

Role of the HIF-1 α /SDF-1/CXCR4 signaling axis in accelerated fracture healing after craniocerebral injury

YONGHUA XUE¹, ZHIKUN LI², YI WANG², XIAODONG ZHU², RUIXI HU² and WEI XU²

¹Department of Neurosurgery, Putuo Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200062; ²Department of Orthopedic Surgery, Tongren Hospital, School of Medicine, Shanghai JiaoTong University, Shanghai 200331, P.R. China

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Abstract. The hypoxic state of the brain tissue surrounding craniocerebral injury induces an increase in the secretion of HIF-1 α during the healing process. HIF-1 α can promote mesenchymal stem cell (MSC) migration to ischemic and hypoxic sites by regulating the expression levels of molecules such as stromal cell-derived factor-1 (SDF-1) in the micro-environment. Stem cells express the SDF-1 receptor C-X-C chemokine receptor type 4 (CXCR4) and serve a key role in tissue repair, as well as a number of physiological and pathological processes. The present study aimed to determine the role of HIF-1 α /SDF-1/CXCR4 signaling in the process of accelerated fracture healing during craniocerebral injury. Cultured MSCs underwent HIF-1 α knockdown to elucidate its effect on the proliferative ability of MSCs, and the effect of SDF-1 in MSCs was investigated. It was also determined whether HIF-1 α could promote osteogenesis via SDF-1/CXCR4 signaling and recruit MSCs. The results indicated that HIF-1 α knockdown suppressed MSC proliferation *in vitro*, and SDF-1 promoted cell migration via binding to CXCR4. Furthermore, HIF-1 α knockdown inhibited MSC migration via SDF-1/CXCR4 signaling. Considering the wide distribution and diversity of roles of SDF-1 and CXCR4, the present results may form a basis for the development of novel strategies for the treatment of craniocerebral injury.

Introduction

Fracture healing is a multi-level and multi-path process regulated by systemic and local factors, involving numerous types of cells and growth factors, such as mesenchymal cells and transforming growth factor- β 1 (TGF- β 1) (1-3).

Fracture non-union or delayed union is a common complication in orthopedics that causes physical and mental pain, as well as a notable economic burden to patients and society. Long-term clinical studies have revealed that, in the process of fracture healing, callus volume and formation rate are higher in patients with fractures combined with traumatic brain injury than in patients with simple fractures. Heterotopic ossification occurs in patients with craniocerebral trauma combined with a fracture, and fracture healing is accelerated (4,5). Numerous cytokines and neuropeptide factors, such as bone morphogenetic protein (BMP), TGF- β 1, vascular endothelial growth factor, insulin-like growth factor, fibroblast growth factor and calcitonin gene-related peptides, can significantly promote osteoblast proliferation, thereby accelerating fracture healing (6-9). Although there have been advances in the study of osteoblasts in accelerated fracture healing, the underlying mechanisms remain unclear. Elucidating the mechanism by which fracture healing is accelerated may facilitate the development of effective clinical treatments for patients with delayed fracture healing.

During craniocerebral injury, hypoxia promotes the expression of hypoxia-inducible factor-1 α (HIF-1 α), which can increase cell viability, and promote adhesion, migration and angiogenesis. HIF-1 α is the most important hypoxia receptor and hypoxia-induced transcription factor known at present (10,11). An increase in HIF-1 α activity can increase the viability of cells in hypoxic environments and promote cell adhesion, migration and angiogenesis (12,13). HIF-1 α can promote stem cell migration to ischemic and hypoxic sites by regulating the expression levels of surface molecules as a result of interaction between a number of ligands and receptors including stromal cell-derived factor-1 (SDF-1), a downstream gene of HIF-1 α , which binds to its specific receptor C-X-C chemokine receptor type 4 (CXCR4) to form a pair of coupling molecules (14,15). SDF-1 is a CXC-type chemokine produced by mesenchymal stem cells (MSCs). SDF-1 and its unique receptor CXCR4 constitute the biological axis of SDF-1/CXCR4 (16). SDF-1 and CXCR4 are expressed in numerous types of cells and tissues, where they serve a key role in tissue repair and a variety of physiological and pathological processes, including organogenesis, revascularization and response to tissue injury (17,18). However, their role in the process of accelerated fracture healing during craniocerebral

Correspondence to: Dr Wei Xu, Department of Orthopedic Surgery, Tongren Hospital, School of Medicine, Shanghai JiaoTong University, 1111 Xianxia Road, Shanghai 200331, P.R. China
E-mail: xw2107@shtrhospital.com

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injury and the regulatory effect of HIF-1 α on SDF-1/CXCR4 in MSCs remains unclear. In the present study, MSCs, which serve a key role in fracture healing, were used as a model to investigate the role of the HIF-1 α /SDF-1/CXCR4 signaling axis in accelerated fracture healing during craniocerebral injury *in vitro*.

Materials and methods

Mouse bone marrow mesenchymal stem cell (BMSC) culture. A total of 10 C57 mice (male; age, 4 weeks; weight, 18–20 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. Mice were housed in specific-pathogen-free conditions at room temperature (22 \pm 1 $^{\circ}$ C) with relative humidity (50 \pm 5%), 12-h light/dark cycles, and free access to food and water. BMSCs were isolated as previously described (19). C57 mice were sacrificed via cervical dislocation and soaked in 75% alcohol for 10 min at room temperature. Under sterile conditions, the bilateral leg bones were extracted and separated from the surrounding muscle. The harvested tissue was placed in cold complete DMEM/F12 (DMEM/F12+10% FBS+1% penicillin/streptomycin/amphotericin B solution; Gibco; Thermo Fisher Scientific, Inc.). The culture medium was aspirated repeatedly with a needle and syringe to rinse the marrow cavity. Cells were dispersed by repeated pipetting, and the cell suspension was passed through a 400-mesh sterile mesh and centrifuged at 200 \times g for 3 min, after which the supernatant was discarded. The cells were resuspended in complete DMEM/F12, and the density was adjusted to 5 \times 10⁵ cells/ml. Subsequently, the cell suspension (4 ml) was inoculated into a T-25 flask and cultured in a 5% CO₂ incubator at 37 $^{\circ}$ C. After 24 h, the flask was shaken horizontally to suspend unattached cells and the supernatant was discarded. Fresh medium was then added to the culture flask and changed every 2–3 days until the primary cells grew to a confluence of 80–90%.

Cells were then sub-cultured as follows: Supernatant media was aspirated, and the cells were washed with 1 ml PBS and treated with 1 ml 0.25% trypsin for 2 min at 37 $^{\circ}$ C. Cell morphology was observed by light microscopy (magnification, \times 100). When the cells were rounded, 1 ml complete medium was added to terminate digestion, and the cells were gently pipetted to detach them from the bottom of the bottle completely. The cell suspension was transferred to a centrifuge tube and centrifuged at 200 \times g for 3 min at room temperature, and the supernatant was removed. The cells were resuspended in complete DMEM/F12 and inoculated into a new culture flask at a ratio of 1:2. After passage, the cells were cultured in the same manner as the primary culture until the next generation.

Flow cytometry and MSC sorting. Third generation MSC cells were digested with 0.25% trypsin for 2 min at 37 $^{\circ}$ C, and a single cell suspension was obtained by adjusting the cell density to 1 \times 10⁶ cells/ml. The cell suspension was incubated for 30 min at room temperature with PBS, anti-CD29-FITC (1.5 μ l; cat. no. ab21845; Abcam), anti-CD44-FITC (2 μ g; cat. no. ab25064; Abcam) or anti-CD45-FITC (10 μ l; cat. no. ab27287; Abcam), and analyzed using a Coulter Epics XL-MCL flow cytometer (BD Biosciences) with EXPO32 software (version 1.2; BD Biosciences).

HIF-1 α siRNA transfection. MSC cells were seeded in 6-well culture plates (5 \times 10⁵ cells/well) overnight. The inoculated cells were washed twice with PBS, and 500 μ l serum-free DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc.) was added. The HilyMax-HIF-1 α siRNA transfection mixture (Dojindo Molecular Technologies, Inc.) and siRNA control were premixed (0.2 μ g/ μ l), added to the MSCs and mixed. siRNA and siRNA control were obtained from Sangon Biotech Co., Ltd. After incubating the cells in the cell culture incubator for 6 h at 37 $^{\circ}$ C, the medium was replaced with fresh medium. The cells were trypsinized and collected for subsequent experiments after transfection for 48 h. The sequences of siRNAs used are presented in Table I.

Reverse transcription-quantitative (RT-q) PCR analysis. Total RNA was extracted from cells with TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA synthesis was performed using the TOYOBO ReverTra Ace qPCR RT kit, according to the manufacturer's protocol. qPCR was performed using the KAPA SYBR-Green Supermix PCR kit (Kapa Biosystems). RT-qPCR primers were obtained from Sangon Biotech Co., Ltd.; sequences are listed in Table II. The reaction was started at 95 $^{\circ}$ C for 5 min, followed by 40 cycles of 95 $^{\circ}$ C for 30 sec, 61 $^{\circ}$ C for 30 sec, and 72 $^{\circ}$ C for 30 sec. Relative gene expression levels were measured using the cycle threshold values and the 2^{- $\Delta\Delta$ C_q} method (20).

Western blotting and antibodies. Cells treated with siRNA were harvested for protein extraction using the RIPA reagent (Pierce; Thermo Fisher Scientific, Inc.). The protein concentration was determined via BCA protein assay reagent. After measuring protein concentration, 50–100 μ g protein was subjected to 10% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was blocked with 5% non-fat milk powder for 1 h at room temperature and incubated with primary antibodies against HIF-1 α (1:1,000; cat. no. ab179483; Abcam), SDF-1 (1:1,000; cat. no. ab25117; Abcam), and CXCR4 (1:1,000; cat. no. ab124824; Abcam) at 4 $^{\circ}$ C overnight, followed by goat-anti-rabbit (1:2,000; cat. no. ab205718; Abcam) secondary antibody for 1 h at 37 $^{\circ}$ C. The chemiluminescent signaling was detected via ECL reagents (Pierce; Thermo Fisher Scientific, Inc.).

Cell proliferation assay. Cell proliferation was assessed using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.). Briefly, MSCs were seeded on 96-well microplates at a density of 1 \times 10⁴ cells/well. The cells were transfected with HilyMax-siRNA and cultured for 0, 24 or 48 h at 37 $^{\circ}$ C. A total of 5 μ l CCK-8 solution was then added to each well and incubated at 37 $^{\circ}$ C for an additional 2 h. Optical density (OD) was determined at a wavelength of 450 nm.

Migration assay. Migration assays were performed using 24-well plate with permeable Transwell inserts (pore size 0.4 μ m; Corning, Inc.). BMSCs were suspended in serum-free DMEM/F12 medium (5 \times 10⁴ cells/well) and equal amounts of cell suspension (200 μ l) was added to the upper chambers on the migration plate. The same amount (200 μ l) of complete medium (DMEM/F12+10% FBS) with different treatment was added to the lower chamber of the migration

Table I. Sequences of siRNA.

siRNA	Sequence (5'→3')
HIF-1 α -Mus-762	F: CUGAUAACGUGAACAAAUATT R: UAUUUGUUCACGUUAUCAGTT
HIF-1 α -Mus-1102	F: CAUUCUCAUCCGUCAAAUTT R: AUUUGACGGAUGAGGAAUGTT
HIF-1 α -Mus-1238	F: GGCCGCUCAAUUUAUGAAUTT R: AUUCAUAAAUUGAGCGGCCTT

siRNA, small interfering RNA; F, forward; R, reverse.

Table II. Sequences of reverse transcription-quantitative PCR primers.

Gene	Sequence (5'→3')
Stromal cell-derived factor-1	F: GCATCAGTGACGGTAAACCA R: TCTTCAGCCGTGCAACAATC
C-X-C chemokine receptor type 4	F: CTAAGGAGCATGACGGACAA R: ATTTCCCAAAGTACCAGTCAGC
GAPDH	F: TGACCTCAACTACATGGTCTACA R: CTTCCCATCTCGGCCTTG

F, forward; R, reverse.

plate separately. There were two sets of experiments. In the first, cells were divided into the following treatment groups for 24 h at 37°C: 0 ng/ml SDF-1+0 μ M AMD3100 (a specific antagonist that blocks the binding of SDF-1 to CXCR4; cat. no. A5602; Sigma-Aldrich; Merck KGaA); 10 ng/ml SDF-1+0 μ M AMD3100; 100 ng/ml SDF-1+0 μ M AMD3100; 100 ng/ml SDF-1+1 μ M AMD3100; and 100 ng/ml SDF-1+10 μ M AMD3100. For the second experiment, cells were divided into the following treatment groups for 24 h at 37°C: NC (siRNA-NC), siRNA (HIF-1 α -siRNA), SDF-1 (siRNA-NC+100 ng/ml SDF-1), and siRNA and SDF-1 group (HIF-1 α siRNA+100 ng/ml SDF-1). After 24 h, the migrated cells on the bottom of the insert were fixed with 4% paraformaldehyde solution for 24 h at room temperature and stained with crystal violet (1%) for 15 min at room temperature. The cells were processed as described in the cell migration assay, and migrated cells were counted, and the relative number was calculated with light microscopy (magnification, x100).

Statistical analysis. Results are representative of three independent experiments. GraphPad Prism (version 7.0; GraphPad Software, Inc.) and SPSS software (version 19; IBM Corp.) were used for statistical analysis of cell proliferation, as well as the results of the Transwell assay and RT-qPCR. Differences between two groups were analyzed using an unpaired t-test. Comparisons between multiple groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Morphological identification of MSCs. The present study determined whether the HIF-1 α /SDF-1/CXCR4-axis serves a role in fracture combined with traumatic brain injury in an *in vitro* BMSC model. MSCs exhibited a typical spindle morphology (Fig. 1A). Cells in the third passage were characterized via flow cytometry. The results demonstrated that the expression levels of CD29 and CD44 on the surface of BMSCs were >95%, whereas the expression level of the hematopoietic stem cell marker CD45 was <5% (Fig. 1B), which was consistent with the characteristics of BMSCs.

HIF-1 α knockdown suppresses MSC proliferation *in vitro*. Next, the effects of HIF-1 α on the migration of MSCs were investigated. In order to assess the effect of HIF-1 α knockdown on cell proliferation, CCK-8 was used to detect proliferation at different time-points. The OD value of HIF-1 α -knockdown cells was significantly decreased at 450 nm (Fig. 2), indicating that HIF-1 α knockdown inhibits MSC proliferation.

SDF-1 promotes cell migration by binding to CXCR4. In order to confirm the effect of SDF-1 in MSCs, its impact on cell migration was analyzed. Treatment of MSCs with various concentrations of SDF-1 caused a dose-dependent increase in cell migration (Fig. 3A). Cell migration induced by SDF-1 was abrogated by AMD3100, indicating that AMD3100 inhibits MSC migration via obstructing SDF-1 binding with CXCR4 (Fig. 3B). Collectively, these results indicated that SDF-1 promoted MSC migration via binding to CXCR4, which was impaired by AMD3100.

HIF-1 α knockdown inhibits MSC migration via SDF-1/CXCR4 signaling. It was then determined whether knockdown of HIF-1 α impairs the chemotactic function of SDF-1/CXCR4 signaling in MSCs. The mRNA and protein levels of SDF-1 and CXCR4 were decreased in MSCs stably transfected with siHIF-1 α compared with NC group (Fig. 4A). Moreover, HIF-1 α knockdown inhibited MSC migration; replenishment of SDF-1 increased cell migration (Fig. 4B and C). Thus, HIF-1 α likely regulates MSC migration via SDF-1/CXCR4 signaling.

Discussion

In the present study, the proliferation rate of MSCs transfected with HIF-1 α siRNA significantly decreased at 24 and 48 h. MSCs are essential for the repair of bone defects, and improving the effects of MSCs in bone is of clinical interest. In patients with skull injuries, ischemia and hypoxia surrounding brain tissue often occur, and the skull bone repair process must be adapted to the hypoxic environment (10,21). HIF-1 α can maintain homeostasis of oxygen under hypoxia, allowing the body to adapt to hypoxia (22). HIF-1 α can induce the formation of blood vessels and bone tissue via numerous factors, including vascular endothelial growth factor, angiopoietin, platelet growth factor and transforming growth factor, making it a potential candidate for the treatment of bone defects and other types of disease (23,24). HIF-1 α is a regulator of BMP2-induced chondrogenic differentiation, osteogenic differentiation and

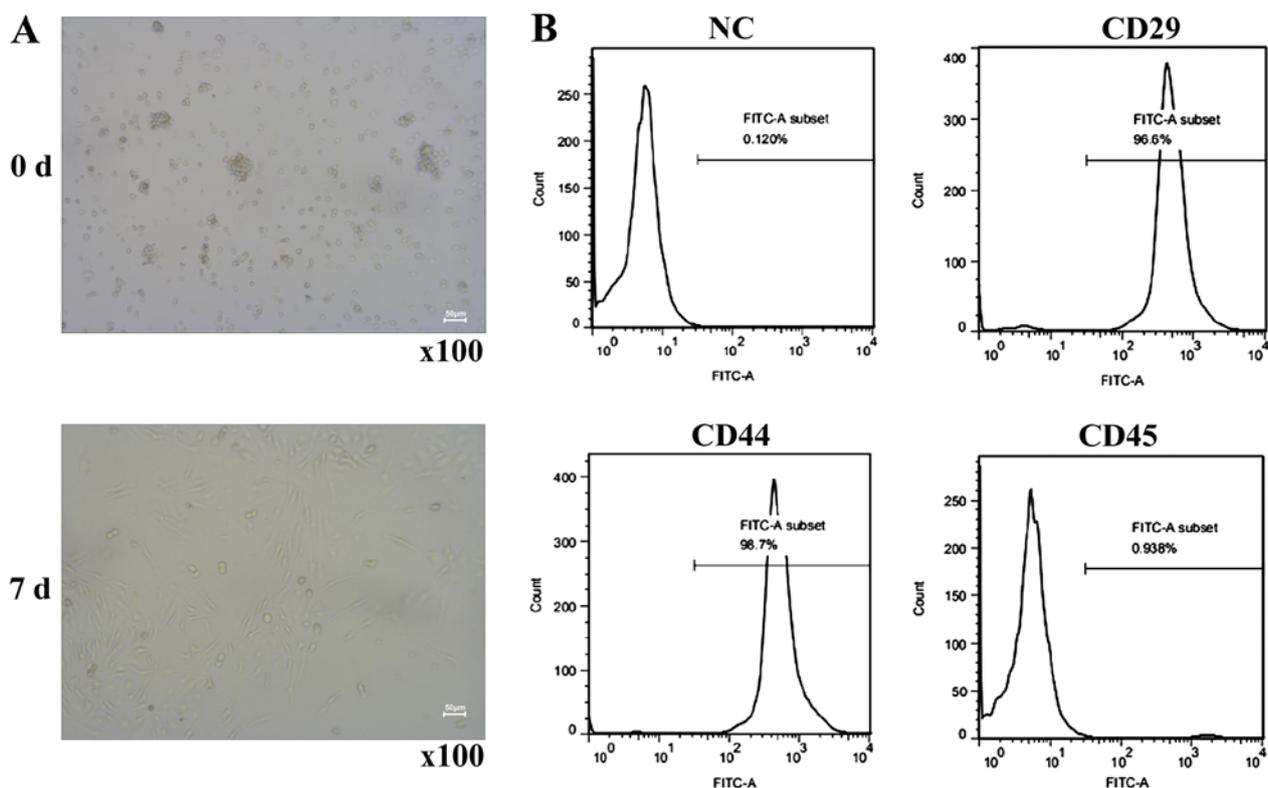


Figure 1. Cell morphology and characterization. Morphology of mouse MSCs at passage 3. (A) MSC morphology observed after one week of primary culture. (B) Immunophenotype of MSCs at passage 3 determined via flow cytometric analysis of CD45, CD29 and CD44 expression levels. MSC, mesenchymal stem cell; NC, negative control.

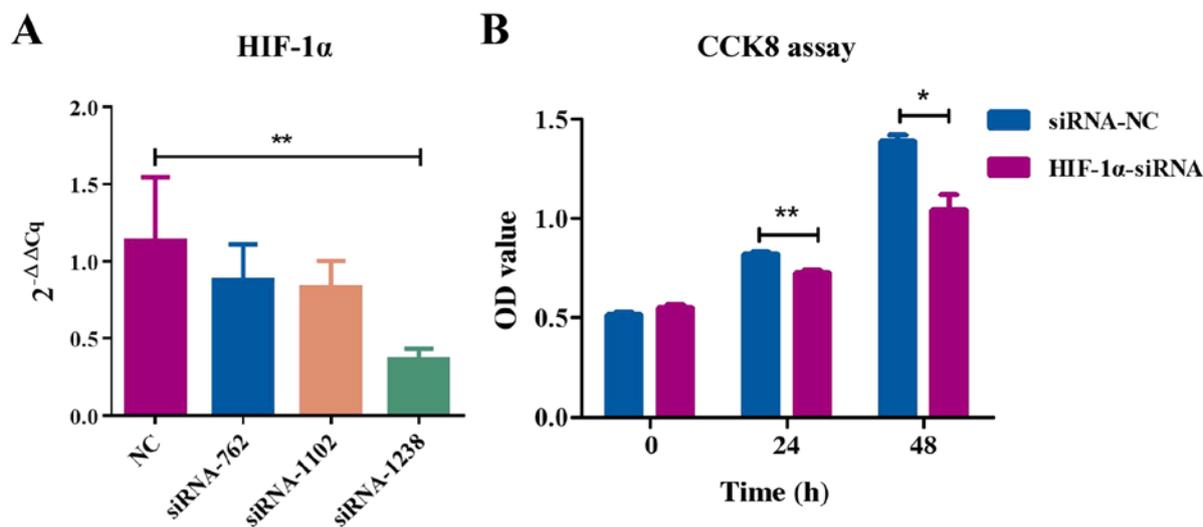


Figure 2. HIF-1 α knockdown inhibits MSC proliferation. (A) HIF-1 α mRNA levels decreased after siRNA-762, siRNA-1102 or siRNA-1238 was transfected into MSCs. The results are representative of three independent experiments. (B) A CCK-8 assay was performed to determine the effects of HIF-1 α knockdown on MSC proliferation. * $P < 0.05$, ** $P < 0.01$. MSC, mesenchymal stem cell; siRNA, small interfering RNA; CCK-8, Cell Counting Kit-8; NC, negative control; OD, optical density.

endochondral bone formation (25). HIF-1 α and BMP2 have been revealed to synergistically induce MSC differentiation and promote the expansion of the proliferating chondrocyte zone (25). HIF-1 also serves an important role in coupling osteogenesis and angiogenesis during skeletal development and bone regeneration, and HIF-1 α overexpression may represent a therapeutic option to improve cellular functions

of MSCs in the treatment of critical-sized bone defects (26). Thus, HIF-1 α may improve the cellular functions of MSCs; however, the underlying molecular mechanisms are still unclear.

SDF-1 is a protein secreted primarily by MSCs. When SDF-1 specifically binds to CXCR4 on a number of types of cell surface, activated SDF-1/CXCR4 can stimulate

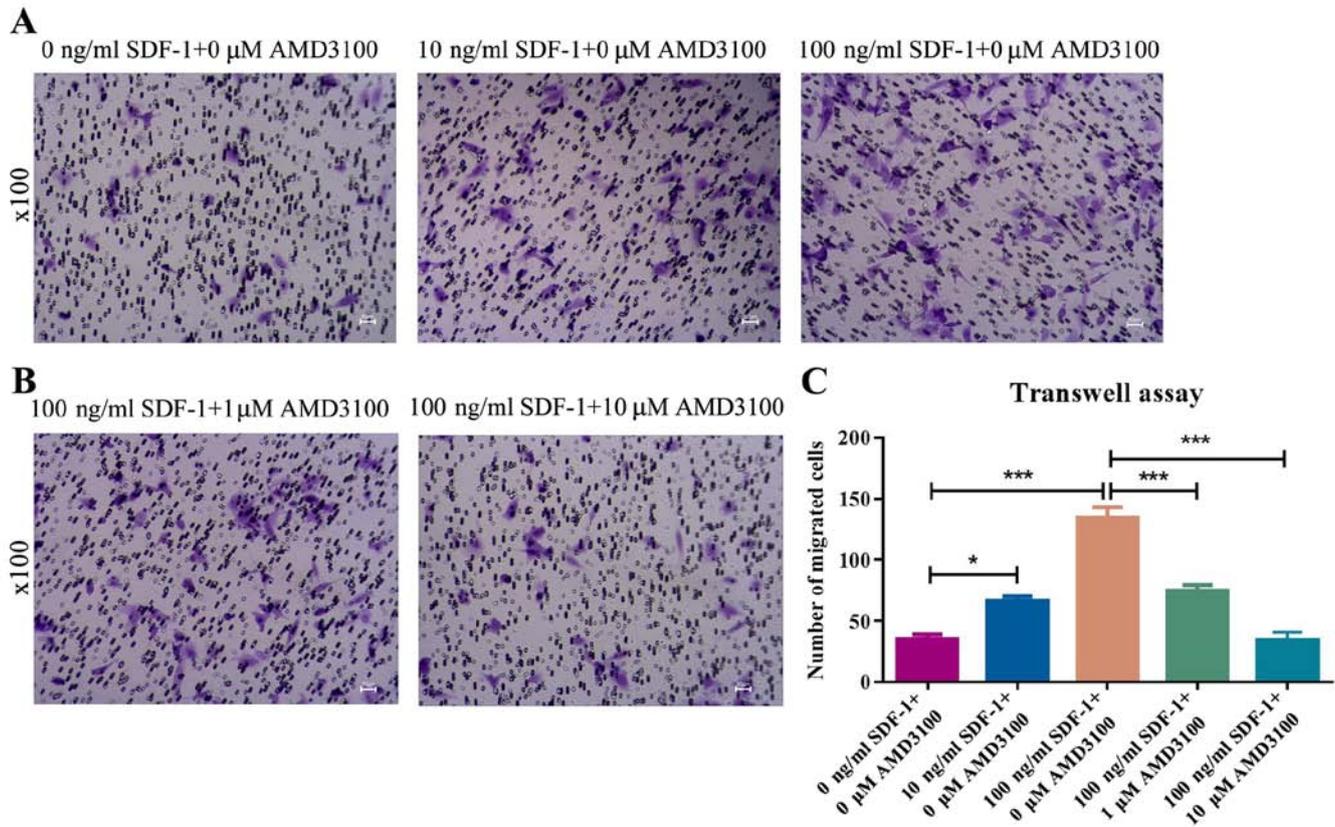


Figure 3. SDF-1 binding to C-X-C chemokine receptor type 4 promotes MSC migration. (A) MSC migration ability was studied via Transwell assay. MSCs were exposed to 0, 10 or 100 ng/ml SDF-1 for 24 h, and migration was observed. (B) MSC migration following exposure to 100 ng/ml SDF-1+1 μM AMD3100 or 100 ng/ml SDF-1+10 μM AMD3100. (C) Graph revealing the average number of migrated cells per field. * $P < 0.05$, *** $P < 0.001$. SDF-1, stromal cell-derived factor-1; MSC, mesenchymal stem cell.

downstream signaling pathways associated with the regulation of stem cell mobilization and migration (27), induce MSC migration to the damaged site and participate in injury repair (28,29). Activation of the ERK-AKT pathway mediates SDF1-induced cell migration. SDF1/CXCR4 signaling is required for MSC homing and retention to their niche in the bone marrow (30). Secretion of SDF-1 has been revealed to increase during bone regeneration, allowing CXCR4-expressing MSCs to enter the stretch region with the concentration gradient of SDF-1 following induction via the SDF-1/CXCR4 molecular axis, and to participate in new bone formation and cardiovascular generation (31-33). In order to confirm the effect of SDF-1 in MSCs, the present study analyzed the chemotactic effect of the SDF-1/CXCR4 axis on MSCs. SDF-1 was demonstrated to promote the migration of MSCs in a dose-dependent manner. This result is similar to that reported by Zhou *et al* (25), who demonstrated that HIF-1 α can regulate cell migration by influencing the expression levels of CXCR4. Additionally, by using AMD3100 to block the binding of SDF-1 to CXCR4, SDF-1-induced cell migration in the present study was abrogated, indicating that AMD3100 inhibits MSC migration by obstructing SDF-1 binding with CXCR4. Therefore, the SDF-1/CXCR4 axis serves a key role in MSC function.

SDF-1/CXCR4 signaling has been revealed to play an important role in cell migration, chemotaxis as well as other

biological behaviors. SDF-1-induced transendothelial behavior has been revealed to be positively associated with the density of cell surface receptor CXCR4 and is affected by a number of factors (34). HIF-1 α is a central transcription factor of hypoxia-specific gene expression levels (30). HIF-1 α can bind to the SDF-1 promoter and specifically regulate the expression levels of SDF-1 (30,35). Hypoxia can upregulate the expression levels of CXCR4 in human monocytes and macrophages, as well as endothelial and tumor cells, via the activation of HIF-1 α (36). The hypoxia/HIF-1 α /CXCR4 pathway may be a mechanism of regulating the migration of different types of cells under hypoxia (37). A number of CXCR4-positive stem cells are involved in angiogenesis in locally damaged areas in a hypoxic environment. The adhesion, migration and homing of these CXCR4-positive stem cells are initiated via binding of SDF-1, containing hypoxia response elements, to CXCR4; the expression levels of SDF-1 are regulated by HIF-1 α (38). The present study therefore determined whether HIF-1 α can recruit MSCs and promote osteogenesis via the SDF-1/CXCR4 molecular axis. Following HIF-1 α knockdown, it was demonstrated that the mRNA and protein levels of SDF-1 and CXCR4 in MSCs were significantly decreased. HIF-1 α gene silencing inhibited the migration of MSCs ($P < 0.05$). This confirmed that SDF-1 promotes the migration of MSC cells and indicated that HIF-1 α acts on MSCs via the SDF-1/CXCR4 molecular axis. However, further investigation is required to elucidate the mechanisms underlying the effect of HIF-1 α on oxidative

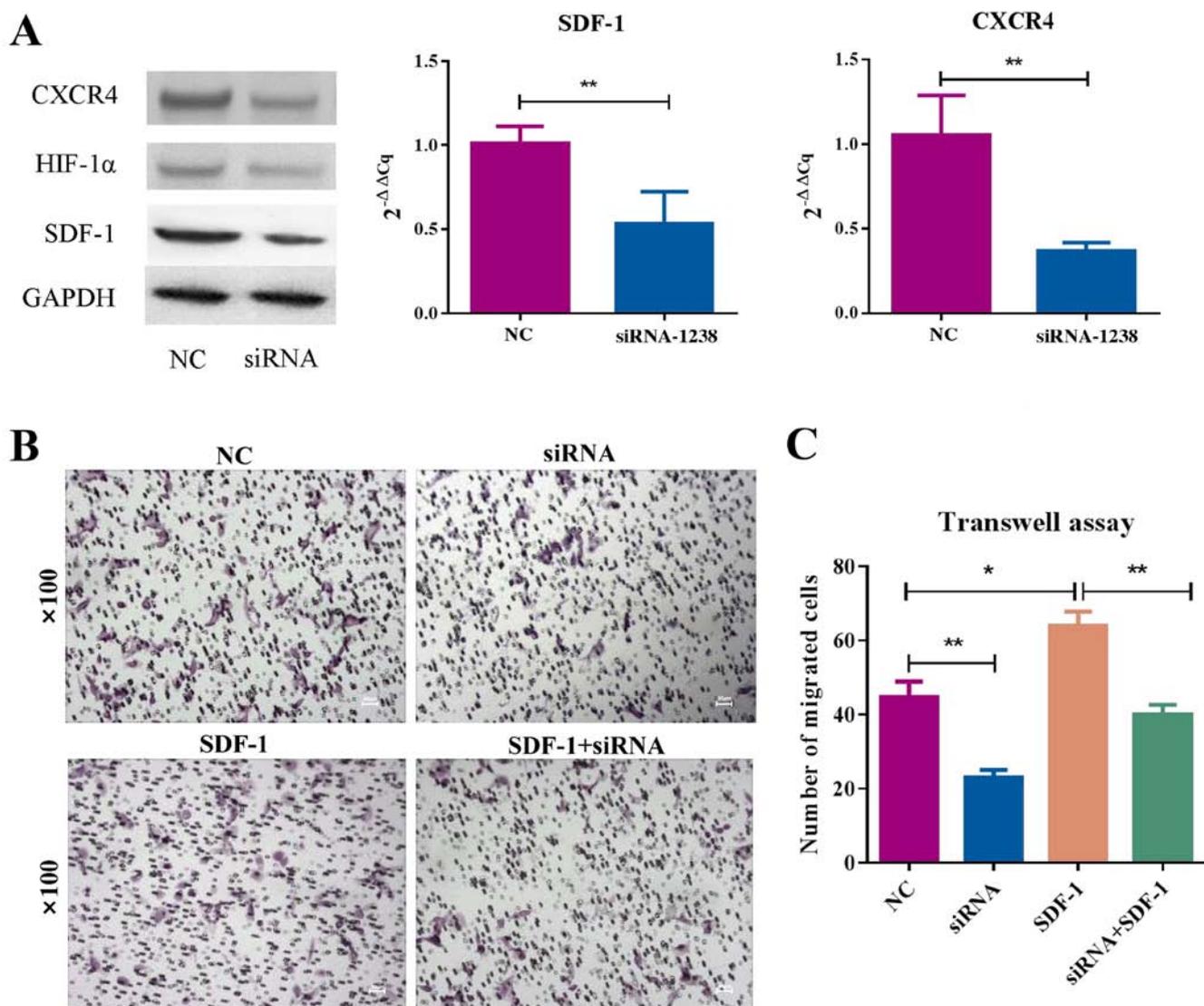


Figure 4. HIF-1 α knockdown inhibits MSC migration via SDF-1/CXCR4 signaling. (A) SDF-1 and CXCR4 in MSCs were identified via western blotting and reverse transcription-quantitative PCR. (B) Migration ability was studied via Transwell assays; MSCs were exposed to siRNA-NC, HIF-1 α -siRNA, siRNA-NC+100 ng/ml SDF-1, or HIF-1 α siRNA+100 ng/ml SDF-1. (C) Graph revealing the average number of migrated cells per field. *P<0.05, **P<0.01. MSC, mesenchymal stem cell; SDF-1/CXCR4, stromal cell-derived factor-1/C-X-C chemokine receptor type 4; siRNA, small interfering RNA; NC, negative control.

stress in the healing process of skull injury, which may involve complex signaling pathways.

During craniocerebral injury healing, the hypoxic state of surrounding brain tissue induces an increase in the secretion of HIF-1 α (39), which accelerates the fracture repair process via chemotaxis due to the SDF-1/CXCR4 axis. In the present study, a gene silencing plasmid was successfully constructed based on the HIF-1 α gene sequence, and the regulatory association between HIF-1 α and SDF-1/CXCR4 was elucidated. Silencing of HIF-1 α decreased MSC migration, as well as the mRNA and protein levels of SDF-1 and CXCR4 in MSCs. Due to the wide distribution and diversity of roles of SDF-1 and CXCR4, they may represent prognostic biomarkers or therapeutic targets for a number of neurological diseases.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WX and YX conceived the study. WX, YX, ZL, RH and XZ performed the experiments and contributed to writing the

manuscript. WX, YX and YW analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Animal Research Ethics Committee of TongRen Hospital, School of Medicine, Shanghai JiaoTong University (approval no. 170004).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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