Characterization of human mesenchymal stromal cell heterogeneity
Maijenburg, M.W.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Characterization of human mesenchymal stromal cell heterogeneity
Chapter 2

Cell cycle and tissue of origin contribute to the migratory behaviour of human mesenchymal stromal cells


Marijke W. Maijenburg
Willy A. Noort
Marion Kleijer
Charlotte J.A. Kompier
Kees Weijer
Jaap D. van Buul
C. Ellen van der Schoot
Carlijn Voermans

1 Department of Experimental Immunohematology, Sanquin Research, Amsterdam, and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands. 2 Laboratory of Experimental Cardiology, Department of Cardiology, Division Heart & Lungs, University Medical Center Utrecht, and the Interuniversity Cardiology Institute of the Netherlands, Utrecht, The Netherlands. 3 Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands. 4 Department of Molecular Cell Biology, Sanquin Research, Amsterdam, and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands. 5 Department of Hematology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.
Summary

Mesenchymal stromal cells (MSC) are potential cells for cellular therapies, in which recruitment and migration of MSC towards injured tissue is crucial. Our data show that culture-expanded MSC from fetal lung and bone marrow, adult bone marrow and adipose tissue contain a small percentage of migrating cells in vitro, but the optimal stimulus was different. Overall, fetal lung-MSC had the highest migratory capacity. As fetal bone marrow-MSC had lower migratory potential than fetal lung-MSC, the tissue of origin may determine migratory capacity of MSC. No additive effect in migration towards combined stimuli was observed, which suggests only one migratory MSC fraction. Interestingly, actin rearrangement and increased paxillin phosphorylation were observed in most MSC upon SDF-1α or PDGF-BB stimulation, indicating that this mechanism involved in responding to migratory cues is not restricted to migratory MSC. The migratory MSC maintained differentiation and migration potential, and contained significantly less cells in S- and G2/M-phase than their non-migrating counterpart.

In conclusion, our results suggest that MSC from various sources have different migratory capacities, depending on the tissue of origin. Similar to haematopoietic stem cells, cell cycle contributes to MSC migration, which offers perspectives for modulation of MSC to enhance efficacy of future cellular therapies.
Introduction

Mesenchymal stromal cells (MSC) consist of a heterogeneous cell population that can be obtained from many adult (1) and fetal tissues (2-4). The MSC population is characterized by plastic adherence, cell surface marker expression and multilineage differentiation into osteoblasts, adipocytes and chondrocytes in vitro (5).

Due to their multilineage differentiation and immunosuppressive capacities (6;7), MSC are increasingly being considered a potential cell source for regenerative medicine and immune therapies (8;9). When injected into irradiated mice, MSC seem to preferentially home to damaged tissue (10), although the observed frequency of engraftment was very low and many MSC were found trapped in the lungs after intravenous injection. Nevertheless, in pre-clinical and clinical settings, transplanted MSC have been shown to improve haematopoietic stem cell (HSC) engraftment (11;12), reduce clinical symptoms of osteogenesis imperfecta (13) and graft-versus-host disease (14) and further stimulate repair of pancreatic islets (15) and the infarcted myocardium (16;17). The migration of MSC towards the site where they are required is crucial for most applications.

Many studies on leukocyte (18;19) and HSC (20;21) 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 integrins on the cell surface, enabling cells to interact with the endothelium. Cells subsequently adhere and transmigrate across the endothelial layer into tissues. The homing efficiency of expanded MSC in vivo is very low (10), and therefore high cell number are needed. Better insight in the migratory mechanisms in MSC is important in order to provide strategies for future, more efficient therapies.

The migratory capacity of bone marrow-derived MSC has been previously studied (22;23). Stromal cell-derived factor-1α (SDF-1α) (24;25), platelet-derived growth factor (PDGF-BB) (26;27), hepatocyte growth factor (HGF) (28) and basic fibroblast growth factor (bFGF) (29) have been reported as migratory stimuli for these cells. For MSC derived from adipose tissue, SDF-1α has also been described as a potent chemokine (30). However, comparing the various studies on MSC migration is difficult because the MSC are often cultured under different conditions and the set up of migration assays varies. Moreover, the properties of the migratory cell fraction(s) and the underlying mechanism involved in MSC migration remain unclear.

This study evaluated the migratory potential of MSC derived from adult bone marrow, adult adipose tissue, fetal lung and fetal bone marrow, whilst performing the cell culture and migration experiments under identical conditions. In addition, the migratory MSC population and the molecular mechanism of migration were investigated. As ongoing clinical trials have indicated that large cell numbers need to be transplanted, the studies
mentioned above and the experiments described in the current paper were performed using culture expanded MSC. Our results indicate that MSC derived from various tissues contain a cell fraction that is able to migrate, although the optimal stimulus and the percentage of migrating cells varied. The migratory MSC were observed to maintain differentiation and migration potential and they contained fewer cells in S- and G2/M-phase of the cell cycle as compared to non-migrating counterparts.
Materials and methods

Isolation and culture of MSC
Fetal lung and fetal bone were obtained after informed consent from legally terminated second trimester pregnancies. The protocol for collecting fetal tissues for research purposes was approved by the medical ethical review board of the Academic Medical Centre (AMC) (MEC: 03/038). Fetal lung MSC (FLMSC) were derived from magnetic bead selected CD34+ fetal lung cells, which were subsequently cultured in M199 containing 10% FCS, Penicillin streptomycin, ECGF and heparin as described by Noort et al (12). To obtain fetal bone marrow MSC (FBMSC), fetal bones were flushed with IMDM (Lonza, Verviers, Belgium) containing 10% FCS (Bodinco, Alkmaar, The Netherlands) and 1% penicillin-streptomycin (Gibco, Paisley, UK). The remaining erythrocytes in the cell suspension were lysed using NH₄Cl for 10 minutes on ice. Subsequently, cells were rinsed in PBS. 1.6 x 10⁶ cells were seeded per well in 6 well dishes in M199 (Gibco) supplemented with 10% FCS, 1% penicillin-streptomycin, 20 µg/ml ECGF (Roche diagnostics, Indianapolis, IN) and 8 IU/ml heparin (Leo Pharma, Breda, The Netherlands). The obtained cells were considered to be FBMSC.

Adipose tissue derived MSC (ASC) were a kind gift from Dr. FJ. van Milligen and were derived as previously described (31). Briefly, adipose tissue obtained from healthy donors was enzymatically digested. The obtained cell suspension was neutralized by DMEM containing glucose and FBS and then centrifuged. The cell pellet was resuspended in PBS and subjected to Ficoll density centrifugation. The cell containing interface was harvested. 1x 10⁵ cells/cm² were seeded in Dulbecco’s Modified Eagle medium (DMEM) containing 10% FBS, penicillin streptomycin and L-glutamine (31).

Adult bone marrow MSC (BMSC) were isolated from bone marrow aspirates obtained after informed consent from the sternum of patients undergoing cardiac surgery, according to the protocol for collecting bone marrow for research purposes approved by the medical ethical review board of the AMC (MEC:04/042#04.17.370). Briefly, MSC were isolated by density gradient centrifugation (Ficoll-paque, 1.077 g/ml, GE Health care Bio-Sciences AB, Uppsala, Sweden). 5 x 10⁶ cells were seeded per well in a 6 well dish in M199 containing 10% FCS, 1% penicillin-streptomycin, 20 µg/ml ECGF and 8 IU/ml heparin.

After 48 hours, the non-adherent cells were removed. The remaining cells were cultured for an additional 12 days or until reaching 80-90% confluency.

Upon reaching 80-90% confluency after initial plating, MSC derived from all sources were replated and further cultured under identical conditions in T80 tissue culture flasks at an initial density of 2500 cells/cm². For all experiments, 80-90% confluent passage 4 to passage 8 MSC were used.
Flow cytometry

All sources of MSC were characterized for surface expression of various receptors by flow cytometry. Cells were rinsed trypsinized, washed and resuspended in PBS containing 0.2% bovine serum albumine (BSA) prior to incubation (20 minutes at RT) with the following monoclonal antibodies. Antibodies purchased from BD, San Jose CA: CD73 (clone AD2), CD90 (clone 5E10), CD45 (clone HI30), CD14 (clone M5E2), CD68 (clone Y1/82A), CD44 (clone G44-26), CD49e (clone VC5), CD54 (clone HA58), CD106 (clone 51-10C9), CD146 (clone 8G12), CXCR4 (clone 12G5). Purchased from Sanquin, Amsterdam, The Netherlands: CD3 (clone CLB-T3/2), CD19 (clone CLB-B4/1), CD34 (clone CLB-HEC/75), CD38 (clone CLB-CLB-1D5), CD11a (clone CLB-LFA-1/2), CD11b (clone CLB-mon-gran/1), CD18 (clone CLB-LFA-1/1), CD49b (clone 10G11), CD49f (clone GoH3). Antibodies from other companies: CD271 (clone ME20.4-1.H4, Miltenyi Biotec, Gladbach, Germany), CD105 (clone SN6, Ancell, Bayport MN), CD34 (clone 58t, IQ-products, Groningen, The Netherlands), CD29 (clone P4C10, Chemicon), CD49d (clone 44H6, Imgen, ITK diagnostics, Uithoorn, The Netherlands), CD166 (clone 3A6, RDI, Concord MA), CXCR7 (Clone 358426, R&D systems), Santa Cruz biotechnology; PDGFRα (Clone 16A1) and PDGRβ (Clone P-20). Secondary antibodies; Goat anti mouse IgG (Dako), goat anti rabbit IgG (Invitrogen molecular probes).

As a negative control, cells were labeled with isotype controls IgG1, IgG2, IgG2a monoclonal antibodies (Sanquin, BD). A minimum of 10,000 events was recorded, using a FACS LSR II flow cytometer (BD).

Differentiation experiments

To study the multilineage differentiation capacity, MSC were cultured under conditions promoting differentiation towards osteoblasts or adipocytes as previously described (12). For differentiation experiments, MSC were plated in a 24 well dish at a plating density of 2.5 x 10^4 cells/cm² in α-minimal essential medium (α-MEM - Gibco). For osteogenic induction, the α-MEM was supplemented with 10% FCS and penicillin-streptomycin to which ascorbic acid (50 μg/ml, Sigma, St Louis, MI) and dexamethasone (10^-7 M, Sigma) were added. From day 7 onwards, β-glycerophosphate (5 mM, Sigma) was added. Cultures were incubated in a humidified atmosphere of 5% CO₂ at 37°C. Medium was replaced every 4th and 7th day of the week. For induction of adipogenesis, indomethacine (50 μM, MP Biomedicals, Solon, OH), IBMX (0.5 mM, Sigma) and insulin (1.6 μM, Sigma) were used.

At day 21, the cells induced towards osteogenic differentiation were stained for alkaline phosphatase and calcium deposition. Cells were incubated with a substrate solution (0.2 mg/ml α-naphthyl-1-phosphate (Sigma), 3 mg/ml sodium borate, 0.3 mg/ml magnesium sulphate and 0.8 mg/ml fast blue RR acid (Sigma)) for 15 minutes, resulting in the formation of an insoluble purple reaction product. To detect calcium deposition, cells were fixed with 3.7% formaldehyde (Merck, Darmstadt, Germany) for 10 minutes, and stained with 2% Alizarin Red S (ICN Biomedicals, Aurora, OH) and 0.1 NH₄ OH (pH 5.4) for 1 minute. Mineralization was indicated by the presence of red depositions.
To demonstrate the presence of adipocytes, expanded cells were fixed as described above. Cytoplasmic inclusions of neutral lipids were stained with Oil-Red-O (3 mg Oil-Red-O/ml 60% isopropanol, Sigma) for 10 minutes.

**In vitro migration experiments**

Migration experiments were performed using 12 µm pore size Transwell plates (Corning Costar, Cambridge, MA). The upper side of the insert was coated overnight at 37°C with fibronectin (20 µg/ml, Sigma) or collagen I (50 µg/ml, BD Biosciences) in PBS. 100,000 cells were seeded into the upper compartment in 500 µl IMDM supplemented with 0.25% BSA, and the stimuli were added to the lower compartment in 1.5 ml IMDM with 0.25% BSA. Optimal concentrations for migration were determined. Stimuli evaluated were; SDF-1α (600 ng/ml, Strathmann/R&D systems/Peprotech), bFGF (100 ng/ml, R&D systems, Minneapolis MN), PDGF-BB (5 ng/ml, R&D systems) and HGF (40 ng/ml, R&D systems/Peprotech, Rocky Hill, NJ). Checkerboard migration assays were performed by adding stimuli or combinations of stimuli in both upper and lower compartment. Treatment of cells and concentrations of stimuli as described above.

After 4h incubation at 37°C, the cells in the upper compartment were removed with a cotton swap. Subsequently, the inserts were carefully rinsed twice in PBS prior to fixation in 3.7% formaldehyde and further stained with Hoechst 33258 (1:500 dilution, Invitrogen). The Transwell filter membranes were cut out and mounted onto glass slides using Vectashield (Vector Laboratories, Burlingame, CA). The total number of migrating cells per view field was counted using fluorescence microscopy by counting nuclei. Data were expressed as the percentage of migrating cells related to the total number of cells loaded into the upper compartment.

**Immunofluorescent microscopy**

Ten thousand MSC were seeded on fibronectin-coated coverslips and cultured for two days. Subsequently, cells were put in serum free IMDM supplemented with 0.25% BSA. MSC were treated with SDF-1α or growth factors as indicated or left untreated. The cells were fixed for 10 minutes on ice, then permeabilized using 0.2% Triton-X-100 (Sigma) and stained for paxillin (Clone 165/Paxillin, BD) and phospho-paxillin (pY31, Rabbit polyclonal, Sigma), followed by incubated with Alexa 488 phalloidin (Invitrogen Molecular Probes, 1 IU/ml) for F-actin staining, goat-anti-mouse Alexa 633 (Invitrogen Molecular probes, 20 µg/ml) and goat-anti-rabbit Alexa 568 (Invitrogen Molecular probes, 20 µg/ml). Subsequently, the cells were stained with Hoechst. Coverslips were then mounted using Mowiol (Sigma). Immunofluorescent staining was detected using a LSM 510 META confocal microscope (Zeiss, Jena, Germany) using a 40x oil-objective. Images were captured by ZEN 2007 confocal software (Zeiss).
Cell lysis and Western blot
MSC were seeded in 6 well culture dishes at a density of 2500 cells/cm² and grown up to 80-90% confluency. Subsequently, cells were serum starved for 30 minutes and treated with SDF-1α, PDGF-BB or FCS as indicated or left untreated. Next, MSC were lysed in 250 µl NP40 buffer (50 mM TRIS, 100 mM NaCl, 10 mM MgCl₂, 1% NP-40, 10% Glycerol, PH 7.4, containing protease and phosphatase inhibitors) for 10 minutes on ice. Lysates were clarified by centrifugation at 14000 rpm at 4°C for 10 minutes. The supernatant was aspirated and further analyzed by Western blot.

For Western blotting, protein samples were separated by electrophoresis using a 10% SDS-polyacrylamide gel and transferred onto polyvinylidene (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were incubated with antibodies for paxillin (Mouse monoclonal, Clone 165/Paxillin) and phospho-paxillin (pY31, Rabbit polyclonal, Sigma; pY118, mouse monoclonal, BD Transduction laboratories) 1 hour in TBST (Tris-buffered saline, Tween 20) containing either 5% BSA (Sigma) for antibodies to detect phosphorylated proteins or 5% nonfat dry milk for others, followed by 45 minutes incubation with horseradish peroxidase-conjugated goat-anti-mouse (Pierce, 1:7000) or goat-anti-rabbit (Dako, 1:5000) secondary antibodies. Immunoreactive bands were revealed using an enhanced chemiluminescence kit (ECL, Pierce).

Cell cycle analysis
MSC were seeded in T80 tissue culture flasks at an initial density of 2500 cells/cm². Upon reaching 80-90% confluency, MSC were trypsinized and 100,000 cells were used for cell cycle analysis. The remaining MSC were allowed to migrate as described above. After migration, migrating and non-migrating MSC were harvested for cell cycle analysis. MSC were fixed in 70% ethanol on ice and subsequently incubated with Ki67 FITC (Clone MIB-1, Dako) for 30 mins at 4°C in PBS containing 0.1% Triton-X-100. Thereafter, the MSC were incubated for 15 mins at 37°C with labeling reagent containing PI (1µg/ml) or Hoechst 33258 (1µg/ml), RNase A (Sigma) and 0.1% Triton-X-100. A minimum of 10,000 cells was analysed by flow cytometry as described above and further analyzed using Modfit LT 3.0 software (Verity Software House, Topsham, ME). Ratios were calculated for each cell cycle phase from the percentage of cells in a certain phase in migrating MSC divided by the percentage of cells in the same phase in non-migrating MSC.

Statistical analysis
Statistical significance was determined by Man-Whitney U Test, using SPSS 15.0 (SPSS Inc, Chicago, IL), except for cell cycle data which were analyzed by One-Sample Kolmogorov-Smirnov Test. Results were considered to be significant when p≤0.05.
Results

Characterisation of MSC
MSC obtained from fetal lung (FLMSC), fetal bone marrow (FBMSC), adult bone marrow (BMSC) and adipose tissue (ASC) all had a spindle shaped morphology, expressed the marker combination CD73, CD90 and CD105, and lacked expression of hematopoietic markers CD34 and CD45 (Supplementary Figure 1a). All MSC sources also lacked expression of other hematopoietic markers (data not shown) in agreement with the definition of the ISCT (5). Osteoblast and adipocyte differentiation was successfully induced in all MSC described. Based on morphology, FLMSC and FBMSC seemed to be less efficient in adipogenic differentiation (Supplementary Figure 1b).

MSC derived from various tissues require extracellular matrix proteins for migration
In order to determine optimal conditions for in vitro migration experiments, FLMSC, BMSC- and ASC were allowed to migrate over 12 µm Transwell membranes coated with fibronectin,
collagen I, or left untreated. Hardly any migration was observed for all MSC sources across bare filters, but coating with fibronectin or collagen significantly increased the percentage of spontaneous migration and SDF-1α-induced migration (Figure 1).

These data indicate that MSC originating from three different tissues all require extracellular matrix proteins for in vitro migration, which is in agreement with previous observations by others (22;32). Coating with fibronectin was used to study the migration dynamics of MSC in more detail.

The expression of integrins, required for binding to extracellular matrix proteins, was evaluated. None of the MSC sources expressed CD11a, CD11b or CD18 (data not shown), while homogenous expression of CD29 and CD49e (VLA-5) was detected for all MSC (table 1). Expression of CD49b (VLA-2), CD49d (VLA-4) and CD49f (VLA-6) was also detected in all sources. Overall, a higher expression of these integrins was observed in fetal-derived MSC (table 1). FBMSC had a significantly higher expression of CD49b and CD49d than BMSC. CD49b was significantly higher expressed on FLMSC as compared to BMSC and ASC. FLMSC also expressed CD49f to a significantly higher level than FBMSC, BMSC and ASC (table 1).

### Migratory capacity of MSC derived from various tissues towards different stimuli

To examine whether MSC originating from different tissues display similar migratory behaviour as compared to the extensively studied BMSC, the different MSC were allowed to migrate towards SDF-1α, PDGF-BB, HGF, bFGF or FCS. As demonstrated in Figure 2, FLMSC showed a significant enhanced migratory capacity towards SDF-1α when compared with BMSC and ASC, whereas ASC showed an increased migratory capacity towards FCS.

<table>
<thead>
<tr>
<th>Integrin expression on MSC</th>
<th>ASC (n=4)</th>
<th>BMSC (n=6)</th>
<th>FBMSC (n=3)</th>
<th>FLMSC (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD49b</td>
<td>22.1±18.8</td>
<td>15.8±6.3</td>
<td>54.9±17.8a</td>
<td>94.8±2.6b</td>
</tr>
<tr>
<td>CD49d</td>
<td>22.9±24.4</td>
<td>4.3±2.0</td>
<td>29.8±2.9a</td>
<td>21.6±15.8</td>
</tr>
<tr>
<td>CD49e</td>
<td>99.8±0.1</td>
<td>99.9±0.1</td>
<td>100±0</td>
<td>99.7±0.2</td>
</tr>
<tr>
<td>CD49f</td>
<td>8.4±11.5</td>
<td>14.6±14.9</td>
<td>15.8±5.2</td>
<td>40.8±5.2c</td>
</tr>
</tbody>
</table>

The data represent the percentage (mean ± SD) of positive cells for the indicated integrin, determined by flow cytometry.

1 significant differences were observed between FBMSC and BMSC, p≤0.05
2 significant difference was observed between FLMSC, and ASC, BMSC, p≤0.05
3 significant differences were observed between FLMSC and ASC, BMSC and FBMSC, p≤0.05

Abbreviations: ASC, adipose tissue-derived MSC; BMSC, adult bone marrow-derived MSC; FBMSC, fetal bone marrow-derived MSC; FLMSC, fetal lung-derived MSC
compared with FLMSC and BMSC. These data show that MSC originating from all tissues are able to migrate, although the percentage of migrating cells and the optimal stimulus differs among MSC sources. Overall, FLMSC showed the highest response to the given stimuli (Figure 2). To determine whether this enhanced migratory capacity was due to its fetal origin, the migratory capacity of adult and fetal bone marrow-derived MSC was compared. No significant differences in migratory potential were observed between adult and fetal BMSC towards any of the stimuli, whereas FLMSC had a significantly increased migratory potential when compared with FBMSC in four out of five stimuli evaluated (Figure 2): SDF-1α, PDGF-BB, HGF and bFGF. These observations suggest that the migratory potential of various MSC is determined by the tissue of origin rather than the maturity of the tissue they have been derived from. The differences in migratory potential of MSC from various tissues could not be explained by chemokine- or growth factor receptor expression. Although slight differences were observed in surface expression of CXCR4, CXCR7, PDGFRα and PDGFRβ, high intracellular expression of these receptors was detected in all MSC (Supplementary table I).

MSC were allowed to migrate for 4 hours towards SDF-1α (600 ng/ml), PDGF-BB (5 ng/ml), HGF (40 ng/ml), and bFGF (100 ng/ml). Medium alone and FCS (20% in medium) served as negative and positive controls respectively. Bars represent the percentage (mean ± SD) of migrated MSC related to the cell number loaded in the upper compartment. *, p<0.05. Abbreviations: ASC, adipose tissue-derived MSC; BMSC, adult bone marrow-derived MSC; FBMSC: fetal bone marrow-derived MSC; FLMSC, fetal lung-derived MSC; FCS, fetal calf serum; bFGF, basic fibroblast growth factor; HGF, hepatocyte growth factor; PDGF-BB, platelet derived growth factor-BB; SDF-1α, stromal derived factor-1α.
No additive effect in migration of FLMSC towards SDF-1α, PDGF-BB and FCS

As it was observed that only a small percentage of the cultured MSC were able to migrate towards the various stimuli, a checkerboard migration experiment was set up to elucidate whether an additive effect could be observed in migration of MSC towards a combination of stimuli. The experiments were performed using FLMSC because they possessed the highest migratory capacity. Optimal concentrations of stimuli were used. As depicted in Figure 3, no additive effect was observed between SDF-1α, PDGF-BB and FCS in migration of FLMSC, when both stimuli were put together in the lower compartment of the Transwell. Interestingly, the presence of SDF-1α in the upper compartment partly inhibited migration towards PDGF-BB in the lower compartment and PDGF-BB in the upper compartment partly blocked migration towards SDF-1α in the lower compartment (not significant). Together, these data suggest that one subset of MSC is able to migrate towards various stimuli.

Figure 3  No additive effect in migration of FLMSC towards SDF-1α and PDGF-BB

FLMSC were allowed to migrate in a checkerboard assay for 4 hours towards SDF-1α, PDGF-BB and FCS or a combination of stimuli. Medium alone or stimuli in the upper compartment only served as negative controls, FCS as positive control. Bars represent the percentage (mean ± SD) of migrated MSC related to the cell number loaded in the upper compartment. No synergistic effect was observed in the percentage of migrating FLMSC between SDF-1α, PDGF-BB and FCS.

Abbreviations: FCS, fetal calf serum; PDGF-BB, platelet derived growth factor-BB; SDF-1α, stromal derived factor-1α.
Actin rearrangement and enhanced paxillin phosphorylation in stimulated MSC
As only a small percentage of expanded MSC was able to migrate, it was evaluated whether only these cells were able to rearrange the actin cytoskeleton and focal adhesions in response to migratory cues, which is required to enable cell migration (33;34). Strikingly, morphological changes and altered F-actin distribution were observed in the majority (80%) of the BMSC after stimulation with PDGF-BB and SDF-1α (Figure 4a). MSC formed top ruffles after 5 minutes (Figure 4a). After 30 minutes, lateral membrane ruffles were observed (Figure 4a). Paxillin is a focal adhesion-associated adapter protein, and its phosphorylation at tyrosine residues Y31 and Y118 is associated with focal adhesion turnover and migration (35;36). After stimulation with FCS and SDF-1α for 60 minutes, increased levels of phosphorylated paxillin were observed at the periphery of the cells (Figure 4b). In addition, the effects of FCS and SDF-1α on the phosphorylation levels of paxillin on the total cell population were studied using Western blot. These data show that FCS and to a lesser extent SDF-1α increased paxillin phosphorylation (Figure 4c). Also PDGF-BB increased paxillin phosphorylation. Similar results were obtained for FLMSC and ASC and were confirmed by pY118 staining (data not shown). These data indicate that, as in other cells, paxillin phosphorylation is enhanced upon stimulation with various migratory cues in MSC.

Migratory MSC maintain differentiation and migratory capacity
In order to study the characteristics of the migratory MSC in more detail, MSC were trypsinized from the Transwell membranes after migration and put in culture as described. Migratory and non-migratory MSC were observed to maintain their proliferation capacity and when seeded in osteogenic or adipogenic supporting medium two weeks after migration, migrating and non-migrating MSC originating from all sources were still able to differentiate (Figure 5a). As a control, MSC from the same passage that were not used for migration experiments were differentiated simultaneously. No variations in differentiation potential could be observed between migrating, non-migrating and the cultured MSC subset in any of the sources.

To evaluate whether the migratory MSC also maintained their migratory capacity, the migrating- and non-migrating MSC obtained from the first migration run were cultured for two weeks before being allowed to migrate again towards a gradient of SDF-1α for 4 hours. FLMSC that had migrated towards SDF-1α in the first experiment had a significant increased migratory potential (p≤0.009) in the second experiment compared to FLMSC that did not migrate in the first run (Figure 5b). This indicates that the heterogeneous population of expanded MSC contains a subset of cells with a higher intrinsic migratory capacity.

To define these MSC, which would enable enriching for migratory MSC, surface expression of the following markers was evaluated: CD44, CD49b, CD49d, CD49f, CD54, CD73, CD106,
Characterization of human mesenchymal stromal cell heterogeneity

Figure 4a,4b  Actin rearrangement and paxillin phosphorylation in MSC.

(A) PDGF-BB and SDF-1α induced morphological changes in the actin cytoskeleton of BMSC. The upper panel depicts cell morphology after 5 minutes stimulation with control serum free medium (left), PDGF-BB (middle) or SDF-1α (right). PDGF-BB and SDF-1α stimulation induced top ruffle formation (arrowheads). The lower panel depicts cell morphology after 30 minutes stimulation with control medium (left), PDGF-BB (middle) or SDF-1α (right), showing lateral membrane ruffles (arrowheads). Green: F-Actin, blue: Nuclei. Of the three independent experiments performed, one representative experiment is shown. (B) Changes in paxillin and phosphorylated paxillin distribution upon stimulation of BMSC with serum free medium (top panel), SDF-1α (middle) or 20% FCS (lower panel) for 60 minutes. After stimulation with FCS or SDF-1α, increased phosphorylated paxillin was observed at the cell membrane (arrowheads). Green: paxillin, red: phosphorylated paxillin. Of the two independent experiments performed, one representative experiment is shown.
CD146, CD166, CD271, CXCR4, CXCR7 and PDGFRα and PDGFRβ. None of these markers was exclusively, neither differentially expressed on migratory MSC (data not shown).

The migratory MSC fraction contains less cells in S- and G2/M-phase Cell cycle has been identified to influence homing and repopulation of HSC (37) and S- and G2/M-phase were found to negatively influence these processes. Thus it was evaluated whether the distribution of the phases of the cell cycle in migratory FBMSC was different from the distribution in non-migrating FBMSC. Immediately after migration, cell cycle analysis was performed for both fractions (Figure 6a). Ratios of migrating versus non-migrating MSC were calculated for each cell cycle phase (Figure 6b). It was observed that migrating MSC contained significantly less cells in S- (0.81±0.13, p<0.028) and G2/M-phase (0.75±0.13, p<0.031; Figure 6b). Expression of the Ki67 antigen, which enables discrimination between G0- and G1-phase, revealed a trend of more cells in G1-phase in migratory MSC (1.34±0.50) as compared to non-migratory MSC, however this did not reach significance (data not shown). These data show that cell cycle is also involved MSC migration and that as in HSC, S- and G2/M-phase negatively influences migration.

Western blot analysis of paxillin phosphorylation

(C) The upper panels depict phospho-paxillin levels after stimulation: control (left panel, left lane), pervanadate (positive control, left panel right lane); control- (right panel, left lane), FCS- (right panel, middle lane) or SDF-1α treatment (right panel, right lane) for 60 minutes. Enhanced paxillin phosphorylation was observed after stimulation with FCS and SDF-1α for 60 minutes. The middle and lower panels show total paxillin levels and an actin loading control for the indicated treatments respectively. Note that the loading control for total paxillin after FCS stimulation is diffuse, due to high phosphorylation levels.

Abbreviations: FCS, fetal calf serum; PDGF-BB, platelet derived growth factor-BB; SDF-1α, stromal derived factor-1α; CTRL, control medium; PV, pervanadate.
Figure 5  Functional analysis of the migratory subpopulation.

(A) After SDF-1α-induced migration, the migratory and non-migratory subpopulations were collected and subsequently cultured for two weeks, prior to being allowed to migrate towards SDF-1α again. Bars represent the percentage (mean ± SD) of migrated MSC related to the cell number loaded in the upper compartment. *, p≤0.05.

(B) After migration, MSC maintained differentiation potential towards adipocytes detected by oil-red-O staining, and osteoblasts, detected by alizarine red and alkaline phosphatase stainings, in vitro. No differences in differentiation potential were observed between the migratory- the non-migratory- and the cultured control population. Representative experiment shown.

Abbreviations: ASC, adipose tissue-derived MSC; FLMSC, fetal lung-derived MSC; SDF-1α, stromal derived factor-1α.
After SDF-1α-induced migration, migrating and non-migrating FBMSC were collected and fixed immediately for cell cycle analysis. (A) Histograms represent the mean fluorescence of the migrating MSC (left panel) and non-migrating MSC (right panel). Percentages per cell cycle phase were analysed using Modfit. One representative experiment shown. (B) Ratios (+ SD) of migrating versus non-migrating MSC were calculated for each cell cycle phase from four independent experiments. Migrating MSC contain significantly less cells in S- and G2/M-phase as compared to non-migrating MSC. * p≤0.05.

Abbreviations: Dip: diploid; FBMSC, fetal bone marrow-derived MSC; PI: propidium iodide.
Characterization of human mesenchymal stromal cell heterogeneity

Discussion

Due to their multilineage differentiation potential, MSC are considered promising cells for tissue engineering and cellular therapies. For successful treatment, MSC have to migrate towards the site of injury, from which migratory cues such as growth factors and chemokines are released. Factors involved in migration of adult BMSC have been studied before (23-29). However, MSC can be derived from various fetal (2-4) and adult tissues (1). It is known from previous studies, that HSC derived from various origins possess different migratory capacities (20;38;39). Therefore, we have compared the in vitro migratory capacity of the well studied adult BMSC to those of MSC derived from fetal lung, fetal bone marrow and adult adipose tissue, all kept under identical conditions.

Similar to BMSC, in all other MSC source evaluated, only a small percentage of the MSC population was able to migrate towards the stimuli provided, although the percentage of migrating cells and the optimal stimulus differed between MSC sources. FLMSC had the highest migratory potential towards all specific stimuli as compared to the other sources tested. This is most probably not due to the fact that they are derived from fetal tissue, because FBMSC had a similar migratory capacity when compared with BMSC. These data suggest that migratory capacity of culture expanded MSC varies with the tissue of origin rather than the maturity of this tissue. The observation that MSC of various origin display differential responsiveness to growth factors and chemokines in vitro, also suggests that these MSC are possibly attracted by different chemokines and/or growth factor combinations released upon injury in vivo and this may be due to conservation of niche-induced characteristics. Similar suggestions have been made by others on differences between MSC sources in terms of colony frequency, differentiation and gene expression (1;40-42).

As all MSC expressed the chemokine or growth factor receptors involved at similar levels, both at the cell membrane and intracellular, this could not explain differences in migratory potential. The large intracellular receptor pools indicate that MSC derived from all sources should be able to rapidly alter surface expression in response to stimuli, however the process of receptor cycling/trafficking may differ between MSC.

In vitro and in vivo experiments have demonstrated that only subpopulation(s) of culture expanded MSC are capable of specific homing (10;23). It was not clear whether one or multiple migratory subpopulations exist. Our experiments suggest that there is one migratory subpopulation because no additional effect was observed when PDGF-BB and SDF-1α were both present in the lower compartment of the Transwell compartment. It is possible that suboptimal concentrations of the stimuli would in act in concert, while maximal levels would not do so.

As only few MSC were able to migrate, it was evaluated whether actin polymerization and focal adhesion formation over in response to migratory cues, required to enable cell migration (33;34) was restricted these cells. In contrast, the majority of the MSC show actin
membrane ruffles in response to SDF-1α or PDGF-BB. Similar observations were made whilst studying the focal adhesion protein paxillin, which has a role in the turnover of focal adhesions formed during migration (43;44). Paxillin has multiple phosphorylation sites that regulate its function (43;45). Enhanced phosphorylation at residues Y31 and Y118 is related to increased focal adhesion turnover and migration (35;46). To our knowledge, this study showed for the first time that stimulation of MSC with migratory cues such as SDF-1α and PDGF-BB increases paxillin Y31 phosphorylation and induces its redistribution to the cell periphery, indicating that also in MSC, phosphorylation at this residue is related to focal adhesion turnover and migration. FCS increased pY31 paxillin to a higher extent than SDF-1α, possibly due to the multiple growth factors and chemokines, including SDF-1α, present in FCS. Therefore, phosphorylation could be induced by several pathways, while SDF-1α only signals through its receptor CXCR4 (47;48). Paxillin has been related to MSC differentiation (49;50) and redistribution of total paxillin was observed upon stimulation with the chemoattractant sphingosine-1-phosphate (51), but a role for phospho-paxillin was not described previously. Phosphorylation at pY31 and actin polymerization were detected in the majority of the MSC, while migration only occurred in a small percentage of MSC, indicating that functionality of the machinery involved in the initial response to migratory cues is not restricted to the migratory MSC subset. Future studies will reveal whether differential phosphorylation of other paxillin residues in migratory and non-migratory MSC causes different paxillin mediated signaling in these cells (43;45).

Studies on the characteristics of the migratory MSC in vitro revealed that they maintained proliferation and differentiation capacity after migration. Moreover, in secondary migration experiments, the migratory subpopulation maintained its migratory capacity and this was significantly higher compared to MSC that did not migrate in the first run. The migration percentages in the second migration run were lower than in the first migration run, which has been described for HSC as well (21). It remains to be established whether the migratory subset also has an enhanced migratory capacity in vivo and whether these cells contribute to a better recovery after injury. To be able to enrich for the migratory MSC from the heterogeneous MSC population, markers that distinguish migratory MSC from the non-migratory MSC are required, but none of the markers evaluated in the current study were exclusively or differentially expressed on migratory MSC. These results, together with observations that many MSC are able to respond to migratory cues, lead to the hypothesis that migratory MSC are not a specific subpopulation that can be identified by surface marker expression, but are in a different intracellular state, which enables these cells to translate the initial response to migratory cues into migratory behaviour.

A better understanding of the (molecular) mechanisms involved in MSC migration will enable modulation of MSC to enhance their homing efficiency (52). Cell cycle has been linked to migration and homing in HSC (37;53). HSC in S- and G2/M phase loose homing and engraftment capacity (37), and SDF-1/CXCR4 is involved in regulating HSC quiescence and cycling (54). Interestingly, mobilized HSC were predominantly in G0/G1-phase of the
cell cycle (55). Here we showed that the cell cycle also contributes to MSC migratory potential. There is a trend of more cells in G1-phase and significantly less cells S- and G2/M-phase in migrating MSC as compared to their non-migrating counterparts. Experiments on modulating the cell cycle in MSC should therefore be considered to enhance in vitro and in vivo migration of MSC. Indeed, when we increased the proportion of FBMSC in G1-phase (34.8±9.4%) by harvesting cells at 50-70% confluency (n=3), as compared to 4.9±1.9% in G1-phase in cells harvested at 80-90% confluency (n=5), both spontaneous and SDF-1α induced migration was significantly higher (spontaneous 13.6±1.1% vs 7.2±0.5%, p<0.025, SDF-induced 20.3±1.2% vs 12.0±0.7%, p<0.025).

In conclusion, our results suggest that ex-vivo expanded MSC derived from various adult and fetal tissues have different migratory capacity towards growth factor and chemokine stimuli, which is likely to be related to the tissue of origin rather than the developmental stage of this tissue. Migratory MSC not only maintain differentiation and migration capacity, but also contain fewer cells in S- and G2/M phase, which shows a relation between cell cycle and MSC migration. Identification and modification of processes that favour MSC migration will make a significant contribution to increasing efficacy of future cellular therapies.
Chapter 2  Migratory behaviour of various human fetal and adult MSC

Reference List

8 Prockop DJ  Marrow stromal cells as stem cells for nonhematopoietic tissues  Science 1997 Apr 4;276(5309):71-4.
18 Butcher EC  Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity  Cell 1991 Dec 20;67(6):1033-6.
20 Voermans C, Gerritsen WR, dem Borne AE, van der Schoot E  Increased migration of cord blood-derived CD34+ cells, as compared to bone marrow and mobilized peripheral blood CD34+ cells across uncoated or fibronectin-coated filters  Exp Hematol 1999 Dec;27(12):1806-14.
Characterization of human mesenchymal stromal cell heterogeneity


Chapter 2 Migratory behaviour of various human fetal and adult MSC


Supplementary data
Supplementary Figure 1A Characterization of MSC

(A) Flow cytometric analysis of MSC derived from various tissues. All MSC were positive for CD73, CD90 and CD105 but lacked expression of CD34 or CD45. The isotype is depicted in red, markers depicted in green. Representative experiment shown.

Abbreviations: ASC, adipose tissue-derived MSC; BMSC, adult bone marrow-derived MSC; FBMSC, fetal bone marrow-derived MSC; FLMSC, fetal lung-derived MSC
(B) MSC derived from various tissues were able to differentiate into adipocytes, detected by oil-red-O staining, and osteoblasts, as indicated by alizarine red and alkaline phosphatase stainings, in vitro.

Abbreviations: ASC, adipose tissue-derived MSC; BMSC, adult bone marrow-derived MSC; FBMSC, fetal bone marrow-derived MSC; FLMSC, fetal lung-derived MSC
Supplementary Table 1  Chemokine- and growth factor receptor expression on MSC

<table>
<thead>
<tr>
<th>Receptor</th>
<th>ASC (n=3)</th>
<th>BMSC (n=3)</th>
<th>FBMSC (n=4)</th>
<th>FLMSC (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR4 surface</td>
<td>753±251</td>
<td>1141±1020</td>
<td>1986±429</td>
<td>488±311</td>
</tr>
<tr>
<td>CXCR4 intracellular</td>
<td>1272±4105</td>
<td>11647±9212</td>
<td>25901±8899</td>
<td>27606±17705</td>
</tr>
<tr>
<td>CXCR7 surface</td>
<td>504±186</td>
<td>1647±9999</td>
<td>921±187</td>
<td>983±894</td>
</tr>
<tr>
<td>CXCR7 intracellular</td>
<td>804±315</td>
<td>1650±1742</td>
<td>941±294</td>
<td>1724±25</td>
</tr>
<tr>
<td>PDGFRα surface*</td>
<td>1004±64</td>
<td>5733±5330</td>
<td>3071±2230</td>
<td>5201±3211</td>
</tr>
<tr>
<td>PDGFRβ surface</td>
<td>16±72</td>
<td>510±646</td>
<td>278±331</td>
<td>1253±510</td>
</tr>
<tr>
<td>PDGFRβ intracellular</td>
<td>9826±2770</td>
<td>13249±3507</td>
<td>8843±1785</td>
<td>19075±7699</td>
</tr>
</tbody>
</table>

Data expressed as MFI (mean ± SD, corrected for the isotype control) of the total population of MSC for the indicated receptor.
*100% surface expression