HHS Public Access

Author manuscript

Circ Res. Author manuscript; available in PMC 2017 January 08.

Published in final edited form as:

Circ Res. 2016 January 8; 118(1): 95–107. doi:10.1161/CIRCRESAHA.115.305373.

Emerging Concepts in Paracrine Mechanisms in Regenerative Cardiovascular Medicine and Biology

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Abstract

In the past decade, substantial evidence supports the paradigm that stem cells exert their reparative and regenerative effects, in large part, through the release of biologically active molecules acting in a paracrine fashion on resident cells. The data suggest the existence of a tissue microenvironment where stem cell factors influence cell survival, inflammation, angiogenesis, repair and regeneration in a temporal and spatial manner.

Keywords

Stem cell; myocardial infarction; heart disease; regeneration and therapy

Development of the Paracrine Hypothesis

Stem cell therapy for tissue repair and regeneration holds great therapeutic potential[1]. 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 1)[12]. 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 very large and diverse set of diseases; including myocardial infarction, Parkinson's disease, Crohn's disease, cancer, amongst others[3–7]. Currently over 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 1) into the injured heart has beneficial effects. Originally, it was believed that these stem cells engrafted into the damaged tissue and differentiated into cardiomyocytes, vascular or other cells[8, 9]. In vitro,

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DISCLOSURES

None of the authors have any real or apparent conflict(s) of interest to disclose.

MSCs treated with 5-azacytidine were shown to differentiate into cardiac-like muscle cells[10]. Moreover, hematopoietic (hematopoietic lineage negative c-Kit positive) stem cells were reported to regenerate infarcted myocardium by differentiating into cardiomyocytes[9]. A number of 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[11–13].

However, it has been shown that following injection adult stem cells suffered from poor survivability[14]. Moreover, it has not been possible to reproduce the earlier studies which showed that bone marrow derived stem cells differentiate into cardiac cells. Utilizing a GFP 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[15]. 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[16]. 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[17] however again the frequency of this event was found to be relatively low[18, 19].

Based on the above studies, it is now clear that although engraftment can result in improved cardiac function[11–13], 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[20]. 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 following 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 (bFGF), hepatocyte growth factor (HGF), and insulin-like growth factor 1 (IGF1) are found in the heart following 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 a number of mechanisms including cardiomyocyte proliferation, cytoprotection, differentiation of resident stem cells, neovascularization, and by limiting inflammatory and pro-fibrotic 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].Over-expression of the pro-survival 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 al showed that rat bone marrow mononuclear cells release proteins such as VEGF, PDGF, IGF-1 and 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[25]. Moreover, Uemura et al demonstrated that bone marrow stromal cells, which showed up-regulation of Akt following brief anoxia, prevented cardiomyocyte apoptosis in a co-culture model. This study went on to show that bone marrow stromal cells markedly inhibited LV remodeling following myocardial infarction[26].

In the course of our research we have identified a number of novel paracrine factors. **Secreted frizzled related protein 2 (Sfrp2)** showed the highest fold difference in expression between Akt1- and un-modified 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 HASF for **Hypoxic induced Akt regulated Stem cell Factor**. HASF, a relatively novel ~49kDa 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 following myocardial infarction 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 positive nuclei as well as

inhibiting caspase activation and mitochondrial pore opening. The cytoprotective effects of HASF were lost in mice lacking PKCε[31].

Immunomodulation/Inflammation

Adult stem cells when injected into myocardium dampen the inflammatory state associated with injury by down-regulating expression of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and MCP-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 MCP-1[32]. In contrast to MSCs, EPCs actively secrete pro-inflammatory cytokines such as MCP-1. Moreover, these cells can be stimulated to produce the procoagulant protein tissue factor by lipopolysaccharide suggesting under certain conditions EPCs could promote thrombosis[33, 34].

MSCs possess immune-modulatory properties that affect a broad range of cells involved in the immune response. MSCs inhibit T-cell proliferation and cytotoxity; rendering the T-cells unresponsive[35]. Paracrine factors released by MSCs, as well as direct interaction between the two cell types[36], are believed to be important. Paracrine factors released by MSCs, such as TGFβ, HGF, nitric oxide, indoleamine 2,3-dioxygenase, and prostaglandin-E2 (PGE2), inhibit T-cell function[37]. Under certain conditions MSCs release T-cell activators such as IL-6, IL-1 and RANTES[37]. IL-6 may also be important for the effect of MSCs upon B-cells and further underscores the spatial aspect of the paracrine hypothesis which we will discuss below. Depending upon the strength of the stimulus MSCs either promote or inhibit IgG production by B-cells[38]. MSCs also prevent dendritic cell maturation and function via the release of IL-6 and PGE2[39, 40]. The latter molecule is also required for the inhibitory effect of MSCs on Natural Killer (NK) cell proliferation, cytokine production, and cytotoxicity[41]. Finally, MSCs also secrete interleukin 1 receptor antagonist which inhibits the release of the pro-inflammatory cytokine TNFα from activated macrophages[42].

Macrophages can promote angiogenesis and tissue healing through a number of secreted molecules[43–45]. Following transplantation of MSCs into infarcted tissue large numbers of macrophages collect at the sites of injection despite an overall reduction in the population of these cells in the heart[46]. Various reports have indicated that MSCs switch macrophages from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype both in vitro and in vivo[47–49], potentially through secreted factors such as IGF-1[50] and IL-10[51]. These studies suggest that MSCs contribute to the overall recovery of cardiac function following myocardial infarction in part via their effects on macrophages. Indeed the regenerative and reparative effects on transplanted MSCs are reduced following transient depletion of macrophages[52].

Cardiomyocyte proliferation

Following cardiac injury cardiomyocytes are lost in significant numbers. Lower vertebrates such as the zebrafish possess mechanisms to replace these cardiomyocytes[53]. 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[54–57]. Finding strategies which increase cardiomyocyte proliferation is an important endeavor.

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

Fibroblast growth factor 2 (FGF2) promotes cardiomyocyte proliferation in vitro via PKCε, a process also potentially involving connexin-43 phosphorylation[58]. Similarly, again in vitro, platelet-derived growth factor (PDGF) increases the proliferation of cardiomyocytes via Akt activation, inactivation of GSK-3beta and the subsequent down-regulation of the cyclin-dependent kinase inhibitor p27[59]. Neuregulin-1 (NRG1), a member of the EGF family, stimulates DNA synthesis in both neonatal and adult cardiomyocytes through its specific receptor ErbB4[60, 61]. Mononucleated cardiomyocytes were found to be capable of karyokinesis, whereas binucleated cells were not[61]. Pertinently, injection of NRG1 into adult mice promoted regeneration following myocardial infarction[61]. This molecule is now undergoing small scale clinical trials as a therapeutic agent to treat congestive heart failure[62]. Though tempting to speculate de-differentiation as the underlying process by which NRG1 promotes cardiomyocyte proliferation Bersell et al could not find any evidence of sarcomere disassembly for example[61]. Despite this finding it is possible that pro-proliferative paracrine factors promote cardiomyocyte proliferation via de-differentiation, and this process underpins the robust regeneration found in the neonatal mouse heart[57, 63–65]. It is particularly notable that growth factors such as FGF, PDGF and NRG1 mediate cardiomyocyte proliferation through PI3K. Under hypoxic conditions Adipose derived stromal cells secrete the pro-inflammatory cytokine IL-6; and curiously this protein has also been linked with augmentation of cardiomyocyte proliferation via Stat3 and ERK1/2[66].

Fibronectin and collagen, acting via their receptor integrin-β1, are pro-proliferative[67]. Periostin is an ECM protein secreted by adipose-derived MSCs[68], and has been reported to promote cardiomyocyte proliferation via integrin (alphaV, beta1, beta3 and beta5) mediated activation of PI3K[69]. In a myocardial infarction model periostin induced cardiomyocytes to re-enter the cell-cycle and this was associated with improvements in cardiac function[69]. Interestingly, activation of PI3K was sufficient to recapitulate the effects of periostin. Other researchers found that periostin had no effect on cardiomyocyte proliferation[70]. Periostin also appears to be associated with increased myocardial fibrosis[71, 72] though it should be noted that delivery of the protein into the pericardial space improved cardiac function following myocardial infarction[71].

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 to FGF. Importantly, evidence of cytokinesis was observed in a murine model[73]. The proliferative effects of HASF were found to be mediated by PI3K and the cell-cycle regulator cyclin-dependent kinase 7 (CDK7)[73]. We are currently investigating in more detail the molecular pathways by which HASF promotes cardiomyocyte proliferation. Utilizing yeast two-hybrid and co-immunoprecipitation assays we identified a direct interaction

between the Insulin-like Growth Factor-1 Receptor (IGF1R) and HASF[74]. Subsequent studies with pharmacological inhibitors and siRNA mediated knockdown showed that the beneficial effects of HASF are mediated by the IGF1R[74]. 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[75], HASF does not[73]. This finding itself highlights the potential clinical benefits of the HASF protein.

Cardiac remodeling

Cardiac injury with significant cell loss and functional imparment 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 a number of animal models MSCs decrease fibrosis in a number of tissues including the heart[22], lung[76], liver[77] and kidney[78]. Stem cells, such as MSCs, express a number of proteins that regulate the extracellular matrix such as metalloproteinases (MMPs), serine proteases, and serine protease inhibitors, suggesting that transplanted MSCs can inhibit fibrosis through a paracrine action[79]. MSC transplantation has been shown to inhibit post-MI increases in the expression of collagens-1 and –III as well as the tissue inhibitor of metalloproteinase (TIMP)-1[80]. Conditioned media prepared from MSCs strongly inhibits cardiac fibroblast proliferation and inhibits the production of collagen-I and –III from these cells[81]. Growth differentiation factor-11 (GDF11), a circulating protein that was revealed as a rejuvenating factor for the aging heart by parabiosis experiments, actively prevents fibrosis following myocardial injury[82].

We have demonstrated that Sfrp2 prevents fibrosis. Injected into infarcted rat myocardium 2 days after injury Sfrp2 inhibited MI-induced collagen type-I deposition as well as left ventricular fibrosis. Activity of Bmp1, a key enzyme involved in the regulation of collagen biosynthesis and maturation, was repressed by a high concentration of Sfrp2[83]. Despite our findings there are a number of reports which have ascribed a pro-fibrotic role for Sfrp2. Kobayashi et al found that fibrosis was reduced in Sfrp2-null mice following myocardial infarction[84]. Similarly, Mastri *et al* reduced fibrosis and improved cardiac function following the intraperitoneal delivery of a Sfrp2 neutralizing antibody into cardiomyopathic hamsters[85]. Why is there a discrepancy between the studies? Sfrp proteins have biphasic effects depending upon their concentration; with Wnt[86] and BMP1[85] 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[87]. 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 Sfrp2 to behave in anti-fibrotic 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[88]. Moreover, in the infarct border zone the phosphocreatine-to-ATP ratio increases[89]. These changes influence infarct size and remodeling.

Injection of MSCs into the hearts of pigs following MI partially prevented the metabolic changes in the heart associated with injury. Due to the low engraftment of the injected cells it was proposed that the MSCs were thwarting metabolic changes via paracrine factors[90]. 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[91].

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[23]. 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[25]. 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[92].

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[93]. Moreover, the administration of MSCs following permanent occlusion increases capillary density[94, 95]. Only a very small number of these stem cells engraft and differentiate into vascular structures[15, 96].

The molecular pathways that control angiogenesis are well characterized and involve proteins such as VEGF, bFGF, HGF, and angiopoietin, amongst 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 pro-angiogenic factors[95, 97, 98]. Support of this paradigm has come from a number of studies. Tse et al compared a number of 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[99]. Similarly, conditioned media from bone marrow mononuclear cells increases vessel density in a rat model of acute MI[25]. The Epstein laboratory found that injection of MSCs into the

adductor muscle following 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 collaterals indicating that the effects they observed were paracrine in nature[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[98]. Conditioned media from these MSCs enhanced endothelial and smooth muscle cell proliferation in vitro. VEGF is an important pro-angiogenic paracrine factor as ablation of this gene significantly inhibits the ability of MSCs to promote functional recovery in the injured heart[100]. However antibodies targeting VEGF and FGF only partially attenuated the effect of the conditioned media[98]; indicating that MSCs release other pro-angiogenic proteins besides these two growth factors.

Endothelial progenitor cells (EPCs) also promote angiogenesis via paracrine mechanisms. Conditioned media derived from EPCs promotes angiogenesis in ischemic myocardium[101]. VEGF and stromal derived factor 1 (SDF-1) are among the responsible bioactive molecules. Both proteins are secreted by EPCs and promote endothelial cell migration as well as capillary formation via differentiation independent mechanisms[102].

Resident stem cell activation

The heart contains a number of resident stem cells which are defined by a number of 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 believed to be capable of promoting regeneration via mobilization into injured tissue and differentiation into mature cardiac cells. Intracoronary infusion of autologous c-Kit+ CPCs improves ventricular systolic function and reduces infarct size in patients with heart failure after myocardial infarction[103, 104]. These c-Kit+ 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+ 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+ cardiac stem cells[105, 106]. The nature of these paracrine factors remains to be identified.

Recent data suggests that paracrine factors released by adult stem cells significantly augment the ability of resident c-Kit+ cardiac stem cells to differentiate into cardiomyocytes. Conditioned medium derived from cultured MSCs promotes cardiac progenitor cell (CPC) proliferation and differentiation. The growth factor IGF-1, a paracrine factor released by MSCs, promotes resident stem mobilization[107] and commitment to the cardiac lineage[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 a number of anti-tumorigenic properties. We found that Abi3bp formed extensive extracellular matrix deposits when secreted by MSCs[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 chrondogenesis and adipogenesis severely impaired. In addition, Abi3bp inhibited MSC proliferation[110].

Considering the close relationship between MSCs and CPCs we hypothesized that Abi3bp would have similar affects upon CPC proliferation and differentiation. Indeed Abi3bp promoted c-Kit+ CPC differentiation, whilst proliferation was inhibited, both in vitro and in vivo[111]. Integrin-β1 was found to be crucial for the effect of Abi3bp on c-Kit+ CPCs. Genetic ablation of Abi3bp was associated with adverse recovery following MI[111]. This is likely due to the effects on CPC differentiation as cardiomyocyte proliferation was unaffected by the loss of Abi3bp expression[111].

Extracellular vesicles & Exosomes

Recent evidence suggests that the paracrine functions of MSCs, and other cell-types, are potentially mediated by extracellular vesicles (EVs). There are a number of EV subtypes, such as exosomes and microvesicles. Exosomes, the most numerous subtype, are released upon fusion of a multivesicular body with the plasma membrane whereas microvesicles are released directly from the cell membrane[112]. EVs were originally thought to be a mechanism by which cells disposed of waste materials. Many lines of research now point towards EVs as important mediators of cell–cell communication, immunomodulation, proliferation, cell-senescence, and differentiation by transferring various bio-active cargoes such as proteins, lipids, mRNAs, as well as miRNAs, from one cell to another[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[115]. Similarly, exosomes derived from bone marrow $CD34^+$ cells and cardiac progenitor cells promote angiogenesis in cultured endothelial cells[116, 117]. *In vivo*, fractionation studies indicated that only the fraction of MSC conditioned media containing products of greater than 1000 kDa (100–220 nm), the typical size of exosomes, provided protection in a mouse model of myocardial ischemia and reperfusion injury[118]. The same group later purified exosomes from MSCs and found that they reduced infarct size post myocardial injury[119]. The protective effect of exosomes are not limited to the heart, indeed EVs derived from MSCs protect the kidney from ischemia-reperfusion injury[120–122].

Considering that EVs can deliver multiple bio-active molecules at the same time they hold much promise as therapeutic agents to deliver paracrine factors *in vivo*. To this end a number of researchers are actively investigating novel approaches to EV delivery such as incorporating tags to aid in their isolation, over-expression of key cargoes, and designing synthetic EV structures to aid scalability for clinical use[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 multi-phased. The healing process following 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 of the review, 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 a number of 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). Though 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 EGF, FGF, Wingless/Wnt, and BMP generate concentration gradients that provide spatial information to generate distinct cell types in a specific three-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 regarding the role of Sfrp2 upon cardiac fibrosis also suggests that paracrine factor concentration gradients can have a dramatic effect on cell behavior. We have found that administrating a high concentration of Sfrp2 prevents fibrosis following MI[83]. In contrast, genetic deletion of Sfrp2[84] and Sfrp2 neutralizing antibodies[85] reduce fibrosis, suggesting that Sfrp2 is pro-fibrotic. This can be explained by the biphasic properties of Sfrp proteins which augment or inhibit Wnt[86]/BMP1[85] signaling at low or high concentrations respectively.

Concentration gradients provide a conduit to attract cells to a specific site. In the context of myocardial infarction proteins released from dying cells release chemoattractants which mediate the infiltration of pro-inflammatory immune cells into the injured tissue[129]. Modifying MSCs to express proteins such as CCR1[130] allows MSCs to follow the same chemotactic gradients as pro-inflammatory immune cells. This is particularly pertinent as MSCs release a myriad of paracrine factors that inhibit the function of pro-inflammatory 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[132, 133] 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[134]. In contrast, the regenerative processes mediated by stem cell paracrine factors are very 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 non-cardiac 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[132]. Considering that each event that follows myocardial infarction is prolonged, and that for each of these events several stem cell paracrine factors will have a direct influence, temporal and spatial summation has been under-appreciated 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 very 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 following myocardial injury (Figure 2).

As mentioned above we have identified Sfrp2 and HASF as two cytoprotective paracrine factors (Figure 2). These two paracrine factors utilize two different mechanisms. Sfrp2 promotes the protection of cardiomyocytes by binding to the pro-apoptotic protein Wnt3a[29]. In contrast, HASF prevents cardiomyocyte cell-death through PKCε[31]. As noted in a recent editorial HASF has a number of novel features as a cytoprotective factor[136]. Over-expression 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 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 IGF1R 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 appears to utilize common growth-factor receptor tyrosine kinase pathways, though there are significant differences. Whereas IGF-1 promotes cardiac hypertrophy[75], HASF does not[73]. As stated earlier, in addition to promoting

cytoprotection, we have found that Sfrp2 prevents fibrosis by inhibiting Bmp1[83]. Moreover we have recently shown that Sfrp2 inhibits Sca-1 cardiac progenitor cell (CPC) proliferation and primes the cells for differentiation[138]. This switch from proliferation to differentiation occurred via Sfrp2 binding to Wnt6, which inhibited canonical Wnt signaling and activated non-canonical Wnt/Planar Cell Polarity signaling through JNK[138].

We originally identified the protein Abi3bp as an autocrine regulator of MSC biology[110]. This molecule is also pleiotropic. Abi3bp was found to strongly promote resident c-Kit+ cardiac progenitor differentiation both in vitro and in vivo[111]. Abi3bp belongs to the proteoglycan family of extracellular matrix proteins. These proteoglycans modify the fibrillar structure of the extracellular matrix which has significant effects upon cell adhesion, migration, and proliferation[139]. Moreover, proteoglycans regulate the activities of secreted proteins. For example, the heparan sulfate chains of proteoglycans bind to fibroblast growth factors, enabling the growth factor to cross-link and activate their cell-surface receptors[139]. It is possible therefore that Abi3bp, by virtue of being a proteoglycan, not only modifies the extracellular matrix 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, PDGF, 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 of the events that occur following myocardial infarction (Figure 4). Using our own research as an example HASF and Sfrp2 prevent cardiomyocyte cell-death, the first event that occurs following a myocardial infarction. Both Sfrp2 and Abi3bp promote resident progenitor cells to differentiate, an event which begins ~1 week after myocardial infarction. Finally HASF promotes the remaining cardiomyocytes to proliferate. Considering the pleiotropic actions of paracrine factors (Table 2), 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 cells presence in low levels weeks after MI[140, 141].

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 following a myocardial infarction?

The inflammatory response is robustly active immediately following myocardial infarction. Increased expression of the cytokine interleukin-6 (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[142] which as discussed earlier promotes new blood vessel formation in the myocardium. Other pro-inflammatory cytokines released by the dying

myocardium have similar effects to IL-6. TNF-a, IL-1 and IFN-g have all been shown to stimulate MSC secretion of a number of growth factors such as epidermal growth factor (EGF), VEGF, hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF1) and angiopoietin. These growth factors go on to regenerate the myocardium through the formation of new capillaries, cardiomyocyte proliferation and resident progenitor cell differentiation[143]. Taking these findings one step further, pre-activating MSCs with pro-inflammatory cytokines prior to 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[144].

In a similar vein, the extracellular remodeling which occurs post-myocardial infarction has been shown to modulate the secretory profile of MSCs. The remodeled matrix was found to promote the secretion of a number of proangiogenic, anti-fibrotic, and immunomodulatory paracrine factors from MSCs, most notably HGF and SDF-1[145].

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 following brain ischemia injury had markedly different effects, stimulating cell proliferation when injected three days after injury and stimulating axonal remodeling when injected 10 days after ischemia. Pathway-focused PCR array analysis revealed that that a number of genes encoding secreted factors were differentially regulated in the MSCs injected at the two-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

In summary, 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 upon their microenvironment influencing the tissue’s response to injury. As mentioned above, paracrine factors create a specific microenvironment, impacting 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 following 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. Though 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 utilized. 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. Utilizing 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 as well promoting their survival,

It is important to note that in the field of cell therapy consistent and reproducible results among laboratories is 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

SOURCES OF FUNDING

This work was supported by National Heart, Lung, and Blood Institute grants RO1 HL81744, HL72010, and HL73219 (to V.J.D.); and the Edna and Fred L. Mandel Jr. Foundation (to V.J.D., and M.M.). M.M. was also supported by an American Heart Association National Scientist Development Award (10SDG4280011).

NON-STANDARD ABBREVIATIONS AND ACRONYMS

bFGF	basic Fibroblast growth factor
CPC	Cardiac progenitor cell
GFP	Green Fluorescent Protein
HASF	Hypoxic induced Akt regulated Stem cell Factor
HGF	Hepatocyte growth factor
IGF1	Insulin-like growth factor
MI	Myocardial infarction
MSC	Mesenchymal stem cell
Sfrp2	Secreted frizzled related protein 2 (Sfrp2)
VEGF	Vascular endothelial growth factor

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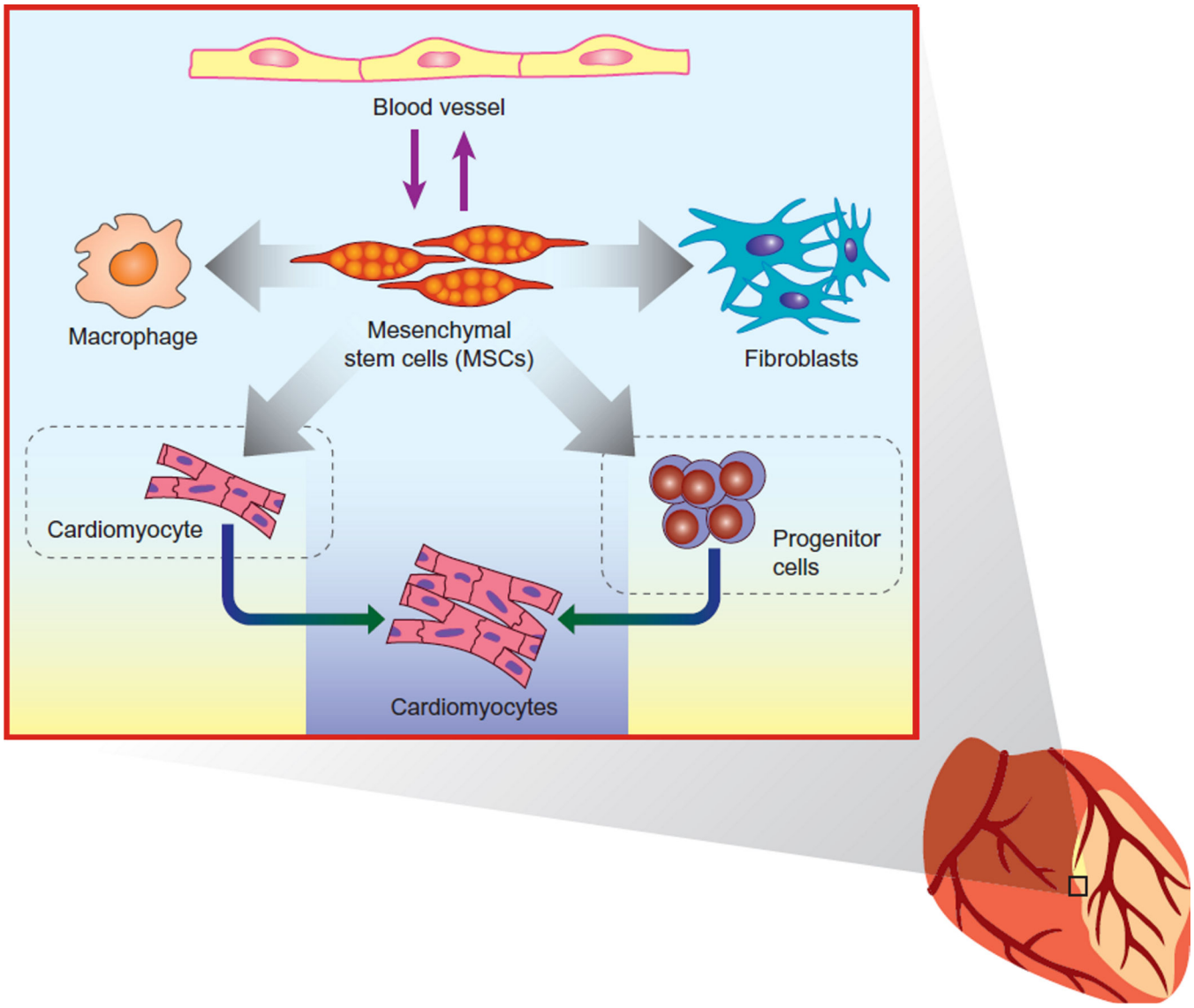

Figure 1.

Paracrine factors affect different cell types and create a microenvironment that is influenced by concentration gradients, with temporal and spatial summation of cellular responses. (This figure is taken from[149] with permission)

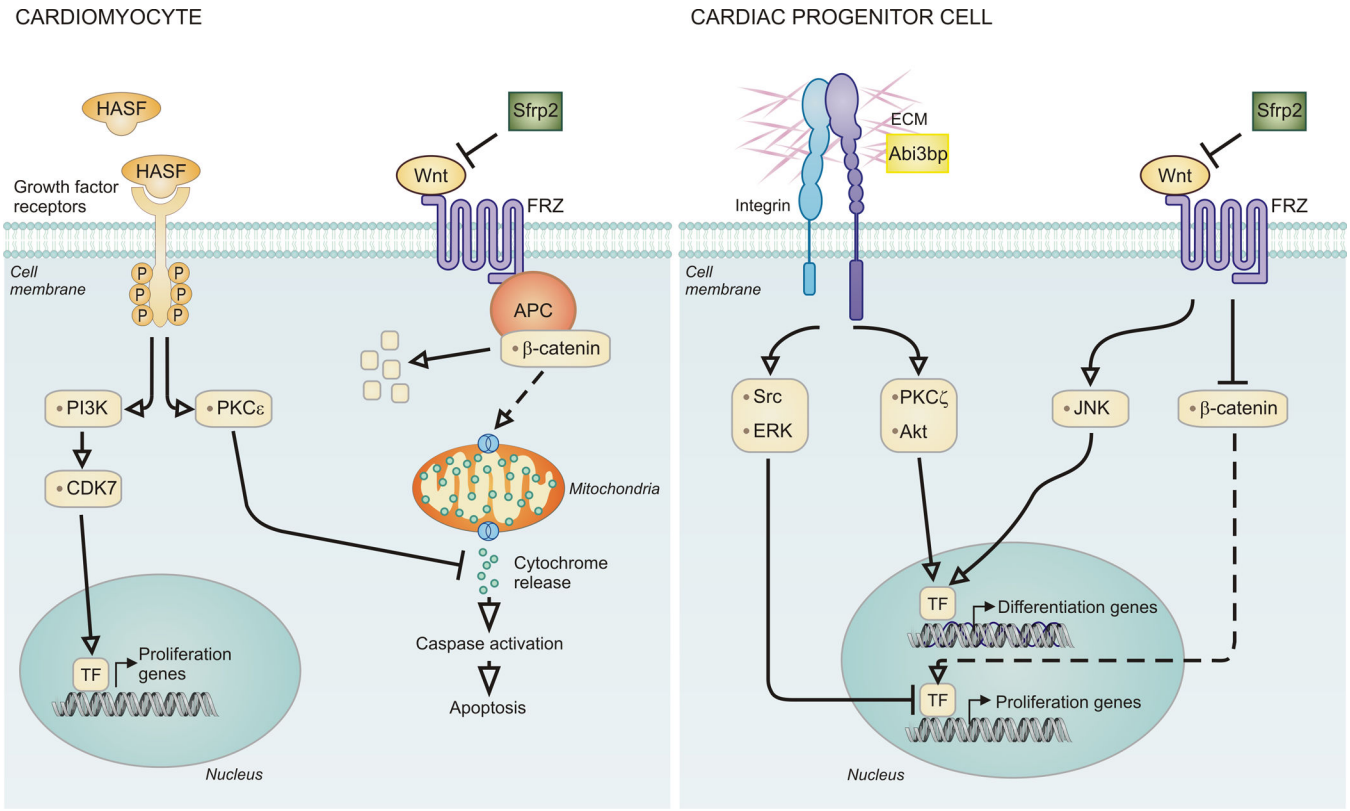

Figure 2. Paracrine factors are pleiotropic

For illustration, we show the cellular effects of 2 selective paracrine factors on the cardiomyocyte. ***Left:*** HASF and Sfrp2 inhibit cardiomyocyte apoptosis through divergent pathways. HASF, following binding to a growth factor receptor, inhibits cytochrome release from mitochondria via PKCe. In contrast Sfrp2 inhibits Wnt activation of Frizzled Receptors. This induces b-catenin degradation via the APC complex. ***Right:*** Abi3bp and Sfrp2 promote cardiac progenitor cell differentiation as well as inhibiting proliferation. Abi3bp activates integrin-b1. Src and ERK activation work together to inhibit proliferation. PKCz and Akt activation switch on cardiac genes. Sfrp2 sequesters Wnt, preventing activation of Frizzled Receptors. This promotes JNK activation and cardiac gene expression. Inhibition of b-catenin blocks the proliferation pathway in these cells.

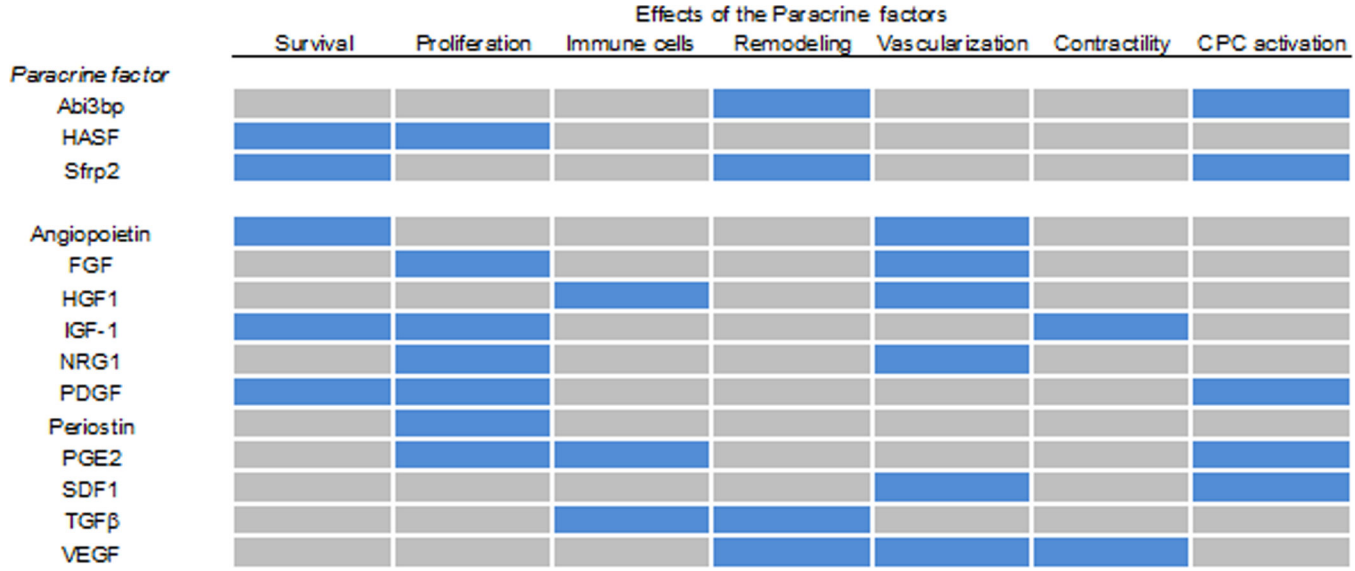

Figure 3.

Paracrine factors described in this review are listed with the effects they have upon the heart post-myocardial infarction. Blue represents an effect, grey no effect.

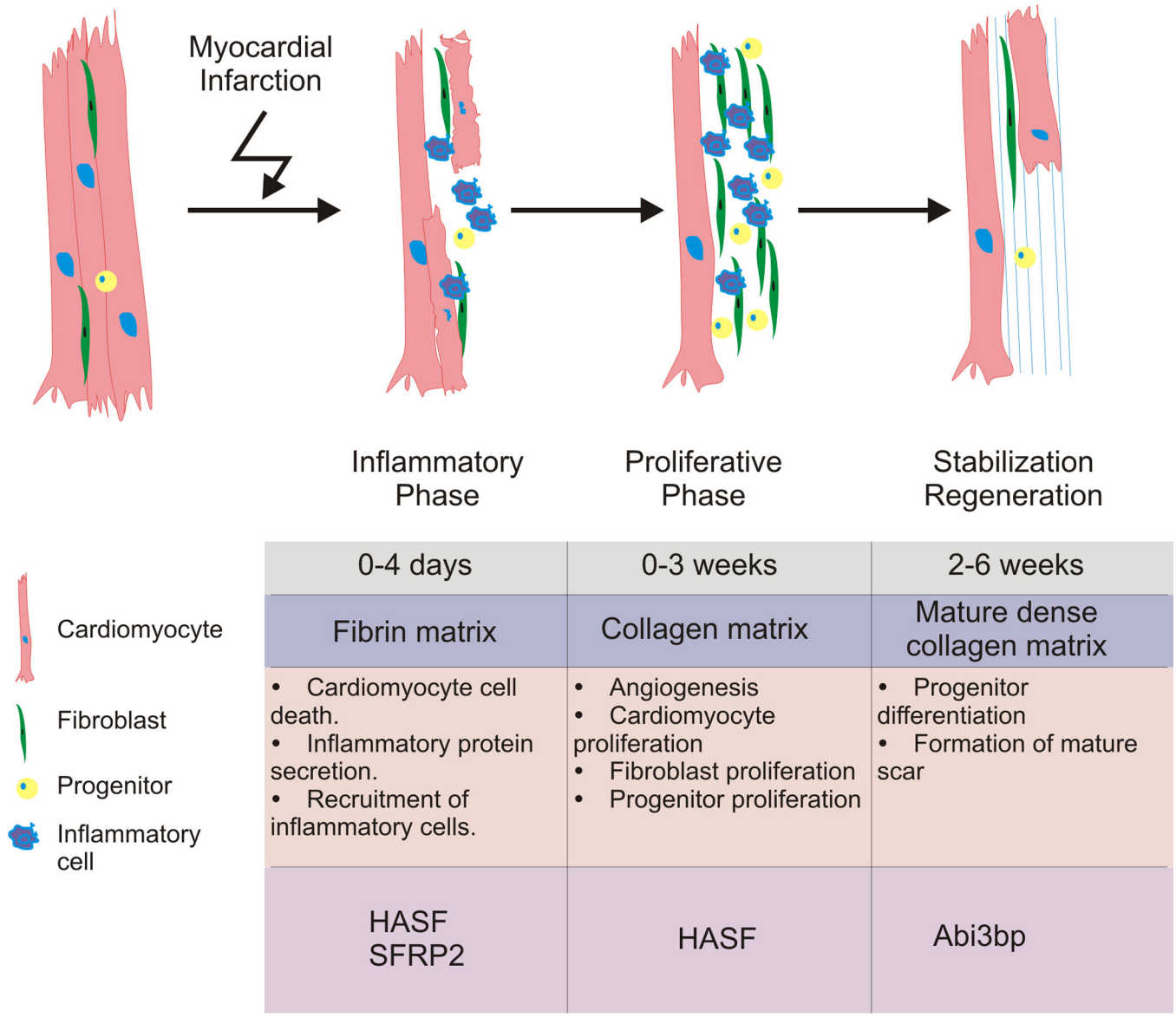

Figure 4.

Paracrine factors affect different temporal events after myocardial injury influencing different stages of the reparative and regenerative processes.

Table 1

Stem cells used in regenerative medicine and for which paracrine effects have been shown.

Tissue	Cell
Bone marrow	mesenchymal stem cells (MSCs)
	hematopoietic stem cells
	bone marrow-derived mononuclear cells
	Unfractionated bone marrow cells
Heart	cardiac progenitor/stem cells
Adipose	mesenchymal stem cells
Skeletal muscle	myoblasts
Circulation	Endothelial progenitor cells