Characterization of human mesenchymal stromal cell heterogeneity

Maijenburg, M.W.
Chapter 7

Summary and general discussion

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

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Summary and general discussion

Mesenchymal stromal cells (MSC) are rare stromal cells that can be derived from a variety of adult and fetal tissues. They represent a very heterogeneous cell population that is currently defined by expression of a combination of markers (1).

Cultured MSC have two remarkable capacities that designate them as suitable candidates for cellular therapy. First, MSC have the capacity to differentiate towards multiple mesenchymal tissues including bone and cartilage. Therefore MSC treatment has been successfully explored to treat the rare bone disorder osteogenesis imperfecta (2;3), and to repair large bone fractures (4). The trilineage differentiation capacity of MSC towards adipocytes, chondrocytes and osteoblasts is well established (1), but the results from studies evaluating the greater differentiation plasticity of MSC for example into cardiomyocytes (5) and even into non-mesodermal tissues (6;7) are still under debate.

The second remarkable MSC characteristic is their ability to suppress the immune response. MSC can do this by both cell-cell contact- and soluble factor-mediated repression of the 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(8)). The discovery of these properties has resulted in the implication of MSC for treatment of graft-versus-host disease (GvHD) (9), an adverse event frequently occurring after allogeneic hematopoietic stem cell (HSC) transplantation (10). MSC express low levels of the MHC class I molecule, and this may partly explain why third-party MSC work just as well as autologous or HLA-matched MSC to treat GvHD (9;11).

Bone marrow-derived MSC are the first source of choice for current clinical application and they are applied in an expanding number of clinical trials (http://clinicaltrials.gov/ct2/results/map?term=MSC). As it is believed that MSC in bone marrow (BM) support and maintain HSC (12), MSC are now also clinically evaluated for the enhancement of HSC engraftment after transplantation, an indication first established in mice (13). Intravenous injection of MSC is the most convenient route of administration in a clinical setting, therefore MSC have to migrate and home towards the site where they are needed. In animal models as well as in patients, it has been observed that the homing capacity of culture-expanded MSC is limited, and the majority of the transplanted cells disappeared within a few days (14;15). 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 (16). However the observation that intrabone injections of the MSC (17;18) further enhances hematopoietic recovery suggests that most therapeutic applications can be improved if more MSC reach the target site. Therefore, better insight in the process of MSC migration is required.
Rombouts and Ploemacher demonstrated that uncultured MSC have an enhanced homing capacity compared to cells that have been cultured as short as 24 hours (19). These data indicate that the heterogeneous MSC populations that are obtained as the progeny of tissue resident counterparts may not resemble the functional characteristics of these cells in vivo. Prospective isolation of MSC from tissues has been hampered by the low frequency of these cells in tissues (20) as well as by the lack of unique markers to specifically isolate MSC. It has recently been demonstrated that two markers, the nerve growth factor receptor (NGFR, CD271) (21) and melanoma cell adhesion molecule (MCAM, CD146) (22;23), can be used to isolate MSC from several human tissues. The availability of these markers offers perspectives to study the biological relevance of defined MSC subpopulations and to elucidate in which respect these cells change upon ex-vivo expansion.

In the studies described in this thesis, we have aimed to contribute to the understanding of mesenchymal stromal cells (MSC) migration and to the identification and functional analysis of primary mesenchymal stromal cell populations in fetal and adult bone marrow.

Enhancing MSC migration

Previous studies on MSC migration have demonstrated that common mechanisms of cell migration also apply to MSC. MSC express a broad range of chemokine and growth factor receptors, and are therefore able to migrate in response to many chemotactic stimuli such as stromal cell-derived factor-1α (SDF-1α/CXCL12) (24;25), platelet-derived growth factor (PDGF) (26;27), basic fibroblast growth factor (bFGF) (28) hepatocyte growth factor (HGF) (29) and monocyte chemoattractant protein-1 (MCP-1) (30). In most studies, only BMSC were used, and due to variability in cell culture and assay conditions the studies were hard to compare. In Chapter 2, we have demonstrated that MSC derived from multiple tissues contain a fraction of cells with migratory characteristics, although the proportion of migratory cells and the optimal stimulus for migration differed between the MSC from various sources. Fetal lung-derived MSC had the highest migratory capacity. This was found not to be due to the fetal origin of the cells because adult and fetal BM-derived MSC had a similar migratory capacity. As the chemokine receptors, growth factor receptors, integrins and adhesion molecules could not explain the observed differences in the migratory phenotype, tissue of origin imprinted characteristics may be an underlying cause of the migratory potential. Exclusive transplantation of MSC with enhanced migratory capacity could reduce the large doses of MSC (varying from $0.4 \times 10^6 /kg$ till $10\times 10^6 /kg$ body weight (11;31)) currently infused in clinical trials. In our experiments, no surface markers were found exclusively expressed on migratory cells; but in agreement with previous findings in HSC (32;33), we found a relation between the cell cycle and MSC migration. The migratory MSC fraction contained less cells in S- and G2/M- and a trend towards more cells in G1-phase of the cell cycle compared to non-migratory MSC. In line with these results, MSC from cultures harvested at 50-70% confluency compared to those from >80% confluent cultures
had a better migratory response. Therefore, a clinically applicable strategy to enhance MSC migration could be simply harvesting the cells at a lower confluency level.

To further define the characteristics of the migratory MSC subset, we took a gene expression profiling approach to identify genes involved in MSC migration. This opens possibilities to identify and modulate signaling pathways or genes that can be targeted to enhance MSC migration.

We identified a small set of 12 genes that were differentially expressed between migratory and non-migratory MSC (Chapter 3). The two most prominent genes were nuclear orphan receptors: Nur77 and Nurr1. These genes belong to the NR4A family of nuclear orphan receptors, which are mainly studied for their role in brain development and apoptosis (reviewed in (34)). We confirmed the migration enhancing effect of Nur77 and Nurr1 expression in MSC with a lentiviral overexpression approach. As NR4A family members are believed to act as ligand independent transcription factors and their downstream targets have not been identified yet (35), we could not unravel the mechanism by which Nur77 and Nurr1 enhance MSC migration. Nur77 and Nurr1 are involved in cell cycle regulation in many cell types (36;37), and indeed overexpression of Nur77 and Nurr1 decreased the proportion of cells in S-phase of the cell cycle. These data suggest that the observed difference in cell cycle distribution between migratory and non-migratory MSC observed in Chapter 2 may be related to enhanced Nur77 and Nurr1 expression in the migratory cells. Also others have found a correlation between cell cycle and migration in MSC, as increased culture confluence was shown to inhibit transendothelial migration in MSC by increasing the production of a natural matrix metalloproteinase (MMP) inhibitor, TIMP-3 (38).

Nur77 and Nurr1 expression affects the cytokine profile of macrophages and vascular smooth muscle cells (37;39), and we also observed increased release of interleukin-6 (IL-6) and IL-8 protein and an increase expression of HGF. As MSC based treatment of GvHD is at least partially cytokine dependent, it was crucial to show that overexpression of Nur77 or Nurr1 did not affect the immunemodulatory capacity. Furthermore, our data indicate that targeting Nur77 and Nurr1 expression to enhance MSC migration in a clinical setting seems to be feasible, because expression is rapidly induced after exposure to SDF-1α, PDGF, tumor necrosis factor-α (TNF-α) or interferon gamma (IFNγ).

As homing capacity and expression of homing receptors decreases upon culture-expansion (19;40), manufacturing of MSC for clinical application will be a trade-off between obtaining sufficient cell number for transplantation and maintaining their migratory and other designated properties. The data in Chapter 2 and 3 point out that confluence level of the cultures and cytokine pre-treatment to target Nur77 and Nurr1 may be feasible approaches to enhance migration of cultured MSC. These findings are supported by the current literature, and together with other approaches to enhance MSC migration, they are summarized in Table 1. Several lines of evidence indicate that culture confluence and plating density are also
an important factor in expanding MSC whilst maintaining clonogenicity, differentiation potential and a normal karyotype (Table 1). Low seeding densities favored long term proliferation of MSC. Plating at 200 cells/cm² generated an optimal number of rat MSC (41). In addition, low plating densities results in better preservation of clonogenic rat and human MSC compared to higher densities (41;42). Adipogenic differentiation of human MSC is favored during short term expansion, whereas chondrocyte yield was increased after prolonged cell culture (38).

To assist MSC expansion, addition of epidermal growth factor (EGF) (43;44) or antibodies against type 1 interferon receptors (IFNAR1) (45) to culture media have been explored. Short term expansion in the presence of EGF results in increased proliferation and maintains 25% more CFU-F compared to control treated cells (43), suggesting that EGF acts as a mitogen and survival factor for clonogenic MSC. IFNAR1 reversibly controls the quiescence of MSC. At initial stages of expansion, IFNAR addition increases CFU-F outgrowth and over 2 months culture period it leads to faster and greater amplification of MSC without losing differentiation potential (45).

At present, serum free medium is not available for the generation of clinical grade MSC. Alternatives to fetal calf/bovine serum are human AB serum and/or platelet lysates (46;47), and reviewed in (48). To reduce risk of contamination with animal or human infections diseases, it is important to develop chemically defined media supplemented with recombinant growth factors that allow derivation and efficient expansion of MSC. Such media were established for embryonic stem several years ago (49). To date, it has not been studied whether the above described culture procedures influence the migratory capacities of MSC.

A suitable chemically defined medium should meet a few criteria. First, it has to allow efficient generation of MSC from adipose tissue or 10-20 ml of BM aspirate. Second, it has to enable optimal expansion of the obtained MSC. Finally, the capacities to differentiate, to modulate the immune response and to migrate and specifically home have to be preserved. Although the in vitro migratory capacity of MSC is not a release criterion for the clinic at the moment (11;50), its inclusion may be considered.

MSC are applied in an expanding number of clinical trials. Although administration of a large dose of MSC to patients with a wide variety of diseases seems to benefit clinical outcome, lack of understanding of MSC homing to injured tissues is restricting efficacy of the therapies. As several routes of delivering MSC explored in murine models may be unfavorable to apply in a clinical setting, such as delivering cells into the heart, bone or brain, in future studies one should focus on improving migration of intravenously administrated MSC.
**Table 1  Strategies to enhance MSC migration**

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<td>Enzymatic conversion of native CD44 on MSC into HCELL</td>
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<td>IFNγ</td>
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<td>IFN-β</td>
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<td>Copaxone</td>
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### Chapter 7 Summary and general discussion

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<td>IL-1β</td>
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**Cell culture strategies**

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<th>Hung, 2007 (55)</th>
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<th>(55): Exposure to hypoxia increases CXCR4 and CX3CR1 expression and increased migration in response to SDF-1α and fractalkine, which could be blocked by receptor antagonists. Increased engraftment in early chick embryo’s.</th>
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<td>Liu, 2010 (56)</td>
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<td>Human (56;57)</td>
<td>(56): Hypoxia increases expression of CXCR4 and CXCR7 and resulted in increased Transwell migration towards SDF-1α</td>
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<td>De Becker, 2007 (38)</td>
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<td>(38): Expression of MMP inhibitor TIMP3 is increased in highly confluent cultures compared to lower confluency. Reduced transendothelial migration in matrigel invasion assays</td>
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**Abbreviations:** BM, bone marrow; CCR3, C-C chemokine receptor 3; CCR8, C-C chemokine receptor 8; CD44, hyaluronan receptor; CX3CR1, fractalkine receptor; CXCL10, C-X-C motif chemokine 10; CXCR4, CXCR4, SDF-1α receptor; CXCR7, scavenging SDF-1α receptor; Flk1; vascular endothelial growth factor receptor 2; GFP, green fluorescent protein; HCELL, hematopoietic cell E-selectin/L-selectin ligand; HGF, hepatocyte growth factor; IFN-β, interferon-β; IFNγ, interferon γ; IL-1β, interleukin 1β; IL-3, interleukin 3; IL-6, interleukin-6; MMP2, matrix metalloproteinase-2; MMP9, matrix metalloproteinase-9; MT-MMP1, membrane type I matrix metalloprotease; NOD/SCID mice, non-obese diabetic/severe combined immunodeficient mice; SCF, stem cell factor; SDF-1α, stromal cell-derived factor 1-α; TGF-β1, transforming growth factor-β1; TNF-α, tumor necrosis factor-α; TIMP1, tissue inhibitor of metalloproteinase 1; TIMP2, tissue inhibitor of metalloproteinase 2; TIMP3, tissue inhibitor of metalloproteinase 3
Several studies point out that cytokine pre-treatment (51-54) and/or exposure to hypoxia (55-57) increases MSC migration in vitro and in animal models (Table 1). A clinical grade cytokine cocktail should enhance expression of homing receptors and increase expression of genes that were found to stimulate MSC migration. Increased homing and engraftment of MSC may then result in lower doses of transplanted cells and thereby decreasing the culture expansion time, costs, availability and the risk of transformations during culture expansion.

It is still under debate whether the few engrafted MSC are all the progeny of one or multiple MSC clones which may have a better migratory capacity than other cells. Recently a cellular barcoding tool was introduced to enable clonal tracking in the hematopoietic system (58). This retroviral system inserts a small unique sequence or barcode in the genome of each individual transduced cell. Since all daughter cells will carry the same barcode, the progeny of all labeled cells can be traced (58). This method will enable to measure how heterogeneous MSC cultures actually are, because the number of clones that contribute to the culture can be enumerated. It can also help to determine preferential outgrowth of specific MSC clones, which may possess enhanced migratory properties. Upon transplantation, it can be used to illustrate how many MSC clones are capable of sustained engraftment in targeted tissue. If an approach to enhance MSC migration is successful, it may be expected that more unique barcodes, thus more MSC clones, can be recovered from the targeted tissue. If MSC are injected or loaded onto a transplatable scaffold, the barcoding tool can be used to determine the proportion of MSC that migrate into adjacent or more distant tissues.

With respect to all future MSC-based cellular therapies, it will be crucial to develop GMP-grade ‘next generation’ MSC that have enhanced in vivo homing capacity. This should lead to a reduced culture-expansion time and lower doses of transplanted cells, thereby reducing the costs, the probability of transformations during culture and the risk of transfusion related adverse events.

Native MSC
Rombouts and Ploemacher demonstrated that culturing MSC dramatically decreases their homing capacity (35). Therefore, identification of the native MSC and studying the biological properties of these cells may result in more efficient clinical applications and eventually it may even show that expanding MSC prior to transplantation is not necessary if uncultured MSC are indeed more potent than their culture-expanded progeny.

Due to the lack of exclusive MSC markers, studying and identifying the native, tissue resident MSC is a major hurdle. Buhring and co-workers identified the nerve growth factor receptor CD271 as a candidate marker for prospective isolation of MSC (21). CD271\textsuperscript{bright} cells contained all assayable colony forming units-fibroblast (CFU-F, a measure for the amount of clonogenic MSC) in human BM. This fraction could be further divided into
CD271<sup>bright</sup>MSCA1<sup>-</sup>CD56<sup+</sup> and CD271<sup>bright</sup>MSCA1<sup+</sup>CD56<sup+</sup> populations (MSCA1: mesenchymal stem cell specific antigen 1; CD56: N-CAM) (59). Others have employed sorting for CD146-positive cells (22;23). These reports also state that perivascular localized CD146<sup+</sup> cells contain all CFU-F in human BM. Subcutaneously transplanted CD146<sup+</sup> cells were able to generate an ectopic hematopoietic microenvironment (23). The crucial role of MSC in hematopoiesis was demonstrated by Mendez-Ferrer et al, who showed that depletion of Nestin<sup+</sup> MSC from murine BM resulted in reduced HSC retention in the BM and impaired homing of transplanted HSC (60), indicating that these cells are a prerequisite for hematopoiesis. CD271 and CD146 seem promising candidates for isolating MSC from human BM, but so far it was unknown whether these markers were co-expressed on identical cells or whether different subpopulations could be discriminated by these two markers. In addition, because native MSC are crucial for the support of life spanning hematopoiesis, it may be expected that changes in the BM hematopoietic compartment that occur during development and aging correlate with changes in the stromal cell microenvironment.

In Chapter 4 we demonstrate for the first time that the distribution of defined human MSC subsets is correlated to donor age. Three putative MSC subpopulations were detected in human BM, which could generate CFU-F at distinct moments during development and aging. These subsets were CD271<sup>bright</sup>CD146<sup+</sup>, CD271<sup>bright</sup>CD146<sup+</sup> and CD271 CD146<sup+</sup>. Only the two CD271<sup>bright</sup> fractions contained CFU-F in adult BM. and they co-expressed the classical MSC markers CD90 (Thy-1) and CD105 (Endoglin). These subsets also had trilineage differentiation capacity as well, thus they meet all criteria that define bona-fide MSC (31). Previous studies never excluded the possibility that other populations may contain MSC that require support from BM cells for CFU-F formation. We show that depletion of CD271<sup+</sup> CD146<sup+</sup> cells for BM mononuclear cells did not reduce the number of CFU-F in the depleted MSC, whereas depletion of CD271<sup>bright</sup>CD146<sup+</sup> and CD271<sup>bright</sup>CD146<sup+</sup> populations, resulted in a complete loss of CFU-F. Thus CFU-F are only present in CD271<sup>bright</sup> cells and clonogenic MSC do not depend on support from BM-derived cells. In addition, the CD271<sup>bright</sup>CD146<sup+</sup> and CD271<sup>bright</sup>CD146<sup+</sup> cells express high levels of Nestin mRNA, a marker that was recently described to mark murine MSC (60), indicating that Nestin may also be a good marker for human MSC.

In elderly adults (>55 years old), the dominant fraction was CD271<sup>bright</sup>CD146<sup+</sup>, while in younger adults the CD271<sup>bright</sup>CD146<sup+</sup> and CD271<sup>bright</sup>CD146<sup+</sup> occurred at similar frequency. The age-related change in MSC subset distribution was confirmed in pediatric and fetal bone marrow samples. The dominant CFU-F containing fraction in children was CD271<sup>bright</sup>CD146<sup+</sup>. In fetal bone marrow, CFU-F were observed in CD271<sup>bright</sup>CD146<sup+</sup> and remarkably also in CD271 CD146<sup+</sup>.

Because it was reported that CD271<sup+</sup>CD146<sup</b></sub> in vivo localize to endosteal or perivascular niches respectively (61), our data suggests that the relative size of specialized BM niches is dynamic, and that distinct phases in life require different MSC subtypes. The
The increase of CD271<sup>bright</sup>CD146<sup>-</sup> MSC in aged BM may therefore correspond to the increase in long-term HSC in aged murine BM (62;63), which predominantly localize in the endosteal niche.

Several groups have reported circulating MSC in patients with major skin burns (64) or other major injuries (65;66), but contrasting data exist on the presence of MSC in G-CSF mobilized peripheral blood (reviewed in (67)). We have not been able to detect CD271<sup>bright</sup>CD146<sup>-/lo</sup> and CD271<sup>bright</sup>CD146<sup>+</sup> MSC subpopulations in G-CSF mobilized peripheral blood from healthy donors nor from patients (Maijenburg et al, unpublished results). In mice, MSC defined by CFU-F did not significantly mobilize into the peripheral blood upon G-CSF stimulation, but were mobilized upon pre-treatment with vascular endothelial cell growth factor (VEGF) followed by administration of the CXCR4 antagonist AMD3100 (68). Analysis of mobilized peripheral blood from patients treated with alternative mobilization inducing drugs such as clinical grade variants of CXCR4 antagonists will reveal if more MSC are recruited into the blood using this method and if so which subsets are involved.

MSC circulate in first trimester fetal peripheral blood (69). These cells were only phenotyped after culturing, so it is unknown whether these fetal MSC were CD271<sup>-</sup>CD146<sup>+</sup> or CD271<sup>bright</sup>CD146<sup>+</sup> like native fetal BMSC.

**Wnt-signaling in primary MSC subsets and culture-expanded counterparts**

Studies on CD146<sup>+</sup> and Nestin<sup>+</sup> MSC in human and murine bone marrow have demonstrated that MSC are a key component in the hematopoietic niche. Upon ectopic transplantation in mice, CD146<sup>+</sup> MSC orchestrated the formation of a complete hematopoietic niche, in which human derived stromal cells and ossicles were colonized by murine hematopoietic stem and progenitor cells (HSPC) (23). Nestin<sup>+</sup> MSC were closely associated with HSC and they expressed many genes involved in HSC maintenance. Transplanted HSC homed close to or adjacent to Nestin<sup>+</sup> MSC. Depletion of Nestin<sup>+</sup> cells resulted in a reduced number of HSC in the bone marrow (60). In addition, it was recently demonstrated that CD271<sup>bright</sup>CD146<sup>-/lo</sup> and CD271<sup>bright</sup>CD146<sup>+</sup> MSC localize to distinct niches in vivo (61), indicating that these subsets may have distinct roles in hematopoietic support.

Wnt-signaling pathway is crucial for hematopoiesis, and stromal cells play a pivotal role to support HSC through Wnt-signals. Overexpression of Dickkopf-1 (DKK1) or loss of secreted frizzled related protein-1 (SFRP1), two Wnt-inhibitors, in stromal cells resulted in impaired HSC self-renewal and maintenance (70;71). Culture-expanded adult BMSC are known to produce several Wnt-proteins and inhibitors (72;73), and they can support HSC. The Wnt-signature of native MSC subsets directly after sorting from tissues, such as the BM-derived adult CD271<sup>bright</sup>CD146<sup>-/lo</sup> and CD271<sup>bright</sup>CD146<sup>+</sup> MSC and their role in hematopoietic support has not been studied before. Therefore, we have generated and compared the
Wnt-signaling profile of uncultured CD271brightCD146-, CD271brightCD146+ and CD271 CD146- cells directly after sorting, as well as from or conventionally cultured MSC from unsorted BM mononuclear cells (MNC) from the same donor (Chapter 5).

Wnt-related gene expression appeared to be clearly distinct in the two sorted uncultured MSC subsets compared to conventionally MSC derived from unsorted bone marrow mononuclear cells. Expression of CCND1, WISP1 WNT5A and WNT5B were strongly increased in the unsorted cultured MSC, whereas sorted unculturedCD271brightCD146- and CD271brightCD146+ expressed higher levels of JUN, LEF1, WIF1 and WNT3A. The differences in Wnt-gene expression was more subtle between CD271brightCD146- and CD271brightCD146+; Wnt receptors frizzled 7 (FZD7) and low-density lipoprotein-receptor related protein 6 (LRP6), were significantly higher expressed in CD271brightCD146- and a trend towards increased expression in this subset was observed for Wnt11, MYC and beta-catenin. Upon culturing the two sorted subsets for one passage, the differences in Wnt-related gene expression between the subsets as well as between the unsorted sorted subsets and MSC from BM MNC were lost. Interestingly, Wnt3a was undetected in both populations while Wnt5a expression was acquired. These data suggests that the initial differences in Wnt-gene expression observed between CD271brightCD146- and CD271brightCD146+ cells may not be a intrinsic, and that they may rather be a reflection of localization to separate bone marrow niches as recently reported by Tormin et al (61).

Despite the loss of a distinct Wnt-signature, co-culture experiments combining the sorted MSC subsets with CD34+ cells show a trend towards enhanced long-term hematopoietic support in CD271brightCD146- compared to CD271brightCD146+ cells (Chapter 5). The difference in hematopoietic support disappeared upon prolonged culture-expansion, suggesting that expression or secretion of the factor(s) that were responsible for maintaining the colony forming capacity of the CD34+ cells are influenced by the cell culture process.

Taken together, our data suggests that Wnt-expression in cultured unsorted MSC subsets is distinct from the Wnt-signature from conventionally cultured MSC from BM MNC. Culturing native MSC dramatically affects Wnt-signaling in these cells and therefore our results question the quality of culture-expanded MSC as model system for Wnt-signaling in MSC in vivo.

The notion that culturing sorted MSC subsets affects their characteristic has also been made by others. Under standard culture conditions, cells that were sorted as CD271+ rapidly loose expression of this marker (32;36), while expression of CD146 was found to appear on cells that initially had a CD271brightCD146+ phenotype (61). It was demonstrated that the upregulation of CD146 was induced by exposure to increased oxygen levels and that this process was reversed by culturing under hypoxic conditions (61). Murine Nestin-GFP cells could not maintain expression of GFP under standard culture conditions (60). These data indicate that to better preserve the characteristics of native MSC, the culture procedures
need to be optimized. Mendez-Ferrer et al pursued an approach adapted from culturing neural crest cells in non-adherent clonal spheres. They allowed the Nestin+ cells to grow in so-called mesenspheres, in which GFP-expression of Nestin+ cells was maintained. Using this method, the authors were even able to demonstrate that clonogenicity was maintained and the cells appeared to self-renew in vivo in serial transplantation experiments (60).

Another suspension culture method was described by Baksh et al. In cultures of BM MNC in stirred suspension spinner flasks, MSC were able to better conserve the capacity to form CFU-F, as well as the capacity to differentiate towards osteoblasts and adipocytes compared to conventional plastic adherent cells. The enhanced clonogenic capacity observed in suspension was further enhanced by administration of Wnt3a or Wnt5a (74). As culturing in mesenspheres or suspension cultures may require extensive technical optimization, testing hypoxic culture conditions may be a first step towards maintaining native MSC characteristics.

Wnt-signaling in adult and fetal BMSC

Bone marrow composition changes during development (75) and aging (20;75–77). MSC or MSC-like cells can be derived from all hematopoietic sites during murine development (78), and for most tissues MSC are already present before the migrating HSC colonize these tissues (79). As different niches can influence the phenotype and characteristics of the cells derived from these tissues, it is not surprising that besides sharing several MSC characteristics, there are also clear differences between adult and fetal MSC. There are indications from the murine system that the first MSC have a neurectodermal origin, and these cells were transiently replaced by cells with another ontogeny (80). We also found a CFU-F containing MSC subset that was exclusively present in fetal bone marrow (Chapter 4). In addition, Fetal MSC proliferate faster and are more prone to differentiate towards osteoblasts (81;82). Understanding the underlying mechanisms that enhance proliferation in FBMSC could be beneficial for cellular therapy, as targeting these signaling pathways may result in a reduced expansion period for ABMSC.

Therefore, we further explored the differences between adult and fetal bone marrow derived MSC (ABMSC and FBMSC) in a gene expression profiling approach. In an array screen described in Chapter 6, we found 687 genes differentially expressed between ABMSC and FBSMC. Functional enrichment analysis revealed that genes associated with limb- and skeletal muscle development, cell cycle regulation and DNA-repair were enriched in both FBMSC compared to ABMSC. Genes involved in these processes were previously also reported to be enriched in an array screen comparing fetal liver-derived MSC compared to ABMSC (83). Thus, fetal MSC share some intrinsic characteristics that discriminate them from ABMSC, which are independent of the fetal tissue of origin.

Because Wnt-signaling is important for human and animal development, as well as for MSC proliferation and differentiation (72;84) and hematopoietic support (85), we focused on
the 16 differentially expressed Wnt genes. This list of genes included Wnt5a, five Frizzled receptors, the Dickkopf family members DKK1 and DKK2, and the secreted Wnt-inhibitor SFRP4. ABMSC and FBMSC produce large quantities of Wnt-inhibitors, such as DDK family members that can either occupy the LRP/FZD receptors or that bind to Wnt to prevent its receptor binding, like secreted frizzled related-proteins. Expression of several Wnt-related genes is dependent on the confluency level of the culture (73). Also in our hands, the expression of DKK1 was strongly influenced by cell culturing, thus the difference in DKK1 expression between ABMSC and FBMSC cannot be considered an intrinsic difference.

In contrast, the differences in SFRP4, Wnt5a and DKK2 were consistently differentially expressed during culture. Because the ABMSC and FBMSC were cultured under similar circumstances, it may be assumed that general effects caused by cell culturing equally affects both MSC. Therefore, at least part of the differentially expressed genes may be a reflection of the tissue of origin, which may be supported by the functional enrichment of genes involved in developmental processes such as tissue morphogenesis. It is known that cultured MSC maintain several tissue of origin imprinted characteristics and even the expression of Hox genes that reflect anterior to posterior patterning in the animal kingdom (86;87).

The observed differences in expression of Wnt-inhibitors and Frizzled receptors were shown to lead to profound differences in Wnt-signaling between adult and fetal BMSC. Although both MSC seem to have a similar low basal canonical Wnt-signaling, the abrogation of autocrine Wnt-production by the small molecule Inhibitor of Wnt-production-2 (IWP2) (88) leads to a strong decrease in expression of downstream targets of canonical Wnt-signaling in FBMSC only and it even inhibited the response to exogenous Wnt3a in these cells. In contrast, in ABMSC autocrine canonical signaling was found to be prevented probably by production of soluble Wnt-inhibitors and possibly for this reason IWP2 treatment had no effect on endogenous Wnt-signaling. ABMSC had a faster response and were more sensitive to low concentrations Wnt3a, and remained responsive to Wnt3a after incubation of IWP2 (Chapter 6). Except for Wnt5a, no other Wnt-proteins were differentially expressed between ABMSC and FBMSC. As it has been observed that the combination of different LRP, FZD co-receptors or alternative Wnt-receptors Ryk and Ror2, dictate the intracellular route and thereby the result of the Wnt signal induced (reviewed in (89)), the differences in the response to Wnt3a and the distinct response to inhibition of endogenous Wnt-production may be explained by the differential expression of FZD1, FZD2, FZD7 and FZD8 detected in our micro array. This might lead to a different net balance in autocrine Wnt-signaling between these cells established through distinct intracellular mechanisms. Therefore, mapping of the Wnt-Wnt-receptor interactions in MSC will complete the current knowledge on the role of Wnt-signaling in MSC differentiation, proliferation and hematopoietic support.
Final remarks
Whereas the clinical applications of culture-expanded MSC have gained most attention in the past decade, many fundamental questions about the origin and function of native MSC remained unanswered. Recent studies have highlighted several new MSC markers that enable studying the characteristics of freshly isolated MSC and resident MSC in vivo. More insight in the function and capacities of MSC in situ may provide clues about what can be expected from their culture-expanded progeny applied in the clinic.

The ontogeny of MSC during development is still unclear. Observations that the first murine MSC arise from Sox1+ neuroepithelial cells (80) and that primary murine and human MSC express the neuronal marker Nestin (Chapter 4) and (60;61)) raise questions about the current hypothesis that all MSC originate from the mesoderm. Answers to these questions will provide insight in the feasibility of MSC-based application for regeneration of tissues and cell types derived from non-mesodermal germ layers.

The relation between the different MSC subsets in BM remains to be elucidated. Are these subsets organized in a hierarchical system similar to HSC, are these subsets just similar cells that arise independently from the same germ layer during development, or are the phenotypic differences between MSC derived from multiple tissues (90-92) only a result of localization to different niches and if so, is this phenotype within a tissue or niche fixed, or do cells temporally change their phenotype and function if they migrate from one niche to the other? The latter hypothesis would be supported by our own observations in Chapter 5 as well as from others that after several passages, the subsets can no longer be discriminated by expression of CD271 and/or CD146 (61;93). Studies by Tormin et al suggest that the expression of CD146 reversibly influenced by the local oxygen tension (61). Data from Battula et al would support the hypothesis of two intrinsically different subsets, because chondrocytes and pancreatic-like cells were exclusively obtained from sorted CD271brightMSCA1+CD56+ cells whereas adipocytes could only be differentiated from CD271brightMSCA1+CD56- cells (59).

If the subsets would be hierarchically related, which subset can be considered to be the most ‘primitive’? One may speculate that CD271brightCD146+ cells could be the ‘true’ stem cells because they are present in fetal, pediatric and adult BM. In addition, CD271brightCD146+ cells have an increased expression of FZD7 and Wnt11, which are associated with maintenance of self renewal capacity in embryonic stem cells (94;95). It is clear that not all MSC subsets are present in all stages of life (Chapter 4 and (80)). If a similar system of waves of MSC generation exist in human compared to mice, this could suggest that fetal CD271 CD146+ CFU-F, that are no longer detected after birth, are transiently replaced by CD271brightCD146+ cells in the bone marrow. Alternatively, CD271 CD146+ cells may acquire CD271 expression at a later stage in fetal or neonatal development. Because these fundamental questions can only be addressed in animal models, identification of more putative MSC markers that are expressed on both murine and human cells will increase the significance of these studies.
MSC derived from various tissues may well appear similar in their capacity to adhere to tissue culture plastic, trilineage differentiation and in the expression of classical MSC markers CD73, CD90 and CD105, but MSC also maintain some tissue of origin imprinted characteristics as we also observed in Chapter 2 and 6. Therefore, further functional characterization of native MSC subsets in various tissues may be helpful to determine which cells or tissue to choose for each clinical indication, as well as how to expand these cells ex-vivo without losing specific subsets or intrinsic capacities. Conventional protocols for MSC expansion may result in selection or preferential outgrowth of a specific subset or cells with specific functions, which may result in the loss of several MSC capacities. Although we demonstrate in Chapter 3 and 4 that CD271brightCD146- and CD271brightCD146+ cells can both be expanded independent of each other in vitro, this does not exclude the possibility that in ‘competitive’ cultures started from mononuclear cells, some subpopulations may be preferentially expanded. In addition, although the currently defined subsets are enriched for CFU-F, they most likely still represent a heterogeneous cell population. As different clinical indications require other MSC properties like immune modulation or regenerative properties, defining the biological properties MSC subsets may help to develop optimal culture protocols per therapy.

In conclusion, answering fundamental questions on the functional relevance of defined MSC subsets in vivo may provide cues to improve clinical application of MSC. It may also result in well balanced choices of MSC sources and specialized culture-expansion techniques to maintain the characteristics required for each indication. This may ultimately lead to more straightforward and tailored MSC transplantation procedures for disease specific applications.
Reference List


19 Rombouts WJ, Ploemacher RE Primary murine MSC show highly efficient homing to the bone marrow but lose homing ability following culture Leukemia 2003 Jan;17(1):160-70.


The surface adhesion molecule CXCR4 stimulates mesenchymal stem cell migration to stromal cell-derived factor-1 in vitro but does not decrease apoptosis under serum deprivation. 

Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-cell-derived factor-1 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases.

BMP-2, BMP-4, and PDGF-bb stimulate chemotactic migration of primary human mesenchymal progenitor cells.

To go or not to go: Migration of human mesenchymal progenitor cells stimulated by isoforms of PDGF.

Basic fibroblast growth factor controls migration in human mesenchymal stem cells.

Efficient homing of multipotent adult mesenchymal stem cells depends on FROUNT-mediated clustering of CCR2.

Hepatocyte growth factor effects on mesenchymal stem cells: proliferation, migration, and differentiation.

Efficient homing of multipotent adult mesenchymal stem cells to promote engraftment in pediatric recipients of unrelated donor umbilical cord blood: results of a phase I-II clinical trial.

Human hematopoietic stem cells stimulated to proliferate in vitro lose engraftment potential during their S/G(2)/M transit and do not reenter G(0).

Ex vivo treatment of proliferating human cord blood stem cells with stroma-derived factor-1 enhances their ability to engraft NOD/SCID mice.

Nuclear receptors Nurr77, Nurr1, and NOR-1 expressed in atherosclerotic lesion macrophages reduce lipid loading and inflammatory responses.

Transplantation of ex vivo culture-expanded parental bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality.

The CD34-like protein PODXL and α6-integrin stimulates migration of culture-expanded parental bone marrow progenitor cells: proliferation, migration, and differentiation.

Efficient homing of multipotent adult mesenchymal progenitor cells depends on FROUNT-mediated clustering of CCR2.

Basic fibroblast growth factor controls migration in human mesenchymal stem cells.

Hepatocyte growth factor effects on mesenchymal stem cells: proliferation, migration, and differentiation.

Efficient homing of multipotent adult mesenchymal stem cells to promote engraftment in pediatric recipients of unrelated donor umbilical cord blood: results of a phase I-II clinical trial.

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Ex vivo treatment of proliferating human cord blood stem cells with stroma-derived factor-1 enhances their ability to engraft NOD/SCID mice.

Nuclear receptors Nurr77, Nurr1, and NOR-1 expressed in atherosclerotic lesion macrophages reduce lipid loading and inflammatory responses.

Transplantation of ex vivo culture-expanded parental bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality.

Efficient homing of multipotent adult mesenchymal progenitor cells depends on FROUNT-mediated clustering of CCR2.
Characterization of human mesenchymal stromal cell heterogeneity


57 Battula VL, Tremil S, Bareiss PM, Gieseke F, Roelofs H, de ZP, et al Isolation of functionally distinct mesenchymal stem cell subsets using antibodies against CD56, CD271, and mesenchymal stem cell antigen-1 Haematologica 2009 Feb;94(2):173-84.


Chapter 7  Summary and general discussion


74 Baksh D, Tuan RS  Canonical and non-canonical Wnts differentially affect the developmental potential of primary isolate of human bone marrow mesenchymal stem cells  J Cell Physiol 2007 Sep;212(3):87-26.

75 Mikkola HK, Orkin SH  The journey of developing hematopoietic stem cells  Development 2006 Oct;133(19):3733-44.


86 Ackema KB, Charite J  Mesenchymal stem cells from different organs are characterized by distinct topographic Hox codes  Stem Cells Dev 2008 Oct;17(5):979-91.


