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ORIGINAL ARTICLE

# Stromal-derived factor-1 deficiency in the bone marrow of acute myeloid leukemia

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**Abstract** Chemokine stromal-derived factor-1 (SDF-1) and its receptor CXCR4 have been shown to play an important role in the migration and homing of the transplanted hematopoietic stem cells (HSCs). Mesenchymal stem cells (MSCs) express these molecules. This study is to test the hypothesis that acute myeloid leukemia (AML) alters the expression of SDF-1/CXCR4 in human bone marrow MSCs. Expression of both CXCR4 and SDF-1 was found to be increased, but excessively retained, in the MSCs in AML. In contrast, the SDF-1 level in bone marrow plasma and supernatant of cultured MSCs from AML patients were reduced, while the SDF-1 was able to efficiently induce a dose-dependent migration of MSCs in vitro. Our results demonstrate that altered expression and distribution of SDF-1/CXCR4 in MSCs may contribute to SDF-1 deficiency in the plasma of AML patients. The migration of MSCs may be negatively affected by the SDF-1 deficiency.

**Keywords** Mesenchymal stem cells · Bone marrow · Stromal-derived factor-1 · Acute myeloid leukemia · Migration

## 1 Introduction

Mesenchymal stromal cells in the bone marrow are non-hematopoietic multipotent cells due to their capacity differentiating into lineages of mesenchymal tissues, such as bone, cartilage, fat, muscle, and marrow stroma [1]. Thus, these cells are often referred as bone marrow derived-mesenchymal stem cells (BM-MSCs). MSC has become an attractive candidate for transplantation therapy to facilitate the engraftment of hematopoietic stem cells (HSCs) [2]. Indeed, systemic infusion of allogeneic MSCs in patients with acute myeloid leukemia (AML) or other types of cancer accelerated bone marrow recovery after high-dose chemotherapy [3, 4].

Chemokines have been well documented to mediate cell migration and homing. Two of extensively studied chemokines are stromal-derived factor-1 (SDF-1 or CXCL12), and its receptor is CXCR4. CXCR4 can be found on the cytoplasmic membrane of MSCs, and thus subject to interact with SDF-1 [5]. SDF-1/CXCR4 signaling axis appears to play an important role in migration and homing of HSCs and MSCs [6, 7]. Recent studies have shown that MSCs also express CXCR7, a new receptor of SDF-1. However, unlike CXCR4, CXCR7 does not mediate the migration of MSCs [8]. Cell migration may also be regulated by CD26/dipeptidylpeptidase IV (DPPIV), a membrane-bound extracellular peptidase capable of cleaving SDF-1. Indeed, SDF-1 with truncated n-terminal by CD26 loses its ability to induce migration of $CD34^+$ cord blood cells [9].

Despite the functions of SDF-1/CXCR4 in regulating migration of MSCs, changes of SDF-1/CXCR4 expression in the MSCs of AML patients, including those treated with high-dose chemotherapy, have not been explored. This issue is clinically relevant. Studies indicate that high-dose chemoradiotherapy may damage marrow stroma and thereby impede MSC's effect in hematopoietic engraftment [3]. Interestingly, infused healthy MSCs have been found to facilitate the engraftment of HSCs, but the transplanted MSCs are often not engrafted into recipients' bone marrow [10, 11]. As an initial step to understand these clinical

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phenomena, we speculate that important insights might be gained by investigating expression of SDF-1/CXCR4.

The present study has found an excessive retention of SDF-1/CXCR4 in the MSCs of AML, and a SDF-1 deficiency in bone marrow plasma of patients with AML that could negatively affect migration of transplanted MSCs and HSCs. Co-infused healthy MSCs increased the level of SDF-1 in the peripheral blood of AML patients. These findings reveal a new molecular target that could be manipulated in patients with AML.

## 2 Materials and methods

### 2.1 Patients' samples and cell cultures

Bone marrow samples were obtained by iliac crest aspiration from healthy donors and patients diagnosed with AML after informed consent. Ten samples were from AML patients (AML-c; subtypes: M1, 1; M2, 4; M4, 2; M5, 3) who had been given chemotherapy, including standard dose of cytarabine-daunorubicin for induction and high dose of cytarabine for consolidation to achieve a complete remission and be ready for HSC transplantation. Another 10 samples were obtained from healthy donors. The remaining ten samples were from newly diagnosed AML (AML-n; subtypes: M2, 5; M4, 3; M5, 2). Diagnosis of AML was established using standard morphological, cytochemical, immunophenotypic criteria plus cytogenetic evaluation.

MSCs from bone marrow were isolated by plastic adherence [11]. Mononuclear cells were separated by centrifugation using Percoll density gradient (Amersham Biosciences, Sweden). Plasma of bone marrow from each subject was collected and stored at −80°C. The cells were counted and seeded at a density of $4 \times 10^5$ cells per $cm^2$ in low glucose Dulbecco Modified Eagle Medium (LG-DMEM, Gibco BRL life Technologies, USA) supplemented with 10% fetal bovine serum (Gibco BRL life Technologies, Australia), penicillin, and streptomycin. Cultures were maintained at 37°C in a humidified atmosphere containing 5% $CO_2$. The medium change was first done after 72 h to remove all non-adherent cells, and refreshed twice weekly thereafter. When adherent layer became near-confluent, cells were detached with 0.25% trypsin–EDTA (Gibco BRL life Technologies, USA) and re-plated as passage one. MSCs at the 3rd passage were used for all experiments.

### 2.2 In vitro differentiation assay

MSCs from the cultures were induced to differentiate to adipogenic and osteogenic lineages. They were cultured in different differentiation medium (NH AdipoDiff Medium, NH OsteoDiff Medium, Miltenyi Biotec, China), and stained according to the manufacturer's instructions.

### 2.3 Flow cytometry analysis

Expression of *cell-surface* chemokine receptors was analyzed by flow cytometry. MSCs were treated with 0.25% trypsin–EDTA and washed twice with phosphate-buffered solution. Before analysis, every sample was incubated with its own plasma of bone marrow at 37°C for 1 h to recover expression of surface proteins. Cells were then incubated at 4°C for 30 min with anti-human CXCR4-phycoerythrin (PE; BD Bioscience, Clone-12G5) and anti-human CD26-fluorescein isothiocyanate (FITC; BD Bioscience, Clone M-A261). To verify molecular phenotype on the surface of MSCs, the following antibodies were used: anti-CD73-PE, anti-CD105-PE, anti-CD166-PE, anti-CD29-allophycocyanin, anti-CD44-FITC, anti-CD86-FITC, anti-CD45-peridinin-chlorophyll-protein, anti-CD34-FITC, and anti-CD80-PE. Isotype-matched controls were used as indicated. Cells were analyzed with a FACScalibur system (BD Bioscience). Flow cytometry data were analyzed using the FCS Express software (De Novo Software, Canada).

### 2.4 Enzyme-linked immunosorbent assay (ELISA) for detection of SDF-1α

SDF-1α in plasma of bone marrow and in supernatant of MSCs was detected by Quantikine Human Immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. For the supernatant, the third passage MSCs were washed three times with LG-DMEM and cultured for 72 h at the concentration of $2 \times 10^5$ cells/3 ml of cultured medium. The supernatant or plasma together with standards was pipetted into wells pre-coated with a monoclonal antibody specific for SDF-1. After washing, an enzyme-linked (conjugated to horseradish peroxidase) polyclonal antibody specific for SDF-1 was added. Optical density was measured using a microplate reader set to 450 nm with correction at 570 nm. All assays were performed in duplicate.

### 2.5 Migration assay

MSC migration was performed in transwell plates (Costar Coring, NY, USA) of 6.5 mm diameter and a pore size of 8 μm. Before adding cells, the transwells were saturated with assay medium (LG-DMEM supplemented with 10% FBS) at 37°C for 1 h. Lower chamber was filled with 600 μl assay medium in the presence or absence of recombinant human SDF-1α (0–500 ng per mL; R&D Systems). MSCs ($5 \times 10^4$) in 100 μl assay medium were added to the upper chamber and incubated overnight at

37°C. For some experiments, MSCs were pre-incubated with CXCR4 antagonist, AMD3100 (0–100 μg/ml; Sigma) for 30 min or with the CD26 inhibitor, Diprotin A (0–50 mM; Sigma) for 15 min. Cells migrated across the inserted membrane were stained using 1% crystal violet. The migrated cells were counted manually in five microscopic fields at high-power (×400). Results were expressed as the mean of the net migrated cells over control cells (basal migration without chemotactic stimulus). Each experiment was performed in triplicate.

### 2.6 Real-time RT-PCR

Total RNA from MSCs was extracted using TRIzol reagent (Invitrogen). Reverse transcription was carried out using a RevertAid First Strand cDNA Synthesis Kit (K1622, Fermentas, EU). Real-time PCR was performed using the ABI Prism 7300 Sequence Detector System (Applied Biosystems, USA). The reaction mixture contained Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), template and primers for each gene (Invitrogen, Shanghai, China). The total reaction volume was 25 μL. After activation of the AmpliTaq Gold DNA Polymerase (Applied Biosystems, Warrington, UK) for 10 min at 95°C, we ran 42 cycles, 15 s for each at 94°C, 20 s for 58°C, and 30 s at 72°C. Data were collected by the Sequence Detection Software (SDS; Version 1.3.1, Applied Biosystems) and analyzed using the threshold cycle relative quantification method [12]. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference gene was used to normalize the data. The dissociation curve for amplification was analyzed to confirm that there were no non-specific PCR products. The primer sequences were as follows: human CXCR4 (sense, 5′-CACCGCATCTGG AGAAC-3′; antisense, 5′-GATGGTGGGCAGGAAG-3′); human SDF-1 (sense, 5′-ACTGTGCCCTTCAGATTG-3′; antisense, 5′-TTGGCTGTTGTGCTTACT-3′); human CD26 (sense, 5′-AGTGGCGTGTTCAAGTGT-3′; antisense, 5′-GTCTTCTGGAGTTGGGAG-3′); human GAPDH (sense, 5′-TGG TAT CGT GGA AGG ACT CA-3′; antisense, 5′-GCA GGG ATG ATG TTC TGG A-3′). Experiments were done in triplicate for each sample. The mean of the triplicate values was used to represent the final level of each sample.

### 2.7 Western blot

Cells ($10^5$) were lysed on ice with RIPA lysate buffer supplemented with protease inhibitor cocktail (Beyotime, China). Total protein concentration was determined using the BCA protein assay kit (Pierce). Proteins were denatured by boiling for 5 min in SDS loading buffer supplemented with $\beta$-mercaptoethanol. Equal amount of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred onto nitrocellulose membranes. After blocking for 1 h at room temperature with 5% fat-free milk in Tris-buffered saline and 0.05% Tween 20, membranes were incubated overnight at 4°C with the following primary antibodies: rabbit anti-human CXCR4 (1:1000; Abcam), goat anti-human SDF-1 (1:500; R&D Systems), goat anti-human dipeptidyl peptidase IV/CD26 (1:1000; R&D Systems), mouse antibody to GAPDH (1:4000; Santa Cruz). Secondary

**Table 1** Characteristics of the AML patients in HSCT

| | Co-infusion group ($n = 13$) | Control group ($n = 12$) |
|---|---|---|
| Age, median (range) | 31 (14–43) | 32.5 (15–48) |
| Sex: male/female | 5/8 | 8/4 |
| Disease subtypes and stage | | |
| CR1 | 11 (M2, 4; M4, 4; M5, 3) | 10 (M2, 6; M4, 1; M5, 3) |
| CR2 | 2 (M3, 1; M5, 1) | 2 (M4, 2) |
| Transplanted cells: median (range) | | |
| MNC × $10^8$/kg | 7.10 (4.70–8.45) | 6.88 (5.20–8.68) |
| CD34+ cells × $10^8$/kg | 2.90 (1.08–3.63) | 2.69 (1.18–3.99) |
| MSCs × $10^5$/kg | 4.09 (2.67–5.22) | 0 |
| Engraftment days: median (range) | | |
| ANC ≥ 0.5 × $10^9$/L | 11 (10–19) [$n = 13$] | 11.5 (11–16) [$n = 12$] |
| Platelets ≥ 50 × $10^9$/L | 19 (12–58) [$n = 11$]* | 26 (16–95) [$n = 10$] |
| Relapse | 1 | 1 |
| Mortality | 4 | 3 |

Data are numbers unless otherwise indicated

*M* male, *F* female, *CR* complete remission, *MNC* mononuclear cell, *MSCs* mesenchymal stem cells, *ANC* absolute neutrophil count

*$P < 0.05$

antibodies were rabbit anti-goat, goat anti-rabbit and rabbit anti-mouse IgG conjugated to horseradish peroxidase (HRP; ZSGB-BIO, China). Immunoreactive bands were enhanced using the SuperSignal West Pico Chemiluminescent Substrate and SuperSignal West Femto Trial Kit (Thermo Scientific, Pierce), and quantified by 1D Image Analysis Software (version 3.5.4, Kodak, NY, USA).

### 2.8 Patients' samples from clinical trial

To determine whether infusion of normal MSCs improve the level of SDF-1 in patients with AML, we have analyzed the samples that were collected from a clinical trial. This trial is briefly summarized below.

Patients with AML lacking an HLA-matched donor were enrolled in the study between 2007 and 2008. The patients in remission by chemotherapy were randomized into two groups for haploidentical hematopoietic stem cells transplantation (HSCT). The control group received a routine procedure [13] (including conditioning regimen, evaluation of engraftment and chimerism, prophylaxis and treatment of GVHD), whereas the co-infusion group was pre-infused with ex vivo-expanded MSCs 24 h prior to HSC infusion (Table 1).

This project has been approved by the Institutional Review Board and Ethical Committee in Peking University Health Science Center. Informed consent was obtained from patients and donors. From June 2007 to September 2008, a total of 25 AML patients completed the entire study (13 in treatment group vs. 12 in control group) and followed up until September 2010. The plasma of patients was collected and anti-coagulated with EDTA every 4 days from −4th day pre-transplantation to the 28th day after transplantation. The level of SDF-1 in the peripheral blood was measured by ELISA.

### 2.9 Statistical analysis

Statistical differences between two groups were evaluated by Student's *t* test. For multiple group comparison, data were analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni post hoc test using SPSS software 13.0. Differences were considered significant when $P$ value was $<0.05$.

## 3 Results

### 3.1 Characteristics of MSCs from patients with AML

MSCs were obtained from the bone marrow of AML patients or healthy donors, and cultured to confluence by the third passage. A vast majority of MSCs exhibit a spindle shape under light microscopy (Fig. 1a-a).

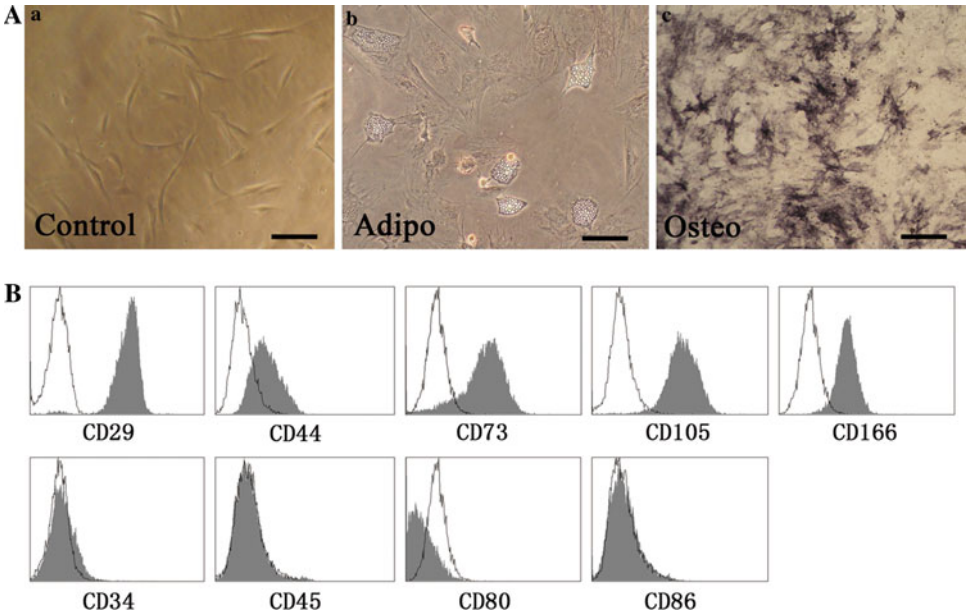


**Fig. 1** Characteristics of BM-MSCs from donor and patients with AML. **a** Cultured BM-MSCs were induced to differentiate into adipogenic and osteogenic lineages in appropriate medium. (*a*) Human MSCs derived from an acute myeloid leukemia (AML) patient exhibit a typical fibroblastic shape. (*b*). MSCs were induced to adipocytic lineage in culture. Adipocytes were round and filled with lipid droplets which might fuse to form vacuoles. (*c*) Osteogenic lineage was confirmed by BCIP/NBT (SIGMA) staining for alkaline phosphatase activity. *Scale bars* 125 μm. **b** Flow cytometry analysis: *Shaded curves* were from specific antibody signals. *Open curves* were from isotype control signals

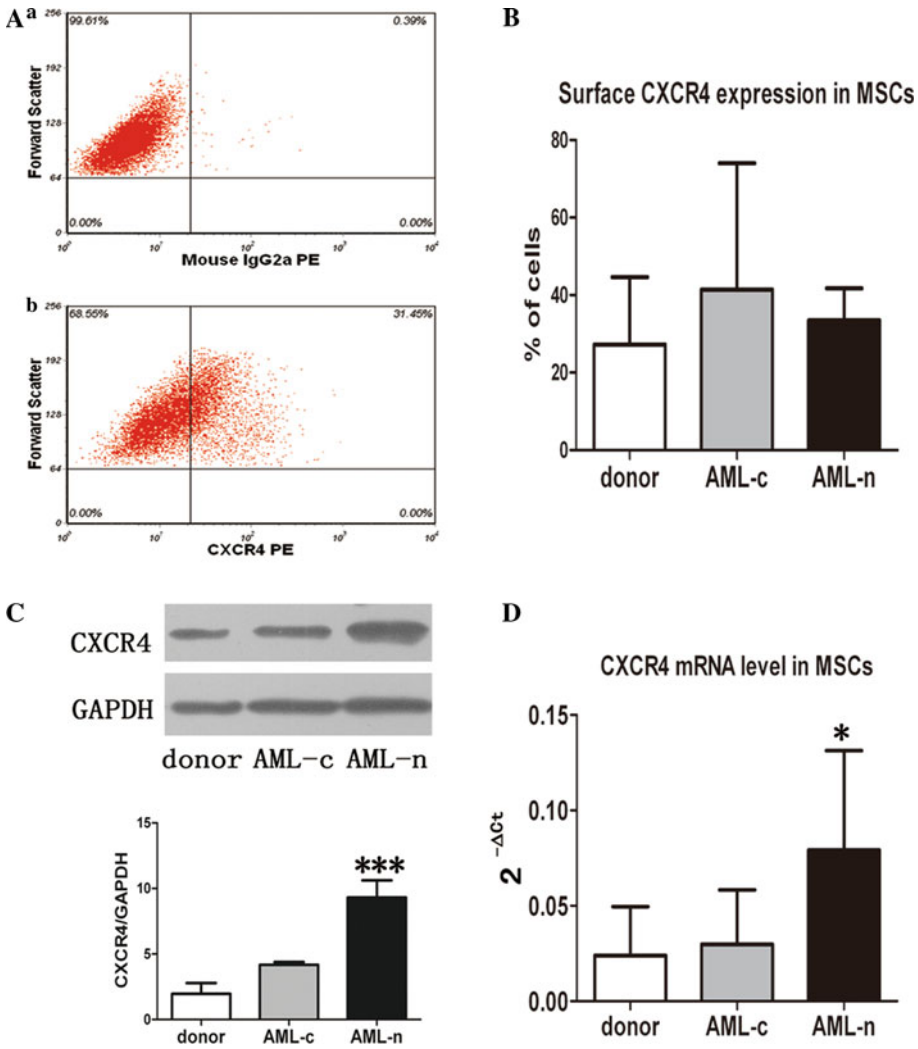


**Fig. 2** Expression of CXCR4 in BM-MSCs. **a** Multivariant flow cytometry was performed on the MSCs derived from heathy donors or patients with AML-c or AML-n. Scatter plot of representative isotype or sample is shown in (*a*) and (*b*). Signals are well clustered in the upper-right quadrant. **b** Surface-CXCR4 expression was analyzed by flow cytometry. There was no significant difference between the three types of MSCs ($n = 10$). **c** Total CXCR4 in the MSCs was measured by western blot. CXCR4 expression in the AML-n MSCs was significantly higher than that in either healthy donors or AML-c MSCs (statistically significant; $***P < 0.001$). Each western blot experiment was repeated three times using cells isolated from different samples. **d** CXCR4 mRNA levels were quantified using SYBR Green real-time PCR technique (Applied Biosystems), and normalized to the level of GAPDH. Transcription level of each gene is represented by $2^{-\Delta C_T}$, where $\Delta C_T = C_T$ (gene X) $- C_T$ (GAPDH). The same difference was also found at the transcriptional level ($*P < 0.05$)

The MSCs were multi-potent and differentiated to adipocytic and osteogenic lineages in respective differentiation medium (Fig. 1a-b, c). To determine the molecular phenotype of these MSCs, flow cytometry was performed. These cells were positive for CD29, CD44, CD73, CD105 and CD166; but negative for CD34, CD45, CD80, and CD86 (Fig. 1b). No difference was found between the three groups (healthy donor, AML-c and AML-n).

### 3.2 Intracellular retention of CXCR4 in the MSCs from patients with AML

CXCR4 regulates migration of MSCs [5, 6]. Its expression could be altered in patients with leukemia or by chemotherapy. We have first determined the expression of cell-surface CXCR4 in the MSCs by flow cytometry (Fig. 2a). We compared the percentages of MSCs positive for CXCR4 in different types of MSC. There was no significant difference between the three groups (healthy donor, AML-c and AML-n) (Fig. 2b).

We next performed western blot to evaluate total CXCR4 in the MSCs. CXCR4 level in the AML-n group was significantly higher than that in either donors or AML-c (Fig. 2c, $P < 0.001$). The same difference was also found at the transcriptional level by real-time PCR (Fig. 2d). These data suggest an increase of CXCR4 production in the MSCs of AML-n, but a significant portion of CXCR4 failed to reach cytoplasmic membrane. Chemotherapy partially reversed the increased production in the AML-c group, but did not alter the surface expression of CXCR4 (Fig. 2).

### 3.3 Secretion of SDF-1 from the MSCs was reduced in patients with AML

SDF-1 with its binding partner, CXCR4, has been suggested to critically regulate MSC migration [6, 14]. Since levels of surface CXCR4 showed no change in different types of MSCs, we examined the level of SDF-1 in the bone marrow plasma by ELISA. SDF-1 from AML patients was significantly lower than that from donors

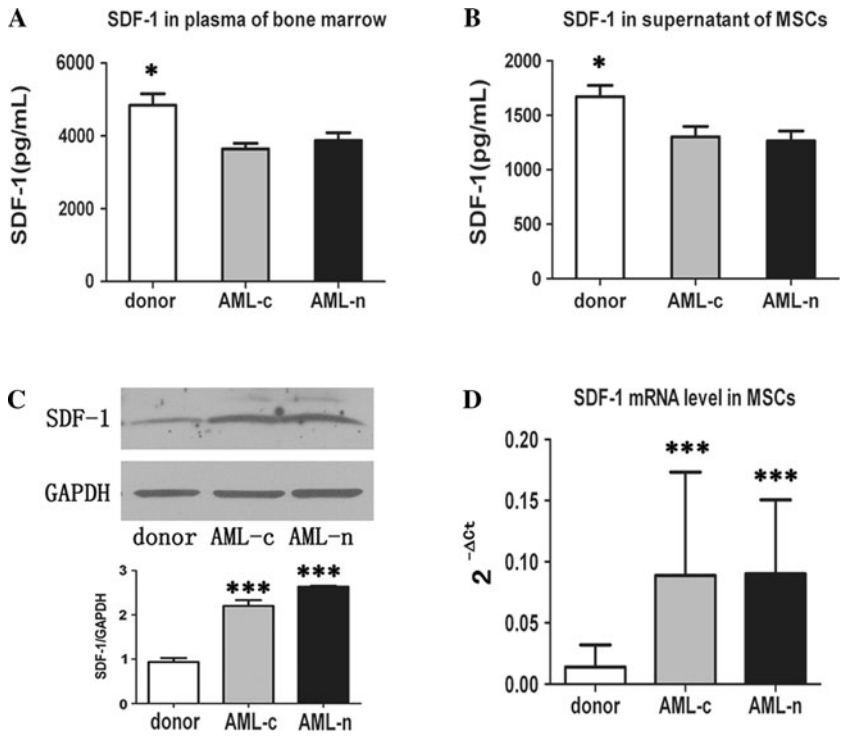


**Fig. 3** Secretion and expression of SDF-1 in BM-MSCs. **a** SDF-1 level was measured by ELISA technique. The bone marrow plasma level of SDF-1 from donor was significantly higher than that from AML ($P < 0.05$, $n = 10$). **b** SDF-1 levels were measured in the supernatants from $2 \times 10^5$ MSCs cultured in three ml medium for 72 h. Bone marrow plasma was not present in these experiments. SDF-1 from healthy donors was significantly higher than that from AML-c and AML-n (*$P < 0.05$; $n = 10$). **c** Western blotting was performed to quantify total SDF-1 in the lysates of MSCs. SDF-1 expression in the MSCs of AML-c or AML-n was significantly higher than that in the MSCs of healthy donors (***$P < 0.001$). **d** Expression of SDF-1 mRNA in MSCs. The difference detected by western blot was also found at transcriptional level by real-time PCR (***$P < 0.001$, $n = 10$)

(Fig. 3a; $P < 0.05$; $n = 10$). There was no significant difference between the AML-n and AML-c.

To explore potential mechanisms for this reduction of SDF-1, we first measured the SDF-1 levels in the supernatants of MSC cultures that were not treated with plasma. These levels would indicate the secretion of SDF-1 from the MSCs. SDF-1 from patients with AML was significantly lower than that from healthy donors (Fig. 3b; $P < 0.05$; $n = 10$). There was no significant difference between the AML-c and AML-n. In contrast, level of total SDF-1 in the MSCs of AML, by western blot, was markedly higher than that in healthy donors (Fig. 3c; $P < 0.001$). This difference was also found at the transcriptional level by real-time PCR (Fig. 3d). This finding suggests that SDF-1, like CXCR4, is abundantly expressed but retained in the MSCs while extracellular level of SDF-1 is reduced in patients with AML.

SDF-1 level is known to be regulated by degrading enzymes, such as CD26 to cleave its N-terminal [9]. We therefore performed flow cytometry to determine the level of CD26 in the surface of MSCs (Fig. 4a). This analysis showed that a small fraction of MSCs were positive for CD26 ($3.65 \pm 5.98$, $3.11 \pm 2.51$, $3.19 \pm 3.99\%$ for the three groups, respectively). No significant difference was found among the three groups (Fig. 4b). This finding does not exclude an altered expression of CD26 from other non-adherent bone marrow cells [9].

Taken together, these results suggest that there is a SDF-1 deficiency in patients with AML. This reduction is not due to a down-regulation of expression in the MSCs of AML since SDF-1 synthesis is increased in the MSCs of AML-c and AML-n (Fig. 3c); this reduction is not related to an increased degradation of SDF-1 by up-regulation of CD26 on the surface of MSCs (Fig. 4b). Instead, our results show a decreased secretion of SDF-1 from the MSCs of AML, which may, at least partially, contribute to the reduction of SDF-1 level in plasma.

### 3.4 Infusion of normal MSCs increases SDF-1 level in vivo

SDF-1 deficiency in the plasma of patients with AML raised an important question whether this deficiency is amendable by supplement of healthy MSCs in vivo. We thus extracted data from a clinical trial that has been conducted by our group to address this question.

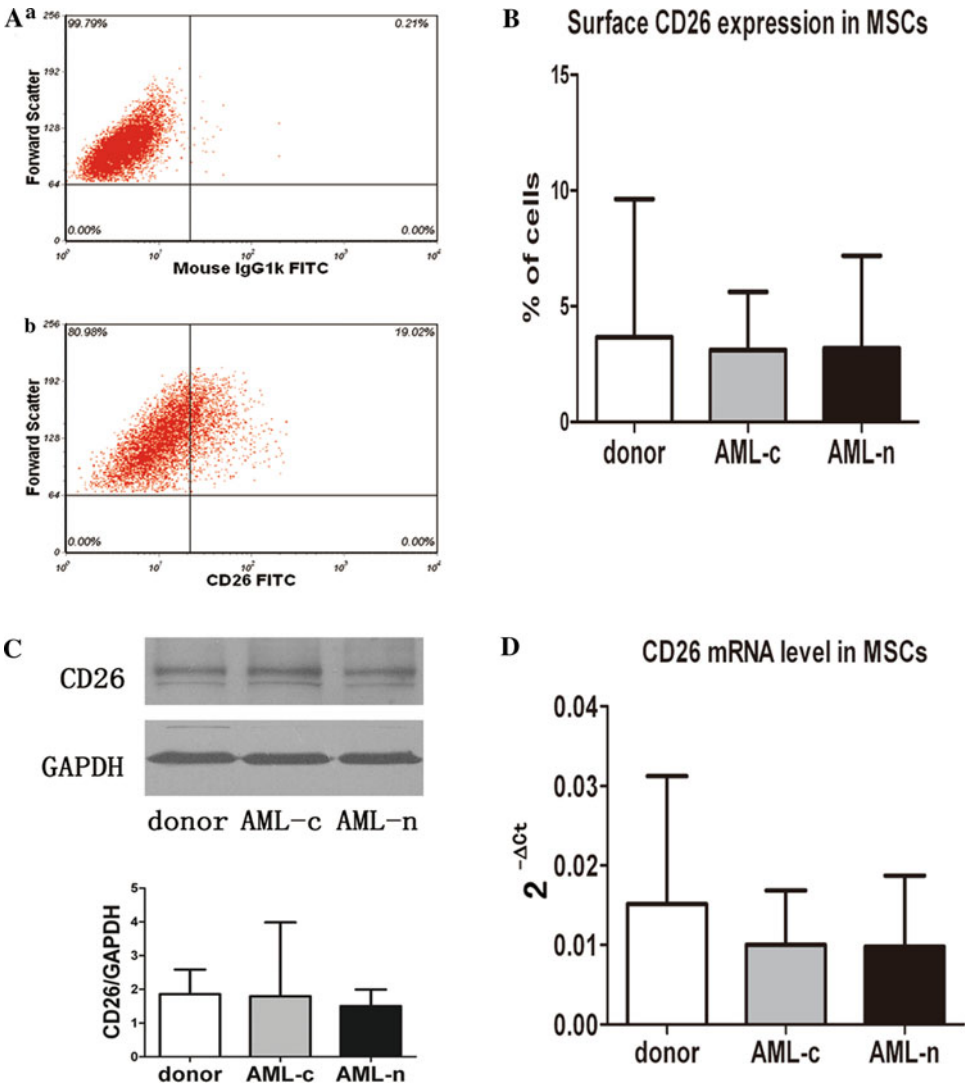


**Fig. 4** Expression of CD26 in MSCs. **a** Scatter plots of representative isotype or sample (CD26-FITC) by flow cytometry were shown in (*a*) and (*b*). Signals of CD26 were clustered in the upper-right quadrant. **b** Surface-CD26 expression by flow cytometry was not significantly different among the three types of MSCs ($n = 10$). **c** Total CD26 was measured by western blot in the MSCs. No significant difference was found among the three types of MSCs. **d** Quantitative expression of CD26 mRNA in each of the MSCs. No significant difference was found among the three types of MSCs

AML patients who received HSCT were randomized into two groups, *with* and *without* co-infusion of healthy MSCs (Table 1). There was no significant difference in the number of infused mononuclear cells and CD34+ cells between the two groups (Table 1). The transplanted MSCs appeared not engrafted into bone marrow. A donor-predominant mixed chimeric pattern was detected in only one case at day +60 after transplantation and disappeared shortly after. Within 100 days after HSCT, interval to reach platelets $\geq 50 \times 10^9$/L was significantly shorter for the treated group ($n = 11$), compared to that in the control group ($n = 10$) (median 19 days [range 12–58 days] vs. 26 days [16–95 days]; $P < 0.05$; Table 1). This delay on the platelet recovery in the control group suggests that engraftment was not optimal after the transplantation. However, after a median of 26 months of follow-up, no difference was found in the relapse rate between the two groups (7.8 vs. 8.3%). Overall survival rate was not significantly different between the two groups (69.2% in the co-infused group vs. 75% in the control group; $P > 0.05$).

The level of SDF-1 in peripheral blood peaked at day +8 (3091.07 ± 413.36 pg/mL) in treated group, which was earlier and higher than the peak level in control group at day +16 (2581.65 ± 404.86 pg/mL). From day +8 to +12 after transplantation, the level of SDF-1 was significantly higher in the treated group than that in the controls (Fig. 5; $P < 0.05$). Together, this result is consistent with the finding in vitro that normal MSCs secret more SDF-1 than that by the MSCs from patients with AML. This suggests that normal MSCs co-infused can increase SDF-1 level extracellularly in patients with AML. It remains to be determined whether this increase of SDF-1 in the peripheral blood can be efficiently delivered into the bone marrow.

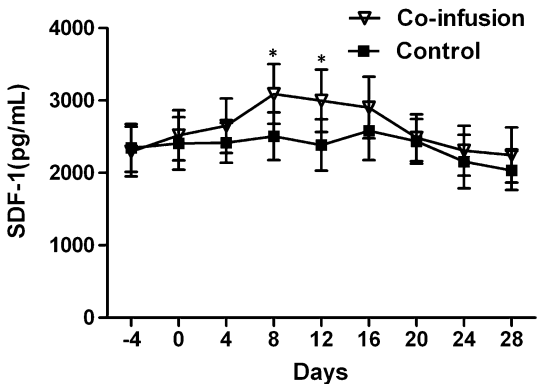


**Fig. 5** SDF-1 level in peripheral blood after HSCT. SDF-1 concentration in peripheral blood was measured by ELISA. Samples were obtained from AML patients every 4 days from days −4 to 28 after HSCT. The level of SDF-1 in MSC co-infusion group ($n = 13$) reached a peak value at day +8, which was earlier and higher than the peak level in control group ($n = 12$) at day +16. From days +8 to +12 after transplantation, the level of SDF-1 was significantly higher in the co-infusion group than that in the controls (*$P < 0.05$)

### 3.5 Signaling of SDF-1/CXCR4 axis affects the migration of MSCs in vitro

Because SDF-1 is known to be important for MSCs and HSCs migration [14], a reduced level of SDF-1 in the plasma of AML could negatively affect this function. However, other defects intrinsic to the MSCs could also affect their migration in AML. To test this issue, chemotaxis assay was performed to test SDF-1-induced migration of MSCs. Transwell chamber was filled with MSCs placed in the upper chamber and chemo-attractant (SDF-1) in the lower chamber. SDF-1 induced migration of normal MSCs and was dose-dependent with a maximal effect achieved at 150 ng/mL (Fig. 6a; $n = 10$).

Next, exogenous SDF-1 was applied in an equal amount (150 ng/mL) for each type of MSCs, so that any difference in migration would have to be resulted from factors intrinsic to the MSCs, but not SDF-1 level. After overnight incubation, migrated cells that remained on the lower face of the filters were stained by crystal violet (Fig. 6d) and counted. Four sets of experimental groups were assigned (1. MSCs without pre-treatment of any inhibitors and no SDF-1; 2. MSCs without pre-treatment + SDF-1; 3. MSCs pre-treated with AMD3100 + SDF-1; 4. MSCs pre-treated with Diprotin A + SDF-1). Migration of MSCs in the absence of SDF-1 was minimal in all three types of MSCs (Fig. 6e-1). In the presence of SDF-1, there was a robust increase of MSCs migration to the lower chamber after 12 h (Fig. 6e-2–4), compared with those without SDF-1 (Fig. 6e-1; $P < 0.05$; $n = 10$ for each group). However, difference between the three types of MSCs was not statistically significant (Fig. 6). Moreover, selective CXCR4 inhibitor, AMD3100, strongly suppressed the migration of MSCs (Fig. 6e; comparison between 6e-2 and e-3; $P < 0.05$) and was dose-dependent (Fig. 6b). Pre-treatment with CD26 inhibitor, Diprotin A, did not change the migratory response of MSCs to SDF-1 (Fig. 6e; comparison between e-4 and e-2; $P > 0.05$).

Together, these data confirm that SDF-1/CXCR4 signaling is able to induce migration of human MSCs. MSCs from patients with AML did not show intrinsic defect in migration as long as sufficient levels of SDF-1 were present. Thus, SDF-1 deficiency in AML would negatively affect the migration of MSCs.

## 4 Discussion

In this study, we have demonstrated an excessive intracellular retention of CXCR4 and SDF-1 in the MSCs from patients with AML. This retention is associated with a decrease of SDF-1 level in the bone marrow plasma and supernatant of cultured MSCs from patients with AML. This finding is consistent with previous studies that leukemic cells change hematopoietic niche in vivo in mice by decreasing SDF-1 levels in perivascular areas where MSCs are usually located. Whether this down-regulation of SDF-1 is directly related to the abnormal retention of SDF-1 in MSCs is not investigated in the study [15]. Together, these data support our hypothesis that AML alters expression and distribution of CXCR4/SDF-1 in human MSCs. This deficiency might be amendable since infusion of healthy MSCs increases levels of SDF-1 in the peripheral blood of AML patients, which could secondarily improve the level of SDF-1 in the bone marrow through circulation.

SDF-1/CXCR4 signaling axis has been reported to play an important role in the migration of MSCs [5, 6] and HSCs [7]. Indeed, our data show a robust effect on human MSCs migration in vitro by application of SDF-1. Because of a SDF-1 deficiency in our AML patients' bone marrow plasma, this reduction of SDF-1 should represent the integrated consequence of all cell types in the patients with AML, including reduced secretion from the MSCs of AML. Furthermore, our in vitro observation suggests that MSCs in patients with AML do not have intrinsic defects affecting their migration. Therefore, SDF-1 deficiency in AML is likely to be deleterious to the cell migration in the bone marrow. We understand that MSC migration could be regulated by other factors, other than SDF-1. Thus, it is unclear whether the cell-migration effect by SDF-1 deficiency will be compensated or modified in vivo since other cell types, including osteoblasts or endothelial cells, can also produce SDF-1 in the bone marrow.

We have explored the mechanisms responsible for the SDF-1 deficiency in the patients with AML. The deficiency is not due to a decrease of SDF-1 synthesis in the MSCs of

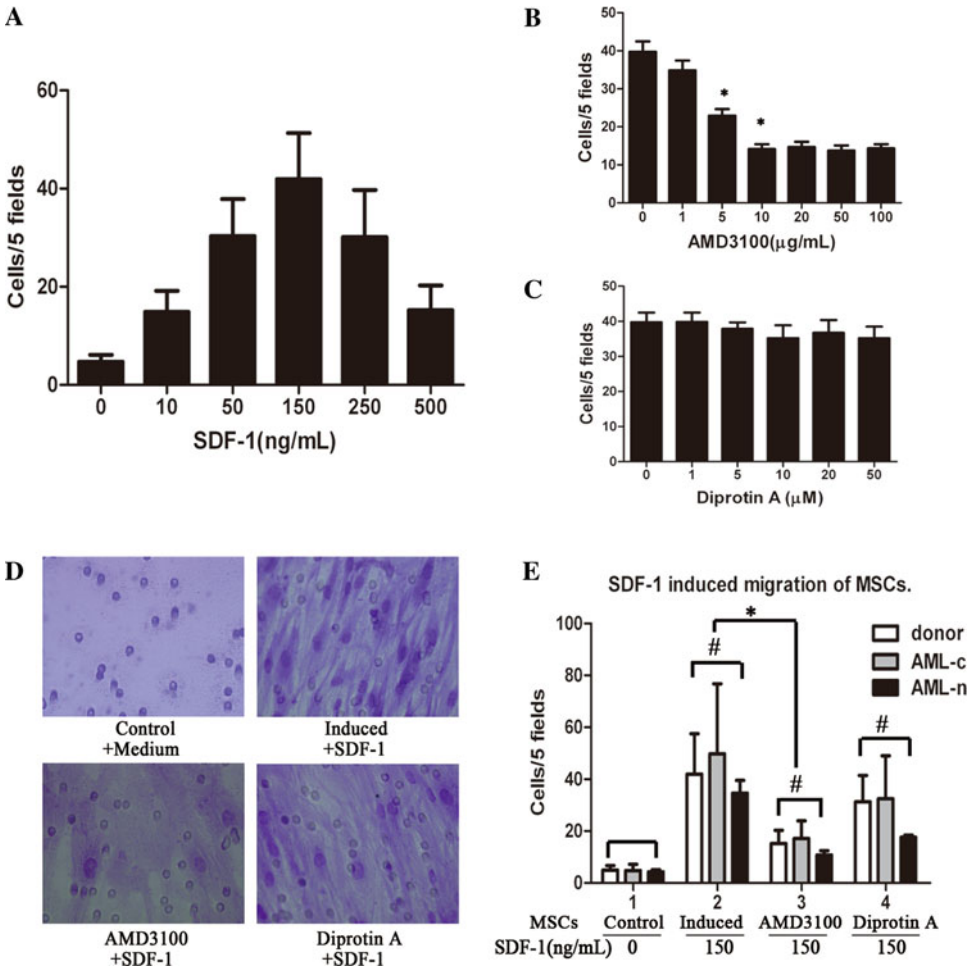


**Fig. 6** SDF-1 induced migration of BM-MSC of AML patients. **a** Chemotaxis assay demonstrating the migration of normal MSCs in response to serial concentrations of SDF-1 (0–500 ng/mL). SDF-1 induced migration of normal MSCs and was dose-dependent with a maximal effect achieved at 150 ng/mL ($n = 10$ for data point). **b** Migration assay using CXCR4 inhibitor AMD3100 (0–100 μg/mL). AMD3100 (10 μg/mL) drastically inhibited the migration of MSCs compared with control (*$P < 0.05$; $n = 10$ for each group). All wells contained 150 ng/mL SDF-1 in the lower chambers. **c** Migration assay using CD26 inhibitor, Diprontin A (0–50 μM). Diprontin A with different concentration could not increase the migration of MSCs ($P > 0.05$; $n = 10$ for each group). All wells contained 150 ng/mL SDF-1 in the lower chambers. **d** Crystal violet-stained membrane. Representative stained filters of control MSCs toward medium alone, MSCs toward SDF-1, AMD3100-incubated MSCs toward SDF-1, and Diprontin A-incubated MSCs toward SDF-1. **e** Differences in SDF-1-induced migration among MSCs from donors, AML-n and AML-c ($n = 10$ for each group). Migration in the presence of SDF-1 was markedly increased compared with those in absence of SDF-1 (**e**-*2–4* compared with **e**-*1*, $^{\#}P < 0.05$). The migration of MSCs was drastically decreased by pre-incubating with AMD3100 (*$P < 0.05$)

AML (Fig. 3). However, we have observed excessive intracellular retention of SDF-1 and reduced secretion of SDF-1 from the MSCs of AML, which may partially contribute to the SDF-1 deficiency. This finding is consistent with previous studies that have demonstrated SDF-1 expression in bone marrow stromal cells (or MSCs). These cells have been considered as one of the main sources of SDF-1 in bone marrow [16].

However, detailed mechanism for the secretion of SDF-1 by the stromal cells is unknown [17]. In addition, how extracellular SDF-1 to be degraded is not fully understood either. Although there is no significant difference of CD26 level on the surface of MSCs among the three groups, an increase of SDF-1 degradation in extracellular space by non-CD26 mechanisms could still contribute to the SDF-1 deficiency. For instance, there are other enzymes, such as matrix metalloproteinase-2 (MMP-2), MMP-9, cathepsin G and leukocyte elastase, who are potential candidates to degrade SDF-1 [18]. Irrespective of the mechanism involved in regulating SDF-1 level, our finding suggests a defect during the process of SDF-1 secretion from intracellular to extracellular space in AML.

SDF-1 deficiency might be amendable if patients receive infusion of normal MSCs that secret SDF-1 in blood and may be subsequently circulated into the bone marrow. It remains to be clarified whether the peripheral SDF-1 can be

efficiently delivered into the bone marrow. Our data also show that elevation of the peripheral SDF-1 level is not sufficient to change the overall survival rate in the clinical trial, suggesting additional factors that need to be identified and modified to improve the overall survival in AML.

Our results show that, like SDF-1, CXCR4 are also abundantly translated, but failed to reach cytoplasmic membrane of MSCs in the patients with AML (Fig. 2). One might argue that the increased expression of CXCR4 in the MSCs of AML is due to a higher proliferative index in the cells. This would increase overall levels of transcription or translation of CXCR4. We believe that this mechanism is unlikely since another chemokine RANTES and its receptor CCR3 or CCR5 in the MSCs of AML showed levels comparable to those in the MSCs from normal controls (data not shown). Expression pattern of CXCR4 in MSCs appears parallel to CXCR4 expression in HSCs. CXCR4 can also be induced to express on the cell-surface of HSCs to mediate cell migration [19]. Together, these observations suggest that, like SDF-1, there is also an excessive retention of CXCR4 in the MSCs of AML, which would be unfavorable for SDF-1 to bind with CXCR4 and induce migration.

In summary, our study demonstrates a deficiency of SDF-1 in the bone marrow plasma of the patients with AML. Chemotherapy could not rescue this defect, but infusion of normal MSCs does improve the SDF-1 level in the peripheral blood. This deficiency of SDF-1 may negatively affect migration of human MSCs, a pathogenic mechanism to be considered in future development of therapies in patients with AML.

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