

## Targeted migration of mesenchymal stem cells modified with CXCR4 to acute failing liver improves liver regeneration

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### Abstract

**AIM:** To improve the colonization rate of transplanted mesenchymal stem cells (MSCs) in the liver and effect of MSC transplantation for acute liver failure (ALF).

**METHODS:** MSC was modified with the chemokine CXC receptor 4 (*CXCR4*) gene (*CXCR4*-MSC) or not (Null-MSC) through lentiviral transduction. The characteristics of *CXCR4*-MSCs and Null-MSCs were determined by real-time quantitative polymerase chain reaction, Western blotting and flow cytometry. *CXCR4*-MSCs and Null-MSCs were infused intravenously 24 h after administration of CCl<sub>4</sub> in nude mice. The distribution of the MSCs, survival rates, liver function, hepatocyte regeneration and growth factors of the recipient mice

were analyzed.

**RESULTS:** *In vitro*, *CXCR4*-MSCs showed better migration capability toward stromal cell-derived factor-1 $\alpha$  and a protective effect against thioacetamide in hepatocytes. *In vivo* imaging showed that *CXCR4*-MSCs migrated to the liver in larger numbers than Null-MSCs 1 and 5 d after ALF. Higher colonization led to a longer lifetime and better liver function. Either *CXCR4*-MSCs or Null-MSCs exhibited a paracrine effect through secreting hepatocyte growth factor and vascular endothelial growth factor. Immunohistochemical analysis of Ki-67 showed increased cell proliferation in the damaged liver of *CXCR4*-MSC-treated animals.

**CONCLUSION:** Genetically modified MSCs expressing *CXCR4* showed greater colonization and conferred better functional recovery in damaged liver.

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**Key words:** Acute liver failure; Cell transplantation; Chemokine CXC receptor 4; Mesenchymal stem cells; Cell mobilization

**Core tip:** Mesenchymal stem cell (MSC) transplantation is an effective therapy for acute liver failure (ALF), and is expected to be an alternative to liver transplantation. Many current studies have exhibited poor efficiency of MSC transplantation due to low colonization in the failing liver. We modified MSCs with the chemokine CXC receptor 4 (*CXCR4*) gene to promote the colonization of MSCs in failing liver depending on the stromal cell-derived factor-1 $\alpha$ /*CXCR4* axis. *In vivo* imaging showed that *CXCR4*-MSCs migrated to the liver in higher numbers than Null-MSCs 1 and 5 d after ALF. Greater colonization led to a longer lifetime and better liver function.

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## INTRODUCTION

In recent years, mesenchymal stem cells (MSCs) have shown paracrine and immunoregulatory effects to repair damaged tissues<sup>[1,2]</sup>. A large number of studies based on stem cell transplantation has achieved impressive results and has provided new ideas for treatment of various diseases. MSC transplantation has also been used to treat a variety of end-stage liver diseases, including acute liver failure (ALF)<sup>[3-5]</sup>. However, many researchers have also found the phenomenon of poor efficacy of cell transplantation. Retrospective studies have revealed that low colonization of transplanted MSCs in the liver was the main reason restricting the efficacy of MSC transplantation<sup>[6]</sup>.

A series of studies has confirmed the accelerative effect of stromal cell-derived factor (SDF)-1 $\alpha$  in homing and survival of stem cells<sup>[7-11]</sup>. SDF-1 $\alpha$  is a chemoattractant protein of the CXC family produced by bone marrow stromal cells. SDF-1 $\alpha$  and its receptor, chemokine CXC receptor 4 (CXCR4), are widely expressed in a variety of cells and tissues, including immune cells, brain, heart, liver, kidney, lung and spleen<sup>[12]</sup>. A large number of studies have shown that the SDF-1 $\alpha$ /CXCR4 axis plays an important role in stem cell homing, chemotaxis, expression of adhesion molecules, engraftment, proliferation and survival<sup>[13,14]</sup>. SDF-1 $\alpha$  can mobilize bone marrow stem cells and promote their migration to damaged tissue by binding to CXCR4 distributed on stem cell membranes<sup>[9-11]</sup>. Zhang *et al.*<sup>[15]</sup> have shown that the SDF-1 $\alpha$ /CXCR4 axis can significantly improve the number of transplanted stem cells in the infarcted myocardium and cardiac function. Liu *et al.*<sup>[16]</sup> have also found that hypoxic stimulation can improve CXCR4 expression of MSCs, which enhances functional recovery, accelerates the mitogenic response, and reduces apoptotic cell death in the kidneys. Moreover, Du *et al.*<sup>[17]</sup> have recently shown improved homing of CXCR4-transduced MSCs to the liver post-transplantation, with improved liver function.

CXCR4 expression is endogenously regulated by tissue environmental factors such as cytokines, chemokines, stromal cells, adhesion molecules, hypoxia, tissue damage and proteolytic enzymes<sup>[18]</sup>. SDF-1 $\alpha$  is secreted by cells within the damaged liver, and is a potent chemoattractant for cells expressing CXCR4. Overexpression of CXCR4 appears to be an effective strategy to accelerate mobilization of these cells toward the damaged area. Due to low native levels of CXCR4 expression in MSCs, they migrate sluggishly towards an SDF-1 $\alpha$  gradient following ALF. We hypothesized that overexpression of CXCR4 in MSCs would enhance their engraftment in the damaged liver and improve liver regeneration.

## MATERIALS AND METHODS

### Ethics statement

This study was approved by the Institutional Animal Care and Use Committee of Nanjing University, China under the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize suffering.

### Animals

Male 7-wk-old nude mice (*nu/nu*) were purchased from the Laboratory Animal Center of the Affiliated Drum Tower Hospital of Nanjing University Medical School, and housed individually, placed in a ventilated cabinet under controlled air pressure and temperature conditions, and under daily cycles of alternating 12 h light/dark. They had sterile water and sterile standard pelleted rodent diet. The mice were sacrificed by cervical spine dislocation.

### Lentiviral vector design and virus production

The human CXCR4 gene sequence was synthesized by Invitrogen (Shanghai, China) and digested by *PacI/AscI*. The identity of the gene was confirmed by sequencing, and was subsequently cloned into the same restriction sites on the shuttle vector pLenti6.3-hCXCR4-IRES2-EGFP, which contains enhanced green fluorescence protein (EGFP). The resulting shuttle vector was mixed with Packaging Mix (pLP1, pLP2 and pLP/VSVG; Invitrogen). The mixed vector was added into the Opti-MEM (Invitrogen) culture medium and transfected into the 293T packaging cell line. Recombinant lentivirus expressing both EGFP and CXCR4 was harvested after 2 d and viral titers were determined.

### CXCR4 labeling of MSCs

The human bone marrow MSCs, UE7T-13 cells<sup>[19,20]</sup>, were used. These cells were immortalized by infection with a retrovirus carrying human telomerase reverse transcriptase (hTERT) and one of the early genes of the human papilloma virus, E7. Although hTERT has been introduced into UET7T-13 cells, it is reported that the differentiation potential of the cells is not affected<sup>[19]</sup>. MSCs were cultured overnight in six-well plates ( $1.0 \times 10^5$  cells/well). The lentivirus with CXCR4 gene (Lenti6.3-hCXCR4-IRES2-EGFP) or without CXCR4 gene (Lenti6.3-eGFP) were diluted by culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, United States), 2% (v/v) fetal bovine serum (Invitrogen) and 8  $\mu$ g/mL polybrene (Invitrogen). MSCs were transduced using dilutions of concentrated virus equivalent to  $5 \times 10^6$  infectious units (MOI = 50). MSCs transduced with Lenti6.3-eGFP served as a control. Blasticidin (Invitrogen) was added to the culture medium 48 h later. Culture medium with Blasticidin was changed every 2-3 d.

### Quantitative real-time reverse transcriptase polymerase chain reaction

Total RNA was isolated from fresh cells using TRIzol

reagent (Invitrogen). First-strand cDNA was synthesized using the Superscript II Reverse Transcriptase Kit (Invitrogen). Quantitative polymerase chain reaction (qPCR) was performed using Power SYBR Green PCR Master Mix (ABI, United States). The relative level of gene expression was normalized to an internal control (level of GAPDH) and calculated using the  $2^{-\Delta\Delta CT}$  method. The sequences of primers was: human CXCR4 sense primer sequence: 5'-CCACGCCACCAACAGTCAGA-3' and antisense primer sequence: 5'-AGGCAGGATAAGGC-CAACCAT-3'; human GAPDH sense primer sequence: 5'-GAAGGTCGGAGTCAACGGATT-3' and antisense primer sequence: 5'-CGCTCCTGGAAGATGGT-GAT-3'. Biological repeats were performed using three different samples for each genotype, and technical triplicates were carried out for each gene expression analysis. The mean relative expression of each gene between groups was used for statistical significant analysis.

#### **Western blotting for membrane CXCR4 expression**

Membrane proteins were obtained by membrane proteins extraction kit according to the manufacturer's instructions (Beyotime, China). About  $5 \times 10^7$  cells were centrifuged at 600 *g* for 5 min at 4 °C. The pellet was washed with 2 mL ice-cold PBS. The supernatant was removed without disturbing the pellet and discarded. Phenylmethylsulfonyl fluoride (protease inhibitor) was added to membrane proteins extraction reagent A 2 min before use. One milliliter of membrane proteins extraction reagent A was added to the wall of the tube and the cell pellet was mixed, resuspended and incubated for 10 min at 4 °C under gentle agitation. Cell nuclei and undisturbed cells were sedimented at 700 *g* and 4 °C for 10 min. The supernatant was collected without sedimentation. The cell membrane fragments were sedimented at 14000 *g* for 30 min at 4 °C. The supernatant was removed. Two hundred microliters of membrane proteins extraction reagent B was added to the tube and the sediment was resuspended with 5 s vortex agitation. The tube was kept on ice for 10 min. Membrane protein was extracted by centrifugation at 14000 *g* for 5 min at 4 °C. The supernatant (membrane fraction) was collected and stored at -20 °C until used for western blotting analysis. Membrane protein extract (20 µg) was separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% milk in Tris-buffered saline solution (pH 7.6) containing 0.05% Tween-20, and incubated with primary antibodies for CXCR4 (Abcam, Cambridge, United Kingdom) overnight at 4 °C. The membrane was incubated for 1 h with horseradish-peroxidase-conjugated secondary antibody at room temperature, washed and developed with the ECL plus kit (Millipore, Billerica, MA, United States).

#### **Flow cytometry**

The rate of CXCR4 expression was determined by flow cytometry. CXCR4-MSCs were labeled by APC Mouse Anti-Human CD184 (BD Pharmingen, San Diego, CA,

United States) according to manufacturer's instructions. About  $1 \times 10^6$  cells were transferred into a flow cytometry tube and centrifuged at 453 *g* for 5 min at 4 °C. The cells were washed with 1 mL PBS and centrifuged at 453 *g* for 5 min at 4 °C. The cells were resuspended in 1 mL PBS and mixed with 20 µL APC Mouse Anti-Human CD184. Incubation was carried out with the antibody at room temperature in the dark. The tube was centrifuged at 453 *g* for 5 min at 4 °C. The supernatant was removed and the cells were washed with 1 mL PBS and centrifuged at 453 *g* for 5 min at 4 °C twice. The cells were resuspended in 500 µL PBS and measured by flow cytometry.

#### **ELISA for SDF-1 $\alpha$**

SDF-1 $\alpha$  was measured in liver tissue collected after injecting CCl<sub>4</sub>. For detection of SDF-1 $\alpha$  in liver tissue, frozen tissue samples were weighed before homogenization. One hundred milligrams of tissue was minced and homogenized in 1 mL PBS with a glass homogenizer on ice. The homogenates were centrifuged at 13400 *g* for 5 min and the supernatants were stored at -80 °C prior to analysis. The concentration of SDF-1 $\alpha$  was determined using ELISA kits according to the manufacturer's instructions (R and D Systems, Minneapolis, MN, United States), and the wells were read at 450 nm on an optical plate reader. Standard curves were prepared using purified cytokine standards. Each experimental sample was run in duplicate.

#### **Transwell migration assay**

Migration assays were carried out in a six-well Transwell using polycarbonate membranes with 8-µm pores (Millipore, Germany). Null-MSCs or CXCR4-MSCs at a density of  $2 \times 10^5$  cells/mL in 100 µL medium ( $\alpha$ -minimal essential medium + 0.5% fetal bovine serum) were placed in the upper chamber of the Transwell assembly. The lower chamber contained 600 µL medium with 30 ng/mL SDF-1 $\alpha$  (Sigma, St Louis, MO, United States). After incubation at 37 °C and 5% CO<sub>2</sub> for 10 h, the upper surface of the membrane was scraped gently to remove nonmigrating cells and washed with PBS. The membrane was fixed in 4% paraformaldehyde for 15 min and stained with 0.5% crystal violet for 10 min. The number of migrating cells was determined by counting five random fields per well under the microscope at 400  $\times$  magnification.

#### **Hepatocyte injury by thioacetamide and recovery of cell viability**

Hepatocytes were isolated from nude mice by a two-step collagenase perfusion procedure. The cells were seeded in the lower level of Transwell chambers at a density of  $1 \times 10^5$  cells/cm<sup>2</sup> in hepatocyte RPMI 1640 medium. On day 2, thioacetamide (TAA) (Sigma-Aldrich, Poole, Dorset, United Kingdom), a liver toxin, was added to DMEM at a final concentration of 0.2 µg/µL. At 6 h after addition of TAA,  $1 \times 10^5$  Null-MSCs or CXCR4-MSCs preseeded in Transwell chambers were placed into the well containing TAA-treated hepatocytes for subsequent coculture. An empty Transwell chamber served as the control. Cell



viability was determined with the WST-1 kit (Roche, Burgess Hill, West Sussex, United Kingdom) and the caspase-3 activity was determined using a Caspase-3 kit (Invitrogen, Paisley, United Kingdom).

### Experimental groups and MSCs infusion

Seventy-five nude mice were randomly divided into three groups. On day 1, 20% v/v CCl<sub>4</sub> (8 μL/g, dissolved in olive oil) was administered intraperitoneally to establish an ALF model. On day 2, Group A (*n* = 25) was injected with PBS *via* the tail vein. Group B (*n* = 25) received 1 × 10<sup>6</sup> Null-MSCs and Group C (*n* = 25) was treated with 1 × 10<sup>6</sup> CXCR4-MSCs *via* the tail vein. In each group, 10 mice were picked randomly for survival analysis. The remaining mice were sacrificed on days 1, 3, 5, 7 and 14. Serum was used for biochemical analyses. The livers were dissected out and frozen in liquid nitrogen.

### Labeling MSCs with DiR

The MSCs were washed three times with PBS, trypsinized with 0.05% trypsin-EDTA (Gibco, United States). The MSCs were incubated with 50 μmol/L DiR buffer for 20 min at 37 °C according to the manufacturer's protocol (Fanbo Biochemicals, Beijing, China). The DiR-labeled MSCs were centrifuged at 453 *g* for 5 min and the cells were resuspended in PBS. This procedure was repeated twice to ensure complete removal of any unbound dye.

### Histological analysis and serum transaminase levels

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in mouse blood were measured with an automated biochemical analyzer. For histology, livers were fixed in 4% formaldehyde for 24 h and embedded in paraffin. Five-micrometer-thick liver sections were deparaffinized and fixed. Sections were stained with hematoxylin and eosin (Sigma-Aldrich).

### Tracking of the transplanted MSCs

The nude mice were anesthetized with chloral hydrate and placed in the *In Vivo* Imaging System (IVIS Spectrum, Caliper Life Sciences, Runcorn, Cheshire, United Kingdom) and images were acquired using the CCD camera at 1 and 5 d. Data analysis was performed using Living Image version 4.3.1 (Caliper Life Sciences).

### Cytokine levels

Serum samples were collected from nude mice at 14 d after administration of PBS, Null-MSCs, or CXCR4-MSCs. Quantification of serum levels of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) was determined by ELISA as per the manufacturer's instructions (R and D Systems).

### Observation of hepatocyte proliferation

Liver tissues were harvested at 14 d and made into paraffin sections for Ki-67 immunohistochemical staining. Anti-Ki67 antibody (Abcam) was used to detect Ki-67 protein expression in hepatocytes, and hematoxylin was

used to indicate the nucleus of the hepatocytes.

### Statistical analysis

All experiments were replicated a minimum of three times. Statistical analysis was performed with SPSS version 19.0, and data were expressed as means ± SD. For survival analyses, a Kaplan-Meier method was used. All other data were analyzed by the independent-sample test. *P* < 0.05 was considered statistically significant.

## RESULTS

### CXCR4 expression in genetically modified MSCs

Expression of the CXCR4-EGFP fusion protein was driven by virus long terminal repeat (Figure 1A). MSCs lentivirally transduced with the Null-eGFP gene (Null-MSCs) and CXCR4-eGFP fusion gene (CXCR4-MSCs) were analyzed for expression of CXCR4 by fluorescence microscopy (Figure 1B). CXCR4 overexpression in MSCs was verified by qRT-PCR (Figure 1C). qRT-PCR showed that CXCR4 expression was > 10000-fold higher in CXCR4-MSCs compared with Null-MSCs. Overexpression of CXCR4 was confirmed by Western blotting using membrane extraction (Figure 1D). Flow cytometry showed the fraction of MSCs expressing CXCR4 and it was > 200-fold greater (83.9%) in the CXCR4 group than the Null group for CXCR4 (0.4%, Figure 1E).

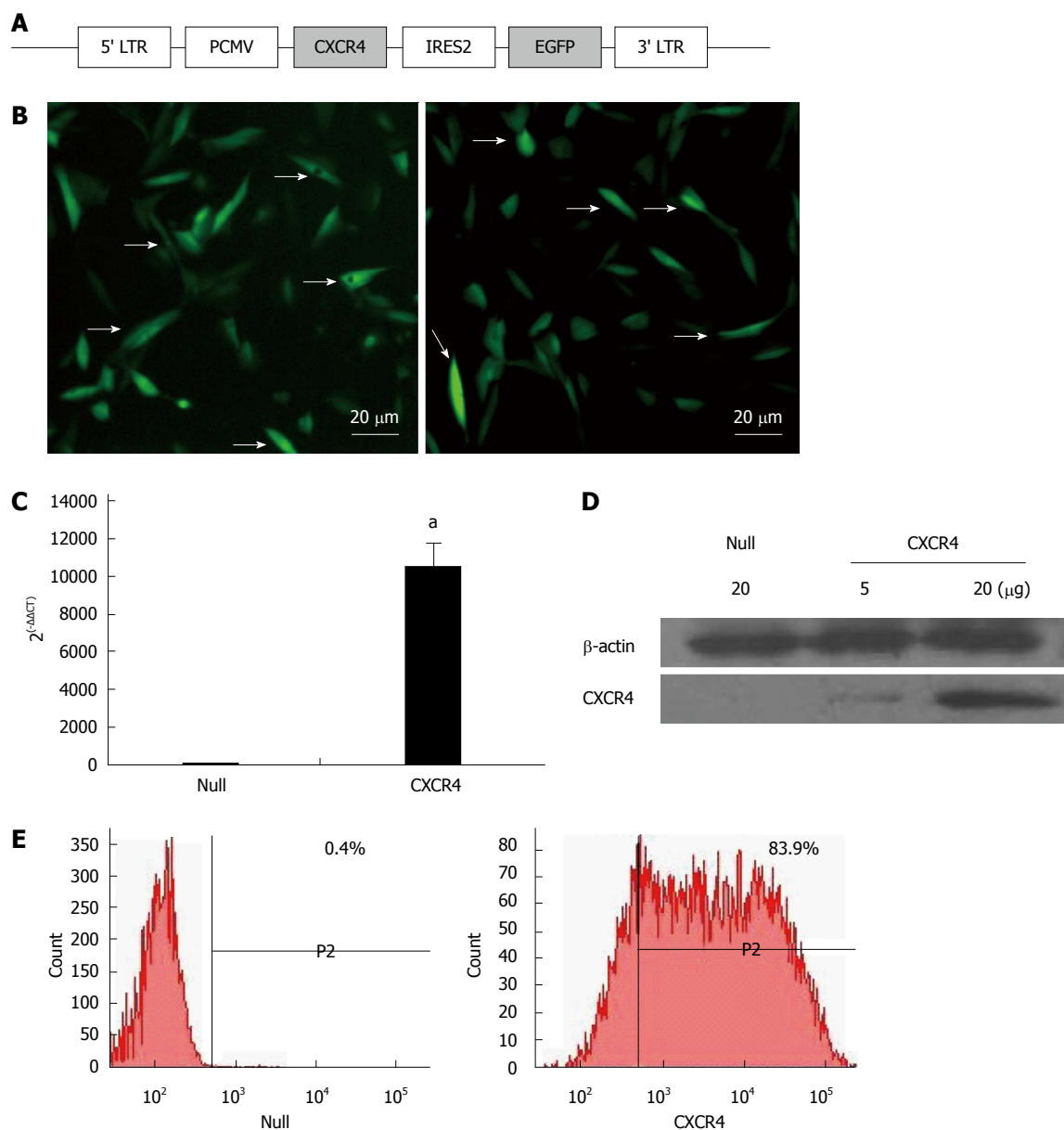
### CXCR4 gene enhances MSC migration depending on SDF-1α

CCl<sub>4</sub> (20% v/v, dissolved in olive oil) was administered to nude mice at a concentration of 8 μL/g. SDF-1α concentration in liver tissue homogenate was detected at 3, 5 and 7 d, and 2 and 3 wk after administration of CCl<sub>4</sub>. The results revealed that compared with the normal group, during day 3 to week 3, SDF-1α concentration increased significantly and was maintained at a relatively stable level (Figure 2A) (*P* < 0.05 *vs* normal group).

We investigated whether exogenous overexpression of CXCR4 enhanced chemotaxis of MSCs toward an SDF-1α gradient in a Transwell migration assay. CXCR4 modification of MSCs significantly increased the number of cells migrating toward SDF-1α. The number of migrating CXCR4-MSCs was more than threefold greater than the number of migrating Null-MSCs (*P* = 0.03) (Figure 2B and C), indicating that overexpression of CXCR4 enhanced the ability of MSCs to respond to SDF-1α-induced chemotaxis.

### MSCs prevent cell death in liver cells

It has been reported that MSCs can secrete a broad variety of cytokines, chemokines and growth factors<sup>[21]</sup>, which may prevent the adjacent cells from death and promote regeneration of injured tissue. Therefore, we tested the ability of CXCR4-MSCs and Null-MSCs to inhibit indirectly TAA-induced death of hepatocytes. We found that hepatocytes treated with TAA displayed low viability, while those administered CXCR4-MSCs or Null-MSCs



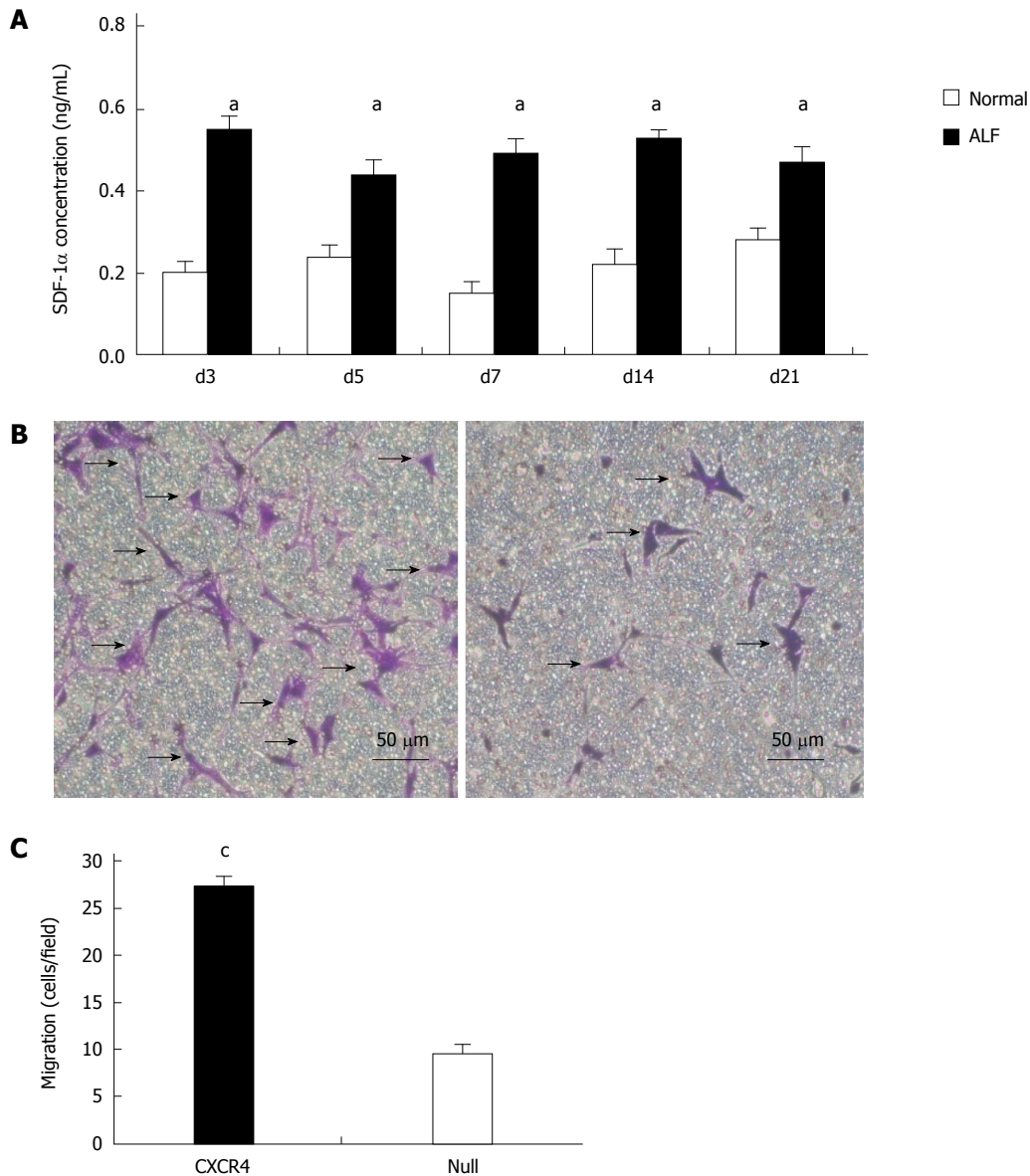
**Figure 1 Chemokine CXCR4 expression in genetically modified mesenchymal stem cells.** A: Schematic representation of the lentiviral vector constructs; B: Fluorescence microscopy of green fluorescence protein (GFP)/Null-mesenchymal stem cells (MSCs) and GFP/chemokine CXCR4 (CXCR4)-MSCs. Most MSCs were uniformly transfected with GFP gene (arrows). Scale bar = 20  $\mu\text{m}$ ; C: CXCR4 gene relative expression by quantitative real-time polymerase chain reaction; D: CXCR4 expression on the membrane was determined by Western blotting using MSC membrane extraction. CXCR4: CXCR4/GFP group; Null: null/GFP group; E: Flow cytometry analysis of CXCR4 expression in Null-MSCs (left) and CXCR4-MSCs (right). Horizontal bar of each label in panel represents subpopulation of MSCs. LTR: Long terminal repeat. <sup>a</sup> $P < 0.05$  vs Null group, data are shown as mean  $\pm$  SD.

showed higher viability (Figure 3A). It is known that TAA can activate the caspase-3 signaling pathway<sup>[22]</sup>, and we analyzed cells for caspase-3 activity. A reduced level of caspase-3 activity in hepatocytes treated with either CXCR4-MSCs or Null-MSCs was observed (Figure 3B). These results suggested that MSCs can secrete factors that prevent cell death.

**MSCs improve survival rate and liver histopathology and inhibit liver enzyme release**

During the 4 wk after cell transplantation, most of the mice (8/10) that received only PBS died within 4 d, and 5/10 mice that received Null-MSCs survived up to 4 wk.

However, 8/10 mice infused with CXCR4-MSCs survived up to 4 wk (Figure 4A). To investigate the liver histology of the mice with ALF after transplantation of MSCs, hematoxylin and eosin staining was performed. The liver histology of the normal mice showed uniform cellular morphology (Figure 4B). In contrast, failing liver showed extensive hepatocyte necrosis with hemorrhaging involving entire lobules, and the hepatocytes present had a swollen cytoplasm (Figure 4B). In the images of mice transplanted with Null-MSCs, there were many light-staining areas, indicating the presence of damaged hepatocytes (Figure 4B). However, this tendency was improved in the images of mice transplanted with CXCR4-MSCs (Figure



**Figure 2 Chemokine CXCR4 gene enhances mesenchymal stem cells migration depending on stromal cell-derived factor-1 $\alpha$ .** A: Stromal cell-derived factor (SDF)-1 $\alpha$  expression in acute liver failure (ALF). Values are mean  $\pm$  SD,  $n = 6$  for each group; B: Images of transmigrated chemokine CXCR4 (CXCR4)-mesenchymal stem cells (MSCs) (left) and Null-MSCs (right) in response to 50 ng/mL SDF-1 $\alpha$  in Transwell assay; C: Average number of cells migrated in Transwell assay. Results are mean values  $\pm$  SD of five different fields from four independent experiments. <sup>a</sup> $P < 0.05$  vs normal group; <sup>c</sup> $P < 0.05$  vs Null group, data are shown as mean  $\pm$  SD.

4B). Serum ALT and AST were detected at 7 d after cell infusion. When compared with the mice in the Null-MSCs group, the mice receiving CXCR4-MSCs showed better improvement of liver function, and liver function in both the CXCR4-MSC and Null-MSC groups was significantly better than in the PBS cohort (Figure 4C).

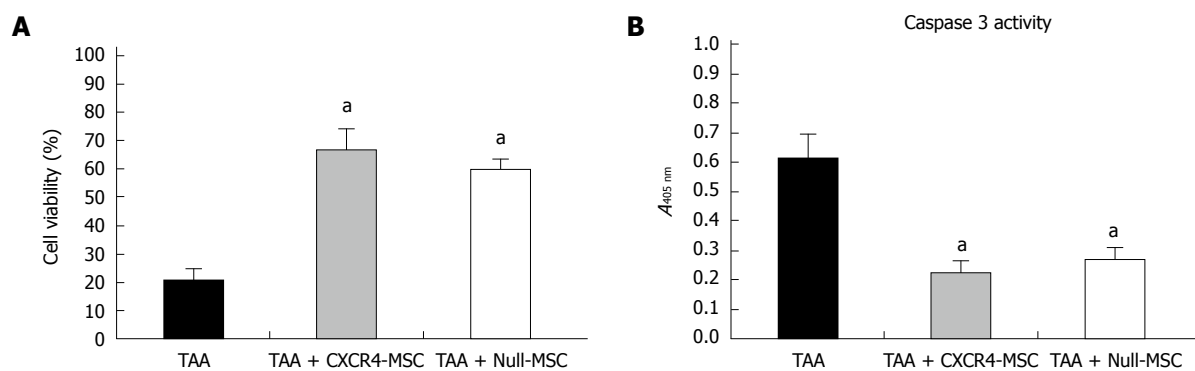
#### Homing and distribution of transplanted MSCs

We used IVIS to identify *in vivo* distribution of MSCs labeled with DiR. At 1 d post-transplantation in the CXCR4 group, a strong fluorescence signal was emitted from the liver, while the fluorescence intensity was low in the spleen. In the Null group, the liver and spleen both exhibited a strong fluorescence signal and the intensity was almost the same. At 5 d following transplantation, the

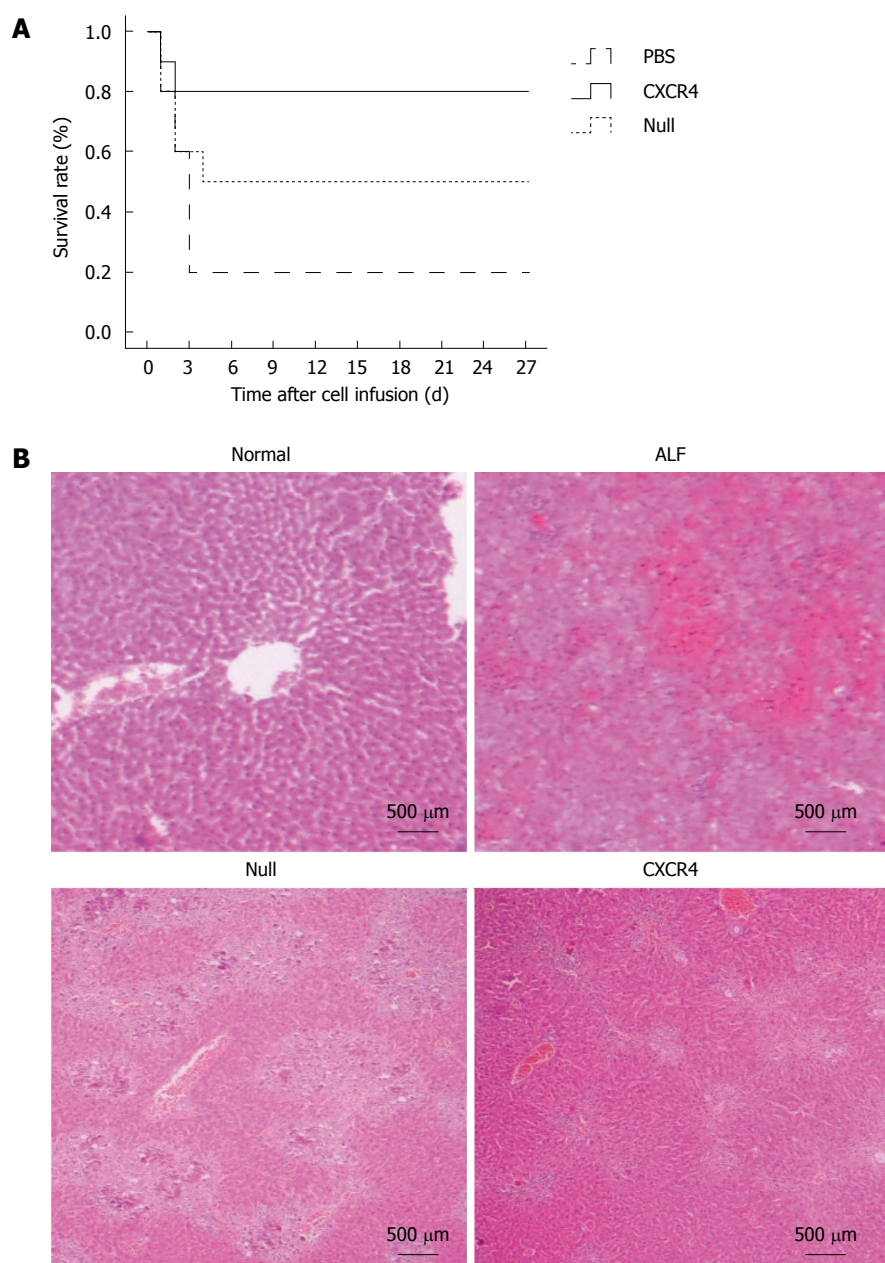
fluorescence signal emitted from the spleen faded in the CXCR4 group, and in the Null group, the signal intensity of the liver and spleen was also nearly the same (Figure 5). No signal could be detected in any other organs.

#### Growth factor levels and hepatocyte proliferation

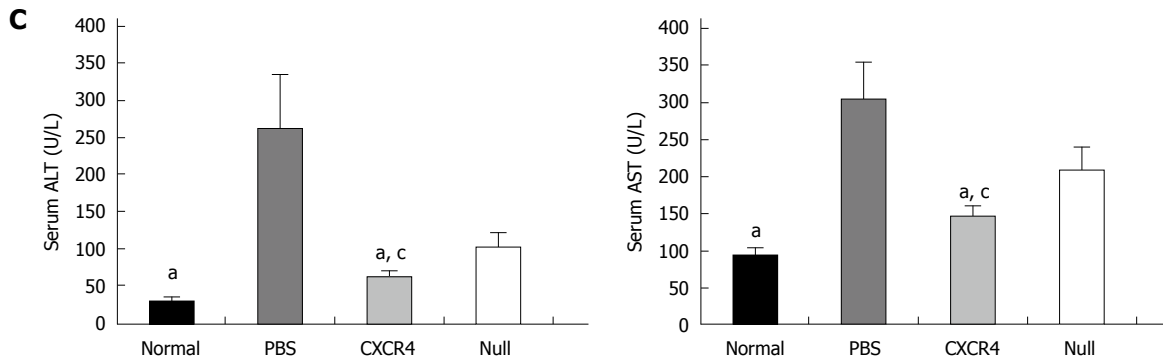
Two weeks after cell transplantation, serum showed a significant increase in HGF and VEGF in the cell transplantation group when compared with the PBS group. The level of HGF and VEGF in the CXCR4 group was approximately 1.5-fold higher than that in the Null group (Figure 6A). To evaluate whether stem cell transplantation enhanced the proliferation of hepatocytes in failing liver, the Ki67 expression level was assessed by immunohistochemistry. In the cell transplanted livers, the number



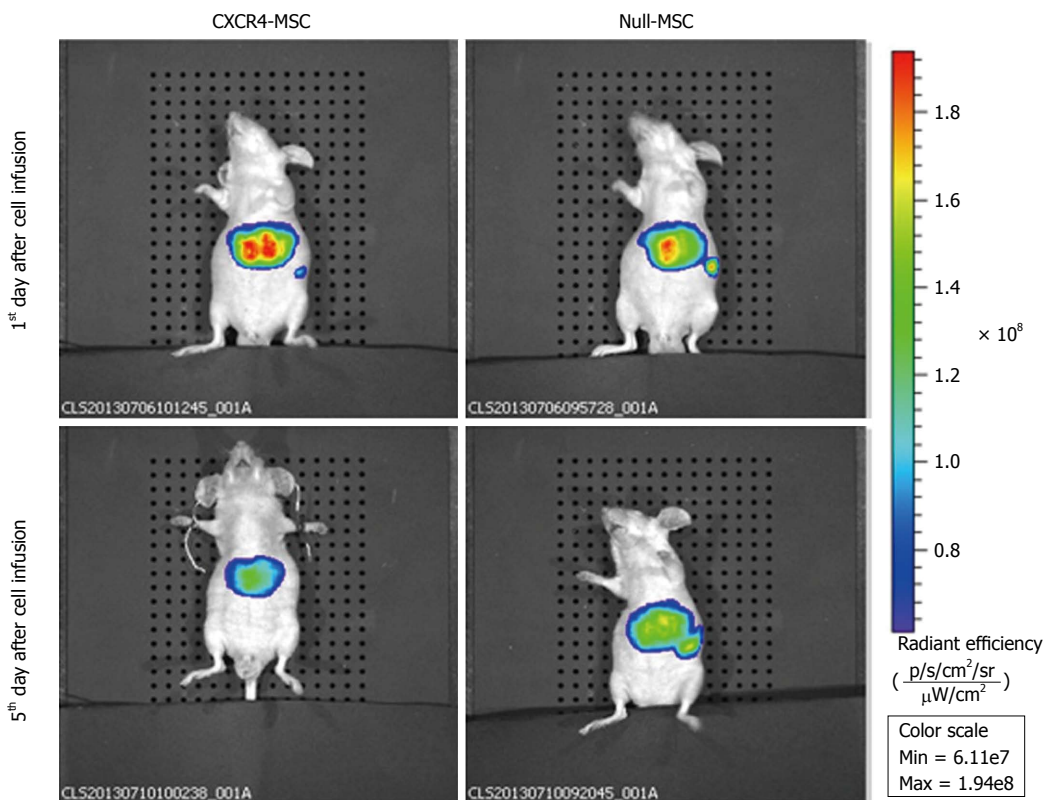
**Figure 3** Indirect coculture with mesenchymal stem cells enhanced viability of the thioacetamide-damaged hepatocytes. A: Viability of damaged mouse hepatocytes cocultured with chemokine CXC receptor 4 (CXCR4)-mesenchymal stem cells (MSCs) or Null-MSCs; B: Caspase-3 activity of damaged mouse hepatocytes cocultured with CXCR4-MSCs or Null-MSCs. <sup>a</sup>*P* < 0.05 vs thioacetamide (TAA) group, data are shown as the mean ± SD.







**Figure 4** Transplantation of chemokine CXCR4-mesenchymal stem cells or Null-mesenchymal stem cells improved survival rate and histopathological recovery, and inhibited liver enzyme release in acute liver failure. A: Survival analysis of CCl<sub>4</sub>-treated nude mice; B: HE staining: normal liver tissue; acute liver failure (ALF) mice showed typical histology with extensive hepatocyte necrosis and hemorrhage involving entire lobules; ALF mice transplanted with Null-mesenchymal stem cells (MSCs); ALF mice transplanted with chemokine CXCR4 (CXCR4)-MSCs; C: Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzyme levels in peripheral blood samples collected at 7 d after transplantation. <sup>a</sup>*P* < 0.05 vs phosphate-buffered saline (PBS) group; <sup>c</sup>*P* < 0.05 vs Null group, data are shown as mean ± SD.



**Figure 5** *In vivo* fluorescence images of nude mice at 1 and 5 d after injecting DiR-labeled mesenchymal stem cells via the tail vein. At 1 d after transplantation in the chemokine CXCR4 (CXCR4) group, a strong fluorescence signal was detected in the liver, whereas the fluorescence intensity was low in the spleen. In the Null group, the liver and spleen both exhibited a strong fluorescence signal and the intensity was almost the same. At 5 d following transplantation, the fluorescence signal in the spleen of the CXCR4 group could not be detected, and in the Null group, although the fluorescence intensity decreased compared with 1 d after transplantation, the liver and spleen showed nearly the same signal intensity. MSC: Mesenchymal stem cells.

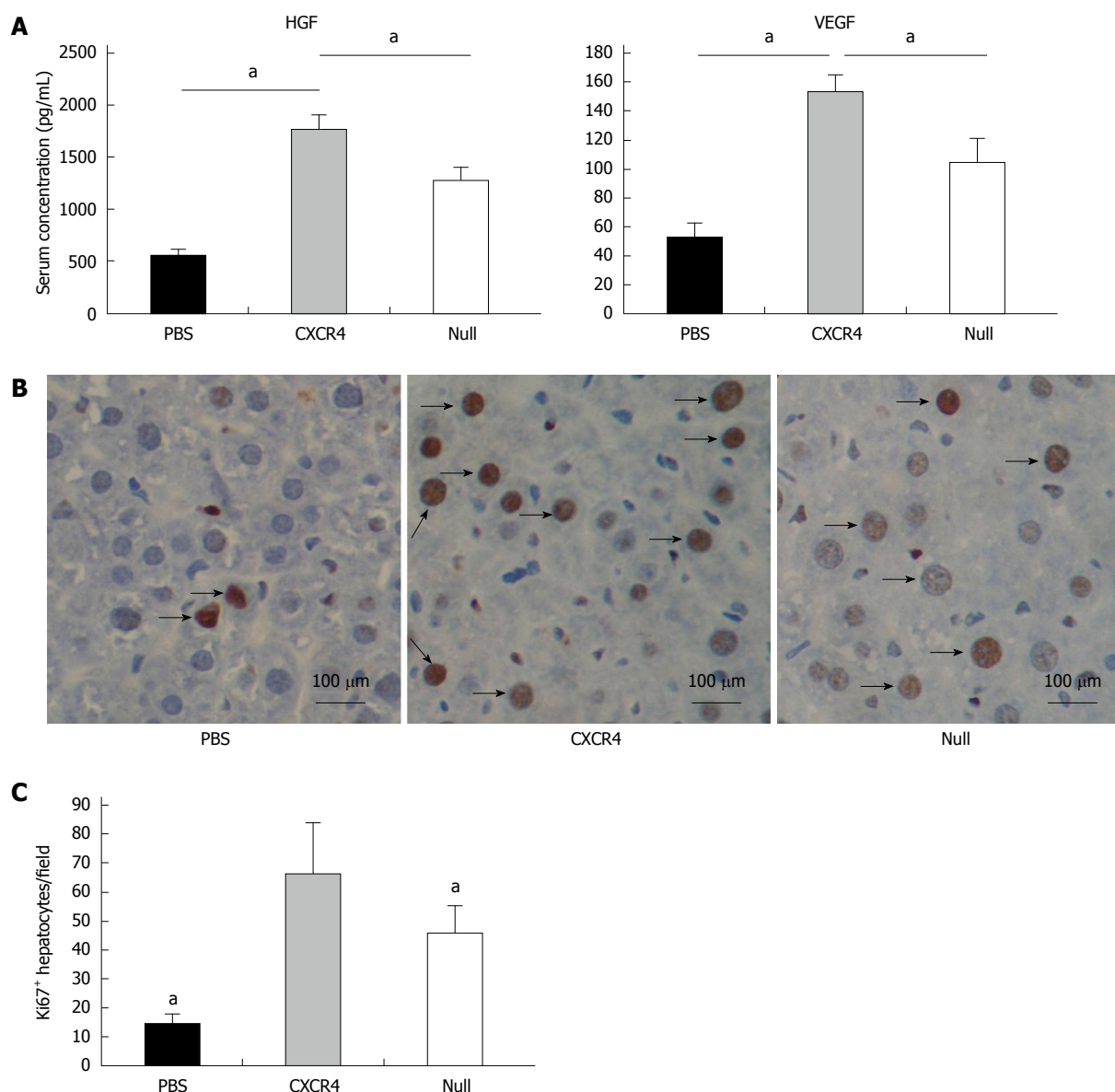
of Ki67<sup>+</sup> cells increased significantly when compared with the PBS group. More Ki67<sup>+</sup> cells were observed in the CXCR4-MSC group than the Null-MSC group (Figure 6B and C).

## DISCUSSION

Hepatocyte damage induced by liver failure stimulates the secretion of various cytokines and chemokines that are

involved in mediating the process of tissue repair. SDF-1 $\alpha$  plays an important role in the homing and recruitment of MSCs to the liver. Increased SDF-1 concentrations in the liver following induction of ALF have been reported previously<sup>[23]</sup>. In our study, we detected that the SDF-1 $\alpha$  concentration in liver homogenate increased after liver failure and was maintained at a high level for 3 wk (Figure 2). CXCR4 is the receptor for SDF-1 $\alpha$  and the SDF-1 $\alpha$ /CXCR4 axis participates in the process of





**Figure 6 Cell transplantation improved hepatocyte proliferation via growth factors.** A: Levels of hepatocyte-generating factors (HGF) and vascular endothelial growth factor (VEGF); B: Immunohistochemistry analyses of Ki67 expression in liver tissues; C: Quantitative image analyses of the number of Ki67<sup>+</sup> cells. <sup>a</sup>*P* < 0.05 vs CXC receptor 4 group, data are shown as mean ± SD.

MSC recruitment. However, culture and proliferation of MSCs gradually downregulate the expression of CXCR4 and the cells lose their ability to migrate toward a gradient of SDF-1 $\alpha$  concentration<sup>[24]</sup>. Thus, we used gene modification for CXCR4 overexpression to strengthen the homing of systemically delivered MSCs toward the injured liver. We used the membrane extraction kit and flow cytometry to determine the localization of CXCR4 on the cell membrane. The results showed that CXCR4-MSCs highly expressed CXCR4 compared to the unmodified MSCs (Figure 1). *In vivo* imaging dramatically demonstrated that ectopic distribution was negligible by use of gene modification, as opposed to unmodified MSCs (Figure 5). Therefore, it is anticipated that with genetic engineering, CXCR4-MSCs can disperse to the damaged liver and improve its function.

Stem cells have low immunogenicity, are readily cryo-

preserved, and show multipotent differentiation, so large numbers of trials of MSCs have been published worldwide. Many researchers have found poor efficacy of cell transplantation. An ongoing clinical trial of stem cell therapy has demonstrated that the engraftment efficiency is < 5% when transplanted into the liver *via* the portal vein<sup>[25]</sup>. Another study has shown that the efficiency can be improved to 20%-30%, if the stem cells are transplanted into the hepatic artery as opposed to the portal vein<sup>[26]</sup>. Even with this improvement, the majority of the cells escape to ectopic sites. To overcome this limitation, grafting strategies focus on gene modification or matrix biomaterials. We chose lentivirus to make MSCs overexpress CXCR4. *In vivo* imaging yielded a macro-image and showed that CXCR4-MSCs were nearly all distributed in the liver at 5 d after transplantation, whereas Null-MSCs migrated to the liver and spleen in equal proportions.

This difference confirmed that our strategy enhanced the localization of cells to the liver.

To date, the mechanism of MSC-based cell therapy in liver regeneration remains controversial. Some researchers have reported that stem cells facilitate liver regeneration by transdifferentiation into primary hepatocytes<sup>[27,28]</sup>. However, this property has been challenged in subsequent reports. Some researchers have reported that only limited donor-derived cells are detected *in vivo*<sup>[29]</sup>. Such limited cells are not sufficient to replace the damaged hepatocytes. Therefore, there must be other mechanisms that are likely to mediate the effect of MSCs. Recently, the concept of stem cell transplantation exerting a paracrine proliferative effect on endogenous hepatocytes has gained support<sup>[21,30,31]</sup>. It has been demonstrated that MSCs can secrete a broad variety of cytokines, chemokines and growth factors, including VEGF-1, insulin-like growth factor-1, epidermal growth factor, NO, HGF, keratinocyte growth factor, angiopoietin-1, SDF-1, macrophage inflammatory protein-1a and -1b, and erythropoietin<sup>[21]</sup>. The released factors are known to be important for cell survival, proliferation and neovascularization during tissue repair. In this study, we demonstrated that the level of HGF and VEGF in mouse serum increased significantly 2 wk after cell transplantation compared with that in the PBS group. We also discovered more hepatocyte proliferation in the MSC groups relative to the PBS group. Therefore, the improvement of liver function *via* MSC transplantation was partially due to enhanced hepatocyte proliferation.

In summary, we showed in this study that genetic modification with CXCR4 markedly increased the homing of systemically delivered MSCs toward the failing liver, leading to reduced mortality and improved liver regeneration. We further demonstrated that MSCs exerted paracrine effects by secreting HGF and VEGF, which may have stimulated endogenous liver regeneration. The strategy of intravenous delivery of genetically modified MSCs expressing CXCR4 may be a useful and noninvasive cell therapy for ALF.

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## COMMENTS

### Background

Recent studies of mesenchymal stem cell (MSC)-based therapies on hepatic diseases have achieved remarkable advances. However, *in vivo*, their reparative capability is limited due to low colonization in the liver. To overcome this limitation, the authors genetically modified MSCs overexpressing chemokine CXC receptor 4 (CXCR4), the cognate receptor for stromal cell-derived factor (SDF)-1 $\alpha$ , in order to maximize the capability of cell migration depending on SDF-1 $\alpha$ .

### Research frontiers

MSCs are being considered as an optimal cell source for stem cell transplantation because of their capacity for self-renewal, immunoregulation and paracrine effects. The research hotspot is how to improve the colonization of MSCs in the

target organs. SDF-1 $\alpha$  and its receptor, CXCR4, are the most important chemokines required for homing of MSCs to injured tissues.

### Innovations and breakthroughs

A large number of studies have shown that the SDF-1 $\alpha$ /CXCR4 axis plays an important role in stem cell homing, chemotaxis, expression of adhesion molecules, engraftment, proliferation and survival. The authors constructed CXCR4-modified MSCs by lentivirus to improve the migration and homing capacity of the cells. This is believed to be the first study in which CXCR4-MSCs were used to treat acute liver failure (ALF). In order to track the distribution of MSCs *in vivo*, the authors labeled them with DiR, a fluorochrome with a wavelength of 700-800 nm. *In vivo* imaging showed that CXCR4-MSCs migrated to the damaged liver in greater numbers than Null-MSCs. The results exhibited that CXCR4-MSCs can migrate to acute failing liver and promote the recovery of liver function.

### Applications

This study suggested that intravenous delivery of genetically modified MSCs expressing CXCR4 may be a useful and non-invasive cell therapy for ALF.

### Terminology

SDF-1 $\alpha$ /CXCR4 axis: this axis implicates multiple signaling pathways in activating a range of cell secretion and cell adhesion molecules that take part in the homing of stem cells.

### Peer review

The low number of MSCs colonizing a particular diseased organ upon intravenous infusion is a concern, and a stumbling block in moving MSCs forward for clinical use. This study showed that CXCR4-modified MSCs homed better to damaged liver and improved recovery. This was a good study and the results are interesting. CXCR4-MSC transplantation could be a potential cell therapy for ALF.

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