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Review

Mesenchymal Stem Cell Homing: The Devil Is in the Details

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The study of MSC trafficking is clinically relevant for minimally invasive cell therapy to promote regeneration of damaged tissue, to treat inflammation, and to promote angiogenesis. However, these studies are complicated by the diverse methods used to culture, characterize, and deliver MSCs and by the variety of methods used to assess homing events. This review provides a critical analysis of the methods used to track homing of exogenously infused MSCs and discusses strategies for enhancing their trafficking to particular tissues.

Introduction

Mesenchymal stem cells (MSCs), also referred to as connective tissue progenitor cells or multipotent mesenchymal stromal cells (Dominici et al., 2006), have demonstrated significant potential for clinical use. This clinical utility is due to their convenient isolation, their lack of significant immunogenicity permitting allogenic transplantation without immunosuppressive drugs, their lack of ethical controversy, and their potential to differentiate into tissue-specific cell types (Sasaki et al., 2008; Toma et al., 2002) with trophic activity (Zhang et al., 2007), to promote vascularization (Martens et al., 2006), and to promote potent immunosuppressive effects (reviewed in Nauta and Fibbe, 2007). Thus, MSCs have been the focus of a regime of emerging therapeutics to regenerate damaged tissue and treat inflammation resulting from cardiovascular disease and myocardial infarction (MI), brain and spinal cord injury, cartilage and bone injury, Crohn's disease, and graft-versus-host disease (GVHD) during bone marrow transplantation (Phinney and Prockop, 2007). Although local transplantation or injection of MSCs represents a potential approach that may be useful in certain settings (Bantubungi et al., 2008; Petite et al., 2000), the potential for minimally invasive delivery of MSCs via systemic infusion is of particular interest (Lee et al., 2008; Sackstein et al., 2008; Wang et al., 2008b). However, a significant barrier to the effective implementation of MSC therapy is the inability to target these cells to tissues of interest with high efficiency and engraftment.

The study of MSC homing following mobilization of host MSCs, or following systemic infusion of exogenous MSCs, is extremely complex. The challenges facing these efforts are due to a number of factors, including the lack of universally accepted criteria for defining the MSC phenotype and their functional properties, by the rare presence of MSCs within blood, and by the diverse methods used to culture MSCs and study their homing potential. Critical questions pertinent to all studies in the MSC trafficking field include the following: (1) Can host MSCs be mobilized into peripheral blood? (2) Can exogenously delivered MSCs home to ischemic tissues or sites of inflammation from peripheral blood, and what is the efficiency of this process? And (3), can host MSCs be mobilized into peripheral blood and then target ischemic tissues? The different routes for MSC trafficking represented in these questions are illustrated in Figure 1. The third question is the most technically difficult to address and, hence, the least discussed in the current literature. The first question has already been reviewed elsewhere (He et al., 2007) and thus will only be briefly discussed.

This review will focus on providing a critical analysis of the methods developed to track the homing of exogenously infused MSCs. Pertinent considerations that will be emphasized include (1) how MSCs are cultured, (2) methods used to deliver MSCs, (3) potential mechanisms for MSC engraftment, (4) methods used to quantify MSC homing, and (5) methods used to characterize the MSCs following a homing event. We will also discuss strategies that have been employed to enhance trafficking of MSCs to particular tissues, and the hurdles hindering their translation to the clinical setting.

Definition of MSCs and MSC Homing

MSCs may be defined as multipotent cells capable of self-renewal that can give rise to a number of unique, differentiated mesenchymal cell types (da Silva Meirelles et al., 2008). Despite this definition, many researchers use different methods to culture MSCs, assess their differentiation potential, and evaluate their capacity for self-renewal. Although MSCs may be derived from multiple tissues, it is critical to consider that significant phenotypic differences in MSCs exist which may reflect distinct functional properties (Bianco et al., 2008), and this heterogeneity may be a function of their tissue microenvironment (da Silva Meirelles et al., 2006). Also, it is critical to consider that MSCs exhibit a striking similarity to vascular mural cells called pericytes that are embedded within the vascular basement membrane of microvessels and capillaries throughout the body (Crisan et al., 2008; da Silva Meirelles et al., 2008).

Given the lack of universally accepted criteria for defining a MSC, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed

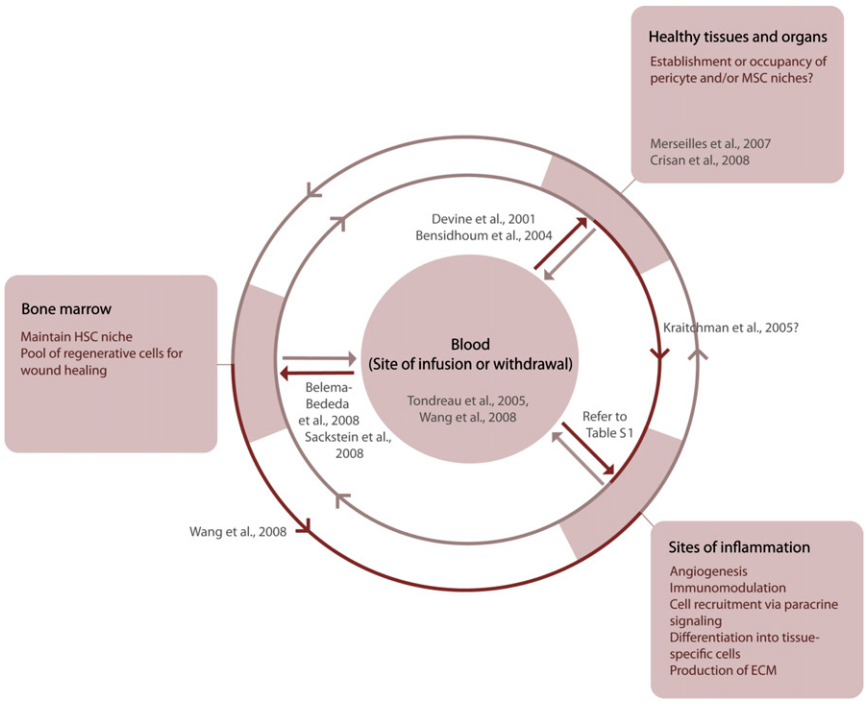

Figure 1. The Active MSC Homing Circuit
MSCs play several roles (red text within pink boxes) depending on their anatomic location. Studies have shown their presence in both peripheral blood and healthy tissues and organs (listed in gray), in addition to the bone marrow, from which they have historically been isolated. Numerous active homing routes exist for MSCs (arrows). Red arrows represent paths that have been substantiated by published studies. Sites of inflammation include acute inflammation due to injury, chronic inflammation (e.g., GvHD), and tumors.

a set of standards to define human MSCs for both laboratory-based scientific investigations and preclinical studies (Dominici et al., 2006). As part of the minimal criteria, human MSCs must adhere to tissue culture plastic; be positive for CD105, CD73, and CD90 and negative for CD45, CD34, CD14 or CD11b, CD79a, or CD19 and HLA-DR; and must be able to differentiate to osteoblasts, adipocytes, and chondroblasts under standard in vitro differentiating conditions. Given the heterogeneity of typical MSC culture procedures and a lack of enforcement of the above-mentioned characterization criteria, definitive conclusions based on the literature are often difficult to surmise.

MSC homing is defined as the arrest of MSCs within the vasculature of a tissue followed by transmigration across the endothelium. Such a nonmechanistic definition is appropriate, given the current absence of a definitive MSC homing mechanism, unlike the well-characterized leukocyte adhesion cascade that defines leukocyte homing. The lack of data describing the exact positioning of the MSCs following infusion makes it difficult to determine if the cells have arrested within the vessels (localization) or have undergone transendothelial migration (homing).

Can Host MSCs Be Mobilized into Peripheral Blood?

The potential for MSC trafficking under physiological steady-state conditions is contentious, with reports in the literature of diametrically opposed findings. Studies that support the presence of MSCs within blood have reported only minute quantities of circulating MSCs (Kuznetsov et al., 2001), whereas several studies report the inability to locate any circulating MSCs at all (reviewed in He et al., 2007). The failure to harvest sufficient numbers of circulating MSCs at steady-state conditions is complicated by the need to access blood via venapuncture, which in theory could release small quantities of pericytes or other connective tissue cells into the circulation. Interestingly, da Silva Mereilles et al. were consistently unable to derive a long-term culture of MSCs from portal vein-accessed blood, a technique that reduced the possibility of pericyte or other connective tissue cell contamination of the blood sample (da Silva Mereilles et al., 2006). However, the success of isolating MSCs is also likely dependent on the methods of mobilizing MSCs into peripheral blood, eliminating contaminating cells, and methods of culturing the isolated MSCs. It is important to note that MSCs isolated from peripheral blood have shown heterogeneous marker expression. Specifically, an early study isolated adherent, fibroblast-like stem cells with osteogenic and adipogenic potential from the blood of four mammalian species including human, which were distinguished from BM-derived human MSCs by the absence of Stro-1 and endoglin (Kuznetsov et al., 2001). Other studies report the isolation of MSCs from peripheral blood using preselection methods for CD133+ cells in G-CSF-mobilized peripheral blood (Tondreau et al., 2005). These cells have potential to differentiate into adipocytes, osteoblasts, chondrocytes, and neuronal/glial cells (Rochefort et al., 2006).

Of particular significance, increased numbers of MSCs have been isolated from peripheral blood cells of injured mice (to induce intimal hyperplasia) compared to noninjured controls (Wang et al., 2008a). This trend correlated with significant increases in peripheral blood concentrations of the cytokines VEGF and G-CSF. Moreover, MSCs from injured animals were cultured up to ten passages and had trilineage differentiation potential in vitro, compared to MSCs from noninjured animals, which could only be passaged twice. These results need to be repeated by several groups before a central dogma—that the presence of circulating MSCs occurs only in response to injury—can be developed.

Exogenously Delivered MSCs

Culturing of MSCs

There are several factors regarding the MSC culture conditions that should be reported for homing experiments, as the culture condition may have a significant impact on MSC function. For example, the confluency of MSCs cultured under laboratory conditions before being infused can affect their migration

potential. Increased culture confluence was shown to inhibit transendothelial migration in MSCs by increasing the production of a natural matrix metalloproteinase (MMP) inhibitor, TIMP-3 (De Becker et al., 2007). The passage number of MSCs used is also important, as MSCs have been shown to gain or lose certain surface receptors during culture, which might influence their homing capability. Freshly isolated MSCs have been shown to display enhanced homing ability compared to their culture-expanded counterparts (Rombouts and Ploemacher, 2003). Homing receptors, such as CXCR4, a chemotactic receptor for SDF-1 that is upregulated in the bone marrow and in ischemic tissues, is usually absent on the surface of culture-expanded MSCs (Phinney and Prockop, 2007; Ruster et al., 2006; Sackstein et al., 2008; Wynn et al., 2004). However, treating MSCs with a cocktail of cytokines in culture has been shown to induce high surface expression of CXCR4 that enhanced homing ability (Shi et al., 2007). Given that the expression of CXCR4 and other homing receptors is typically observed on a subset of MSCs and often lost with culture expansion, it is plausible that these may be naturally present on endogenous MSCs but lost after culture (Wynn et al., 2004). Interestingly, simulating ischemic environments in culture, which some believe is representative of the MSC niche, may also increase MSC motility. Hypoxic preconditioning increased MSC migration through Matrigel by upregulating MMPs (Annabi et al., 2003) and on tissue culture plastic (Rosova et al., 2008) compared to MSCs maintained in normoxic environments. In addition to the passage number, confluency of the passaged cells, site of isolation, and properties of the media and incubation environment, it is critical to consider the heterogeneity in MSC surface receptor expression and resulting MSC behavior that has been observed both within and between studies (Jones et al., 2002; da Silva Meirelles et al., 2006; Simmons and Torok-Storb, 1991; Sordi et al., 2005; Wynn et al., 2004). Such variability of MSC properties emphasizes the importance of comprehensive characterization of MSCs within each study. It is especially important to have an accurate assessment of MSC properties prior to injection or implantation of MSCs into the highly complex and varying microenvironments that exist within the body.

Delivery of MSCs

For MSC trafficking experiments, the timing of delivery, number of cells delivered, and site of MSC infusion may impact the engraftment efficiency and the destination of exogenously delivered cells. Both higher numbers of infused MSCs and early delivery of MSCs following an event causing ischemia (e.g., MCAO) have been shown to improve engraftment rates (Chen et al., 2001). MSCs were found to engraft in the myocardium at higher rates 1 day after MI as compared to 14 days after MI, suggesting that MSCs engraft specifically in response to acute MI (Schenk et al., 2007). Although it may be expected that higher infused numbers of MSCs should result in higher numbers of engrafted MSCs and better functional outcomes, there may be a plateau beyond which additional delivered cells may not improve the outcome. For example, in a rat model of brain injury, although neurological function after the systemic infusion of MSCs was improved for a dose of 1×10^6 cells, no additional enhancement was observed when 3×10^6 MSCs were infused (Wu et al., 2008). Studies that have attempted to optimize the protocol for MSC delivery in terms of numbers and timing found that higher numbers of MSCs and MSCs delivered sooner after presentation of ischemia resulted in higher engraftment rates, though differences in the extent of functional outcome were not apparent (Omori et al., 2008).

The site of MSC delivery may impact the route MSCs travel to reach the target organ. Systemic administration can be achieved by intravenous (IV) injection, intraperitoneal (IP) injection, intra-arterial (IA) injection, or intracardiac (IC) injection. IV delivery is the least invasive; however, IC and IA delivery have led to higher engraftment rates than IV delivery in certain models of MI (Barbash et al., 2003; Freyman et al., 2006). IA injection close to the target site (extracranial right internal carotid artery) in a model of brain injury was shown by MRI imaging of radiolabeled cells to significantly enhance homing to the brain versus distant IV injection (femoral vein) (Walczak et al., 2008). IA injection may reduce accumulation of MSCs within filtering organs such as the lung, liver, or spleen that is often observed following IV delivery (Barbash et al., 2003; Kraitchman et al., 2005; Sackstein et al., 2008); however, IA may also lead to increased probability of microvascular occlusions (Walczak et al., 2008), which is termed "passive entrapment." Since IC and IA delivery bypasses the initial uptake by the lungs, more MSCs are available to engraft at the ischemic site. IP delivery is rarely used but has been employed to deliver MSCs to murine fetuses in a mouse model of muscular dystrophy, since IV delivery was deemed inappropriate for this application (Chan et al., 2007). Following birth, the donor cells were found in both muscle and nonmuscle organs. It is unknown why IV delivery resulted in the consistent death of the fetuses. A final method of delivery is local infusion, which entails injecting MSCs directly into the tissue of interest. DiI-labeled MSCs intravenously transfused into baboons were undetectable in limb muscles compared to detection of DiO-labeled MSCs following direct injection into the muscle (Beggs et al., 2006). However, local infusion is likely not clinically feasible in many cases due to its potentially high degree of invasiveness (e.g., into the heart or brain), and locally administered cells often die before significantly contributing to the healing response due to diffusion limitations of nutrients and oxygen (Muschler et al., 2004).

The First E in MSC Engraftment: Efficacy

It is presumed that therapeutic efficacy of infused MSCs relies on extravasation and engraftment of systemically infused MSCs where they may exhibit local trophic or paracrine activity or where MSCs may inhabit a tissue and release paracrine factors into the vasculature for a systemic effect. However, few studies have provided insight into the mechanisms of homing. Specifically, it is unclear if the MSCs actively home to tissues using leukocyte-like cell-adhesion and transmigration mechanisms (reviewed in Ley et al., 2007) or become passively entrapped in small-diameter blood vessels. Instead of selectin and integrin-mediated cell arrest on inflamed endothelium (Ley et al., 2007), it is possible that MSCs become passively arrested in capillaries or microvessels including arterioles and postcapillary venules (Sackstein et al., 2008; Walczak et al., 2008) (refer to Figure 2). Passive entrapment is likely a function of the cell's size and overall deformability. The mode of arrest is thus of particular importance for MSCs, since they are known to enlarge during in vitro cell culture (Chavakis et al., 2008). Expansion of cell numbers in culture is a necessary step during MSC therapy; however,

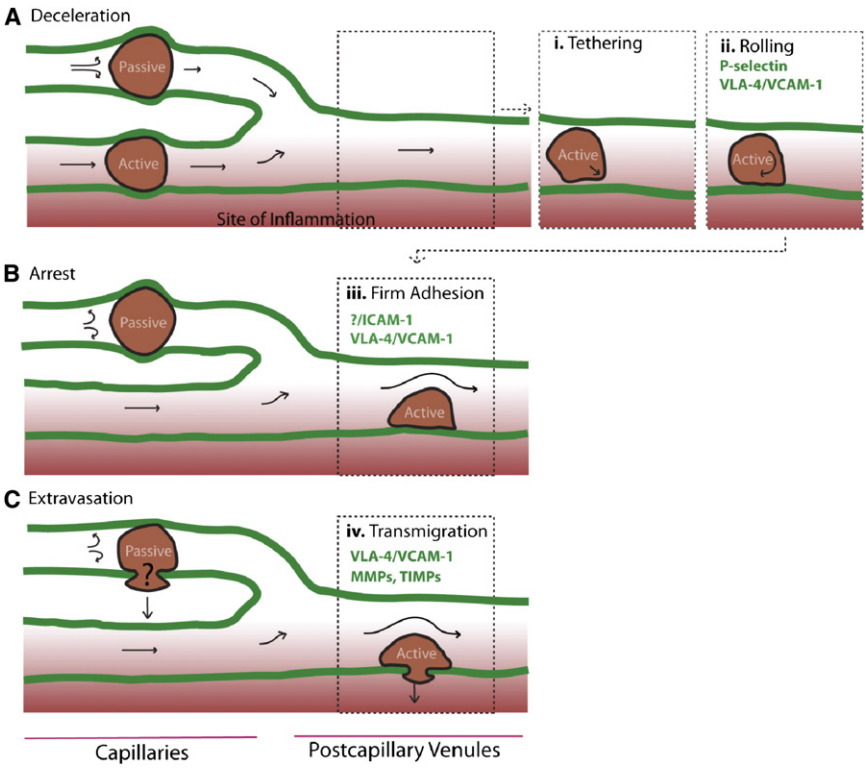

Figure 2. Model for Passive versus Active Homing

(A) There are two potential mechanisms for how MSCs may decelerate within the vasculature during the homing process. The large size of MSCs and/or narrower capillaries may reduce the cell velocity due to physical interactions leading to passive entrapment (top cell). Alternatively, MSCs that deform likely pass through capillaries to postcapillary venules similar to leukocyte homing (von Andrian, 1997) can (1) tether and (2) roll on activated vaculature at sites of inflammation, where a chemokine gradient (red gradient) is established.

(B) During passive arrest, an altered blood flow (arrows) may be observed. In contrast, during active arrest, cells quickly flatten and spread on the underlying endothelium, and blood flow is virtually unchanged. Although ICAM-1 expression on ECs has been implicated in active arrest of MSCs, it is not known which ligands present on MSCs interact with this receptor.

(C) After active arrest, MSCs may transmigrate, but the fate of passively arrested MSCs is unclear. The molecular interactions that regulate MSC homing are listed in green. A third possibility for MSC engraftment within inflammatory tissues (data not shown) involves passive arrest within the vasculature proximal to the site of inflammation, followed by transmigration in response to a chemokine gradient in the surrounding tissue. It is also possible that the physical properties of culture-expanded MSCs (i.e., increased size) reduce the cell velocity enough within postcapillary venues to permit engagement of firm adhesion receptors (negating the need for rolling receptors), thus leading to a proposed mechanism that incorporates both aspects of active and passive homing. VLA-1, very late antigen-4; VCAM-1, vascular cell-adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitor of metalloproteinases.

this practice may elevate the risk of entrapment of cells within nonspecific tissues including the lung (Barbash et al., 2003; Sackstein et al., 2008). Geometrical and mechanical entrapment of cells has been previously described after systemic injection of MSCs (Barbash et al., 2003; Sackstein et al., 2008) and after injection of endothelial progenitor cells into the tumor microvasculature (Vajkoczy et al., 2003). Passive arrest may be distinguished from active arrest by the observation of an altered blood flow (i.e., through blocking a vessel) (Walczak et al., 2008). Cells that home via leukocyte adhesion mechanisms quickly flatten and spread on the underlying vascular endothelium in preparation for transmigration (Diacovo et al., 1996), which reduces the possibility for altered blood flow. Understanding the mechanisms of passive and active arrest will likely be essential for developing more-effective MSC delivery strategies.

Methods Used to Probe the Active Arrest of MSCs. There is substantial evidence that infused MSCs have higher engraftment efficiencies within sites of inflammation or injury. An interesting study by Francois et al. examined this through subjecting mice to total body irradiation (TBI) and in some cases in combination with additional local irradiation within the abdominal area or hind leg (Francois et al., 2006). The engraftment of systemically infused MSCs was measured 15 days later, and engraftment levels were found to be higher in mice subjected to TBI compared to nonirradiated mice. TBI induced a 2.8-fold increase on engraftment levels of MSCs in the brain, 3-fold in the heart, 2.5-fold in the liver, 2.6-fold in BM, and 1.7-fold in muscles, while levels of engraftment in the lung were not affected. This pattern suggests that MSCs engrafted in response to radiation damage except in the lung, in which engraftment rates remained the same with or without radiation. Presumably, this lack of change is because their presence in the lung resulted from a passive process. However, we cannot discount the possibility that in addition to integrin upregulation, especially that of VCAM-1 (Mazo et al., 2002), irradiation or the induction of MI also leads to local changes in microvessel diameter (i.e., constriction) (Eder et al., 2004; Freas et al., 1989), affecting passive arrest and thus explaining the accumulation of MSCs in inflamed tissue.

The most significant evidence for active arrest of MSCs within inflamed tissues is supported by methodology involving integrin blocking (Ip et al., 2007) and knockout studies (Ruster et al., 2006) that show a dependence on selectin and integrin interactions. For example, Ip et al. showed that blocking the β1-integrin on MSCs, a component of the adhesion molecule VLA-4 that governs the arrest of leukocytes on activated endothelium, reduced their engraftment in ischemic myocardium (Ip et al., 2007). Furthermore, using P-selectin knockout mice, Ruster et al. showed via intravital microscopy that fewer MSCs slowed down in postcapillary venules compared to wild-type mice (Ruster et al., 2006). These results suggest that the engraftment of MSCs within target tissues depends on specific molecular interactions prior to the transmigration step, rather than a nonspecific passive steric phenomena. These molecular interactions can readily be studied in vitro via standard leukocyte adhesion assays.

To date, only one study has examined MSC rolling on endothelial cells in vitro. Ruster et al. showed that MSCs bind to endothelial cells in a P-selectin-dependent manner and that rolling MSCs interact with VLA-4/VCAM-1 that promotes firm adhesion on the endothelial cells (Ruster et al., 2006). However, the rolling velocities reported were ~100–500 μm/s at shear stresses of 0.1–1.0 dynes/cm^2. To provide context, leukocyte rolling has been typically observed to be less than 5 μm/s at shear stresses up to 4 dynes/cm^2 (Goetz et al., 1994; Ley et al., 2007). The observation of high rolling velocities of MSCs is likely explained by the lack of in vitro activation (e.g., by TNF-α) required to mimic endothelium within inflamed or injured tissues, which promotes the expression of cell-adhesion receptors that regulate cell rolling and firm adhesion. Also, it is important to note that interacting leukocytes are typically defined by velocities lower than 50% of the free stream velocity (Hong et al., 2007). However, this study used a more generous criterion; namely, cells traveling at less than the free stream velocity were considered to be rolling. Although velocities of MSCs traveling on activated endothelium were not reported, experiments that examined retention of firmly adherent MSCs under shear showed a significant increase in the number of MSCs that remained adhered to endothelial cells after activation with TNF-α at shear stresses between 0.1 and 2.0 dynes/cm^2. Nevertheless, given the generous criteria for an interacting cell, the implications of this study into the mechanisms of MSC homing are limited. Clearly, studies that examine MSCs rolling on activated endothelium, at velocities and shear rates that are physiologically relevant, are essential to further elucidate potential MSC homing mechanisms. A study by Segers et al. examined the firm adhesion of MSC under static and various shear stress conditions on activated endothelium (Segers et al., 2006). Similar to the study by Ruster et al., this study highlighted the dominant role of VLA-4 and VCAM-1 as effectors of firm adhesion. Interestingly, firm adhesion receptor/ligand interactions including the VLA-4/VCAM-1 axis mediates rolling due to changes in their tertiary conformations (Alon et al., 1995; Salas et al., 2002; Sigal et al., 2000). Similar studies to elucidate the actual mechanisms of MSC arrest on vasculature demands attention in the field of MSC trafficking.

Methods Used to Probe the Transmigration of MSCs. Very few studies have examined the transmigration of MSCs. Steingen reported that MSCs can transmigrate through nonactivated endothelial monolayers via VCAM-1/VLA-4 interactions, but rather than undergoing complete diapedesis, as is observed for leukocytes, MSCs tended to integrate with the endothelial layer, perhaps as embedded pericytes (Steingen et al., 2008). The time course for transmigration was long compared to leukocytes, which take 5–20 min (Ley et al., 2007)—specifically, the endothelial monolayer resealed over the integrated MSCs after 240 min, leaving the MSCs beneath the monolayer. This deviance from previously studied leukocyte transmigratory behavior might have resulted from the use of nonactivated endothelium, rather than a physiologic inability of MSCs to transmigrate. Lymphocytes, for example, exhibit significantly reduced transmigratory activity on nonactivated endothelium due to the absence of pertinent cell-surface adhesion molecules. Such subtleties are important to consider when interpreting and comparing results between studies. Results thus far suggest that specific MSC-endothelium interactions regulate transmigration, although further studies are required to examine this phenomenon under conditions that mimic an active inflammatory state.

Methods Used to Promote Chemotaxis of Systemically Infused MSCs. In addition to adhesive interactions that mediate MSC homing to specific sites, chemokines released from tissue or endothelial cells may promote activation of adhesion ligands, transendothelial migration, chemotaxis, and/or subsequent retention in surrounding tissue (Belema-Bedada et al., 2008; Hordijk, 2003; Ponte et al., 2007). For example, systemically infused GFP-labeled MSCs that express the MCP-1 receptor CCR2 on their surface were infused into transgenic mice with MCP-1 specifically expressed in the myocardium. MCP-1 is typically expressed at sites of inflammation and thus represents a model homing chemokine (Belema-Bedada et al., 2008). GFP-positive cells were found in the myocardium at high frequencies of 20 cells/microscopic field compared to none in the hearts of control mice 7 and 14 days later. These frequencies were also 20 times higher than those for GFP-positive cells found in skeletal muscle, brain, and kidney as detected by immunofluorescence, and approximately eight times higher as detected by real-time polymerase chain reaction (real-time PCR) analysis of whole organs for eGFP mRNA. Unfortunately, the number of MSCs found in the lung was not reported, a likely destination for infused MSCs (Kraitchman et al., 2005). It is possible that indirect effects, rather than the interaction of MSCs with MCP-1 via the CCR2 receptor, was responsible for difference in the distribution. For example, MCP-1 is known to upregulate adhesion molecules on the endothelial surface and increase endothelial permeability (Stamatovic et al., 2003). Hence, MSCs were transfected with a vector expressing a truncated version of FROUNT (DN-Frount). FROUNT binds to CCR2, enabling CCR2-mediated chemotaxis toward MCP-1, but not HGF, SDF-1, or VEGF. DN-Frount competes with endogenous FROUNT for CCR2 binding and acts as a dominant-negative effector of CCR2-mediated chemotaxis. DN-Frount-transfected MSCs lacked the capability to home to the hearts of the MCP-1 transgenic mice compared with nontransfected MSCs. Hence, the direct interaction of CCR2 with MCP-1 was crucial to the engraftment of MSCs in ischemic heart tissue in this model. In a similar study, systemically administered MSCs detected in the myocardium doubled after the expression of another inflammatory chemokine, MCP-3 (a ligand for CCR2 and CCR1), was induced in the myocardium compared to nontransfected controls (Schenk et al., 2007). This study, unlike Belema-Bededa et al., did not present evidence that the response was directly due to the interactions between CCR2 or CCR1 and MCP-3.

Methods Used to Assess the Role of Enzymes in MSC Homing. In addition to chemokines and adhesion molecules, invasive cells often secrete enzymes that are essential for their migratory activity. MSCs secrete proteases that regulate transmigration and invasion of the basement membrane of endothelium and degrade extracellular matrix (ECM) during chemotaxis. Both blocking antibodies toward MMP2 and SiRNA knockdown of MMP2 in MSCs reduce transendothelial migration in vitro (De Becker et al., 2007). The role of MMP2, as well as MT1-MMP and TIMP-2, in MSC invasion was further confirmed by Ries et al., who also showed that chemotactic invasion of MSCs through human ECM-coated transwell chambers could be

hindered by inhibition of the proteases (Ries et al., 2007). Downregulation of MMP-2, MT1-MMP, and TIMP-2 via RNAi significantly impaired the migration of MSCs by 72%, 75%, and 65%, respectively, when compared with control cells that had received a non-target-directed siRNA. Steingen et al. also perfused MSCs through isolated mouse hearts and detected the presence of gelatinases at sites of MSC invasion through in situ zymography (Steingen et al., 2008). Thus MSCs possess the ability to break down endothelial basement membrane and migrate toward chemotactic factors.

The substantial migratory properties of systemically infused MSCs were also demonstrated in a recent study using a rat model of middle cerebral artery occlusion (MCAO). Specifically, Feridex-labeled human MSCs were directly infused into either the ipsilateral or contralateral hemisphere of the injured brain (Kim et al., 2008) and imaged via MRI once a week up to 10 weeks. Regardless of the site of infusion, MSCs were found to migrate and localize in both the boundary and core of the infarcted tissue. This result was confirmed by Prussian blue staining and immunohistochemistry using a human nuclei-specific antibody, in which cells positive for both stains were exclusively found in the infarcted region. The time series imaging that showed the gradual movement of the MSCs toward the site of inflammation, in the case of contralateral infusion, supports the notion that MSCs possess extensive migratory capabilities within a tissue. Such capabilities are likely a function of their responsiveness to chemotactic factors and production of ECM-degrading enzymes.

The Second E in MSC Engraftment: Efficiency

Although it has been well established that systemically infused MSCs localize within injured, inflamed, and cancerous tissues, their efficiency of homing as a function of local tissue properties is unclear, and the method of detection, method of quantification, and timing of quantification can significantly impact the result. MSCs are often detected in vivo using radioactive labeling (Barbash et al., 2003; Freyman et al., 2006; Gao et al., 2001b; Kraitchman et al., 2005), fluorescent labeling (Kawada et al., 2004), transduction of MSCs with reporter genes (Barbash et al., 2003; Devine et al., 2001), species mismatch (i.e., injection of human MSCs into a rodent), and probing for sex mismatch (i.e., injection of female cells into a male rodent) via specific genes by fluorescent in situ hybridization (Jiang et al., 2006; Pereira et al., 1998) or real-time PCR (Belema-Bedada et al., 2008; Kumar and Ponnazhagan, 2007). These tracking and assessment methods have been used for models such as acute MI, cerebral ischemic stroke (Chen et al., 2001), brain injury (Wu et al., 2008), pulmonary fibrosis (Ortiz et al., 2003), intimal hyperplasia (Wang et al., 2008a), and chronic graft rejection (Wu et al., 2003) and are described in Table S1, available online. The different sensitivities between methods may account for some of the variability that is often observed (Bensidhoum et al., 2004; Kraitchman et al., 2005).

The quantification of homing efficiency within a target tissue is typically assessed by one of two techniques: (1) quantification of the relative level of radioactivity in excised tissues and organs (Barbash et al., 2003) or (2) averaging the number of fluorescently labeled cells present in a fixed number of microscopic fields per tissue sample (Barbash et al., 2003; Jiang et al., 2006; Kawada et al., 2004). Of particular interest is the capability for real-time in vivo tracking of MSCs rolling along the vascular endothelium (Ruster et al., 2006) and diapedesis of MSCs through the endothelium within specific tissues (Sackstein et al., 2008). The available methods for assessing MSC trafficking have shown that systemically infused MSCs can (1) preferentially target, with limited efficiency, inflammation, sites of injury, tumors, and specific tissues such as the bone marrow (Belema-Bedada et al., 2008; Devine et al., 2001; Sackstein et al., 2008), and (2) nonspecifically distribute throughout various tissues and organs (Devine et al., 2003) including the lung, liver, kidney, and spleen, where a high percentage of infused cells are often observed (Barbash et al., 2003; Kraitchman et al., 2005). However, the methods used to assess MSC homing efficiency are often relative, comparing between the densities of engrafted MSCs in experimental and control groups versus quantifying the total number of MSCs which have homed to a particular tissue. Furthermore, no robust positive controls for high homing efficiencies exist. Emerging techniques for tracking MSCs, which include labeling with super paramagnetic iron oxide nanoparticles (Hsiao et al., 2007; Song and Ku, 2007) or quantum dots (Shah et al., 2007), may enhance the assessment of MSC homing, although the utility of these techniques requires further analysis.

Methods for Imaging the Precise Location of MSCs as a Function of Time. It is important to consider that systemically infused MSCs may redistribute after their initial localization in tissues. Using SPECT/CT imaging, Kraitchman et al. showed that the initial concentration of MSCs in the lung posttransfusion decreased after 24 hr, with a simultaneous increase in MSCs found in the infarcted heart tissue (Kraitchman et al., 2005). Gao et al. also found that MSCs gradually moved from the lung to the liver, spleen, kidney, and bone marrow (Gao et al., 2001a). This observation suggests that the amount of time between transfusion and detection must be considered when interpreting such studies, as the relative distribution among tissues and organs will vary depending on when the detection takes place. Also, at earlier time points (e.g., less than 24 hr), it becomes especially important to distinguish the local position of MSCs within the tissues with respect to blood vessels. Presumably, MSCs that remain inside the vasculature of the tissue in which they are detected cannot be assumed to have engrafted until they have extravasated through the vessel wall. It is critical to know if MSCs are transiently residing within vessels in the tissue, passively entrapped in vasculature (Vajkoczy et al., 2003), or have extravasated. Discriminating between these options requires high-resolution imaging with relevant staining for blood vessels and specific tissue structures, as is performed in Charles Lin's laboratory (Sackstein et al., 2008). One must also be cautious and consider the possibility that the localization of observed donor MSCs may be due to fusion with endogenous cells (Spees et al., 2003).

Characterization of MSCs Postdelivery

Characterization of engrafted MSCs following systemic infusions is a big unmet need in the field of MSC trafficking. Achieving progress in this area would be useful to determine if these exogenous MSCs can form an "MSC niche" following engraftment. It is important to consider that data generated during the 80 s showed engraftment of donor stromal cells following human bone marrow transplantation (Keating et al., 1982) but was later

refuted (Simmons et al., 1987). More recently, within a clinical setting, using sex-mismatched bone marrow T cell-depleted allografts, a limited reconstitution of marrow mesenchymal cells was demonstrated. Specifically, stromal layers containing donor-type cells were observed in 14 out of 41 patients in one study (Cilloni et al., 2000) and 4 out of 14 patients in another study (Tanaka et al., 1994). (For a more detailed examination of MSC engraftment following bone marrow transplantation, please see Koc and Lazarus [2001], Rombouts and Ploemacher [2003], and Svennilson [2005].) In another study of significant interest, MSCs from eGFP transgenic mice were isolated from the BM, expanded in vitro, and systemically infused into wild-type mice (Belema-Bedada et al., 2008). eGFP-positive cells were isolated from the BMs of the wild-type mice 3 and 6 months later, expanded, and reinfused into an additional, secondary recipient. Again, eGFP-positive cells could be isolated from the BM of this third mouse and expanded, albeit at lower numbers. Unfortunately, one limitation of the study was the lack of thorough characterization of the MSCs after each isolation. Ideally, labeled cells should have been isolated from the bone marrow after infusion, cultured, and characterized as suggested by the ISCT (Dominici et al., 2006). The stemness of the isolated MSCs was confirmed by only assaying their ability to express myocyte markers in response to appropriate inductive cues. Therefore, it remains open to question whether the isolated cells were indeed MSCs or differentiated progeny, and thus it is critical that future experiments include thorough characterization of the MSC phenotype.

Given the lack of sufficient characterization methods of systemically infused MSCs following engraftment, it is unknown whether they engraft in their native state or differentiate to replenish the parenchymal and stromal cells at an ischemic site. Delineation between MSCs and their differentiated progeny has been attempted by assaying for markers unique to mature cell types that MSCs were expected to differentiate into based on the tissue of interest (Chan et al., 2007; Wu et al., 2003; Zhang et al., 2008b). For example, infused cells or their progeny have been found to express dystrophin in a muscular dystrophy model (Chan et al., 2007), cytokeratin in a model of intestinal epithelium irradiation damage (Zhang et al., 2008b), and osteocalcin in a model of MSC homing to healthy bone marrow (Sackstein et al., 2008). The current challenges associated with characterization of MSCs following systemic infusion are a consequence of the combined complexity of defining what an MSC is, with sensitive means for detection and isolation of MSCs within an in vivo system.

Methods for Engineering of MSCs to Enhance the Homing Response

Methods of improving the trafficking and engraftment of MSCs and other cell types are a high priority for cellular therapies. Although culture-expanded MSCs express certain cell-surface receptors that mediate aspects of homing including VLA-4 (Ruster et al., 2006) and certain chemokine receptors (Ponte et al., 2007), they do not express PSGL-1(Sackstein et al., 2008) and have low expression levels of other pertinent adhesion and chemokine receptors (e.g., CXCR4), which typically governs tethering and rolling of circulating cells on activated vascular endothelium. Retrovirus vectors encoding homing receptors such as CXCR4 have been recently used to enhance homing and engraftment of HSCs and MSCs through increasing cell invasion in response to SDF-1, the ligand for CXCR4, which is typically present at inflammatory sites (Brenner et al., 2004; Cheng et al., 2008; Zhang et al., 2008a). In one example, genetically modified MSCs overexpressing CXCR4 on their surface homed to ischemic myocardium following systemic administration and enhanced postmyocardial infarction recovery of left ventricular function in a rat model (Cheng et al., 2008). In another study, the α4 subunit of the VLA-4-integrin was similarly upregulated on MSCs using an adenovirus vector, found to successfully dimerize with β1-integrin to form VLA-4 and increase the homing of MSCs to the bone marrow by more than 10-fold as compared to nontransduced MSCs (Kumar and Ponnazhagan, 2007). Interestingly, the engraftment of MSCs in the lung simultaneously decreased. An alternative approach to gene therapy, which may present potential safety concerns, involves chemical engineering of cell-surface glycans to initiate cell rolling (Sackstein et al., 2008). A critical initial step in the cascade of events during cell homing involves cell rolling, during which cells engage shear-resistant, low-affinity interactions with vascular endothelial cells (Butcher, 1991). Specifically, Sackstein and colleagues enzymatically engineered an E-selectin binding motif that is responsible for hematopoietic stem cell homing onto the surface of MSCs (Sackstein et al., 2008). Since E-selectin is highly expressed in bone marrow, substantial bone marrow engraftment of systemically administered MSCs was achieved along with rare foci of osteoid juxtaposed to the endosteal surface. A similar approach has been applied to improve engraftment of cord blood-derived HSCs (Xia et al., 2004). However, these methods require complex sugar chemistry, and the scope of potential alterations is limited to modification of existing cell-surface ligands. Another approach that could be applied to MSCs involves the conjugation of antibodies to the cell surface via bispecific antibodies (Lee et al., 2007) or palmitated protein G or protein A, which permits cell-surface functionalization by potentially any antibody bearing an accessible Fc region (Dennis et al., 2004). However, it is unclear how targeting based on these firm adhesion approaches (i.e., with antibodies) would compare to approaches that promote cell rolling at the target site. We have recently demonstrated that a robust MSC rolling response can be induced on P-selectin substrates in vitro by chemically attaching ligands to the surface of MSCs. The method, which involves covalent attachment of biotin to the cell surface followed by streptavidin and a biotinylated ligand, can be used to attach potentially any adhesion ligand to the surface of any cell type to enhance targeting to specific tissues following systemic infusion (Sarkar et al., 2008). In addition, culture conditions may be used to stimulate the expression of certain homing receptors, such as CXCR4 (Chavakis et al., 2008; Potapova et al., 2008). Taken together, these approaches provide significant potential for enhancing the homing of MSCs to specific tissues.

Summary and Prospective

There are several clinical trials being performed worldwide to examine the systemic administration of MSCs to treat a variety of diseases and tissue defects. Despite the general excitement about these trials and the promising results thus far, there is a major lack of understanding of how MSCs target specific tissues. This gap in our knowledge may be why current clinical

Problem	Recommended Action
Heterogeneity in MSCs used within, and between studies	Stem cell societies together with reviewers of research manuscripts that involve MSCs should consider and emphasize that particular MSC characterization should be performed. A good guideline can be found in the following citation (Cytotherapy (2006) Vol. 8, No. 4, 315 317)
Lack of standard methods for determining homing efficiencies and correlation with functional impact	Reviewers (and potentially journals) should mandate that research papers in this field compare and contrast their results, in form of tables or figures, with previously published results to clearly emphasize potential reasons for similarities and differences reported. The following information should be included:
	- Number of cells infused
	- Infusion site
	- Characterization of MSCs
	- Time interval between MSC infusion and assessment of homing
	- Methods used to assess homing
	- Homing efficiency
	- Functional outcome
Inability to determine the relative contributions of active and passive entrapment leading to specific homing efficiencies	Systematic studies are needed to determine if using larger MSCs can increase non-specific homing. This may involve construction of in vitro models to better understand (i) how size and deformability of MSCs changes with passage number and (ii) how this impacts their travel under relevant shear stress conditions through model systems that mimic particular vasculature beds.
Difficult to determine which cell type has actually homed (especially if a heterogeneous population of cells are systemically infused)	Methods to assess the properties of MSCs that have homed should be developed. Also, methods to characterize and label individual MSCs with tracers should be developed. It is insufficient to assume that a perceived homing event correlates with a general phenotypic property of the MSCs which have been characterized based on population versus on single cell characteristics.
Premature to construct a definitive and mechanistic definition of MSC homing	The resolution of the methods used to assess homing should be reported. For example, can the methods used differentiate between MSCs that are in blood vessels versus those that have transmigrated?
Lack of standard methods for determining presence of MSCs in peripheral blood	Reviewers (and potentially journals) should mandate that research papers in this field compare and contrast their results, in form of tables or figures, with previously published results to clearly emphasize potential reasons for similarities and differences reported. The following information should be included:
	- Method of MSC mobilization or infusion
	- Method of withdrawing blood
	- Withdrawal site
	- Method of reducing potential contaminating cells
	- Method of culturing isolated MSCs
	- Characterization of MSCs
MSCs that are locally infused may enter the blood stream during infusion as many anatomic locations are highly vascularized	Methods to limit the possibility that MSCs escape into the bloodstream should be developed in addition to methods for quantifying this during local transplantation.
Absence of proper antibody controls to prove that interactions between specific receptors and ligands are required for MSC homing	Control experiments showing that antibodies bound to receptors that are not involved in cell rolling (at the same density as the antibody of interest) do not impact the homing response.

Figure 3. Problems Faced in Field of MSC Trafficking

Given the complexities involved in identifying MSCs and tracking their position and the lack of standardized methods for culturing and characterizing them, new studies in this area should consider the common problems/challenges that are experienced and the available methods to address them.

dosing includes high numbers of cells that may range from 150 million to 300 million MSCs administered twice per week over the course of 2 weeks (National Institutes of Health, 2008). Furthermore, the balance between the beneficial effects from locally engrafted MSCs versus systemic effects from secreted paracrine factors that diffuse into target tissues is unclear. Typical problems faced by those in the MSC trafficking field and recommended actions are discussed in Figure 3.

Whether MSCs mobilize and home under steady-state conditions remains a common topic of debate. Progress in this area has been stifled by the difficulties in identifying and isolating native MSCs; most studies utilize culture-expanded MSCs that do not express many of the cell-adhesion or chemokine receptors that are responsible for the homing of leukocytes and hematopoetic stem cells. Furthermore, tracking of MSCs after local transplantation or systemic infusion has relied on techniques that have inherent disadvantages, including indirect methodology, significant manipulation of the host biology (e.g., bone marrow reconstitution), or use of an exogenous MSC source.

Based on the knowledge derived from existing studies, we can conclude the following:

(1) There is mounting evidence that host MSCs appear to mobilize in response to inflammation or injury and target specific tissues via active mechanisms; however, more work is required to substantiate this model, and the origin and mechanisms of trafficking of the mobilized MSCs remain unresolved.

(2) Systemically infused MSCs are frequently observed in significant concentrations within the bone marrow compartment, or within an injury or inflammatory site, and these cells have potential to reduce inflammation and promote tissue regeneration. However, the exact location of the MSCs (within the vessel or tissue) and their phenotype remain elusive, and thus broad conclusions cannot be substantiated regarding their engraftment or mechanisms that mediate their functional properties.

(3) Direct methods of assessing native MSCs and their trafficking properties is a big unmet need required to conclusively elucidate mechanisms of MSC trafficking during physiological and pathological states. Detection of infused MSCs that remain in an undifferentiated state compared to their differentiated progeny also presents a significant challenge.

(4) Homing of culture-expanded MSCs is inefficient compared to leukocytes and HSCs, which apparently is due to a lack of relevant cell-adhesion and chemokine receptors; however, engineering strategies are available to enhance the homing response. The increased size of MSCs likely promotes passive cell entrapment and reduces the number of MSCs that reach the target site.

As our understanding of the mechanisms of MSC trafficking grows, the ability to enhance homing to specific tissues through engineered approaches should significantly reduce the number

of cells required to achieve a therapeutic effect, and presumably provide better outcomes for patients. Accumulating evidence suggests that MSCs have a significantly larger role in regulating wound healing and inflammatory diseases than previously thought. Given the systemic nature of many diseases and the desire to have minimally invasive therapies, systemic infusion of MSCs that can promote tissue regeneration and immunosuppressive effects represents an attractive therapeutic approach. The number of potential therapeutic applications and their efficiency and efficacy will continue to grow as the fundamental biology that is responsible for the MSC regenerative properties and homing responses continues to be elucidated.

SUPPLEMENTAL DATA

The Supplemental Data include one table and can be found with this article online at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(09)00056-3.

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