and early phase clinical trials document their safety when delivered as either autologous or allogeneic forms in a range of cardiovascular diseases, but also importantly define parameters of clinical efficacy that justify further investigation in larger clinical trials. Here, we review the biology of mesenchymal stem cells, their interaction with endogenous molecular and cellular pathways, and their modulation of immune responses. Additionally, we discuss factors that enhance their proliferative and regenerative ability and factors that may hinder their effectiveness in the clinical setting. (Circ Res. 2015;116:1413-1430. DOI: 10.1161/CIRCRESAHA.116.303614.)

Key Words: cell differentiation ■ mesenchymal stem cell ■ regeneration ■ stem cells

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easily isolated, expanded, and immunologically tolerated, allowing for allogeneic, off-the-shelf transplantation. The ability to use preprepared allogeneic cells for cell-based therapy allows for a level of quality control and scalability that far exceeds autologous strategies. In this review, we describe the biology, the mechanisms of action, the emerging preclinical and clinical trial results, and discuss potential strategies that will refine the development of MSCs as a therapeutic tool.

What Constitutes an MSC?
In 1970, Friedenstein discovered a rare (0.0001%-0.01% of nucleated cells in human BM) population of plastic adherent stromal cells residing in the BM. These cells, which readily expand in culture, are now commonly called mesenchymal stem or stromal cells and are recognized to play an integral role in the hematopoietic niche. In 2006, the International Society for Cellular Therapy established minimal requirements for MSC definition: (1) adherence to plastic in standard culture conditions; (2) expression of the surface molecules CD73, CD90, and CD105 in the absence of CD34, CD45, HLA-DR, CD14 or CD11b, CD79a, or CD19 surface molecules, and (3) a capacity for differentiation into osteoblasts, adipocytes, and chondroblasts in vitro (Figure 1). The rationale behind the selection of these criteria was to facilitate the comparison of different studies, to foster a more uniform characterization of MSC, and render the exchange of data among investigators easier. However, these markers represent a range of MSC differentiation potential. Furthermore, these criteria apply to human MSCs, but do not necessarily extend to other species, and also after culture, these markers may be lost or new markers may arise. Some reports fail to meet these criteria, thus making an across-the-board comparison difficult. The convention of referring to human BM MSCs as stem cells results from their proven self-renewal capability and capacity for multilineage differentiation (Figure 1).

Sources and Types of MSCs
MSCs are broadly distributed throughout the body outside BM and reside in adipose tissue, gut, lung, liver, placenta, amniotic fluid, dental pulp, periodontal ligament and recently in the heart. The cells most commonly used in clinical trials to date originate from BM (MSCs and mesenchymal precursor cells [MPCs]), adipose tissue, and umbilical cord (Table). Umbilical cord blood–derived MSCs have been extensively studied in models of heart disease, but their isolation can be difficult. Wharton’s jelly of the umbilical cord is also a rich source of MSCs, but mainly studied in the context of heart valve tissue engineering. Cells that share some of the characteristics of MSCs can be identified in peripheral blood.

Figure 1. Schematic overview of signaling molecules and transcription factors involved in the regulation of differentiation of mesenchymal stem cells (MSCs). BMP-2 indicates bone morphogenetic protein-2; EGF, epidermal growth factor; FGF-2, fibroblast growth factor-2; LRPS/6, low-density lipoprotein receptor-related protein-5/6; MEF2, myocyte enhancer factor-2; MRF, myogenic regulatory factors; Oax, Osterix; PDGF, platelet-derived growth factor; RUNX2, runt-related transcription factor-2; TGF-β, transforming growth factor-β; and VEGF, vascular endothelial growth factor.
Amniotic fluid–derived MSCs possess some of the characteristics of embryonic stem cells. In’t Anker et al. described amniotic fluid–derived MSCs, with full in vitro multipotential differentiation capacity. In addition, amniotic fluid–derived MSCs have a ckit+ subpopulation that also expresses embryonic stem cell markers (Oct-4, Nanog, and SSEA-4). Amniotic fluid–derived MSCs express proteins and genes of HLA-ABC (MHC class I), but not those of HLA-DR (MHC class II). Recently, heart-derived MSC-like cells have been identified and have been used both in animal models of heart disease and in the Cardiosphere-Derived Autologous Stem Cells to Reverse Ventricular Dysfunction (CADUCEUS) study. It has also been shown that cardiac stromal cells treated with a cocktail of epigenetic drugs differentiate into functional cardiovascular precursors. Cardiac stromal cells exhibit many phenotypic similarities to their bone marrow counterpart; however, they demonstrate an ability to acquire a cardiomyocyte phenotype more efficiently. In 2002, Jiang et al. isolated adult cells from rodent bone marrow and suggested that under some conditions may have broad differentiation potencies. They named those cells multipotent adult progenitor cells. Multipotent adult progenitor cells have been shown under appropriate circumstances to be able to differentiate into hematopoietic cells, osteoblasts, hepatocytes, neurons and also possess immunomodulatory capabilities.

### Tissue Sources of MSCs

Adipose and BMMSCs are the most heavily investigated and tested. The abundance of MSCs derived from adipose tissue and the ease of acquiring them with a liposuction makes this source attractive and feasible for clinical trials (Table; Online Table V). However, there has been a degree of uncertainty on whether adipose-derived MSCs are truly MSCs; hence, they are often termed as adipose tissue stem cells. Adipose derived and BMMSCs share many biological characteristics; however, there are some differences in their immunophenotype, differentiation potential, transcriptome, proteome, and immunomodulatory activity (Table). Some of these differences may represent specific features, whereas others are suggestive of the inherent heterogeneity of both populations.

### Isolation and Expansion of MSCs

It is possible to obtain (after expansion) 50 to 400 million or even more cells from a BM aspirate of 10 mL. The 3 critical steps that allow MSCs to be isolated from other BM cells are (1) use of density gradient centrifugation (i.e., Ficoll or Percoll) to separate non-nucleated red blood cells from nucleated cells or cell mobilization and isolation; (2) the ability of MSCs to adhere to plastic; and (3) the ability of monocytes to be separated from MSCs by trypsinization. To isolate MSC from a BM aspirate, cord blood, or peripheral blood, the samples are fractionated by density gradient for mononuclear cell isolation, resuspended in appropriate culture medium containing selected batches of fetal bovine serum, and allowed to adhere to plastic dishes for 2 days; then, nonadherent cells are removed and the remaining cells allowed to grow for 2 to 3 weeks. Cells initially generate a heterogeneous adherent cell layer, including fibroblast-like and small round-shaped cells, whereas they seem uniformly spindle-shaped after several passages in culture. Confluent cells are trypsinized and allowed to expand for as many as 40 generations without loss of characteristics.

### Table. Comparison of Characteristics of MPC, BMMSC, ATMSC, and UCMSC

<table>
<thead>
<tr>
<th>Feature</th>
<th>MPC</th>
<th>BMMSC</th>
<th>ATMSC</th>
<th>UCMSC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Yield</strong></td>
<td></td>
<td>1%–3% BM mononucleated cells</td>
<td>0.001%–0.01% of total marrow nucleated cells</td>
<td>0.5%–5% of adipose tissue</td>
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<td><strong>Immunophenotype</strong></td>
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<td>CD90</td>
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<td>CD105</td>
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<td>CD54</td>
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<td>MHC I</td>
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<td>CD80</td>
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<td>STRO-1/STRO-3</td>
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<td><strong>Differentiation potential</strong></td>
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<tr>
<td>Adipogenic</td>
<td>+</td>
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<tr>
<td>Osteogenic</td>
<td>+</td>
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<tr>
<td>Chondrogenic</td>
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<tr>
<td>Smooth muscle cells</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Endothelial</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Transcriptome and proteome†</td>
<td>IFN-γ, WNT11, WNT7B, CCL3, FGFR9, Vimentin, SOX6, IL1R2, KDR, nph3, PAX3, gelsolin, SP1, ZNF45, prohibitin</td>
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</table>

ATMSC indicates adipose tissue–derived mesenchymal stem cell; BM, bone marrow; BMMSC, BM-derived mesenchymal stem cell; IFN-γ, interferon-γ; MPC, mesenchymal precursor cell; and UCMSC, umbilical cord–derived mesenchymal stem cell.

†Uniquely expressed or in significantly higher levels in each population.
of multipotentiality. A panel of monoclonal antibodies against epitopes expressed on their surface is used for phenotypic characterization.11

**Biochemical Cross-Talk and Other Factors Regulating MSC Differentiation**

Colony-forming units (CFU), once isolated, can commit to particular cell lineages through treatment with bioactive factors in vitro or in vivo (Figure 1). When vascular endothelial growth factor (VEGF)34 is present, CFUs preferentially take up vascular endothelial fate. Similarly, 5-azacytidine treatment induces their cardiac differentiation in vitro.35 However, other factors shown to induce CFU differentiation into cardiac cells recently, including dexamethasone and ascorbic acid,36 bone morphogenetic protein-2, and fibroblast growth factor-4,37 represent alternative avenues of CFU pretreatment. Treating CFU with dexamethasone, 13-glycerol phosphate, and ascorbic acid prompts differentiation into osteogenic cells that forms a mineralized extracellular matrix.38 Chondrogenic differentiation is achieved by using dexamethasone and transforming growth factor-β (TGF-β)3,39 adipogenic through dexamethasone, insulin, indomethacin, and 1-methyl-3-isobutylxanthine.11 Exposed to basic fibroblast factor, dimethyl-sulfoxide, β-mercaptoethanol, and butylated hydroxyanisole, MSC differentiate into cells expressing neuronal phenotype.40 MSCs also differentiate into other mesoderm-derived tissue, notably myocytes, as shown in some but not all studies.41

MSCs also secrete several cytokines. Many cytokines pertinent to hematopoietic cell proliferation and differentiation are among them (interleukin-6, Flt-3 ligand, G-CSF, GM-CSF). MSCs express several adhesion-related antigens (CD166, CD54, CD31, CD106) and integrins (CD49, CD29, CD11, b4-integrin).11 Mechanical loading, as shown by Pijnappels et al.42 may explain the increased propensity for MSC differentiation to occur in vivo or in coculture situations that add mechanical forces.

When mouse MSC and rat ventricular myocytes were cocultured, MSCs became actin-positive and formed gap junctions with the native myocytes.33 On the contrary, when a semipermeable membrane divided the 2 cell types, there were no such findings, suggesting that differentiation also requires direct cell–cell contact.43 Although this is not always true,44 in a similar experiment, the MSCs started to contract, express SERCA2 and ryanodine receptor, and were positive for troponin, sarcemeric actinin, and desmin.41 All these suggest that cell–cell contact, cardiac microenvironment, and factors secreted by myocytes play intertwined roles in MSC transdifferentiation to cardiomyocytes.

Two main molecular pathways govern MSC differentiation: the Wnt and the TGF-β superfamily pathway44 (Figure 1). The Wnt pathway is critical in skeletogenesis by promoting osteoblast proliferation45 and by suppressing chondrocyte formation. Activation of Wnt receptors on MSCs leads to downstream signal transduction that regulates cell proliferation and differentiation.45 The TGF-β pathway is involved in skeletal tissue growth and regulation of MSC differentiation into chondrocytes.9 This is achieved by upregulation of gene expression in MSCs via several intracellular cascades, including extracellular signal–regulated kinase (1/2), SMAD proteins, mitogen-activated protein kinases, p38, and c-Jun N-terminal kinases (JNK).9

**Immunomodulatory Properties of MSCs**

MSCs are potent modulators of the immune system by suppressing white blood cells and triggering anti-inflammatory subsets (Figure 2). MSCs were used to treat therapy-resistant severe graft-versus-host disease47 based on the fact that MSCs inhibit T-cell proliferation in vitro.48 The success of MSCs has sparked vigorous investigation of their immunomodulatory capabilities. Moreover, the immunomodulatory properties in addition to the immunosuppressive properties further underlie the ability of MSCs to be used as an allograft.5

MSCs when cocultured with T-cells upregulate indoleamine-pyrrrole-2,3-dioxynase, leading to tryptophan depletion and accumulation of metabolites, such as kynurenine, both of which reduce T-cell proliferation.49 MSCs also express PD-L1 and PD-L2 ligands that activate PD-1 receptor on a T-cell, resulting in decreased production of the proinflammatory cytokines interferon-γ, tumor necrosis factor-α, and interleukin-2. In addition, MSCs have been shown to secrete TSG-6 (tumor necrosis factor-stimulated gene 6 protein), a powerful anti-inflammatory factor.50 Moreover, MSCs also downregulate the activating receptors of natural killer cells Nkp30, Nkp44, and NKG2D.51 MSCs are able to arrest B-cell maturation in G0/G1 phase and simultaneously reduce the chemotactic activity of these cells and block maturation of dendritic cells, resulting in reduced expression of antigens and costimulatory molecules necessary to activate T-cells.52

After an MI, 2 major types of macrophages can be found in the heart: (1) M1 (inducible nitric oxide synthase, MHC class II, CD80, CD86) that clears the debris and produces proinflammatory interleukin-1β, tumor necrosis factor-α, and interferon-γ, and after 5 days, (2) M2 (arginase, macrophage mannose receptor CD206), which has an anti-inflammatory phenotype reducing the release of proinflammatory cytokines and at the same time stimulating scar formation and angiogenesis.53 In the presence of MSCs, differentiation into the M2 subtype was boosted,53 whereas the debris-cleaning function remained intact.54

The effects described earlier were observed in controlled in vitro studies. In vivo, transplanted cells first have to survive in order to then suppress or modulate the immunologic milieu. Their survival is aided by the fact that MSCs express moderate levels of HLA class I and lack HLA class II, B7, and CD40 ligand expression. In some55 but not all56 in vivo preclinical studies, allogeneic MSCs may lose immunoprivilege. The most important assessment of MSC immunology in humans is the Perucutaneous Stem Cell Injection Delivery Effects on Neomyogenesis (POSEIDON; NCT01087996) clinical trial where allogeneic MSCs did not induce an immunologic reaction5 12 months after transplantation. That was also confirmed in the clinical trial by Ascheim et al.,50 in which MPCs were administered to left ventricular assist device patients.

**In Vivo Mechanism of Action**

There is substantial accumulating data from in vitro,61,62 preclinical,63–66 and clinical67–69 settings supporting a multifactorial mechanism of action for the cardio reparative effects of
MSCs. Three main mechanisms of action underlie the favorable actions of MSCs in disease: (1) reduction of fibrosis, (2) stimulation of angiogenesis, and (3) restoration of contractile function through engraftment, differentiation, and stimulation of endogenous cardiac stem cells to proliferate and differentiate (Figure 3). These effects occur in concert and together lead to the replacement of scarred or dysfunctional myocardial tissue with contractile and perfused tissue.

Most studies that examine engraftment of cell therapy reveal low retention rates. Toma and colleagues reported that after 4 days, 0.44% of transplanted MSCs resided in the myocardium. This low retention rate prompted the exploration of other mechanisms of action driving the recovery in cardiac structure and function.

Cardioprotection

Many early studies revealed that the border zone of an experimentally induced myocardial infarction was characterized by a reduction in apoptotic myocytes and an augmentation of vascularity. Scar tissue reduction and cardioprotection after MSC transplantation is well described in both preclinical models and clinical trials. Amado et al. using serial computed tomography imaging showed in-vivo reappearance of myocardial tissue and restoration of contractility after MSC implantation in swine. Gneccchi et al. showed that cell culture medium conditioned by hypoxic MSC reduces apoptosis and necrosis of isolated rat cardiomyocytes exposed to low oxygen tension. These investigators interpreted the findings as consistent with paracrine signaling, given the use of cell culture medium as opposed to cells themselves. This cardioprotective effect could be enhanced when MSCs were engineered to overexpress Akt-1. This was later confirmed in vivo by a study showing enhanced survival of Akt-MSCs post transplantation. Additionally, Akt-MSCs secrete a protein (Sfrp2) that exerts a prosurvival effect through modulation of Wnt signaling. Rehman et al. showed that adipose-derived stem cells secrete angiogenic and antiapoptotic factors providing further evidence of cardioprotection. The responsible pathways may include activation of inflammation and stress response-associated signaling pathways mediated by insulin-like growth factor 1 (IGF-1) and inhibition of transcription factor NF-kB (nuclear factor

Figure 2. Immunomodulatory capabilities of mesenchymal stem cells (MSCs). Schematic overview of the interactions between MSC and the immune system. Via multiple pathways, MSCs suppress proliferation of both T helper (T<sub>H</sub>) and cytotoxic T cells (T<sub>C</sub>). In addition, differentiation to T<sub>1,2</sub> and regulatory T-cells (T<sub>reg</sub>) is triggered, resulting in an anti-inflammatory environment. Maturation of dendritic cells (DC) is inhibited via interleukin (IL)-6, blocking upregulation of CD40, CD80, and CD86, which in turn reduce T-cell activation. Monocytes are induced by MSC to differentiate preferentially toward the M2 phenotype. IL-10 produced by the M2 macrophages can boost the formation of T<sub>reg</sub> whereas simultaneously reducing tissue migration of neutrophils. Neutrophils (polymorphonuclear granulocytes; PMN) are allowed longer life span but reactive oxygen species production is decreased. Natural Killer (NK) cell proliferation is suppressed as well as their cytotoxic activity. B-cell proliferation is inhibited and the production of antibodies is reduced. Modified from van den Akker et al. HGF indicates hepatocyte growth factor; IDO, indoleamine-pyrrole-2-3-dioxygenase; PGE2, prostaglandin E2; and TGF-β, transforming growth factor-β.
kappa-light-chain-enhancer of activated B cells). The cardioprotective effect was further enhanced by preconditioning the MSCs with TGF-β by or by activating the tumor necrosis factor receptor-2. In vivo, MSC cardioprotection leads to reduced number of apoptotic cells around the MSC injections. Thus, together, these findings support the idea that in addition to other key cardioreparative effects, MSCs also create a milieu that protects or reduces stimuli, driving ongoing loss of myocytes.

Neangiogenesis

The formation of new vessels is the cornerstone of any meaningful cardiac repair. There are 3 mechanisms of postnatal neovascularization: (1) angiogenesis, (2) arteriogenesis, and (3) postnatal vasculogenesis, where endothelial precursors originating from the BM assemble to create new blood vessels. It is still under debate whether the observed increase in capillary density and tissue perfusion is caused by differentiation of MSCs to endothelial cells and vascular smooth muscle cells or because of secretion of paracrine mediators and generation of new pericytes. There is evidence that MSCs act as pericytes, perivascular cells that are essential to vascularization by stimulating the endothelial cells to form tube-like structures, and subsequently vascular networks. Expression of MSC markers was also detected at the surface of native, noncultured perivascular cells. Thus, blood vessel walls harbor a reserve of progenitor cells that may be integral to the origin of the MSCs and other related adult stem cells.

In vitro, MSCs express α-smooth muscle actin and β-actin filaments, whereas in in vivo studies, it is shown that MSCs express an endothelial phenotype that enhanced microvascular density. Despite this evidence, several groups suggest that only a small number of vessels contain donor cells. Therefore, it is proposed that it is the release of proangiogenic and proartheriogenic factors from the MSCs that play the most important role in neovasculogenesis. In their experiments, there was a significant increase in the levels of VEGF and bFGF (basic fibroblast growth factor) in the MSC-treated animals, and that was also documented in a gene expression profiling of MSCs under hypoxia. Further supporting this theory, Markel and colleagues showed that MSCs underexpressing VEGF have significantly less cardioreparative capabilities. Therefore, MSCs either directly through exosomes or indirectly through soluble factors are strong proangiogenic factors.

Antifibrosis Triggers Neomyogenesis

The dominant lesion in the remodeling heart after infarction is the replacement of necrotic myocardium with scar tissue. It is the scar burden that leads to both infarct expansion and extension, and the former sets the stage for the overall remodeling and transformation of the shape of the ventricle from ellipsoid to spherical. In a fibrotic environment, type 1 collagen accumulates, resulting in decreased expression of a wide array of genes, growth factors, and cytokines that inhibits endogenous reparative potential of muscle. In addition, degradation of extracellular matrix components play a key role in regulating muscular tissue regeneration. Thus, reducing the fibrotic tissue directly enhances endogenous myogenesis. Willems et al showed that a 1,4-dihydropyridine inducer of type 2 TGF-β receptor degradation-1 selectively enhanced the differentiation of uncommitted mesodermal cells to cardiomyocytes. Extensive studies in the field of bone and cartilage regeneration have shown us that mechanical forces and extracellular matrix components significantly influence MSCs, but also MSCs modulate the matrix by secreting metalloproteinases and their tissue inhibitors by shifting the balance toward domination of matrix-degrading effects. This may require the secretion of hepatocyte growth factor and heme oxygenase-1 by MSCs. However, MSCs do not only interact with the matrix directly. It is reported that MSCs suppress the proliferation of fibroblasts and promote their metalloproteinase secretion.

Direct MSC Stimulation of Endogenous Repair

MSC transplantation has now been shown by multiple groups to stimulate proliferation and differentiation of endogenous cardiac stem cells in (Figure 4). This discovery provides a highly plausible explanation for the replacement of scarred tissue with new contractile myocardium. Neomyogenesis occurs by 2 related mechanisms: stimulation of endogenous cardiac stem cells (c-kit+ and other lineages) and enhancement of myocyte cell cycling. In the first demonstration of this phenomenon, GFP+ allogeneic MSCs were injected in infarcted swine hearts and led to the formation of chimeric clusters containing immature MSCs and endogenous c-kit+ cardiac stem cells. These clusters exhibited cell–cell interactions mediated by connexin-43-mediated gap junctions and N-cadherin mechanical connections. Importantly, there was a 20-fold increase in the endogenous c-kit+ population in MSC-treated animals relative to controls, and the c-kit+ cells had much greater capacity for myocyte lineage commitment. Beltrami and colleagues cocultured MSCs and resident cardiac stem cells and they reported maturation and increased viability of

![Figure 3. Mechanisms of action of mesenchymal stem cells (MSCs). The proposed mechanism of action of MSCs form an intertwined cycle of paracrine, autocrine, and direct effects that include vascular regeneration, myocardial protection, cardiomyocyte regeneration that ultimately lead to cardiac repair. MiR-1 indicates mitochondrial Rho-GTPase.](image-url)
the latter. Loffredo et al. used a lineage tracing mouse to examine the capacity of cell therapy to stimulate endogenous myogenesis and compared the ability of BM-derived c-kit+ stem cells and BM-MSCs to induce proliferation of endogenous cardiopoietic stem cells (CSCs) after MI. Mice were genetically modified to express GFP in cardiomyocytes. At 8 weeks after transplantation, BM c-kit+ stem cells led to a significant reduction in the GFP+ cardiomyocyte pool and parallel increases in β-galactosidase+ cardiomyocytes compared with control, suggesting increased progenitor activity induced by BM c-kit+ stem cells. However, BMMSCs did not exhibit similar findings, suggesting that MSCs may not stimulate endogenous progenitors. This murine finding contrasts with several studies in pigs and may reflect species differences. Suzuki et al. found that in pigs with hibernating myocardium induced by left anterior descending artery stenosis, treatment with intracoronary injections of autologous MSCs led to an improvement in regional wall thickening at both 2 and 6 weeks after injection compared with the control. They also noted a 4-fold increase in c-kit+ and CD133+ populations that also coexpressed GATA-4 in MSCs- vs non-MSCs-treated hearts. The markers of proliferation (Ki67) were significantly increased in hibernating myocardium in MSC-treated animals. More rarely, fusion of transplanted MSC with resident cells may take place as proposed by certain groups. In a preclinical study by our group, the combination of human MSCs and c-kit+ CSCs led to enhanced cardio reparative than that seen in either cell type alone. Together, these findings exhibit important biological interactions between c-kit+ CSCs and MSCs.

**Cellular Effect of MSCs**

Although the paracrine theory seems to play a role, it is the direct cellular mechanisms (exosomes, mitochondrial transfer, connexin43, etc.) that convincingly explain the effects observed in preclinical and clinical studies (Figure 3).

**Extracellular Vesicles and Exosomes**

MSCs exert a host of direct cellular effects by transmitting exosomes and mitochondria into recipient cells (Figure 3). Cells continuously secrete a large number of extracellular vesicles (EV), microvesicles, macromolecular complexes, and small molecules called exosomes. Exosomes have been reported to contain significant amounts of microRNA, other noncoding RNAs, as well as mRNA. Valadi et al. reported that some full-length molecules are present and translated extracted RNA to identifiable full length. Several papers indicate that the RNA content of exosomes differs from that of the parental cell. They facilitate immune responses and participate in antigen presentation, play roles in programmed cell death, angiogenesis, inflammation, and coagulation. Exosomes’ most unique function might be specific interaction with a target recipient cell, enabling cell–cell communication, putatively between widely separated locations in the body. More recent studies have demonstrated that exosomes are not only specifically targeted to recipient cells to exchange proteins and lipids...
or to trigger downstream signaling events, but also deliver specific nucleic acid cargo.⁹⁰ Embryonic stem cells secrete EVs containing Wnt-3⁹⁰, the protein Oct-4, but also mRNA. Those EVs derived from endothelial progenitor cells triggered angiogenesis in endothelial cells by horizontal transfer of mRNA.¹⁰⁹ In addition to mRNA, those vesicles can also transfer microRNA, further supporting the pivotal role of EVs in signaling within the stem cell niche.¹¹⁰ Both mRNA and microRNA contained in EVs have been suggested to mediate a bidirectional exchange of genetic information between the stem cells and the injured cells.¹¹¹ EVs of endothelial origin that were released by ischemic muscle were able to induce the differentiation of BM-derived mononuclear cells into endothelial cells and thus promote postnatal vasculogenesis.¹¹² MSC-derived exosomes were first investigated in 2010 in a mouse model of ischemia/reperfusion injury.¹¹³ MSCs produce increased quantities of exosomes relative to myoblasts and human embryonic kidney cell line.¹¹⁴ MSC-derived exosomes express both the common surface markers of exosomes and markers expressed on MSCs (CD29, CD44, and CD73). Pretreating MSCs with RNase completely abolishes their renal protective effect.¹¹⁵ Treatment of neurons and astrocytes with MSC-derived exosomes leads to an increase of miR-133b in these cells, which promotes functional recovery in Parkinson’s disease and spinal cord injury. This finding suggests that MSCs regulate neurite outgrowth at least partly by transferring miR-133b to neurons and astrocytes via the release of exosomes.¹¹⁶ Purified exosomes administered to a mouse ischemia–reperfusion injury model revealed that MSCs mediate their cardioprotective paracrine effect by exosome secretion.¹¹⁷ Thus, MSC-derived exosomes play a key role in cell–cell interactions, regulating everything from immune responses to neoangiogenesis in their surrounding tissue.

**Mitochondrial Transfer**

In addition to exosomes, MSCs may transfer mitochondria through tunneling nanotubes to injured cells. Spees et al.¹¹⁷ co-cultured somatic cells depleted of their mitochondrial DNA with MSCs and demonstrated rescue of respiration via the transfer of mitochondria from the healthy cells to the respiratory-deficient cells. Similar results can be reproduced using cardiomyocytes.¹¹⁸ Although full details of the molecular mechanism remain to be elucidated, it seems to be mediated by actin-based extensions named tunneling nanotubes and by gap junctions containing connexin 43.¹¹⁹ Ahmad et al.¹²⁰ showed that mitochondrial Rho-GTPase plays a key role mediating the mitochondrial transfer between cells. Interestingly, an association between mitochondrial Rho-GTPase levels and mitochondrial transfer was also reported when comparing different cell types with different mitochondrial transfer capacity, with MSCs expressing higher levels of mitochondrial Rho-GTPase compared with lung epithelial cells and fibroblasts. Thus, MSCs through tunneling nanotubes and gap junctions are able to directly rescue impaired cardiomyocytes.

**Improvement of Cardiac Function by Reconstituting the Cardiac Stem-Cell Niche**

Stem cell niches are described in the bone marrow,¹²¹ hair follicles,¹²² intestinal epithelium,¹²³ and in the heart.¹²⁴ In the heart, the niche is comprised of supporting cells and cell-cell interactions that have crucial regulatory roles. MSC transplantation triggers chemokine and cytokine cascades that initiate and boost an endogenous repair mechanism through the restoration of a cellular and molecular collective with the properties of a stem cell niche.¹²⁵ One key axis that modulates the niches is the SDF-1/CXCR4 axis that also regulates the homing of hematopoietic stem cells to the injured myocardium. Shi and colleagues.¹²⁶ reported high levels of CXCR4 expression both on the surface and intracellularly in BMSCs even after several passages. When the MSCs were exposed to F-3 ligand, stem cell factor, interleukin-6, hepatocyte growth factor, and interleukin-3, they upregulated the expression of CXCR4. Cardiac myocytes can be induced to reenter the cell cycle after treatment with peristin, fibroblast growth factor-1/p38 mitogen-activated protein kinase inhibitor,¹²⁷ neuropeptide, insulin, and TGF-β. The fact that some of these factors are secreted by MSCs underlines their role in activating the niche’s proliferative potential and in fact modulating the cardiovascular microenvironment toward a reparative mode.

**Harnessing and Enhancing the Therapeutic Potential of MSCs**

Numerous efforts to further enhance the therapeutic potential of MSCs by genetically modifying or pretreating MSCs with various drugs and cytokines are under way. Although combining MSCs and simvastatin¹²⁸ did not affect the reduction in perfusion defect, scar size, or end-diastolic volume, it did potentiate increases in ejection fraction (EF) and systolic wall thickening, as well as improved MSC retention and survival. Triptazidine-preconditioned MSCs decreased LDH (lactate dehydrogenase) levels and enhanced production of survival proteins, including HIF-1α, survivin, phosphorylated Akt, and Bcl-2 protein levels and Bcl-2 gene expression.¹²⁹ Guo and colleagues.¹³⁰ pretreated MSCs with IGF-1 that led to overexpression of CXCR4, resulting in improved wall thickness and left ventricular function as compared with native MSCs alone. Similarly, preconditioning MSCs with VEGF resulted in increased activation of the Akt pathway, lower expression of cell aging markers, and cell cycle inhibitors (p16 and p21).¹³¹ In another study, heme oxygenase-1 was used that improved MSC survival in hypoxic conditions and upregulated the secretion of various cytoprotective cytokines by MSCs.¹³² The combination of various factors was also quickly put to test by various research groups. One such combination¹³³ was a cocktail of fibroblast growth factor-2, IGF-1, and bone morphogenetic protein-2 to pretreat MSCs before transplantation that led to increased cardiac-specific markers and also interestingly enhanced expression of phosphorylated Akt and phosphorylated cAMP (cyclic adenosine monophosphate) response by native cardiomyocytes. Surprising Akt activation not only enhanced the survival of the transplanted MSCs, but also decreased the apoptosis of the surrounding myocytes.¹³⁴

Another intriguing and more difficult approach is that of genetic modification of MSCs. MSCs that were modified to overexpress Bcl-2 had reduced apoptosis and increased secretion of VEGF that led to longer survival even at 6 weeks post transplantation in vivo.¹⁰ Transfected MSCs to overexpress SDF-1 receptor, a chemoattractant factor for lymphocytes,
resulted in both increased MSC retention and expression of SDF-1 by the ischemic myocardium that triggered increased intracellular activation of Akt. Genetic modification of MSCs, although tremendously interesting, will have greater challenges in reaching the clinic because of ethical and safety concerns.

**Preclinical Trials of MSC Therapy**

In a pioneering study by Toma et al., human MSCs were injected in murine hearts and were shown to adopt a cardiac fate. The immunoprivileged capabilities of MSCs were shown by Amado et al., where allogeneic MSCs were transplanted into 3-day-old immunocompetent porcine infarcted hearts that resulted in long-term engraftment and a large decrease of scar tissue without any inflammatory response.

Subsequent to those early groundbreaking studies, MSCs have been tested in numerous cardiovascular settings. In acute MI, the inflammatory microenvironment and the necrotic/apoptotic signals are the dominant opposing forces to the therapeutic activities of MSCs. However, the presence of homing signals and the antiinflammatory milieu may be of benefit. Another study, where MSCs were intracoronarily infused into porcine heart 5 days after infarction, showed improvement in EF and scar reduction in MSC-treated animals. In another study, where different doses of MSCs were used 3 days after MI, there was no dose-dependent effect on scar size reduction or EF improvement 12 weeks post transplantation. In contrast, in a study where 4 different doses of BM-derived STRO-3陽 MPCs were directly injected into sheep hearts 1 hour post MI, there were improvements in end-diastolic volume only in the 2 lower doses, although EF increased universally. These seemingly contradicting reports suggest that there might be a therapeutic threshold in the total number of cells that can be injected, although other factors certainly play a role. A preclinical study, where Str-3陽 MPCs were intracoronarily infused in sheep’s hearts immediately after MI showed a 40% decrease of infarct size, attenuation of remodeling, and a 50% increase of blood vessel density in border and remote zones.

In the chronic setting, the repair processes have been completed, the scar has stabilized, and the newly formed network of blood vessels is disorganized and inadequate to halt any disease deterioration. Silva and colleagues injected MSCs into canine hearts that resulted in increase in EF, vascular density, and decrease in scar tissue. Schuleri and colleagues in a similar setting reported that autologous MSCs produce reverse remodeling and functional recovery. Our group has transendocardially injected allogeneic MSCs in swine hearts 3 months post infarction. Although adverse remodeling is nearly complete in that setting, we have observed significant decrease of scar tissue and improved contractility in MSC-treated animals 3 months post transplantation. Left ventricular global function was also improved, thus indicating that MSCs may be able to reverse chronic adverse remodeling.

The intermediate setting between chronic and acute, the subacute model also presents with its own challenges. Although there are fewer preclinical studies in this setting, the accumulating data seem consistent with a benefit of MSCs. Our group administered allogeneic MSCs to the border and infarct zones of infarcted porcine myocardium 3 days after MI via intramyocardial injections. Eight weeks after transplantation, there was a 50% reduction in scar size that was coupled with improvements in EF, left ventricular end-diastolic pressure, relaxation time, and systolic compliance in the treated animals. Another group directly injected autologous MSCs into 2-week-old swine infarcts. Four weeks after transplantation, a trend toward improved wall thickness and systolic thickening was observed in the MSC-treated animals. Online Table I summarizes the preclinical trials described here.

**Non-Ischemic Heart Disease Models**

Ohnishi and colleagues in a rat model of nonischemic dilated cardiomyopathy reported that MSCs attenuate myocardial injury and ventricular dysfunction. In canines, Plotnikov and colleagues suggest that MSCs may provide a platform for sustained biological pacemaker function. That experiment was rather provocative because whether cell therapy exerts pro- or antiarrhythmic effects is crucial to address. These early concerns though have been largely resolved, especially after the encouraging results from clinical trials supported the antiarrhythmic effects.

Autologous MSCs were even used to recellularize tissue-engineered heart porcine valves that were then transplanted to lambs. Four months later, the MSC valves exhibited better transvalvular and distal gradients, as well as less inflammatory reaction and structural deterioration than the BM-derived mononuclear cell counterparts.

**Safety of MSC Therapy**

Although the vast amount of preclinical and clinical data suggest that MSC possess a great potential in treating cardiac diseases, and in spite of all the evidence for the safety of MSC transplantation, it is important to point out that long-term safety requires additional study. There are 2 main theoretical concerns regarding MSC therapy, arrhythmogenicity, and tumorogenicity. The former, as discussed earlier, has been proven not to be an issue in all the clinical trials that have been completed. Miura and colleagues showed that there is accumulating chromosomal instability in murine BM MSC that may lead to malignant transformation. Although seen in rodent models, this observation has yet to be described in numerous preclinical and clinical studies.

**Clinical Trials of MSC Therapy for Cardiac Repair**

**Acute Myocardial Infarction**

Several studies have examined both autologous and allogeneic MSCs for acute MI. In a phase I randomized study, 53 patients were randomized to receive either allogeneic MSCs or placebo 7 to 10 days after MI and in different doses. The intravenous infusion of allogeneic MSCs in this study resulted in improvement in overall clinical status 6 months after infusion, fewer arrhythmic events, and improved EF. The success of this pilot study led to a phase II trial, where as it was preliminary reported by Osiris, the trial sponsor that intravenous infusion of allogeneic MSCs within 7 days of an acute MI resulted in significantly reduced cardiac hypertrophy, stress-induced...
ventricular arrhythmia, heart failure, and rehospitalizations for cardiac complications.\textsuperscript{145} Chen and colleagues\textsuperscript{146} administered autologous MSCs intracoronaroly in patients with subacute MI and observed decreased perfusion defect, improved left ventricular ejection fraction, and left ventricular remodeling 3 months after therapy. In CADUCEUS trial\textsuperscript{25} where autologous cardiospheres (that included MSC-like cardiac cells) were injected 2 to 4 weeks after MI, there were scar reduction, increase in viable heart mass, and regional contractility. However, changes in end-diastolic volume, end-systolic volume, and left ventricular ejection fraction did not differ between groups by 6 months.

In addition to BMMSCs, adipose-derived MSCs have also been tested for acute MI in the APOLLO trial,\textsuperscript{147} a trial of 14 patients, which exhibited the safety of intracoronary infusion of freshly isolated adipose-derived MSCs in the acute setting of an STEMI (ST segment elevation myocardial infarction). This study also demonstrated a trend toward improved cardiac function, accompanied by a significant improvement of the perfusion defect and a 50% reduction of myocardial scar formation (Figure 5). Thus, these studies suggest a potential role for MSCs in the setting of acute MI.

**Chronic Ischemic Cardiomyopathy**

Currently, chronic ischemic cardiomyopathy is the setting in which there is the highest level of consensus for MSC efficacy. There are 6 published or ongoing clinical trials that together show that MSCs have antibiobotic effects, induce neoangiogenesis, enhance contractility, and improve the quality of life of the recipient patients. Thus, the mechanisms of actions identified preclinically seem to be operative in humans with chronic ischemic cardiomyopathy. The most consistent finding is that of a 30% to 50% decrease in size of the MI scar in BMMSC trials\textsuperscript{5,27,68} (Figure 6). Importantly, a decrease in MI size is not evident in the one published trial of adipose MSCs for ischemic cardiomyopathy (Figure 5). Currently, no clinical study has directly compared head-to-head BMMSCs with ADMSCs (adipose-derived mesenchymal stem cells).

Importantly, scar reduction is shown to be accompanied by reperfusion and restoration of contractile performance. Perfusion was also preserved or increased in the PRECISE\textsuperscript{148} (A Randomized Clinical Trial of Adipose Derived Stem and Regenerative Cells In the Treatment of Patients With Non Revascularizable Ischemic Myocardium) and in the PROMETHEUS trials.\textsuperscript{67} These effects also led to a restoration of contractile function as measured by echocardiography\textsuperscript{149} or Eulerian circumferential strain using MRI at the site of injection.\textsuperscript{57,68} The last is also an interesting point and highlights the importance of imaging studies in the assessment of stem cell efficacy. In 2 studies from our group, a substudy of POSEIDON\textsuperscript{141} and the PROMETHEUS trial,\textsuperscript{67} it was shown that MSC therapy exerts its strongest effect at the site of injection, and there is a drop off effect in the adjacent myocardial segments that drive the global functional improvement (Figure 6). Maybe one of the most interesting findings that the clinical trials have offered is the improvement in functional capacity and quality of life these patients experience after cell therapy\textsuperscript{5,68,149}.

There is also an ongoing clinical trial using adipose-derived MSCs, the Mesenchymal STEMORAL CELLTherapy in Patients With Chronic Myocardial Ischemia (MySTromalCell),\textsuperscript{151} that is using culture-expanded adipose tissue–derived MSCs, and is designed to investigate the safety and efficacy of intramyocardial delivery of VEGF-A165-stimulated autologous adipose tissue–derived MSCs to improve myocardial perfusion and exercise capacity and reduce symptoms in patients with chronic ischemic cardiomyopathy.

In a recently published clinical trial\textsuperscript{50} where allogeneic MPCs were injected in the hearts of patients who were undergoing left ventricular assist device implantation, 50% of
the patients were successfully temporarily weaned from their left ventricular assist device at 90 days post transplantation. Although this trial was small and exploratory, it demonstrated the safety of MPC implantation. The efficacy end points though were derived only from a comparison of 10 patients in the MPC group and only 2 in the control group. In addition, an ongoing clinical trial, the Allogeneic Mesenchymal precursor cell Infusion in myoCardial Infarction (AMICI) trial, aims to prove safety, feasibility, and efficacy of MPC therapy in the acute ST-elevation MI (NCT01781390). Online Tables II–V summarize important completed and ongoing clinical trials.

Phase III Clinical Trials

There are currently 2 phase III clinical trials using MSCs. In the C-CURE trial,149 where MSCs were treated ex-vivo with cytokines to enhance their commitment to cardiopoietic lineage (Figure 5). Bartunek and colleagues reported significant improvements in EF, end-systolic volume, and 6-minute walk distance compared with controls. Another phase III study is currently underway aiming to enroll 1730 patients with chronic heart failure, in which MPCs will be injected intramyocardially (NCT02032004).

Non-Ischemic Cardiomyopathy

The Percutaneous Stem Cell Injection Delivery Effects On Neomyogenesis in Dilated Cardiomyopathy (The POSEIDON-DCM; NCT01392625) ongoing clinical trial is testing transcendocardial injections of autologous and allogeneic MSCs in a nonischemic setting of dilated cardiomyopathy.

Demographic and Biological Factors Affecting MSC Therapy Efficacy

When the results of preclinical studies are compared with those of clinical trials, there is a relative decrease of the effect seen in the former. Simply the fact that the animals used in preclinical trials are younger, healthier, and followed up in a closed and controlled environment compared with humans enrolled in clinical trials could account for the best part of this discrepancy in translation. But there are many lessons to be drawn from this phenomenon that will lead to further optimizing the cell therapy itself, but also detecting the patient population that will benefit the most from it.

The cell products produced in different laboratories and the treated population are heterogeneous, heavily influencing the
clinical outcome. Timing, delivery method, and optimal dose are still open to debate, although already there are hints for the existence of time windows and maximal and minimal effective doses.

Sethe and colleagues\(^{153}\) reported that the proportion of MSCs in the BM decreases with age, and the MSC yield is lower in direct association with the age of the donor. Although the inherent ability to differentiate in different cell lineages seems to be preserved in older MSCs, there are quantitative differences between older and younger cells.\(^{154}\) Zhang and colleagues\(^{155}\) in a rat model found that increasing donor age adversely affects the beneficial effect of MSCs. Khan et al\(^{156}\) suggested that only MSCs derived from young and healthy donors can effectively regenerate senescent rat hearts. Understanding the effect of aging on MSCs is crucial for autologous therapy for older patients, who are typically afflicted by cardiovascular diseases. In a recently published substudy\(^{157}\) from the TAC-HFT and POSEIDON trials, it was reported that older individuals did not have an impaired response to MSC therapy.

Sex may influence the biology of MSCs. Some studies suggest that females have a lower reservoir of MSCs, but compared with male, female MSCs exhibit greater resistance to injury.\(^{158}\) But different conditions in the patients themselves may play a role. Less injury and inflammation is found in female patients compared with males after acute cardiac injuries. Estradiol contributes by decreasing the expression of the Bcl-2 family of genes and JNK pathways.\(^{159}\)

It is also important to understand whether and what differences exist between MSCs obtained from normal and diseased individuals. Zhao and colleagues\(^{160}\) described the characteristics of MSCs derived from various malignant hematopoietic diseases. The morphology and phenotype of MSCs remained unchanged even after 20 passages. Patients with rheumatoid arthritis, on the other hand, although having normal reserves of MSCs, seem to display decreased proliferative and clonogenic potential compared with normal.\(^{161}\) Some studies suggest that in diabetic patients, MSCs become exhausted and lose their differentiation potential. These changes seem to be caused by induction of apoptosis and senescence by advanced glycation end-products.\(^{162}\) Interestingly, the changes in MSCs caused by the diabetic environment are probably permanent because culturing MSCs in medium without glucose does not reverse the effect. Cigarette smoking, another risk factor for cardiovascular disease, may adversely affect MSCs. A study demonstrated that nicotine decreased the proliferation of MSCs in a dose-dependent manner.\(^{163}\)

**Future of MSC Therapy**

**Indications, Timing, Delivery of MSCs**

MSCs have been used to treat pediatric cardiomyopathy,\(^{164}\) congenital heart diseases (hypoplastic left heart syndrome),\(^{165}\) refractory angina,\(^{166}\) acute MI,\(^{99}\) and chronic ischemic cardiomyopathy.\(^{5,67,68,148,153}\) are being investigated in nonischemic dilated cardiomyopathy,\(^{152}\) and have been proposed for doxorubicin-induced cardiomyopathy.\(^{168}\) In the setting of refractory angina and in acute MI, the chemokines, cytokines, and growth factors released by the injured myocardium provide migratory cues for endogenous resident stem cells, as well as bone marrow–derived stem cells;\(^{169}\) hence, the rationale behind locally (intracoronary) and systemically (intravenous) administered stem cells, respectively, in these settings. However, both delivery methods have limitations. After intravenous infusion, there is wide distribution of MSCs throughout the body, with entrapment of these cells in the lungs, spleen, and liver,\(^{170-172}\) whereas the intracoronary technique requires a transient ischemic period through the inflation of a balloon to give the cells the chance to be distributed and not washed out. In addition, there is the potential for microvascular obstruction by the infused cells, which can result in myocardial necrosis. The intracoronary delivery technique is also limited by the inaccessibility of some myocardial distributions in patients with advanced coronary artery disease. In acute MI, intramyocardial injections have not been a preferred mode of delivery because of concerns about perfusion in the setting of acute ischemia and necrosis. However, Perin et al\(^{173}\) have shown that transendocardial delivery was more efficacious than intracoronary delivery in a canine model of acute MI.

On the other hand, in the chronic setting of heart failure caused by chronic ischemic and nonischemic heart disease, the migratory cues for stem cells are thought to be absent or minimal and a more targeted approach, namely intramyocardial injection, has been investigated. In an analysis from the POSEIDON\(^{150}\) clinical trial, transendocardial injection of MSCs reduced scar size in both injected and noninjected myocardial segments, but segmental contractility improved only in the injected scar segments and was greatest in those territories with severe baseline dysfunction. Moreover, analysis of the effects of direct surgical myocardial injection in the PROMETHEUS\(^{67}\) clinical trial found a concordant reduction in scar size and improvement in perfusion and contractility in MSC-injected myocardial segments when compared with revascularization only and non-MSC-treated segments in patients who underwent coronary artery bypass graft surgery. In light of the PROMETHEUS trial\(^{67}\) and POSEIDON substudy\(^{150}\) findings, special attention should be invested in designing the strategy of the precise placement of the intramyocardial injections to cover as much of the impaired myocardium as possible. When there is no ischemia, as in the setting of nonischemic dilated cardiomyopathy, this strategy makes even more sense, and effort should be made to inject as much area of the left ventricle as possible.

There is evidence\(^{5}\) that autologous and allogeneic sources of MSCs produce similar beneficial effects in patients with ischemic cardiomyopathy, and this is currently being investigated in patients with nonischemic cardiomyopathy as well.\(^{152}\) Given these findings, the allogeneic source provides an off-the-shelf therapeutic option that is advantageous over the autologous source, especially in the urgent setting of an acute MI. Although there are clinical trials (TIME,\(^{174}\) LateTIME\(^{175}\)) that were designed to detect the optimal window of delivery of bone marrow mononuclear stem cells after an acute MI, the effect of timing of MSC delivery on therapeutic efficacy has not been specifically investigated. However, MSCs have been used in the chronic setting of ischemic cardiomyopathy in the PROMETHEUS, POSEIDON, and TAC-HFT studies\(^{69}\) and have been shown to produce favorable outcomes.
strategies offer clinical benefits to patients. These trials based on substantial basic, preclinical, and phase I/II data will take the next step in addressing the appropriate use of cell-based therapy in the armamentarium of approaches for chronic heart disease.

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