

# Critical role of SDF-1/CXCR4 signaling pathway in stem cell homing in the deafened rat cochlea after acoustic trauma

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## Abstract

Previous animal studies have shown that stromal cell-derived factor-1 (SDF-1)/CXC chemokine receptor-4 (CXCR4) signaling pathway plays an important role in the targeted migration of bone marrow-derived mesenchymal stem cells (BMSCs) to the injured area. In the present study, we aimed to investigate the potential role of chemotactic SDF-1/CXCR4 signaling pathway in the homing of transplanted BMSCs to the injured cochlea after noise-induced hearing loss (NIHL) in a rat model. White noise exposure (110 dB) paradigm was used for hearing loss induction in male rats for 6 hours in 5 days. Distortion-product otoacoustic emission (DPOAE) responses were recorded before the experiment and post noise exposure. Hoechst 33342-labeled BMSCs and CXCR4 antagonist (AMD3100)-treated BMSCs were injected into the rat cochlea through the round window. SDF-1 protein expression in the cochlear tissue was assayed using western blot assay. The number of labeled BMSCs reaching the endolymph was determined after 24 hours. SDF-1 was significantly increased in the cochlear tissue of rats in the noise exposure group than in the control group. The number of Hoechst 33342-labeled BMSCs reaching the endolymph of the cochlea was significantly smaller in the AMD3100-treated BMSCs group than in the normal BMSCs group. Our present findings suggest that the SDF-1/CXCR4 signaling pathway has a critical role in BMSCs migration to the injured cochlea in a rat model of noise-induced hearing loss.

**Key Words:** nerve regeneration; stem cells; migration; SDF-1/ CXCR4 axis; noise-induced hearing loss; neural regeneration

## Introduction

Noise-induced hearing loss (NIHL) is a common occupational illness in industrialized countries (Nelson et al., 2005). It is a hearing impairment that results from damage to the hair cells in the inner ear (Chen and Fechter, 2003). Permanent hearing loss likely occurs when mammalian cochlear hair cells are not able to regenerate themselves (Matsui et al., 2005). There is no definitive treatment for irreversible damage to the auditory system. However, cell therapy has recently been identified as a promising approach to treat several diseases (Dunnett et al., 2001; Hussain and Theise, 2004; Hanna et al., 2007; Segers and Lee, 2008; Abbaszadeh et al., 2017). Stem cell therapy reportedly exhibits therapeutic effects on injury of the auditory system (Li et al., 2004). However, the mechanism by which the transplanted mesenchymal stem cells (MSCs) migrate to the injured organ remains unclear. There is strong evidence that MSCs have the ability to migrate to the injured tissue in myocardial ischemia (Barbash et al., 2003), brain stroke (Lu et al., 2001), spinal cord injury (Abbaszadeh et al., 2015), renal ischemia/reperfusion injury (Hagiwara et al., 2008), pulmonary fibrosis (Ortiz et al., 2003) and liver injury (Chen et al., 2010; Xiao Ling et al., 2016). The primary factor that regulates cell mobilization

is chemotactic signaling molecules called ‘chemokines’. MSCs express chemokine receptors on their cell surface, and cell migration is induced by chemotaxis signaling pathway (Wynn et al., 2004). SDF-1/CXCR4 signaling pathway has been considered as an important factor in the stem cell migration to the injured area (Tögel et al., 2005; Kitaori et al., 2009; Carbajal et al., 2010; Okada et al., 2016; Li et al., 2017; Liu et al., 2017; Park et al., 2017). SDF-1/CXCR4 interaction plays an important role in embryogenesis and angiogenesis (Murdoch, 2000). SDF-1 is a member of the CXC chemokine family and it is expressed by damaged cells in large quantities (Honczarenko et al., 2006). CXCR4, the family of G-protein receptor binding, is a known receptor for SDF-1 on the surface of stem cells (McGrath et al., 1999; Honczarenko et al., 2006). Recent studies have also found that the expression of SDF-1 is increased in the injured cochlea (Kilpatrick et al., 2011; Zhang et al., 2013). Migration of transplanted neural stem cells to the auditory nerve increases in rats subjected to an augmented acoustic environment (Zhang et al., 2013). Based on the above data, the present study was designed to investigate the role of SDF-1/CXCR4 signaling pathway as a migratory mechanism of stem cells in noise-induced cochlear injury.

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## Materials and Methods

### Experimental animals

Thirty male Wistar rats, aged 2–3 months and weighing 180–250 g, were obtained from the Laboratory Animal Center of Shahid Beheshti University of Medical Sciences, Iran and included in this study. Animals were housed at constant temperature ( $21 \pm 1^\circ\text{C}$ ), with 12/12-hour light/dark cycle. These rats were randomly divided into five groups ( $n = 6$  per group): control, sham-operated, noise exposure, normal BMSCs (noise exposure followed by treatment with MSCs only) and AMD3100-treated BMSCs (noise exposure followed by AMD3100-treated BMSCs treatment) groups. The present study was approved by the Research Ethics Committee of Shahid Beheshti University of Medical Sciences (approval number: IR. SBMU. REC.1395.20) based on National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1985).

### Cell isolation and culture

Rats were anesthetized using intraperitoneal (IP) injections of ketamine (90 mg/kg, Rotexmedica, Trittau, Germany) and xylazine (10 mg/kg, Alfasan Chemical Co, Woerden, Netherlands); and then bone marrow was aspirated from femur and tibia using a 22 gauge needle. Bone marrow-derived mesenchymal stem cells (BMSCs) were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplementing with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Then cells were plated in 25 cm<sup>2</sup> flasks filling with 4–5 mL of DMEM culture medium, incubated in a humidified 5% CO<sub>2</sub> incubator at 37°C, and washed with PBS after 48 hours. Cell culture medium was changed every 2 to 3 days. All tests were performed on cells at passages 3 to 4. BMSCs were then incubated with CXCR4 antagonist AMD3100 (5 µg/mL, Sigma-Aldrich, St. Louis, MO, USA) in DMEM culture medium prior to use. Cultures without CXCR4 antagonist were considered as controls.

### Flow cytometry

Markers of MSCs were analyzed using flow cytometry (Harting et al., 2008; Zhang and Chan, 2010). Passage 4 cells were detached by Trypsin/EDTA, washed twice in PBS, incubated with phycoerythrin (PE) and fluorescein isothiocyanate (FITC)-conjugated monoclonal rat anti-mouse CD44, CD45 CD73, CD90, and CD34 antibodies (Becton Dickinson, San Diego, CA, USA) for 40 minutes at 4°C in the dark. The labeled cells were analyzed using a FACS Caliber (Becton Dickinson, Franklin Lakes, NJ, USA) and Cell Quest software (BD Biosciences, San Jose, CA, USA). Rat anti-mouse IgG1 was used as a negative isotype control. Since the expression of cell surface marker CD184 (CXCR4) is critical for BMSCs homing, surface CXCR4 expression was assayed using flow cytometry on cultured cells (Bing et al., 2016). For CXCR4, passage 3 cells were stained with anti-CXCR4 antibody (Abcam, Cambridge, UK) or IgG isotype control, and the secondary antibody used was mouse anti-goat IgG-FITC (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

### *In vitro* and *in vivo* cell migration assay

The Chemicon QCM™ 96-well migration assay system with 8 µm pore size (Millipore, Billerica, MA, USA) was used to determine the migration ability of cells (Mirzamohammadi et al., 2015). BMSCs suspension ( $4 \times 10^4$  cells /100 µL) was cultivated and treated with or without AMD3100 in the upper chamber. Various doses of recombinant rat SDF-1 (0, 100 and 500 ng/mL; Enzo Life Sciences, Farmingdale, NY, USA) were added to the lower chamber according to the manufacturer's instructions. Then, the plates were incubated for 24 hours at 37°C. Migratory cells were lysed and stained with CyQuant GR dye (Kit Components, (Part No. 90132)). Fluorescence of migrated cells was measured using a 480 nm /520 nm filter set. For *in vivo* chemotaxis analysis,  $1 \times 10^5$  BMSCs were incubated with PBS (20 µL) or AMD3100 (5 µg/mL) for 30 minutes before injection and then perfused for 10 minutes at a rate of 2 µL/min into the inner ear (left) through the round window niche of rats. Hoechst 33342-labeled BMSCs were assayed under the fluorescence microscope (Nikon Inc., Melville, NY, USA).

### Noise exposure protocol

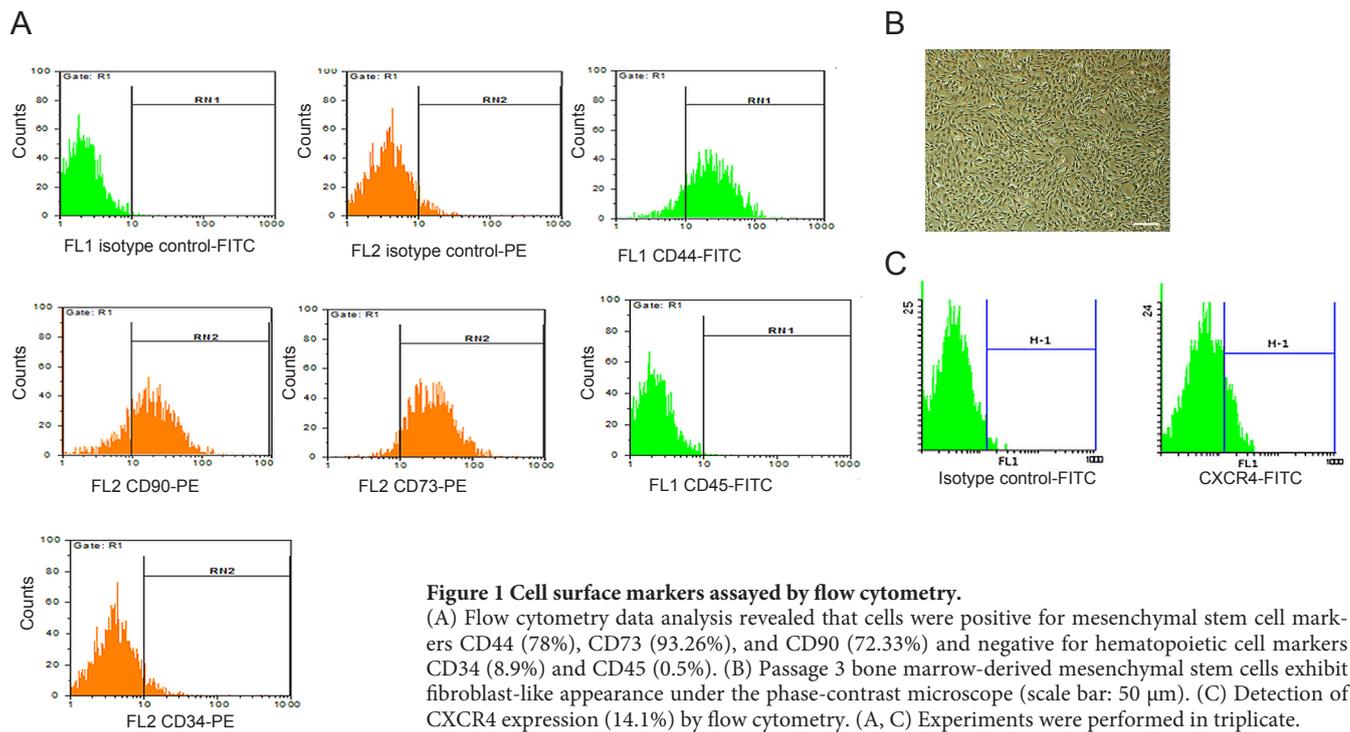
Rats were subjected to DPOAE to assess their cochlear hearing function before noise exposure. The DPOAE test was administered to the control ( $n = 6$ ) and sham-operated ( $n = 6$ ) groups on days 0 (baseline audiometry) and 6 (with no noise exposure). The test was also administered to noise exposure (noise exposure without any treatment,  $n = 6$ ), normal MSCs (noise exposure + treatment with normal BMSCs;  $n = 3$ ), and AMD3100-treated BMSCs (noise exposure + treatment with AMD3100-treated BMSCs;  $n = 3$ ) on days 0 (before) and 6 (after) of the noise exposure protocol. DPOAEs (in dB SPL) were measured at stimulus frequencies of 2, 3, 4, 5, 6, 7, 8, 9, and 10 kHz. Rats that had abnormal auditory function were excluded from the experiment. Rats with normal auditory function were exposed to 110 dB white noise, 6 hours/day for 5 successive days in a glass chamber. Sound intensity was measured using a sound level meter (TES-1358 Sound Analyze, Taiwan).

### Auditory assay

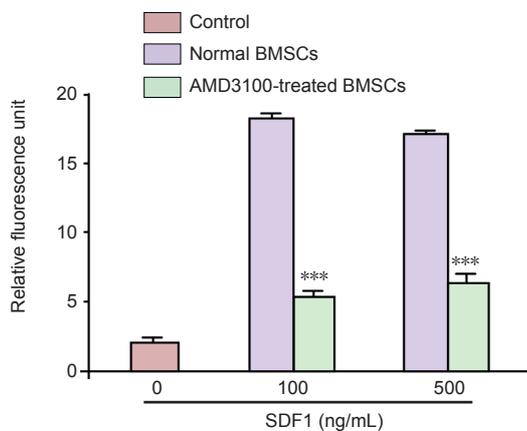
The DPOAE test was performed in a sound-attenuated chamber (San Diego Instruments, San Diego, CA, USA) covered with sound-absorbing foam. Before each recording session, rats were anesthetized using intraperitoneal injections of ketamine (90 mg/kg) and xylazine (10mg/kg). In all animals, probe was placed in the left ear canal. DPOAEs were recorded with the Neuro-Audio (NEUROSOFT, Russia) System. DPOAEs were generated by simultaneously presenting two sinusoids differing in frequency (f1 and f2) (Lonsbury-Martin and Martin, 1990).

### Hematoxylin-eosin staining of the cochlear tissue

After decapitation, the left cochlea was dissected in ice-cold 10 mM phosphate-buffered saline (PBS). Round window and oval window of the cochlea were opened by a fine



**Figure 1 Cell surface markers assayed by flow cytometry.** (A) Flow cytometry data analysis revealed that cells were positive for mesenchymal stem cell markers CD44 (78%), CD73 (93.26%), and CD90 (72.33%) and negative for hematopoietic cell markers CD34 (8.9%) and CD45 (0.5%). (B) Passage 3 bone marrow-derived mesenchymal stem cells exhibit fibroblast-like appearance under the phase-contrast microscope (scale bar: 50 μm). (C) Detection of CXCR4 expression (14.1%) by flow cytometry. (A, C) Experiments were performed in triplicate.



**Figure 2 Effect of chemotaxis inhibitor (AMD3100) on *in vitro* migration of BMSCs.** BMSCs were treated with or without AMD3100 (a specific antagonist of CXCR4) for 24 hours. Control wells were not incubated with a chemoattractant. All experiments were performed in triplicate. \*\*\* $P < 0.001$ , vs. normal BMSCs group (Student's  $t$ -test). SDF-1: Stromal cell-derived factor-1; BMSCs: bone marrow-derived mesenchymal stem cells.

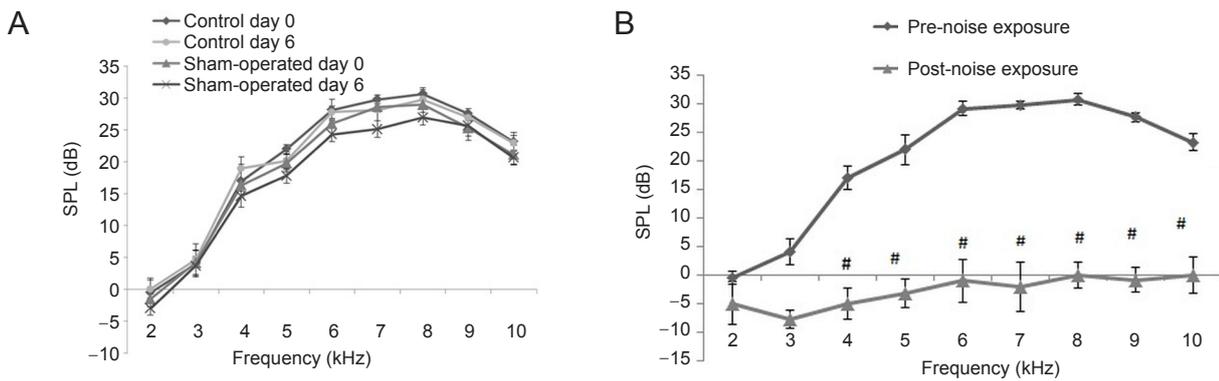
needle, and then 4% paraformaldehyde was perfused. The cochlea was fixed in 4% paraformaldehyde overnight and then decalcified in 5% ethylenediaminetetraacetic acid (pH 7.4) containing 5% sucrose for 15 days and then embedded in paraffin. Next, 5 μm-thick sections were cut on a sliding microtome and stored in a cryoprotectant solution. For the staining procedure, paraffin-embedded blocks were deparaffinized and rehydrated; and sections were stained later with hematoxylin-eosin (Sigma-Aldrich, St. Louis, MO, USA).

### Western blot analysis

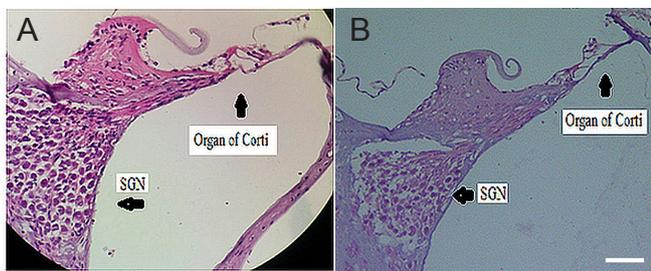
After decapitation of the animals, the bulla was quickly removed and placed in ice-cold 10 mM PBS. The bony capsule and the lateral wall of the cochlea were removed. Cochlea sample included the organ of corti, and the modiolus was dissected. Specimen was homogenized in a RIPA lysis buffer (sc-24948, Santa Cruz Biotechnology). Homogenate was centrifuged, and supernatant solution was collected. Total protein amount was determined according to the Bradford method (Bradford, 1976) using bovine serum albumin as a protein concentration standard. Protein was separated using SDS gel and transferred into a nitrocellulose membrane. After blocking, the membrane was incubated with primary antibodies for rabbit polyclonal to SDF-1 (AB 25117, 1:1,000; Abcam, Cambridge, UK) and β-actin (AB, ab8227, 1:2,000; Abcam) (as an internal control) at 4°C overnight. After rinsing to eliminate unbound primary antibody, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (AB, 6721, 1:2,000; Abcam) for 2 hours at room temperature. The protein bands were visualized using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) and quantified by TotalLab software (Wales, UK) (Niknazar et al., 2016, 2017).

### Hoechst labeling

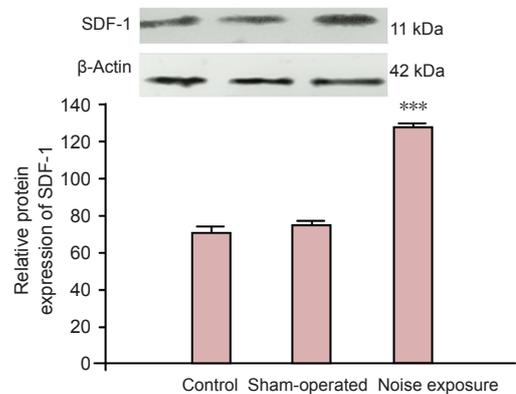
To evaluate BMSCs migration to the endolymph of cochlea, Hoechst 33342-labeled BMSCs were injected into the perilymph through the round window niche. Deaf rats were divided into two groups, which were transplanted with Hoechst 33342-labeled untreated BMSCs ( $1 \times 10^5$ ) ( $n = 3$ ) and AMD3100-treated BMSCs ( $1 \times 10^5$ ) ( $n = 3$ ). Ani-



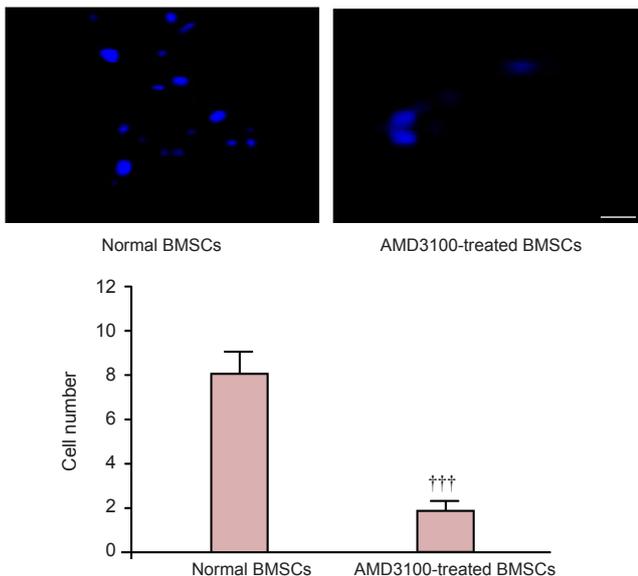
**Figure 3 Auditory function of rats assessed by DPOAE assay.** DPOAE responses (in dB SPL) on days 0 and 6 in the control and sham-operated groups (A) and in the noise exposure group (B). Experiments were performed in triplicate. # $P < 0.001$ , vs. day 0 (one-way analysis of variance followed by Tukey's test). SPL: Sound pressure level.



**Figure 4 Effect of noise exposure on the structure of rat cochlea.** Abnormal morphology of auditory hair cells and spiral ganglion neurons (SGN) in noise induced deaf rats (B) compared to intact organ of Corti and SGN structure in control rats (A). Scale bar: 60  $\mu$ m.



**Figure 5 Effects of noise exposure on SDF-1 protein expression in the cochlea of rats.** Quantitative results are shown as the mean  $\pm$  SEM. \*\*\* $P < 0.001$ , vs. control group (one-way analysis of variance followed by Tukey's test),  $n = 6$  cochlear samples in each group.  $\beta$ -Actin was used as loading control. Experiments were performed in triplicate. SDF-1: Stromal cell-derived factor-1.



**Figure 6 Hoechst 33342-labeled BMSCs migration to the endolymph of injured cochlea.** AMD3100 (a specific antagonist of CXCR4) reduced MSCs homing (blue) in deaf rat cochlea. Scale bar: 100  $\mu$ m. ††† $P < 0.001$ , vs. cochlear tissue of deaf rats receiving normal BMSCs ( $n = 3$ , Student's  $t$ -test). Experiments were performed in triplicate. BMSCs: Bone marrow-derived mesenchymal stem cells

mals were decapitated and the cochleae were removed 24 hours after BMSCs transplantation. The cochleae were fixed through intrascalar perfusion of 4% paraformaldehyde and then decalcified in 5% ethylenediaminetetraacetic acid (pH 7.4) containing 5% sucrose for 15 days. Next, the cochlea was embedded in paraffin and then cut into 5  $\mu$ m-thick sections. The number of Hoechst (Hoechst 33342; Thermo Fisher Scientific, Waltham, MA, USA) -labeled BMSCs in the cochlea was assayed using a fluorescence microscope (Nikon Inc., Melville, NY, USA). Data were analyzed using ImageJ software (NIH, Bethesda, MD, USA).

### Statistical analysis

All data are expressed as the mean  $\pm$  SEM and were statistically analyzed with GraphPad Prism version 5.04 Windows (GraphPad Software, San Diego, CA, USA). Statistical significance was determined by the Student's  $t$ -test or one-way analysis of variance followed by Tukey's test. A level of  $P < 0.05$  was considered statistically significant.

## Results

### Characterization of BMSCs

Cell surface markers were assayed using flow cytometry. Results analysis revealed that cells were positive for CD44, CD73, and CD90, while they were negative for CD34 and CD45. In addition, 14.1% passage 3 BMSCs expressed surface CXCR4 (Figure 1).

### In vitro BMSCs migration

Migration of AMD3100-treated BMSCs in response to SDF-1 at different concentrations was assayed. AMD3100-treated BMSCs revealed a significantly lower migration capacity in response to SDF-1 (100 and 500 ng/mL) after 24 hours compared to normal BMSCs ( $P < 0.001$ ) (Figure 2).

### Cochlear hearing function assessment

DPOAEs were measured to assess cochlear function. DPOAE test results showed that there was no significant difference in DPOAE responses at any frequency between the control and sham-operated groups on days 0 and 6 (Figure 3A). In the noise exposure group, DPOAE responses were significantly decreased on day 6 than those on day 0 (Figure 3B).

### Pathological changes in cochlear tissue

Hematoxylin-eosin staining of the cochlear tissue sections revealed an irregular arrangement of the auditory hair cells and degeneration of spiral ganglion neurons in the cochlea of rats ( $n = 3$ ) (Figure 4B) following chronic exposure to 110 dB white noise compared to control rats ( $n = 3$ ) (Figure 4A).

### SDF-1 protein expression

SDF-1 protein expression in normal ( $n = 6$ ), sham-operated ( $n = 6$ ), and noise induced ears ( $n = 6$ ) was determined using western blot assay. Antibodies against SDF-1 detected a band on the blot at about 11 kDa. Western blot assay results revealed that SDF-1 protein expression was significantly increased in the cochlea of rats in the noise exposure group compared to the control group ( $P < 0.001$ ). These results confirmed that SDF-1 protein expression was increased following cochlear injury (Figure 5).

### In vivo BMSCs migration to the injured cochlea

To evaluate the migration of BMSCs to cochlear endolymph, Hoechst 33342-labeled BMSCs were injected through the round window into noise-induced deaf rats. About 24 hours after BMSCs transplantation, rats were decapitated. The number of Hoechst-stained BMSCs per cochlear section was counted, and three sections per animal were used in each group. The number of transplanted BMSCs was significantly lower in the CXCR4-treated group than in the normal BMSCs group ( $n = 3$ ) ( $P < 0.001$ ; Figure 6).

## Discussion

In this study, we investigated the mechanism by which the SDF-1/CXCR4 signaling pathway regulates the homing of

MSCs in a rat model of noise-induced hearing loss. We used CXCR4 antagonist to inhibit CXCR4 activity on the surface of BMSCs to influence the homing ability of BMSCs to the damaged site of cochlea. For this purpose, adult male rats subjected to the chronic white noise exposure were used, and then electrophysiological and molecular alterations of the cochlea were evaluated. DPOAE responses were attenuated after noise exposure. SDF-1 expression was increased in the cochlea of rats in the noise exposure group. Normal and AMD3100-treated BMSCs were injected into the perilymph through the round window. A smaller number of transplanted BMSCs (~5%) was observed in the cochlear endolymph in the AMD3100-treated MSCs group than in the normal MSCs group.

BMSCs can produce several molecules that provide further guidance for effective delivery and homing of injected cells to the injured tissue and contribute to tissue repair (Augello et al., 2010; Méndez-Ferrer et al., 2010). It is demonstrated that the expression of CXCR4 and this ligand SDF-1 in BMSCs support their survival and proliferation through the autocrine-paracrine loop (Nagasawa et al., 1996; Kortessidis et al., 2005). SDF-1 is a chemotactic factor produced by various pathological conditions. In the present study, chronic exposure to white noise increased the SDF-1 protein expression in the cochlear tissue of rats. This is consistent with findings from previous studies demonstrating SDF-1 upregulation in the auditory nerve following spiral ganglion neuron degeneration due to ouabain application in mice (Guyon and Nahon, 2007; Tan et al., 2008; Kilpatrick et al., 2011) and acoustical trauma in rats (Zhang et al., 2013). Several factors including cytokines, integrins, and chemokines associated with their receptors are involved in the migration of BMSCs (Li et al., 2012; Sohni and Verfaillie, 2013; Esfahani et al., 2015; Kamiya, 2015). CXCR4 is a chemokine receptor specific to SDF-1 $\alpha$ , which is normally expressed on the surface of BMSCs (Liu et al., 2011; Yu et al., 2015). SDF-1/CXCR4 system plays an important role in stem cells mobilization to the damaged area. Previous studies have demonstrated that SDF-1/CXCR4 pathway has been upregulated in numerous injured organs and tissues of the experimental models such as spinal cord injury, ischemic brain damage, myocardial infarction, burn wounds, acute pancreatitis, and acute kidney injury (Marquez-Curtis and Janowska-Wieczorek, 2013). Recently, several researches have shown that AMD3100 can inhibit CXCR4 activity. There is evidence that in renal ischemia, migration of MSCs to ischemic tissue was mainly dependent on CXCR4 and completely inhibited by the CXCR4 antagonist AMD3100 (Liu et al., 2013a, b). Li et al. (2015) found that SDF-1/CXCR4 signaling pathway has a critical role in the mobilization of transplanted BMSCs to the lesion site following spinal cord injury. They indicated that CXCR4 expression was enhanced by erythropoietin, but attenuated significantly after AMD3100 treatment.

Our data revealed that expression of SDF-1, which was induced in the injured cochlea, can promote BMSCs migration to this area. AMD3100, as a SDF-1/CXCR4 signaling

pathway antagonist, significantly attenuated BMSCs mobilization to the endolymph of injured cochlea, which confirms that interaction of production of SDF-1 in the cochlea with CXCR4 expression in BMSCs has a crucial role in BMSCs migration towards the noise-induced damaged cochlea in rats.

In conclusion, migration and homing of transplanted stem cells is an essential mechanism for cell delivery to the damaged target area. The present findings demonstrated that SDF-1/CXCR4 signaling pathway plays an important role in the migration of transplanted cells to the injured cochlea following NIHL. Thus, the activation of SDF-1/CXCR4 signaling pathway may be used as a cell-based therapy approach for hearing recovery after acoustic trauma.

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**Data sharing statement:** Datasets analyzed during the current study are available from the corresponding author on reasonable request.

**Plagiarism check:** Checked twice by iThenticate.

**Peer review:** Externally peer reviewed.

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