available at www.sciencedirect.com

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Fractalkine and CX3CR1 are involved in the migration of intravenously grafted human bone marrow stromal cells toward ischemic brain lesion in rats

*Jie Zhu, Zhujuan Zhou, Yong Liu, Jian Zheng**

Department of Neurology, Xinqiao Hospital, Third Military Medical University, Xinqiao Street, Chongqing 400037, PR China

ARTICLE INFO

Article history:
Accepted 23 June 2009
Available online 27 June 2009

Keywords:
Human bone marrow stromal cells
Cerebral infarct
Fractalkine
CX3CR1
Migration
RNA interference

ABSTRACT

Recent research has shown that transplanted bone marrow stromal cells (MSCs) migrate to the injured regions and exert their therapeutic effects in cases of intracranial trauma, stroke, inflammation and degenerative disease. The specific mechanisms involved in their migration to lesions are still to be fully elucidated. In the present study, a rat model of transient middle cerebral artery occlusion (MCAO) was established. At 24 h after reperfusion, human bone marrow stromal cells (hMSCs) were transplanted by intravenous injection to explore the effects of fractalkine/CX3CR1 on the migration of transplanted MSCs to lesions. In vitro study using real-time PCR and western blot revealed that CX3CR1, the only known receptor of fractalkine, was expressed in cultured hMSCs. The expression of fractalkine in the ischemic brain was significantly increased. The directional migration of transplanted hMSCs to the damaged region was observed through detection of green fluorescence protein (GFP). The results indicated the cells were mainly distributed in the ischemic boundary zone with high fractalkine expression. In a further study, lentivirus-mediated RNA interference of CX3CR1 expression was employed. The results of these experiments indicated that CX3CR1 knock-down dramatically decreased the migration of hMSCs to the ischemic brain. The present study suggests that fractalkine and its specific receptor CX3CR1 are involved in the directional migration of transplanted MSCs to the ischemic damaged brain region.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Bone marrow stromal cells (MSCs) can be easily obtained and massively proliferate in vitro. Under certain conditions, MSCs can be induced to differentiate into multiple types of cells. The transplantation of cultured autologous bone marrow cells can avoid post-transplant immune rejection. Therefore, MSCs may be the optimal candidate for transplantation and have good prospects in clinical applications (Prockop, 1997; Prockop, 1998; Prockop et al. 2003). Recently, an increasing number of studies have shown that transplanted MSCs tend to migrate in the direction of an injured lesion in the central nervous system (Chen et al. 2008; Shichinohe et al., 2004; Shyu et al., 2007; Yano et al., 2005a; Yano et al., 2005b), and that the aggregation of transplanted cells in damaged regions contributed to the repair of neurofunction. These results provide

* *Corresponding author*. Fax: +86 23 68755613.
E-mail address: jzheng12@hotmail.com (J. Zheng).

Abbreviations: MSCs, bone marrow stromal cells; MCAO, middle cerebral artery occlusion; hMSCs, human bone marrow stromal cells; GFP, green fluorescence protein; MCP-1, monocyte chemoattractant protein-1; SDF-1, stromal cell-derived factor-1

0006-8993/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2009.06.068

us with a new strategy in the treatment of central nervous system injury. However, the mechanism of the directional migration of transplanted MSCs to the lesion remains unclear. Exploration of the specific mechanism will enable us to take active measures to promote the migration of transplanted cells to lesions and enhance their therapeutic effects.

Chemokines form a superfamily that shares a common structure and function-related peptide. The basic function of a chemokine is to induce the directional chemotaxis of cells with corresponding receptors (Campbell et al., 2003). MSCs express certain chemokine receptors, including CXCR4, CXCR5, CXCR6, CCR1, CCR7 and CCR9 (Honczarenko et al., 2006), which suggests that chemokines and their receptors may be involved in the promotion of directional migration of transplanted MSCs in vivo. It has been reported that the expression of monocyte chemoattractant protein-1 (MCP-1) and stromal cell-derived factor-1 (SDF-1) is up-regulated in injured brain tissue, which promotes the migration of transplanted MSCs to the lesions (Shichinohe et al., 2007; Wang et al., 2002a; Wang et al., 2002b; Wang et al., 2008). Fractalkine is one of the few chemokines that is expressed in the central nervous system (Harrison et al., 1998). Studies have shown up-regulated expression of fractalkine in select central nervous system lesions, such as those caused by inflammation, ischemia and hypoxia (Hughes et al., 2002; Pan et al., 1997; Sunnemark et al., 2005). Through interaction with its receptor, CX3CR1, it promotes the activation of microglial cells and their migration to the lesions, elevates the number of mononuclear cells, natural killer cells and T lymphocytes in the blood, plays a critical role in the clearance of necrotic tissue and promotes neurofunction repair (Mizuno et al., 2003; Re and Przedborski, 2006). In other situations, it may aggravate the injury (Soriano et al., 2002). Researchers have observed expression of CX3CR1 in MSCs by RT-PCR, flow cytometry and immunocytochemistry techniques (Sordi et al., 2005). These results indicate that fractalkine/CX3CR1 may be involved in the induction of directional migration of transplanted MSCs to injured brain regions.

This study aimed to explore whether fractalkine and its receptor CX3CR1 play an important role in the induction of directional migration of transplanted hMSCs to ischemic brain tissue. Furthermore, we aimed to explore the possible mechanisms involved in MSCs migration. A transient MCAO model was established in rats to observe the changes in fractalkine expression in the ischemic brain and the relationship between the migration of intravenously transplanted hMSCs and fractalkine expression. An RNA interference technique was adopted to investigate the effect of CX3CR1 knock-down on the migration of hMSCs to the damaged brain region.

2. Results

2.1. Up-regulated fractalkine expression in the infarcted brain after transient MCAO

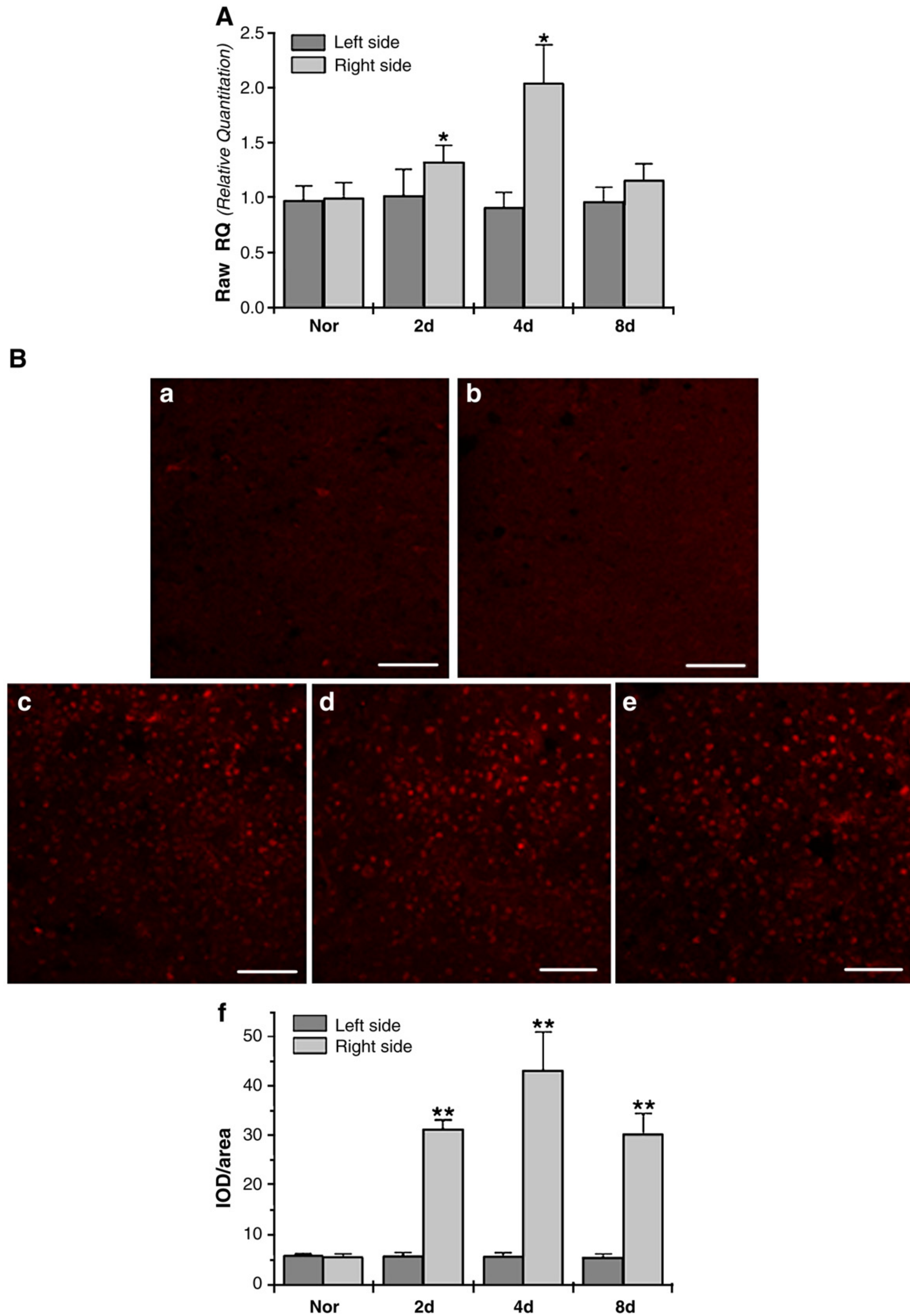
We examined fractalkine mRNA and protein expression in the infarcted hemisphere at 2, 4 and 8 days after ischemia. Using real-time PCR of RNA extracted from homogenized brain tissues, fractalkine mRNA expression was found to be up-regulated in the cortex of injured hemisphere on days 2 and 4, in comparison with the contralateral hemisphere and normal control tissues ($P<0.05$, Fig. 1A). Similarly, compared with the weak fractalkine expression observed in the contralateral hemisphere (Fig. 1Ba) and in the brains of control rats (Fig. 1Bb), fractalkine expression was dramatically up-regulated in the injured hemisphere at 2 (Fig. 1Bc), 4 (Fig. 1Bd), and 8 (Fig. 1Be) days after ischemia, as determined by immunofluorescence analysis. Quantification revealed that the density of fractalkine-immunoreactive cells in the ischemic hemisphere was significantly ($P<0.01$) increased at 2, 4, and 8 days after ischemia (Fig. 1Bf). In the brains of normal rats, fractalkine immunoreactivity showed a weak but wide distribution pattern in many brain areas, including the hippocampus (not shown), cortex (Fig. 1Ba) and striatum (not shown). In ischemic brains, fractalkine immunoreactivity was strongly increased in the ischemic boundary zone, and was nearly absent from the infarcted core (Fig. 2). The double immunostaining for fractalkine and NeuN revealed that fractalkine-positive cells were reactive for the neuronal marker (Fig. 3).

2.2. shRNA-mediated downregulation of CX3CR1 expression in hMSCs

CX3CR1, which is the only known receptor of fractalkine, was recently found to be expressed in hMSCs at the mRNA and protein levels. In our study, CX3CR1 mRNA was detected by real-time PCR (Fig. 4B), and CX3CR1 protein was detected by western blot (Fig. 4C). We constructed a lentiviral vector encoding shRNA directed against human CX3CR1 (Lv-rCX3CR1) or encoding a non-silencing shRNA as control (Lv-CON). To demonstrate that the Lv-rCX3CR1 construct was able to knock-down the expression of CX3CR1 in hMSCs, we sorted these cells 5–7 days after transduction and evaluated CX3CR1 expression. Transduction efficiencies were about 90%, as indicated by the frequency of GFP-positive cells by flow cytometry (Fig. 4A). Real-time PCR analysis revealed that, the CX3CR1 mRNA level in cells expressing the CX3CR1 shRNA construct was about 70-fold lower than those of non-transduced and control-transduced cells (Fig. 4B). Also, in

Fig. 1 – Expression of fractalkine in the brain. (A) Real-time PCR analysis revealed that fractalkine mRNA expression significantly ($P<0.05$) increased in the cortex at 2 and 4 days after cerebral ischemia. The mean value and S.E. of triplicate measurements of samples are shown. The presented results are representative of three independent experiments. *$P<0.05$ compared to the normal and the left side. (B) Fractalkine immunostaining was up-regulated in the ischemic hemisphere at 2(c), 4(d), and 8(e) days after cerebral ischemia compared with the normal brain (a) and the contralateral hemisphere (b). Quantification revealed that the mean intensity of fractalkine-specific fluorescence was significantly ($P<0.01$) increased in the ischemic hemisphere 2, 4, and 8 days after brain ischemia compared with the normal brain and the contralateral hemisphere (f). **$P<0.01$ compared to normal brain and the left side. Scale bar = 100 μm.

the hMSCs transduced with CX3CR1 siRNA, a reduced level of CX3CR1 protein was detected by western blot (Fig. 4C). No obvious effect on cell growth or survival of the transduced cells was observed in vitro, as determined by the MTT assay (not shown). These data show that a lentivirus produced siRNA can provide highly efficient and specific CX3CR1 knock-down in hMSCs, and does not affect cell proliferation or survival.



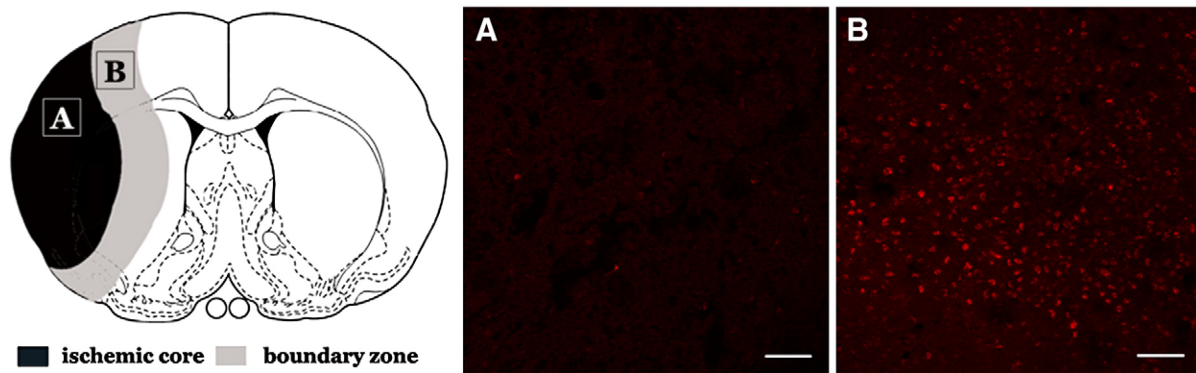


Fig. 2 – The schematic diagrams for distributions of the fractalkine-positive cells. The black and gray shade in the right hemisphere show the ischemic core and ischemic boundary zone respectively. (A) Fractalkine immunoreactivity was nearly absent from the ischemic core. (B) Fractalkine immunoreactivity was strongly increased in the ischemic boundary zone. Scale bar = 100 μm.

2.3. *Selective migration of transplanted hMSCs into the damaged brain region*

At 1, 3, and 7 days after transplantation, rats were sacrificed, and their brains were processed. Within the coronal slides stained with cresyl violet, the overall pale staining of the neuropil and the generalized presence of shrunk neurons were observed in the ischemic core of all rats subjected to MCAO, with and without hMSC injection. No significant reduction in the volume of ischemic damage was detected in control-transduced hMSCs-treated rats (229.29 ± 16.15 mm^3) or CX3CR1 knock-down hMSCs[Float1]-treated rats (235.24 ± 13.49 mm^3) compared with rats injected with PBS (243.49 ± 18.64 mm^3).

To determine whether transplanted hMSCs possess the directed migratory capacity to target impaired sites in the brain, we investigated their migration in the ischemic brain. Examination of frozen sections by laser-scanning confocal microscopy revealed that, after cell transplantation, GFP-labeled hMSCs were distributed throughout the ischemic damaged brain of recipient rats (Fig. 5). No GFP-labeled cells were found in the brains of the PBS-treated rats. Although GFP-labeled cells were found in multiple areas in the ipsilateral hemisphere, including the cortex and striatum, most of the cells were located in the ischemic boundary zone (about 59% of the total cells, 475.4 ± 57.1, 508.0 ± 76.3, 449.8 ± 64.8, respectively, for GFP-labeled cells counting at 1, 3, and 7 days), where fractalkine immunoreactivity was strongly increased, as seen by double immunostaining of GFP and fractalkine (Fig. 6).

2.4. *Down-regulation of CX3CR1 expression in hMSCs inhibits migration of transplanted hMSCs to the damaged brain*

To determine whether fractalkine/CX3CR1 signaling plays an essential role in hMSCs migration, we transduced hMSCs with an CX3CR1 shRNA construct by lentivirus-mediated gene transfer, and observed significant CX3CR1 suppression using both real-time PCR and western blot. Notably, Lv-rCX3CR1 transduction did not negatively impact hMSCs survival or proliferation, GFP-labeled cells were more abundant ($P < 0.01$) in the ischemic hemisphere of rats injected with control-transduced hMSCs (Figs. 7A–C), than in that of rats injected with CX3CR1 knock-down hMSCs (Figs. 7D–F) at 1 (761.8 ± 118.2

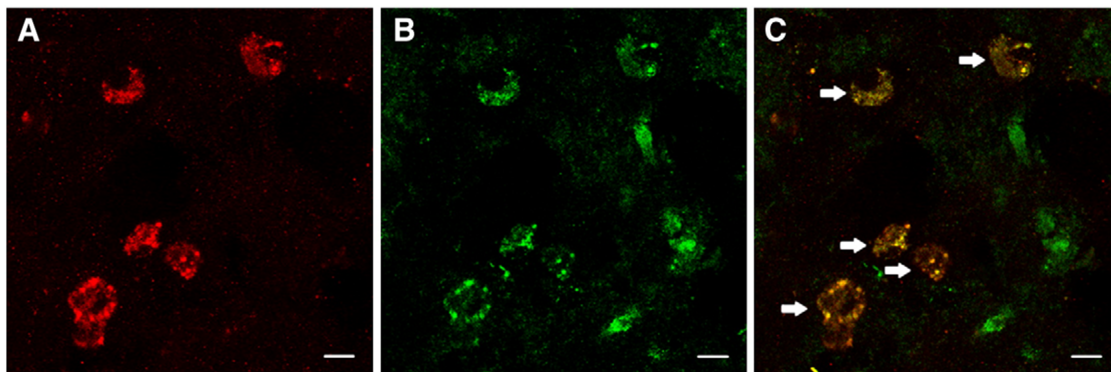


Fig. 3 – Double immunostaining for fractalkine and NeuN. Photomicrographs show fractalkine (red, A and C) and NeuN (green, B and C) positive cells in the ischemic boundary zone. The arrow shows the host NeuN-positive cells expressing fractalkine. Scale bar = 20 μm.

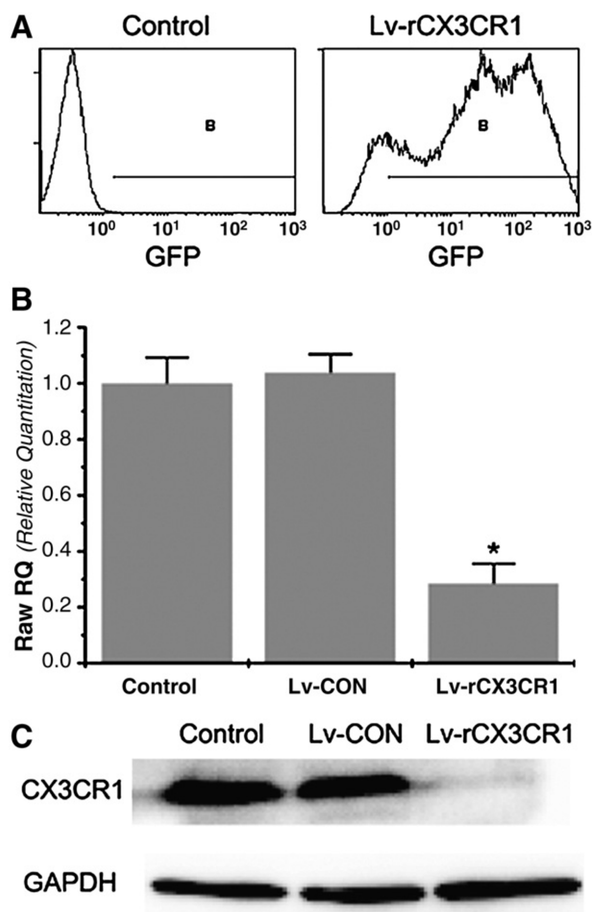


Fig. 4 – CX3CR1 knock-down by lentivirus-mediated RNA interference in hMSCs. (A) hMSCs were transduced with the indicated siRNA vectors at an MOI of 20. At 5 days post-transduction, >90% of the cells were GFP-positive. (B) Real-time PCR analysis revealed an efficient reduction of CX3CR1 mRNA in hMSCs by siRNA. *$P<0.01$ compared with normal hMSCs and Lv-CON-transduced hMSCs. The mean value and S.E. of triplicate measurements of samples are shown. The presented results are representative of three independent experiments. (C) A decreased CX3CR1 protein level in Lv-rCX3CR1-transduced hMSCs relative to the levels in control cells was revealed by western blot. The presented western blot is representative of the six replicates that were performed.

and 487.0±53.2, respectively), 3 (856.7±129.2 and 461.7±61.3, respectively) and 7 days (804.7±124.5 and 556.4±74.1, respectively) after cell transplantation. These data show that CX3CR1 knock-down reduces the potential of hMSCs to migrate toward a brain lesion.

3. Discussion

Previous studies have documented that MSCs transplantation promoted the repair and improvement of neurological function after central nervous system injury (Chen et al., 2008; Lu et al., 2006; Parr et al., 2007; Seyfried et al., 2006; Shen et al., 2006; Wu et al., 2008; Zhang et al., 2006). MSCs transplantation studies (including local transplantation, intravenous transplantation and intracarotid transplantation) have indicated that transplanted MSCs tend to migrate toward lesions (Liu et al., 2008; Shen et al., 2006; Wu et al., 2008). This targeted migration is considered to be related to the niche after injury, but the exact mechanisms remain unclear. Exploring the specific mechanisms will enable us to take active measures to promote the migration of transplanted MSCs to the lesions and enhance the therapeutic effects of transplantation.

Chemokines are secreted proteins with low molecular weight, consisting of only 70–100 amino acids. Their basic function is to induce the directional chemotaxis of cells with corresponding receptors (Campbell et al., 2003). Pro-inflammatory cytokines, including hypoxia inducible factor-1 (HIF-1) and vascular endothelial cell growth factor (VEGF), which are induced by inflammation, ischemia and hypoxia may promote the secretion of chemokines in damaged local neurons, glial cells and endothelial cells (Cartier et al., 2005). These chemokines increase the aggregation of chemotactic inflammatory cells, promote the up-regulation of adhesion molecules, and induce the repair of neurofunction and neurogenesis, which further aggravate the injury or protective activity (Constantin, 2008; Li and Ransohoff, 2008; Mines et al., 2007; Mizuno et al., 2003). Studies have indicated SDF-1α up-regulation in the ischemic brain and expression of its receptor,

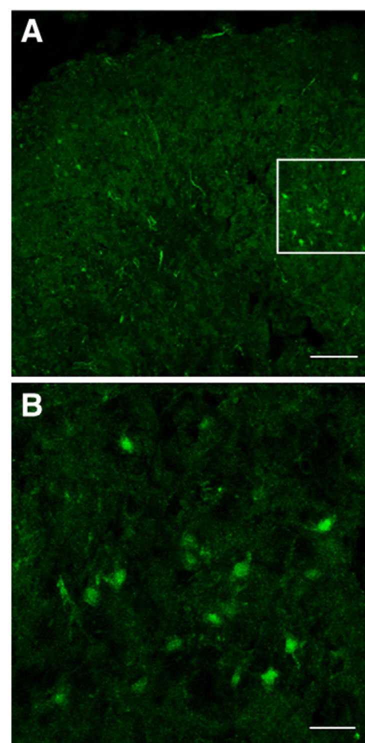


Fig. 5 – Fluorescence immunohistochemistry for GFP. Photomicrographs show the distribution of GFP-positive cells (green, A and B) in the boundary zone of the lesion area of the rats that received control-transduced hMSCs. Panels B show high-power findings in the square areas in panels A. A scale bar indicates 100 μm in panels A or 50 μm in panels B, respectively.

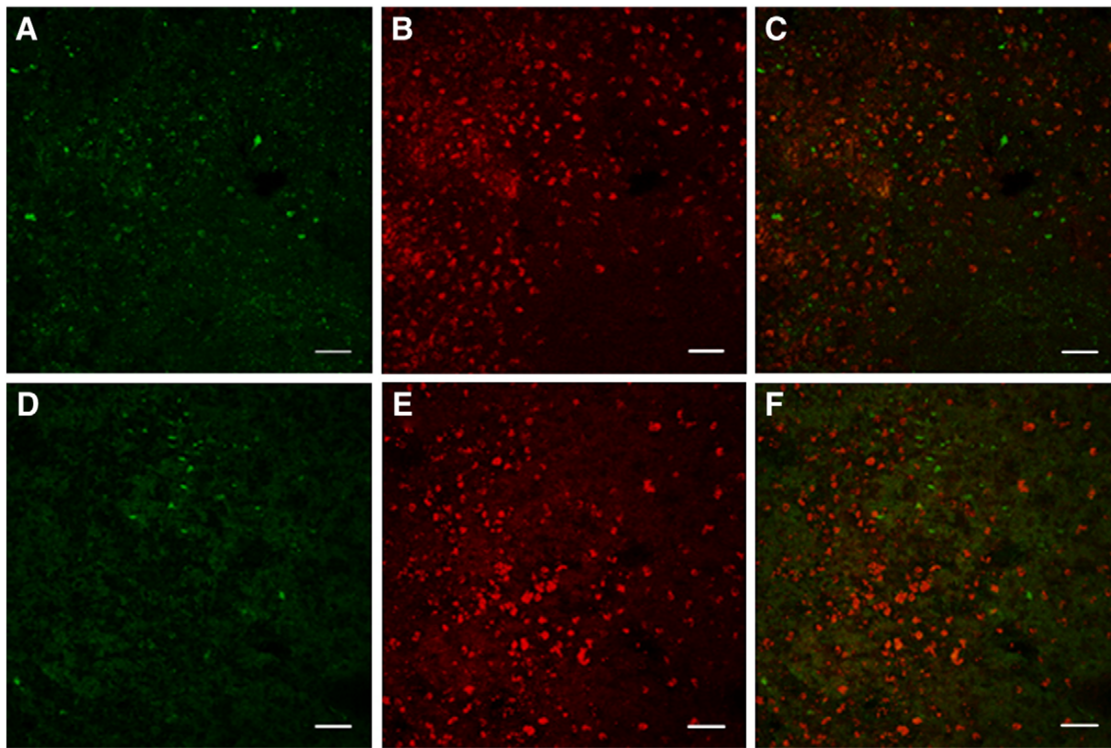


Fig. 6 – Fluorescence immunohistochemical analysis of GFP and fractalkine in the cortex and striatum of the ischemic brain. Photomicrographs show the distribution of GFP-positive (green, A and D) and fractalkine-expressing (red, B and E) cells in the cortex (A–C) and the striatum (D–F) at 3 days after transplantation. Scale bar = 50 μm.

CXCR4, in MSCs (Shichinohe et al., 2007; Wang et al., 2008). Intravenously transplanted MSCs could migrate to the ischemic brain, and become concentrated in the boundary zone in response to elevated SDF-1α. AMD3100, an antagonist of CXCR4, was found to significantly decrease the migration of MSCs to the injured brain (Wang et al., 2008). In vitro, Wang et al. (2002a; 2002b) found that MCP-1, IL-8 and ischemic brain extracts could induce the migration of MSCs. These results suggest that elevated chemokines levels at lesion sites might induce the migration of transplanted MSCs.

Fractalkine is the only member of the chemokine CX3C subfamily. It interacts with its specific receptor, CX3CR1, and hereby mediates inflammation and participates in wound healing by improving the adhesion of CX3CR1-positive cells and promoting cell migration. Fractalkine is one of the few constitutive chemokines that is expressed in brain tissue and is widely distributed in the central nervous system, with notably high expression in neurons (Harrison et al., 1998). Studies have showed that CX3CR1 is mainly expressed in bone marrow-derived cells (Imai et al., 1997). In the brain, it is mainly expressed in microglia (Harrison et al., 1998). Characteristically, fractalkine is a transmembrane glycoprotein and exists in both membrane-bound and soluble forms. It can promote migration and adhesion of leukocytes, which indicates that it has the functional characteristics of a chemokine and intercellular adhesion molecule (Fong et al., 1998). Lee et al. (2006) found that fractalkine induced the directional migration of MSCs, and that CX3CR1 antibody inhibited this migration, suggesting that the interaction between fractalkine and CX3CR1 induces directional migration of MSCs. They also found that transplanted hMSCs that expressed CX3CR1 and the SDF-1 receptor, CXCR4, could migrate to the hippocampal structure of normal mice, while cells lacking expression of CX3CR1 and CXCR4 could not migrate into the cerebral parenchyma, indicating that the expression of fractalkine and SDF-1 was associated with the migration of MSCs. Ji et al. (2004) found that the expression of fractalkine was increased in the nucleus of the hypoglossal nerve in rats with hypoglossal nerve injury, and that intraventricularly-transplanted MSCs could migrate to the injured hypoglossal nucleus region, suggesting that fractalkine might induce the migration of transplanted MSCs to the secreting site. Although the results described above indicate that association of fractalkine with CX3CR1 induces the migration of MSCs, and that fractalkine expressed in the brain promotes migration of transplanted MSCs to the parenchyma, there is a lack of direct evidence that increased expression of fractalkine in the brain after injury induces the directional migration of transplanted MSCs to the lesion sites.

In the present study, a rat transient MCAO model was established and these rats received hMSCs by intravenous tail injection to determine whether there was a change in fractalkine expression in the ischemic region, and whether the migration of transplanted hMSCs to the ischemic brain was related to fractalkine expression. An shRNA expression construct targeting CX3CR1 was introduced into hMSCs by lentivirus-mediated gene transfer in order to evaluate the effect of CX3CR1 knock-down on hMSCs migration. The aim of

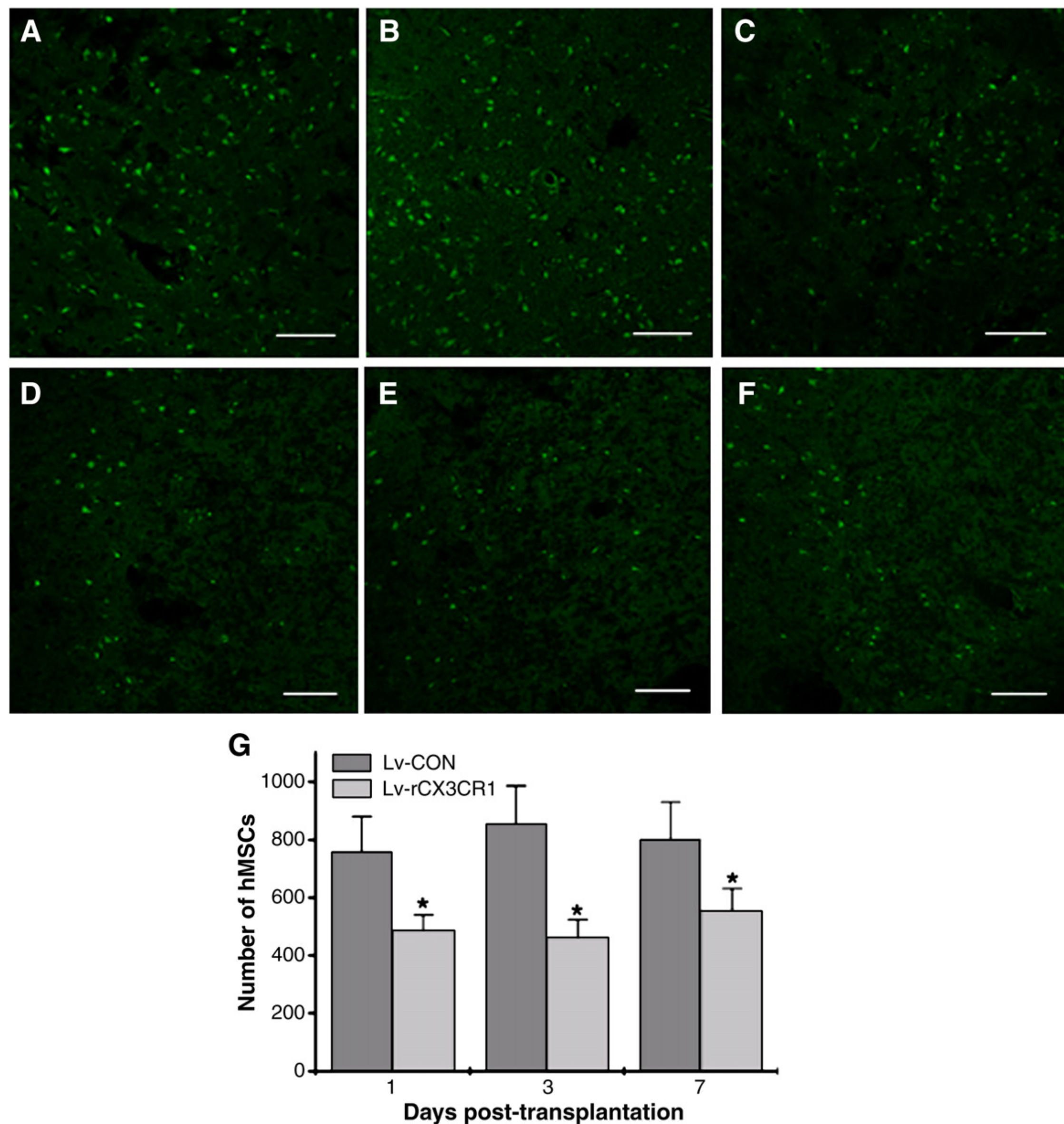


Fig. 7 – Distribution of transplanted hMSCs labeled by GFP in the ischemic brain. Photomicrographs showing the presence of GFP-positive cells in the boundary zone of the lesion area of the rats that received control-transduced hMSCs (A–C) and CX3CR1 knock-down hMSCs (D–F) at 1 (A, D), 3 (B, E), and 7 days (C, F) after injection. Quantification revealed that significantly more ($P < 0.01$) hMSCs were present in the control-transduced hMSCs (Lv-CON) injected group than in the CX3CR1 knock-down hMSCs (Lv-rCX3CR1) injected group (G). *$P < 0.01$ compared to Lv-CON on the same day. Scale bar = 100 μm. The presented results are the mean ± S.E.

these experiments was to explore the role of fractalkine and its receptor CX3CR1 in the migration of transplanted hMSCs to an injured brain region.

First, up-regulation of fractalkine in the ischemic brain was observed by real-time PCR and immunofluorescence analysis. The real-time PCR analysis revealed that fractalkine mRNA expression increased dramatically in the injured cerebral cortex at 2 and 4 days after reperfusion. We also found that fractalkine was extensively expressed in the peri-infarct area after MCAO and the fractalkine-expressing cells were positive for neuronal marker NeuN, this co-expression also meant the fractalkine-expressing cells were neurons, which were consistent with the previous studies. Tarozzo et al. (2002) found that fractalkine was highly expressed in ischemic penumbral neurons and vascular endothelial cells after MCAO via in situ hybridization and immunohistochemistry. Our results confirmed the changes in fractalkine expression in the ischemic brain.

Next, an in vitro study, real-time PCR and western blot were employed to evaluate the expression of CX3CR1 in hMSCs cultured in vitro. Our results were consistent with the findings of Sordi et al. (2005). The proliferating GFP-labeled hMSCs harvested at the 3rd to 5th passages were transplanted through tail intravenous injection at 24 h after reperfusion.

We observed that the transplanted hMSCs migrated to the parenchyma of the ipsilateral hemisphere and were mainly distributed in the ischemic boundary zone, especially in the region with high fractalkine expression. The results showed that fractalkine which was highly expressed in the ischemic brain, and its receptor CX3CR1 induced the migration of transplanted hMSCs to the damaged region. No significant reduction in the infarct volume was detected in hMSCs-treated rats relative to the PBS group. This finding may be explained by the fact that transplantation was carried out 24 h after reperfusion, by which time the brain cells had already been irreversibly damaged. Also, the experiment was performed within 7 days after transplantation, which may not have been sufficient to observe the full therapeutic effect of hMSCs transplantation.

To further confirm the role of fractalkine/CX3CR1 in the induction of directional migration of transplanted hMSCs, RNA interference was applied to suppress the expression of CX3CR1 in hMSCs. The results indicated that CX3CR1 knock-down significantly decreased the migration of transplanted hMSCs to ischemic brain regions, which indirectly suggested that fractalkine/CX3CR1 signaling induced directional migration of transplanted hMSCs to lesions. Real-time PCR and western blot confirmed that the lentiviral vector encoded shRNA mediated human CX3CR1 knock-down and efficiently transduced hMSCs. As best as we know, this is the first study that employed RNAi to confirm the effects of fractalkine and CX3CR1 on the directional migration of transplanted hMSCs to lesions in the ischemic brain.

Overall, this study demonstrates that intravenously transplanted hMSCs migrate to the ischemic brain. The increased expression of fractalkine in the ischemic injured brain and its receptor CX3CR1 played an important role in the directional migration of the transplanted hMSCs. The present study provides an intervention strategy for the promotion of directional migration of transplanted MSCs to brain lesions and may provide improvement of therapeutic outcomes of MSCs-mediated cell therapy.

4. Experimental procedures

4.1. *Transient middle cerebral artery occlusion model*

Adult male Sprague–Dawley rats (270 to 300 g) were purchased from the Laboratory Animal Center of the Third Military Medical University. We induced transient middle cerebral artery occlusion (MCAO) using a method of intraluminal vascular occlusion, based on the method described by Nagasawa and Kogure (1989). Briefly, rats were anesthetized with 10% chloral hydrate (300 mg/kg, i.p.). A length of 4–0 monofilament nylon suture (18.5 to 19.5 mm), with a 0.25 mm diameter silicone tip, was introduced through the stump of the external carotid artery into the lumen of the internal carotid artery until it blocked the origin of the middle cerebral artery. 2 h after MCAO, animals were reanesthetized by ether inhalation, and reperfusion was performed by pulling out of the nylon filament until the tip cleared the lumen of the external carotid artery. Once the animals awoke, behavioral changes were evaluated according to the standard described by Longa et al. (1989). Rats with scores of 2–3 were included in the next experiments. This study was approved by the Animal Ethics Committee of the Third Military Medical University. All experiments conformed to the guidelines of ethical use of animals, and all efforts were made to minimize animal suffering and to reduce the number of animals used.

4.2. *Culture and amplification of hMSCs*

Cells harvested from the iliac heparinized bone marrow were obtained by iliac crest aspiration from healthy adult volunteers, aged 18 to 45 years old, after informed consent and in accordance with guidelines approved by the Ethics Committee of the Third Military Medical University. Mononuclear cells were isolated from bone marrow specimens by Percoll (1.073 g/ml; Pharmacia, New Jersey, USA) gradient centrifugation and allowed to adhere to cell culture flasks in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Grand Island, NY). After incubation at 37 °C in a 5% CO_2 atmosphere for 24 h, the nonadherent cells were removed by replacement of the medium. The remaining adherent cells were defined as passage 0. The adherent layer reached 90% confluence within 10–14 days. Cells were then detached with 0.25% trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA; Invitrogen), split at 1:2 or 1:3 and replated as passage 1. The plastic-adherent hMSCs were split every 5–7 days thereafter to assess cell growth and cell yield. The cells used in the following experiments were harvested after 3 to 5 passages.

Before use, the cells were analyzed for morphology and marker expression. All of the cells had a fibroblast-like morphology in culture and were uniformly positive for CD105 and CD29 and negative for hematopoietic markers CD34 and CD45, as determined by flow cytometry.

4.3. *Transduction of hMSCs with human CX3CR1 shRNA-expressing lentiviral vectors*

A human CX3CR1 small hairpin RNA (shRNA)-expressing lentivirus gene transfer vector, encoding the green fluorescent protein (GFP) sequence, was constructed by Genechem Co., Ltd, Shanghai, China. The target sequence of the shRNA was 5′-GCCTGTCTCTTCCATATGA-3′ (GenBank accession no. NM001337). The identity of the vector was confirmed by PCR and sequencing analysis. The recombinant CX3CR1 shRNA-expressing lentivirus (Lv-rCX3CR1) and the control lentivirus (Lv-CON) were prepared at a titer of 10^9 TU (transfection unit)/ml.

hMSCs from passages 3 to 5 were transduced with lentiviral vectors at a multiplicity of infection (MOI) of 20 in complete medium containing polybrene (5 μg/ml) at 37 °C and 5% CO_2 for 18–24 h. The cells were then washed and cultured in fresh medium containing 10% FBS. Transduction efficiency was measured by determining the frequency of green flourescent protein (GFP)-positive cells. Cells were harvested 5–7 days following transduction, and CX3CR1 expression was determined by real-time PCR and western blot.

Cell viability was determined by colorimetric measurement of the reduction product of MTT (Sigma-Aldrich, St. Louis, USA). Cells transduced with the shRNA-expressing lentivirus were seeded in 96-well plates containing DMEM supplemented

with 10% FBS (100 μl/well, 1×10^4 cells/well) and divided into 7 groups. After incubation at 37 °C in 5% CO_2 for 1, 2, 3, 4, 5, 6, and 7 days, 10 μl MTT was added to each well and the cells were incubated for another 4 h at 37 °C. Then, 100 μl dimethyl sulfoxide was added to each well and the suspension was thoroughly mixed for 10 min. The optical density of each well was measured at 570 nm. Non-transduced cells and control-transduced cells were used as controls. All experiments were performed in triplicate.

4.4. *hMSCs transplantation procedures*

At 24 h after the onset of cerebral ischemia, randomly selected animals received transplantation. Animals were anesthetized by ether inhalation. Approximately 2×10^6 hMSCs in 1 ml phosphate buffered saline (PBS) were injected slowly into the tail vein. The rats were divided into 3 groups: rats in Group 1 ($n=18$) received 2×10^6 CX3CR1 shRNA transduced hMSCs; rats in Group 2 ($n=18$) received 2×10^6 control-transduced hMSCs; rats in Group 3 ($n=18$) received PBS as a control. Healthy rats ($n=9$) also received control-transduced hMSCs as a control. Rats were sacrificed at 1, 3, and 7 days after transplantation (2, 4, and 8 days after transient cerebral ischemia) and analyzed by immunohistochemistry.

4.5. *Determination of fractalkine and CX3CR1 mRNA levels by real-time PCR*

The fractalkine mRNA expression level in the ischemic brain, and CX3CR1 mRNA expression level in hMSCs were determined by real-time PCR. At 2, 4 and 8 days after cerebral ischemia ($n=6$ at each time point), the model animals were anesthetized with 10% chloral hydrate (300 mg/kg, i.p.), the brains were quickly removed, and whole cerebral cortices were carefully dissected from each rat. Tissues were snap-frozen in liquid nitrogen and stored at −80 °C. Healthy rats ($n=3$) were used as control subjects. At 5–7 days following transduction, hMSCs transduced with CX3CR1 shRNA were harvested for analysis. Non-transduced cells and control-transduced cells were used as controls.

Total RNA obtained from brain biopsy tissues and hMSCs was extracted using Trizol RNA extraction reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription (RT)-PCR assays were performed using 1 μg of RNA (A260/280 ratio 1.8–2.0). The transcribed cDNA was amplified using CX3CR1 and fractalkine primer pairs: CX3CR1 forward, 5′-GGC CTT GTC TGA TCT GCT GTT TG-3′; CX3CR1 reverse 5′-AAT GCT GAT GAC GGT GAT GAA GAA-3′; fractalkine forward, 5′-TGG TGG CAA GTT TGA GAA GC-3′; fractalkine reverse, 5′-CTG GGA AAT AGC AGT CGG TT-3′. GAPDH served as an internal standard and control for reverse transcription efficiency. The GAPDH primers were forward, 5′-GAC CTG ACC TGC CGT CTA-3′; reverse, 5′-AGG AGT GGG TGT CGC TGT-3′. All PCR primers were designed using Premier Primer 5.0 software, and BLAST searches were performed for each primer to avoid sequence homology with other genes. cDNA was analyzed by real-time PCR (ABI 7500 Sequence Detector, Applied Biosystems) using SYBR Green PCR reagents (TaKaRa, Dalian, China). Reactions were incubated at 95 °C for 10 s, and were then submitted to 40 cycles at 95 °C for 5 s followed by 60 °C for 34 s.

4.6. *Examination of CX3CR1 in hMSCs by western blot analysis*

CX3CR1 expression in hMSCs was determined by western blot analysis. At 5–7 days after transduction, CX3CR1 shRNA transduced hMSCs[Float1] were harvested by trypsinization and subjected to analysis. Non-transduced cells and control-transduced cells were used as controls. Total cellular extracts were prepared by lysing the cells in RIPA buffer for 30 min on ice. Equivalent amounts of protein, as determined by use of the BCA Protein Assay Kit (Pierce, IL, USA), were loaded onto 10% sodium dodecyl sulfate (SDS) PAGE gels. CX3CR1 protein was detected by use of rabbit anti-CX3CR1 polyclonal antibody (Abcam, MA, USA) at a dilution of 1:1000, and GAPDH, which served as a loading control, was detected by use of rabbit anti-GAPDH polyclonal antibody (Santa Cruz Biotechnology, CA, USA) at a dilution of 1:2000. After incubation with the appropriate secondary antibody for 1 h at room temperature, an enhanced chemiluminescence kit (Pierce) was used for chemiluminescent detection of the proteins.

4.7. *Determination of infarct volume, hMSCs migration and fractalkine expression*

At 1, 3, and 7 days after cell transplantation (2, 4, and 8 days after transient cerebral ischemia), animals were deeply anesthetized with chloral hydrate, and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Brains were carefully removed and post-fixed for 12 h in 4% paraformaldehyde in 0.1 M PB (pH 7.4), and then stored in 30% sucrose in 0.1 M PB (pH 7.4). Each brain was cut into seven equally sized (2 mm) coronal blocks. The tissues were processed, and a series of 40-μm-thick coronal sections from each block was cut on a cryostat.

One of each of the frozen coronal sections from seven blocks was stained with cresyl violet. The cross-sectional area of infarction in each brain slice was examined with a dissecting microscope and was measured using image analysis software (Adobe Photoshop 7.0). The total infarct volume of each brain was calculated by multiplying the sum of the infarct area by the distance between sections.

Immunostaining was performed on the frozen sections of the brain. To identify the distribution of grafted hMSCs, the distribution of GFP-positive cells was analyzed. Briefly, after blocking in normal serum, all frozen coronal sections were treated with mouse polyclonal GFP antibody (Beyotime, Shanghai, China) diluted at 1:200 in PBS overnight at 4 °C, followed by sequential incubation with FITC-conjugated goat anti-mouse IgG (1:100; Zhongshan Golden Bridge Biotechnology, Beijing, China). To define the location of fractalkine and GFP-positive hMSCs, double staining was used. Each coronal slide was sequentially treated with GFP antibody, FITC-conjugated secondary antibody, rabbit polyclonal fractalkine antibody (1:200; BioVision, CA, USA), and CY3-conjugated secondary antibody (1:500; Beyotime). To visualize the cellular colocalization of fractalkine, double staining was used on some frozen sections. Each coronal slide was treated with the first primary antibody, fractalkine, as described above and then was treated with cell-type-specific antibodies: a neuronal nuclear antigen (NeuN, 1:200; Chemicon, CA, USA), glial

fibrillary acidic protein (GFAP, 1:300; Cell Signaling, MA, USA), The sections were observed under a laser scanning confocal microscope (Leica SP-5, Germany). Negative control slides were processed as describe above, except that primary antibodies were omitted.

To determine the number of GFP-positive cells in the ischemic brain, an average number of cells in five equally spaced sections (at approximately 200-μm intervals) were determined in each brain block, and the number of GFP-positive cells was counted within the seven 2-mm-thick blocks. To reduce bias introduced by the sampling parameters, all of the sections obtained from the rats were stained for GFP simultaneously. All analyses were conducted by observers who were blinded to the treatment conditions.

For semi-quantitative measurement of fractalkine expression, Image Pro® Plus 5.0 software (Media Cybernetics, Inc., Bethesda, MD, USA) was used. Using a laser scanning confocal microscope (Leica SP-5, Germany), a series of random images of each section (6 from the ischemic hemisphere and 6 from the contralateral hemisphere), was taken for each immunostained parameter. Fractalkine expression in the infarcted brain was expressed as the mean fluorescence intensity. Data are presented as the sum of integrated optical density (IOD)/sum area. All analyses were conducted by observers who were blinded to the treatment conditions.

4.8. Statistical analysis

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

REFERENCES

Campbell, D.J., Kim, C.H., Butcher, E.C., 2003. Chemokines in the systemic organization of immunity. Immunol. Rev. 195, 58–71.

Cartier, L., Hartley, O., Dubois Dauphin, M., Krause, K.H., 2005. Chemokine receptors in the central nervous system: role in brain inflammation and neurodegenerative diseases. Brain Res. Brain Res. Rev. 48 (1), 16–42.

Chen, J.R., Cheng, G.Y., Sheu, C.C., Tseng, G.F., Wang, T.J., Huang, Y.S., 2008. Transplanted bone marrow stromal cells migrate, differentiate and improve motor function in rats with experimentally induced cerebral stroke. J. Anat. 213, 249–258.

Constantin, G., 2008. Chemokine signaling and integrin activation in lymphocyte migration into the inflamed brain. J. Neuroimmunol. 198, 20–26.

Fong, A.M., Robinson, L.A., Steeber, D.A., Tedder, T.F., Yoshie, O., Imai, T., Patel, D.D., 1998. Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. J. Exp. Med. 188 (8), 1413–1419.

Harrison, J.K., Jiang, Y., Chen, S., Xia, Y., Maciejewski, D., McNamara, R.K., Streit, W.J., Salafranca, M.N., Adhikari, S., Thompson, D.A., Botti, P., Bacon, K.B., Feng, L., 1998. Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. Proc. Natl. Acad. Sci. U. S. A. 95, 10896–10901.

Honczarenko, M., Le, Y., Swierkowski, M., Ghiran, I., Glodek, A.M., Silberstein, L.E., 2006. Human bone marrow stromal cells express a distinct set of biologically functional chemokine receptors. Stem Cells 24, 1030–1041.

Hughes, P.M., Botham, M.S., Frentzel, S., Mir, A., Perry, V.H., 2002. Expression of fractalkine (CX3CL1) and its receptor, CX3CR1, during acute and chronic inflammation in the rodent CNS. Glia 37, 314–327.

Imai, T., Hieshima, K., Haskell, C., Baba, M., Nagira, M., Nishimura, M., Kakizaki, M., Takagi, S., Nomiyama, H., Schall, T.J., Yoshie, O., 1997. Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. Cell 91 (4), 521–530.

Ji, J.F., He, B.P., Dheen, S.T., Tay, S.S., 2004. Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury. Stem Cells 22, 415–427.

Lee, R.H., Hsu, S.C., Munoz, J., Jung, J.S., Lee, N.R., Pochampally, R., Prockop, D.J., 2006. A subset of human rapidly self-renewing marrow stromal cells preferentially engraft in mice. Blood 107, 2153–2161.

Li, M., Ransohoff, R.M., 2008. Multiple roles of chemokine CXCL12 in the central nervous system: a migration from immunology to neurobiology. Prog. Neurobiol. 84, 116–131.

Liu, W., Jiang, X., Fu, X., Cui, S., Du, M., Cai, Y., Xu, R., 2008. Bone marrow stromal cells can be delivered to the site of traumatic brain injury via intrathecal transplantation in rabbits. Neurosci. Lett. 434, 160–164.

Longa, E.Z., Weinstein, P.R., Carlson, S., Cummins, R., 1989. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 20, 84–91.

Lu, J., Moochhala, S., Moore, X.L., Ng, K.C., Tan, M.H., Lee, L.K., He, B., Wong, M.C., Ling, E.A., 2006. Adult bone marrow cells differentiate into neural phenotypes and improve functional recovery in rats following traumatic brain injury. Neurosci. Lett. 398, 12–17.

Mines, M., Ding, Y., Fan, G.H., 2007. The many roles of chemokine receptors in neurodegenerative disorders: emerging new therapeutical strategies. Curr. Med. Chem. 14, 2456–2470.

Mizuno, T., Kawanokuchi, J., Numata, K., Suzumura, A., 2003. Production and neuroprotective functions of fractalkine in the central nervous system. Brain Res. 979, 65–70.

Nagasawa, H., Kogure, K., 1989. Correlation between cerebral blood flow and histologic changes in a new rat model of middle cerebral artery occlusion. Stroke 20, 1037–1043.

Pan, Y., Lloyd, C., Zhou, H., Dolich, S., Deeds, J., Gonzalo, J.A., Vath, J., Gosselin, M., Ma, J., Dussault, B., Woolf, E., Alperin, G., Culpepper, J., Gutierrez Ramos, J.C., Gearing, D., 1997. Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. Nature 387, 611–617.

Parr, A.M., Tator, C.H., Keating, A., 2007. Bone marrow-derived mesenchymal stromal cells for the repair of central nervous system injury. Bone Marrow Transplant. 40, 609–619.

Prockop, D.J., 1997. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276, 71–74.

Prockop, D.J., 1998. Marrow stromal cells as stem cells for continual renewal of nonhematopoietic tissues and as potential vectors for gene therapy. J. Cell. Biochem., Suppl. 30–31, 284–285.

Prockop, D.J., Gregory, C.A., Spees, J.L., 2003. One strategy for cell and gene therapy: harnessing the power of adult stem cells to repair tissues. Proc. Natl. Acad. Sci. U. S. A. 100 (Suppl. 1), 11917–11923.

Re, D.B., Przedborski, S., 2006. Fractalkine: moving from chemotaxis to neuroprotection. Nat. Neurosci. 9, 859–861.

Seyfried, D., Ding, J., Han, Y., Li, Y., Chen, J., Chopp, M., 2006. Effects of intravenous administration of human bone marrow stromal cells after intracerebral hemorrhage in rats. J. Neurosurg. 104, 313–318.

Shen, L.H., Li, Y., Chen, J., Zhang, J., Vanguri, P., Borneman, J., Chopp, M., 2006. Intracarotid transplantation of bone marrow stromal cells increases axon-myelin remodeling after stroke. Neuroscience 137, 393–399.

Shichinohe, H., Kuroda, S., Lee, J.B., Nishimura, G., Yano, S., Seki, T., Ikeda, J., Tamura, M., Iwasaki, Y., 2004. In vivo tracking of bone marrow stromal cells transplanted into mice cerebral infarct by fluorescence optical imaging. Brain Res. Brain Res. Protoc. 13, 166–175.

Shichinohe, H., Kuroda, S., Yano, S., Hida, K., Iwasaki, Y., 2007. Role of SDF-1/CXCR4 system in survival and migration of bone marrow stromal cells after transplantation into mice cerebral infarct. Brain Res. 1183, 138–147.

Shyu, W.C., Chen, C.P., Lin, S.Z., Lee, Y.J., Li, H., 2007. Efficient tracking of non-iron-labeled mesenchymal stem cells with serial MRI in chronic stroke rats. Stroke 38, 367–374.

Sordi, V., Malosio, M.L., Marchesi, F., Mercalli, A., Melzi, R., Giordano, T., Belmonte, N., Ferrari, G., Leone, B.E., Bertuzzi, F., Zerbini, G., Allavena, P., Bonifacio, E., Piemonti, L., 2005. Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. Blood 106, 419–427.

Soriano, S.G., Amaravadi, L.S., Wang, Y.F., Zhou, H., Yu, G.X., Tonra, J.R., Fairchild Huntress, V., Fang, Q., Dunmore, J.H., Huszar, D., Pan, Y., 2002. Mice deficient in fractalkine are less susceptible to cerebral ischemia-reperfusion injury. J. Neuroimmunol. 125, 59–65.

Sunnemark, D., Eltayeb, S., Nilsson, M., Wallstrom, E., Lassmann, H., Olsson, T., Berg, A.L., Ericsson-Dahlstrand, A., 2005. CX3CL1 (fractalkine) and CX3CR1 expression in myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis: kinetics and cellular origin. J. Neuroinflammation 2, 17.

Tarozzo, G., Campanella, M., Ghiani, M., Bulfone, A., Beltramo, M., 2002. Expression of fractalkine and its receptor, CX3CR1, in response to ischaemia-reperfusion brain injury in the rat. Eur. J. Neurosci. 15, 1663–1668.

Wang, L., Li, Y., Chen, X., Chen, J., Gautam, S.C., Xu, Y., Chopp, M., 2002a. MCP-1, MIP-1, IL-8 and ischemic cerebral tissue enhance human bone marrow stromal cell migration in interface culture. Hematology 7, 113–117.

Wang, L., Li, Y., Chen, J., Gautam, S.C., Zhang, Z., Lu, M., Chopp, M., 2002b. Ischemic cerebral tissue and MCP-1 enhance rat bone marrow stromal cell migration in interface culture. Exp. Hematol. 30, 831–836.

Wang, Y., Deng, Y., Zhou, G.Q., 2008. SDF-1alpha/CXCR4-mediated migration of systemically transplanted bone marrow stromal cells towards ischemic brain lesion in a rat model. Brain Res. 1195, 104–112.

Wu, J., Sun, Z., Sun, H.S., Wu, J., Weisel, R.D., Keating, A., Li, Z.H., Feng, Z.P., Li, R.K., 2008. Intravenously administered bone marrow cells migrate to damaged brain tissue and improve neural function in ischemic rats. Cell Transplant 16, 993–1005.

Yano, S., Kuroda, S., Lee, J.B., Shichinohe, H., Seki, T., Ikeda, J., Nishimura, G., Hida, K., Tamura, M., Iwasaki, Y., 2005a. In vivo fluorescence tracking of bone marrow stromal cells transplanted into a pneumatic injury model of rat spinal cord. J. Neurotrauma. 22, 907–918.

Yano, S., Kuroda, S., Shichinohe, H., Hida, K., Iwasaki, Y., 2005b. Do bone marrow stromal cells proliferate after transplantation into mice cerebral infarct?—a double labeling study. Brain Res. 1065, 60–67.

Zhang, J., Li, Y., Lu, M., Cui, Y., Chen, J., Noffsinger, L., Elias, S.B., Chopp, M., 2006. Bone marrow stromal cells reduce axonal loss in experimental autoimmune encephalomyelitis mice. J. Neurosci. Res. 84, 587–595.