Regulation of CXCR4 expression in human mesenchymal stem cells by cytokine treatment: role in homing efficiency in NOD/SCID mice

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ABSTRACT

Background and Objectives

The use of mesenchymal stem cells (MSC) for cell therapy relies on the capacity of these cells to home and engraft long-term into the appropriate target tissue(s). Homing of MSC to bone marrow (BM) post-transplantation can occur, but does so with only poor efficiency. This study was designed to evaluate the role of the SDF-1/CXCR4 axis in the homing of Flk1$^+$ MSC derived from human fetal BM.

Design and Methods

We investigated the expression of CXCR4 in Flk1$^+$ MSC stimulated with a cytokine cocktail and explored their homing ability 24 hours after intravenous infusion into sub-lethally irradiated NOD/SCID mice. The peripheral blood was analyzed and human cells in recipients' BM were quantified from 2 to 6 months after transplantation.

Results

We found that Flk1$^+$ MSC harbored intracellular CXCR4 which can be rapidly induced to the cell surface within a few hours. Short-term (24 hours) stimulation with the cocktail of cytokines resulted in up-regulation of both cell surface and intracellular CXCR4, increasing *in vitro* migration capacity to SDF-1 and homing to the BM of irradiated NOD/SCID mice. Moreover, compared to non-treated cells, transplantation of cytokine-treated Flk1$^+$ MSC resulted in faster hematologic recovery and higher levels of donor chimerism in BM. Neutralization of CXCR4 significantly reduced homing and engraftment of Flk1$^+$ MSC in murine BM.

Interpretation and Conclusions

These results suggest that the SDF-1/CXCR4 axis plays an important role in the regulation of motility of Flk1$^+$ MSC. Increasing CXCR4 expression might be a potential strategy to improve engraftment of MSC in BM and accelerate the recovery of hematopoiesis.

Key words: mesenchymal stem cells, homing, transplantation, CXCR4.

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At least two different types of stem cells have been identified in the hematopoietic compartment: hematopoietic stem cells (HSC) and mesodermal-derived mesenchymal stem cells (MSC). MSC represent an important cellular component of the bone marrow (BM) microenvironment that supports hematopoiesis. They have the capacity to differentiate into not only mesenchymal lineage cells, but also endothelium, neuroectodermal and endodermal tissues *in vitro*.[1-4] Furthermore, MSC can migrate to injured tissues and differentiate into tissue-specific cell types in response to local cues.[5-7] They can also contribute to most somatic tissues when injected into an early blastocyst.[8] In addition, MSC have been shown to secrete cytokines and growth factors, supporting expansion of hematopoietic and embryonic stem cells.[9-11] Clinically, MSC may be used to enhance HSC engraftment post-transplantation, correct inherited disorders of bone and cartilage or act as vehicles for gene therapy.[12-15] Thus, MSC are of great potential in the context of tissue engineering and cell-based therapy.

As MSC have been demonstrated to support hematopoiesis, it would be desirable to co-transplant donor MSC with HSC to promote the rate of engraftment. Unfortunately, the transplant ability of marrow stromal elements remains controversial, with most studies showing that MSC, or stromal cells, have a limited capacity to reconstitute the marrow microenvironment.[16-20] The fact that methods for isolation and amplification of MSC have been well studied and MSC home to BM with poor efficiency prompted us to find approaches to promote MSC homing and elucidate the mechanisms that guide homing of implanted MSC.

A number of studies have proven that stem cell migration and organ-specific homing are regulated by chemokines and their receptors. The expression of functional CXCR4 has been observed on the surface of embryonic stem cells[21] and several tissue committed stem/progenitor cells, such as HSC,[22] retinal pigment epithelium progenitors,[23] liver oval/stem cells,[24] primordial germ cells,[25] skeletal muscle satellite progenitor cells[26] and neural stem cells.[27] Similarly, it has been reported that the specific CXCR4 ligand, stromal cell-derived factor-1 (SDF-1), is expressed/secreted by several tissues/organs in the body, especially BM. Proper functioning of the SDF-1/CXCR4 axis plays an essential role in directing engraftment of HSC into BM after transplantation.[28,29] Mice that lack SDF-1 or do not express CXCR4 exhibit defects in hematopoiesis in the fetal BM and blood vessel development.[30,31] The SDF-1/CXCR4 axis may also be involved in regulating the metastatic behavior of several tumor cells.[32] Most recently, CXCR4 was reported to be expressed on the surface of MSC and BM stromal cells.[33-36] Wynn *et al*. reported that a small proportion of MSC expressed CXCR4, which contributed to their migration *in vitro*.[33] Sordi *et al*. found that CXCR4 expressed on MSC was capable of promoting migration to pancreatic islets.[34] It was also reported that SDF-1 could induce migration of human BM stromal cells *in vitro*,[35] and that CXCR4 might play a role in the engraftment of these cells in brain tissue of immunodeficient mice.[36] However, the role of the SDF-1/CXCR4 axis in homing and engraftment of MSC into BM has not been evaluated *in vivo*. Previously, we identified a rare cell population (adherent fetal liver kinase-1–positive [Flk1$^+$] CD31$^-$CD34$^-$ cells) within human fetal BM MSC culture which possesses multipotent capacity. These cells can migrate efficiently to BM and contribute to hematopoietic reconstitution in sublethally irradiated non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice.[4,37] In this study, we investigated whether the SDF-1/CXCR4 axis may also regulate the homing and engraftment of Flk1$^+$ MSC.

Design and Methods

Animals

NOD/SCID mice were bred and maintained under defined flora conditions in individually ventilated (high-efficiency particle-arresting filtered air) sterile microisolator cages (Techniplast, Milan, Italy). All experiments were approved by the animal care committee of the Chinese Academy of Medical Sciences. At 6 to 8 weeks old, the mice were sublethally irradiated (300 cGy) with a cesium source (MDS Nordion; Gammacell, Ottawa, QC, Canada) prior to transplantation.

Cell culture and treatment with cytokines

MSC were isolated from human fetal BM as described previously.[4,37] (*For details see online supplementary Appendix 1 at www.haematologica.org*). Purified Flk1$^+$ MSC were cultured at 37°C in 5% CO_2 for 24 or 48 hours (h) in serum-free media containing 300 ng/mL Flt-3 ligand, 50 ng/mL stem cell factor (SCF), 50 ng/mL interleukin (IL)-6, 50 ng/mL hepatocyte growth factor (HGF) and 10 ng/mL IL-3 (all from Sigma). Cultures without cytokines served as controls. After incubation, cells were harvested, washed, and used for different assays.

Flow cytometry and real-time quantitative RT-PCR analysis of CXCR4 expression

Expression of cell surface CXCR4 on Flk1$^+$ MSC was detected with phycocerythrin (PE)-conjugated monoclonal anti-CXCR4 (BD Biosciences Pharmingen, San Diego, USA). For intracellular staining, cells were first blocked with non-conjugated anti-human CXCR4 monoclonal antibody (clone 12G5, 10 μg/mL, 1 h, 4°C), then fixed with 2% paraformaldehyde (15 min, 4°C) and permeabilized with 0.1% saponin (Sigma) for 1 hour at room temperature. Monoclonal antihuman CXCR4-PE was used to label the cells for flow cytometry. The cells were washed with PBS containing 0.5% BSA (Sigma) after each step and analyzed on a FACSCalibur flow cytometer (Becton-Dickinson) with CellQuest software. We further analyzed the expression of CXCR4 expression in Flk1+ MSC at the mRNA level (*protocols available in online Supplementary Appendix 2*).

Long-term bone marrow culture

We examined the supportive function of Flk1$^+$ MSC on CD34$^+$ hematopoietic cell proliferation and differentiation using a long-term BM culture system. Flk1$^+$ MSC were grown to confluence in 35 mm plastic dishes. Freshly prepared BM CD34$^+$ cells (10^4) were suspended in 4 mL of IMDM supplemented with 10% FCS and inoculated onto the MSC layer which had or had not been pretreated with cytokines for 24 h, as described above. A total of 3 mL of the supernatant with hematopoietic cells was replaced with fresh growth medium every 5 days.[38] An aliquot of the cells harvested was assayed for the numbers of total hematopoietic cells and CFU-GM.

Chemotaxis assay

Migration of Flk1$^+$ MSC toward a gradient of SDF-1 was determined by a Transwell (Corning Costar, Cambridge, MA, USA) assay as described elsewhere,[29] with minor modifications. Dilutions of 0, 100, 200, 300 and 400 ng/mL of recombinant SDF-1 (Peprotech, Rocky Hill, NJ, USA) in 1.5 mL of media were placed to the lower side of the membrane (12 mm diameter, 12 μm pore size) pre-coated with fibronectin. Seventy thousand cells in 500 μL of media were added to the upper side of the membrane. For neutralization studies cells were incubated with anti-human CXCR4 monoclonal antibody (10 μg/mL; BD Biosciences Pharmingen). Data are presented as the average number of migrated cells in five high-power fields (×400) after 15 h incubation at 37°C in 5% CO_2. Each experiment was performed in triplicate, and then the data were averaged for statistical analysis.

Short-term homing

Flk1$^+$ MSC were labeled with PKH26 dye (Sigma) according to the manufacturer's instructions with some modifications. Briefly, after 5 minutes of incubation with PKH26 (5 μmol/L), Flk1$^+$ MSC were washed extensively and resuspended in culture medium. The total number of viable cells was determined using the trypan blue dye exclusion method, and the extent of labeling was determined by flow cytometry. Three hours before transplantation, NOD/SCID mice were exposed to 300 cGy total body irradiation (TBI). Six million PKH26-labeled Flk1$^+$ MSC were infused intravenously through the tail vein of the irradiated recipients. Where indicated, human cells were preincubated with neutralizing anti-human CXCR4 monoclonal antibody (10 μg/10^6 cells) before transplantation. Then incubated cells, together with the incubation solution containing unbound anti-CXCR4 monoclonal antibody, were infused intravenously. Mice were kept in the isolator under controlled environment until they were sacrificed at 24 h post-transplantation. Cells were recovered from the BM and were analyzed for the presence of PKH26+ cells by flow cytometry acquiring 10^6 cells per sample. Cells obtained from non-transplanted mice were used to exclude false positive cells. Propidium iodide staining was used to exclude dead cells.

Hematopoietic recovery and long-term engraftment

Transplantation experiments were performed to evaluate the long-term engraftment potential of Flk1$^+$ MSC and their effect on the hematopoietic recovery. Sublethally irradiated NOD/SCID mice (300 cGy) were injected intravenously with 10^6 Flk1$^+$ MSC. Where indicated, MSC were preincubated with antihuman CXCR4 monoclonal antibody (10 μg per mouse). Control mice received the same volume of saline. Mice were bled from the lateral tail vein 3, 6, 9, 12, 15, 22, 29, 36 and 43 days after transplantation. Until day 15, only half the mice in each cohort were analyzed alternately at each time so that no individual animal was bled more frequently than every 7 days. Circulating leukocyte, erythrocyte, and platelet counts were measured by analysis of 20 μL blood using a Sysmex SE-9000 Haematology analyzer (Sysmex Corporation, Kobe, Japan). Mice were killed by cervical dislocation 2 or 6 months after transplantation. Their BM cells were collected for analysis. To demonstrate the engraftment of donor cells, we used quantitative real-time PCR. The presence of human-specific DNA within the BM of transplanted mice was analyzed by TaqMan-chemistry based real-time PCR amplifying a 480 bp fragment of the α-satellite region of the human chromosome 17 as described by Becker and his colleagues,[39] with minor modifications. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA, USA). The primers and the probe used are listed in *Supplemental Table 1*. PCR reactions were performed in a volume of 50 μL containing 1.5 U AmpliTaq Gold enzyme (Applied Biosystems), 200 μM each of the respective nucleotides, 2 mM $MgCl_2$, 250 nM of each primer, 10 nM TaqMan probe, and 250 ng of genomic DNA template, and analyzed in an ABI 7500 Real-Time System. Reactions were performed at 94°C for 10 min, followed by 45 cycles of 94°C for 15 s, 60°C for 50 s. Each sample was assessed in triplicate. Serial dilution steps of human Flk1$^+$ MSC in mouse NIH3T3 cells served to construct the Taqman-calibration curves.

Statistical analysis

Statistical analyses were performed using SPSS software. Results are presented as mean ± SEM (standard error of the mean). Statistical significance was evaluated with an unpaired Student's *t*-test for comparisons between two groups or by ANOVA for multiple comparisons. A value of $p<0.05$ was considered statistically significant.

Results

Cytokines enhance CXCR4 expression in Flk1$^+$ MSC

Recent studies by Wynn and Sordi *et al.*[33,34] showed that MSC express functional CXCR4 protein. In this study, we first verified the expression of CXCR4 in Flk1$^+$ MSC using a commercial monoclonal antibody against CXCR4. We documented that most of the cells (87.4-97.8%, n=5) harbored intracellular CXCR4 (Figure 1B), whereas almost no

CXCR4 was detectable on the cell surface (Figure 1A). Interestingly, short-term (24-48 h) incubation in medium containing five cytokines (Flt-3 ligand, SCF, IL-6, HGF and IL-3) resulted in up-regulation of both cell surface and intracellular CXCR4 (Figures 1 A and B). Spontaneous up-regulation of surface CXCR4 expression within 24 h was also observed when Flk1$^+$ MSC were cultured in serum-free media without additional cytokines, suggesting up-regulation of CXCR4 by autocrine cytokines may happen, although cell viability was significantly reduced (*data not shown*). When SCF, IL-6, Flt-3 ligand, HGF or IL-3 was added separately, or a combination of SCF and IL-6 was added to the culture, CXCR4 was up-regulated to a lesser extent than when they were used together. Thus a cocktail of the five agents was used in the study. We further confirmed the up-regulation of CXCR4 expression in Flk1$^+$ MSC at the mRNA level. Real-time quantitative PCR experiments showed that the increase in the surface CXCR4 protein was accompanied by an increase in CXCR4 mRNA expression as shown in Figure 1C.

Flk1$^+$ MSC migrate in response to SDF-1 gradients

The ability of Flk1$^+$ MSC to migrate towards a SDF-1 gradient was determined by Transwell assays. Flk1$^+$ MSC migrated in a dose-dependent manner to different concentrations of SDF-1 (Figure 2A). Compared with untreated cells, Flk1$^+$ MSC treated with cytokines for 24 h showed enhanced migration capacity in response to SDF-1 induction ($p<0.01$). Treatment of Flk1$^+$ MSC with a monoclonal antibody blocking CXCR4 (10 μg/mL) abrogated the cell migratory response, confirming the specificity of the migration toward SDF-1.

Cytokine stimulation does not influence the supportive function of Flk1$^+$ MSC in hematopoiesis

We examined the supportive function of Flk1$^+$ MSC in CD34$^+$ hematopoietic cell proliferation and differentiation using a long-term BM culture system as described in the *Design and Methods* section. No significant difference was found between the non-stimulated group and the cytokine-stimulated group (*Supplemental Table 2*). These data suggest that the increase of CXCR4 expression does not influence the effect that MSC have of supporting hematopoiesis.

Short-term homing capabilities of Flk1$^+$ MSC into BM are enhanced by up-regulation of CXCR4

To assess recruitment of transplanted cells to hematopoietic organs, PKH-26-labeled Flk1$^+$ MSC were infused through the tail vein into sublethally irradiated NOD/SCID mice. Using the staining method described in the *Design and Methods* section, 99% of Flk1$^+$ MSC were brightly stained by PKH26, yielding a fluorescence intensity at least one log higher than that of unlabeled control cells (*data not shown*). In consideration of the sensitivity of flow cytometry, a relatively high dose (6×10^6 cells per mouse) was used. At 24 h after transplantation, the recipients were sacrificed and PKH26$^+$ cells in the BM were quantitated by flow cytometry. The up-regulation of both intracellular and cell surface CXCR4 induced by cytokine stimulation within 24 h (Figures 1 A and B) correlated well with improved CXCR4-dependent homing capacities. As shown in Figure 2B, cytokine stimulation increased the homing levels of Flk1$^+$ MSC by about 7-fold in the BM 24 h after transplantation. Preincubation of MSC with anti-CXCR4 monoclonal antibody significantly hindered the homing of the cells (Figure 2B). These findings suggest that the SDF-1/CXCR4 axis plays an important role in short-term homing of Flk1$^+$ MSC into BM.

Flk1$^+$ MSC accelerate hematopoietic recovery following irradiation injury

One million Flk1$^+$ MSC were transplanted into sublethally irradiated NOD/SCID recipients. Circulating blood cells were counted once or twice a week from day 3 to more than 1 month after transplantation to measure the supportive effect of Flk1$^+$ MSC on hematopoiesis. As in previous studies,[14,40,41] MSC promoted hematopoietic recovery with a pronounced enhancement of peripheral leukocyte, erythrocyte, and platelet recovery (Figure 3). Transplantation of cytokine-stimulated Flk1$^+$ MSC resulted in faster hematologic recovery than did non-stimulated cells. Neutralization of CXCR4 significantly reduced the effect on hematopoietic recovery.

Flk1$^+$ MSC engraft NOD/SCID mice in a CXCR4-dependent manner

Next we examined whether up-regulation of CXCR4 also enhanced long-term residence of MSC in BM. The recipient mice were killed 2 or 6 months after transplantation and their BM cells were collected for analysis. As the fluorescence of PKH26 may be too weak to be detected, we quantified engraftment levels of human-derived cells in the BM using real-time PCR. No human DNA was detected in BM isolated from control mice injected with saline. In NOD/SCID recipients that received unstimulated Flk1$^+$ MSC, human DNA accounted for about 6.9% and 7.5% of total BM DNA at, respectively, 2 and 6 months after transplantation. In recipient mice transplanted with cytokine-pretreated MSC, the percentages of human DNA increased to 14.1% and 15.6% at, respectively, 2 and 6 months after transplantation. This result means a 2-fold increase in the engraftment level of donor-derived cells ($p<0.05$) (Figure 4). The levels of human DNA in BM of recipients were significantly decreased when Flk1$^+$ MSC were coinjected with neutralizing anti-CXCR4 monoclonal antibody, indicating that the increase in Flk1$^+$ MSC engraftment is dependent on CXCR4.

Discussion

In recent years, MSC have generated a great deal of interest because of their potential uses in regenerative medicine and tissue engineering, which have been illustrated in both

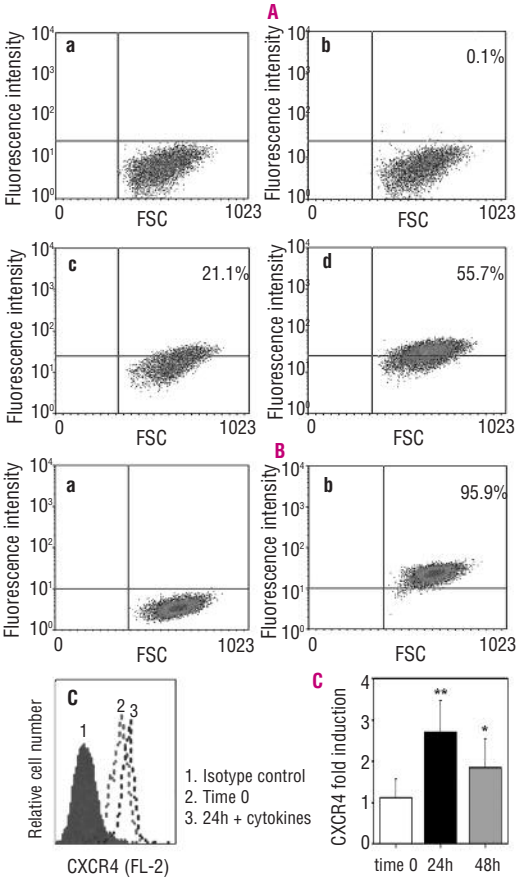

Figure 1. Up-regulation of cell surface and intracellular CXCR4 expressed by Flk1⁺ MSC. A. Cell surface staining. Flk1⁺ MSC were stained with control antibody (Aa) or antibody to CXCR4 before (time 0, Ab) or after 24 (Ac) and 48 (Ad) hours of treatment with indicated cytokines. **B.** Intracellular CXCR4 expression. (Ba) isotype control antibody; (Bb) time 0, high levels of receptor expression were found in 95.9% of cells; (Bc) up-regulation after 24 hours' culture with 5 cytokines. A representative experiment of five performed is shown. **C.** Real-time PCR analysis of CXCR4 mRNA expression in Flk1⁺ MSC before or after 24 or 48 hours' treatment with cytokines. RT-PCR was performed as described in the *Design and Methods* section. Threshold cycle values were normalized to GAPDH expression, and the levels of induction of CXCR4 expression were calculated relative to the 0-h untreated control. Results are shown as mean ± SEM of three independent experiments conducted in triplicate, **p*<0.05, ***p*<0.01 *vs* time 0.

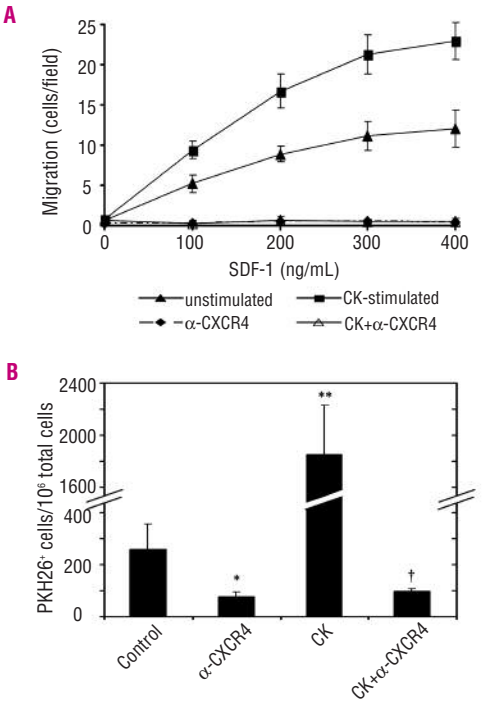

Figure 2. Cytokine stimulation increases SDF-1 chemotaxis and CXCR4-dependent short-term homing of Flk1⁺ MSC. A. Flk1⁺ MSC were incubated for 24 h with indicated cytokines and assayed for migration levels. Their chemotactic activity towards various doses of SDF-1 was determined. Cytokine-stimulated Flk1⁺ MSC had enhanced SDF1-induced migratory response compared to unstimulated cells ($p<0.01$). Treatment of Flk1⁺ MSC with a neutralizing anti-CXCR4 monoclonal antibody (α-CXCR4, 10 μg/mL) abrogated the cell migratory response, confirming the specificity of the migration toward SDF-1. Results are expressed as mean ± SEM. (n=3). **B.** NOD/SCID mice were transplanted with cytokine-stimulated (CK) or unstimulated cells (control). PKH26-labeled Flk1⁺ MSC (6×10^6 cells/mouse) were injected with or without anti-CXCR4 monoclonal antibody. The number of PKH26⁺ cells in the murine BM 24 h after transplantation is shown. Differences of homing between control and cytokine-stimulated cells were statistically significant (**$p<0.01$). Anti-CXCR4 pretreatment significantly reduced the number of human cells in the BM 24 h following transplantation. Data represent mean±SEM from three experiments, n=5 mice/group. *$p<0.05$ *vs* control; †$p<0.01$ vs cytokine-stimulated group.

pre-clinical and clinical studies. Most clinical and experimental protocols transplant MSC intravenously into recipients preconditioned with TBI and/or chemotherapy. Systemic applications of MSC, including hematopoiesis support, gene delivery and treatment of musculoskeletal diseases, require an understanding of the mechanism underlying the migration of these cells to the target tissues.

Although a great deal is known about the mechanisms and key regulators of hematopoietic stem / progenitor cells homing to the BM, reports on the homing and engraftment of MSC are few and controversial. Most studies that focused on BM stroma chimeras in human and experimental animal models point to a host origin after allogenic transplantation.[16-18,42] However, Cilloni *et al.*[19] reported that marrow stromal progenitors reinfused into patients receiving a T-cell-depleted allograft have the capacity to reconstitute marrow mesenchymal cells, although to a limited extent. Rombouts *et al.*[43] demonstrated that primary murine MSC could home to BM with high efficiency but lost homing ability following culture, most likely because of the modulation of homing molecules, such as chemokine receptors or adhesion molecules, during *in vitro* expansion. In five of six patients with osteogenesis imperfecta, Horwitz *et al.*[12] demonstrated that donor-derived MSC resided in the recipients' BM after two infusions of allogeneic mesenchymal cells. Pozzi *et al.*[44] found that MSC of donor origin may engraft in pediatric patients undergoing allogeneic HSCT, suggesting that BM of children might be a more favorable milieu than that of adults. Most recently, Reyes's group reported that following syngeneic BM

transplantation into lethally irradiated C57BL6 mice, multipotent adult progenitor cells (MAPC) isolated within the BM adherent cell component were of donor origin.[45] These data suggest that there might be some cell populations within BM adherent cells which have a multipotent capacity and could home and engraft into BM after transplantation. Controversial results so far may be attributed to the different cell populations and cell doses infused, methodological differences in detecting donor-derived cells, the heterogeneity of conditioning regimens and the characterization of the different recipients. Previously we reported that fetal dermis-derived Flk1$^+$ MSC can promote hematopoietic recovery in irradiated NOD/SCID mice, but infused MSC were absent in bone marrow 7 days after transplantation.[40] This indicated that the cell populations derived from different tissues might also have different homing abilities. Overall, under current conditions MSC can only home and engraft into BM to a very restricted degree. Substantial improvements are necessary to enable greater clinical benefits.

In previous studies,[4,37] we identified the multipotent Flk1$^+$ MSC and found that they could home to the BM upon intravenous injection into NOD/SCID mice, but the mechanism of their homing is still unknown. Numerous studies have demonstrated that the SDF-1/CXCR4 axis is essential for human HSC homing.[28,29] Recently it was reported that functional CXCR4 is also expressed on MSC in humans[33,34] and rats.[46] Sordi *et al.*[34] showed that the SDF-1/CXCR4 interaction promotes MSC migration to pancreatic islets. We hypothesize that the SDF-1/CXCR4 axis may also play essential roles in the migration, development, and anchorage of Flk1$^+$ MSC in the BM. The data presented in this study demonstrate that levels of CXCR4 on the cell surface of MSC were low, with most found intracellularly. This is consistent with Wynn's report.[33] It has been reported that cytokine treatment can up-regulate the expression of CXCR4 on hematopoietic stem cells. So we supposed that the CXCR4 expression of MSC might also be regulated by cytokines. We chose a specific cytokine cocktail described by Rosu-Myles *et al.*[22] with some modifications, e.g. HGF instead of G-CSF, because HGF was reported to be able to increase the expression of CXCR4 on human CD34$^+$ stem cells.[47] We found that when SCF, IL-6, Flt-3 ligand, HGF or IL-3 was added separately, or a combination of SCF and IL-6 was added to the culture, CXCR4 was up-regulated to a lesser extent than when the cytokines were all used together. Thus a cocktail of the five agents was used in the study. Treatment of Flk1$^+$ MSC with the cytokines induced CXCR4 expression, which significantly enhanced the cells' migration to SDF-1 and short-term homing to BM and promoted hematopoietic recovery after transplantation in NOD/SCID mice. Neutralization of CXCR4 obviously reduced the homing of the human cells into murine BM and their effect on hematopoietic recovery. To our knowledge, this is the first report showing the importance of regulating CXCR4 in the homing of MSC into BM after transplantation. However, we cannot exclude that other cytokine panels exert more powerful effects on stimulation of CXCR4 expression than the one we used in this study. More emphasis should be placed on optimizing the cytokine cocktails for possible clinical application in the future. Moreover, it has been reported that hypoxia can regulate CXCR4 expression in several cell types.[48,49] Based on these findings, we speculate that short-term hypoxia preconditioning *in vitro* might up-regulate the functional expression of CXCR4 on Flk1$^+$ MSC and contribute to homing and engraftment in recipient BM. These studies are currently underway in our laboratory.

It was recently reported that intra-BM injection of allogeneic grafts can result in more efficient marrow homing and engraftment than intravenous injection.[50,51] In a NOD/SCID xenograft model, Yahata *et al.*[50,51] showed that CXCR4 also plays a role in human HSC transplantation by the intra-BM route. However Mahmud *et al.*[53] found that the intra-BM route was not more effective than the intravenous route in delivering MSC grafts. The intra-BM strategy seems to hold promise for future clinical application and deserves further investigation. Interestingly we found that neutralization of CXCR4 further reduced the homing levels of Flk1$^+$ MSC without cytokine stimulation, despite the fact that no significant surface expression of CXCR4 was detected. These findings suggest that during the homing process additional CXCR4 receptors may be functionally expressed on the cell surface, which may provide an explanation for why Flk1$^+$ MSC have the ability to seed the BM efficiently. The transplantation of Flk1$^+$ MSC accelerat-

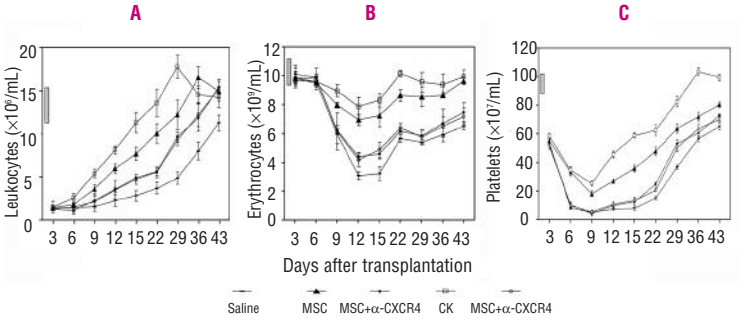

Figure 3. **Flk1$^+$ MSC expressing elevated CXCR4 enhance hematopoietic recovery after transplantation. Flk1$^+$ MSC (10^6 cells per mouse) were either injected directly or treated with the indicated cytokines for 24 h before being injected into sublethally irradiated NOD/SCID mice. Control mice received the same volume of saline. The figure shows the mean±SEM number of peripheral blood leukocytes (A), erythrocytes (B), and platelets (C) counted on the indicated days after transplantation. The vertical bars along the y-axis define the ranges of blood counts in normal NOD/SCID mice. Note that the time after transplantation is not depicted on a linear scale. Results are expressed as means for three mice per group at each time point.**

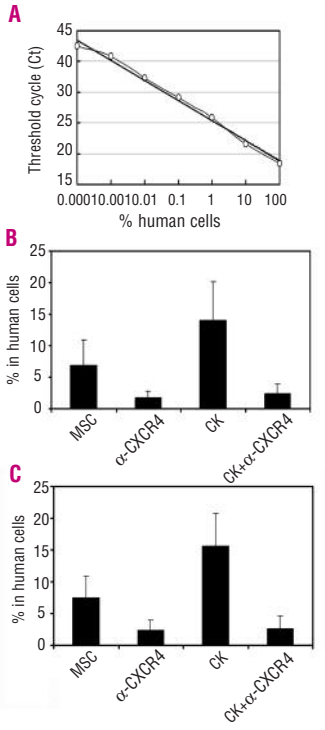

Figure 4. Quantification of human cell engraftment in mouse BM by real-time PCR. **A.** The relationship between threshold cycle number and the percentage of human genomic DNA within the BM of recipients after transplantation. The standard curve was generated using a dilution series of human Flk1$^+$ MSC in mouse NIH3T3 cells. **B.** Detection of human cells in murine BM tissue 2 months after transplantation with cytokine-stimulated (CK) or unstimulated Flk1$^+$ MSC with or without co-injection of anti-CXCR4 monoclonal antibody (α-CXCR4). Increased human-cell engraftment into BM was observed with cytokine-pretreatment ($p<0.05$, n=8). The levels of human DNA in BM of recipients decreased significantly when Flk1$^+$ MSC were co-injected with neutralizing anti-CXCR4 monoclonal antibody. **C.** Similar results were observed in recipient mice 6 months after transplantation (n=5).

ed hematologic recovery in NOD/SCID mice. The mechanism is not yet clear. The beneficial effects are considered to be mediated by two factors: (i) human MSC may secrete hematopoietic, angiogenic and anti-apoptotic factors, and/or increase endogenous hematopoietic, angiogenic and anti-apoptotic factors secreted by host stromal cells in BM; (ii) Flk1$^+$ MSC themselves could directly differentiate into both hematopoietic and endothelial cells as we and others reported previously.[4,8,37] In this study we did not determine whether other chemokines and their receptors also participated in the regulation of MSC homing. We showed that neutralization of CXCR4 could not completely abolish the homing and engraftment of Flk1$^+$ MSC. This suggests that there may be other factors involved in the specific migration of MSC. As we know, MSC, after intravenous infusion, are capable of specific migration not only to BM but also to other sites of injury. This extraordinary ability of implanted cells to seek damaged tissue has been demonstrated in the case of bone fracture,[54] myocardial infarction,[5,55] liver injury[7,56] and cerebral ischemia.[57] The mechanisms that guide the homing of implanted cells are unclear. If the SDF-1/CXCR4 axis is also involved in these situations, pretreatment of MSC with these cytokines may facilitate migration of infused MSC to the site of injury and promote tissue repair. In summary, this study shows that the SDF-1/CXCR4 axis plays an important role in the regulation of motility of MSC in NOD/SCID mice, and increasing CXCR4 expression can improve homing of MSC to BM and accelerate hematologic recovery. This information may be used to good advantage for modulating these cells in the clinical setting, particularly for enhancing the effectiveness of transplantation.

Authors' Contributions

MS and JL designed the research, performed the experiments, analyzed the data and wrote the manuscript; LL and BC participated in the design of the research and revised the manuscript; BL, LC and HJ performed the experiments and collected and analyzed the data; RCZ designed the research, analyzed the data and wrote the final version of the paper.

Conflict of Interest

The authors reported no potential conflicts of interest.

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