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Role of stromal-derived factor-1 in the hematopoietic-supporting activity of human mesenchymal stem cells

Van Overstraeten-Schlögel N, Beguin Y. Gothot A. Role of stromal-derived factor-1 in the hematopoietic-supporting activity of human mesenchymal stem cells.

Abstract: Mesenchymal stem cells (MSC) have the ability to support and maintain hematopoiesis *in vitro*. However, mechanisms implicated in this support are not fully characterized. In the present study, the role of stromal-derived factor-1 (SDF-1)/CXCR4 axis in the interactions between MSC and hematopoietic stem/progenitor cells (HSPC) was studied. Human bone marrow MSC were plated as feeder layers in Dexter-type long-term cultures (LTC) with human cord blood $CD34^+$ HSPC. Cultures were supplemented weekly with neutralizing antibodies against CXCR4 or SDF-1 for 5 wk. LTC-initiating cell (IC) activity was strongly dependent on the SDF-1/CXCR4 axis, as both antibodies significantly decreased secondary colony-forming cell production. To assess the effect of SDF-1/CXCR4 axis on progenitor cell proliferation, LTC-IC killing assays were carried out: in LTC of $CD34^+$ cells in contact with MSC, treatment with anti-CXCR4 antibody significantly reduced the number of cycling progenitors. These results indicate that the SDF-1/CXCR4 axis promotes HSPC proliferation in contact with MSC. Interestingly, when HSPC were separated from MSC by a semi-permeable membrane, LTC-IC activity became CXCR4 independent. Multiplex analysis of MSC-conditioned medium revealed that in addition to SDF-1, MSC produced stimulatory and inhibitory factors, such as interleukin (IL)-6, IL-11, granulocyte macrophage-colony stimulating factor as well as monocyte-chemoattractant protein-1. Altogether, human MSC support hematopoiesis in Dexter-type cultures through the activation of the SDF-1/CXCR4 axis. Our data further suggest that SDF-1 stimulates retention of HSPC in MSC niches which expose them to stimulatory and inhibitory factors in a paracrine manner.

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Human bone marrow (BM) mesenchymal stem cells (MSC) have the capacity to give rise to adipocytes, osteocytes and chondrocytes (1). Recent findings indicate that they may have a broader differentiation potential and develop into myoblasts, endothelial cells as well as non-mesodermal cells such as hepatocytes or neural cells (2). *Ex vivo*-expanded MSC can be reproducibly generated from adult BM and maintained in undifferentiated state for extended periods of time. Interestingly, undifferentiated MSC support the proliferation and differentiation of hematopoietic stem/progenitor cells (HSPC) *in vitro* (3). These findings may be translated into the possible use of autologous MSC in *ex vivo* expansion of HSPC. However, mechanisms underlying the hematopoietic-supporting ability of MSC are not fully understood.

Stromal-derived factor-1 (SDF-1) is a critical factor in the regulation of hematopoiesis. Acting on its receptor CXCR4, SDF-1 is essential for normal hematopoietic development through its action on fetal BM seeding by HSPC (4). In postnatal hematopoiesis, SDF-1 mediates selective BM homing and retention of HSPC transplanted by intravenous infusion (5). In addition, SDF-1 may also stimulate *ex vivo* survival and proliferation of purified HSPC (6, 7). SDF-1 is secreted by BM immature cells located in the endosteal region as

well as by BM endothelial cells (8). Interestingly, SDF-1 is highly expressed by immature pre-osteogenic MSC and rapidly downregulated in MSC cultured in osteo-inductive conditions (9). Altogether, these findings suggest that SDF-1 may be a critical factor involved in the stimulation of hematopoiesis by undifferentiated MSC.

In the present study, we investigated the contribution of SDF-1/CXCR4 interactions in the hematopoietic-supporting ability of MSC *in vitro*. Dexter-type long-term cultures (LTC) of human cord blood (CB) $CD34^+$ cells were established with human MSC as feeder layers. Neutralization of endogenously produced SDF-1 was achieved with specific antibodies blocking SDF-1 or its receptor CXCR4. The effect of SDF-1/CXCR4 inhibition on the proliferative potential and cycling status of very primitive HSPC was determined in 5-wk LTC assays.

Materials and methods

Human hematopoietic and mesenchymal cells

Umbilical CB blood samples were collected aseptically following full-term vaginal delivery according to the guidelines established by the ethical committee of the University of Liège. A Ficoll-Hypaque (Pharmacia Amersham, Uppsala, Sweden) gradient centrifugation was used to isolate mononuclear low density cells. $CD34^+$ cells were purified using MACS CD34 isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) as per manufacturer's instructions. The immunomagnetic separation was performed twice to achieve maximal purity of the $CD34^+$ fraction, which was always $>97\%$.

Cryopreserved human BM MSC were obtained from Cambrex (Verviers, Belgium) and subcultured in mesenchymal stem cell growth medium (Cambrex). Cells were pretested for differentiation capacity into osteogenic, chondrogenic and adipogenic lineages. As for antigenic phenotype, cells were pretested for absence of CD45 and CD34, and presence of CD105, CD29 and CD44, which was confirmed in our laboratory prior to the experiments described above.

Long-term cultures

Dexter type LTC of CB $CD34^+$ cells were performed over feeder layers of MSC. MSC were plated at the concentration of 15×10^3 cells/cm^2. Within a week, $CD34^+$ cells were seeded in 24-well plates at 1000 cells/well at 33°C in a 100% humidified atmosphere and 5% CO_2 in 1.5 mL LTC medium. LTC medium consisted of αMEM (Cambrex) supplemented with 12.5% horse serum (Stem Cell Technologies, Meylan, France), 12.5% fetal bovine serum (FBS; Invitrogen, Paisley, Scotland), 0.2 mM i-inositol (Sigma, Bornem, Belgium), 10^{-4} M 2-mercaptoethanol (Invitrogen) and 2 mM L-glutamine (Cambrex). Cultures were maintained by weekly half-medium changes. After 5 wk, the medium was gently aspirated and cells were harvested by trypsinization before being transferred into semisolid medium as described below to measure secondary colony-forming cell (CFC) production. In some experiments, HSC and MSC co-cultures were supplemented weekly with 1/100 dilution of anti-CXCR4 (BD Biosciences, Erembodegem, Belgium), anti-SDF-1 (R&D, Abingdon, UK) or CD3 (BD Biosciences) neutralizing antibodies to assess the role of SDF-1/CXCR4 axis on progenitor cell output. No azide – low endotoxin antibody formulations were used in these assays.

Non-contact LTC were set up by plating 500 $CD34^+$ cells in the upper chamber of 12-mm diameter 0.4-μm pore Transwells^{TM} (Costar, Cambridge, MA, USA). The lower chamber was seeded with 15×10^3 MSC/cm^2 in 2 mL LTC medium. Medium feeding and secondary CFC assays were done as described above.

Colony-forming cell (CFC) assays

Hematopoietic progenitors were assayed in semisolid media composed of 1.3% methylcellulose (Stem Cell Technologies), 30% FBS (Cambrex), 100 ng/mL rh stem cell factor (SCF), 10 ng/mL rh interleukin-3 (IL-3), 10 ng/mL rh interleukin-6 (IL-6), 5 ng/mL rh granulocyte macrophage-colony stimulating factor (GM-CSF; all from Peprotech, Boechout, Belgium) and 2 U/mL erythropoietin (EPO, Amgen, Brussels, Belgium) suspended in Iscove's modified Dulbecco's medium. After 2 wk, numbers of burst-forming unit-erythroid (BFU-E), colony forming unit-granulocyte macrophage (CFU-GM) and mixed colonies (CFU-mix) were scored with an inverted microscope with ×40 magnification.

LTC killing assays

At the end of 5-wk LTCs, cells were trypsinized and half of the collected cells was treated with 2 mg/mL of hydroxycarbamide (HC) for 1 h at 37°C while the other half was saved as control. Cells were washed and transferred in semisolid medium for progenitor assays. The proportion of cycling LTC-initiating cells (IC) was calculated from the number of secondary CFC killed by HC treatment compared to the number of secondary CFC produced by control cells (10).

Flow cytometric determination of CXCR4 and SDF-1 expression

Expression of SDF-1 in $CD34^+$ cells and MSC was determined by intracellular labeling. Cells were washed in phosphate-buffered saline (PBS) without bovine serum albumin (BSA) and suspended in 1 mL of 0.4% paraformaldehyde before adding 1 μL of Brefeldin A (GolgiPlug; BD Biosciences). After a 30 min incubation at 4°C, 1 mL of 0.2% Triton X100 (Sigma) was added. Cells were incubated for another 30 min at 4°C before washing in PBS 1% BSA. Once permeabilized, cells were incubated with biotinylated anti-SDF-1 (R&D) or isotype-matched control IgG (BD Biosciences) for 60 min at 4°C and with streptavidin-allophycocyanin (Molecular Probes, Leiden, the Netherlands) for another 60 min at 4°C. For the determination of surface expression of CXCR4, cells were washed in PBS 1% BSA and incubated with PE–conjugated anti-CXCR4 antibody or isotypic control IgG (BD) for 20 min at 4°C in the dark. Cells were washed in PBS 1% BSA and fixed in PBS 1% paraformaldehyde. Flow cytometric data were acquired on a FACSort flow cytometer (BD Biosciences) and analyzed by the CELLQUEST software (BD Biosciences).

Multiplex analysis of MSC-conditioned medium

Quantification of IL-3, IL-6, IL-11, leukemia-inhibitory factor (LIF), GM-CSF, G-CSF, monocyte chemoattractant protein (MCP)-1, SDF-1, macrophage inflammatory protein (MIP)-1α, interferon (IFN)-γ, transforming growth factor (TGF)-β, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, E-selectin, L-selectin and matrix metalloprotease (MMP)-9 was performed by multiplex enzyme-linked immunosorbent assay (ELISA) as per manufacturer's instructions (Searchlight™ proteome array, Perbio, Erembodegem, Belgium). Briefly, 16 capture antibodies were bound to each well of a microplate. After addition of culture supernatants and removal of unbound proteins, plates were incubated with biotinylated detection antibodies, followed by streptavidin-horseradish peroxydase (all reagents supplied by Perbio). Chemiluminescent signals were detected with a cooled CCD camera (Photometrics, Zeiss, Brussels, Belgium).

Results

To assess the hematopoietic-supporting ability of MSC, Dexter-type LTC of CB $CD34^+$ cells were established for 5 wk using MSC as feeder layers. Secondary CFC production per 10^3 $CD34^+$ cells plated at LTC initiation was used as a measure of primitive progenitor cell activity. In the presence of MSC, progenitor output was 240.7 ± 51.3 secondary CFC while no hematopoietic activity was detected after LTC in the absence of MSC. To determine the possible role of SDF-1/CXCR4 axis in mediating MSC supporting ability, LTC were supplemented weekly with neutralizing antibodies against CXCR4 or SDF-1. Anti-CD3 was used as control (Fig. 1A). Neutralization of CXCR4 decreased the output of secondary progenitors by more than 50% compared to control cultures ($n = 6$; $P = 0.002$). Neutralization of SDF-1 induced a similar inhibitory effect ($n = 3$; $P = 0.04$).

We next analyzed the expression of CXCR4 by MSC and CB $CD34^+$ progenitor cells. Flow cytometric analysis revealed the presence of CXCR4 on the surface of the $CD34^+$ cells while MSC tested CXCR4 negative. SDF-1 was

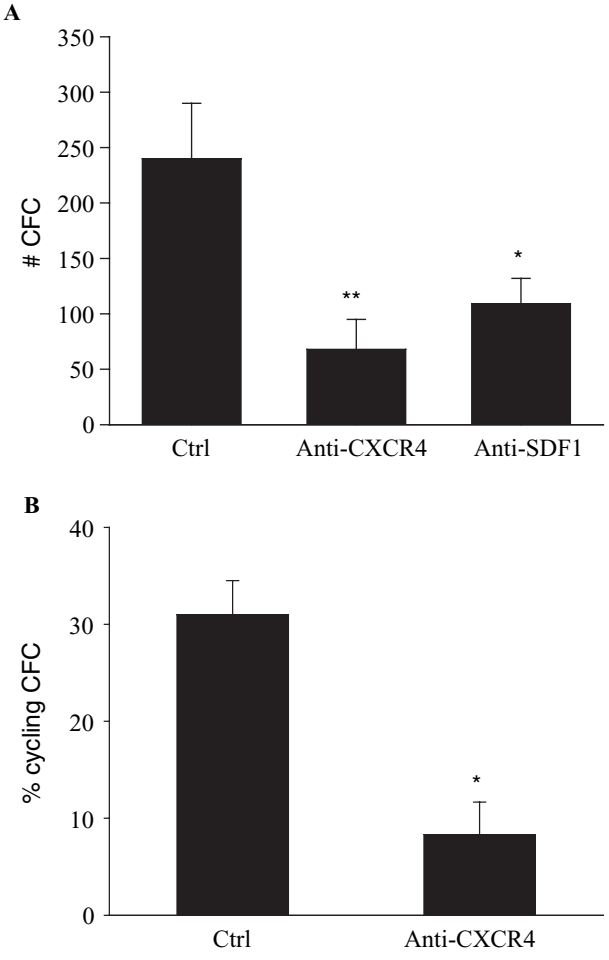

Fig. 1. Neutralization of stromal-derived factor-1 (SDF-1) and CXCR4 in mesenchymal stem cell (MSC)-supported human long-term cultures (LTC). LTC were treated with neutralizing antibodies anti-SDF-1 or anti-CXCR4 for 5 wk. (A) Colony-forming cell (CFC) output per 1000 $CD34^+$ cells plated at LTC initiation. (B) % cycling CFC at the end of 5 wk LTCs was determined by killing assays.

measured in MSC- and $CD34^+$ cell-conditioned medium by ELISA. Supernatant of MSC cultures contained 726.3 ± 140.9 pg/mL SDF-1 ($n = 4$) whereas no SDF-1 was detected in medium conditioned by $CD34^+$ cells. As measures of secreted SDF-1 may be underestimated by autocrine cell consumption, intracellular production of SDF-1 (iSDF-1) was measured by flow cytometry after cell permeabilization and treatment with Brefeldin A. While SDF-1 production was absent in $CD34^+$ cells, the chemokine was readily detected in MSC (Fig. 2). By combining functional and phenotypical data it appears that SDF-1 is produced by MSC, binds CXCR4 receptor on the surface of $CD34^+$ cells and mediates important signals implicated in maintaining hematopoietic activity in Dexter-type LTC cultures.

It has been previously reported that SDF-1 stimulates the proliferative status of purified hematopoietic progenitors in suspension cultures (6). However, such effect of SDF-1 has not been examined so far in cultures of hematopoietic progenitors plated in contact with human MSC. The cycling status of hematopoietic progenitors was determined by killing assays: cells harvested from LTC after 5 wk were treated with hydroxycarbamide to eliminate progenitors in S phase, prior to transfer in secondary progenitor assays. The proportion of cycling progenitors was calculated by comparison with untreated control cells (Fig. 1B). In LTC supplemented with an irrelevant antibody (anti-CD3), the proportion of cycling progenitors was 31.0 ± 3.5% ($n = 3$). In contrast, in the presence of anti-CXCR4 neutralizing antibody, progenitor proliferation index decreased to 8.3 ± 3.3% ($P = 0.009$). These data demonstrate that CXCR4 stimulates the cell cycle activity of hematopoietic progenitors cultured in contact with human BM-derived MSC.

We next examined whether the stimulation of hematopoietic activity by MSC through CXCR4-mediated signals could be elicited by soluble factors produced by MSC. To this end, we set up 'non-contact' LTC in which $CD34^+$ cells were separated from MSC feeder layers by a Transwell filter (Fig. 3). In that fashion, neither neutralization of CXCR4 (266.2 ± 27.6 CFC/1000 $CD34^+$ cells) nor blocking of SDF-1 (239.2 ± 13.8) significantly affected secondary CFC production compared to control cultures (302 ± 13.8; $P > 0.05$). Thus, MSC may support hematopoiesis in the absence of direct contact with $CD34^+$ cells but this

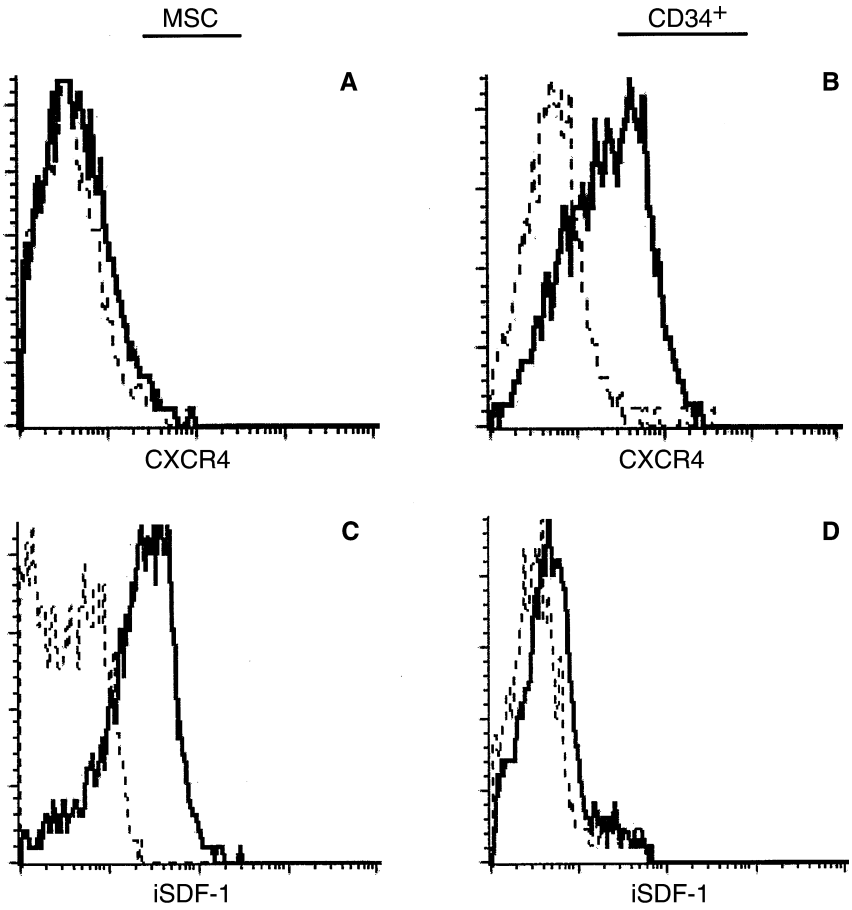

Fig. 2. CXCR4 and stromal-derived factor-1 (SDF-1) expression in $CD34^+$ cells and mesenchymal stem cells (MSC). Membrane CXCR4 was measured in MSC (A) and cord blood (CB) $CD34^+$ cells (B). Intracellular SDF-1 was measured in MSC (C) and $CD34^+$ cells (D) after Brefeldin treatment and cell permeabilization. Staining with control IgG is shown in dotted lines.

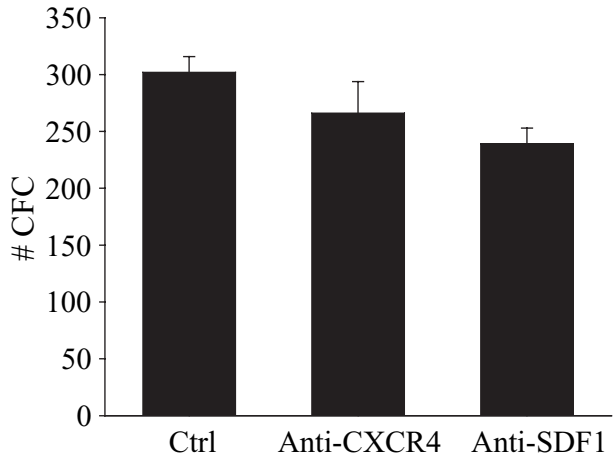

Fig. 3. Neutralization of stromal-derived factor-1 (SDF-1) and CXCR4 in non-contact mesenchymal stem cell (MSC)-supported long-term cultures (LTC). $CD34^+$ cells were separated from MSC by a Transwell filter. Colony-forming cell (CFC) output per 1000 $CD34^+$ cells plated at LTC initiation was measured.

supporting activity does not involve the SDF-1/CXCR4 axis.

Finally, to identify soluble factors potentially involved in the maintenance of hematopoiesis in non-contact cultures, we carried out a multiplex analysis of 16 cytokines or adhesion molecules in MSC-conditioned medium (Table 1). Various activating cytokines were actively produced by MSC notably IL-6, IL-11 and GM-CSF. Among inhibitory factors IFN-γ, TGF-β and MIP-1α were not detected, while high concentrations of MCP1 were secreted. Among adhesion molecules, both soluble VCAM-1 and ICAM-1 were present in MSC-conditioned medium, suggesting their active role in mediating binding of $CD34^+$ cells to MSC. MSC secreted also MMP-9 which might be important for the release and activation of membrane-bound cytokines (11). Altogether, MSC produce many factors which might be implicated in the maintenance of hematopoietic activity even in the absence of direct contact with progenitor cells.

Table 1. Multiplex enzyme-linked immunosorbent assay (ELISA) analysis of mesenchymal stem cells (MSC)-conditioned medium

Molecule	Concentration (pg/mL)
IL-3	ND
IL-6	6612.1*
IL-11	112.1*
LIF	214.3
G-CSF	92.2
GM-CSF	69.6*
IFN-γ	155.4
TGF-β	757.7
MCP1	13533.0*
SDF1	726.3*
MIP-1α	81.2
ICAM	44.1*
VCAM	56784.7*
E-selectin	ND
L-selectin	ND
MMP9	252.3*

Concentrations of indicated proteins were measured in MSC-conditioned medium after 3 d at confluence ($n = 4$). *$P < 0.10$ compared to medium only.
ND, not detected; IL, interleukin; LIF, leukemia-inhibitory factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN, interferon; TGF, transforming growth factor; MCP, monocyte chemoattractant protein; SDF, stromal-derived factor; MIP, macrophage inflammatory protein; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; MMP, matrix metalloprotease.

Discussion

Although its primary role in hematopoiesis is to regulate homing and retention of stem cells in the bone marrow microenvironment (12), SDF-1 may be implicated in other processes. The effect of SDF-1 on the proliferative status of HSPC has been studied previously but conflicting results were reported. First, it was described that SDF-1 acts as a survival and mitogenic factor in suspension cultures of purified $CD34^+$ cells (6, 13). Our results are consistent with these studies since neutralization of CXCR4 was associated with impaired progenitor cell cycling and decreased CFC output by LTC-IC. In other reports, SDF-1 was found to inhibit HSPC cycling in Dexter-type cultures. This discrepancy with the present study may be due to methodological differences. Cashman *et al.* studied the effect of a single addition of neutralizing anti-SDF-1 in LTCs that had been initiated 1 wk previously, while we studied progenitor cycling and production in LTCs after a 5-wk treatment with blocking antibodies. Extension of LTC duration enabled us to determine the effect of SDF-1/CXCR4 neutralization on more primitive hematopoietic progenitor cells, in which CXCR4 signaling is reportedly higher in comparison to more mature ones (12). Also, feeder cells used by Cashman *et al.* consisted of mouse marrow fibroblasts engineered to secrete SCF, IL3 and G-CSF, which may produce a largely different set of cytokines and chemokines, compared to human MSC (14).

The stimulatory effect of SDF-1 on hematopoietic progenitor cell growth in LTC established on MSC layers may be direct or indirect. We observed that CXCR4-mediated hematopoietic-supporting ability of MSC requires contact with $CD34^+$ cells. In non-contact LTC, MSC are still able to maintain hematopoietic progenitor activity but in a CXCR4 independent fashion. This may indicate that the role of SDF-1/CXCR4 axis is indirect, by stimulating retention of hematopoietic progenitors in contact with specialized MSC niches and exposing

them to adhesion molecule-mediated signals in a paracrine fashion.

While we did not detect CXCR4 receptor on the cell surface of MSC, it remains possible that CXCR4 is indeed expressed in MSC but internalized in response to autocrine binding of SDF-1 (15). It was recently reported that SDF-1 promotes growth and survival of bone marrow stromal cells in an autocrine manner (9). Neutralization of CXCR4 or SDF-1 could then possibly affect the viability of MSC feeder layers in LTC and consequently their hematopoietic-supporting ability. However, in non-contact cultures, hematopoietic activity was maintained during CXCR4 neutralization, which argues against a direct effect of SDF-1 on MSC function.

In conclusion, we have demonstrated that SDF-1/CXCR4 is critical in mediating the maintenance of the hematopoietic niche provided by cultured undifferentiated MSC. This finding could have important implications for designing *ex vivo* expansion procedures in valuable clinical applications. We suggest that careful monitoring of SDF-1 secretion by cultured MSC might be used as a predictive assay for the efficiency of MSC lines to support growth of HSPC in contact cultures. Further investigation of the hematopoietic support provided by subpopulations of undifferentiated and committed MSC may help define other important regulators of hematopoiesis.

Acknowledgements

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