

# Harnessing the Mesenchymal Stem Cell Secretome for the Treatment of Cardiovascular Disease

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The broad repertoire of secreted trophic and immunomodulatory cytokines produced by mesenchymal stem cells (MSCs), generally referred to as the MSC secretome, has considerable potential for the treatment of cardiovascular disease. However, harnessing this MSC secretome for meaningful therapeutic outcomes is challenging due to the limited control of cytokine production following their transplantation. This review outlines the current understanding of the MSC secretome as a therapeutic for treatment of ischemic heart disease. We discuss ongoing investigative directions aimed at improving cellular activity and characterizing the secretome and its regulation in greater detail. Finally, we provide insights on and perspectives for future development of the MSC secretome as a therapeutic tool.

## Introduction

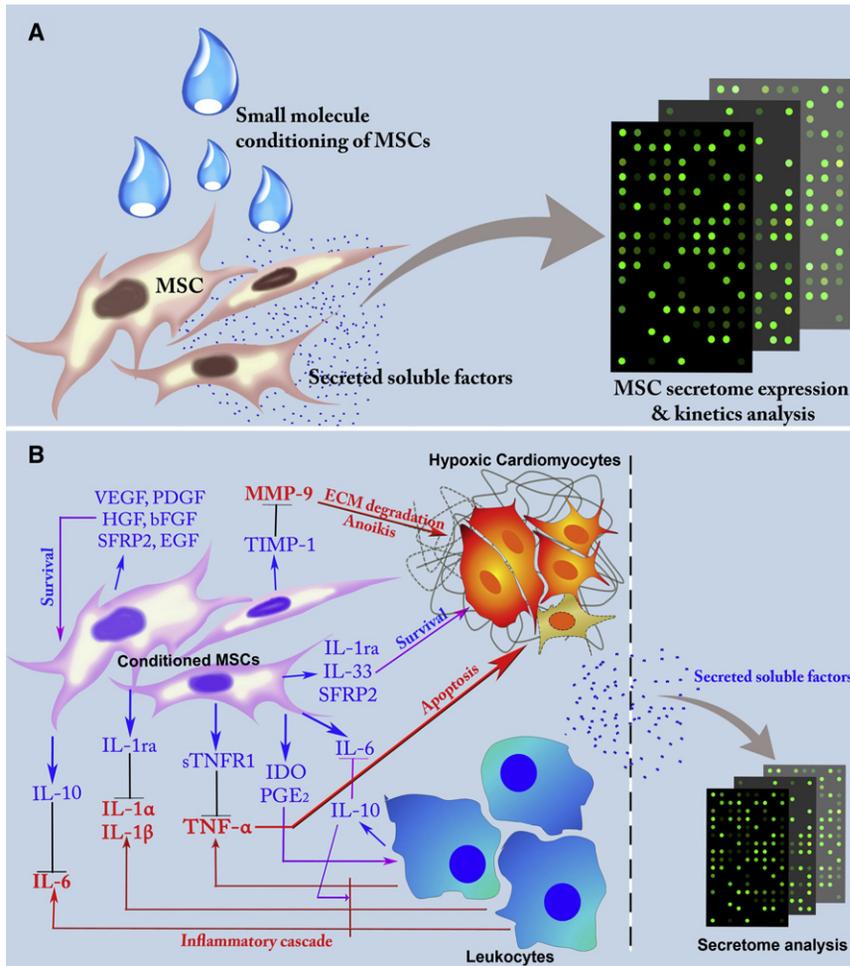
Ischemic heart disease is the leading cause of human mortality globally, resulting in about 7.25 million deaths each year (World Health Organization, 2011). Acute myocardial infarction (AMI) is the most common cause of heart failure. AMI triggers a series of cellular and molecular changes leading to apoptosis, necrosis, and hypertrophy of cardiomyocytes; impaired neovascularization; interstitial fibrosis and inflammation; reduced contractility; and pathological remodeling. Current therapies have failed to address the devastating aftermath of AMI. Most clinically approved therapeutics focus on modulating hemodynamics to reduce early mortality but do not facilitate cardiac repair in the way that would be needed to reduce the incidence of heart failure (Velagaleti et al., 2008). It is now widely accepted that treatment of the complex pathology resulting from AMI will require taking approaches designed to enhance tissue regeneration via cell transplantation or co-opting local mechanisms that promote healing and inhibit pathological remodeling (Wollert and Drexler, 2010).

Regeneration of an infarcted heart necessitates massive cell replenishment, possibly in the order of a billion cardiomyocytes, and functional integration together with supporting cell types (Lafamme and Murry, 2005). While the search for cardiac-progenitor cells (CPCs) that can readily engraft within damaged tissue and differentiate into functioning cardiomyocytes continues (Xu et al., 2011), regenerative therapy using bone-marrow-derived mononuclear cells (BM-MNCs) and mesenchymal stem cells (MSCs) has shown considerable promise in preclinical studies (Chavakis et al., 2010; Mirotsoy et al., 2011). The first stem-cell-based clinical trials for MI (initiated between 2002 and 2005) used unfractionated, easily accessible, and highly heterogeneous adult BM-MNCs. Despite initial positive results indicating safety of BM-MNC transplantation and

improved cardiac function, the differences in trial design, treatment methods, outcome evaluation, and cell isolation have prevented general conclusions, and all of these studies require long-term follow-up analysis (Wollert and Drexler, 2010).

Recent clinical trials have looked at relatively homogenous MSCs expanded in culture after isolation from bone marrow (containing 0.001%–0.01% MSCs) as potential cell therapy candidates for AMI owing to their immunomodulatory properties, ready availability, and cardiac stem cell (CSC) niche-regulatory ability. The first clinical trial for AMI using human MSCs (hMSCs) demonstrated the safety of hMSC transplantation and provisional efficacy (Hare et al., 2009). However, the improved cardiac function observed in preclinical studies is without long-term MSC engraftment (Iso et al., 2007), and, in animal studies, systemically administered MSCs exhibit low (~2%) engraftment levels and limited capacity for transdifferentiation into cardiomyocytes posttransplantation (Leiker et al., 2008). Thus, it seems unlikely that MSCs contribute directly to replenishing cardiomyocyte populations in the heart, and this notion motivated MSC-induced immunomodulatory and remodeling effects to be proposed as mechanisms of cardiovascular repair. Although the trophic and immunomodulatory properties of MSCs represent a primary mechanism of therapeutic action that is referred to in many current clinical trials (Ankrum and Karp, 2010; Wollert and Drexler, 2010), it is important to note that these functions of MSCs have not yet been optimized in preclinical models to maximize their therapeutic potential.

The spectrum of regulatory and trophic factors secreted by MSCs, including growth factors, cytokines, and chemokines, is broadly defined as the MSC secretome. A thorough in vivo examination of this MSC secretome and strategies to modulate it are still lacking, but seem essential for rational therapy design and improvement of existing therapies. Despite the absence of



**Figure 1. A Proposed Approach for Small-Molecule-Mediated Regulation of the MSC Secretome**

(A) Conditioning MSCs with small molecules can stimulate the production of a customized secretome that can be optimized and characterized in vitro.

(B) The effect of small molecule conditioning of MSCs can be tested under highly dynamic, simulated conditions, mimicking microenvironments before and after the onset of MI, and this may include coculture assays with hypoxic cardiomyocytes and inflammatory cells. Proinflammatory cytokines such as TNF- $\alpha$  and IL-6 can be introduced to an MSC culture, for example, and MSC-secreted factors (in blue) such as sTNFR1 and IL-10 can be tracked, as can their ability to modulate the release of inflammatory cytokines from activated leukocytes. MSCs may secrete molecules such as IDO and PGE<sub>2</sub> that induce leukocytes to produce anti-inflammatory cytokines such as IL-10 that attenuate the effects of activated leukocytes and inhibit the proinflammatory activity of constitutively secreted IL-6 (from MSCs) and/or IL-6 already expressed in the myocardium. Likewise, to attenuate pathological remodeling, one can test if small molecules can boost MSC secretion of TIMP-1 to inhibit ECM-degrading proteases such as MMP-9. In addition to paracrine effects, conditioned MSCs may act through autocrine signaling to improve cell survival in hypoxic conditions.

### Profiling the MSC Secretome: Tools and Critical Parameters

To define the specific roles of MSC-secreted factors in cardiovascular regeneration, one should start with biomolecular profiling or secretome analysis of cultured primary MSCs (Figure 1A). A

such in vivo data, current MSC-based approaches have shown some promise in preclinical models. In these cases the secretome was modulated by physiological (hypoxic or anoxic), pharmacological (small molecule), cytokine, or growth factor preconditioning and/or genetic manipulations (Afzal et al., 2010; Kamota et al., 2009; Shi et al., 2009; Tang et al., 2010) prior to transplantation. Nevertheless, several questions regarding MSC secretome function and regulation remain unanswered, including the following: (1) what are the most effective approaches to study the MSC secretome in vivo, and are new technologies required to achieve this? (2) How do the properties of the MSC secretome (composition and sustainability) change in vitro and after transplantation, and how does it evolve as a function of the dynamic local microenvironment? (3) What are the best methods to achieve sustainability of the secretome and control over its composition posttransplantation?

Here we discuss current understanding of the MSC secretome and put in perspective its application to cardiovascular therapy. We also review tools for MSC secretome profiling and current preconditioning strategies that aim to transiently control the secretome posttransplantation. Finally, we suggest approaches that could exploit the MSC secretome for cardiovascular therapy.

typical MSC secretion profile comprises growth factors, cytokines, extracellular matrix (ECM) proteases, hormones, and lipid mediators (typically in low abundance). Thus, MSC in vitro secretome analysis must consider the effect of serum, which contains many overlapping components and can interfere with detection. To circumvent this problem, MSCs can be cultured for a short time frame in serum-free medium or medium with defined serum replacements. It is critical to consider that secretome expression in vitro is likely very different from what would be expected in vivo where cells within different microenvironments would exhibit unique secretome expression profiles. Conversely, as microenvironments are highly dynamic, they would in turn impact the kinetics of secretome expression. Thus, moving forward it will be critical to examine secretome expression in vitro under conditions that model several relevant in vivo microenvironments.

The tools available for studying secretome expression in vitro include multiplex antibody-based techniques, such as antibody arrays that offer high sensitivity (typically 1–10 pg/ml) as well as high specificity, reproducibility across a broad range of concentrations, and the potential for massively parallel experimentation. High-throughput analysis of the hMSC secretome using a human cytokine antibody array, for example, identified at least 40 proteins with high expression levels varying from 10% to 110% spot intensity relative to the negative control and normalized to

a positive control (Parekkadan et al., 2007). Antibody arrays have also been employed to assess the contribution of MSC-derived factors such as VEGF, TIMP-1, TIMP-2, and TSP-1 in cardiac improvement in swine MI models (Nguyen et al., 2010). The impact of hMSC tissue origin on secretome characteristics (bone marrow versus umbilical cord) has also been examined (Park et al., 2009) using antibody arrays. IL-8 was secreted at higher concentrations in umbilical-cord-blood-derived-MSCs (UCB-MSCs), while IGBP class cytokines were specific to UCB-MSCs compared with BM-MSCs, indicating a potential origin-specific hMSC secretome.

In addition to antibody-based approaches, Liquid Chromatography with Tandem Mass Spectrometry Detection (LC-MS/MS) is useful for characterizing the secretome profile. For example, preconditioning of human adipose-tissue-derived MSCs with TNF- $\alpha$  had a profound impact on the secretome detected using LC-MS/MS (Lee et al., 2010), and led to increased expression of cytokines and chemokines such as IL-6, IL-8, MCP-1, MMPs, PTX3, and Cathepsin L. However, LC-MS/MS was unable to detect many cytokines and growth factors that were present in low concentrations. A more systematic integrated approach for hMSC secretome analysis included LC-MS/MS detection, antibody arrays, microarrays, and bioinformatics (Sze et al., 2007), and identified 201 unique proteins (132 using LC-MS/MS and 72 using antibody arrays). Importantly, Sze et al. used computational analysis to predict the roles of the secretome components in metabolism, immune response, and development.

While current techniques have been useful to identify factors expressed at high levels such as IL-6, IL-8, TIMP-2, VEGF, and MCP-1, suggesting constitutive secretion from BM-hMSCs (Park et al., 2009), a complete list of constitutively expressed MSC secretome factors remains to be generated. Despite recent advances in the characterization of the MSC secretome, current techniques suffer from multiple deficiencies. Gel-based and LC-MS/MS techniques have limited sensitivity to molecules in low concentrations (10–20 fmol), and antibody-based techniques (e.g., ELISA and antibody arrays) are limited by the availability of antibodies to detect secreted proteins. Hence, comprehensive *in vitro* secretome profiling requires an integrated approach employing multiple techniques. Although determining the mechanism regulating the expression of the secretome is important, the task is made more challenging given that some of the proteins are released during cell death (Skalnikova et al., 2011). Perhaps the most important goal will be to move toward methods to profile the secretome *in vivo* that can distinguish between factors released from the host versus those secreted by the transplanted MSCs. Reaching this point will require the development of new techniques that can directly quantify the dynamic expression profile of MSC-secreted factors both locally and systemically.

### Close to the Heart? Relevance of MSC Paracrine Signaling to Cardiovascular Therapy

Recent studies have suggested four potential mechanisms for how exogenous-culture-expanded MSCs may contribute to cardiovascular repair: MSC transdifferentiation into cardiomyocytes (Hatzistergos et al., 2010), fusion of MSCs with native cells (Noiseux et al., 2006), MSC-induced stimulation of endogenous

CSCs via direct cell-cell interaction (Mazhari and Hare, 2007), and MSC-paracrine (or endocrine) signaling (Gnecchi et al., 2005; Lee et al., 2009). MSC transdifferentiation into contractile cardiomyocytes is inefficient at best (Toma et al., 2002) and occurs only in the presence of native cardiomyocytes (Hatzistergos et al., 2010; Loffredo et al., 2011; Mazhari and Hare, 2007). Cell fusion is a rare event, which rules out substantial involvement in MSC-mediated cardiovascular regeneration (Loffredo et al., 2011). Nevertheless, there is strong evidence emerging that rat BM-derived MSCs (rMSCs) secrete trophic factors that may induce activation and proliferation of endogenous CPCs *in vitro* (Nakanishi et al., 2008). Although it is possible that resident CSCs may differentiate into mature and functional cardiomyocytes upon interaction with transplanted MSCs (Hatzistergos et al., 2010), evidence suggests that CSCs possess only a limited capacity to differentiate into fully mature cardiomyocytes with an adult phenotype (Beltrami et al., 2003; Urbaneck et al., 2005). Despite evidence of preferential accumulation of MSCs at sites of myocardial ischemia (Williams and Hare, 2011), exogenously administered MSCs show poor survival and do not persist at the site of AMI (Iso et al., 2007; Terrovitis et al., 2010), probably because of the harsh ischemic microenvironment, characterized by oxidative stress, inflammation, cytotoxic cytokines, and in some instances an absence of ECM for MSC attachment (Rodrigues et al., 2010; Song et al., 2010). Such a hostile microenvironment could hinder the interaction of MSCs with endogenous CSCs.

A more plausible explanation for MSC-mediated cardiovascular repair is an effect on host cells and the microenvironment via MSC-secreted growth factors, cytokines, and other signaling molecules. This proposal is supported by recent preclinical studies (Kanki et al., 2011; Timmers et al., 2011) that demonstrated improved cardiac function upon infusion of cytokines or MSC-conditioned medium (without cell transplantation) (Beohar et al., 2010). Therefore, identifying key MSC-secreted factors and their functional roles in cardiovascular therapies seems a useful approach for rational design of next-generation MSC-based therapeutics.

### Effects of the MSC Secretome on Cardiovascular Repair

The functional roles reported for MSC-secreted factors are both impressive and confusing. MSCs are known to be the source of multiple immunomodulatory agents plus trophic factors involved in repair and regenerative processes (Nauta and Fibbe, 2007; van Poll et al., 2008). This broad array of secreted factors suggests possible stress response regulatory roles for MSCs, such as homing of c-kit<sup>+</sup> cells to injured myocardium (Tang et al., 2010). It is not known whether cytokines released from stressed or dying MSCs make a therapeutic contribution. The hMSC secretome includes multiple factors (Lee et al., 2010; Parekkadan et al., 2007; Park et al., 2009; Sze et al., 2007) known to promote cardiovascular repair (Table S1 available online) and factors that negatively modulate cardiomyocyte apoptosis, inflammation, and pathological remodeling (Table S2). Although several factors in the MSC secretome have shown utility for influencing cardiac repair when delivered exogenously in the absence of MSCs (factors listed in Tables S1 and S2 not marked by an [\*]), it is still critical to demonstrate the direct functionality of such factors when secreted from MSCs and the potential synergy that may exist with other secreted factors.

In the context of cardiovascular repair, the array of potential therapeutic mechanisms offered by MSC secretome components spans tissue preservation (antiapoptotic and promitotic), neovascularization, cardiac remodeling (ECM alteration and strengthening of the infarct scar), anti-inflammatory responses (antifibrosis and suppression of inflammatory cells), and the highly contentious endogenous regeneration (activation of CPCs and CSCs). MSCs induce myocardial protection by promoting cardiomyocyte survival and preventing apoptosis through activation of PKC, PI3K/Akt, NF- $\kappa$ B, and STAT3 signaling (Gnecchi et al., 2008; Mirosou et al., 2011). In ischemic animal models, MSCs mediate neovascularization via paracrine signaling (Kinnaird et al., 2004a, 2004b; Matsumoto et al., 2005; Tang et al., 2005) and have antiapoptotic, anti-inflammatory, and antifibrotic effects on cardiomyocytes and endothelial cells (Bartosh et al., 2010; Berry et al., 2006; Iso et al., 2007; Lee et al., 2009; Shabbir et al., 2009). MSC-induced immunomodulation and antiapoptosis of cardiomyocytes that has been observed in inflammatory heart diseases such as acute myocarditis in mice (Van Linthout et al., 2011) and sepsis in rats (Weil et al., 2010) are likely mediated via paracrine effects. In addition, MSCs exert immunomodulatory effects by inducing neighboring cells to secrete relevant cytokines (Aggarwal and Pittenger, 2005; François et al., 2012; Németh et al., 2009; Prockop and Oh, 2012), which may be useful in inhibiting excessive inflammation and pathological remodeling under MI settings.

#### **MSC Homing to the Injured Myocardium: The Role of MSC Secretome**

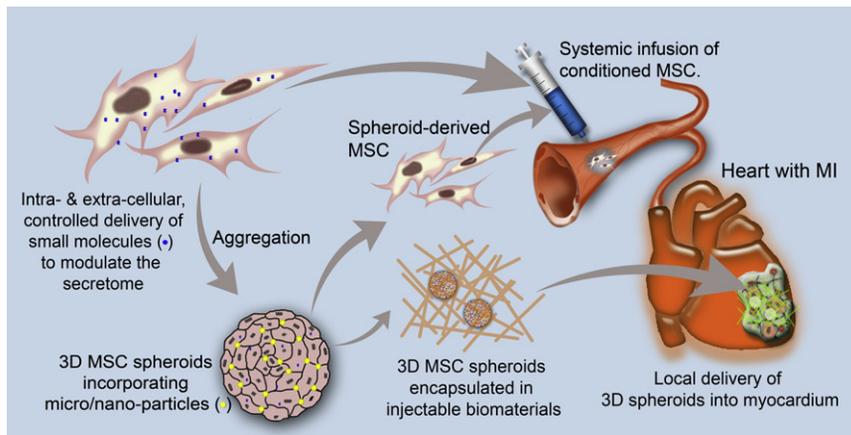
There is significant debate about whether MSCs need to engraft at the target site of injury or can exert their effects systemically. Engraftment at the target site would in principle seem beneficial due to the potential for cell-cell contact and increased concentrations of immunomodulatory and trophic factors. In the context of cell homing following systemic infusion, sites of MI exhibit increased expression and secretion of selective chemokines, cytokines, and cell adhesion molecules, including ICAM-1, IL-6, SDF-1, VCAM-1, and FN-1 (Ip et al., 2007). However, culture-expanded MSCs exhibit limited homing capacity, probably because of poor expression of receptors for chemokines and adhesion ligands such as CXCR4 and CCR1. As the number of transplanted MSCs homing to the infarcted heart rapidly declines following intravenous infusion (Assis et al., 2010) due to entrapment in the microvasculature, there is a significant need to improve circulation times and homing efficiency of systemically administered cells (Karp and Leng Teo, 2009). For instance, genetic engineering of MSCs has been employed to overexpress key chemokine receptors such as CXCR4 (Cheng et al., 2008) and CCR1 (Huang et al., 2010), and growth factor preconditioning has been used (Hahn et al., 2008; Son et al., 2006) to increase MSC homing to injured myocardium and improve cardiac performance. In addition, bioengineering approaches offer significant potential for chemically modifying the hMSC surface to improve homing to sites of inflammation (Sarkar et al., 2011b). Interestingly, MSCs secrete mobilizing factors such as HGF, LIF, SDF-1, SCF, and VE-Cadherin (Table S1) and thus, optimizing the transplanted MSC secretome could also be beneficial for mobilization and homing of host MSCs.

#### **Striking a Balance between Positive and Negative Factors**

Some factors in the MSC secretome, depending on the concentration and release kinetics, may exert inhibitory effects on the cardiac microenvironment, such as apoptosis of cardiomyocytes, inflammation, pathological remodeling, or scar formation. For instance, the TGF- $\beta$  class of cytokines secreted from poly(I:C)-treated, TLR3-primed hMSCs (Waterman et al., 2010) are known to mediate pathological remodeling during MI and their repressed secretion likely results in decreased collagen deposition. MMP-2, a factor known to mediate ECM degradation during MI (Matsumura et al., 2005) resulting in pathological remodeling via cardiomyocyte anoikis and macrophage infiltration, is endogenously secreted by hMSCs, and the activity of hMSC-secreted MMP-2 can be inhibited by treating hMSCs with TNF- $\alpha$  or hypoxia (Lozito and Tuan, 2011). Additionally, MSC-secreted factors such as MMP-9 and IL-6, responsible for pathological remodeling and proinflammatory responses, respectively, should ideally be maintained at minimum levels because these factors are upregulated in the myocardium during MI (Biswas et al., 2010; Liu et al., 2011). Inhibition of negative factors using antagonists (produced by MSCs) such as TIMP-1 (for MMP-9) and IL-10 (for IL-6) via either intracellular or extracellular targets is one possible strategy for alleviating these effects. Hence, it seems important to not only consider upregulating anti-inflammatory or proangiogenic factors, but also to strive to achieve an appropriate balance between stimulatory and inhibitory factors produced by MSCs as depicted in Figure 1B. In addition to achieving such a balance through iterative *in vitro* experiments, ultimately the response will need to be preserved following *in vivo* transplantation, perhaps through bioengineering approaches (Sarkar et al., 2011a) and strategies illustrated in Figure 2.

#### **Bench to Bedside: Practical Considerations for Harnessing the MSC Secretome in Clinical Settings**

The first clinical trial for AMI using hMSCs was a randomized, double-blinded, placebo-controlled, dose-escalation study of allogenic hMSCs (Prochymal, Osiris Therapeutics, Inc., Baltimore, MD) (Hare et al., 2009). This study demonstrated the safety of intravenous hMSC transplantation and provisional efficacy (increased left ventricular ejection fraction [LVEF], reduced cardiac arrhythmias, and reverse remodeling compared to placebo) in AMI patients. Results from a phase II multicenter, randomized, double-blind, placebo-controlled study to evaluate Prochymal for safety and efficacy are anticipated in the near future. The ongoing MSC-based trials for treatment of cardiovascular diseases listed in Table 1 reveal an interesting trend in trial designs, in which MSC paracrine mechanisms for improving angiogenesis, cardio-myogenesis, stimulating endogenous cardiac progenitors, and inhibiting remodeling have been highlighted as the primary modes of action. The interim follow-up of two ongoing trials (NCT00677222 and NCT00721045) has reported significant improvement in cardiac functions such as LVEF and stroke volume, and a reduced number of patients with major adverse cardiac events. Nevertheless, the performance of MSCs in these clinical trials has not uniformly met expectations, because positive results and statistical significance were not achieved for all output measures, the



**Figure 2. A Proposed Engineering Solution for Sustaining a Customized MSC Secretome In Vivo and Facilitating Cardiovascular Repair**

Bioengineering strategies may be employed to control and sustain the expression of the customized MSC secretome through smart-biomaterials-based, intra- and/or extra-cellular, controlled release of stimulating molecules. MSCs, either as single cells or aggregates, may be systemically infused or locally transplanted to facilitate cardiovascular repair with greater control over cell fate and function.

mechanism of action is not fully understood, and the MSC formulations are not fully optimized in terms of delivery methods, secretome composition, cell survival/persistence, and engraftment efficiency. Furthermore, little is known regarding the effect of time of MSC administration on the prevention of cardiomyocyte necrosis/apoptosis, which develops rapidly within 30 min to 12 hr following the onset of MI (Schoen, 2007).

Direct involvement of factors secreted by MSCs in cardiac functional improvement in humans is difficult to demonstrate. Although identification and quantification of the myocardial tissue concentrations of paracrine factors is not feasible, their plasma levels could be indicative of their presence. Future clinical trials should therefore incorporate systematic analysis of the patient plasma not only to elucidate the presence/absence of MSC-secreted paracrine factors but also to investigate whether the impact on host tissue is sustained after elimination of the transplanted MSCs. Although it is challenging to determine whether paracrine factors originate from host cells or transplanted MSCs and to characterize their impact on regulating cytokine expression from host cells (new techniques may be required), a comparative analysis of the patient plasma before and after MSC treatment may provide some insight (and be useful for establishing biomarkers for MSC therapy). Approaches for upregulating specific paracrine factors may help to elucidate (indirectly) mechanisms responsible for the MSC-mediated clinical outcomes (Mirotsov et al., 2007). Although some studies have shown that endocrine activity of dying MSCs can promote regeneration of distant ischemic tissues (Lee et al., 2009), the impact of the MSC secretome on cardiovascular repair can likely be improved through enhancing the survival of the transplanted cells and improving their homing to the target site (Karp and Leng Teo, 2009). Hence, MSC modifications that lead to improved survival and facilitate a sustained and regulated secretome should be considered.

#### **“Cell-free” Therapy: An Alternate to Using MSCs?**

Several clinical trials have investigated cytokine therapy approaches for treating cardiovascular diseases (Beohar et al., 2010), and this is further motivated by the improvement in cardiac function seen in preclinical studies from administration of MSC-conditioned medium (Gnecchi et al., 2006). For example, VEGF protein delivery has been shown to improve angiogenesis in coronary artery disease patients (Henry et al.,

2003). G-CSF, a cytokine known to mobilize progenitor cells from the bone marrow, was subsequently explored in a series of AMI clinical trials. Despite evidence of safety and feasibility of G-CSF administration in MI patients (Valgimigli et al., 2005), treatment with G-CSF with 5 or 10  $\mu\text{g}/\text{kg}/\text{day}$  via subcutaneous injection 5 to 6 days after percutaneous coronary intervention (PCI) did not yield any significant increase in LVEF (Engelmann et al., 2006; Ripa et al., 2006). Other cytokines such as GM-CSF, EPO, and IGF-1 have also been tested in clinical trials of cardiovascular diseases (Beohar et al., 2010). To date, single cytokine therapy trials have not met expectations, and there are several possible explanations for why this is the case. Multiple cytokines/growth factors may need to be administered simultaneously at different concentrations and time points to act synergistically to achieve a therapeutic effect. Side effects due to high doses of certain cytokine/growth factors, which may be required due to challenges in protein delivery, can lead to the formation of aberrant and leaky vessels (Carmeliet, 2005), hypotension (Henry et al., 2001), and tumor angiogenesis (Epstein et al., 2001). Controlling the local levels of exogenously delivered cytokines is critical given limitations of pharmacokinetics and stability of proteins in vivo. For instance, intramyocardially delivered, protease-resistant SDF-1 was undetectable after 1 day, yet controlled-release, protease-resistant SDF-1 tethered to self-assembling peptide nanofibers was retained within the myocardial tissue even at day 7 in a rat MI model, and this persistence translated into significant improvement in capillary density and LVEF. Controlled local release of SDF-1 also led to a substantial increase in  $c\text{-kit}^+$  cell recruitment into the myocardium (Segers et al., 2007). Recombinant periostin exhibited enhanced tissue distribution and persistence via controlled local delivery from Gelfoam patches in a rat MI model (Kühn et al., 2007). Compared to the delivery of single growth factors or cytokines, the use of cells such as MSCs to supply these agents offers significant potential for sustained pharmacokinetics, synergy from multiple factors, and an opportunity for systemic infusion, which is less invasive than local injection and thus amenable to repeated dosing.

#### **Secrets of the MSC Secretome: Underlying Signaling Pathways**

Elucidation of the molecular pathways mediating MSC secretome expression is a crucial step toward improving our understanding of the secreted factor profile and its clinical utility.

**Table 1. Ongoing MSC-Based Clinical Trials for Cardiovascular Diseases Registered at [clinicaltrials.gov](http://clinicaltrials.gov)**

Clinical Trial ID	Phase	Condition	No. of Patients	Outcome Measure	Cell Delivery Route	Basis of Trial Design
NCT01394432	III	AMI	50	LVSV	endocardial	reduction in scar formation and increased reverse remodeling
NCT00877903	II	MI	220	ESV, LVEF, infarct size	intravenous	improvement in myocardial remodeling and reduction in incidence of CHF
NCT00790764	II	SCI	60	safety	intracoronary and transendocardial	development of mature and stable vessels and improved cardiac function via combinatorial effect of BM-MNCs and MSCs
NCT00555828	I/II	MI	25	safety, feasibility	transendocardial	transdifferentiation of mesenchymal precursor cells (MPCs) into cardiomyocytes
NCT00677222	I	AMI	28	safety, efficacy	space surrounding target vessel (perivascular)	improvement in cardiac function via MSC paracrine actions
NCT01291329	II	AMI	160	myocardial metabolism, perfusion, LVEF	intracoronary	transdifferentiation of MSCs into cardiomyocytes
NCT00768066 (TAC-HFT)	I / II	IHF	60	safety	transendocardial	stimulation of endogenous cardiac stem cells by the transplanted MSCs
NCT00644410	I / II	CHF	60	LVEF	intramyocardial	development of new myocardium and blood vessels
NCT00587990 (PROMETHEUS)	I / II	LVD	45	safety, LVEF, infarct size, ESV	intramyocardial	combinatorial effects of bypass surgery and MSC transplantation
NCT00721045	II	HF	60	safety, efficacy	transendocardial	MPC-induced large blood vessel formation and cardiac repair
NCT00418418	II	MI	60	LVEF, safety	intramyocardial	combinatorial effects of bypass surgery and MSC transplantation
NCT00883727	I / II	MI	20	myocardial perfusion, infarct size	intravenous	transdifferentiation of MSCs into cardiomyocytes and production of new blood vessels
NCT01087996 (POSEIDON)	I / II	LVD, MI	30	safety, efficacy	transendocardial	neo-myogenesis induced by transplanted allogenic and autologous MSCs
NCT01076920 (MESAMI)	I / II	MI, LVD	10	safety, efficacy	transendocardial	transdifferentiation of MSCs to produce new blood vessels
NCT01449032	II	CMI	60	safety, efficacy	not specified	angiogenesis
NCT01442129	II	HF	30	safety, efficacy	intramyocardial	MPC-induced angiogenesis via paracrine signaling combined with LVAD implantation
NCT01392625	I / II	NDC	36	safety, efficacy	transendocardial	neomyogenesis via MSC-CSC interaction
NCT01270139 (NANOM)	I / II	CAD	180	plaque volume	stenting	functional restoration of blood vessels via nanoburning and MSC paracrine effects
NCT01436123 (NANOM2)	I	CAD	120	plaque volume	stenting	reduction of plaque via paracrine signaling in combination with burning effects from Si-Fe NPs

LVSV, left ventricular systolic volume; SCI, severe coronary ischemia; IHF, ischemic heart failure; CHF, congestive heart failure; LVD, left ventricular dysfunction; ESV, end systolic volume; LVEF, left ventricular ejection fraction; CMI, chronic myocardial ischemia; LVAD, left ventricular assist device; NDC, nonischemic dilated cardiomyopathy; CAD, coronary artery disease.

Although further research is required to fully delineate the signaling mechanisms involved in the expression of the MSC secretome, a wide array of signaling pathways have been implicated in paracrine-mediated cardiac repair by MSCs (Gnecchi et al., 2008).

The PI3K/Akt pathway is believed to be involved in the production and secretion of paracrine factors by rMSCs (Gnecchi et al., 2005, 2006). Genetically modifying rMSCs to overexpress the *Akt* gene resulted in the upregulation of the *Akt* target genes *VEGF*, *FGF-2*, *IGF-1*, *HGF*, and *Thrombospondin-4* (Gnecchi

et al., 2006). PI3K signaling and ERK1/2 signaling have also been implicated in VEGF production by mMSCs in response to exogenous IL-6 in vitro (Herrmann et al., 2011), although it is unclear whether the effect is solely from the exogenous IL-6 treatment because mMSCs constitutively secrete IL-6. In another study, AngII-stimulated VEGF expression and secretion from rMSCs was mediated by ERK1/2 and the Akt pathway via angiotensin II type 1 (AT<sub>1</sub>) receptor in vitro (Shi et al., 2009). Another important signaling pathway is the p38 mitogen-activated protein kinase (p38 MAPK), which mediates hMSC

paracrine activity. p38 MAPK was activated as a stress (TNF- $\alpha$ -induced) response, and led to increased in vitro production of VEGF, HGF, and IGF-1 by hMSCs (Wang et al., 2006). The p38 MAPK pathway, along with MEK and PI3K, has also been implicated in mediating TGF- $\alpha$ -induced in vitro HGF production in hMSCs via EGF receptor (EGFR) (Wang et al., 2009). The involvement of p38 MAPK in TGF- $\alpha$ -induced in vitro VEGF production in mMSCs via EGFR (Herrmann et al., 2010) and serum-free-medium-induced in vitro production of IL-6, IL-8, and CXCL1 (Yew et al., 2011) in hMSCs have also been reported.

The JAK-STAT cascade is thought to be a central regulatory pathway in MSC paracrine factor expression. For instance, STAT3 and p38 MAPK were shown to mediate the TNF- $\alpha$ -stimulated VEGF production by mMSCs in vitro (Wang et al., 2007). However, whether VEGF production is independently controlled by p38 MAPK and STAT3 or via a crosstalk between these pathways is not yet clear. STAT3 and MAPK were also activated by treating hMSCs with IL-6, leading to improved in vitro hMSC migratory potential, likely via paracrine activity (Rattigan et al., 2010). In another study, knockout of toll like receptor-4 (TLR4) in mMSCs resulted in an increased in vitro secretion of angiogenic factors and chemokines and decreased secretion of inflammatory chemokines via STAT3 activation (Wang et al., 2010), further highlighting the role of STAT3 signaling in the MSC secretome expression. The transcription factor GATA-4 has also been implicated in the increased rMSC production of angiogenic paracrine factors (VEGF, IGF-1, and bFGF) and was shown to possess antiapoptotic effects on MSCs under stress via GATA-4 overexpression (Li et al., 2010).

The transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) is considered a central regulator of stress response and a key mediator of immune responses, regulating the expression of more than 150 target genes (Pahl, 1999) that code for cytokines, chemokines, growth factors, cell adhesion proteins, and cell surface receptors. NF- $\kappa$ B function in hMSCs has been investigated under stress conditions, such as TNF- $\alpha$ , lipopolysaccharide (LPS), and hypoxia (Crisostomo et al., 2008), and its activation was implicated in the increased in vitro production of several growth factors, such as VEGF, FGF-2, and HGF, by hMSCs in response to stress conditions. The involvement of NF- $\kappa$ B via TLR4 receptor activation is demonstrated in the production of prostaglandin E<sub>2</sub> in hMSCs upon treatment with LPS, leading to a reduction in inflammation in a cecal ligation and sepsis (CLP) model in mice (Németh et al., 2009). Recently, more evidence of the involvement of NF- $\kappa$ B signaling in rMSC paracrine factor expression has been reported (Afzal et al., 2010). Diazoxide (DZ), a K<sub>ATP</sub>-channel-opening small molecule, concomitantly augmented the phosphorylation of PI3K/Akt, glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), and NF- $\kappa$ B in rMSCs, resulting in elevated expression levels of growth factors such as IGF, bFGF, HGF, Ang-2, and VEGF in vitro.

Although the mechanistic studies conducted so far have provided some key insights, a more comprehensive understanding of the signaling networks responsible for the unique MSC secretome is still required. The network of signaling pathways involved in constitutive expression of the MSC secretome has not been elucidated yet, but clearly there is a major role for stress signaling that may represent the MSCs' ability to sense and respond to specific stimuli and allow the cells to cope with

changing environmental conditions. A comparative study of evolutionarily conserved signaling pathways that mediates MSC secretome expression could be informative. The activation/inhibition of multiple pathways could be essential to obtain an appropriately customized balance of secreted factors. The interaction of kinases with transcription factors is also not well understood. Additionally, two or more transcription factors, for example GATA and STAT, could also interact at the transcriptional level to mediate paracrine secretion (Wang et al., 2005). Considering the complexity of signaling networks, a holistic approach must be used to establish the specific role of receptors, kinases, and transcription factors in the MSC secretome. Such an approach could provide a useful axis for enhanced control over the secretome profile, leading to the development of precisely regulated MSC therapies.

### The Secretome Switches: Preconditioning Strategies for Stimulating MSC Paracrine Secretion

A number of preclinical studies have focused on transplanting MSCs into the infarcted heart with the hope that relevant signaling cues from the injury would regulate the MSC secretome (Iso et al., 2007; Lee et al., 2009; Nagaya et al., 2005; Shabbir et al., 2009). However, most of the signaling molecules (such as TNF- $\alpha$ , IL-6, IL-1 $\beta$ , IFN- $\gamma$ , MCP-1, Fractalkine, and others) secreted by macrophages, monocytes, fibroblasts, and cardiomyocytes during MI are transient. For example, the TNF- $\alpha$  level in the myocardium of rat infarcted hearts peaked at 7–8 days after MI, followed by a decline to basal level in the plasma within 48 hr (Berthonneche et al., 2004; Moro et al., 2007). Thus, even though these signaling molecules could produce a brief extension of MSC survival and an improved response to the highly dynamic and heterogeneous signaling cues during MI, they are unlikely to achieve long-lasting, controlled MSC paracrine action. Hence, other longer-lasting means of improving transplanted MSC function through extending MSC survival or via improved control of the secretome composition have been investigated. Most of these strategies are performed ex vivo and are referred to as preconditioning strategies.

#### Physiological Preconditioning

Subjecting MSCs to physiological conditions of hypoxia (<5% O<sub>2</sub>) and anoxia in vitro and in the ischemic heart has been reported to improve the survival of transplanted MSCs, cardiomyocytes, and endothelial cells via paracrine effects. For example, Kinnaird et al. demonstrated a significant increase (>1.5-fold) in the secretion of several arteriogenic cytokines, including VEGF, bFGF, PIGF, and TGF- $\beta$ , after subjecting hMSCs to 72 hr hypoxia compared with normoxic conditions (Kinnaird et al., 2004a). However, the increased levels of VEGF and bFGF in hMSC-conditioned medium could only partially account for the improved endothelial cell proliferation response in vitro. A systematic gene expression analysis showed that at least 165 genes, including *vegf*, *egf*, and *mmp-9*, were upregulated >3-fold in rMSCs following 24 hr hypoxic preconditioning (Ohnishi et al., 2007). However, this study did not report secreted protein levels and related functional assays to establish a correlation between secretion levels and function. Moreover, there is considerable variation between studies in terms of the hypoxia exposure time and the resulting secretion levels of paracrine factors. It is also not clear how long hypoxia preconditioning

effects last both in vitro and in vivo. While serum deprivation, yet another in vitro model for ischemia, has also been shown to induce secretion of angiogenic factors by hMSCs (Oskowitz et al., 2011), the observed effect could have been due to differences in cell proliferation rates. In general, physiological preconditioning via hypoxia exposure induces MSCs to activate survival pathways and secrete factors to counteract hypoxic effects. However, given its short duration, it is unclear if this transient response could produce a clinically relevant outcome.

### Genetic Manipulation

MSCs can also be engineered with transgenes for conditional gene expression (typically a single gene) with the aim of improving cell survival and controlling the MSC secretome posttransplantation. Transplanting *Akt1*-transfected rMSCs intramyocardially in rat MI models was advantageous and restored a 4-fold increase in myocardial volume (Mangi et al., 2003), but whether this effect was a result of improved rMSC survival or paracrine effects (or both) was not investigated. *Akt*-overexpressing rMSCs showed upregulated transcript levels of cytoprotective genes *vegf*, *bfgf*, *hgf*, *igf1*, and *tb4* in vitro, suggesting their involvement in mediating the early improvement in cardiac function seen in a rat MI model, including significant reduction in infarct size and improved (1.42-fold versus control) ventricular function <72 hr after rMSC transplantation (Gnecchi et al., 2006). *Akt* overexpression in mMSCs substantially upregulated SFRP2, a paracrine factor that was demonstrated to be responsible for the improved cardiomyocyte survival and reduced infarct size (3-fold versus PBS control) following transplantation (Mirotsoiu et al., 2007). MSCs have also been genetically modified to overexpress factors such as VEGF (Yang et al., 2010), IGF1 (Haider et al., 2008), and SDF-1 (Tang et al., 2010). When harnessed for cardiovascular applications, these modified MSCs improved angiogenesis, LVEF, c-kit<sup>+</sup> and CD31<sup>+</sup> cell mobilization, and contractile function, and reduced LV remodeling effects, primarily through paracrine actions. Overexpression of the transcription factor *GATA-4* (Li et al., 2010) and knockout of *TLR4* (Wang et al., 2010) in MSCs resulted in the increased secretion of VEGF. However, these manipulations also resulted in increased (for MSC-*GATA-4*) or decreased (*TLR4*KO-*MSC*) IGF-1 secretion levels versus wild-type MSCs; the discrepancy is likely due to the genetic targets manipulated and MSC sources (rMSCs for *GATA-4* and mMSCs for *TLR4* knockout). Therefore in these two studies, the role of IGF-1 in the observed cardioprotective effects in rat MI models is not clear. In general, genetic approaches could be harnessed to directly or indirectly upregulate specific MSC paracrine factors via upregulation of established target genes, even though overexpression of certain genes could lead to undesired effects (Fierro et al., 2011). Nonviral modifications should be sought due to the limitations of viral approaches, including the potential for insertional mutagenesis and increased regulatory hurdles. Furthermore, while there have been many attempts to improve MSC function via genetic manipulation, aside from immunomodulatory factors (e.g., IL-10, IDO, and PGE2) and proangiogenic factors (e.g., VEGF), strong candidates worthy of future pursuit have yet to be identified. The lack of such candidates is probably due to an absence of deep understanding of the underlying pathways and a lack of replicated studies by multiple laboratories.

### Molecular Preconditioning Using Proteins

Cytokines, chemokines, and growth factors represent key signaling cues during MI (Debrunner et al., 2008) and hence have been used to control MSC paracrine secretion in vitro (Croitoru-Lamoury et al., 2007). Stimulation of MSCs with TNF- $\alpha$  (50 ng/ml) for 24 hr (Wang et al., 2007), SDF-1 (50 ng/ml) for 1 hr (Pasha et al., 2008), or TGF- $\alpha$  (250 ng/ml) and TNF- $\alpha$  (50 ng/ml) for 24 hr (Herrmann et al., 2010) resulted in increased production of VEGF in the conditioned medium compared with unstimulated MSCs. Table 2 highlights the available in vitro data including peak concentration of the secreted paracrine factors as a function of dose and duration of stimulation. So far, however, these studies have not characterized the impact of time and/or dose of stimulation on paracrine factor secretion and cardiac functional improvement in vivo, which would be important for assessing the potential utility in clinical settings.

In an attempt to exploit synergistic effects, cocktails of cytokines, conditioned medium, or serum have been employed to stimulate MSCs. For example, when transplanted into NOD/SCID mice, hMSCs stimulated in vitro by a cytokine cocktail (Flt-3 ligand, SCF, IL-6, HGF, and IL-3) expressed higher levels of *CXCR4* mRNA and showed improved SDF-1-induced migration capacity (>20-fold versus unstimulated MSCs) to the bone marrow 24 hr after transplantation and enhanced homing (>2-fold versus unstimulated MSCs) to the bone marrow of irradiated mice 2–6 months after transplantation (Shi et al., 2007). In another study, hMSCs exposed to LPS-stimulated rat serum for 24 hr responded by secreting higher levels (4.5-fold versus normal serum) of sTNFR1 (Yagi et al., 2010). Importantly, intramuscularly injected serum-stimulated hMSCs attenuated inflammation via paracrine actions of sTNFR1 and other anti-inflammatory cytokines. Another approach that has been tested is coculture of MSCs with other cell types. For example, TSP-1 was upregulated in rMSCs cocultured with retinal ganglion cells (RGCs) (Yu et al., 2008). Block et al. cocultured hMSCs with apoptotic fibroblasts for 48 hr, thus exposing the hMSCs to apoptotic cytokines (Block et al., 2009) and leading the stimulated hMSCs to secrete STC-1, a peptide with antiapoptotic effects on lung epithelial cells. Clearly more comprehensive studies are required to examine the impact of protein-based preconditioning regimens on MSC-based therapeutic approaches, including applications for cardiovascular diseases. A better understanding of how the cytokines expressed in cardiac ischemic or inflammatory microenvironments in vivo modulate MSCs to exert a therapeutic effect could be very helpful for developing more effective protein-based preconditioning approaches.

### Pharmacological Preconditioning

Another promising approach for pretreating MSCs prior to transplantation involves small molecules, which have the advantages of ease of synthesis, cost effectiveness, and specific actions on cellular signaling. The availability of small molecule libraries enables high throughput screening to identify molecules for modulating specific cellular functions. However, there is currently no clearly demonstrated evidence of efficient MSC secretome regulation by small molecules. Some studies have suggested that small molecules can increase rMSC survival under ischemic conditions and can bring about a moderate

**Table 2. Representative Time- and Dose-Dependent Release Profiles of Paracrine Factors from Preconditioned MSCs In Vitro and Related Functional Improvements**

Stimulating Molecule	Dose and Time of Stimulation	Paracrine Factors Upregulated	Peak Concentration <sup>a</sup> (ng/ml)	Fold Change in Concentration versus Nonconditioned MSCs	Functional Improvements	Reference
TNF- $\alpha$	0.1, 0.1, 0.5, 2, 5, 10 ng/ml for 0, 6, 12, 24 and 48 hr	IL-6	19.0	15	dose-dependent improvement in monocyte migration upon treatment with varying doses of TNF- $\alpha$ -stimulated, MSC-conditioned medium	Lee et al., 2010
	(as above)	IL-8	38.0	30	(as above)	(as above)
	(as above)	MCP-1	18.0	15	(as above)	(as above)
	(as above)	CXCL6	0.3	30	(as above)	(as above)
TNF- $\alpha$	100 IU/ml for 6, 24, 72 hr	IL-8	100.0	6	enhanced migration of preconditioned hMSCs in response to chemokines such as SDF-1	Croituru-Lamoury et al., 2007
	(as above)	MCP-1	75.0	3.75	(as above)	(as above)
IFN- $\beta$	100, 1000, 2000 UI/ml for 6, 24, 72 hr	MCP-1	10.0	4	(as above)	(as above)
	100, 1000, 2000 UI/ml for 6, 24, 72 hr	IP-10	0.42	4	(as above)	(as above)
LPS	0, 0.01, 0.1, 1, 10 $\mu$ g/ml for 48 hr	VEGF	1.35 pg/10 <sup>5</sup> cells	2	enhanced VEGF levels in myocardium and improved survival of transplanted mMSCs	Yao et al., 2009

<sup>a</sup>Peak concentrations have resulted from the duration and dose of stimulation (in *italics*) in the respective studies.

improvement in cardiac function in MI models by upregulating intracellular levels of rMSC-expressed paracrine factors (Afzal et al., 2010; Mias et al., 2008; Wisel et al., 2009). In addition to small molecules, LPS treatment enhanced paracrine factor secretion (dose dependently) from mMSCs (Table 2), thereby improving survival of transplanted mMSCs (>1.5-fold versus control) via increased VEGF levels in the myocardium (>2.2-fold versus control) observed 3 weeks after cell transplantation in rat infarct hearts (Yao et al., 2009). If we are looking to apply preconditioning with agents such as LPS that would exert a detrimental systemic impact on the host, it will be important to minimize the concentration delivered with MSCs after preconditioning to prevent pharmacological effects on the host.

#### **Preconditioning through Cell-Cell Interactions and Physical Preconditioning**

Promotion of cell-cell interactions between MSCs can also have a profound impact on the MSC secretome. For example, Potapova et al. developed a simple strategy of organizing hMSCs into 3D spheroids of varying sizes using a hanging drop method to increase secretion levels of paracrine factors (Potapova et al., 2007). Using this approach, they observed high concentrations (5–20 times) of IL-11, as well as the proangiogenic cytokines VEGF, bFGF, and angiogenin, in the conditioned medium from hMSC spheroids compared with conditioned medium from hMSC monolayers. The onset of hypoxia in the core of the cell aggregates was proposed to be the driving force for the increased secretion levels. A similar study demonstrated that culturing hMSCs as 3D spheroids restored CXCR4 functional expression, demonstrated by 35%

of the cells derived from day 3 spheroids being CXCR4<sup>+</sup> (Potapova et al., 2008). In a separate study, hMSC spheroids grown in suspension cultures were found to secrete 60-fold more TSG-6 protein than adherent monolayered hMSCs (Bartosh et al., 2010). More importantly, the size of the spheroid-derived cells was significantly smaller than hMSCs from adherent cultures, allowing them to more readily escape lung entrapment in vivo following systemic infusion. Another approach for increasing secretion of paracrine factors involves culturing monolayers of rat-adipose-tissue-derived MSC sheets, prepared by incubating rMSC monolayers within temperature-responsive dishes at 20°C for about 40 min and detaching them into monolayers of rMSC sheets (Miyahara et al., 2006). Although promising, it is unclear how MSC preconditioned by promoting assembly into 3D aggregates or 2D monolayers will retain their secretion profiles following transplantation as single cells or 2D/3D constructs.

Microenvironmental cues such as shear stress and substrate compliance have also been used to control MSC paracrine activity. For example, human adipose-tissue-derived MSCs subjected to laminar shear stress (10 dyn/cm<sup>2</sup> up to 96 hr) secreted higher amounts of VEGF (2-fold versus static hMSCs) (Bassaneze et al., 2010). In another study, hMSCs grown on hydrogel substrates mimicking hard and soft tissue secreted differential levels of VEGF, IL-8, and uPA for up to 14 days (Seib et al., 2009).

#### **Summary of MSC Preconditioning Strategies**

Preconditioning via controlled cell-cell interactions has shown promise for increased secretion of pertinent factors, but may

**Table 3. Unaddressed Critical Issues for Current MSC Preconditioning Strategies**

Preconditioning Mode	Issues	Recommended Actions
Physiological	highly varied hypoxia exposure time (4 to 72 hr); nonspecific activation of signaling pathways leading to uncontrolled secretome; duration of preconditioning effects is not well understood	optimize hypoxia exposure time to maximize MSC survival; investigate different modes of hypoxia such as brief exposures mimicking ischemic preconditioning to better understand effect of hypoxia on MSC survival and sustained paracrine action in vivo
Genetic	activates single target gene; gene expression levels do not correlate with the concentration of secreted factors; limited control over local pharmacokinetics of expressed protein; limited understanding of the temporal expression of proteins; safety posttransplantation	multiple gene activation leading to expression/release of a cocktail of proteins that act in synergy; overexpression studies to establish correlation between gene and protein expression levels; optimize the mode of gene and cell delivery (e.g., combine with a biomaterials approach); carefully examine the literature for comparable approaches, perform relevant safety analysis in animal models, and consider nonviral approaches
Protein/cytokine	effects of incubation time and protein concentration on promoting sustained effects are not well understood; high concentrations required to stimulate the MSC due to transport limitations	kinetic studies of pathway activation and factor release in vitro and in vivo; transport limitations could be overcome by intracellularly or extracellularly controlled delivery of proteins/cytokines using polymeric micro/nano-systems
Pharmacological	effects of incubation time and drug concentration on promoting sustained effects are not well understood; agents may exhibit a negative impact on the host	kinetic studies of pathway activation and factor release in vitro and in vivo; identifying highly specific activators/inhibitors; ensuring concentration of agents is minimized in cell suspension prior to transplantation
Cell-cell interaction	the mechanism by which 3D MSC aggregates retain high secretome expression is not clear	perform studies to reveal the involvement of MSC adhesion ligands or ECM in activating specific signaling pathways
Physical	the signaling pathways activated by physical stimuli are not well understood	elucidation of the mechanisms by which physical stimuli influence the MSC secretome; kinetic studies of pathway activation and factor release both in vitro and in vivo

offer less control than other approaches over the components of the secretome. Although physiological and molecular (cytokine/small molecule) preconditioning is simple and more targeted, the impact is transient due to self-regulatory mechanisms, and it is likely that these approaches will have a limited duration of impact posttransplantation. Genetic manipulation has the advantage of a sustained response; however, it is often limited to a single target gene and clinical translation may be challenging due to higher regulatory hurdles if viruses are utilized. Regardless of the preconditioning approach applied, MSCs typically exhibit limited persistence following transplantation, and thus, achieving sustained secretion following a single MSC dose will require new techniques to boost MSC survival. While a proof of concept for this has been achieved using virally transfected MSCs (Mangi et al., 2003; Mirotsov et al., 2007), much-desired nonviral approaches are currently being developed to achieve rapid and safe translation (Yang et al., 2010). Coadministration of drugs such as Atorvastatin with MSCs may also improve the cardiac microenvironment after MI to achieve better survival of the implanted MSCs (Yang et al., 2008). Likewise, immunoselection aimed to enrich the cell population with stromal precursor antigen-1 (STRO-1)<sup>+</sup> mesenchymal progenitor cells (MPCs) could also improve cardiovascular paracrine activity (Psaltis et al., 2010).

While the majority of preconditioning strategies promote expression/secretion of a narrow class of cytokines that are constitutively secreted by MSCs at basal levels, it would be useful to examine if preconditioning of MSCs could be utilized to express therapeutic factors that are not secreted by MSCs under basal conditions. Despite the promise of several preconditioning strategies, there are critical unmet needs and uncer-

tainties yet to be addressed, and these are listed and discussed in Table 3. However, better control over the MSC secretome posttransplantation could be achieved through customization strategies as depicted in Figure 1, which could be translated into clinics via engineering the MSC secretome using controlled release approaches as depicted in Figure 2.

### Summary and Perspectives

Harnessing the MSC secretome for cardiovascular repair seems in principle to have significant clinical potential given the innate immunomodulatory and trophic properties of many of the factors secreted by MSCs. While some groups are directly employing MSC-derived therapeutic paracrine factors in the absence of cells, approaches employing a cocktail of secreted factors will require GMP manufacturing protocols with reproducible batch-to-batch secretome properties (that may be impacted by several factors including the MSC donor), and will require a defined regulatory pathway. Also, in general, cytokine-based approaches have not performed well in clinical trials due to inherent limitations in tissue transport, pharmacokinetics, and protein stability in vivo. These issues can likely be addressed for individual cytokines through the development of appropriate controlled release strategies; however, delivery approaches for complex cocktails of therapeutic agents, such as the isolated MSC secretome, will be a significant challenge.

The majority of MSC-based clinical trials for cardiovascular therapy focus on the potential benefits of the immunomodulatory and trophic properties of MSCs rather than their potential to generate new tissues directly. Although it is still early to draw conclusions, the available trial results are not as promising as has been hoped based on preclinical animal studies. This relative

lack of success is likely due to the use of nonoptimized MSC formulations and poor understanding of how MSCs induce cardiovascular repair. Importantly, signaling pathways mediating the expression and secretion of relevant MSC factors and the mechanism of how they synergistically impact cardiovascular repair are beginning to be elucidated. Nevertheless, the relevance of the MSC secretome to the treatment of cardiovascular disease is still controversial, and hence, identifying and characterizing additional MSC-secreted factors that can either facilitate cardiomyogenesis or activate endogenous CSCs seems crucial. The molecular events responsible for altering the MSC secretome in vivo, as a function of microenvironmental stimuli, remain elusive. Clear understanding of the in vivo MSC secretome and its potential functional benefits is still far from being resolved, and this is a key prerequisite to harnessing this potentially powerful tool for maximal therapeutic benefit.

The currently favored approach for regulating cells after transplantation involves preconditioning MSCs with the aim of improving homing, survival, and secretome control. Clarifying the underlying signaling pathways should enable development of more effective preconditioning regimens to activate/inhibit relevant pathways to maximize the therapeutic effect. As the biology mediating the therapeutic benefit of MSC secretome becomes more defined, targeted preconditioning and genetic manipulation approaches will likely be useful to enhance the therapeutic benefit.

Looking to the future, state-of-the-art bioengineered materials offer the potential for enhanced control of cells and presentation of MSC secretome after transplantation. For example, paracrine factors from hypoxia-conditioned MSCs bound to nano-structured materials have yielded significant hemodynamic functional preservation within an infarcted heart model (Webber et al., 2010), and the transplantation of cells such as CPCs with immobilized IGF-1 on nano-fibers exhibited dual effects from IGF-1-mediated activation of resident cardiac cells and protection of transplanted CPCs (Padin-Iruegas et al., 2009). Cardiac-specific decellularized matrices (Godier-Furnémont et al., 2011; Singelyn and Christman, 2010) and biopolymers (Danoviz et al., 2010) may also serve as injectable biomaterials to deliver MSCs in a more sustainable and effective manner. Recently, we employed a polymer-based controlled drug release strategy to program MSC fate through engineered intracrine-, paracrine-, and endocrine-like mechanisms (Sarkar et al., 2011a). In addition to controlling cell fate, this biomaterials approach provides an opportunity to control the MSC secretome posttransplantation—for example, through sustained intracellular release of small molecules that target specific pathways. As an alternative to transplantation of single-cell suspensions, the MSC secretome may also be exploited through transplantation of engineered MSC spheroids that have shown potential for enhanced paracrine levels in vitro. Given the relatively harsh microenvironment presented at a site of injury or ischemia, it may be of interest to transplant MSCs at a distant site where paracrine factors can reach damaged heart tissue through systemic endocrine effects (Lee et al., 2009). Although challenges remain, harnessing the MSC secretome for meaningful therapeutic outcomes will likely be realized in the near future by capitalizing on customization strategies as depicted in Figure 1 and Figure 2 for controlling and sustaining the MSC secretome posttransplantation.

## SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Tables S1 and S2 and can be found with this article online at doi:10.1016/j.stem.2012.02.005.

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