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REVIEW ARTICLE

Mesenchymal Stem Cells for Cardiovascular Regeneration

Drew Kuraitis · Marc Ruel · Erik J. Suuronen

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Abstract Despite recent studies suggesting that the heart has instrinsic mechanisms of self-regeneration following myocardial infarction, it cannot regenerate itself to an optimal level. Mesenchymal stem cells (MSCs) are currently being investigated for regeneration of mesenchyme-derived tissues, such as bone, cartilage and tendon. In vitro evidence suggests that MSCs can also differentiate into cardiomyogenic and vasculogenic lineages, offering another cell source for cardiovascular regeneration. In vivo, MSCs may contribute to the regrowth and protection of vasculature and cardiomyocytes, mediated by paracrine actions, and/or persist within the myocardium in a differentiated state; although proof of cardiomyocytic phenotype and functional integration remains elusive. Herein, we review the evidence of MSCs as a cell source for cardiovascular regeneration, as well as their limitations that may prevent them from being effectively used in the clinic.

Key words Cardiovascular Regeneration · Cell Therapy · Mesenchymal Stem Cell · Neovascularization

Cardiovascular regeneration

Recent decades have seen refinements in life-saving cardiovascular therapies, such as percutaneous and surgical treatments, implantable defibrillators, cardiac resynchronization therapy, left ventricular assist devices, and others. Although these treatments are now considered commonplace and are widely available, they do not actively restore or regenerate the injured myocardial tissue. The fact remains that after a myocardial infarction, the resulting necrotic and scar tissue cannot be restored. The use of stem cells for myocardial regeneration attempts to amplify and/or recreate the natural processes of tissue formation. It is expected that therapeutic cells will augment the creation of myocardium de novo, and even replace damaged or dead cells. The ultimate goal is to restore perfusion, contractility, lusitropy and conduction by repopulation with normal, healthy cells [1, 2].

The heart has classically been thought of as a post-mitotic organ, without potential for regeneration. Recent reports have shown that cardiomyocytes are replaced in the adult heart [3, 4], with the cardiomyocyte's lifespan being approximately 4.5 years. In addition to the lack of regenerating cardiomyocyte pools, another major hurdle for myocardial regeneration is that in order for whole-tissue regeneration to occur, perfusion must first be restored. Neovascularization is required to perfuse the regenerating myocardium; without it, new muscle formation will not occur to any significant level. To complicate matters, as described in the following section, the post-ischemic myocardium can be a hostile environment, not necessarily permissive for healthy, regenerative processes.

Endogenous responses to infarction

Temporally, immediately following a myocardial infarction (MI), cardiomyocytes downstream of occluded arteries undergo apoptosis within minutes. Local ischemic zones

D. Kuraitis · M. Ruel · E. J. Suuronen (✉)
Division of Cardiac Surgery, University of Ottawa Heart Institute,
40 Ruskin Street,
Ottawa, ON K1Y 4W7, Canada
e-mail: esuuronen@ottawaheart.ca

D. Kuraitis · M. Ruel · E. J. Suuronen
Department of Cellular & Molecular Medicine,
University of Ottawa,
Ottawa K1H 8M5, Canada

accumulate dead cells and toxic by-products, and subsequent reperfusion brings the generation of reactive oxygen species [5]. From this, a major inflammatory response occurs, as summarized in Fig. 1. The natural response is for the myocardium to secrete cytokines (such as tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), and interleukins-1β, -6, and -8 (Il-1β, IL-6, Il-8)) that will activate an inflammatory response, signaling for the invasion of leukocytes [6]. To augment the inflammatory response, myocardial endothelial cells upregulate cell adhesion molecules, such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), which promote inflammatory cell engraftment into the ischemic tissue. This effort is followed by a wave of monocytes that home towards the source of the inflammatory signal cascades, where they mature into macrophages and have the role of removing debris and dead cells from extracellular matrix (ECM). Within days, myofibroblasts enter the infarcted zone and contribute to the remodeling process [6]. The deposition of collagen scar tissue, or fibrosis, is a known cause of diastolic dysfunction [7].

As tissue turnover proceeds, inflammatory cytokines and proteases accumulate in the heart, which are harmful to adjacent surrounding cells [8]. Concurrent with these processes, neovascularization occurs in an attempt to re-supply the ischemic zones with a blood supply. Much of these actions are initiated by the release of soluble stromal cell-derived factor-1 (SDF-1), which is a ligand for CXCR4, a receptor on many endothelial progenitor cells (EPCs)[11]. (Note: the phenotypic definition of what constitutes a EPC is a highly-debated topic [9, 10]; this review will use the term 'EPC' as a broad-spectrum population consisting of mononuclear cells that are derived in the bone marrow, can be mobilized to the circulation, and have pro-vasculogenic properties.) $CXCR4^{+}$ cells use the generated SDF-1 gradient to home to the site of ischemia, wherein they engraft and support many pro-angiogenic processes before myofibroblasts begin to repopulate the ischemic zones [11]. To this effect, it is believed that neovascularization of the dysfunctional myocardium from paracrine/humoral factors and secondary recruitment of host stem/progenitor cells are the likely mechanisms leading to functional improvement observed in studies of

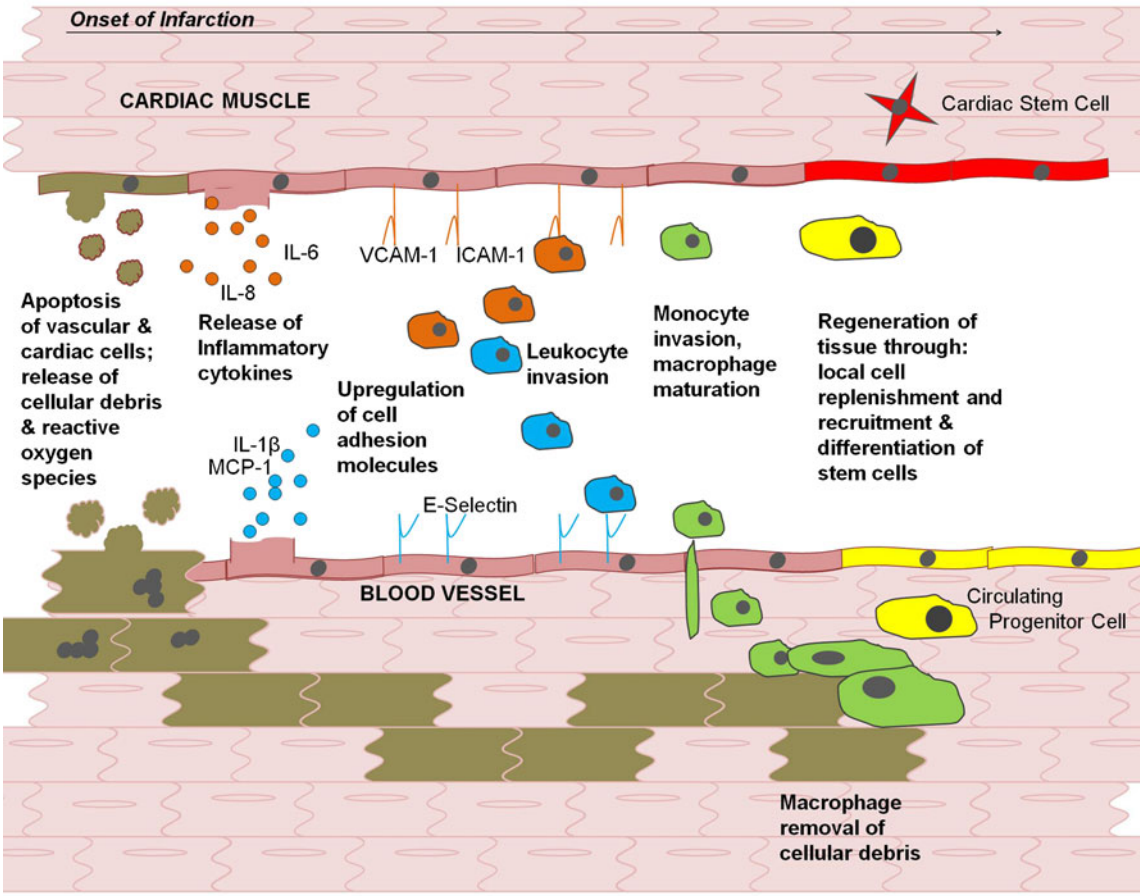

Fig. 1 Temporal events of the inflammatory response following a myocardial infarction

cardiac regeneration, as opposed to regeneration of the muscle itself [12].

The endogenous regenerative process is thought to be a combination of recruited EPCs and resident cardiac stem cells (CSCs). Following infarction, EPCs are recruited to the heart via the well-characterized SDF-1/CXCR4 axis, which peaks early and returns to baseline levels within 1 week [13, 14]. In addition, pools of CSCs are activated within the heart. EPCs are able to home to sites of ischemia, where they may augment regenerative processes, in particular vasculogenesis, via paracrine signaling [15]. Clinical trials have already begun using autologous bone marrow-derived mononuclear cells. Transplantation of EPCs into ischemic hearts has shown modest improvements in cardiac function [16–19], which are largely thought to result from angiogenesis and improved perfusion. The formation of new blood vessels is considered the first step towards regeneration; however, studies have thus far failed to demonstrate cardiomyogenic commitment of EPCs [20, 21]. In 2002, the description of a pool of progenitors residing in cardiac tissue suggested that endogenous mechanisms for post-natal myocardial turnover exist [22]. However, the low number of CSCs (~1 of 30,000 heart cells) perhaps explains why endogenous repair by CSCs is insufficient to reverse major injury [23]. Currently, CSCs are thought to be responsible for turnover of cardiomyocytes, smooth muscle and endothelial cells [24]. Specifically, annual cardiomyocyte turnover in the adult human heart occurs at an estimated rate of 1–2%, and ~40% of the mature heart is composed of postnatally-generated myocytes [25]. Two recent studies have assessed CSC transplantation into ischemic myocardium, both producing data supportive of functional improvement post-transplantation. Tang et al. showed that the observed improvement was related to stimulation of endogenous CSC populations [26]; while Hosoda et al. demonstrated that cardiomyocytes and endothelial cells of transplanted cell origin were engrafted and incorporated into the recovering tissue, 4–6 weeks post-treatment [27].

As described above, EPCs and CSCs are promising as cells for therapeutic applications because of their endogenous regenerative potential. Despite the underwhelming results produced by clinical trials using EPCs, a global, yet mild, effect was observed, suggesting an overall improvement in recovery [28, 29]. It is possible that the cell-derived signals may only need to be amplified to better restore myocardial function. However, another cell population of interest is the Mesenchymal Stem Cell (MSC). Typically, MSCs are thought to be supportive of differentiation into and growth of bone, cartilage and adipose tissues. Although research has made great progress in these respective fields, the application of MSCs for cardiovascular regeneration has received less attention when compared with that of the EPCs. Despite the fact that MSC research predates the study of EPCs by many years, and despite the fact that publications investigating cardiovascular regeneration have been increasing over time (Fig. 2A), there are at least twice as many publications regarding myocardial regeneration using EPCs rather than MSCs (Fig. 2B). Recently, MSCs have been shown to be a stem cell population with great promise for cardiovascular regeneration and the treatment of ischemic heart disease, as described in the sections that follow.

Mesenchymal stem cells

Since the first description of MSCs in 1991 as a population of bone marrow-derived cells able to give rise to osteoblasts and adipocytes [30], MSCs have classically been thought of as marrow cells that have capacity for self-renewal, and can yield tissue of mesenchymal origin (adipose, bone, cartilage)[31]. MSCs isolated from traditional cell culture techniques have been shown to have the ability to differentiate into neural (non-mesenchymal) tissue [32–34] suggesting that the term *mesenchymal* stem cell is no longer appropriate, or that current isolation techniques are not producing pure *mesenchymal* stem cell populations. Most studies produced MSCs from culturing unfractionated bone marrow and collecting culture plate-adherent cells [35], which produces a highly heterogeneous population [36]. These heterogeneous cultures often contain hematopoietic contaminants; efforts to reduce this hetereogeneity have been recently published, and modify the original plate-adherent method using enzymatic digestion of compact bones, where contaminating cell populations are rare [37].

The classical adherent MSC population is thought to express cell surface markers CD73 and CD105 [38]. Over time, this definition has expanded to include CD29, CD44, and CD90 [36, 39], as well as a lack of CD34 and CD45 expression [36, 40]. It is probable that cell surface marker alone is insufficient in identifying MSCs, and that functional assays may be required in order to demonstrate their stem cell plasticity and potential for differentiation [41–43].

Since MSCs lack major histocompatability complex II and B7 co-stimulatory molecule expression, they are able to evade much of the T-cell responses [44, 45]. MSCs can also reduce IFN-γ production and decrease the expression of markers typical of activated lymphocytes, such as CD25, CD38, and CD69 [46, 47]. This may be mediated, in part, by their ability to arrest stimulated T-cells at the G_0-G_1 checkpoint of the cell cycle via the inhibition of cyclin D_2 [48]. Additionally, studies have shown that MSCs have an inherent ability to avoid rejection [49–51]. Although estimated to only constitute 0.001–0.01% of bone marrow cells [36, 52], MSCs can be expanded for 4–10 population

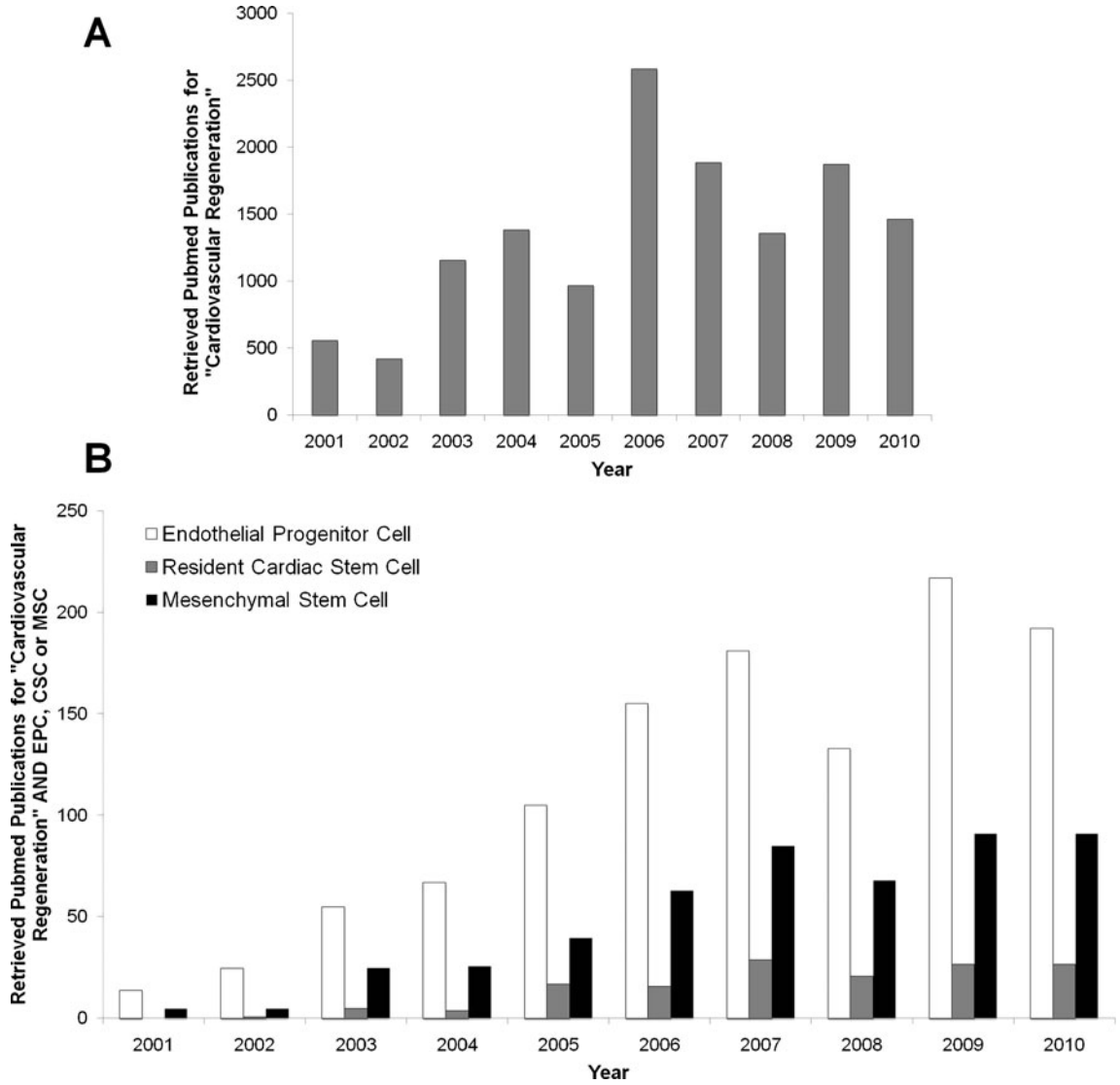

Fig. 2 Number of publications from 2001 – 2010 indexed with NIH-Pubmed regarding Cardiovascular Regeneration and/or keywords for EPC, CSC or MSC populations

doublings [52] to achieve therapeutically-relevant cell counts. Taken together, these observations (minimal elicited immune responses, non-autologous source, and easy production of sufficient cell numbers) support the MSC as a suitable cell type for transplantation, and may even hold promise as a future off-the-shelf product.

In vitro differentiation of therapeutic populations

In the presence of 5-aza-cytidine, MSCs have been observed to differentiate into cardiomyocyte-like cells [53, 54]. These cells were similar to fetal cardiomyocytes as determined by transcription factor profiling, which detected the expression of cardiac transcription factors GATA4, Nkx2.5; however, their morphology resembled rod-like myotubes of skeletal, not cardiac muscle. Spontaneous and synchronous beating of MSC-derived cardiomyocyte-like cells occurs in culture, and these cells can integrate with neonatal myocytes via the formation of intercalated discs [55]. Additionally, when generated cardiomyocytes are exposed to β-adrenergic blockers, their contraction decreases, and contraction rate increases when treated with isoproterenol [56]. Cardiomyocyte-like differentiation of MSCs using 5-aza-cytidine is now a well-known protocol, even though its mechanisms are poorly understood, and many researchers have demonstrated alternative results of this treatment, including failure to induce cardiomyocytes phenotypes [57, 58], generation of extremely low numbers of differentiated cells [59], generation of adipocytes and chondrocytes [60], and even induction of uncontrollable expression of various genes [61], an obvious limitation for clinical usage. Regardless, 5-aza-cytidine may have potential to generate

a cardiomyocyte-like phenotype, and after a single exposure, cells still retain their multipotency [62].

To address the uncertainty regarding the effects of 5-aza-cytidine, other protocols for differentiation have been investigated. Cardiomyocyte-like cells can be generated from MSC fractions simply by supplementing medium with insulin, dexamethasone, and ascorbic acid [63]. Early on, these cells began to express cardiac-specific GATA-4 and MEF2, and after 5–6 passages, more than 90% of cells were positive for cardiac troponin I, connexin-43, and cardiac-specific titin, while being negative for skeletal muscle markers myosin heavy chain (MHC) and MyoD. These results are highly supportive of successful MSC-to-cardiomyocyte differentiation; however, these populations lacked spontaneous beating in culture, suggesting that the generated cells may be cardiomyocytes in phenotype, but not in function [63]. Regardless, protocols for furthering stem cells towards a differentiated phenotype may prove useful, as pre-differentiation is likely to be a method for enhancing a cell's ability to survive and engraft following transplantation—therapies may benefit from reliable, safe, and robust methodologies for this phenomenon.

Although vasculature is not a mesenchyme-derived tissue, some evidence exists to support the notion that an MSC can become a vascular cell. MSCs grown in cell culture media for endothelial cells, supplemented with vascular endothelial growth factor (VEGF), stained positively for von Willebrand Factor (vWF) after 1 week of culture [64]. Similar methods can produce cells that express endothelial nitric oxide synthase (eNOS), with the ability to bind Ulex europaeus agglutinin I (UEA-1), both of which are hallmark features of endothelium [65]. These endothelial-"primed" cells can rapidly generate tubules, similar to small diameter vessels, in a 3-dimensional ECM assay, while the raw, "unprimed" MSC populations cannot [64, 65]. Another route towards vascular differentiation is to modify the cell culture substrate. Culture on native endothelial cell matrix can generate populations of MSCs that express CD31, a platelet-binding indicator of functional endothelium [66, 67]. These cells, however, also produced smooth muscle actin, a marker of another vascular lineage: smooth muscle cells. In MSC-to-endothelial literature to-date, generated populations may share some similarities with endothelium, but they do not always express classical markers (vWF, CD31, CD144), have the classical spindle-cobblestone morphology, or maintain the same functional characteristics (proliferative, migratory) of native endothelial cells. Other studies that have investigated MSC administration and vasculogenesis have shown that MSCs do not actively constitute de novo vasculature; rather, they may facilitate vasculogenesis by acting as a pericyte, as evidenced by increased vessel density and localization of MSCs in the perivascular niche [68]. Other investigations have also correlated MSC presence with increased vascular density, without evidence of MSC-to-vascular lineage transdifferentiation [69]. It has also been proposed that pericytes, which reside adjacent to native vasculature, are marrow-exuded MSCs that are maintained in this adventitial niche, readily available for entry into the circulation if required (reviewed by da Silva Meirelles et al. [70]). A summary of major findings regarding the cardiomyogenic and vasculogenic differentiation of MSCs is presented in Table 1.

MSC: most social cell?

Much of what we know about MSCs has been learned using co-culture systems. MSCs appear to have a potent ability to communicate with other cell types, modifying

Table 1 Summary of the evidence for vascular and cardiomyogenic differentiation of MSCs

MSC Differentiation	Conditions	Evidence
… into vascular cells	Endothelial growth factor supplementation	vWF expression [64]; eNOS expression & UEA-1 binding [65]; tubule generation [64, 65]
	Growth on native endothelial matrix	CD31 & SMA expression [66, 67]
	Physical contact with ECs	Expression of vWF, CD31, CD144 [76]
	Myocardial injection	Expression of both EC & SMC factors [88, 90, 93, 94]
	Growth factor pre-conditioning	Enhanced tubule formation [112]
…into cardiomyocytes	Presence of 5-aza-cytidine	Gata4 & Nkx2.5 expression [53, 54]; integration with neonatal myocytes [56]
	Insulin, dexamethasone, ascorbic acid supplementation	Gata4, Nkx2.5, cardiac troponin I & connexin-43 expression [63]
	Co-culture with cardiomyocytes	α-sarcomeric actin, troponin I, MEF-2C expression [79]
	Myocardial injection	α-actinin, MHC, troponin-T expression(88, 94, 95); Z-band formation, MHC & desmin expression [97]
	Growth factor pre-conditioning	Gata4 & Nkx2.5 expression [112, 113]

their own as well as their neighbours' differentiation pathways. A powerful tool for studying vasculogenesis with MSCs has been a 3-dimensional ECM assay whereby the total capillary network formed serves as an indication of the potential for in vivo angiogenesis. Depending on culture conditions, MSCs are able to form such a network [64, 65]. Experiments examining communication with endothelial cells in this assay has shown that MSCs can stabilize these tubular networks and increase their size [71, 72]. Using silencing RNAs, ablation of pro-angiogenic insulin-like growth factor-I (IGF-1) and VEGF eliminated the potential of tubule formation in an endothelial co-culture [73]. Such a co-culture can generate MSCs that express Flk-1, a stem cell marker for endothelial lineages [74]. MSCs were observed to express vWF only in the co-culture, and only if they were already Flk^{+}. Physical interactions between endothelium and MSCs are complex and dynamic [72, 75]. Co-culture experiments have shown that direct contact may be associated with increased endothelial activity [71], and that physical interactions between endothelium and MSCs are required for endothelial differentiation [76]. Only MSCs that were allowed direct contact to endothelial cells in co-culture expressed vWF, CD31 and CD144. Additionally, when media is supplemented with pro-inflammatory cytokines, such as TNF-α or IL-1β, physical adhesion of MSCs to endothelial cells is greatly increased, even compared to supplementation with protective cytokines, such as IL-6, SDF-1, or stem cell factor (SCF)[77]. This may be an endogenous response, whereby under hostile, inflammatory conditions, local vasculature becomes adhesive and permissive for stem cells. Interestingly, treatment with vascular cell adhesion molecule-1 (VCAM-1) antibodies ablated the TNF-α-mediated adhesion [77]. VCAM-1 is a marker of functional endothelium, used to communicate and adhere with other vascular cells, suggesting that MSCs again may acquire endothelial characteristics.

Freshly isolated cardiomyocytes have a tendency to form spheroid aggregates in suspension. When MSCs are added to such a mixture, they appear to co-localize with regions rich in fibronectin, a pro-angiogenic ECM component, and express CD31, followed by vWF [78]. Similar studies examining MSC and cardiomyocyte interactions have demonstrated cardiomyocyte differentiation of MSCs when co-cultured with cardiomyocytes [79]. Of note, differentiation can be modulated in vitro by varying the ratio of stem-to-adult cells. With an increased frequency of cardiomyocytes, MSC-to-cardiomyocyte differentiation, indicated by cardiac specific α-sarcomeric actin, Troponin I, and MEF-2C, increased significantly in a dose-response manner [79]. Differentiation also increased when MSCs were co-cultured with injured, apoptotic cardiomyocytes, compared to healthy cells [79]. Regardless of the cardiomyocyte's viability, it has been shown that co-culture with neonatal cardiomyocytes is key to differentiating MSCs: co-culture with adult cells did not augment differentiation [80]. In support of these observations, an attempt to establish a functional cardiomyocytic co-culture with MSCs and adult cardiomyocytes failed to produce functionally differentiated cells, despite showing interactions between cell types via connexin43 [81]. One convincing study of note examined a co-culture in which the cells were not able to physically associate, separated by a semipermeable membrane [82]. MSCs acquired the ability to spontaneously contract, and some of these cells were positive for cardiac troponin-T, cardiac troponin-I, and sarcomeric α-actinin. These cells developed hallmark ultrastructural organization of sarcomeres, and functionally, they developed inward rectifier potassium currents and expression of Ryanodine receptor-2, all of which are characteristics of native cardiomyocytes. It remains to be seen whether or not the cells produced by these methodologies will have therapeutic effects in vivo. To contrast this, when MSCs were supplemented with the conditioned medium of neonatal cardiomyocytes, levels of differentiation were negligible, suggesting that the phenomenon of cell-mediated differentiation is a product of direct cell contact interactions, and not a result of a paracrine signaling [80]. Similarly, MSCs did not express endothelial markers vWF, CD31, or CD144 in an endothelial co-culture unless they were allowed direct physical contact, and not separated by a semi-permeable membrane [76]. Another co-culture study that used separated populations was able to generate cardiomyocyte-like cells from MSCs [82], suggesting the alternative; that is, that paracrine mechanisms are involved in MSC differentiation in co-culture. To date, paracrine effects are believed to be potent mechanisms of MSC signaling, but these mechanisms remain phenomena, without clear explanations of how they are affected.

In an attempt to explain the phenomena observed in literature where cardiomyocyte-like populations are generated, but are not considered pure cardiomyocytes, Rose et al determined that although MSCs can acquire many cardiomyocyte-like properties, they also retain many properties of MSCs [83]. These experimentss suggest that in vitro, current methods can force MSCs towards a cardiomyocytes phenotype, but cannot force MSCs past a differentiation threshold in which they become fully differentiated cardiomyocytes.

MSCs improve cardiac function post-infarction

Across different animal models of myocardial infarction, evidence supports that MSC transplantation can significantly improve cardiac function [84–89], as measured by end systolic and diastolic volumes, and left ventricular ejection

fraction. Related to improved cardiac function, MSC-treated animals have a reduced mortality rate [84]. The mechanisms behind these beneficial effects are not entirely clear, but appear to be a combination of: increased myocardial perfusion, reduced scar formation, regeneration of cardiomyocytes, and recruitment and activation of endogenous progenitor populations, as summarized in Fig. 3.

Vasculogenesis

Perhaps one of the best documented mechanisms of myocardial recovery is the increase in vasculature in the ischemic myocardium. Restoration of perfusion is a key step for myocardial regeneration—other tissues require a stable vascular network for significant regeneration and maturation to occur. Direct transplantation of MSCs has been shown to significantly improve the vascular density in canine [90], murine [91], porcine [92], and rat [87, 93] models of MI; however, what is not clear is whether or not the fate of transplanted MSCs includes incorporation into new vasculature. Incorporation of MSCs into vascular structures has been observed, but it is not always clear whether or not these cells have differentiated into smooth muscle or endothelium, despite expressing markers of both cell types [88, 90, 94]. One study noted that transplanted MSCs can differentiate and incorporate into vasculature as either smooth muscle cells *or* endothelium in differential amounts [93]. Despite these promising results, other studies have failed to demonstrate MSC differentiation and incorporation into vasculature [95]. It has been estimated that around 3% of successfully engrafted cells will acquire an endothelial phenotype [96].

Cardiomyogenesis

Based on a great amount of in vitro evidence suggesting cardiomyogenesis, a plausible hypothesis is that MSCs can generate new myocardium. There have been some studies wherein transplanted MSCs were seen to express muscle-specific proteins [88, 94, 95] such as α-actinin, myosin heavy chain, and troponin-T. Toma et al. tracked transplanted MSCs for up to 60 days post-treatment, showing cardiovascular commitment, indicated by Z-bands, MHC and desmin expression [97]. These cells also became rod-shaped and aligned with host cardiomyocytes. Similarly to MSC-augmented vasculogenesis, other studies have failed to produce results demonstrating such commitment [86]. One study noted that MSCs have a strong potential to differentiate into vascular lineages, but not cardiomyocytic ones [90], and another study noted that cell arrangement and incorporation was inconsistent and not in alignment with host myocardium [98]. Between these two polar differences, it has been noted that in vivo, engrafted MSCs

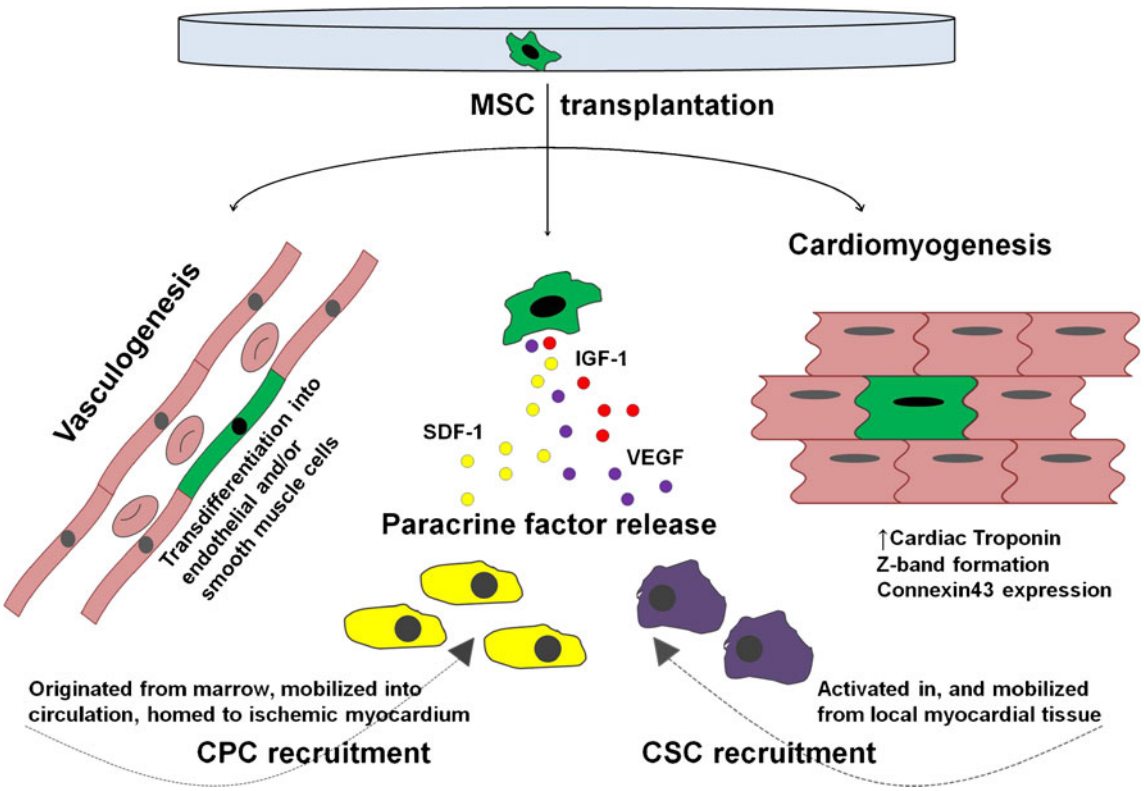

Fig. 3 Major mechanisms by which transplanted MSCs can contribute to cardiac repair and regeneration

can express cardiac-specific genes and have a molecular profile similar to that of native cardiomyocytes, but these cells may not be functionally integrated, as indicated by immature myofibril organization [87]. Despite much research claiming cardiomyogenic commitment of MSCs by molecular profiles, successful and functional integration is not always co-observed, highlighting that cardiomyogenic differentiation is still not entirely clear.

Paracrine mechanisms of MSCs

Despite controversial and incongruent results attempting to define mechanisms of regeneration of the post-MI myocardium with MSCs, thus far, the most accepted explanation is to attribute the beneficial effects to paracrine signaling. Literature regarding paracrine signaling is well-established and can be used to partially explain the vasculogenic, myogenic, and stem cell-mediated observations contributing to myocardial regeneration [15, 99]. Convincing evidence for this comes from studies whereby MSCs were cultured, and the culture media was applied as a therapeutic. In vitro, this cytokine concoction can stimulate the migration and differentiation of non-MSC stem cell populations [100] and reduce cell death in ischemic conditions [101]. In IGF-1- and VEGF-silenced cardiomyocyte co-cultures, these beneficial effects were negligible, and the frequency of apoptotic cells was similar to that of controls when exposed to hypoxia [73] showing the cardioprotective paracrine potential of MSCs. In vivo, conditioned medium alone reduced the death of cardiomyocytes, and even reduced the infarct size in a rat MI model [102]. Moreover, application of MSCs or MSC-conditioned medium both led to increases in ventricular function in a model of hamster heart failure when they were administered not in the myocardium, but in skeletal limb muscle [103]. Interestingly, MSCs were recently demonstrated to produce a significant amount of SDF-1 [104], a key signaling molecule for EPCs, suggesting EPC mobilization and homing as another mechanism by which MSCs can amplify the regenerative response. The field of stem cell-produced paracrine factors is vast and has been a resource for many years. From these experiments, novel approaches to paracrine signaling have emerged: transplantation of cytokine pre-conditioned stem cells, and genetically modified MSCs that over-produce cardiomyogenic cytokines, which are discussed later in this review.

Post-MI scar formation is inevitable. Large scars can hinder normal cardiac function, making the heart work harder to maintain normal cardiac function. MSC treatment may be able to reduce scar formation, thereby improving cardiac function. As early as 2 weeks after implantation, MSC treatment can reduce both the infarct size and the degree of fibrotic scar [94]. Many other studies have shown similar results of reduced fibrotic scar after direct MSC injection [88–91]. This effect has been hypothesized to be mediated by paracrine signaling; and the supporting evidence has come from a study in which the addition of MSCs to fibroblast cultures in vitro led to reduced transcription of collagen I and III and reduced the proliferation of fibroblasts [105]. This study similarly noted an improvement in heart function after MSC treatment, which was correlated with a reduction in collagen deposition/fibrosis. Interestingly, Wang et al. [106] delivered MSCs through an intra-coronary approach, and MSCs that localized to the heart had different patterns of differentiation depending on the site of engraftment: cells within the scar tissue appeared as a fibroblast phenotype, and non-infarct localized cells developed a cardiomyocyte phenotype.

Some studies have identified transplanted MSCs as mediators that can activate endogenous stem cell populations for a regenerative response. MSC treatment can lead to an increase in c-kit-expressing cells in the myocardium [88]. C-kit is the receptor for stem cell factor (SCF), a potent cytokine that is able to activate a variety of progenitor cells. Hatzistergos et al. [89] also identified clusters of c-kit$^+$ cells, believed to be CSCs, after MSC treatment in a porcine MI model. These cells were only localized within the infarcted regions, and not in the healthy myocardium. Their numbers increased 20-fold by 2 weeks post-treatment, and they interacted with native adult cardiomyocytes via connexin-43 and N-cadherin connections. MI can induce the production of systemic SCF and the mobilization of EPCs [86]; however, MSC treatment can augment both of these responses, maintaining a longer duration of SCF, and increasing the rate and numbers of mobilized EPCs in the circulation [86]. This evidence of endogenous stem cell populations' activation is plausibly mediated by paracrine mechanisms, whereby transplanted MSCs may modulate other cells via the secretion of soluble cytokines.

Another notable paracrine-mediated mechanism of MSC transplantation is the protection of diseased cardiomyocytes. When MSCs are simply added to cardiomyocyte cultures under hypoxic conditions, the frequency of viable cardiomyocytes is greatly increased [107]. Direct transplantation of MSCs into ischemic myocardium also reduced cell death [108], although the specific paracrine signals that are responsible for this phenomenon have not yet been deduced. A recent study by Lai et al. has suggested that the pro-survival signaling may be mediated not solely by secreted cytokines, but by newly identified exosomes (phospholipid particles) that were identified in MSC-conditioned medium [107]. Results of this study and others have shown that direct transplant of MSC-conditioned medium alone, whether mediated by secreted cytokines or exosomes, can improve recovery and reduce cell death in ischemic hearts [107, 109, 110].

Novel MSC therapies

Pre-conditioning of MSCs

Various pre-conditioning strategies with different growth factor mixtures have been employed to enhance the therapeutic potential of MSCs. Pre-treatment with IGF-1 can force sca-1$^+$ cells towards a cardiomyogenic phenotype [111]. Combination of IGF-1 and fibroblast growth factor-2 (FGF-2) generated a pro-angiogenic phenotype with an improved ability to form tubules in vitro and a greater potential to produce other growth factors [112]. These cells also upregulated the pro-survival gene Akt, as well as cardiac transcription factors GATA-4 and Nkx2.5. The addition of bone morpogenic protein-2 (BMP-2) to IGF-1 and FGF-2 pre-conditioned cultures also generated cells positive for cardiac markers GATA-4 and Nkt2.5, and had an anti-apoptotic effect on native cardiomyocytes in a rat MI model [113]. These pre-treated cells further reduced fibrotic scar areas and enhanced MSC engraftment, when compared to untreated MSCs. SDF-1 pre-treatment generated a robust MSC population that was resistant to peroxide-induced cell death and also produced significant amounts of VEGF [114]. Subsequent treatment showed these cells home to infarcts, where they proliferate extensively and appear to undergo cardiac differentiation, contribute to an accumulation of c-kit$^+$ cells, increase vascular density, and improve cardiac function.

Protein over-expressing MSCs

Early experiments of genetic overexpression were performed in 2003, whereby Akt, an anti-apoptotic pathway member, was over-expressed, creating cells termed Akt-MSCs [115]. These cells were resistant to apoptosis both in vitro and in vivo, and had an enhanced ability to develop into cardiomyocytes-like cells post-transplantation. Similarly to unmodified MSC transplantations, this experiment also normalized cardiac function of infarcted hearts. Subsequent experiments showed that Akt-MSCs could reduce infarct size and incorporate into the damaged myocardium [85]. Using cell tracking methods, the authors elucidated that cardiomyocytes were generated from cell fusion between Akt-MSCs and native cells, and not through differentiation. Furthermore, experiments using conditioned medium from Akt-MSCs not only protected cardiomyocytes from apoptosis, but its injection into infarcted hearts significantly limited the infarct size and improved cardiac function [102]. Akt-MSCs also upregulated several growth factor genes (IGF-1, FGF-2, VEGF), suggesting that the beneficial effects of these cells is mediated by paracrine factors.

Over-expression of SDF-1 by MSCs did not induce a regenerative response, but a highly cardioprotective one [104]. These cells increased cardiomyocyte survival post-infarct, and also led to an increase in vascular density. Similar approaches investigated the over-expression of VEGF (VEGF-MSCs). These cells surpassed unmodified MSCs in preserving heart functions, improving ejection fractions, and reducing infarct area, again thought to be partially mediated by increases in myocardial vasculature [116]. Similar work has also shown that VEGF-MSCs will improve heart function and increase vascular density [117, 118]. Although engrafted VEGF-MSCs can acquire a cardiomyocyte phenotype in vivo, they do not appear to be a cell population that incorporates into vasculature as terminally differentiated cells [118].

Limitations of MSC therapy & future directions

The ability to evade and somewhat suppress host immune responses make MSCs ideal candidates for transplantation, compared to EPCs, CSCs, and embryonic stem cells. All stem cells, however, maintain risks of unwanted differentiation into, and formation of, undesirable tissue. Short-term culture, or expansion of up to 8 weeks ex vivo, is considered a potentially safe method for MSC expansion. MSCs that were expanded past this time experienced accelerated growth rates, acquired many karyotypic abnormalities, and subsequent in vivo injection into immune deficient mice produced tumors in most organs [119]. Other studies have described the potential for MSCs to generate tumors after significant ex vivo culture [120]. Besides the formation of malignant growth, a major concern with MSC use is the generation of osteoblasts from MSCs, and the subsequent calcification that can occur. MSCs have been observed to deposit calcium, indicating the beginning of bone formation, within the scar tissue of mice post-MI [121]. Interestingly, these abnormalities were greatly reduced when the transplanted cell population was a heterogeneous mixture of unfractionateed bone marrow isolate, compared to purified MSCs. In order to proceed towards clinical application, we must be confident in the safety and efficacy of MSC therapy. Wolf et al. [122] performed a study demonstrating that autologous MSCs had a superior ability to reduce infarct size, compared to allogenic cells. One of the 24 pigs receiving cell transplants developed a cardiac tumor of mesenchymal tissue, severely limiting their study, but also greatly highlighting the risks of MSC usage for regenerative cardiac therapy. Our understanding of MSC usage would be greatly benefitted if, in addition to myocardial regeneration, reports also examined mesenchymal differentiation and/or tumor formation in treated animals, providing a clearer picture of the safety of MSC transplantation.

Another universal limitation of stem cell therapy is the poor engraftment and persistence of transplanted cells. Intracoronary infusion of cells has shown that there is ~5% in the myocardium after 2 h, and only ~1% after 18 h [123]. Intra-myocardial application may be a superior method for mid-to-long-term persistence, but the number of engrafted cells has been observed to be as low as 0.3% after 6 weeks [124]. Deployment of progenitor cells within a delivery vehicle, such as an injectable matrix, into muscle has greatly improved the engraftment and persistence of these cells, compared to cell-only injections [125]. Although the cells were only tracked for 3 h post-injection, the delivery matrix also prevented undesirable accumulation of cells in non-target tissues [125]. Delivery of cells using an injectable matrix has also been shown to augment their efficacy; specifically, material-based deployment of cells was superior to bolus cell injections in improving perfusion recovery and preventing necrosis in hindlimb ischemia [126]. Similarly, increased vessel densities in the ischemic hindlimb of rats have been observed with treatment of cell-impregnated materials, and this was correlated with a >2-fold improvement in injected cell persistence after 2 weeks [127]. Based on the ability of cell-delivery materials to augment both the efficacy and the engraftment of transplanted cells, this novel application method may serve as a future field for expanding our therapeutic repertoire using MSCs for the treatment of ischemic heart disease.

Although not consensually considered a limitation, a concern using MSCs in the myocardium is the risk of arrhythmogenesis. In vitro studies have shown that MSCs can induce arrhythmic phenotypes of cardiomyocytes, evidenced by a reduced conduction velocity which was not observed in cardiomyocyte mono-cultures [128]. In vivo, MSC transplantation increased the activation time and activation time dispersion, and these observations were positively correlated with the number of stem cells within the pacing site [129]. A 3-month follow-up after intravenous MSC administration in post-infarct pigs showed a reduction in the epicardial effective refractory period, suggesting arrhythmic development [130]. Despite this, it has been suggested that intramyocardial transplantation of stem cells is of greater risk for arrhythmia development, compared to intravenous administration [131]. Krause et al. have claimed that MSC administration is not arrhythmogenic [132], based on their porcine study. A similar study on humans also claimed no arrhythmogenic risk from MSC application [133], while another porcine study showed a reduction in arrhythmogenic risk after intracoronary MSC application [134]. Despite these results, there is not a clear consensus regarding the generation of arrhythmia following MSC transplantation.

Future research will ideally elucidate the optimal time and conditions for MSC transplantation. In one attempt at this, MSCs were transplanted into rat hearts either 1 h, or 1 or 2 weeks following infarction [135]. Animals that received MSC transplantation 1 week post-MI demonstrated the greatest cardiomyocyte function, as well as the greatest induction of angiogenesis and persistence of transplanted cells. Despite these phenotypic results, cardiac function, represented by fractional shortening measurements, was unchanged between groups receiving cell transplantation immediately- or 1-week post-MI [136]. To date, the "optimum time" for MSC transplantation post-infarction has not yet been elucidated. Poor persistence of transplanted MSCs is believed to be attributed to a combination of apoptosis/anoikis and necrosis [137].

Transplantation of MSCs into ischemic myocardium may improve cardiac function, mediated by the growth of new vasculature and paracrine effects on stem cell recruitment and on cardioprotection, and possibly also through de novo generation of cardiomyocytes. These cells hold promise for regenerative therapies; however future research will better elucidate the mechanisms of these phenomena and improve current methodologies for maximal efficacy. Before clinical application, a better understanding of the safety and risks of MSC transplantation will also be needed.

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