Migration of culture-expanded human mesenchymal stem cells through bone marrow endothelium is regulated by matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-3

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Acknowledgments: we would like to thank Nicole Arras, Veerle De Greef and Wim Renmans for their technical support.

Funding: our research work is supported by grants from the Foundation for Scientific Research (FWO) and the Vrije Universiteit Brussel (HOA), Belgium.

Manuscript received June 30, 2007. Manuscript accepted February 12, 2007.

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ABSTRACT

Background and Objectives

Mesenchymal stem cells (MSC) are adult stem cells that can be expanded many fold *in vitro* and have the therapeutic potential to restore the bone marrow microenvironment and support hematopoietic recovery after myeloablative conditioning for hematopoietic stem cell transplantation. Successful homing to the target tissue, such as bone marrow, implies that MSC are able to extravasate after systemic administration. However, the extravasation capacity of MSC and the underlying mechanisms are poorly understood to date. We studied *in vitro* the capacity of MSC to migrate through bone marrow endothelium.

Design and Methods

In vitro invasion and transendothelial migration assays were performed. The expression of matrix metalloproteinase (MMP) was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) and zymography. Migration of cells cultured at high or low confluence was compared and differential gene expression in these conditions was analyzed with microarray and real-time RT-PCR. The functional involvement in MSC migration was assessed using neutralizing anti-MMP-2 antibody, MMP-2 short interfering RNA or recombinant tissue inhibitor of metalloproteinase (TIMP-3).

Results

We demonstrated that MSC can invade reconstituted basement membrane and that bone marrow endothelial cells stimulate this process. We also showed that the transendothelial migration of MSC is at least partially regulated by MMP-2. High culture confluence was found to increase production of the natural MMP-inhibitor TIMP-3 and decrease transendothelial migration of MSC.

Interpretation and Conclusions

We show that MSC have the potential to migrate through bone marrow endothelium and that this process involves MMP-2. Moreover, the migration of MSC is significantly influenced by the level of culture confluence. Increased culture confluence impairs migration and is related to an upregulation of TIMP-3. The therapeutic use of MSC would benefit from a selection of culture conditions that allow optimal extravasation of these cells.

Key Words: mesenchymal stem cell, migration, culture confluence, MMP-2, TIMP-3

Haematologica 2007; 92:440-449

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Mesenchymal stem cells (MSC) are multipotent, adult stem cells predominantly residing in the stromal compartment of hematopoietic bone marrow. They were first described by Friedenstein[1] as fibroblast-like, adherent cells that can be expanded *in vitro* after selection through plastic adherence. It has been shown that a single MSC can give rise to different mesodermal cell types such as adipocytes, chondrocytes and osteocytes, if the appropriate stimuli are present in the growth medium.[2] In recent years it has become clear that MSC might have an even broader differentiation capacity. There are reports of differentiation into neural cells,[3,4] cardiac muscle cells,[5] endocrine cells,[6] hepatocytes,[7] hepatocytic cells[8] and epithelial cells.[9,10] MSC also create the stromal environment that supports the growth of hematopoietic progenitor cells.[11] Initially MSC were thought to be only present in hematopoietic bone marrow. However, over the past few years, several groups have reported other sources of MSC including umbilical cord blood,[12] umbilical vein,[13] peripheral blood,[14] non-hematopoietic bone marrow,[15] adipose tissue[16] and the post-partum placenta.[17]

Because of their wide differentiation potential and accessibility many research groups are investigating the therapeutic potential of these stem cells. It has been reported that MSC can support hematopoietic recovery after co-transplantation with autologous hematopoietic stem cells (HSC) and that they also express the capacity to suppress graft-versus-host disease after allogeneic HSC grafting.[18-23] Other therapeutic applications of MSC are related to tissue replacement therapy and are currently being studied in different domains including cardiology and neurology.[24-27]

For most therapeutic purposes, systemic infusion of MSC seems to be the most practical mode of administration. However, this means that MSC must be capable of migrating and homing to the targeted tissue. Once they have arrived here, the cells must be able to cross the endothelial barrier and integrate into the tissue. For regeneration of bone and restoration of bone marrow stroma, this means that the systemically infused MSC must migrate through bone marrow endothelium. At present little is known about the homing of MSC. Several groups have reported that small numbers of systemically administered human MSC can be found in different tissues, including bone marrow.[28-31] However so far there are no conclusive data on the specificity of this process or the molecules involved in human MSC homing. It can be assumed that the MSC homing process is similar to homing of normal lymphocytes as described by Butcher and Picker in 1996.[32] These authors distinguished four steps: (a) initial contact with and rolling over the endothelial cells, (b) activation of the lymphocyte, (c) activation dependent arrest and (d) diapedesis or the actual extravasation. This multistep process involves several types of molecules such as chemokines and their receptors, adhesion molecules as well as proteases. Matrix metalloproteinases (MMP) are proteolytic enzymes that depend on binding with a Zn^{2+} ion for their catalytic activity. They are synthesized as pre-pro-enzymes and most are secreted as inactive pro-enzymes or zymogens that are activated by cleavage of the pro-domain.[33] Based on substrate specificity and structure they are divided into several subgroups: collagenases, stromelysins, matrilysins, gelatinases and membrane-type MMP. The gelatinases, MMP-2 and MMP-9, have the same basic structure as the other MMP but in the catalytic domain they have three repeats of the fibronectin type II domain. These repeats interact with gelatin and collagen type IV, two major constituents of the basement membrane.[34] They have been shown to be involved not only in cancer cell migration but also in leukocyte homing.[35,36] In this study we analyzed the transendothelial migration capacity of culture-expanded MSC as well as the involvement of the gelatinases in this process. In addition we studied at which level the migration of MSC is affected by the culture confluence at the moment of harvest.

Design and Methods

Primary culture of MSC

Bone marrow samples, aspirated from the sternum, were obtained from healthy donors, following informed consent. All tested samples were derived from different donors. Bone marrow mononucleated cells (BMMNC) were isolated by Ficoll-Hypaque (Nycomed, Lucron Bioproducts, De Pinte, Belgium) density gradient centrifugation. BMMNC were initially plated in 10 mL Mesenchymal Stem Cell Growth Medium (MSCGM, Cambrex, Verviers, Belgium) at a density of 60×10^6 cells per 75 cm^2 in Nunclon culture flasks (Nunc, VWR International, Leuven, Belgium) and incubated at 37°C with 5% humidified CO_2. After 4 hours, growth medium and non-adherent cells were discarded. The adherent cell fraction was rinsed with RPMI (GibcoBRL, Invitrogen, Merelbeke, Belgium) and 20 mL MSCGM was added to the culture flask. Cells were cultured for five passages. At each passage cells were detached with trypsin (0.05%)/ EDTA (0.02%) (Cambrex): 5 min incubation at 37°C. After adding 10% fetal calf serum (FCS, A&E Scientific, Marcq, Belgium) in RPMI the cells were washed once in DPBS (Cambrex). After the first passage, one part of the cells was harvested when the cultures reached complete culture confluence while the other part of the cells was harvested at low culture confluence. Low confluence was defined as 50% coverage of the culture surface area and high confluence as 100% coverage. Cell counts after trypsin revealed that this corresponded to a cell density at harvest of less than 800 cells/cm^2 for low confluent cultures and more than 2000 cells/cm^2 for high confluent cultures. Cells from low confluent cultures were passaged every 3-4 days and cells from high confluent cultures were passaged every 10-12 days.

Immunophenotyping

Approximately 10^5 MSC were incubated with 5 or 10 μL monoclonal antibody conjugated with phycoerythrin (PE) or fluorescein isothiocyanate (FITC): CD34-FITC (Becton

Dickinson (BD), Erembodegem, Belgium), CD45-PE (DAKO. Leuven, Belgium), CD73-PE (PharMingen, BD), CD90-PE (PharMingen, BD), CD105-FITC (Ancell-10P's, Zandhoven, Belgium), and CD166-PE (PharMingen, BD). After 10 min unbound antibody was washed with 2 mL FACS flow (BD). The cell pellet was resuspended in 0.5 mL FACS flow. The samples were analyzed with a flow cytometer (Coulter Epics® XL-MCL, Analis, Namur, Belgium) with 10,000 events recorded for each condition.

Differentiation cultures

Adipogenic differentiation

In order to induce adipogenic differentiation 2×10^5 *in vitro* expanded MSC were seeded into a well of a 6-well plate (Nunc). They were first grown to confluence in MSCGM and the medium was changed every 2-3 days until a confluent cell layer was formed. The cells were stimulated to differentiate into the adipogenic lineage by submitting them to three cycles of alternating culture in Adipogenic Induction Medium (Cambrex) and Adipogenic Maintenance Medium (Cambrex) according to the manufacturer's instructions. At the end of these cycles cells were grown for another 7 days in Adipogenic Maintenance Medium. As a control MSC were grown in Adipogenic Maintenance Medium only to exclude spontaneous adipogenic differentiation. To visualize adipocytes, cells were stained with Oil Red O (Sigma, Bornem, Belgium), while cell nuclei were counterstained with hematoxylin (Sigma).

Osteogenic differentiation

Osteogenic differentiation was induced by exposing 3×10^4 MSC to Osteogenic Induction Medium (Cambrex). The medium was changed every 3 to 4 days. As a negative control, cells were cultured in MSCGM and medium was changed at the same frequency as that for the differentiating MSC. To verify osteogenic differentiation, Von Kossa's method was used to stain calcium deposits.

Chondrogenic differentiation

In order to induce chondrogenic differentiation 2.5×10^5 MSC were washed twice in Incomplete Chondrogenesis Induction Medium (Cambrex). Cells were then sedimented by centrifugation at 150 g for 5 minutes and 0.5 mL Complete Chondrogenesis Induction Medium (Cambrex) was added to the cell pellet. Complete medium was made by adding 5 μL TGFβ3 (Cambrex) to 1 mL incomplete medium. Cells were cultured for 2-3 weeks and the medium was changed every 3 to 4 days. Pellets were harvested and fixed in formalin and embedded in paraffin. Sections of 4 μm were made, transferred onto a slide and dried for 1 hour. The sections were immunohistochemically stained for collagen type II. A rabbit polyclonal anti-collagen II antibody (NCL-COLL-IIp, NovoCastra, Prosan, Merelbeke, Belgium) was used.

In vitro migration assays

Prior to the migration assay cells were labeled with the carbocyanine fluorochrome DiI (1,1'dioctadecyl-3,3,3', 3'tetramethylindocarbocyanine perchlorate) (Molecular Probes, Invitrogen, Merelbeke, Belgium). DiI is a lipophilic molecule that incorporates in the cell membrane, and has the following spectral characteristics: absorption maximum at 549 nm and an emission maximum at 565 nm. MSC were incubated with 10 μg/mL DiI in culture for 72 hours at 37°C with 5% humidified CO_2. The cells were harvested after incubation with trypsin/EDTA. Since MSC were labeled with a fluorescent dye, we used BD Falcon™ HTS FluoroBlok Inserts (BD). They have a polyethylene terephthalate (PET) membrane that blocks light transmission from 490 to 700 nm. This allows detection of cells present in the lower compartment only. Once cells migrate through the pores of the membrane they are no longer shielded from the light source and can be detected with a fluorescence plate reader. Data were assembled with the Fluoroskan Ascent plate reader and software (Thermo-Labsystems, VWR International, Leuven, Belgium).

Invasion assays were performed using filters coated with 50 μg Matrigel (gelled at 37°C for 1 h). To analyze transendothelial migration, *in vitro* filters were prepared using Matrigel and human bone marrow-derived endothelial cells (4LHBMEC-line) as described previously.[37] MSC (5×10^4) in 100 μL RPMI were added to the upper compartment, the lower compartment; contained 10% FCS in RPMI as a source of chemoattractants. Samples were incubated overnight at 37°C. Percentage migration was calculated as the ratio of signal intensity of migrated cells versus signal intensity of total input number of cells. In additional experiments, cell migration was measured after 2, 4, 6, 8, 20 and 22 h to determine the migration kinetics.

In some transendothelial migration experiments recombinant human tissue inhibitor of metalloproteinase-3 (rhTIMP-3) (R&D systems, Abingdon, UK) (1-12 nM) was added to the upper compartment. In other experiments a blocking anti-MMP-2 antibody (CA-4001, Chemicon International, Biognost, Heulen, Belgium) or control antibody of the same isotype (mouse myeloma IgG1, Serotec, DPC, Humbeek, Belgium) was added to the upper compartment (final concentration 10 μg/mL). Finally migration assays were performed using MSC in which MMP-2 was knocked down by short interfering RNA (siRNA) transfection (*see below*).

Short interfering RNA transfection

Two days before the migration assay MSC were transfected with MMP-2 Stealth™ RNAi (Invitrogen). The MMP-2 Stealth™ RNAi provides three non-overlapping Stealth™ RNAi duplexes for this gene to obtain high knock-down efficiency. These duplexes were transfected into the MSC with Lipofectamine RNAiMAX (Invitrogen); each MMP-2 Stealth™ RNAi duplex was added at a concentration of 10 nM. To exclude aspecific knock-down, MSC were also transfected with Stealth™ RNAi Negative Control Duplex (Invitrogen).

RNA extraction, microarray and real-time reverse transcriptase polymerase chain reaction (RT-PCR)

RNA extraction

Total RNA was extracted with Trizol reagent (Invitrogen) and purified on RNeasy mini spin columns (Qiagen, Westburg, Leusden, Netherlands). The RNA was then quantified by spectrophotometry (Gene Quant II, Pharmacia, Diegem, Belgium). An optical density (OD) of 1 corresponded to 40 μg/mL RNA; all 260/280 ratios were between 1.7 and 2.0.

Microarray

MSC from three different donor samples were selected for microarray analysis. MSC RNA extracts from high and low confluent cultures from each donor were directly compared against each other on dual color Agilent's Human 1A Oligo Microarray (Agilent, Diegem, Belgium) that contains over 20,000 60-mer oligonucleotide probes representing more than 18,000 well annotated human genes. The integrity and purity of the total RNA extracts were controlled using an Agilent Bioanalyzer and Nanodrop spectrophotometer, respectively. None of the samples showed signs of degradation or impurities (260/280 and 260/230 >1.8). Probes were prepared from 1 μg total RNA using the Agilent Low RNA input Fluorescent Linear Amplification Kit (Agilent). Briefly, from total RNA, poly-A RNA was reverse transcribed using a poly dT-T7 primer and labeled by incorporating cyanine 3-CTP or cyanine 5-CTP (Perkin Elmer) during a T7 *in vitro* transcription amplification. The resulting amplified and labeled RNA probes were purified separately with RNeasy purification columns (Qiagen, Germany). Probes were verified for amplification yield and incorporation efficiency by measuring the DNA concentration at 280 nm, Cy3 incorporation at 550 nm and Cy5 incorporation at 650 nm using a Nanodrop spectrophotometer. A good aRNA probe had a labeling efficiency of 1 fluorochrome every 300 bases. For each color 10 pmol incorporated dye was fragmented and resuspended in 500μL hybridization solution (Agilent 60-mer oligo microarray processing protocol, Agilent). The arrays were hybridized in Agilent microarray hybridization chambers (Agilent) and incubated overnight in a rotisseri oven at 60°C. Slides were treated according to the *Agilent 60-mer oligo microarray processing protocol* (Agilent). Slides were scanned with an Agilent DNA microarray scanner and images were processed using Agilent Feature Extraction Software version 7. Each hybridization was repeated by swapping the dyes. Therefore, this microarray analysis resulted in six data points per condition including data from three biological samples each analyzed in duplicate with reversal of color. The list of differentially expressed genes was conservatively selected based on two sample t-testing with $p<0.01$ and ratios larger than 1.8 fold.

Real-time RT-PCR

Differential expression of TIMP-3 was verified with real-time PCR in the three samples used for microarray analysis and three additional MSC samples. cDNA was synthesized from 1 μg mRNA using the Thermoscript System® (Invitrogen). Fluorescence was measured using the iCycler Thermal Cycler (Biorad, Nazareth, Belgium). Real-time PCR cycles for TIMP-3 were performed incorporating 12.5 μL SYBR Green mix (Biorad), 0.75 μL forward primer (10 μM), 0.75 μL reverse primer (10 μM), 5 μL cDNA and 6 μL water. Real-time PCR reactions for quantification were incubated for 3 min. at 95°C followed by 45 cycles of 95°C for 15 sec. and 60°C for 60 sec. As a reference the Abelson (*Abl*) housekeeping gene was amplified; real-time PCR cycles were performed with a mixture of 25 μL containing 12.5 μL UDG Supermix, 1 μL $MgCl_2$ (50 mM), 1 μL primer mix (forward primer, reverse primer and probe), 5 μL cDNA and 5.5 μL water. Samples were incubated for 3 min at 50°C and 95°C followed by 45 cycles at 95°C for 15 sec and 60°C for 60 sec. The melting curve was obtained by incubating the reactions at 95°C for 60 sec and 55°C for 60 sec followed by 80 cycles increasing the temperature by 0.5°C every 10 sec, starting from 55°C.

RT-PCR

For MMP-2, MMP-9, CD45 and actin, RT-PCR samples were amplified for 40 cycles, with each cycle consisting of 30 sec at 94°C, 30 sec at the annealing temperature and 30 sec at 72°C. The resulting fragments were separated on a 1.5% agarose gel and stained with ethidium bromide. As positive controls for MMP-2, MMP-9 and CD45 we used cDNA from human umbilical vein endothelial cells, the myeloma cell line 8226/RPMI and normal peripheral blood lymphocytes, respectively. Primers and probes used in these reactions are listed in Table 1.

Enzyme-linked immunosorbent assays (ELISA)

TIMP-3 ELISA

Since TIMP-3 binds to the extracellular matrix and is not, therefore, abundant in conditioned medium we prepared cell lysates. A lysis buffer was prepared containing 50mM Tris (Invitrogen), 50 U/mL penicillin (Invitrogen) and 50 μg/mL streptomycin (Invitrogen). Cells grown at high and low confluence were incubated with the buffer for 2 h on ice. Subsequently the samples were centrifuged at 10000 rpm for 5 min and the supernatant was diluted 1:20 and used for ELISA. A 96-well plate (Costar, Elscolab, Kruibeke, Belgium) was coated overnight at 4°C with 20 μg/mL polyclonal anti-TIMP-3 antibody (R&D systems, Abingdon, UK). The plate was washed twice with PBS and then aspecific binding sites were blocked by incubation with 3% BSA (Invitrogen) in PBS at room temperature in a humid atmosphere. The following day the wells were washed twice with PBS and 50 μL of diluted supernatant were added. Additionally a serial dilution (50 ng/mL – 0.78 ng/mL) of rhTIMP-3 (R&D Systems) was used to obtain a standard curve, allowing quantification of TIMP-3 in the different samples. The antigen was allowed to bind to the antibody for 2h at room temperature in a humid atmosphere. Wells were washed four times with PBS, then 1

Table 1. Real time RT-PCR primer pairs and Taqman probes.

	Forward primer	*Reverse primer*
TIMP-3	CTACCTGCCTTGCTTTGTGAC	ACTCTCGGTACCAGCTGCAG
Abl	TGGAGATAACACTCTAAGCATAACTAA AGGT	GATGTAGTTGCTTGGGACCCA
MMP-2	GGGACAAGAACCAGATCACATAC	CTTCTCAAAGTTGTAGGTGGTGG
MMP-9	CGCAGACATCGTCATCCAGT	GGATTGGCCTTGGAAGATGA
CD45	GGCTGACTTCCAAGATATGACCATGTAT	GGTTGTTTCAGAGGGATTAAGGTAGG
	Taqman probe	
Abl	FAM-CCATTTTTGGTTTGGGCTTCACACCATT-TAMRA	

Overview of the primer pairs and probes used for RT-PCR and real-time RT-PCR analyses.

μg/mL monoclonal anti-TIMP-3 antibody (R&D systems) was added to each well and incubated for 2 h at room temperature in a humid atmosphere. Subsequently the wells were washed four times with PBS and incubated with 1:1000 horseradish peroxidase (HRP) labeled anti-mouse IgG antibody (R&D systems) for 2 h at room temperature. HRP substrate reagent (R&D systems) was prepared according to the instructions of the manufacturer and 200 μL were added per well and incubated in the dark. After 30 min 50 μL Stop solution (R&D systems) were added and the optical density was determined at 450 nm with wavelength correction at 540 nm with the Multiskan Ascent plate reader (Thermo LabSystems).

MMP-2 ELISA

The amount of MMP-2 secreted by MSC was determined using the Quantikine Human/Mouse MMP-2 Immunoassay (R&D systems). Conditioned medium was collected after incubating MSC cultures for 48h with RPMI. ELISA was performed according to the manufacturer's instructions. Optical density was determined at 450 nm with wavelength correction at 540 nm with the Multiskan Ascent plate reader.

Gelatin zymography

Gelatin zymography was performed using supernatant from MSC, cultured overnight on Matrigel®. The samples were stored at –20°C until use. Supernatant from the fibrosarcoma cell line HT1080 was used as a positive control. The gelatinolytic activity of the supernatant was assayed under non-reducing conditions. Equal volumes of sample and 2x Novex Tris-Glycerine SDS Sample Buffer (Invitrogen) were mixed and applied to a precast sodium dodecylsulphate (SDS) polyacrylamide gel containing 0.1% gelatine (Invitrogen). Electrophoresis was performed in the X-cell sure lock system (Novex, Quickpoint, Invitrogen) at a constant voltage of 125 V for 2-4 hours. The gels were then incubated with Zymogram Renaturing Buffer (Invitrogen) and Zymogram Developing Buffer (Invitrogen) respectively for 30 minutes. The Zymogram Developing Buffer was replaced with fresh developing buffer and the gels were incubated overnight at 37°C. The following day the gels were stained with a Coomassie Blue solution containing 40% methanol (Filter Service, Eupen, Belgium), 10% acetic acid (Merck, VWR International), 0.05% Coomassie Blue (Merck) and deionised water. The gels were destained in 40% methanol, 10% acetic acid and deionised water and dried with the DryEase Mini Gel drying system (Invitrogen). A clear band against a blue background represents enzyme activity.

Statistical analysis

Microarray data were analyzed using t-tests with the level of statistical significance set at $p<0.01$. Results from migration assays are presented as mean + standard error of mean; data were analyzed for differences with Student's t-test, with the levels of statistical significance set at $p<0.05$.

Results

Characterization of in vitro-expanded MSC

MSC were cultured from the mononuclear cell fraction of bone marrow samples obtained from normal donors. Cells were selected based on plastic adherence and passaged five times prior to further use to ensure removal of contaminating hematopoietic cells.

Through FACS analysis we examined the expression of some MSC markers CD73 (SH3), CD90 (Thy-1), CD105 (endoglin, SH2) and CD166 (ALCAM, activated leukocyte cell adhesion molecule) as well as the hematopoietic membrane antigens CD34 (hematopoietic precursor cells) and CD45 (LCA, leukocyte common antigen). Culture-expanded MSC were positive for CD73, CD90, CD105 and CD166, but showed no expression of CD34 and CD45 (Figure 1A).

Next we tested the potential of the expanded cells to differentiate *in vitro* into the adipogenic, osteogenic and chondrogenic lineages. At the fifth passage, cells were detached with trypsin and cultured in media containing additives to induce differentiation into one of these three cell types. Simultaneously MSC were cultured in regular medium to exclude spontaneous differentiation. Adipogenesis was demonstrated by staining the lipid droplets with Oil Red O stain (Figure 1B). Calcium deposits in differentiated osteogeneic cells were stained black using Von Kossa's method (Figure 1C). Immunohistological staining for collagen type II confirmed chondrogenic differentiation (Figure 1D). These data indicate that the culture-expanded cells show all characteristics of MSC: they were selected based on plastic adherence, they express surface antigens found on MSC and are capable of differentiation into three mesenchymal cell types.

In vitro migration capacity of MSC

Fifth passage cells from low confluent cultures were seeded on filters coated with the reconstituted basement membrane, Matrigel®, and were allowed to migrate to the lower compartment overnight (n=10). They showed limit-

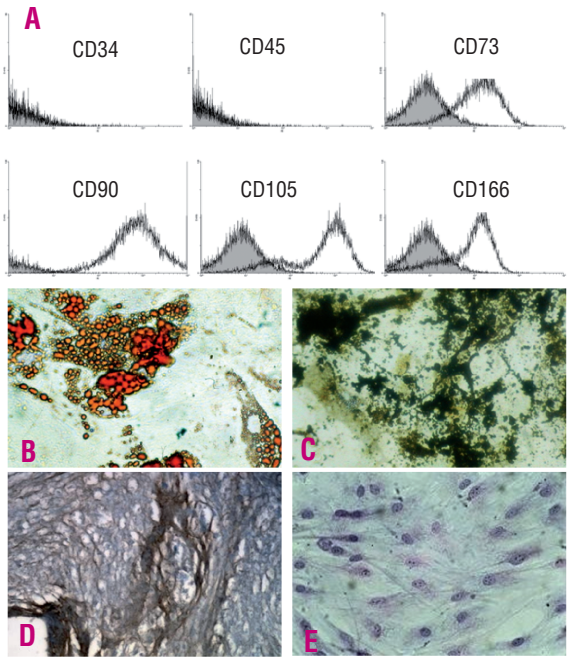

Figure 1. Characterization of expanded human MSC. Histogram overlays of FACS-analysis of one representative sample. The gray peak represents the negative control antibody, the transparent peak the antigen-specific antibody. MSC express CD73, CD90, CD105 and CD166 whereas CD34 and CD45 were not expressed (A). Photographs showing *in vitro* differentiation of MSC from one representative culture into the adipogenic (B), osteogenic (C) and chondrogenic (D) lineages. Undifferentiated MSC (E) (hematoxylin).

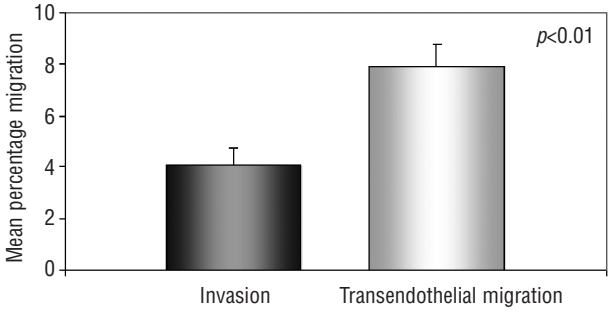

Figure 2. Invasion and transendothelial migration of MSC. Bar graph of migration of expanded MSC through Matrigel® or Matrigel® covered with bone marrow endothelial cells. Data are represented as mean ± SEM.

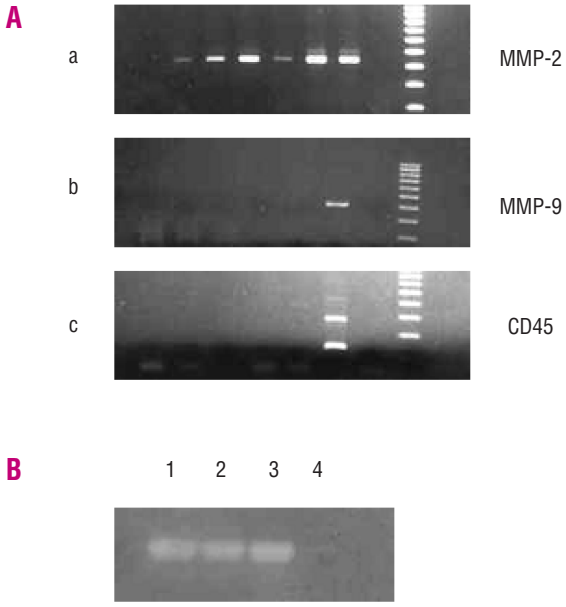

Figure 3. MMP-2 expression by RT-PCR and zymography. (A) Lane 1-5: MSC cDNA from five different cultures. Lane 6: positive control. Lane 7: no template control. Lane 8: 100 bp ladder. (B): Gelatin zymography of supernatants of three different MSC samples cultured on Matrigel®.

ed migratory capacity but when a confluent layer of bone marrow endothelial cells was established on Matrigel®-coated filters, the migratory capacity of MSC increased significantly (Figure 2) ($p<0.05$). The mean percentage migration through Matrigel®-coated filters without and with bone marrow endothelial cells was 4.04% and 7.91%, respectively (Figure 2).

Role of gelatinases MMP-2 and MMP-9 in MSC migration

Using RT-PCR, we demonstrated that culture-expanded MSC express MMP-2 mRNA while no transcripts could be detected for MMP-9. Since no CD45 transcripts could be found in the tested cDNA samples, it is unlikely that the MMP-2 transcripts were derived from contaminating hematopoietic cells (Figure 3A). In addition the supernatant of cultured MSC also contained secreted MMP-2 protein, as demonstrated by gelatin zymography (Figure 3B). To determine the role of MMP-2 in MSC migration we performed migration assays in the presence of a blocking anti-MMP-2 antibody (n=4). We observed that MMP-2 inhibition significantly reduced the transendothelial migration capacity of MSC (Figure 4A). To further confirm the functional involvement of MMP-2 in MSC transendothelial migration we transfected cells with MMP-2 siRNA. ELISA of conditioned media 48h after transfection showed a 96% knock down of MMP-2 compared to that in untreated MSC and MSC transfected with negative control siRNA (0.97 ng/mL vs 24 ng/mL and 23 ng/mL, respectively). Migration assays performed with MMP-2 siRNA transfected MSC show a similar decrease of *in vitro* migration capacity as observed with the inhibitory antibody (n=3) (Figure 4B).

Culture confluence influences *in vitro* transendothelial migration

Transendothelial migration assays were performed with fifth passage MSC from either high or low confluent cultures (n=12) and demonstrated a different migratory potential. MSC cultured at high confluence (mean density: 2200 MSC/cm^2) showed a significantly lower migration capacity than MSC cultured at low confluence (mean density: 660 MSC/cm^2) ($p<0.05$). Mean percentage migration of MSC harvested at high and low culture confluence was 5.02% and 14.3%, respectively (Figure 5A). We also assessed the

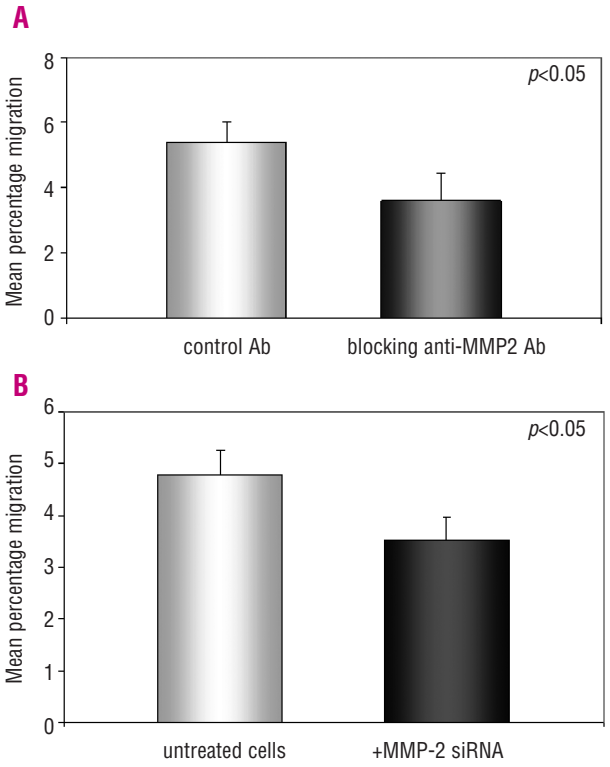

Figure 4. Role of MMP-2 in MSC migration. A. Bar graph showing transendothelial migration in the presence of a neutral antibody or a blocking anti-MMP-2 antibody. Blocking MMP-2 results in a significant decrease of migration. B. Bar graph showing transendothelial migration of MSC transfected with MMP-2 Stealth™ RNAi and untreated cells. This MMP-2 knock-down results in a similar decrease of migration capacity as observed with the blocking antibody. Data are represented as mean ± SEM.

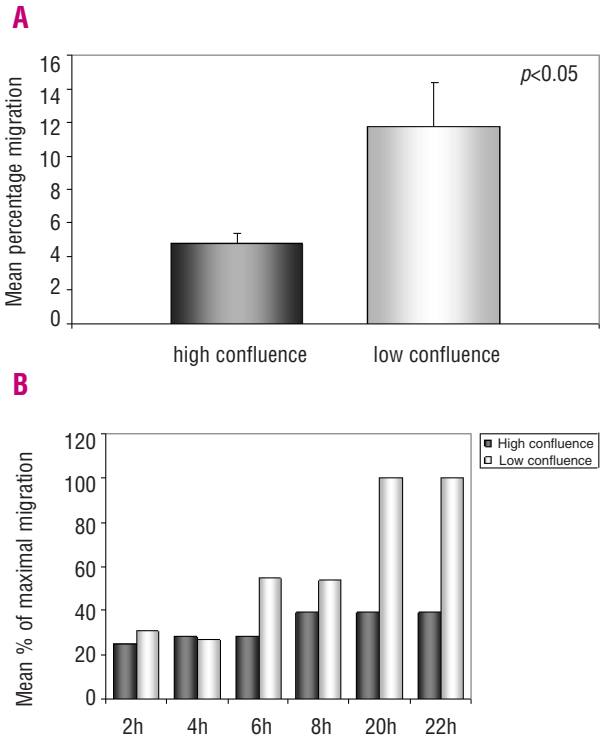

Figure 5. Transendothelial migration of MSC from high and low confluent cultures. A. Bar graph of transendothelial migration of MSC from high and low confluent cultures (n=12). Culturing MSC at high confluence significantly impairs their transendothelial migration capacity. Data are represented as mean ± SEM. B. Bar graph showing kinetics of migration of MSC grown at both levels of culture confluence. Data are represented as percent migration compared to maximal migration (i.e. migration of MSC harvested at low confluence after 22 h).

migration kinetics of MSC grown at high and low confluence (n=6). Figure 5b shows that MSC harvested at low confluence reached maximal migration after 20 h while the migration of MSC from high confluent cultures reached its endpoint after 8 h.

Culture confluence influences MSC gene expression

MSC from three different normal donors were cultured both at high and low confluence. At the fourth passage the cells were collected and RNA was extracted. These samples were tested with microarray analysis using the commercially available Human 1A chip from Agilent Technologies. For each donor the genetic profile of MSC cultured at low confluence was compared to that of the cells cultured at high confluence. Data from the microarray experiments were analyzed with Student's t-test and significant differential expression was set at $p<0.01$. Genes were further selected based on fold change: only genes with ratios larger than 1.8 fold were retained. We found that 466 genes were differentially expressed. Of these 217 were upregulated and 249 were downregulated in high confluent cultures. Most of the differentially expressed genes were related to cell cycle control, proliferation and differentiation (*data not schown*). There was also one gene that could be linked directly to MMP regulation of cellular migration; tissue inhibitor of metalloproteinase-3 (TIMP-3). We verified the results obtained from the microarray analysis for this gene by real-time RT-PCR in the samples used for microarray and three additional samples. The housekeeping gene *Abl* was not differentially expressed and was amplified for each sample as an internal reference. The microarray and real-time RT-PCR data for *TIMP-3* are shown in Table 2. The level of differential expression varied between the different samples tested and was more pronounced in the real-time RT-PCR analysis, which is a finding commonly reported in the literature. Additionally we assessed whether this differential gene expression was also translated into different levels of TIMP-3 protein expression. We performed ELISA on cell lysates from MSC cultured at high or low confluence. We found a significantly lower concentration of TIMP-3 (11±2.3 ng/mL/10^5 cells) in MSC from low confluent cultures than in MSC cultured at high confluence (56±3.5 ng/mL/10^5 cells).

Effect of TIMP-3 on migration of MSC from low confluent cultures

The natural MMP inhibitor TIMP-3 is significantly upregulated in high confluent cultures as shown by microarray and confirmed by real-time RT-PCR. To assess whether TIMP-3 has a functional role in the migration of MSC we

Table 2. TIMP-3 RT-PCR and microarray data.

	Real-time RT-PCR					Microarray	
Sample	mean Ct TIMP-3	mean Ct Abl	Δ^{ct}	$\Delta\Delta^{ct}$	H vs L (%)	log_2 ratio	p value
donor 1 H	24.7	31.6	-6.9	-1.9	373	1.13	0.00160
donor 1 L	28.3	33.3	-5.0	–	–	–	–
donor 2 H	24.3	30.1	-5.8	-2.3	492	1.28	–
donor 2 L	23.5	27.0	-3.5	–	–	–	–
donor 3 H	26.1	32.1	-6.0	-0.3	123	0.94	–
donor 3 L	30.7	36.4	-5.7	–	–	–	–
donor 4 H	22.6	34.8	-12.2	-2.7	650	–	–
donor 4 L	22.5	31.97	-9.5	–	–	–	–
donor 5 H	23.2	36.7	-13.5	-0.1	107	–	–
donor 5 L	22.8	36.2	-13.4	–	–	–	–
donor 6 H	20.3	32.5	-12.2	-1.9	382	–	–
donor 6 L	21.0	31.2	-10.2	–	–	–	–
overall	–	–	–	–	–	354	1.12

Overview of real-time RT-PCR and microarray results for TIMP-3. (H: high confluence; L: low confluence).

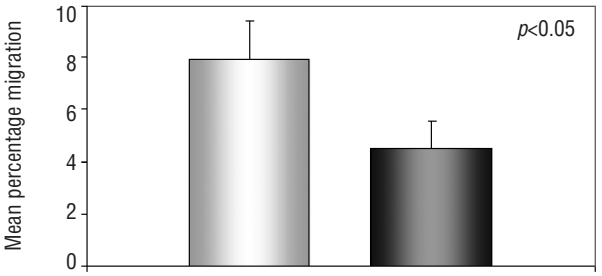

Figure 6. Impact of TIMP-3 on transendothelial migration of low confluent MSC. Bar graph showing transendothelial migration in the presence of 6 nM rhTIMP-3 (n=3). The presence of TIMP-3 significantly impairs the transendothelial migration capacity of MSC from low confluent cultures. Data are represented as mean ± SEM.

performed *in vitro* migration assays (n=3) in the presence of exogenously added TIMP-3. MSC from low confluent cultures were incubated with rhTIMP-3 (1-12 nM) during transendothelial migration assays. This resulted in a significant decrease of migration capacity with a maximal effect at 6 nM rhTIMP-3. At this concentration a decrease in migration 43% ($p<0.05$) was observed (Figure 6).

Discussion

MSC are non-hematopoietic, bone marrow-derived stem cells that can be expanded *in vitro* and that express a broad differentiation capacity. Because of these characteristics MSC are regarded as possible effectors for tissue repair *in vivo*, tissue engineering and as target cells for gene therapy.[38-40] In recent preclinical and clinical studies culture-expanded MSC were systemically administered to animals or patients. The establishment of a biological/clinical effect following this route of administration necessitates that MSC are capable of crossing the endothelial barrier lining blood vessels in order to reach a specific tissue. Several groups have shown that MSC can be found in a wide range of tissues after systemic administration.[28-31,41,42] It has been shown, both in animal models and in patients, that MSC can also extravasate to bone marrow although the numbers of MSC that integrated in the marrow compartment were found to be low.[42,43] What limits MSC bone marrow homing and engraftment is unclear. The exact molecular mechanisms underlying MSC homing to bone marrow also remain undefined. In this study MSC were isolated from bone marrow samples and selected based on plastic adherence and cultured in a medium containing 10% FCS. Immunophenotyping showed that the cultured cells expressed CD73, CD90, CD105 and CD166 while they did not express the hematopoietic markers CD34 and CD45. This phenotypic profile corresponds to the previously published profiles.[2,44] It is generally accepted that a key feature of MSC is their potential to differentiate into adipogenic, osteogenic and chondrogenic lineages.[2] Therefore, we exposed the culture-expanded cells to specific signals that induce differentiation into these three lineages and concluded that the cultured cells possessed the properties of true MSC. Next we analyzed the *in vitro* migration capacity of the cultured MSC using invasion and transendothelial migration assays. It was found that MSC have the capacity to invade a reconstituted basement membrane and that bone marrow endothelial cells enhance this process.

So far there is little if any information available about the molecular mechanisms involved in the homing of MSC. In analogy to the homing of normal leukocytes and hematopoietic stem cells, it can be assumed that this process involves different types of molecules, including adhesion molecules, chemokines and proteases. As we confirmed in this study, MSC express different adhesion molecules including CD44 and integrins that might allow MSC adhesion to endothelial ligands (*data not shown*). Some recent reports indicated that culture-expanded MSC express transcripts for chemokine receptors, including CCR1,[45,48] CCR2,[46] CCR4,[48] CCR7,[45,48] CCR10,[48] CXCR5,[48] CXCR6[45] and CXCR4,[45,47,49] although the functional role of these last receptors remains unclear. In this study we demonstrated that MSC express a gelatinase, i.e. MMP-2, which has the capacity to degrade collagen IV, a major constituent of the basement membrane. Previous studies demonstrated that this protease is involved in the extravasation of both normal and malignant cells. Concordant with our observation, Son *et al.* very recently showed that MSC secrete MMP-2 and not MMP-9. Moreover the authors of that study showed a functional involvement of MT1-MMP in MSC migration *in vitro.*[49] To our knowledge we are the first to show that there is also a functional involvement of MMP-2 in MSC migration. We found that neutralizing MMP-2 with a blocking antibody impaired *in vitro* MSC transendothelial migration capacity. Moreover, when MSC were transfected with MMP-2 siRNA, this resulted in a near complete knock-down of MMP-2 and impaired *in vitro* migration capacity. Interestingly, we observed that the migratory capacity of MSC was strongly affected by the level of culture confluence at the moment

the cells were harvested for analysis. There was a significant decrease of migration for cells cultured at higher confluence. This observation is similar to what has been reported in a previous study using a murine model. In this murine study it was found that culturing MSC for just 24-48 hours at 80% confluence was sufficient to cause significant impairment of *in vivo* homing to the bone marrow and other lymphohematopoietic tissues.[50] One of the genes that was significantly up-regulated in MSC cultured at high confluence as shown by micro-array analysis, real-time PCR on the mRNA level and by ELISA on the protein level, is *TIMP-3*. *TIMP-3* is one of the natural inhibitors of MMP-2.[51] Four different TIMP have been characterized so far. Their basic structure contains two domains: a C-terminal domain and an N-terminal domain responsible for inhibition of MMP. Every member of the TIMP-family can inhibit the active form of all MMP but differences in substrate specificity have been noted.[52] TIMP-3 differs from other TIMP, which are soluble proteins, in its binding to the extracellular matrix. Several studies have demonstrated that TIMP-3 affects cell migration and reduces cancer cell migration and invasion.[53] Overexpression of TIMP-3 has been found to inhibit migration of vascular endothelial cells, through strong binding to the extracellular matrix, thereby protecting the vascular basement membrane from proteolysis.[54] TIMP-3 also impairs homing of leukocytes through inhibition of L-selectin shedding.[55] In this study, we provide functional evidence that TIMP-3 can impair the transendothelial migration of MSC; the observed increase in TIMP-3 expression is related to a lower migration capacity of MSC cultured at high confluence. In preclinical and clinical settings, harvesting culture-expanded MSC at high culture confluence might result in a higher cell yield but our data indicate that this approach might considerably reduce the homing and engraftment capacity of MSC when infused intravenously.

In conclusion, we have demonstrated that human MSC have the capacity to migrate through bone marrow endothelium and that this process involves the metalloproteinase MMP-2. Since *in vitro* migration is not completely blocked by inhibiton of MMP-2 (expression) other factors must be involved in this process. An active role for MT1-MMP has been reported recently.[49] However, we found that the level of culture confluence has an important effect on the transendothelial migration capacity of expanded MSC, and that TIMP-3 has a definite role in inhibition of this migration. These data suggest that the homing potential of MSC which are systemically delivered for therapeutic purposes, is significantly affected by the *in vitro* expansion conditions of these cells. The therapeutic use of MSC could benefit from a better understanding of MSC homing mechanisms and selection of culture conditions that allow optimal extravasation of these cells.

Authors' Contributions

ADB: performed most of the experimental work, analyzed the data and wrote the paper; PVH: performed part of the experimental work, analyzed data and reviewed the final version of the pape; MB: performed part of the experimental work and reviewed the final version of the paper; IVB: provided important experimental tools; JDW: performed part of the experimental work; MDW: provided technical support and contributed to data analysis; IVR: designed research, analyzed data and critically reviewed the paper.

Conflict of Interest

The authors reported no potential conflicts of interest.

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