

## MESENCHYMAL STEM CELLS: A PERSPECTIVE FROM *IN VITRO* CULTURES TO *IN VIVO* MIGRATION AND NICHES

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### Abstract

Mesenchymal Stromal Progenitor/Stem Cells (MSCs) are a rare population of non-hematopoietic stromal cells, present in the bone marrow and most connective tissues of the body. They are capable of differentiation into mesenchymal tissues such as bone, cartilage, adipose tissue and muscle. In the absence of specific markers, MSCs have been defined following isolation and culture expansion, by their expression of various molecules including CD90, CD105 and CD73 and absence of markers like CD34, CD45, and CD14. MSCs have extensive proliferative ability in culture in an uncommitted state while retaining their multilineage differentiation potential, which make them attractive candidates for biological cell-based tissue repair approaches. However, their identity in their tissues of origin is not clear and the niches in which they reside are not defined. This review addresses the current state of MSC research including the differentiation potency of culture expanded MSCs, expression of chemokines and their receptors in MSCs – both relevant issues for the advocated use of MSCs for tissue repair and their systemic delivery to the affected tissues. It also reviews current knowledge of MSC niches in their native tissues, addressing the relationship with pericytes. Finally, it provides a scientific basis for the requirement of a thorough characterisation of the endogenous MSC niches within their native tissues *in vivo*. The knowledge of MSC niches will instruct development of innovative therapeutic measures such as producing pharmacological substances that target endogenous MSCs and their niches in order to activate and guide intrinsic repair and to improve disease outcomes.

**Keywords:** Stem cells, mesenchymal stromal progenitors, niche, pericytes, regenerative medicine, migration, chemokines, differentiation, cartilage, bone.

### Mesenchymal Stromal Progenitor/Stem Cells

After placing whole bone marrow cells in plastic culture dishes with medium supplemented with 10% foetal calf serum, Friedenstein *et al.* (1970) demonstrated that bone marrow contains hematopoietic non-adherent cells along with a rare population of plastic-adherent cells (approximately 1 in 10,000 nucleated cells in the bone marrow). These cells were able to form colonies derived from single cells. After a few days, these adherent cells, of heterogeneous appearance, start to proliferate and can differentiate into mature cells of mesenchymal lineages such as osteoblasts (Friedenstein *et al.*, 1970; Friedenstein *et al.*, 1976). The initial clones of adherent cells expanded into round-shaped colonies composed of fibroblastoid cells, thus the term of Colony Forming Unit – fibroblasts (CFU-f). Friedenstein also found that some of the colonies could differentiate into aggregates resembling small areas of bone or cartilage. Other groups then extended these initial observations, studying CFU-f proliferative abilities and phenotypic characteristics (Castro-Malaspina *et al.*, 1980; Prockop, 1997; Caplan and Bruder, 2001), and it was established that these cells were multipotential and could differentiate into osteoblasts, chondrocytes, adipocytes, and even myoblasts. Each bone marrow donor shows a specific frequency of CFU-f, which is dependent on the age and health of the donor. The current categorization of these cells is either mesenchymal stem cells (MSCs) as proposed by Caplan (1991), because of their ability to differentiate into cells of the mesenchymal lineages, or stromal cells because they belong to the stroma that is believed to have a physical supporting role to the hematopoietic stem cell (HSC) niche (Devine and Hoffman, 2000; Wilson and Trumpp, 2006). Whether these cells should be considered true stem cells at all or as multipotent progenitors of mesenchymal lineages has been the focus of intense debate. It has therefore been proposed the term “multipotent mesenchymal stromal cells” is adopted in place of “mesenchymal stem cells” (Horwitz *et al.*, 2005; reviewed in Bianco *et al.*, 2008).

### MSCs: Definition and Tissue Sources

The definition of MSCs relies solely on the analysis of *in vitro* culture-expanded cell populations. Despite years of intense investigation, the location and role of the native MSCs within their tissue of origin *in vivo* are not known, mainly because of the lack of specific markers allowing their unambiguous identification (Bianco *et al.*, 2008; Jones and McGonagle, 2008; Morikawa *et al.*, 2009). The possibility exists that the MSC phenotype and abilities

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vary between *in vivo* and *in vitro* settings due to the removal from their natural environment and the use of chemical and physical growth conditions that might alter their characteristics. MSCs are known to undergo phenotypic rearrangements during *ex vivo* manipulations, losing expression of some markers while also acquiring new ones (Jones *et al.*, 2002). Due to the growing interest in using MSCs in cell-based therapy (Barry and Murphy, 2004; De Bari and Dell'Accio, 2008), the need to identify MSCs in a definitive way is not only a scientific interest but also derives from clinical and regulatory requirements (Sensebé, 2008; Sensebé *et al.*, 2010). As noted above, no unique markers can unequivocally identify a MSC and distinguish it from other cell types. In 2006, the International Society for Cell Therapy proposed the following criteria for the minimal identification of human MSCs (Dominici *et al.*, 2006): adherence to plastic in standard culture conditions; CD73<sup>+</sup>, CD90<sup>+</sup>, CD105<sup>+</sup>, CD34<sup>-</sup>, CD45<sup>-</sup>, HLA-DR<sup>-</sup>, CD14<sup>-</sup> or CD11b<sup>-</sup>, CD79a<sup>-</sup> or CD19<sup>-</sup> cell phenotype as assessed by FACS analysis; *in vitro* differentiation into osteoblasts, adipocytes and chondroblasts (demonstrated by staining of *in vitro* cell culture). These criteria allow only a retrospective definition of a cell population containing MSCs but do not allow prospective purification of MSCs. In addition, these criteria are not entirely valid across and intra species. In mouse models, MSCs differ frequently not only from the human MSCs, but also between strains in marker expression and behaviour in culture (Peister *et al.*, 2004; Sung *et al.*, 2008), the major differences being in the expression of CD34 and CD105 (for CD105 and other markers see also Fiorina *et al.*, 2009). These criteria are overly dependent on culture conditions for derivation and expansion of MSC populations and, therefore, are unlikely to be extrapolated to the native cells. Indeed, MSCs are expanded under conditions that maintain the typical MSC differentiation potency but do not preserve what is currently considered to be the native MSC phenotype (Jo *et al.*, 2007; Morikawa *et al.*, 2009).

Isolation of MSCs has been performed in several species (Friedenstein, 1970) including humans (Castro-Malaspina, 1980; Haynesworth, 1992; Bruno *et al.*, 2009; Yoo *et al.*, 2009) and mice (Gindraux *et al.*, 2007; Sung *et al.*, 2008), and from many tissues other than the bone marrow, including peripheral blood (Zvaifler *et al.*, 2000), cord blood (Erices *et al.*, 2000), cord Wharton's jelly (Sarugaser *et al.*, 2005), adipose tissue (Zuk *et al.*, 2002), amniotic fluid (In't Anker *et al.*, 2003), compact bone (Guo *et al.*, 2006), periosteum (Nakahara *et al.*, 1991; De Bari *et al.*, 2001a; De Bari *et al.*, 2006a), synovial membrane (De Bari *et al.*, 2001b; De Bari *et al.*, 2003) and synovial fluid (Jones *et al.*, 2004), articular cartilage (Dowthwaite *et al.*, 2004) and foetal tissues (Campagnoli *et al.*, 2001; Miao *et al.*, 2006). Cells derived from different tissues show phenotypic heterogeneity and different growth abilities, but they also show similarities, with the potential to differentiate into the classical mesenchymal lineages and the expression of common surface markers (Baksh *et al.*, 2007). However, there is increasing evidence that marked differences exist in the biology of MSCs that are

dependent on the tissue of origin, which appears to be the main source of variation in the biological properties of MSCs (De Bari *et al.*, 2008). Within each tissue source, single-cell-derived clonal MSC populations are known to be highly heterogeneous in their proliferative and differentiation potential (De Bari *et al.*, 2008; Phinney and Prockop, 2007). The resulting variability limits standardization of MSC-based bone repair strategies and impedes the comparison of clinical study outcomes. There is, therefore, an unmet clinical need for assays that allow quantitative estimation of the differentiation potency of MSC preparations. Such potency assays would allow development of quality controls for efficacy of MSC preparations (De Bari *et al.*, 2006c; De Bari and Dell'Accio, 2007), a vital prerequisite for their routine use in clinical practice.

Cells with properties of MSCs have also been isolated from tissues in several pathological conditions, sometimes with distinctive features. For instance, in the rheumatoid arthritic joint, MSC-like cells appear to express bone morphogenetic protein (BMP) receptors (Marinova-Mutafchieva *et al.*, 2000). In the peripheral blood of acute burns patients, Mansilla *et al.* (2006) reported increase in circulating MSC-like cells compared with healthy donors, with greater numbers found among younger patients with more extensive burns. It is postulated that MSCs are mobilized into the bloodstream following acute burn signals which have not yet been elucidated. In other pathological conditions, such as obstructive apnoeas and bone sarcomas, studies provide evidence of possible mobilization of MSCs which increase in their circulating numbers compared to healthy individuals (Carreras *et al.*, 2009; Bian *et al.*, 2009); these reports are initial studies, often imprecise in the definition of MSC phenotype, and therefore they warrant further more accurate studies to understand the mechanisms underlying MSC mobilization *in vivo*, its biological significance and possible clinical impact in terms of recruitment to tissue and wound healing.

Intense investigation on MSC isolation studies the use of monoclonal antibodies in order to pre-select cells with an MSC surface phenotype; the methods vary from negative selection, where other cell types, such as hematopoietic cells, are removed (Baddoo *et al.*, 2003), to positive selection, when MSCs are directly enriched from a pool of other cells in which they are known to be present (Jones *et al.*, 2002; Quirici *et al.*, 2002; Deschaseaux *et al.*, 2003; Jones *et al.*, 2006; Buhning *et al.*, 2007; Gindraux *et al.*, 2007; Battula *et al.*, 2009). This proves very challenging in view of the lack of specific markers and the phenotypic plasticity that MSCs demonstrate *in vitro* (Jo *et al.*, 2007). There are ongoing efforts to come up with marker sets that would include positive and negative selection, in order to obtain enrichment and, ideally, purification from native tissues of MSC subsets with a consistent and clinically desired potency, which is a prerequisite for development of standardized, GMP-compliant cell therapy in a clinical setting. Currently, the intense investigation of prospective MSC isolation markers has led to the identification of a variety of molecules that could prove useful in the *in vivo* identification and

purification of MSC-like cells (reviewed in Jones and McGonagle, 2008); prominently among them are LNGFR (CD271), a neural marker repeatedly found to be expressed by MSCs (Jones *et al.*, 2002; Quirici *et al.*, 2002; Jones *et al.*, 2006; Buhning *et al.*, 2007; Battula *et al.*, 2009), and CD49a (Deschaseaux *et al.*, 2003; Jones *et al.*, 2006), together with the markers already indicated by the International Society for Cell Therapy (Dominici *et al.*, 2006). Recently, PDGFR $\alpha$ + Sca-1+ CD45- TER119- cells have been isolated from murine BM with abilities and characteristics consistent with conventional plastic-adherent MSCs (Morikawa *et al.*, 2009). It is anticipated that the adoption of MSC purification procedures will allow a one-stop therapeutic approach, involving rapid production of uncultured MSCs for immediate administration to patients.

### Differentiation Potency of Culture-Expanded MSCs

#### General concepts

Among the criteria used to define MSCs is the ability to differentiate *in vitro* into the three mesenchymal lineages, i.e. bone, cartilage and fat. The classical osteogenic differentiation of human MSCs (Jaiswal *et al.*, 1997; Pittenger *et al.*, 1999) requires incubation of cell monolayers with ascorbic acid,  $\beta$ -glycerophosphate, and dexamethasone (added to medium containing FBS), resulting in increase in alkaline phosphatase and calcium deposition. The chondrogenic differentiation requires a high cell-density pellet or micromass culture in conjunction with the use of transforming growth factor- $\beta$  in a chemically defined serum-free medium; the histological analysis reveals production of cartilage-specific highly sulphated proteoglycans and type II collagen. The adipogenic differentiation requires treatment with dexamethasone, insulin, isobutyl methyl xanthine, and indomethacin (added to medium containing FBS), and is revealed by the appearance of lipid vacuoles detected with oil red O staining. In Pittenger's report, some of the clonal-derived populations were able to differentiate into all three lineages, but other clonal populations were lacking differentiation into at least one lineage. Notably, all clonal populations were able to undergo osteogenesis (Pittenger *et al.*, 1999). In following studies, many of the human MSC populations were reported as readily differentiating into the three lineages, and they were undergoing a sequential loss of lineage potential with the osteogenic precursors as residual cells (Muraglia *et al.*, 2000), indicating the possible existence of a hierarchical model of differentiation.

There is increasing evidence to indicate that MSC populations are heterogeneous with coexisting subsets having varying potency, and this applies to bone marrow MSCs as well as those from other tissues. In this regard, we recently reported that human synovium-derived clonal MSCs were all capable of osteogenic and chondrogenic differentiation though with varying potency, where only 30% of the clonal populations tested were able to differentiate into adipocytes (Karystinou *et al.*, 2009).

Under appropriate conditions, MSCs have been shown to differentiate also into other mesenchymal lineages such

as skeletal myocytes and tenocytes (Wakitani *et al.*, 1995; De Bari *et al.*, 2003; Hoffmann *et al.*, 2006). Notably, there are reports indicating the capacity of MSCs to differentiate into non-mesenchymal lineages such as neurons (Woodbury *et al.*, 2000). The clinical relevance of the presumptive non-mesenchymal potency of MSCs is however questioned since MSC-derived neuron-like cells were unable to generate action potentials and therefore function as neurons (Hofstetter *et al.*, 2002).

#### MSC-derived cartilage and bone

Particular attention has been devoted to the chondrogenic and osteogenic abilities of MSCs. This is because it is hypothesized that MSCs with their natural mesenchymal potency would primarily be used for the biological repair of articular cartilage and bone. The osteogenic potential of whole bone marrow population first (Friedenstein *et al.*, 1966; Luria *et al.*, 1987) and of culture-expanded MSCs later (Friedenstein, 1976; Ashton *et al.*, 1985) has been studied extensively in *in vitro* and *in vivo* experiments. The first *in vivo* experiments were performed using diffusion chambers loaded with whole bone marrow (Friedenstein *et al.*, 1966) or with culture-expanded cells (Ashton *et al.*, 1980). Later, the adoption of bioscaffolds, such as hydroxyapatite (HA) implanted in immunocompromised mice has proved useful to help understanding the mechanisms of MSCs differentiation *in vivo* (Ohgushi and Okumura, 1990). In studies in nude mice, Muraglia *et al.* (1998) were able to develop donor-derived bone by subcutaneous implantation of HA scaffolds seeded with human MSCs. Later, it became possible to repair large bone defects *in vivo* by using autologous MSCs, in loaded conditions, both in large animals (Kon *et al.*, 2000) as well as in humans in a proof-of-concept study (Quarto *et al.*, 2001). In another set of *in vivo* human studies, Horwitz *et al.* (1999) reported that bone marrow transplantation in three children affected by osteogenesis imperfecta (OI) resulted in enhancement of bone structure with differentiation of donor MSCs into functional osteoblasts. In a subsequent study (Horwitz *et al.*, 2002), allogeneic bone marrow-derived MSCs were safely administered to children with severe OI, and were shown to engraft in genetically defective bone and differentiate into osteoblasts.

Aslan *et al.* (2006) purified CD105+ cells from human bone marrow that were able to differentiate *in vitro* and *in vivo* toward the osteogenic lineage. Such an approach highlights the clinically relevant possibility that function-specific cell types could be purified and directly used for tissue engineering/repair purposes, without the need for time-consuming and costly cell culture expansions. Although it cannot be ruled out that a relatively low number of "true" stem cells may be sufficient to provide repair, purification strategies appear unlikely to yield adequate quantities of MSCs at least for repair of large defects, especially in view of their known low frequency.

Another unresolved issue pertains to the MSC type to use in clinical practice, given the plethora of tissue sources. For instance, human periosteum is also known to contain cells that upon enzymatic release and culture expansion display MSC phenotype and capacity at the single cell

level to differentiate into multiple skeletal lineages including bone *in vitro* and *in vivo* (De Bari *et al.*, 2006a). Notably, in a proof-of-concept study we quantified the bone-forming potency of matched human MSCs from synovium and periosteum and analyzed the sources of variability in osteogenic outcome. We identified the tissue of origin of MSCs as the main source of variability, since MSCs from periosteum had significantly greater osteogenic potency than MSCs from synovium. A second source of variability was related to the individual donor, within each tissue. We measured the basal expression levels of osteoblast-lineage genes in clonal MSCs prior to osteogenic treatment, identified biomarkers that correlated with osteogenic outcome and developed a mathematical model that predicts bone-forming potency of clonal MSC preparations, independent of donor and tissue source (De Bari *et al.*, 2008). The development of a biomarker-based model that predicts the osteogenic potency of human MSC preparations is of considerable clinical relevance. A similar approach is likely to increase consistency of therapies that employ MSCs for bone repair. It may also facilitate the selection of individuals that qualify for MSC-based bone repair and help identify the best source and preparation protocol of human MSCs. It remains to be investigated whether the same formula can be applied successfully to MSC-based orthotopic bone repair in a preclinical model, where it is easy to think that in addition to the properties intrinsic to the cell preparation, other factors such as inflammation and biomechanics will influence bone formation.

While bone formation is relatively straightforward when MSCs are loaded onto matrices and then implanted subcutaneously in mice, the formation of stable cartilage appears to be a very challenging task with MSCs. The chondrogenic potential of MSCs is well known *in vitro* in pellet cultures but the key question as to whether this is stable cartilage or a transient cartilage template destined to be replaced with bone in a process of endochondral ossification remains to be addressed. Using a nude mouse assay of ectopic cartilage formation validated with intramuscular injection of adult human articular chondrocytes (Dell'Accio *et al.*, 2001), we demonstrated that the *in vitro* chondrogenic potential of synovial membrane-derived MSCs is not sufficient to predict the *in vivo* outcome at least in this nude mouse model, since the synovial MSCs induced *in vitro* into a chondrocyte-like phenotype failed to form stable cartilage when implanted *in vivo* (De Bari *et al.*, 2004). Of note, Pelttari and colleagues reported that bone marrow MSC-derived cartilage pellets transplanted into ectopic sites in SCID mice underwent endochondral ossification, *via* premature induction of chondrocyte-hypertrophy-related molecules such as type X collagen (Pelttari *et al.*, 2006). These studies, however, do not rule out the possibility that, as opposed to an ectopic site, the joint environment of a cartilage defect may instead be sufficient either to induce a stable cartilage phenotype or stabilize the chondrocyte-like phenotype of *in vitro* pre-committed MSC populations. Uplift of the bone front at the expense of the overlying articular cartilage has been observed in osteochondral repair by bone marrow cells (Qiu *et al.*, 2003). This phenomenon has not been

reported in patients treated with autologous chondrocyte transplantation and therefore one may argue that the phenotypic memory of articular chondrocytes could possibly limit the advancement of the bone front, thus preserving the normal thickness of the repaired cartilage tissue (De Bari *et al.*, 2006b).

### Expression of Chemokines and Their Receptors in MSCs

The knowledge of native MSC biology and interactions with their nearby microenvironment, i.e. the stem cell niche, in healthy *versus* damaged or diseased tissues will provide guidance on future clinical applications employing MSCs. A challenge that the biomedical community will face in regenerative medicine is the re-establishment of a functional niche similar to the physiological one when regenerating or healing damaged tissues. The restoration of a functional niche will indeed be essential to safeguard durable repair and ensure continual replacement of mature cells lost to physiological turnover or subsequent stress or damage.

Stem cell niches have been described so far for a number of tissue types such as the hair follicle, intestine and the bone marrow (Fuchs *et al.*, 2004; De Bari *et al.*, 2006c). The niche of hematopoietic stem cells (HSCs) in bone marrow might serve as a good example for the complexity of the niche functional concept. It is commonly regarded that the HSC niche consists of at least two distinct niches, the endosteal niche where hematopoietic stem cells are in close contact with osteoblasts residing at the bone surface of the trabeculae, and the perivascular niche where the HSCs are found close to the sinusoids in the bone marrow (Mitsiadis *et al.*, 2007). In the endosteal niche signalling events between osteoblasts and HSCs play a crucial role in maintenance and activation of stem cells (reviewed in Kiel and Morrison, 2009). Pathways like Notch and Wnt signalling are known to be involved (Calvi *et al.*, 2003; Reya *et al.*, 2003; Duncan *et al.*, 2007), and the SDF-1/CXCR4 system is part of the complex signalling network.

Chemokines are small (8-10 kDa) proteins able to chemically attract lymphocytes, neutrophils and other immune cell types to the sites of inflammation. Several families of chemokines and their receptors exist, each with different characteristics and abilities. The chemokine SDF-1 is expressed on osteoblasts and endothelial cells and the interaction with CXCR4 is thought to regulate trafficking of HSCs in the bone marrow (Semerad *et al.*, 2005). In the perivascular niche, sinusoidal reticular cells express high levels of SDF-1 and were found to be in close contact with HSCs (Sugiyama *et al.*, 2006). Interestingly, these SDF-1 expressing reticular cells were also located at the endosteal niche. The fact that HSCs express the receptor CXCR4 (Peled *et al.*, 1999) suggests that the SDF-1/CXCR4 system would be crucial for the modulation of activation or quiescence of the HSC niche in bone marrow.

Similarly to HSCs, chemokines and their receptors might be of importance for MSCs in their niche. MSCs have been reported to express varying degrees of

chemokines and chemokine receptors, with differences being likely due to the isolation techniques and *in vitro* culture conditions (Honczarenko *et al.*, 2006; Djouad *et al.*, 2007). Sordi *et al.* (2005) reported that chemokine receptors (namely CXCR4, CX3CR1, CXCR6, CCR1 and CCR7), expressed by a minority (2%-2.5%) of the MSCs, were linked to the *in vivo* migratory abilities of MSCs toward murine pancreatic islets.

Few data are available regarding the existence of a MSC niche *in vivo* (reviewed in da Silva Meirelles *et al.*, 2008), and they suggest a perivascular location of the MSCs (reviewed in Kuhn and Tuan, 2010), although this notion is challenged by the retrieval of MSC-like cells in avascular tissues such as articular cartilage (Barbero *et al.*, 2003; Dell'Accio *et al.*, 2003; Dowthwaite *et al.*, 2004). Tissue-specific MSC niches are likely to exist, since tissue-specific distinct MSC phenotypes and functions have been reported (Hennig *et al.*, 2007; Roubelakis *et al.*, 2007; Meijer *et al.*, 2008; Zhu *et al.*, 2008; Hwang *et al.*, 2009; Ivanova-Todorova *et al.*, 2009), and also embryologically MSC could derive from non-mesodermal progenitors, such as neuro-epithelial cells (Takashima *et al.*, 2007). The study of the migratory abilities of MSCs *in vivo* will elucidate the requirements for homing and engraftment of such cells and therefore underpin common features of a generic MSC niche.

Systemically infused MSCs have been retrieved in multiple organs such as lung, liver, kidney, and spleen (Barbash *et al.*, 2003; Devine *et al.*, 2003; Kraitchman *et al.*, 2005), but also in specific targets, such as sites of inflammation, injury, tumors and tissues already known to contain MSCs such as the bone marrow (Devine *et al.*, 2001; Belema-Bedada *et al.*, 2008; Sackstein *et al.*, 2008). Cultured MSCs used as a therapeutic tool *in vivo* by means of systemic infusion were retrieved in the site of action in some experimental models (Zappia *et al.*, 2005; Sasaki *et al.*, 2008), but not in other models (Augello *et al.*, 2007). Freshly isolated, uncultured MSCs have been reported to migrate to bone marrow and spleen after systemic transplantation in experimental animal models (Rombouts and Ploemacher, 2003; Mahmud *et al.*, 2004; Morikawa *et al.*, 2009). In contrast, culture-expanded MSCs appear unable to migrate and home to the bone marrow (Rombouts and Ploemacher, 2003; Karp and Leng Teo, 2009; Morikawa *et al.*, 2009). Recent reports show that following clinical bone marrow transplantation human MSCs are of host origin (Bartsch *et al.*, 2009). A parabiosis study from Maloney *et al.* (1985) demonstrated that in mice in parabiotic equilibrium where one partner had been X-irradiated, repopulation of the CFU-F compartments of the bone marrow in the irradiated mouse resulted from recovery of the local CFU-F and not from migration of CFU-Fs from the parabiotic, non-irradiated partner. The apparent discrepancy in findings on MSC homing and engraftment may be explained by the differences in model systems, the adoption of freshly isolated MSCs versus culture-expanded MSCs and also the different culture conditions, likely to affect MSC phenotype and hence their migratory patterns. Nonetheless, it is believed that MSCs, when infused intravenously, have potential to migrate to

sites of injury, such as to adult brain (Ji *et al.*, 2004), embryonic brain (Munoz-Elias *et al.*, 2004), infarcted myocardium (Barbash *et al.*, 2003; Schenk *et al.*, 2007), injured skeletal muscle (De Bari *et al.*, 2003) and kidney (Morigi *et al.*, 2004). MSCs have repair and immunomodulatory abilities also in mouse lungs, reducing systemic response to endotoxin when infused intravenously, but disappear from the site of injury after a short time lapse (Xu *et al.*, 2007).

Several reports indicate that the SDF-1 (CXCL12)/CXCR4 axis is present and functional in MSC populations (Wynn *et al.*, 2004; Dar *et al.*, 2005). Recently, it has been demonstrated that this pathway is crucial in the migration of MSCs to injury sites such as bone fractures, with absence of MSC recruitment if SDF-1 signalling was impaired (Kitaori *et al.*, 2009). Ma *et al.* (2005) investigated the time course of myocardial SDF-1 expression and effects of intravenously administered bone marrow-derived MSCs in rats with experimental myocardial infarction (Ma *et al.*, 2005). Myocardial SDF-1 expression was increased only in the early phase post-infarct, and as a result only MSCs intravenously infused in temporal vicinity to the early phase of MI were recruited to injured myocardium, enhancing angiogenesis and improving cardiac function, while MSCs injected when the cardiac SDF-1 expression had already fallen did not home to the heart or have a positive effect on the MI outcome. These findings raise the need for identification of a temporal therapeutic window for intervention with MSCs.

There is evidence that MSCs can respond to chemotactic signalling molecules acting on pathways other than the SDF-1/CXCR4 axis. One of those is the Monocyte Chemotactic Protein-3 (MCP-3). Schenk *et al.* (2007) showed that when systemically infused, MSCs migrated transiently toward the infarcted myocardium in response to MCP-3 signalling. The Authors then induced migration of MSCs to the infarcted area by previous implantation of MCP-3-over-expressing cardiac fibroblasts in the infarct border zone. Structural and functional improvements were reported, mainly due to remodelling of the cardiac collagen matrix, in the absence of angiogenesis and without cardiomyocyte regeneration.

Migrating MSCs may therefore represent a source of multipotent cells that could be available for the repair of damaged tissues and organs. However, the mechanisms that underlie homing of implanted cells are still unclear and may be merely a stochastic event or explainable with the vasodilatation and increased blood supply (and cells) to the injured areas of the body. It is also postulated that MSCs could have the ability to interact with immune cells during inflammation; these interactions could have an impact on the way MSCs contribute to the repair process in recipients *in vivo* (Ohtaki *et al.*, 2008; Constantin *et al.*, 2009). Understanding the underlying mechanisms of action as part of the pharmacology of cell therapy is thus of paramount importance in view of the increasing number of clinical trials with MSCs, as this is anticipated to allow forecasting the outcome of MSC-based treatments.

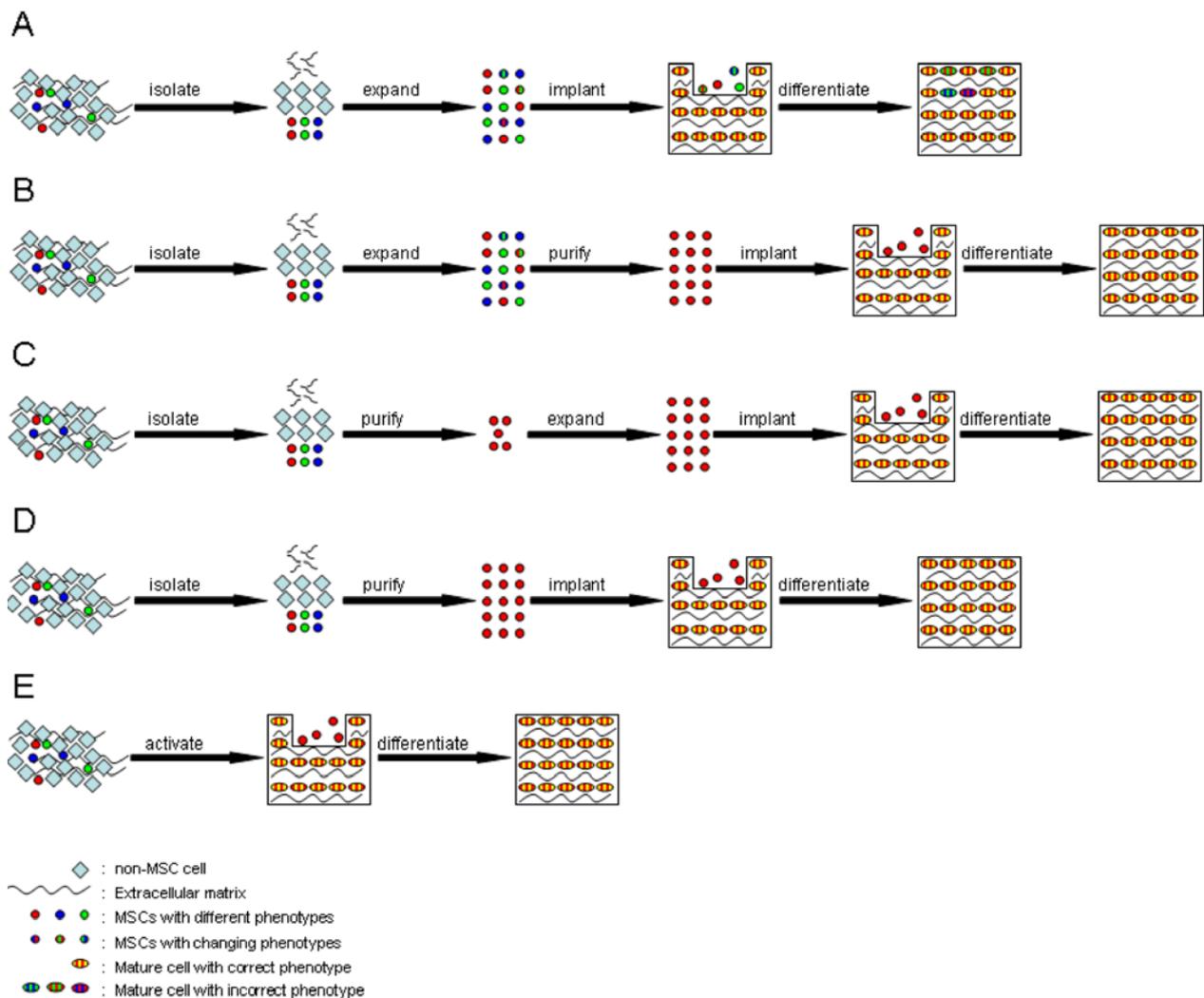
## MSCs and Pericytes: a Catch-22?

A major unresolved question relates to the identity of MSCs within their native tissues. There is growing evidence that pericytes may be the native cells of the *ex vivo* MSCs. Pericytes, also known as mural cells or Rouget cells, are described as branched cells located on the abluminal side of small blood vessels (arterioles, capillaries and venules) and are in close connection with the vessels' endothelial cells. The functions of pericytes include vessel stabilization, synthesis of matrix proteins, macrophage-like properties, activity in immunologic defence and, possibly, mesenchymal potentiality (reviewed in Diaz-Flores *et al.*, 2009). Of special interest in this regard is the perivascular MSC niche hypothesis. A stem cell niche is defined as the microenvironment where the adult stem cell resides and includes surrounding cells and extracellular matrix, both thought to provide signals that keep the stem cells quiescent or instead modulate their activation. In the case of activation, stem cells undergo either symmetric division or asymmetric division, i.e. they give rise to daughter cells that are both stem cells or they produce progeny one of which is a stem cell while the other daughter cell is already committed to its differentiation fate. The proximity to vessels would allow pericytes quickly to enter the bloodstream to replace cells lost due to physiological turnover or repair of local lesions (da Silva Meirelles *et al.*, 2008). Brighton *et al.* (1992) demonstrated that pericytes exhibit *in vitro* a phenotype similar to that of bone marrow-derived bone cells (MSCs). Diaz-Flores and colleagues provided evidence by Monastral Blue staining that pericytes could be involved in generating cartilage and bone (Diaz-Flores *et al.*, 1991; Díaz-Flores *et al.*, 1992). Furthermore, pericytes from various adult and foetal tissues have been shown to express MSC markers such as CD44, CD73, CD90 and CD105. Freshly isolated pericytes from the placenta were shown to be myogenic when injected into SCID-mdx-mice (Crisan *et al.*, 2008). However, despite growing circumstantial evidence that pericytes might be the *in vivo* native cells of the *ex vivo* MSCs, there has been no direct evidence up to now that pericytes have key features of stem cells including proliferation and differentiation into mature cell phenotypes *in vivo* following injury by prospective cell lineage tracking experiments, a requirement that should apply as well to other putative MSC populations. In their recent study, Crisan and colleagues indeed concluded that pericytes might not be the only source of MSCs (Crisan *et al.*, 2008). The question therefore remains open as to whether the MSC is a unique cell type, distinct from the pericyte, with the specific function to replace mature mesenchymal cells lost to physiological turnover, injury or disease, or if there are multiple subsets of MSCs or progenitor cells, which might be functionally distinct. Cell-lineage tracking experiments, now increasingly feasible with modern technologies and detection systems, will shed light on these unresolved issues related to the *in vivo* nature of the native MSCs in the natural environment of their intact tissues.

## Conclusion

The advancement of therapeutic approaches using MSCs is currently somewhat constrained by the lack of data about the *in vivo* properties of the native MSCs within their tissues and niches. The proliferation and differentiation data, as well as the marker definition, are all related to the *in vitro* culture systems, which are likely to alter the natural characteristics that these cells have *in vivo*. Another problem arises from the fact that frequently the data obtained through *in vitro* manipulation are not reproducible when translating to *in vivo* applications even when using the same batch of cells. Bianco and co-workers found that even parallel cultures of cells extracted from the same batch could not demonstrate true multipotency (Bianco *et al.*, 2008). So far, in most of the clinical skeletal tissue engineering applications in which MSCs have been used the investigators applied large numbers of cells in order to physically fill the defects, and therefore a culture expansion stage was unavoidable. However, no conclusive data have been produced showing that a higher number of cells is more adequate and efficacious to repair a large defect than a smaller number of purified and potent stem cells. Moreover, when applying the concepts of tissue repair to some clinical settings such as small cartilage lesions, many investigators have started to indicate that one possible way to use MSCs therapeutically is through pharmacological targeting of endogenous MSCs and related niches, without the need to remove the cells from their environment, culture expand them and then implant them back to patients, thus avoiding culture-related modifications such as possible malignant transformation of the cells and risks of adverse immune reactions e.g. to components of the FBS used in culture (reviewed in Tonti and Mannello, 2008).

The *ex vivo* expansion of mixed cell populations is likely to lead to unsatisfactory tissue repair, e.g. because of possible contamination of undesired cell types that could even interfere with the repair process (Fig. 1A). This is likely to make cell therapy inconsistent and unreliable. There is therefore a clinical and regulatory requirement to devise technologies for prospective purification of cells with the desired potency in order to standardise cell-based therapy, and ensure consistent and reproducible structural and clinical outcome. Such purification could be either from fresh tissue sources or from culture-expanded mixed cell populations (Fig. 1B and C). In some clinical applications, it may be possible to purify the cells of interest with known and predictable potency directly from the tissue sources (e.g., bone marrow) and implant the purified cells intra-operatively using a one-step procedure (Fig. 1D). The purification of the desired cell type could be coupled with the concurrent purification of other cell types, such as endothelial cells, that could prove to be beneficial in assuring a successful outcome of selected MSC-based cellular therapies (Lasala *et al.*, 2010). A valuable option will also be the activation of intrinsic repair or regeneration by targeting endogenous MSCs with bioactive molecules (Fig. 1E). Understanding the *in vivo* MSC niches and their molecular regulation in health and disease is therefore of the utmost importance for the development of novel



**Fig. 1:** Tissue repair strategies using MSCs. **(A)** MSCs of potentially different phenotypes are isolated from their native tissue and culture expanded. Due to culture conditions phenotypes might change and the repair of the defect could fail as a consequence of incorrect phenotypes of mature cells filling the defect. **(B)** MSCs are expanded *in vitro* after isolation. Enrichment based on marker combinations specific for a defined MSC phenotype would lead to differentiation into the required mature cell type. **(C)** MSCs are purified and culture expanded before implantation in the defect. **(D)** MSCs are purified after tissue release by defined selection using combinations of MSC markers. Repair of tissue defects will be of a consistent phenotype of mature cells. **(E)** MSCs of a defined phenotype suitable for tissue defect repair will be stimulated *in vivo* to migrate into the defect and then differentiate into the desired mature cell type.

pharmacological approaches to tissue repair by targeting endogenous stem cells and niches and their regulatory reparative signalling networks. The scientific community needs to devote efforts towards an in-depth understanding of the networking abilities of MSCs *in vivo*, in order to unravel the physiological ways by which these cells exit from the quiescent state and become activated to cope with parapsychological or pathological conditions. There is also a need to investigate the migratory abilities of MSCs, likely to be a function of the adhesion molecules that MSCs express on their surface. With these approaches, we anticipate that one day we will be able to directly influence and guide, using tissue- and cell-specific pharmacological targets, the *in vivo* mechanisms that activate and direct native MSCs towards the sites of inflammation and injury in order to trigger and enhance tissue regeneration by means of directed *in vivo* tissue engineering.

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## Discussion with Reviewers

**Reviewer I:** What are the quantified data indicating that MSCs home?

**Authors:** Several studies, cited throughout our review article, have reported quantification of MSC homing and engraftment in multiple tissues and organs. Chemotactic mechanisms of MSC homing remain largely unknown and the engraftment of MSCs in organs and tissues may be simply stochastic or due to multiple factors including increased blood supply secondary to injury. Hereafter, we briefly report a few selected studies on quantitation of homing and engraftment of MSCs. Results cannot be compared due to differences in experimental systems and detection methods.

Horwitz *et al.* (1999) (text reference) showed engraftment of mesenchymal cells in two children with Osteogenesis Imperfecta (OI) following transplantation of bone marrow (BM) from HLA-identical or single-antigen-mismatched siblings after ablative conditioning therapy. To assess for the engraftment of donor-derived cells in the recipient, osteoblasts were cultured from fresh bone biopsy specimens. In one patient the donor cells were quantified at a frequency of 1.5% by *in situ* hybridization for Y chromosome, while in the other patient the frequency was 2% as determined by DNA polymorphism analysis.

Horwitz *et al.* (2002) (text reference) showed that in five out of six OI patients there were signs of engraftment of retrovirally-infected cultured MSCs. Donor cells were detected by PCR for a retroviral marker. Engraftment in BM stroma and bone of donor cells did not exceed 1%. Despite such low engraftment, clinical improvement ranged from 60% to 94% compared with 0% to 40% over the 6 months immediately preceding the infusions (Horwitz *et al.*, 2002).

In baboons, Devine *et al.* (2001) (text reference) detected by PCR in the BM of recipients retrovirally transduced BM-derived syngeneic or allogeneic MSCs for over 1 year after their co-infusion with autologous HSCs. The Authors also identified donor MSC-derived cells by flow cytometry in the BM aspirate from one experimental animal at day 33 post-infusion, in a percentage of 1%.

De Bari *et al.* (2003) (text reference) quantified by RT-PCR using species-specific primers the numbers of “human cell equivalents” engrafting in nude mouse skeletal muscles after intramuscular injection of human synovial membrane-derived MSCs. They also performed an intravenous administration of  $5 \times 10^6$  human MSCs into nude mice. MSCs homed preferentially to the injured (cardiotoxin-treated) tibialis anterior muscle as opposed to the contralateral uninjured control muscle. At 3 weeks, about  $2 \times 10^3$  human cells were detected in the injured muscles, while they were undetectable in the uninjured control muscles.

Rombouts and Ploemacher (2003) (text reference) performed experiments of intravenous infusion of uncultured (obtained from CD45<sup>-</sup>/low BM cells) or cultured BM-MSCs in irradiated and control syngeneic mice. The Authors, analyzing the ratio of donor vs. host CFU-f from the organs of transplanted mice, assessed in a

quantitative manner the homing, *in vivo* expansion and tissue distribution of donor-derived MSCs in BM, spleen, thymus and lymph nodes.

Belema-Bedada *et al.* (2008) (text reference) showed that in mice overexpressing MCP-1 in the heart, intravenously infused allogeneic eGFP<sup>+</sup> BM-MSCs migrated preferentially toward the MCP-1 overexpressing heart as compared with wild-type animals, in which migration toward heart was negligible. Quantitation was performed by counting eGFP<sup>+</sup> cells on histological sections and by real-time RT-PCR for eGFP on whole hearts. In mice operated to induce cardiac ischemia-reperfusion, there was a requirement for an intact CCR2/Front signalling in the donor MSCs to migrate toward the site of injury.

Sackstein *et al.* (2008) (text reference) injected human MSCs expressing a variant of CD44, HCELL, which permits selective MSC migration toward bone. While human MSCs that did not express HCELL were not retrieved in the BM of recipient mice, the HCELL<sup>+</sup> cells migrated to the murine bone marrow at an endosteal location. Cells positive for human CD44 and for human osteocalcin were quantified by microscopic analysis.

Morikawa *et al.* (2009) (text reference) studied migration of murine uncultured EGFP<sup>+</sup>Sca1<sup>+</sup>PDGFR $\alpha$ <sup>+</sup>CD45.1<sup>-</sup>Ter119<sup>-</sup> MSCs in EGFP<sup>-</sup>CD45.1<sup>-</sup> mice when cotransplanted with HSCs from EGFP<sup>-</sup>CD45.1<sup>+</sup> mice. The EGFP<sup>+</sup>CD45.1<sup>-</sup> MSCs were found in the recipient BM up to 16 weeks after transplantation. By contrast, cultured MSCs were not found to migrate into the recipient BM. Flow cytometry was carried out to detect and quantify donor EGFP<sup>+</sup> MSCs homed into the host BM. The Authors also cultured the host BM as single cells and were able to produce EGFP<sup>+</sup> fibroblastic clones, demonstrating effective engraftment of MSCs in the host BM.

**Reviewer II:** How do the results of Patt and Maloney using mouse symbionts compare to more recent results on MSC homing?

**Authors:** Maloney *et al.* (1985) (text reference) demonstrated that in mice in parabiotic equilibrium where one partner had been X-irradiated, repopulation of the CFU-F compartments of the BM in the irradiated mouse resulted from recovery of the local CFU-F and not from migration of CFU-Fs from the parabiotic, non-irradiated partner. Later studies, using different models and detection systems, have demonstrated engraftment of donor MSCs in the recipient BM (see above). Resident BM-MSCs are known to be resistant to lethal irradiation and, due to their quiescence and permanence in G<sub>0</sub>, they can survive despite their inability to replicate, thus competing for the niche with the donor MSCs (Morikawa *et al.*, 2009). Independent of the model system, the frequency of MSC engraftment is low and difficult to detect, also in view of the lack of MSC specific markers. It is therefore anticipated that the combination of mouse genetics and sophisticated novel detection systems will allow a more detailed analysis of MSC homing and engraftment *in vivo*, as for instance in the study by Morikawa. (Editor’s note: All references cited in the Discussion with Reviewers are text references).