Development of the Paracrine Hypothesis

Stem cell therapy for tissue repair and regeneration holds great therapeutic potential. The ability of stem cells to develop into various cell types, and the ease with which they can be expanded in culture, has led to a great deal of interest in their use as therapeutic agents to treat a wide range of diseases. Various embryonic and adult stem cells, isolated from a variety of different tissues including brain, heart, kidney, and bone marrow, have been assessed for their therapeutic potential (Table). Of these, adult stem cells from the bone marrow have been studied widely in clinical trials. Indeed, bone marrow-derived mesenchymal stem cells (MSCs) have been used to treat a large and diverse set of diseases, including myocardial infarction (MI), Parkinson disease, Crohn disease, cancer, among others. Currently, >100 clinical trials using MSCs are active in the United States alone (from ClinicalTrials.org), and the results of these preliminary studies have been encouraging.

It has been shown that injection of adult stem cells (Table) into the injured heart has beneficial effects. Originally, it was thought that these stem cells engrafted into the damaged tissue and differentiated into cardiomyocytes, vascular, or other cells. In vitro, MSCs treated with 5-azacytidine were shown to differentiate into cardiac-like muscle cells. Moreover, hematopoietic (hematopoietic lineage negative c-Kit positive) stem cells were reported to regenerate infarcted myocardium by differentiating into cardiomyocytes. Several other groups also demonstrated that MSCs possess the ability to differentiate into cardiomyocytes. Indeed, studies have demonstrated that engrafted MSCs in vivo can improve cardiac function and remodeling.

However, it has been shown that after injection, adult stem cells had poor survivability. Moreover, it has not been possible to reproduce the earlier studies which showed that bone marrow–derived stem cells differentiate into cardiac cells. Using a green fluorescent protein mouse, Balsam et al could not find any evidence that bone marrow–derived hematopoietic lineage negative c-Kit positive cells differentiated into cardiomyocytes when injected into infarcted myocardium. Instead, these cells adopted a typical hematopoietic fate. Moreover, using genetic tracing techniques, Murry et al were unable to identify differentiation of hematopoietic stem cells into cardiomyocytes in any of their 145 transplants into normal and injured adult mouse hearts. These findings, and others, have called into question the plasticity of bone marrow–derived stem cells and their direct role in tissue regeneration. Fusion with recipient cells within the tissue was also proposed to be a mechanism by which injected adult stem cells exerted their beneficial effects; however, again the frequency of this event was found to be relatively low.

On the basis of above studies, it is now clear that although engraftment can result in improved cardiac function, the small number of adult stem cells engrafted cannot directly generate sufficient cardiomyocytes to account for the therapeutic benefits observed. How can one explain the apparent tissue reparative and regenerative effects of these cells? Recent evidence suggests the importance of the paracrine mechanism of stem cell action. Our laboratory was among the first to report that the administration of conditioned medium from adult stem cells was sufficient to recapitulate the beneficial effects of the cells in vitro and in vivo. This observation and similar reports from other laboratories have led to the proposal that adult stem cells exert their therapeutic benefits via the release of biologically active proteins, or paracrine factors, acting on resident cells. Indeed, there is now a large body of evidence supporting the hypothesis that paracrine factors are essential for the reparative effects of adult stem cells after delivery into the injured heart. Adult stem cells secrete a wide variety of growth factors and chemokines that can promote cardiac repair. Elevated levels of proteins such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF), fibroblast growth factor (VEGF), basic fibroblast growth factor (FGF), and others, influence cell survival, inflammation, angiogenesis, repair, and regeneration in a temporal and spatial manner.
hepatocyte growth factor (HGF), and insulin-like growth factor-1 (IGF-1) are found in the heart after injection of adult stem cells.21,22

In this review, we will discuss the mechanisms through which paracrine factors released by stem cells promote cardiac repair and regeneration. We will propose the following novel concepts: (1) paracrine factors released by stem cells influence adjacent and distant cells differentially by their concentration gradients and thus creating a tissue microenvironment, (2) paracrine factors are often pleiotropic in nature and act on multiple mechanisms and different cell types, and (3) paracrine factors can influence in temporal and spatial manners, the post–myocardial repair and regenerative responses.

Effects of the Paracrine factors
It has been demonstrated that paracrine factors promote cardiac regeneration through several mechanisms including cardiomyocyte proliferation, cytoprotection, differentiation of resident stem cells, neovascularization, and by limiting inflammatory and profibrotic processes. Below is a review of these paracrine actions.

Survival/Cytoprotection
Adult stem cells in an ischemic environment promote cardiomyocyte survival via the paracrine release of cytoprotective molecules. We have shown that cell culture medium conditioned by hypoxic MSCs reduces rat cardiomyocyte apoptosis and necrosis when exposed to conditions that promote cell death.20 Overexpression of the prosurvival protein Akt1 greatly enhances the cytoprotective capabilities of MSCs. To further validate the cytoprotective potential of MSCs, we studied the effect of the conditioned media in vivo using a rat model of coronary occlusion. We showed that administration of culture media from Akt MSC reduced infarct size and restored cardiac function in the rodent model of MI.23 Our findings have been replicated by others in a large animal model.24 Taken together, these studies validate that MSCs promote cardiomyocyte survival via paracrine factors and that Akt is crucial for this process.23 We are not alone in reporting these paracrine effects of bone marrow–derived adult stem cells.25–27 Takahashi et al25 showed that rat bone marrow mononuclear cells release proteins such as VEGF, platelet-derived growth factor, IGF-1, and interleukin (IL)-1b, some of which were significantly enhanced by hypoxia. The conditioned media of bone marrow mononuclear cells strongly inhibited cardiomyocyte apoptosis and preserved their contractile capacity. Moreover, Uemura et al26 demonstrated that bone marrow stromal cells, which showed upregulation of Akt after brief anoxia, prevented cardiomyocyte apoptosis in a coculture model. This study went on to show that bone marrow stromal cells markedly inhibited left ventricular remodeling after MI.

In the course of our research, we have identified many novel paracrine factors. Secreted frizzled related protein 2 (Sfrp2) showed the highest fold difference in expression between Akt1- and unmodified MSCs. When delivered to hypoxic cardiomyocytes, Sfrp2 inhibited caspase-3 activity and prevented apoptosis.28 On the basis that Sfrp proteins are Wnt antagonists, we analyzed Wnt expression in hypoxic cardiomyocytes, identifying Wnt3a as a potential candidate. Cardiomyocyte apoptosis in response to hypoxia-reoxygenation was significantly augmented by Wnt3a acting via β-catenin. Sfrp2 was found to bind directly to Wnt3a and significantly attenuated Wnt3a-induced caspase activity in a dose-dependent fashion.29 We also identified C3orf58 as a novel paracrine factor secreted from MSCs. By virtue of how the gene was regulated in MSCs, we named C3orf58 as hypoxic-induced Akt regulated stem cell factor (HASF). HASF, a relatively novel 49-kDa protein with no recognizable domains apart from a signal peptide, has been previously associated with human familial autism.30 A single dose of purified HASF protein injected into the heart immediately after MI prevented the loss of cardiac function associated with this type of injury. Analysis of the heart tissue showed that HASF reduced the number of TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling)-positive nuclei and inhibiting caspase activation and mitochondrial pore opening. The cytoprotective effects of HASF were lost in mice lacking protein kinase C-ε (PKCe).31

Immunomodulation/Inflammation
Adult stem cells when injected into myocardium dampen the inflammatory state associated with injury by downregulating expression of proinflammatory cytokines such as tumor necrosis factor-α, IL-1β, IL-6, and monocyte chemoattractant protein-1.32 These effects have a paracrine component;
conditioned media prepared from cultured MSCs was found to inhibit damage to isolated adult rat cardiomyocytes in response to monocyte chemoattractant protein-1. In contrast to MSCs, endothelial progenitor cells (EPCs) actively secrete proinflammatory cytokines such as monocyte chemoattractant protein-1. Moreover, these cells can be stimulated to produce the procoagulant protein tissue factor by lipopolysaccharide, suggesting that under certain conditions EPCs could promote thrombosis.

MSCs possess immunomodulatory properties that affect a broad range of cells involved in the immune response. MSCs inhibit T-cell proliferation and cytotoxicity, rendering the T cells unresponsive. Paracrine factors released by MSCs, as well as direct interaction between the 2 cell types, are thought to be important. Paracrine factors released by MSCs, such as transforming growth factor-β, HGF, nitric oxide, in-doleamine 2,3-dioxygenase, and prostaglandin-E2, inhibit T-cell function. Under certain conditions, MSCs release paracrine factors that mediate cardiomyocyte proliferation. These factors that mediate cardiomyocyte proliferation. These mechanisms are either ab-

Cardiomyocyte Proliferation

After cardiac injury, cardiomyocytes are lost in significant numbers. Lower vertebrates such as the zebrafish possess mechanisms to replace these cardiomyocytes. Specifically, the remaining cardiomyocytes de-differentiate, re-enter the cell-cycle, and proliferate. These mechanisms are either absent or inactive in the adult mammalian heart. Indeed, cardiomyocyte proliferation is known to be relatively infrequent. Finding strategies that increase cardiomyocyte proliferation is an important endeavor.

Recent research has uncovered many MSC paracrine factors that mediate cardiomyocyte proliferation. These paracrine mediators of cardiomyocyte proliferation tend to be either growth factors or extracellular matrix (ECM) proteins.

FGF2 promotes cardiomyocyte proliferation in vitro via PKCe, a process also potentially involving connexin-43 phosphorylation. Similarly, again in vitro, platelet-derived growth factor increases the proliferation of cardiomyocytes via Akt activation, inactivation of GSK-3β (glycogen synthase kinase-3 beta), and the subsequent downregulation of the cyclin-dependent kinase inhibitor p27. Neuregulin-1, a member of the epidermal growth factor family, stimulates DNA synthesis in both neonatal and adult cardiomyocytes through its specific receptor ErbB4. Mononucleated cardiomyocytes were found to be capable of karyokinesis, whereas binucleated cells were not. Pertinently, injection of neuregulin-1 into adult mice promoted regeneration after MI. This molecule is now undergoing small-scale clinical trials as a therapeutic agent to treat congestive heart failure. Although tempting to speculate dedifferentiation as the underlying process by which neuregulin-1 promotes cardiomyocyte proliferation, Bersell et al. could not find any evidence of sarcomere disassembly, for example. Despite this finding, it is possible that proproliferative paracrine factors promote cardiomyocyte proliferation via dedifferentiation, and this process underpins the robust regeneration found in the neonatal mouse heart. It is particularly notable that growth factors such as FGF, platelet-derived growth factor, and neuregulin-1 mediate cardiomyocyte proliferation through phosphoinositide 3-kinase (PI3K). Under hypoxic conditions, adipose-derived stromal cells secrete the proinflammatory cytokine IL-6, and curiously this protein has also been linked with augmentation of cardiomyocyte proliferation via Stat3 and ERK1/2 (extracellular signal regulated kinase).

Fibronectin and collagen, acting via their receptor integrin-β1, are proproliferative. Periostin is an ECM protein secreted by adipose-derived MSCs and has been reported to promote cardiomyocyte proliferation via integrin (αV, β1, β3, and β5)–mediated activation of PI3K. In an MI model, periostin induced cardiomyocytes to re-enter the cell-cycle, and this was associated with improvements in cardiac function. Interestingly, the activation of PI3K was sufficient to recapitulate the effects of periostin. Other researchers found that periostin had no effect on cardiomyocyte proliferation. Periostin also seems to be associated with increased myocardial fibrosis, although it should be noted that delivery of the protein into the pericardial space improved cardiac function after MI.

Our novel paracrine factor, HASF, was found to increase DNA synthesis in cultured rat neonatal ventricular cardiomyocytes by 60%, a level of stimulation comparable in intensity with FGF. Importantly, evidence of cytokinesis was observed in a murine model. The proliferative effects of HASF were found to be mediated by PI3K and the cell-cycle regulator cyclin-dependent kinase 7. We are currently investigating the molecular pathways by which HASF promotes cardiomyocyte proliferation in more detail. Using yeast 2-hybrid and coimmunoprecipitation assays, we identified a direct interaction between the IGF-1 receptor and the HASF. Subsequent studies with pharmacological inhibitors and siRNA-mediated
knockdown showed that the beneficial effects of HASF are mediated by the IGF-1 receptor. HASF is a much larger molecule than IGF-1, and indeed many of the commonly investigated growth factors, and may activate different pathways. Indeed, whereas IGF-1 promotes cardiac hypertrophy, HASF does not. This finding itself highlights the potential clinical benefits of the HASF protein.

Cardiac Remodeling

Cardiac injury with significant cell loss and functional impairment leads to cardiac remodeling, which is mediated by a significant change in the ECM including fibrosis, cardiomyocyte hypertrophy, and changes in ventricular dimension and function. Paracrine factors released by adult stem cells can alter the ECM and prevent post-infarction remodeling. In many animal models, MSCs decrease fibrosis in many tissues including the heart, lung, liver, and kidney. Stem cells, such as MSCs, express many proteins that regulate the ECM such as matrix metalloproteinases, serine proteases, and serine protease inhibitors, suggesting that transplanted MSCs can inhibit fibrosis through a paracrine action. MSC transplantation has been shown to inhibit post-MI increases in the expression of collagen-I and collagen-III as well as the tissue inhibitor of metalloproteinase-1. Conditioned media prepared from MSCs strongly inhibits cardiac fibroblast proliferation and inhibits the production of collagen-I and collagen-III from these cells. Growth differentiation factor-11, a circulating protein that was revealed as a rejuvenating factor for the aging heart by parabiosis experiments, actively prevents fibrosis after myocardial injury.

We have demonstrated that Sfrp2 prevents fibrosis. Injected into infarcted rat myocardium 2 days after injury Sfrp2 inhibited MI-induced collagen type-I deposition and left ventricular fibrosis. Activity of bone morphogenetic protein-1 (Bmp1), a key enzyme involved in the regulation of collagen biosynthesis and maturation, was repressed by a high concentration of Sfrp2. Despite our findings, there are many reports that have ascribed a profibrotic role for Sfrp2. Kobayashi et al. found that fibrosis was reduced in Sfrp2-null mice after MI. Similarly, Mastri et al. reduced fibrosis and improved cardiac function after the intraperitoneal delivery of a Sfrp2-neutralizing antibody into cardiomyopathic hamsters. Why is there a discrepancy between the studies? Sfrp proteins have biphasic effects depending on their concentration; with Wnt and BMP1 signaling being augmented or inhibited at low or high concentrations, respectively. Indeed, Mastri et al. were unable to identify Sfrp2 in the hamster heart, whereas we used a large dose of the protein. As mentioned in a recent commentary, a more detailed analysis of the concentration dependence of the effects of Sfrp2 is needed. The biphasic effects of Sfrp proteins highlight the importance of a spatial component to the paracrine hypothesis. Considering that MSCs secrete Sfrp2, cells in close proximity to the paracrine source will be exposed to high concentrations of Sfrp2. Thus, in the microenvironment formed by the injected adult stem cells, one would expect that Sfrp2 to behave in antifibrotic fashion.

Metabolism and Contractility

Injury alters cardiac metabolism with a switch from the typical fatty acid oxidation to glucose uptake and a shift to lactate production. Moreover, in the infarct border zone, the phosphocreatine:ATP ratio increases. These changes influence infarct size and remodeling.

Injection of MSCs into the hearts of pigs after MI partially prevented the metabolic changes in the heart associated with injury. Because of the low engraftment of the injected cells, it was proposed that the MSCs were thwarting metabolic changes via paracrine factors. This has also been observed in a rat model of MI. Here, Akt overexpression significantly increased the ability of MSCs to inhibit changes in metabolism, sparing phosphocreatine stores and limiting glucose uptake.

There is evidence that the administration of adult stem cells promotes cardiac contractility. Indeed, we witnessed a large increase in spontaneous contractility of adult rat ventricular cardiomyocytes exposed to conditioned media from hypoxic Akt1-MSCs. The strong and synchronized contraction suggested that the conditioned media contained inotropic factors that had a positive effect on cardiomyocyte contractility. Similarly, Takahashi et al. found that conditioned media from bone marrow mononuclear cells maintained fractional shortening and maximal rate of re-lengthening of adult rat ventricular cardiomyocytes in culture. Conditioned media was more effective in preserving contractility if the bone marrow mononuclear cells were exposed to hypoxic conditions. Both of these studies suggest that the release of inotropic paracrine factors is increased by hypoxia. The identity of these inotropic paracrine factors is currently unknown; however, IGF-1, a growth factor released by MSCs, can promote cardiomyocyte contractility in vitro.

Neovascularization

Another important effect of adult stem cells in the ischemic myocardium is neovascularization. For example, injection of bone marrow mononuclear cells into ischemic myocardium resulted in increased regional blood flow and capillary density. Moreover, the administration of MSCs after permanent occlusion increases capillary density. Only a small number of these stem cells engraft and differentiate into vascular structures.

The molecular pathways that control angiogenesis are well characterized and involve proteins such as VEGF, basic FGF, HGF, and angiopoietin, among others. These molecules are also secreted by bone marrow–derived stem cells, suggesting that exogenously delivered adult stem cells promote vessel formation via the paracrine release of known proangiogenic factors. Support of this paradigm has come from many studies. Tse et al. compared many different types of bone marrow–derived cells for their ability to improve cardiac function in a swine model of chronic ischemia. Bone marrow mononuclear cells were the most effective, and the authors ascribed the increased capillary density arising from the injections of these cells to the paracrine release of VEGF and angiopoietin-2. Similarly, conditioned media from bone marrow mononuclear cells increases vessel density in a rat model of acute MI. The Epstein laboratory found that the injection of MSCs into the adductor muscle after distal
femoral artery injection improved distal limb perfusion and increased the number of mid-thigh conductance vessels. The injected MSCs were not observed to incorporate into collateral vessels, indicating that the effects they observed were paracrine in nature.\textsuperscript{95} Using a murine hind-limb ischemia model, they also observed that conditioned media from MSCs enhanced collateral flow recovery and remodeling, improving limb function.\textsuperscript{96} Conditioned media from these MSCs enhanced endothelial and smooth muscle cell proliferation in vitro. VEGF is an important proangiogenic paracrine factor as ablation of this gene significantly inhibits the ability of MSCs to promote functional recovery in the injured heart.\textsuperscript{100} However, antibodies targeting VEGF and FGF only partially attenuated the effect of the conditioned media,\textsuperscript{98} indicating that MSCs release other proangiogenic proteins besides these 2 growth factors.

EPCs also promote angiogenesis via paracrine mechanisms. Conditioned media derived from EPCs promotes angiogenesis in ischemic myocardium.\textsuperscript{101} VEGF and stromal-derived factor-1 are among the responsible bioactive molecules. Both proteins are secreted by EPCs and promote endothelial cell migration and capillary formation via differentiation independent mechanisms.\textsuperscript{102}

**Resident Stem Cell Activation**

The heart contains many resident stem cells, which are defined by several markers including c-Kit and Sca-1. The most heavily researched resident stem cell in the heart is the c-Kit cardiac progenitor cell (CPC). These CPCs are thought to be capable of promoting regeneration via mobilization into injured tissue and differentiation into mature cardiac cells. Intracoronary infusion of autologous c-Kit\textsuperscript{+} CPCs improves ventricular systolic function and reduces infarct size in patients with heart failure after MI.\textsuperscript{103,104} These c-Kit\textsuperscript{+} cells are also beneficial in rodent models, improving cardiac function when injected into infarcted myocardium. However, in all of these studies, it was apparent that donor c-Kit\textsuperscript{+} cell differentiation into mature cardiac cells, such as cardiomyocytes, was too low to account for the functional benefits. The authors concluded that paracrine effects must be responsible for the regenerative effects of the injected c-Kit\textsuperscript{+} cardiac stem cells.\textsuperscript{105,106} The nature of these paracrine factors remains to be identified.

Recent data suggest that paracrine factors released by adult stem cells significantly augment the ability of resident c-Kit\textsuperscript{+} cardiac stem cells to differentiate into cardiomyocytes. Conditioned medium derived from cultured MSCs promotes CPC proliferation and differentiation. The growth factor IGF-1, a paracrine factor released by MSCs, promotes resident stem mobilization\textsuperscript{107} and commitment to the cardiac lineage.\textsuperscript{108,109}

In the course of our research, we identified Abi3bp as a putative paracrine factor released by MSCs. There is little known about Abi3bp except for roles in neural cell differentiation and many antimutagenic properties. We found that Abi3bp formed extensive ECM deposits when secreted by MSCs.\textsuperscript{110} Abi3bp had dramatic effects on MSC differentiation. When we assessed the differentiation of MSCs prepared from Abi3bp knockout mice, we found that osteogenesis was completely ablated with chondrogenesis and adipogenesis severely impaired. In addition, Abi3bp inhibited MSC proliferation.\textsuperscript{110} Considering the close relationship between MSCs and CPCs, we hypothesized that Abi3bp would have similar effects on CPC proliferation and differentiation. Indeed, Abi3bp promoted c-Kit\textsuperscript{+} CPC differentiation, whereas proliferation was inhibited, both in vitro and in vivo.\textsuperscript{111} Integrin-β1 was found to be crucial for the effect of Abi3bp on c-Kit\textsuperscript{+} CPCs. Genetic ablation of Abi3bp was associated with adverse recovery after MI.\textsuperscript{111} This is likely because of the effects on CPC differentiation as cardiomyocyte proliferation was unaffected by the loss of Abi3bp expression.\textsuperscript{111}

**Extracellular Vesicles and Exosomes**

Recent evidence suggests that the paracrine functions of MSCs, and other cell types, are potentially mediated by extracellular vesicles (EVs). There are many EV subtypes, such as exosomes and microvesicles. Exosomes, the most numerous subtype, are released on fusion of a multivesicular body with the plasma membrane, whereas microvesicles are released directly from the cell membrane.\textsuperscript{112} EVs were originally thought to be a mechanism by which cells dispose of waste materials. Many lines of research now point toward EVs as important mediators of cell–cell communication, immunomodulation, proliferation, cell-senescence, and differentiation by transferring various bioactive cargoes such as proteins, lipids, miRNAs, and mRNAs, from one cell to another.\textsuperscript{112-114}

Exosomes derived from stem cells such as MSCs have been shown to protect against injury and promote regeneration in a number of models. In vitro, cardiomyocyte protection from cell death by MSCs is partially mediated by the transfer of miRNA-221 contained within EVs, reducing caspase activity in the target cells.\textsuperscript{115} Similarly, exosomes derived from bone marrow CD34\textsuperscript{+} cells and CPCs promote angiogenesis in cultured endothelial cells.\textsuperscript{116,117} In vivo, fractionation studies indicated that only the fraction of MSC conditioned media containing products of >1000 kDa (100–220 nm), the typical size of exosomes, provided protection in a mouse model of myocardial ischemia and reperfusion injury.\textsuperscript{118} The same group later purified exosomes from MSCs and found that they reduced infarct size post myocardial injury.\textsuperscript{119} The protective effect of exosomes is not limited to the heart; indeed, EVs derived from MSCs protect the kidney from ischemia-reperfusion injury.\textsuperscript{120-122}

Considering that EVs can deliver multiple bioactive molecules at the same time, they hold much promise as therapeutic agents to deliver paracrine factors in vivo. To this end, many researchers are actively investigating novel approaches to EV delivery such as incorporating tags to aid in their isolation, overexpression of key cargoes, and designing synthetic EV structures to aid scalability for clinical use.\textsuperscript{123-125}

**Emerging Concepts in Paracrine Mechanisms: Temporal and Spatial Pleiotropic Actions and the Creation of Microenvironment**

Effects of paracrine factors after myocardial injury are dynamic, multifaceted, and multiphasic. The healing process after a myocardial injury is a complex sequence of time-dependent events involving cell death (apoptosis and necrosis), inflammation, fibroblast proliferation, collagen deposition, neovascularization, cardiac remodeling, and, in a limited manner, cardiac regeneration. In this section, we propose the
following new concepts that (1) paracrine factors released by stem cells influence adjacent and distant cells differentially by their concentration gradients and thus creating a tissue microenvironment, (2) paracrine factors have pleiotropic actions on different cells and multiple mechanisms, and (3) paracrine factors can exert temporal and spatial effects on cardiac repair and regenerative events.

**Release of Paracrine Factors Produces Concentration Gradients and Creates Unique Tissue Microenvironment**

Once the stem cells are established in the injured myocardium via endogenous mobilization or exogenous administration, they release paracrine factors that will form concentration gradients. The concentration gradient of the factors will influence adjacent and distant cells differentially and thus creating a unique microenvironment within the cardiac tissue. These concentration gradients have the potential to impact cardiac repair and regeneration in many ways previously unconsidered. The concentration of the paracrine factor, or in other words, the spatial proximity to the stem cell, may directly influence the response of resident cells to the secreted protein (Figure 1). Although currently undefined in the context of paracrine factors acting in the heart, there are other settings where concentration gradients of secreted proteins have been shown to dictate cell behavior. For example, spatial proximity to an IL-2 source affects the magnitude and direction of the T-cell response.126 Moreover, it is well established that paracrine concentration gradients are critical for the normal development of the embryo.127,128 Proteins such as epidermal growth factor, FGF, Wingless/Wnt, and BMP generate concentration gradients that provide spatial information to generate distinct cell types in a specific 3-dimensional pattern.128 In the developing embryo, these paracrine factors have a specific range that is dependent on their diffusion capacity and interaction with proteoglycans.128 Our own data and that of others on the role of Sfrp2 on cardiac fibrosis also suggest that paracrine factor concentration gradients can have a dramatic effect on cell behavior. We have found that administering a high concentration of Sfrp2 prevents fibrosis after MI.83 In contrast, genetic deletion of Sfrp244 and Sfrp2-neutralizing antibodies85 reduces fibrosis, suggesting that Sfrp2 is profibrotic. This can be explained by the biophysical properties of Sfrp proteins, which augment or inhibit Wnt/BMP185 signaling at low or high concentrations, respectively.

Concentration gradients provide a conduit to attract cells to a specific site. In the context of MI, proteins released from dying cells release chemotactants, which mediate the infiltration of proinflammatory immune cells into the injured tissue.129 Modifying MSCs to express proteins such as CCR1130 allows MSCs to follow the same chemotactic gradients as proinflammatory immune cells. This is particularly pertinent as MSCs release a myriad of paracrine factors that inhibit the function of proinflammatory immune cells. By analogy, it is tempting to speculate that concentration gradients of paracrine factors released by injected stem cells act as a chemoattractant for therapeutically beneficial cells. One possibility would be resident progenitor cells as they migrate in vitro in response to IGF-1,131 a paracrine factor released by MSCs.

Adapting concepts from immunology and neuron function,122,123 we also suggest that stem cell–derived paracrine factor concentration gradients affect cell behavior via temporal and spatial summation. Signaling is typically a transient event, for example, when bound to ligand receptor tyrosine kinases are internalized and degraded.124 In contrast, the regenerative processes mediated by stem cell paracrine factors are prolonged. How transient signaling events initiated by paracrine factors are accumulated over time and integrated into a prolonged biological response is thus a pivotal question. This question has been addressed in noncardiac settings. For example, temporal and spatial summation, where signaling events are built up incrementally, have been proposed to explain the dichotomy between T-cell commitment to cytokine production and proliferation, which requires sustained signaling and the rapid loss of a signal from an activated T-cell receptor.122 Considering that each event that follows MI is prolonged, and that for each of these events several stem cell paracrine factors will have a direct influence, temporal and spatial summation have been underappreciated in the paracrine model.

Once the adult stem cells are in their microenvironment, the secretory profile of the cell is also likely to change with time. Indeed, autocrine/paracrine signals from embryonic stem cells, which are necessary for their self-renewal and differentiation, are temporal in nature.135

**Paracrine Factors Are Pleiotropic**

What has become apparent to us during the course of our research is that paracrine factors released by stem cells are pleiotropic. This pleiotropic ability of stem cell–derived paracrine factors allows them to influence cardiac repair and regeneration at multiple points after myocardial injury (Figure 2).

As mentioned above, we have identified Sfrp2 and HASF as 2 cytoprotective paracrine factors (Figure 2). These 2 paracrine factors use 2 different mechanisms. Sfrp2 promotes the protection of cardiomyocytes by binding to the proapoptotic protein Wnt3a.29 In contrast, HASF prevents cardiomyocyte cell death through PKCε.31 As noted in a recent editorial, HASF has many novel features as a cytoprotective factor.136 Overexpression of PKCε promotes cardiac hypertrophy,137 whereas that of HASF does not.73 Moreover, pharmacological inhibition of PKCε did not affect HASF-mediated activation of Akt, and it is a curious feature of HASF that high levels of Akt activity are not important for the cytoprotective effects of this paracrine factor.136 Certainly protection of the heart without promoting ventricular hypertrophy or dysfunction is a unique and important feature of HASF.136 As described earlier, our recent research has identified that the IGF-1 receptor mediates the beneficial effects of HASF.74 This finding, which implies that HASF is a novel member of the IGF family of growth factors, suggests that HASF may have additional roles beyond those currently discovered, for example, effects on metabolism. Both HASF and Sfrp2 have effects on the injured heart beyond simply protecting against cell death. HASF promotes cardiomyocyte proliferation both in vitro and in vivo. Importantly, evidence of cytokinesis was observed in a murine model.73 HASF seems to use common growth factor receptor tyrosine kinase pathways although there are significant differences. Whereas IGF-1 promotes cardiac hypertrophy,75
Figure 1. Paracrine factors affect different cell types and create a microenvironment that is influenced by concentration gradients, with temporal and spatial summation of cellular responses. Reprinted from Hodgkinson et al\textsuperscript{149} with permission of the publisher. Copyright ©2015, Elsevier.

Figure 2. Paracrine factors are pleiotropic. For illustration, we show the cellular effects of 2 selective paracrine factors on the cardiomyocyte. Left, Hypoxic-induced Akt regulated stem cell factor (HASF) and secreted frizzled related protein 2 (Sfrp2) inhibit cardiomyocyte apoptosis through divergent pathways. HASF, after binding to a growth factor receptor, inhibits cytochrome release from mitochondria via protein kinase C-\(\varepsilon\) (PKC\(\varepsilon\)). In contrast, Sfrp2 inhibits Wnt activation of frizzled receptors. This induces \(\beta\)-catenin degradation via the anaphase promoting complex (APC) complex. Right, Abi3bp and Sfrp2 promote cardiac progenitor cell differentiation and inhibiting proliferation. Abi3bp activates integrin-\(\beta\)1. Src and extracellular signal regulated kinase (ERK) activation work together to inhibit proliferation. PKC\(\zeta\) and Akt activation switch on cardiac genes. Sfrp2 sequesters Wnt, preventing the activation of frizzled receptors. This promotes c-Jun N-terminal kinase (JNK) activation and cardiac gene expression. Inhibition of \(\beta\)-catenin blocks the proliferation pathway in these cells. ECM indicates extracellular matrix; FRZ, frizzled; and TF, transcription factor.
HASF does not. As stated earlier, in addition to promoting cytoprotection, we have found that Sfrp2 prevents fibrosis by inhibiting Bmp1. Moreover, we have recently shown that Sfrp2 inhibits Sca-1 CPC proliferation and primes the cells for differentiation. This switch from proliferation to differentiation occurred via Sfrp2 binding to Wnt6, which inhibited canonical Wnt signaling and activated noncanonical Wnt/planar cell polarity signaling through JNK.

We originally identified the protein Abi3bp as an autocrine regulator of MSC biology. This molecule is also pleiotropic. Abi3bp was found to strongly promote resident c-Kit+ cardiac progenitor differentiation both in vitro and in vivo. Abi3bp belongs to the proteoglycan family of ECM proteins. These proteoglycans modify the fibrillar structure of the ECM, which has significant effects on cell adhesion, migration, and proliferation. Moreover, proteoglycans regulate the activities of secreted proteins. For example, the heparan sulfate chains of proteoglycans bind to FGFs, enabling the growth factor to cross-link and activate their cell-surface receptors. It is possible, therefore, that Abi3bp, by virtue of being a proteoglycan, not only modifies the ECM of the scar in a fashion that promotes repair and regeneration but also regulates the activities of other paracrine factors released by MSCs.

Many other paracrine factors also possess pleiotropic properties. These include IGF-1, FGF, platelet-derived growth factor, and VEGF (Figure 3). Given the fact that response to cardiac injury involves complex, dynamic, and time-dependent events, paracrine factors can influence myocardial pathobiology, a multifaceted temporal manner on different cell types and via different mechanisms as discussed below.

**Paracrine Factors Promote Repair and Regeneration at Multiple Time Points**

As we have described above, paracrine factors released by MSCs affect all the events that occur after MI (Figure 4). Using our own research as an example, HASF and Sfrp2 prevent cardiomyocyte cell death, the first event that occurs after an MI. Both Sfrp2 and Abi3bp promote resident progenitor cells to differentiate, an event that begins ≈1 week after MI. Finally, HASF promotes the remaining cardiomyocytes to proliferate. Considering the pleiotropic actions of paracrine factors (Table), they can influence the post–myocardial injury sequence of responses such as inflammation, fibrosis, neovascularization, remodeling, and cardiomyocyte proliferation. This hypothesis is contingent on the existence of stem cells or other secretory cells albeit at low levels throughout the cardiac repair and regenerative process. Indeed, this is supported by the evidence of stem cell presence in low levels weeks after MI.

One question one can ask is, does the secretory profile of MSCs change in a positive fashion in response to the temporal sequence of events that occur after a MI?

The inflammatory response is robustly active immediately after MI. Increased expression of the cytokine IL-6 is one of the hallmarks of this inflammatory response. Intriguingly, IL-6 modifies MSC paracrine function by stimulating these cells to secrete VEGF, which as discussed earlier promotes new blood vessel formation in the myocardium. Other proinflammatory cytokines released by the dying myocardium have similar effects on IL-6. Tumor necrosis factor-α, IL-1, and IFN-γ have all been shown to stimulate MSC secretion of many growth factors such as EGF, VEGF, HGF, IGF-1, and angiopoietin. These growth factors go on to regenerate the myocardium through the formation of new capillaries, cardiomyocyte proliferation, and resident progenitor cell differentiation. Taking these findings 1 step further, preactivating MSCs with proinflammatory cytokines before their delivery into the heart may have therapeutic applications by stimulating MSC paracrine effects as has recently been shown in a radiation-induced intestinal injury model.

In a similar vein, the extracellular remodeling that occurs post MI has been shown to modulate the secretory profile of MSCs. The remodeled matrix was found to promote the secretion of many proangiogenic, antifibrotic, and immunomodulatory paracrine factors from MSCs, most notably HGF and stromal-derived factor 1.
Studies in brain injury models suggest that MSCs are not passive players but actively sense their environment to affect repair. Administration of MSCs at different time points after brain ischemia injury had markedly different effects, stimulating cell proliferation when injected 3 days after injury and stimulating axonal remodeling when injected 10 days after ischemia. Pathway-focused polymerase chain reaction array analysis revealed that many genes encoding secreted factors were differentially regulated in the MSCs injected at the 2 time points. This led the authors to conclude that the MSCs were actively sensing the microenvironment and changing their secretory profile according to the needs of the milieu, adapting to the specific signals provided by the injured brain.146,147

Conclusions and Future Directions in Paracrine Factor Research

The paracrine hypothesis is a natural extension of the traditional concept of the stem cell niche to include the role of factors released by stem cells on their microenvironment influencing the tissue’s response to injury. As mentioned above, paracrine factors create a specific microenvironment, affecting the biology of cells within that niche. Understanding the temporal and spatial components underlying the regenerative properties of paracrine factors in the injured heart will clarify the complex process of repair and regeneration.

It is apparent from a large number of studies that transplanted adult stem cells fail to integrate and differentiate into mature cardiac cells. Moreover, there is an extensive loss of the cells after transplantation. So why not simply inject more adult stem cells? This is neither practical nor desirable. Some of these adult stem cells are particularly rare, for example, c-Kit+ CPCs. Although these cells can in certain circumstances be amplified ex vivo, the amount of time taken to get a sufficient quantity is considerable, too long to be clinically useful. It should also be borne in mind that cardiac injury tends to occur in mid to late life. Increasing age significantly impairs the ability of stem cells to renew and differentiate,97,148 potentially limiting an individualized treatment strategy using the patient’s own cells. Potentially allogenic cells from young or, notwithstanding ethical concerns, fetal donors could be used. However, immunosuppression would then be necessary to prevent cell rejection.

We, and others, have shown that the release of paracrine factors mediate the majority of the effects of transplanted adult stem cells. Using that knowledge, many researchers, including ourselves, have genetically engineered adult stem cells to augment the paracrine effect or increase cell survival and engraftment.1 For example, our modifications of MSCs include overexpression of Akt1,20 to augment the paracrine effect of these cells and promoting their survival.

It is important to note that in the field of cell therapy, consistent and reproducible results among laboratories are a major issue. There are initiatives being pursued to standardize procedures and nomenclature. However, especially for the rare adult stem cells, growth conditions and the number of passages will give rise to a different population with each isolate. This is less than ideal for a standardized therapeutic modality. Substituting the paracrine factors for the adult stem cells is thus a sensible approach. The treatment strategy can be defined and reproducible; furthermore, any difficult issues involve the use of cells are avoided.

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