



Cell biology / Biologie cellulaire

Overexpression of HIF-1 α in mesenchymal stem cells contributes to repairing hypoxic-ischemic brain damage in rats



Deju Lin^a, Liping Zhou^a, Biao Wang^a, Lizhen Liu^b, Li Cong^c, Chuanqin Hu^a, Tingting Ge^a, Qin Yu^{a,*}

^a College of Life Science, Zhejiang Chinese Medical University, Hangzhou, Zhejiang, China

^b Bone Marrow Transplantation Center, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, China

^c The First Clinical Medical College, Zhejiang Chinese Medical University, Hangzhou, Zhejiang, China

ARTICLE INFO

Article history:

Received 12 May 2016

Accepted after revision 6 November 2016

Available online 7 December 2016

Keywords:

Hypoxic-ischemic brain damage

Mesenchymal stem cell

Hypoxia inducible factor-1 alpha

ABSTRACT

Preclinical researches on mesenchymal stem cells (MSCs) transplantation, which is used to treat hypoxic-ischemic (HI) brain damage, have received inspiring achievements. However, the insufficient migration of active cells to damaged tissues has limited their potential therapeutic effects. There are some evidences that hypoxia inducible factor-1 alpha (HIF-1 α) promotes the viability and migration of the cells. Here, we aim to investigate whether overexpression of HIF-1 α in MSCs could improve the viability and migration capacity of cells, and its therapeutic efficiency on HI brain damage. In the study, MSCs with HIF-1 α overexpression was achieved by recombinant lentiviral vector and transplanted to the rats subsequent to HI. Our data indicated that overexpression of HIF-1 α promoted the viability and migration of MSCs, HIF-1 α overexpressed MSCs also had a stronger therapeutic efficiency on HI brain damaged treatment by mitigating the injury on behavioral and histological changes evoked by HI insults, accompanied with more MSCs migrating to cerebral damaged area. This study demonstrated that HIF-1 α overexpression could increase the MSCs' therapeutic efficiency in HI and the promotion of the cells' directional migration to cerebral HI area by overexpression may be responsible for it, which showed that transplantation of MSCs with HIF-1 α overexpression is an attractive therapeutic option to treat HI-induced brain injury in the future.

© 2016 Académie des sciences. Published by Elsevier Masson SAS. All rights reserved.

1. Introduction

Hypoxic-ischemic (HI) brain damage is a clinical syndrome with high mortality and morbidity [1], and the survivors have suffered widely mental and physical

disabilities [2]. However, there are no effective treatments available for HI brain damage at present.

Mesenchymal stem cells (MSCs), which are easily obtained from many tissues, have aroused great interest in regenerative medicine because of their multi-directional differentiation potential and hypo-immunogenicity properties [3]. In recent years, MSCs have showed a significant neuroprotective effects in experimental models of HI brain damage [4–6]. However, the main problem is that the low number of active cells reaching the target tissues after

* Corresponding author at: 548, Binwen Road, 310053 Hangzhou, Zhejiang, China.

E-mail address: qinyu3587@126.com (Q. Yu).

transplantation blocks its potential therapeutic effects [7,8].

Previous studies suggested that delivering the desired genes into the cells may overcome some limitations of cells and facilitate therapeutic efficiency after cell transplantation [9,10]. Hypoxia inducible factor-1 alpha (HIF-1 α) is one of the most important regulators in response to oxygen levels, and the stabilization of HIF-1 α in cells may promote activation and migration [11–13].

In the present study, we intended to overexpress HIF-1 α in MSCs by recombinant lentivirus and investigate the issue whether HIF-1 α overexpression could promote the therapeutic efficiency of MSCs.

2. Materials and methods

2.1. Animals

All procedures related to the use of animals complied with the guide published by NIH [14]. Adult male Sprague-Dawley rats (80–100 g) were purchased from the Animal Center of Zhejiang Chinese Medical University, Hangzhou, China (Laboratory Animal Certificate: scxk 2008-0016).

2.2. Generation, culture and pretreatment of MSCs

MSCs were obtained from rat femoral and tibia bone marrow as previously described [15]. Briefly, muscles, connective tissue and epiphyses were removed from femur and tibia. Marrow was harvested by 1.0 mL syringe with cell medium that consist of Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12; Invitrogen, Grand Island, NY, USA), 10% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA, USA) and 1% penicillin-streptomycin (HyClone Laboratories, Inc., South Logan, Utah, USA). Cells were then centrifuged for 10 min at 1000 rpm, re-suspended in the cell medium and cultured at 37 °C, 5% CO₂. After 24 h, all non-adherent cells were removed by medium exchange; the fresh medium was subsequently replaced every 2 days. The adherent cells were trypsinized at 80% confluence, and passaged at 1:2. MSCs from passage 3 were used in the subsequent experiments.

MSCs had received a hypoxia pretreatment during 24 h to mimic the microenvironment in the damaged area in order to activate the endogenous gene expression, which is the natural effect of cells in response to hypoxia. Briefly, the cells were placed in a hypoxia incubator and balanced with a gas mixture (1% O₂, 5% CO₂, 94% N₂) for 10 min at a flow rate of 1 L/min. Then, the hypoxia incubator was sealed to maintain a hypoxic environment before being put into a cell incubator under 5% CO₂ at 37 °C.

2.3. Lentivirus-mediated gene transfer and experimental groups of cells

MSCs with HIF-1 α overexpression were constructed by a lentivirus system following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The cells were infected with a lentivirus encoding either GFP alone or

both GFP and HIF-1 α . Then the MSCs were assigned into three experiment groups: HIF-1 α -MSCs (transfection with HIF-1 α and GFP), GFP-MSCs (transfection with GFP), NM-MSCs (undisturbed normal MSCs).

The transfection efficiency of the cells was detected by fluorescence microscopy analysis after transfecting for 48 h. The protein expression level of HIF-1 α in MSCs was detected by Western blotting.

2.4. Western blotting

The expression level of HIF-1 α was evaluated by western blotting after hypoxia pretreatment. Equal amounts of protein from MSCs were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Boston, MA). After being blocked with 5% nonfat dry milk for 2 h, the membranes were incubated with rabbit polyclonal antibodies against either HIF-1 α (1:1000 dilution, Abcam Technology, Cambridge, MA, USA) or GAPDH (1:2000 dilution, Abcam Technology, Cambridge, MA, USA) overnight at 4 °C. The membranes were then incubated with a second antibody (anti-rabbit IgG HRP, 1:3000; Abcam Technology, Cambridge, MA, USA) and ECL detection systems (Thermo Fisher Scientific, Waltham, MA, USA) were used for detection, GAPDH was used as a loading control.

2.5. MTT assay

An assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was performed to determine the influence of HIF-1 α overexpression on MSC viability. Briefly, pretreated MSCs were seeded in 96-well plates at a density of 2000 cells per well, and cultured for 48 h, and NM-MSCs were used as a control. 10 μ L (5 g/L in PBS) of MTT (Sigma Chemical Co., St. Louis, MO, USA) were added to each well and incubated at 37 °C for 4 h. The supernatants were carefully discarded and 150 μ L of dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO, USA) was added. Absorbance was measured at 490 nm by microplate reader (Bio-RAD Model 680, Cal, USA).

2.6. CM-DiI labelling

In order to make it easily observable, MSCs were stained with chloromethylbenzamido dialkylcarbocyanine (CM-DiI, Molecular Probes, USA) at the dose of 1 μ g CM-DiI per million cells, and washed twice after incubation at 37 °C for 20 min. Labeled MSCs were used in the following experiments.

2.7. Migration assay

A cell migration assay was performed in Transwell chambers (pore size: 8 μ m, polycarbonate membrane; Corning Incorporated, NY, USA) to evaluate the migratory capacity of MSCs. Transwell chambers were put into six well plates. CM-DiI labeled cells were harvested in a serum-free medium; the density was adjusted to 2 \times 10⁵ cells/ml. 200 μ L of cell suspension were plated in the upper chamber and 500 μ L of a fresh medium

containing 10% FBS were added to the lower chamber. After incubation at 37 °C under 5% CO₂ for 24 h, the cells on upper membrane layer were gently wiped away using a cotton swab and the cells migrating to the lower membrane were fixed with 4% paraformaldehyde. The number of cells in the lower side of the membranes was counted microscopically.

2.8. Hypoxic-ischemic model and experimental groups of rats

As HI model was established as in the previous method of Rice et al. [16]. The rats were anesthetized with a 10% chloral hydrate solution (0.3 mL/100 g), and the left carotid artery was isolated and ligated doubly. After 2–3 h, the waking rats were put into a transparent container which was ventilated with a constant flow of mixed gas containing 8% oxygen and 92% nitrogen for 2 h.

The animals were randomly assigned into four experimental groups after HI treatment: sham group, HI-vehicle group, HI-GFP-MSC group, HI-HIF-1 α -MSC group. For the rats in the sham group, the carotid artery was isolated but not ligated, and no hypoxia exposure was performed. The rats in the HI-GFP-MSC group and the HI-HIF-1 α -MSC group had received 5×10^5 MSCs re-suspended in 0.5 mL of a physiological saline solution by intravenous injection in the tail 24 h after HI treatment, and the other rats received the same volume of physiological saline solution.

2.9. Morris water maze test

Rats ($n=6$ per group) have been submitted to the Morris water maze test [17] on day 14 after HI to evaluate their spatial learning and memory abilities. The maze consisted of a black circular tank (160 cm diameter \times 50 cm height) filled with water (temperature: 22–24 °C). An automatic tracking system (San Diego Instruments, San Diego, CA, USA) was used to record the swimming path and time. Each rat was tested for five training days and one test day. In the task, the rat was put in every quadrant of the pool sequentially, and swam for 60 s to locate the submerged platform; if it failed, the experimenter would guide it to the platform. Then the rat

was allowed to remain on the platform for 10 s. On the last day, the platform was removed before the test, and the swimming time in the former platform quadrant was recorded.

2.10. Tissue preparation and histological examination

Rats brains were obtained on days 7, 14 and 21 after HI (each time point $n=6$ per group), and each hippocampus was embedded in paraffin. The paraffin sections (4 μ m thick) were prepared for detection of the migration of MSCs and histological examination in the hippocampus. To measure the migrated cells' number, the sections were observed with a fluorescence microscope to count the CM-DiI positive cells. The histological examination was determined by hematoxylin and eosin (HE) staining.

2.11. Statistical analysis

The data obtained were presented as mean \pm standard deviation (SD). Differences between groups were evaluate by analysis of variance for multiple comparisons using SPSS 19.0. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Overexpression of HIF-1 α in MSCs

After transfection for two days, GFP expression observed under the fluorescence microscope reached up to 90% of the cultured MSCs (Fig. 1A). In the meantime, western blotting was performed to investigate the expression of HIF-1 α in MSCs after hypoxia pretreatment. In the present study, the expression of HIF-1 α in HIF-1 α -MSCs was approximately two times that in NM-MSCs and GFP-MSCs, while the expression level in GFP-MSCs was in accordance with NM-MSCs (Fig. 1B and C). The results indicated that HIF-1 α overexpressed MSCs were successfully constructed by a recombinant lentivirus system.

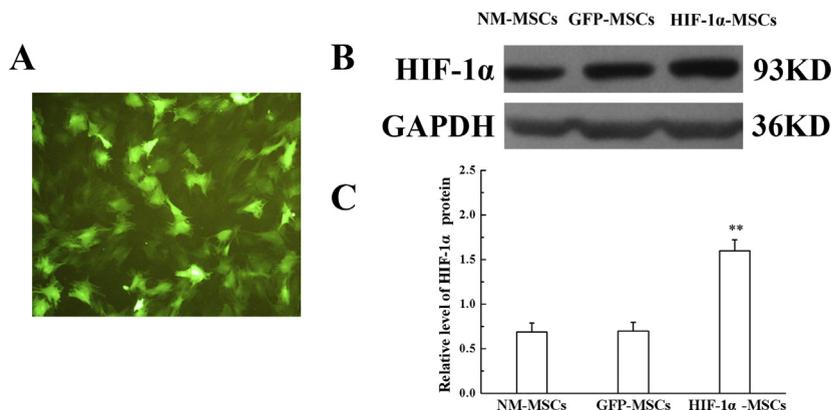


Fig. 1. Transfection efficiency of MSCs. A. The level of GFP expressed in MSCs was observed by fluorescence microscopy (magnification $\times 100$). B. Protein level of HIF-1 α in different groups; GAPDH was used as an inner control. C. Quantification of HIF-1 α protein expression showed in B. ** $P < 0.01$ versus the GFP-MSCs group.

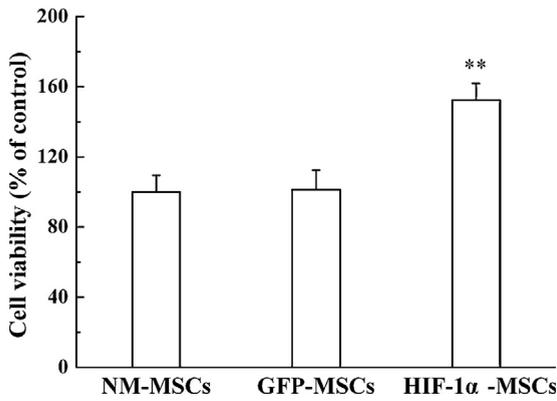


Fig. 2. MTT assay used to quantify the MSCs viability in different group. ** $P < 0.01$ versus the GFP-MSCs group.

3.2. HIF-1 α overexpression improved the viability of MSCs

MSC viability was assessed by an MTT assay to determine the effect of HIF-1 α overexpression. As shown by our results, HIF-1 α overexpression immensely improved the viability of MSCs, while there was no significant difference between NM-MSC and GFP-MSC groups (Fig. 2A). The result implied that MSCs with overexpression of HIF-1 α had better viability.

3.3. HIF-1 α overexpression enhanced migration of MSCs both in vitro and in vivo

The migration of MSCs was analyzed both in in vitro and in vivo experiments. To observe the migration of MSCs

directly, the cells were labeled with CM-Dil, a fluorescence marker. The results showed that more than 95% of MSCs were CM-Dil positive after having been labeled (Fig. 3A).

According to the Transwell assay, the number of cells migrating to the lower compartment in HIF-1 α -MSC group was more than that in NM-MSC and GFP-MSC groups after hypoxia pretreatment, while no significant difference was found between NM-MSC and GFP-MSC groups after culturing for 24 h (Fig. 3B).

One day after HI, rats received an intravenous transplantation of MSCs, and the presence of migrated MSCs in the hippocampus after transplantation was investigated by observing CM-Dil positive cells. CM-Dil positive cells were clearly observed in the hippocampus on day 7 after HI. And from day 7 to day 21 subsequent to HI, the number of CM-Dil positive cells in the hippocampus increased gradually in a time-dependent manner. A remarkable increase in the recruitment of HIF-1 α transduced MSCs was observed in the hippocampus at every time point compared with GFP transduced MSCs (Fig. 3C). Statistical analysis showed that HIF-1 α overexpression improved the migration capacity of MSCs toward the sites of injury.

The quantitation of migrated cells demonstrated significant increases in MSCs motility in HIF-1 α overexpressed MSCs both in in vitro and in vivo experiments in comparison to GFP infected cells.

3.4. HIF-1 α overexpression strengthen the therapeutic efficiency of MSCs on HI rats

In order to investigate the therapeutic effect of HIF-1 α transduced MSCs, the Morris water maze test and HE

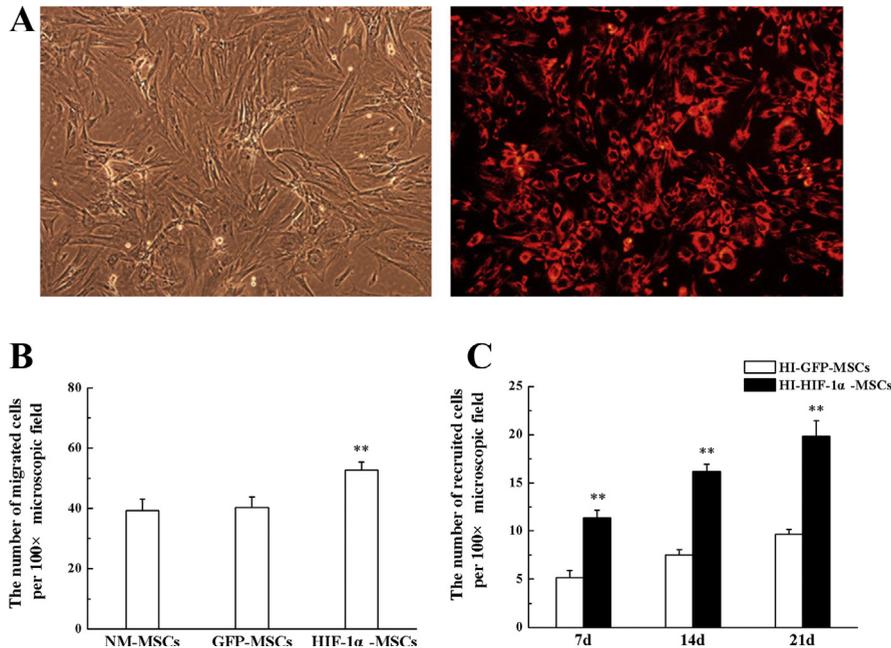


Fig. 3. MSCs migration assay in vitro and in vivo experiments. A. The phase-contrast and fluorescent image of CM-Dil labeled MSCs (magnification: $\times 100$). B. Transwell migration assay, performed in vitro experiments. C. Transplanted MSCs homing to the hippocampus were counted at different time points. ** $P < 0.01$ versus the GFP-MSCs group.

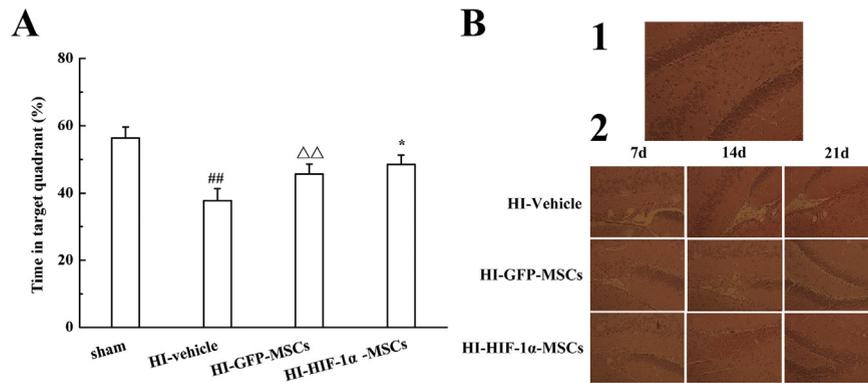


Fig. 4. The therapeutic efficiency of HIF-1 α overexpressed MSCs in HI rats. **A.** Percentage of swimming time in the target quadrant in the Morris water maze. **B.** HE staining in different groups of the hippocampus. Representative images in the sham group (1), the pathological changes in the HI-Vehicle, HI-GFP-MSCs and HI-HIF-1 α -MSCs groups at different time points (2). ## $P < 0.01$ versus the sham group, $\Delta\Delta P < 0.01$ versus the HI-Vehicle group, * $P < 0.05$ versus the HI-GFP-MSCs group.

staining in the hippocampus were performed on HI rats. On day 14 after HI, the spatial performance of four groups was evaluated by the Morris water maze test. In the spatial probe test, spatial learning ability was significantly affected by HI, as HI-treated rats spent less time in the target quadrant compared to the sham group. The results suggested that the rats suffered from cognitive dysfunction due to HI treatment. There was a significant increase of time in the target quadrant in both MSC-transplanted groups compared with HI-vehicle group, but a more increase in the amount of time have been found in HI-HIF-1 α -MSC group (Fig. 4A). These results indicated that the HI-induced rats achieved partial remission on memorial and cognitive functions after MSCs transplantation, while HIF-1 α overexpression could promote this effect.

Some visualized pathological changes were found in HE staining. The data supported that the morphological structures of the hippocampus in the sham group were normal, with uniform dyeing (Fig. 4B1). In contrast with the sham group, the pathological changes in the hippocampus in the HI-vehicle group revealed serious injury, the cells were disordered, and cavitation was visible, suggesting that the HI model had been successfully established. Meanwhile, the rats in HI-GFP-MSC and HI-HIF-1 α -MSC groups showed similar but slight pathological changes at every time point after having been subjected to HI, and the changes in the MSCs with HIF-1 α transfection group were less marked than those of the GFP control group (Fig. 4B2). The results illustrated that the transplantation of MSCs ameliorated pathological changes after HI, while HIF-1 α overexpression could enhance therapeutic efficiency.

4. Discussion

In recent years, abundant researches show that MSCs transplantation has been considered a promising strategy in HI-induced brain-damaged treatment [4,18,19]. However, the insufficient viability as well as the low number of cells transplanted to the target tissues limits the potential therapeutic effects [7,8]. Recent evidences have indicated that HIF-1 α is important in cell viability, proliferation and

migration following HI injury [12,13]. In the present study, we therefore investigated the therapeutic effect of HIF-1 α overexpressed MSCs on HI brain damage. Firstly, overexpression was achieved using a recombinant lentiviral, and a robust overexpression of HIF-1 α at the protein level has been seen. Additionally, the functions of HIF-1 α overexpression in MSCs showed that HIF-1 α overexpression resulted in increased viability and migration of MSCs. Finally, using a HI model, we found that HIF-1 α overexpressed MSCs had a stronger therapeutic efficiency on HI brain-damaged treatment by mitigating the injury on behavioral and histological changes evoked by HI insults, accompanied with more MSCs migrating to the damaged cerebral area.

The hypoxia-inducible factor-1 (HIF-1) is recognized as the master regulator of hypoxia response. It is a heterodimeric transcription factor composed of an inducible HIF-1 α subunit and a HIF-1 β subunit, and HIF-1 α is a crucial mediator of the cellular response to hypoxia in contrast to HIF-1 β , implying that HIF-1 α is a key mediator of the beneficial effect in a low oxygen environment [20]. In this study, just as expected, we have seen the expression of HIF-1 α after hypoxia pretreatment, while overexpression increased the expression.

The present study showed that overexpression of HIF-1 α enhanced MSCs viability in vitro, which was consistent with a previous study [21]. Intriguingly, the results also showed in our present study that HIF-1 α overexpression could further promote the viability of MSCs in vitro in comparison to the effect of HIF-1 α induced by hypoxia pretreatment, indicating that the expression level of endogenous HIF-1 α , which achieved during hypoxia, does not reach the maximum required for HIF-1 α transcription activities, and transfection with exogenous HIF-1 α improves the transcription level.

Cerebral ischemia and hypoxia will result in cognitive dysfunction [22,23] as well as pathological changes [24], especially the changes in the hippocampus [25], which showed in the Morris water maze test and HE staining in our study. Transplantation of MSCs after HI contributed to an improved function recovery of neurological deficits

and reduced the degree of pathological changes [18,19,26]. Meanwhile, it was observed that HIF-1 α overexpression led to a better efficiency of MSC transplantation. This result is not in keeping with studies by Lopez et al. [27], which showed that overexpression of HIF-1 α did not have protective actions upon hypoxia-mediated neuronal death. However, enhanced therapeutic efficiency of cells on brain damage through upregulation of HIF-1 α also been found in other groups [28,29]. These controversial results may be explained by the different damage degree (HI vs. mild hypoxia).

We also investigated the recruitment of MSCs in the hippocampus in time course experiments; the present study results together with that from previous study [30] demonstrated a number of MSCs in the hippocampus increasing continuously three weeks after transplantation. Consistently with *in vitro* experiments, HIF-1 α overexpression also improved MSCs migration to the injured area. The upregulation of HIF-1 α increased the migration of MSCs, as well as it enhanced the therapeutic efficiency of MSCs, which was also found by other groups [31,32]. Thus, enhancing the number of MSCs to the site of injury may improve the therapeutic efficiency of MSCs transplantation. In other words, the improvement of the therapeutic efficiency through overexpression of the HIF-1 α of MSCs could be due to increased cell migration to the damaged area.

The limitation of this study is that the underlying associated mechanisms about HIF-1 α promoting the directional migration of MSCs to the cerebral HI area need to be confirmed, which we will investigate in further studies.

In conclusion, this study demonstrated that HIF-1 α overexpression could increase the therapeutic efficiency MSCs in HI, which will correlate well with the promotion cells' directional migration to the cerebral HI area. We propose that transplantation of MSCs with HIF-1 α overexpression is an attractive therapeutic option to treat HI-induced brain injury in the future.

Acknowledgments

This work was financially supported by National Natural Science Foundation of China (Grant No. 81270566, Grant No. 81500114).

References

- [1] F. Triulzi, C. Parazzini, A. Righini, Patterns of damage in the mature neonatal brain, *Pediatr. Radiol.* 36 (2006) 608–620.
- [2] E.M. Graham, K.A. Ruis, A.L. Hartman, F.J. Northington, H.E. Fox, A systematic review of the role of intrapartum hypoxia-ischemia in the causation of neonatal encephalopathy, *Am. J. Obstet. Gynecol.* 199 (2008) 587–595.
- [3] H.D. Zomer, A.S. Vidane, N.N. Goncalves, C.E. Ambrosio, Mesenchymal and induced pluripotent stem cells: general insights and clinical perspectives, *Stem Cells Cloning* 8 (2015) 125–134.
- [4] E. Diez-Tejedor, M. Gutierrez-Fernandez, P. Martinez-Sanchez, B. Rodriguez-Frutos, G. Ruiz-Ares, M.L. Lara, B.F. Gimeno, Reparative therapy for acute ischemic stroke with allogeneic mesenchymal stem cells from adipose tissue: a safety assessment: a phase II randomized, double-blind, placebo-controlled, single-center, pilot clinical trial, *J. Stroke Cerebrovasc. Dis.* 23 (2014) 2694–2700.
- [5] S.J. Kim, G.J. Moon, W.H. Chang, Y.H. Kim, O.Y. Bang, Intravenous transplantation of mesenchymal stem cells preconditioned with early phase stroke serum: current evidence and study protocol for a randomized trial, *Trials* 14 (2013) 317.
- [6] H. Ding, H. Zhang, H. Ding, D. Li, X. Yi, X. Ma, R. Li, M. Huang, X. Ju, Transplantation of placenta-derived mesenchymal stem cells reduces hypoxic-ischemic brain damage in rats by ameliorating the inflammatory response, *Cell Mol. Immunol.* (2015), <http://dx.doi.org/10.1038/cmi.2015.99>.
- [7] L.H. Shen, Y. Li, J. Chen, A. Zacharek, Q. Gao, A. Kapke, M. Lu, K. Raginski, P. Vanguri, A. Smith, M. Chopp, Therapeutic benefit of bone marrow stromal cells administered 1 month after stroke, *J. Cereb. Blood Flow Metab.* 27 (2007) 6–13.
- [8] Z. Pasha, Y. Wang, R. Sheikh, D. Zhang, T. Zhao, M. Ashraf, Preconditioning enhances cell survival and differentiation of stem cells during transplantation in infarcted myocardium, *Cardiovasc. Res.* 77 (2008) 134–142.
- [9] J. Wang, H. Wang, J. Shi, Y. Ding, Effects of bone marrow MSCs transfected with sRAGE on the intervention of HMGB1 induced immunoinflammatory reaction, *Int. J. Clin. Exp. Pathol.* 8 (2015) 12028–12040.
- [10] X. Si, X. Liu, J. Li, X. Wu, Transforming growth factor-beta1 promotes homing of bone marrow mesenchymal stem cells in renal ischemia-reperfusion injury, *Int. J. Clin. Exp. Pathol.* 8 (2015) 12368–12378.
- [11] T.I. Nathaniel, J.O. Soyinka, A. Adedeji, A. Imeh-Nathaniel, Molecular and physiological factors of neuroprotection in hypoxia-tolerant models: pharmacological clues for the treatment of stroke, *J. Exp. Neurosci.* 9 (2015) 1–5.
- [12] L.A. Cunningham, K. Candelario, L. Li, Roles for HIF-1 α in neural stem cell function and the regenerative response to stroke, *Behav. Brain Res.* 227 (2012) 410–417.
- [13] R.J. Ruthenborg, J.J. Ban, A. Wazir, N. Takeda, J.W. Kim, Regulation of wound healing and fibrosis by hypoxia and hypoxia-inducible factor-1, *Mol. Cells* 37 (2014) 637–643.
- [14] R. National Research Council Institute for Laboratory and Animal, Guide for the care and use of laboratory animals, The National Academy of Sciences, Washington, DC, 1996.
- [15] D.P. Lennon, A.I. Caplan, Isolation of rat marrow-derived mesenchymal stem cells, *Exp. Hematol.* 34 (2006) 1606–1607.
- [16] J.E. Rice, R.C. Vannucci 3rd., J.B. Brierley, The influence of immaturity on hypoxic-ischemic brain damage in the rat, *Ann. Neurol.* 9 (1981) 131–141.
- [17] R. Morris, Developments of a water-maze procedure for studying spatial learning in the rat, *J. Neurosci. Methods* 11 (1984) 47–60.
- [18] C.T. van Velthoven, Y. van de Looij, A. Kavelaars, J. Zijlstra, F. van Bel, P.S. Huppi, S. Sizonenko, C.J. Heijnen, Mesenchymal stem cells restore cortical rewiring after neonatal ischemia in mice, *Ann. Neurol.* 71 (2012) 785–796.
- [19] C.T. van Velthoven, A. Kavelaars, C.J. Heijnen, Mesenchymal stem cells as a treatment for neonatal ischemic brain damage, *Pediatr. Res.* 71 (2012) 474–481.
- [20] Q. Ke, M. Costa, Hypoxia-inducible factor-1 (HIF-1), *Mol. Pharmacol.* 70 (2006) 1469–1480.
- [21] F.M. Lampert, C. Kutscher, G.B. Stark, G. Finkenzeller, Overexpression of hif-1 α in mesenchymal stem cells affects cell-autonomous angiogenic and osteogenic parameters, *J. Cell Biochem.* 117 (2016) 760–768.
- [22] D.A. Tata, I. Markostamou, A. Ioannidis, M. Gkioka, C. Simeonidou, G. Anogianakis, E. Spandou, Effects of maternal separation on behavior and brain damage in adult rats exposed to neonatal hypoxia-ischemia, *Behav. Brain Res.* 280 (2015) 51–61.
- [23] C.Z. Fang, Y.J. Yang, Q.H. Wang, Y. Yao, X.Y. Zhang, X.H. He, Intraventricular injection of human dental pulp stem cells improves hypoxic-ischemic brain damage in neonatal rats, *PLoS One* 8 (2013) e66748.
- [24] Q. Yu, L. Zhou, L. Liu, L. Cong, Y. Wang, T. Ge, D. Lin, Stromal cell-derived factor-1 α alleviates hypoxic-ischemic brain damage in mice, *Biochem. Biophys. Res. Commun.* 464 (2015) 447–452.
- [25] S.K. Hota, K. Barhwal, S.B. Singh, G. Ilavazhagan, Differential temporal response of hippocampus, cortex and cerebellum to hypobaric hypoxia: a biochemical approach, *Neurochem. Int.* 51 (2007) 384–390.
- [26] R.K. Jellema, T.G. Wolfs, V. Lima Passos, A. Zwanenburg, D.R. Ophelders, E. Kuypers, A.H. Hopman, J. Dudink, H.W. Steinbusch, P. Andriessen, W.T. Germeraad, J. Vanderlocht, B.W. Kramer, Mesenchymal stem cells induce T-cell tolerance and protect the preterm brain after global hypoxia-ischemia, *PLoS One* 8 (2013) e73031.
- [27] B. Lopez-Hernandez, I. Posadas, P. Podlesniy, M.A. Abad, R. Trullas, V. Cena, HIF-1 α is neuroprotective during the early phases of mild hypoxia in rat cortical neurons, *Exp. Neurol.* 233 (2012) 543–554.
- [28] W. Wu, X. Chen, C. Hu, J. Li, Z. Yu, W. Cai, Transplantation of neural stem cells expressing hypoxia-inducible factor-1 α (HIF-1 α) improves behavioral recovery in a rat stroke model, *J. Clin. Neurosci.* 17 (2010) 92–95.
- [29] K. Chu, K.H. Jung, S.J. Kim, S.T. Lee, J. Kim, H.K. Park, E.C. Song, S.U. Kim, M. Kim, S.K. Lee, J.K. Roh, Transplantation of human neural stem cells

- protect against ischemia in a preventive mode via hypoxia-inducible factor-1alpha stabilization in the host brain, *Brain Res.* 1207 (2008) 182–192.
- [30] Y.X. Liu, X.M. Guo, J.F. Li, Y. Meng, H.T. Zhang, A.J. Liu, S.C. Li, Y.L. Liu, H. Zhu, J.H. Xue, Y. Zhang, Z.W. Zhang, Restoration of tissue damage, and nerve activity after hypoxia-ischemia by implantation of peripheral blood mononuclear cells, *Brain Res.* 1546 (2014) 34–45.
- [31] X. Yu, C. Lu, H. Liu, S. Rao, J. Cai, S. Liu, A.J. Kriegel, A.S. Greene, M. Liang, X. Ding, Hypoxic preconditioning with cobalt of bone marrow mesenchymal stem cells improves cell migration and enhances therapy for treatment of ischemic acute kidney injury, *PLoS One* 8 (2013) e62703.
- [32] I. Rosova, M. Dao, B. Capoccia, D. Link, J.A. Nolte, Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells, *Stem Cells* 26 (2008) 2173–2182.