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Characterization of human mesenchymal stromal cell heterogeneity

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Chapter 1

Introduction and scope of this thesis

Partially adapted from: “Mesenchymal stromal cell migration, possibilities to improve cellular therapy”

Stem Cells and Development, July 2011 Epub ahead of print

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General introduction

Introduction
Mesenchymal stromal cells (MSC) were originally identified by Friedenstein et al in the sixties as the bone marrow stromal cells supporting hematopoietic stem- and progenitor cells (HSPC) (1). Pittenger (2) and Prockop (3) rediscovered MSC in the nineties. They reported that MSC have the capacity to differentiate into several mesenchymal lineages (2;3). The renewed interest in MSC coincided with the first derivation of pluripotent human embryonic stem cells (ES cells) (4) and the discovery of these potent stem cells raised hope for clinical applications of both MSC and ES cells. To date, numerous studies have focused on the plasticity of ES cells and MSC, with the ultimate goal to develop clinically applicable stem cell based solutions to degenerative diseases or to large scale tissue damage. Due to safety and ethical concerns, only one clinical trial for ES cells has been approved to date. Although the evidence for stemness of MSC is still lacking and the official name of MSC has even been changed from mesenchymal stem cell to multipotent mesenchymal stromal cell (5), MSC have already been evaluated in numerous clinical trials for treatment of multiple diseases.

Currently, extensive expansion (3-6 weeks) is required to obtain enough cells for transplantation. The efficacy of these MSC-based therapies at least partially depends on migration and specific homing of MSC towards the site where they are needed. However, culture-expanded MSC have almost completely lost their engraftment potential. Thus understanding MSC migration will offer perspectives to modulate the expansion protocols to obtain cells that maintain migration and homing capacities.

This introduction highlights MSC biology, current clinical applications, MSC migration, as well as the role of MSC in the bone marrow microenvironment, where they support hematopoiesis.

Mesenchymal stromal cells
Mesenchymal stromal cells (MSC) were first isolated from bone marrow. They represent a very heterogeneous cell population in which some cells have stem cell-like properties. These cells have a high proliferative potential, generating colonies when plated in tissue culture at low density. These colonies are the so-called colony forming unit-fibroblast (CFU-F) (6). Another hallmark of MSC is their ability to differentiate into several mesenchymal cell types, such as osteoblasts, chondrocytes and adipocytes (2;3;7) (Figure 1).

MSC represent a rare population in the bone marrow. The frequency in human BM has been estimated at 0.001-0.01% of the total nucleated cells (7). MSC frequency seems to decline
with age, from 1/10000 nucleated bone marrow cells in a newborn to about 1/1000000 nucleated marrow cells in a 80-year-old person (7). Besides BM, also other tissues contain MSC. They can be obtained from almost every post-natal organ (8;9) including adipose tissue (10;11), the periosteum (12), brain (13), liver (8), skeletal muscle (13), hair follicles (14), peripheral blood (15), umbilical cord blood (16); and Wharton’s Jelly (17) as well as fetal tissues (18-20).

Because no unique marker identifies MSC, the International Society of Cellular Therapy has postulated a definition for ex vivo expanded MSC to be used in clinical studies, based on three main characteristics: 1) their adhesion to plastic 2) their expression of a specific set of membrane molecules CD105, CD90, and CD73 together with lack of expression of the hematopoietic markers CD14, CD34 and CD45 and HLA-DR 3) trilineage differentiation into osteoblasts, adipocytes and chondrocytes in vitro (21). Recently, a new set of MSC markers has been identified (CD140b, CD146, CD271, CD340, and CD349) (22-25) that enable prospective isolation of MSC and enrichment of CFU-F from human BM. The frequency of CFU-F in these enriched fractions is approximately 1/50 (22;23;25). It is currently unknown whether prospectively isolated MSC have different biologic properties, neither is it known whether culture-expansion of MSC from BM mononuclear cells selects for outgrowth of a certain population. For most clinical applications, MSC are cultured from BM mononuclear cells without any selection.

![Mesenchymal stromal cell derivation and differentiation](Figure 1)
Clinical application of MSC

MSC do not only have regenerative potential, they are also potent suppressors of the immune response. MSC can inhibit differentiation and maturation of monocytes towards dendritic cells. In addition, they can suppress the proliferation of T-cells, B-cells and NK-cells (reviewed in (26)). Both the immune-suppressive capacity and the regenerative potential of MSC have raised clinical interest in these cells. Although BM-derived MSC are currently the most frequently used source for a wide range of therapeutic applications, (reviewed in (27;28)) also adipose tissue-derived MSC are regarded a safe and suitable cell type for therapy (29-31). To date, no significant adverse events have been reported in these trials (32). Transplanted MSC have been applied in bone tissue engineering strategies to reduce clinical symptoms of osteogenesis imperfecta (33) and large bone defects (34), in regenerative treatments to further stimulate repair of pancreatic islets (35), and the infarcted myocardium (36-38). Furthermore MSC have been used in several small trials for immunomodulatory treatments of autoimmune diseases including Crohns disease (39;40) and diabetes mellitus (41). However, feasibility for these applications has not been demonstrated yet.

The application of MSC in hematopoietic stem cell transplantation (SCT) is far ahead of other indications, reviewed in (27;28;42). Currently, the two main potential applications of MSC in SCT are 1) prevention and/or treatment of graft-versus-host disease (GvHD) and 2) enhancement of engraftment.

Ad 1) Severe acute GvHD after allogeneic SCT is associated with high morbidity and mortality (43), particularly in corticosteroid resistant patients. Because of the immune modulatory effect of MSC in vivo and in vitro, use of ex vivo expanded MSC can be applied for treatment of GvHD. The first study showed rapid improvement of GvHD after treatment with allogeneic BM-derived MSC (44). Other studies confirmed that infusion of ex vivo expanded adipose or bone marrow derived-MSC can alleviate GvHD. Also third-party mesenchymal stem cells (derived from unrelated HLA-mismatched donors) were as effective as HLA-identical or haplo-identical cells to treat GvHD (45). This finding has practical implications and suggests that third-party cells can be prepared and stored frozen to be used for GVHD therapy. Following these initial findings, treatment of GvHD with MSC has been widely applied in clinical trials, in which Dutch medical centers participate as well.

Ad 2) Since MSC provide support for primitive hematopoietic progenitor cells in vivo, it was postulated that they might enhance engraftment after SCT. Indeed, simultaneous intravenous injection of donor MSC and HSC was found to accelerate recovery of hematopoiesis after myeloablative therapy in animal models (46;47). This effect is further enhanced by intrabone injections of MSC, suggesting that homing of MSC to the bone marrow is relevant (48;49). Koc et al were the first to show rapid hematopoietic engraftment after co-infusion of autologous peripheral blood stem cells and autologous expanded BM-derived MSC in patients (50). Thereafter, MSC-derived from the HSC donor have been investigated for their ability to enhance hematopoietic recovery in allogeneic SCT (51-55),
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and co-transplantation of third party MSC was studied as well (56). The beneficial effects on hematopoietic recovery in these studies were highly variable and they only indicate that MSC could enhance hematopoietic engraftment. To establish this indication, larger randomized trials are required.

Large doses of MSC, varying from $0.4 \times 10^6$ /kg till $10 \times 10^6$ /kg body weight (45;55), are currently required for successful clinical application. Although transplantation of these large doses of MSC in clinical trials seems to be beneficial to the patients, the engraftment potential of MSC in vivo is limited. For example, 23 months after co-transplantation of HSC and MSC, 100% donor chimerism was observed for hematopoietic cells in blood and bone marrow whereas the MSC remain of host origin (57). This does not exclude the possibility that transient MSC engraftment may exert favorable effects through the secretion of cytokines or other paracrine factors, which engage and recruit recipient cells in productive tissue repair (28). This may be the so-called ‘hit and run’ effect. However, the observation that intrabone injections of the MSC (48;49) further enhances hematopoietic recovery suggests that most therapeutic applications can be improved if more MSC reach the target site. In murine models, MSC seem to preferentially home to damaged tissue (58), although the observed frequency of engraftment is highly variable depending on the conditioning regimen and the route of administration but the overall consensus points to limited homing efficiency (0.00023%-0.00030%) of expanded MSC (reviewed in (59)).

Chemokines, cytokines and growth factors related with MSC migration

It is assumed that common mechanisms of cell migration also apply to MSC migration (reviewed in (59)). Studies on leukocyte (60;61) and HSC (62;63) migration have provided insight into common mechanisms of migration. Chemokines, cytokines and growth factors released upon injury provide migratory cues for cells. They induce upregulation of selectins and activation of integrins on the cell surface, enabling cells to interact with the endothelium. Cells subsequently adhere and transmigrate across the endothelial layer into tissues.

MSC express a wide variety of chemokine- and growth factor receptors; amongst others CXCR4 (64-66), platelet-derived growth factor (PDGF) receptors alpha and beta (67), and the hepatocyte growth factor (HGF) receptor cMet (68). In vitro migration studies have demonstrated that several chemokines and growth factors are chemotactic stimuli for MSC, including stromal derived factor-1 (SDF-1) (69;70), PDGF (71;72), HGF (68) and basic fibroblast growth factor (bFGF) (73). These stimuli induce migration of MSC derived from various adult and fetal tissues (67;74-76), however comparing the various studies is hard because cell culture protocols and the migration assays vary. Therefore, is still unclear whether MSC derived from a certain tissue has superior migratory capacity compared to other tissues. MSC were reported to migrate across endothelial cell monolayers (77;78) and
through the underlying extracellular matrix (79;80), which are pivotal capacities since in most clinical trials MSC are administered intravenously. However, in multiple studies it has been shown that only a small fraction of MSC shows strong in vitro migratory characteristics (58;67;74). To understand why only a small proportion of all culture-expanded MSC are able to migrate, the characteristics of migratory MSC have to be studied. These data will be important to explore strategies to improve directed migration of MSC.

MSC have shown to possess the ability to migrate to sites of inflammation and injury for example in an animal model of cerebral ischemia (81). In a model of multiple organ failure, Chapel et al. showed that tagged MSC homed to numerous tissues with localization correlating to the severity and site of injury (82). The homing efficiency of infused MSC has been reported to be greatly influenced by the variety of protocols currently used for isolation and culture-expansion of MSC. Studies by Rombouts and Ploemacher (83) demonstrated that primary, uncultured BM-derived MSC were able to effectively home in irradiated mice, whereas cultured MSC had lost this homing capacity already after 24 hours of culture. Furthermore, culturing of MSC has been associated with a decrease in expression of adhesion molecules, the loss of chemokine receptors and a subsequent lack of chemotactic response (64;70).

It has been reported that a very small number of MSC consistently circulate in the blood. This circulating pool was found increased under hypoxic conditions (84), in case of major injury (37;85), or large skin burns (86), suggesting that in vivo, MSC can be mobilized and can migrate to places where they are needed. However, contrasting reports exist on the presence of MSC in (mobilized) peripheral blood and the success rate of identifying any circulating MSC is low (reviewed in (87)).

Strategies to enhance migration and homing of MSC
Various approaches to modify MSC or to enhance expression of surface markers of MSC have been explored to enhance MSC migration. Sackstein et al showed that modification of CD44 with an E-selectin binding motif increased specific homing of MSC to the bone marrow (88). Many groups have focused on the CXCR4-SDF-1 axis, as this pathway is dominant in trafficking of cells to bone marrow and functional CXCR4 expression is required for MSC to migrate towards SDF-1α (66). Transfection or transduction of MSC with CXCR4 resulted in increased migration towards SDF-1α in vitro (69;89;90) and towards infarcted myocardium in rats, whereas hardly any migration was observed to normal myocardium (90).

Cytokine pretreatment has been shown to enhance homing of HSC (91) and this approach has been explored in MSC as well. Short term stimulation of MSC with Flt-3 ligand, SCF, IL-6, HGF an IL-3 increased surface expression of CXCR4. Correspondingly, in vitro migration and long term engraftment in mice were increased (92). Pre-incubation of MSC
with TNF-α, but not IFNγ, also resulted in upregulation of chemokine receptors (93;94) and increased chemokine mediated migration (67;93;94). TNF-α did not affect growth factor induced chemotaxis (67). Pro-inflammatory cytokines also increased the production of matrix metalloproteinases in MSC, thereby increasing the ability of stimulated MSC to migrate through the extracellular matrix. SDF-1α pre-treatment did not influence matrix metalloproteinase expression or cell invasion (79).

As oxygen levels in body tissues are lower than those under standard cell culture conditions (95), Hung et al. explored the hypothesis that culturing MSC under hypoxic conditions increases homing capacity (96). Exposure of MSC to hypoxic conditions as short as one day increased expression of homing receptors CX3CR1 (96), CXCR4 (96), and CXCR7 (97) and activates AKT and c-MET-signaling (98). When transplanted into chick embryos or in a murine ischemic hind limb model, homing of hypoxia exposed cells was increased compared to cells cultured at normoxia (96;98). Thus short-term exposure to hypoxia may provide an opportunity to enhance MSC homing.

MSC in the bone marrow niche
The bone marrow is the main site of adult hematopoiesis. Hematopoietic stem cells (HSC) are on top of a hierarchical chain of cells that progressively mature towards all fully differentiated blood cell types. To precisely balance between self renewal and differentiation, HSC require tightly regulated signals from their microenvironment. The current consensus is that the most primitive HSC localize to the endosteal niche, whereas more frequently cycling short term HSC are predominantly localized in the vascular niche (99-101). MSC can support hematopoiesis through production of soluble factors such as SDF-1α, stem cell factor, granulocyte monocyte stimulating factor and IL-6 and though expression of membrane bound molecules like Jagged (102-105). In murine bone marrow, MSC are marked by the neuronal marker Nestin, and transplanted HSC home to close proximity of Nestin positive cells. Deletion of Nestin positive MSC from the niche resulted in mobilization of HSC into the peripheral blood (106). Mutations in the stromal compartment of the niche can ultimately result in the development of myelodysplastic syndrome and secondary acute myeloid leukemia (107), underlining the crucial role for MSC in the bone marrow microenvironment.

Wnt signaling
Wnts are a large family of secreted lipid-modified glycol-proteins that are expressed in a variety of tissues. Wnt signaling is crucial for human and animal embryonic development, and defects in the pathway are associated with tumorigenesis (108).

Wnt proteins bind a receptor complex consisting of a Frizzled (FZD) receptor and the LDL receptor-related proteins LRP5 or LRP6 (109) or alternatively to the recently described Wnt
receptors Ryk (110-112) and Ror2 (113-115). The canonical Wnt pathway leads to stabilization of beta-catenin, which accumulates and translocates to the nucleus where it activates target gene expression. Wnt-signaling through non-canonical pathways is largely beta-catenin independent and it induces the release of Ca^{2+}, and the phosphorylation of jun N-terminal-kinase (JNK) or members of the Src-family kinases (Reviewed in(109)). Currently Wnt1, Wnt2, Wnt3 and Wnt3a, Wnt8a and Wnt8b, Wnt10a and Wnt10b are considered canonical Wnts. Wnt4, Wnt5a and Wnt5b, Wnt6, Wnt7a and Wnt7b, Wnt11 and Wnt16 are regarded as non-canonical Wnts (reviewed in(109;116)). Wnt-signaling can be inhibited at various levels. Members of the Dickkopf (DKK) family occupy FZD receptors and thereby prevent binding of Wnt-ligands. Soluble factors such as secreted frizzled related proteins (SFRP) or Wnt inhibitory factors (WIF) are believed to interfere with the secreted Wnt-proteins to prevent signaling (reviewed in (117)), although there are reports showing that several isoforms of these factors may in fact promote Wnt-signaling (reviewed in(118)).

Cultured MSC express a wide variety of Wnt-proteins including Wnt2, Wnt4, Wnt5a, Wnt11 and Wnt16 and Frizzled receptors 2, 3, 4, 5 and 6 (119). Wnt-signaling is essential for MSC proliferation and multilineage differentiation. The Wnt-inhibitor DKK1 is required to allow MSC to re-enter the cell cycle (120) and exogenous administration of Wnt3a promotes MSC proliferation through activation of cyclin D1 and Myc (121). Upon induction of osteogenic differentiation in vitro, the Wnt-expression profile of MSC changes. Wnt11, FZD6, SFRP2, SFRP3 and Ror2 are upregulated, while Wnt3a and FZD7 are downregulated (121). Opposing roles have been reported for Wnt3a and Wnt5a in osteogenic differentiation. Wnt3a seems to suppress osteogenesis, whereas Wnt5a promotes this process and Wnt-5a induced osteogenesis can be partially inhibited by Wnt3a (122). Wnt-signaling also influences differentiation of MSC towards chondrocytes and adipocytes. Canonical signaling is known to block adipogenesis, while an interplay between canonical and non-canonical Wnt-signaling dictates chondrogenesis (reviewed in (116)).

In vitro and in vivo experiments have demonstrated that Wnt-signaling in stromal cells is important for hematopoietic support. Stabilization of beta-catenin in murine stroma was found to be required for the maintenance of HSC during in vitro culture (123;124). Not much is known about Wnt-signaling in native MSC, but mouse experiments point out that niche derived Wnt-signaling is crucial for hematopoiesis. Inhibition of Wnt signaling in the bone marrow niche by the expression of Dickkopf-1 (Dkk1), in the BM microenvironment, results in premature loss of HSC self-renewal activity (125). Similarly, maintenance of HSC is impaired by secreted frizzled-related protein 1 (SFRP1), another inhibitor of the canonical Wnt-pathway (126). Taken together, these studies demonstrate that a balanced interplay between the HSC and the stromal compartment is required for the maintenance of hematopoiesis.
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Scope of this thesis

The aim of the work presented in this thesis was to increase the efficiency of culture-expanded mesenchymal stromal cell (MSC) migration with respect to cellular therapies and to identify and study the properties of primary mesenchymal stromal cell populations in fetal and adult bone marrow.

Previous studies have shown that culture-expanded MSC have migratory capacities, but the experiments are hard to compare due to cell culture and assay variability. In Chapter 2, we have compared the migratory capacity of culture-expanded MSC derived from multiple tissues; adult adipose tissue, adult bone marrow, fetal bone marrow and fetal lung, all cultured and assayed under similar circumstances. In addition, we studied whether migratory MSC can be discriminated from non-migratory MSC by cell surface marker expression and we evaluated the involvement of the cell cycle in MSC migration.

The expanded MSC population only contains a minor fraction with migratory characteristics. To enhance migration of the entire MSC population, it is crucial to target the genes or signaling pathways that are involved in MSC migration. To identify these genes, we performed a micro-array-based gene expression analysis of migratory and non-migratory FBMSC (Chapter 3). We have identified twelve genes that are important for MSC migration. We studied the two most prominent genes, the nuclear orphan receptors Nur77 and Nurr1, in more detail. In this chapter we explored the role of Nur77 and Nurr1 in MSC migration, cytokine production and immune modulation.

Because the culture-expanded MSC currently applied in clinical trials represent a very heterogenous cell population and it is unknown which bone marrow resident cells are responsible for the initiation of the MSC cultures, we characterized different primary MSC populations in human bone marrow during human development and aging (Chapter 4). We designed a six-colour flow cytometry panel to sort the putative MSC subpopulations, based on the expression of two novel MSC markers CD271 (nerve growth factor receptor) and CD146 (Melanoma cell adhesion molecule); the classical MSC markers CD90 (Thy-1) and CD105 (Endoglin); and the absence of the hematopoietic markers CD34 and CD45.

MSC are a crucial constituent of the hematopoietic niche and Wnts produced by the niche are important for hematopoiesis. Expanded MSC express multiple Wnt proteins, but it is unknown whether the Wnt-signature of cultured cells is representative for the native MSC in bone marrow. In Chapter 5 we describe and compare the Wnt-signaling profile of the MSC subpopulations directly after sorting from human adult bone marrow and after culturing. In addition, we evaluated the hematopoietic support provided by the different MSC populations.
Bone marrow composition changes during development and aging. MSC are present in fetal bone marrow before the hematopoietic stem cells migrate and home to this site of definitive adult hematopoiesis. This suggests that fetal and adult bone marrow-derived MSC have different biological functions. In Chapter 6 we describe the gene expression profile of these MSC sources and we have focused on the different balance of Wnt (target)-gene expression observed between adult and fetal MSC.

Finally, in Chapter 7, all results described in this thesis are summarized, discussed and placed in perspective of the current knowledge.
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