Neurosci Bull October 1, 2015, 31(5): 550–560. http://www.neurosci.cn
DOI: 10.1007/s12264-015-1533-1

·Original Article·

Hypoxia-controlled matrix metalloproteinase-9 hyperexpression promotes behavioral recovery after ischemia

Hongxia Cai[1,2], Zhihao Mu[1,2], Zhen Jiang[1,2], Yongting Wang[2], Guo-Yuan Yang[1,2], Zhijun Zhang[2]
[1]*Department of Neurology, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China*
[2]*Neuroscience and Neuroengineering Center, Med-X Research Institute, Shanghai Jiao Tong University, Shanghai 200030, China*
Corresponding authors: Zhijun Zhang and Guo-Yuan Yang. E-mail: zhangzhij@gmail.com; gyyang0626@gmail.com

ABSTRACT

Matrix metalloproteinase-9 (MMP-9) plays a beneficial role in the sub-acute phase after ischemic stroke. However, unrestrained MMP-9 may disrupt the blood-brain barrier (BBB), which has limited its use for the treatment of brain ischemia. In the present study, we constructed lentivirus mediated hypoxia-controlled MMP-9 expression and explored its role after stroke. Hypoxia response element (HRE) was used to confine MMP-9 expression only to the hypoxic region of mouse brain after 120-min transient middle cerebral artery occlusion. Lentiviruses were injected into the peri-infarct area on day 7 after transient ischemia. We found hyperexpression of exogenous HRE-MMP-9 under the control of hypoxia, and its expression was mainly located in neurons and astrocytes without aggravation of BBB damage compared to the CMV group. Furthermore, mice in the HRE-MMP-9 group showed the best behavioral recovery compared with the normal saline, GFP, and SB-3CT groups. Therefore, hypoxia-controlled MMP-9 hyperexpression during the sub-acute phase of ischemia may provide a novel promising approach of gene therapy for stroke.

Keywords: blood-brain barrier; hypoxia response element; matrix metalloproteinase 9; stroke

INTRODUCTION

Ischemic stroke is the leading cause of adult disability and the second most common cause of death worldwide[1]. Over the past two decades, research on brain ischemia has been focused on pathophysiology and therapies including gene therapy, stem cell transplantation, and drug interference[2-4]. Among them, gene therapy has been the most extensively studied in recent years. Non-viral gene delivery is safer than virus-mediated delivery but is limited by its inefficiency[5]. Compared to the adenovirus-associated virus, lentivirus has a large packaging capacity (8 kb) and maintains persistent gene transfer in most tissues[6], so we used lentivirus-mediated gene therapy.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases with over 20 members, which can degrade almost all proteinaceous components of the extracellular matrix[7]. MMPs are produced in a latent form and participate in many physiological and pathological processes after activation[8]. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are expressed in the brain and can be easily identified by zymography during brain injury and repair[8, 9]. The proteolytic property of MMP-9 may result in tissue damage by attacking the basal lamina and extracellular matrix. Furthermore, as part of the neuroinflammatory response, MMP-9 may also lead to vasogenic or cytotoxic edema, hemorrhagic transformation, and neuronal apoptosis[10]. However, recently, MMP-9 has been implicated in tissue repair such as extracellular matrix remodeling, axonal regeneration, remyelination, and the facilitation of neurogenesis and angiogenesis[11-14]. These beneficial roles of MMP-9 may be helpful in treating diseases such as ischemic stroke.

MMP-9 is associated with the progress of stroke and plays opposing roles in the acute and delayed phases of

ischemic stroke[15]. It is up-regulated in the first 3 days after stroke (acute phase) in mouse brain, contributing to blood-brain barrier (BBB) permeability and brain inflammation[15, 16]. However, MMP-9 is also elevated 7–14 days after ischemic stroke (sub-acute phase), and plays a beneficial role in promoting brain angiogenesis and functional recovery[15, 17]. Another study reported that MMP-9 plays key roles in the formation of vascular networks after focal cerebral ischemia[18]. These lines of evidence suggest that strategies to modulate MMP-9 expression at different stages are needed to promote post-stroke recovery. We hypothesized that lentivirus-mediated MMP-9 gene therapy may provide a useful approach for stroke treatment in the sub-acute phase, but uncontrolled MMP-9 expression may induce BBB disruption and brain hemorrhage[15, 16, 19]. Therefore, the safe control of exogenous MMP-9 expression is extremely important. Hypoxia response element (HRE), an enhancer of downstream targeted gene expression, is used for gene regulation based on the focal oxygen concentrations[20-22]. Hypoxia-inducible factor 1 (HIF-1) is a protein that accumulates in hypoxic/ischemic tissue. By combining HRE with HIF-1, we produced a vector containing nine copies of HRE bound to HIF-1 alpha, which allowed the targeted gene expression controlled by local hypoxia levels[20, 21]. Indeed, this vector has been successfully used to control VEGF expression in an ischemic heart model[22]. In conclusion, by using the HRE-HIF system to control MMP-9 expression, we investigated, for the first time, the effect of exogenous HRE-MMP-9 on the BBB and neurological behavior in the sub-acute phase of ischemic stroke.

MATERIALS AND METHODS

HRE-SV40-MMP9 Vector Construction

The SK-H9-SV40 pro plasmid backbone was kindly provided by Dr. Hua Su (University of California San Francisco). After digestion with Hind III and Sma I, nine copies of HRE and the minimum simian virus 40 (SV40) promoter fragment were cut out. In the lentiviral vector pCDH-CMV-MCS-EF1-copGFP (System Biosciences SBI, Mountain View, CA), the cytomegalovirus (CMV) promoter site was replaced by the 9HRE-SV40 fragment, and the mouse proMMP-9 cDNA structure was inserted into the vector, located downstream of the SV40 promoter.

CMV-MMP9 Vector Construction

MMP9 cDNA was inserted downstream of the CMV promoter in the vector pCDH-CMV-MCS-EF1-copGFP without HRE modification. The control was the same vector but without MMP9 insertion.

Lentiviral MMP9 Production, Purification, and Titration

Lentiviral vector was obtained as previously described[23]. Briefly, gene-carrier plasmid vector was co-transfected with pDelta (helper plasmid) and VSV-G (envelope protein vector) into HEK293T cells. After collecting the supernatant, viral vector was purified by ultracentrifugation. The HEK293T cells were transfected with the collected virus, and the virus titer was determined by the percentage of labeled GFP-fluorescent cells 3 days later.

Lentiviral Vector-Mediated MMP9 Gene Transfer into the Mouse Brain

Viral vector was stereotaxically injected into the left striatum of mice 7 days after transient middle cerebral artery occlusion (tMCAO; 2.5 mm left lateral to the sagittal suture, 0.5 mm posterior to the bregma, and 3.5 mm deep into the brain)[24]. Two microliters of lentiviral vector containing 4×10^9 TU/mL particles was injected into the brain at 300 nl/min. The Hamilton needle was withdrawn from the brain after 5 min.

Experimental Design

All animal surgery procedures were approved by the Animal Care and Use Committee of Shanghai Jiao Tong University, Shanghai, China. Mice (a total of 90 mice, excluding deaths and failed models) were randomly divided into four groups (n = 14–17 per group): normal saline group (NS); Lenti-HRE-GFP group (GFP), Lenti-HRE-MMP-9-GFP group, and Lenti-HRE-MMP-9-GFP plus SB-3CT group. The groups of Lenti-CMV-MMP-9 and Lenti-HRE-MMP-9 were used for the BBB assay and were described in the following part. All groups underwent 120-min tMCAO and were given brain injections on day 7 after ischemia. To block MMP-9, we used 2-[[(4-phenoxyphenyl)sulfonyl] methyl]-thiirane (SB-3CT, Sigma), which specifically inhibits MMP-9-driven pathways *in vivo*[25, 26]. SB-3CT was diluted in 10% dimethylsulfoxide/90% NS, and was injected intraperitoneally at 10 mg/kg per day for 3 consecutive days beginning from days 18 and 32, after ischemia[27, 28].

Neurobehavioral testing was performed in each group on days 0 (baseline), 7, 14, 21, 28, and 35 after tMCAO. Mice were sacrificed on days 21 and 35 for zymography assays. BBB damage was assayed in the HRE-MMP9 and CMV-MMP9 groups on day 21. The experimental design is illustrated in Fig. 1.

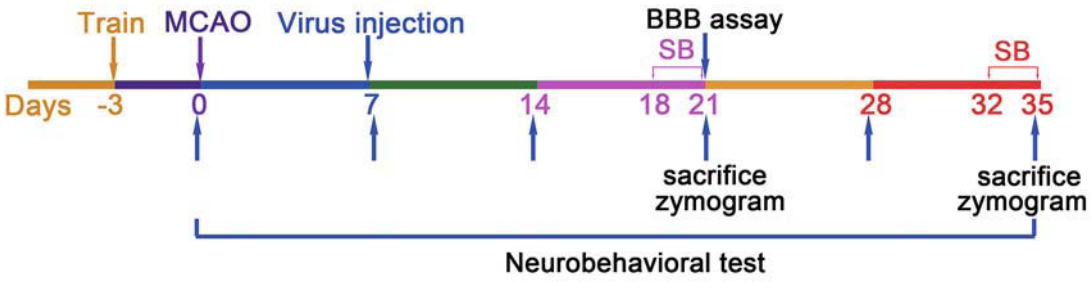

Fig. 1. Experimental design. Mice in four groups (NS, GFP, MMP-9, and MMP-9+SB-3CT) underwent 120-min tMCAO and were given brain injections on day 7 after ischemia. Neurological test was performed on days 0 (baseline), 7, 14, 21, 28, and 35. Mice in each group were sacrificed on days 21 and 35 for zymogram assays. BBB damage was assayed in the HRE-MMP9 and CMV-MMP9 groups on day 21.

Transient Middle Cerebral Artery Occlusion in Mice

Adult male ICR mice (Sippr-BK, Shanghai, China) weighing 25–30 g were used for tMCAO. Based on previous studies, mice were anesthetized with ketamine (100 mg/kg)/xylazine (10 mg/kg, Sigma)[29]. A silicone-coated 6-0 suture (Covidien, MA) was gently inserted from the external into the internal carotid artery to occlude the origin of the middle cerebral artery. Successful occlusion was confirmed by laser Doppler flowmetry (Moor Instruments, Devon, UK) as a >80% decline in surface blood flow compared with the contralateral hemisphere. These animals were included in the next experiments. Reperfusion was achieved by suture withdrawal.

Gelatin Zymography

MMP activity was determined by zymography. Gelatin (0.1%; Sangon Biotech, Shanghai, China) was added to the resolving buffer. Samples (50 μg) were loaded with zymogram sample buffer (Bio-Rad) and electrophoresed in SDS-PAGE for ~2 h, then the gels were removed into renaturing buffer (2.5% Triton X-100, 50 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, pH 7.6) and incubated for 15 min each time and washed 4 times with gentle agitation. The renaturing buffer was decanted and replaced with developing buffer (50 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, 0.02% Brij-35, pH 7.6) for 30 min with gentle agitation at room temperature. After adding fresh developing buffer and incubation for 42 h at 37°C, gels were stained with Coomassie blue (0.05% Coomassie Brilliant Blue, 30% methanol, 10% acetic acid) for 3 h and then de-stained with 30% methanol containing 10% acetic acid for proper color contrast. The final bands were quantified using GelPro-32 densitometer software (Media Cybernetics, Bethesda, MD).

Blood-Brain Barrier Permeability Assay

Two groups were used for BBB damage assay: Lenti-CMV-MMP-9 and Lenti-HRE-MMP-9. The injection site for MMP-9 was the same as for lentivirus injection. Mice were subjected to brain injections on day 7 and sacrificed 3 weeks after ischemia. Immunostaining of the tight junction proteins zonula occludens-1 (ZO-1) and occludin was performed and the percentage of the gap length to the whole tight junction length was calculated[4, 30, 31].

Immunohistochemistry (IHC)

IHC was performed as previously described[32]. Briefly, after fixation in 4% paraformaldehyde for 10 min and 3 washes in phosphate-buffered saline (PBS, pH 7.4), brain sections were treated with 0.3% TritonX-100 (Sigma) for 10 min and then blocked in 10% bovine serum albumin (BSA) for 1 h at room temperature. After that, the sections were incubated with the following primary antibodies at 4°C overnight: NeuN (1:200, Millipore, Billerica, MA), GFAP (1:200, Millipore), NG2 (1:300, Millipore), ZO-1 (1:100, Invitrogen,

Carlsbad, CA), Occludin (1:100, Invitrogen), and CD31 (1:100, R&D). After 3 washes with PBS, the sections were incubated at room temperature for 1 h with fluorescent conjugated IgG secondary antibodies (Alexa Fluor-488/594, Invitrogen; 1:500 in sterile PBS). Excess secondary antibodies were removed. After counterstaining with DAPI, final images were acquired on a Leica microscope (Solms, Germany). In some sections, the secondary antibody Alexa Fluor 647 (1:500, Invitrogen) was used, and analyzed by pseudo-color processing. For each animal, 3 sections and 6 optical fields per slice were randomly selected for counting and analysis.

Rotarod Test for Neurological Outcomes

An operator blinded to the group assignment obtained and analyzed the rotarod test data. In this test, the mice were required to balance on a rod with a gradually increased rotating speed (40 revolutions/min for 2 min). Mice were trained in this motor skill for 3 consecutive days before tMCAO and then the test was performed on day 0 before tMCAO for baseline and on days 7, 14, 21, 28, and 35 after tMCAO. The time mice stayed on the rod was recorded[32, 33]. All rotarod data were analyzed from 3 average trials.

Statistical Analysis

Data are presented as mean ± SD. Statistical analyses were performed using GraphPad Prism version 3.05 (GraphPad Software, Inc., La Jolla, CA). Unpaired two-tailed Student's *t*-test (between two groups) and one-way ANOVA followed by Student–Newman–Keuls (among multiple groups) were used to evaluate statistical significance. $P < 0.05$ was considered statistically significant.

RESULTS

HRE-Controlled MMP-9 Expression Level in the Ischemic Mouse Brain

To take advantage of the beneficial role of MMP-9 in the sub-acute phase of stroke as well as to avoid its deleterious effects including hemorrhage and cell death[10, 34], we used an HRE-HIF system to control the expression of MMP-9 in the sub-acute phase of stroke (Fig. 2A). To measure the MMP-2/9 activities after *in vivo* transduction, we performed zymography in the NS, Lenti-HRE-GFP, Lenti-HRE-MMP-9 and Lenti-HRE-MMP-9 + SB-3CT groups. At 3 and 5 weeks after tMCAO, MMP-9 activity increased in the Lenti-HRE-MMP-9 group compared to the other 3 groups (Fig. 2Bb), but MMP-2 activity remained unchanged in all 4 groups (Fig. 2Bc). In the sham group, no bright bands were found, suggesting no active MMPs in sham-operated mice (Supplementary Fig. 1A). These results indicated successful hyperexpression of MMP-9 without affecting endogenous MMP-2 expression after tMCAO.

To further confirm the dependence of MMP-9 induction on the HRE, we performed another gel zymogram and found that the MMP-9 level induced by the CMV promoter did not differ in the ischemic and normal brain, but was significantly increased when induced by the SV40-HRE promoter in ischemic brain at 5 weeks after tMCAO (Fig. 2C). Therefore, in brain tissue, the HRE allowed MMP-9 expression to be controlled under ischemic conditions.

Furthermore, we calculated the ratio of GFP expression area to the whole section area (stained by DAPI) using an image montage technique. The GFP fluorescence ratio was greater in the ischemic brain than in the normal brain at each time point (3, 7, 14, and 28 days after lentivirus injection) after SV40-HRE injection. The GFP fluorescence area was smaller in the SV40-HRE group than in the CMV group, perhaps because the CMV promoter is stronger than SV40 (Fig. 3). This result suggested that the ischemic environment together with exogenous HRE enhancer regulated and accelerated downstream gene expression, causing the ischemic brain to exhibit more GFP signal than the normal brain.

MMP9-GFP Was Mainly Expressed in Neurons and Astrocytes *in vivo*

To clearly observe the cellular morphology of viral infection *in vivo*, three fields including the corpus callosum and striatum were observed (Fig. 4A). To identify the lentivirus-infected cell types, we used immunostaining with cell-specific markers. The results showed that both GFAP (marker of astrocytes) and NeuN (marker of neurons) -labeled cells, but not $CD31^+$ endothelial cells, were GFP-positive. In addition, the GFP^+ cells surrounding the blood vessels were mainly astrocytes. Only a few $NG2^+$ oligodendrocyte progenitor cells were GFP-positive (Fig. 4B–C). These data indicated that lentivirus-mediated gene transfer was mainly expressed in neurons and astrocytes *in vivo*.

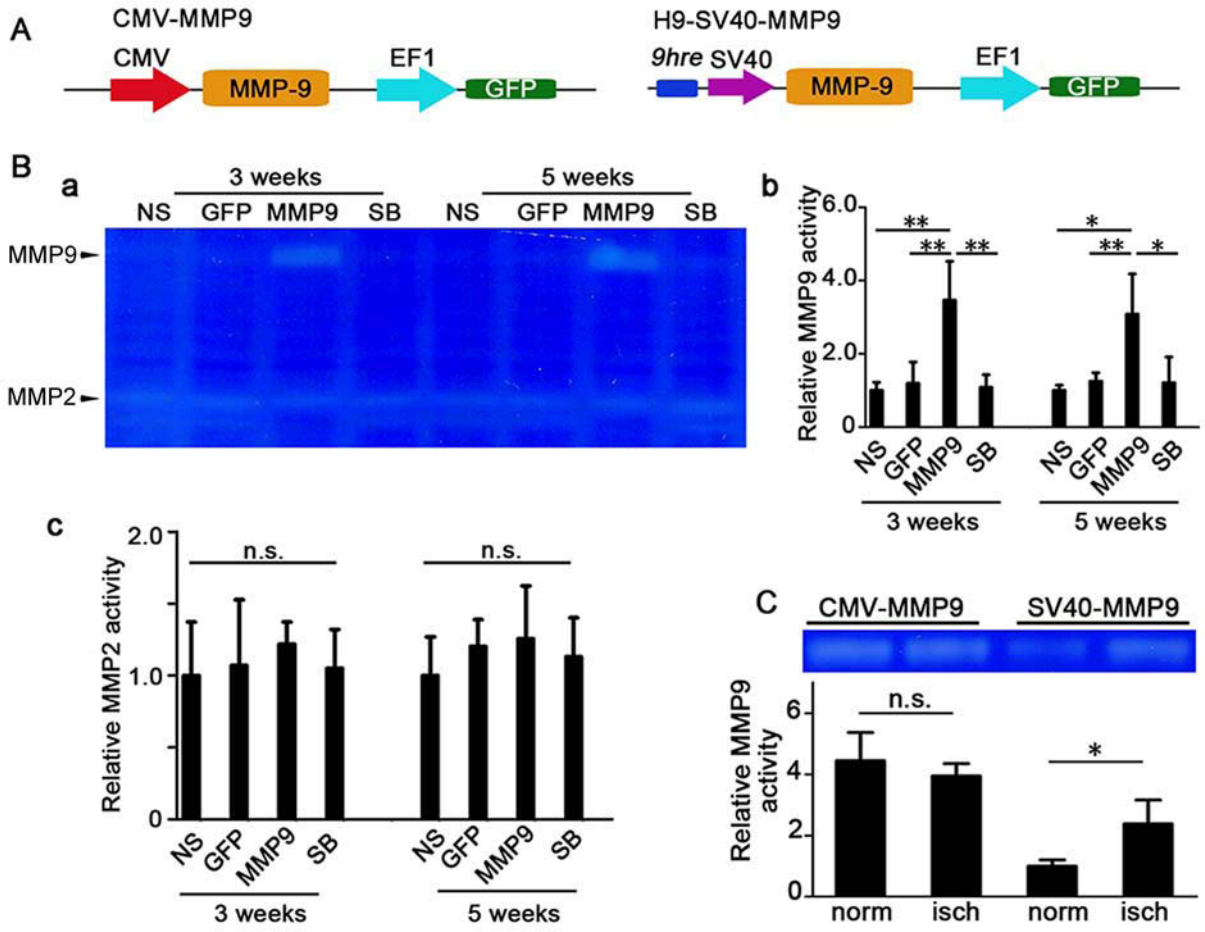

Fig. 2. Hypoxia-controlled MMP-9 hyperexpression using HRE viral vector. (A) Cartoons showing the key fragments of plasmid vectors for CMV-MMP-9 and HRE-MMP-9. (B) Zymographs (a) and quantification of active MMP-9 and MMP-2 levels (b, c) in mouse brain from the 4 groups at 3 and 5 weeks after tMCAO (P* <0.05 and ***P* <0.01, MMP-9 group *vs* the other 3 groups; *n* = 3/group; mean ± SD). (C) Zymogram and its quantification showing MMP-9 levels of both vectors in normal and ischemic brains (5 weeks after tMCAO; *n* = 3/group; mean ± SD; **P* <0.05 and ***P* <0.01; n.s., no significant difference).**

HRE-MMP-9 Did Not Severely Damage Tight Junctions in the BBB

To determine whether exogenous HRE-MMP-9 affected BBB permeability, we immunostained for the tight-junction proteins ZO-1 and occludin to detect gaps in the Lenti-CMV-MMP-9 and Lenti-HRE-MMP-9 brain 3 weeks after tMCAO. The results showed fewer gaps in the HRE-MMP-9 group than in the CMV-MMP-9 group (Fig. 5).

HRE-MMP-9 Reduced Brain Atrophy and Improved Behavioral Recovery

Brain atrophy volume and behavioral outcome determination are commonly used to evaluate therapeutic effects in animal models of stroke[35]. In this study, we found that the HRE-MMP-9 group exhibited less brain atrophy than the CMV group at 3 weeks after ischemia (Fig. 6A). In the rotarod test, the fall latencies were comparable among the 4 groups until 2 weeks after ischemia. However, the time on the rotarod was longer in the MMP-9 group than in the other 3 groups at 3 and 5 weeks after tMCAO (Fig. 6B). In the sham group, there was no difference among time points (Supplementary Fig. 1B). These data indicated that HRE-controlled MMP-9 hyperexpression improved behavioral recovery.

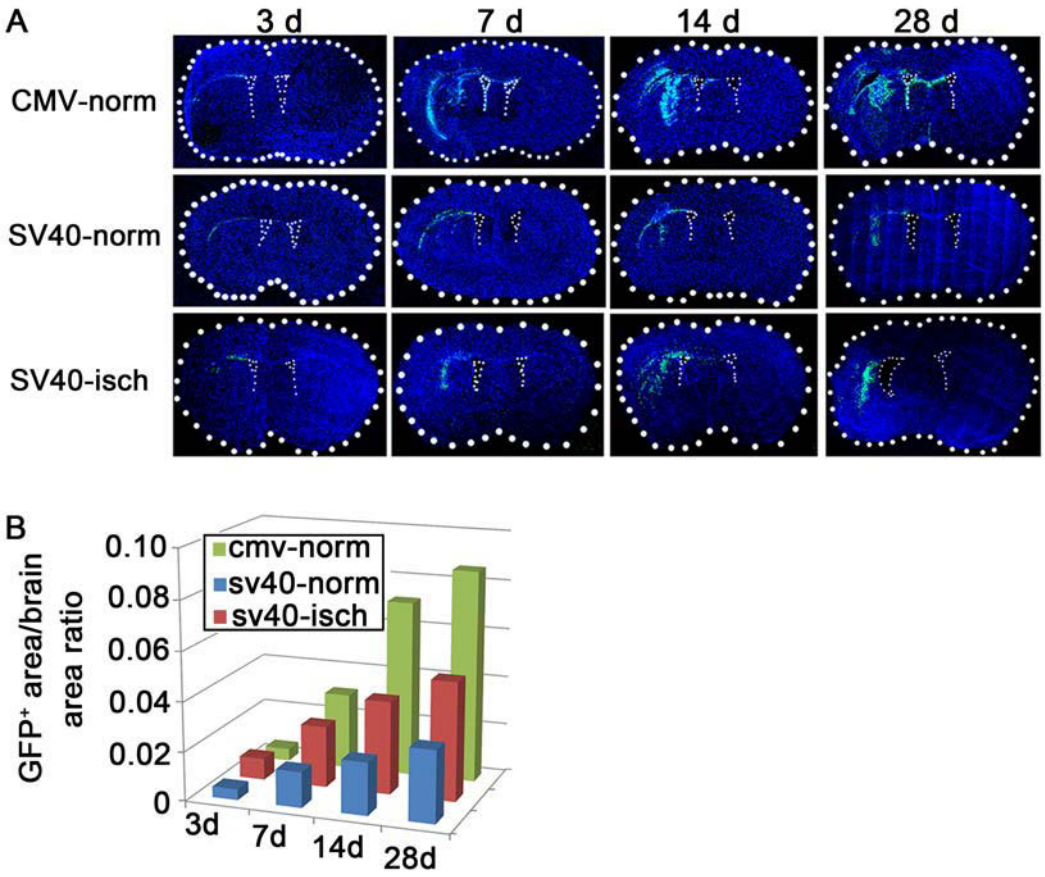

Fig. 3. HRE regulation made MMP-9 more controllable. (A) Representative images of different viral transductions in brain sections shown by GFP counterstained with DAPI at 3, 7, 14, and 28 days after gene transfer in normal (upper and middle panels) and ischemic brains (bottom panel). (B) Ratios between GFP$^+$-transduced areas and whole brain areas at each time point.

DISCUSSION

Our results provide the first evidence that MMP-9 expression can be controlled by hypoxia using the HRE-HIF system, and that HRE-regulated MMP-9 hyperexpression can improve the outcome of ischemia in the sub-acute phase without aggravating BBB injury.

MMP-9 plays dual roles after ischemia, up-regulation being detrimental in the acute phase but beneficial in the sub-acute phase[15, 36]. On the one hand, MMP-9 can degrade almost all components of ECM, and its abnormal expression may contribute to brain injury and BBB breakdown in the acute phase of cerebral ischemia[15, 16]. On the other hand, MMP-9 promotes functional recovery in the repair phase after stroke[15]. These consideration prompted us to maximize the beneficial role of MMP-9 while circumventing its disadvantages during stroke therapy. In the brain, to achieve the optimal enzymatic activity, MMP expression and activity are tightly regulated at the transcriptional and post-translational levels[37]. If unrestrained MMP-9 was delivered into the brain, excessive disruption of extracellular matrix and disorganized branching of blood vessels might be induced[38, 39]. It is known that in acute ischemic stroke (the first 3 days), brain tissue suffers a series of detrimental cascades such as energy failure, excitotoxicity, spreading depression, the generation of free radicals, BBB disruption, inflammation, and neuronal apoptosis, which may last for several days[1]. MMP-9, one of the productions of these reactions, inevitably worsens the neurological deficits. Hence, in this study, we induced MMP-9 gene expression 7 days after brain ischemia, which means that edema and

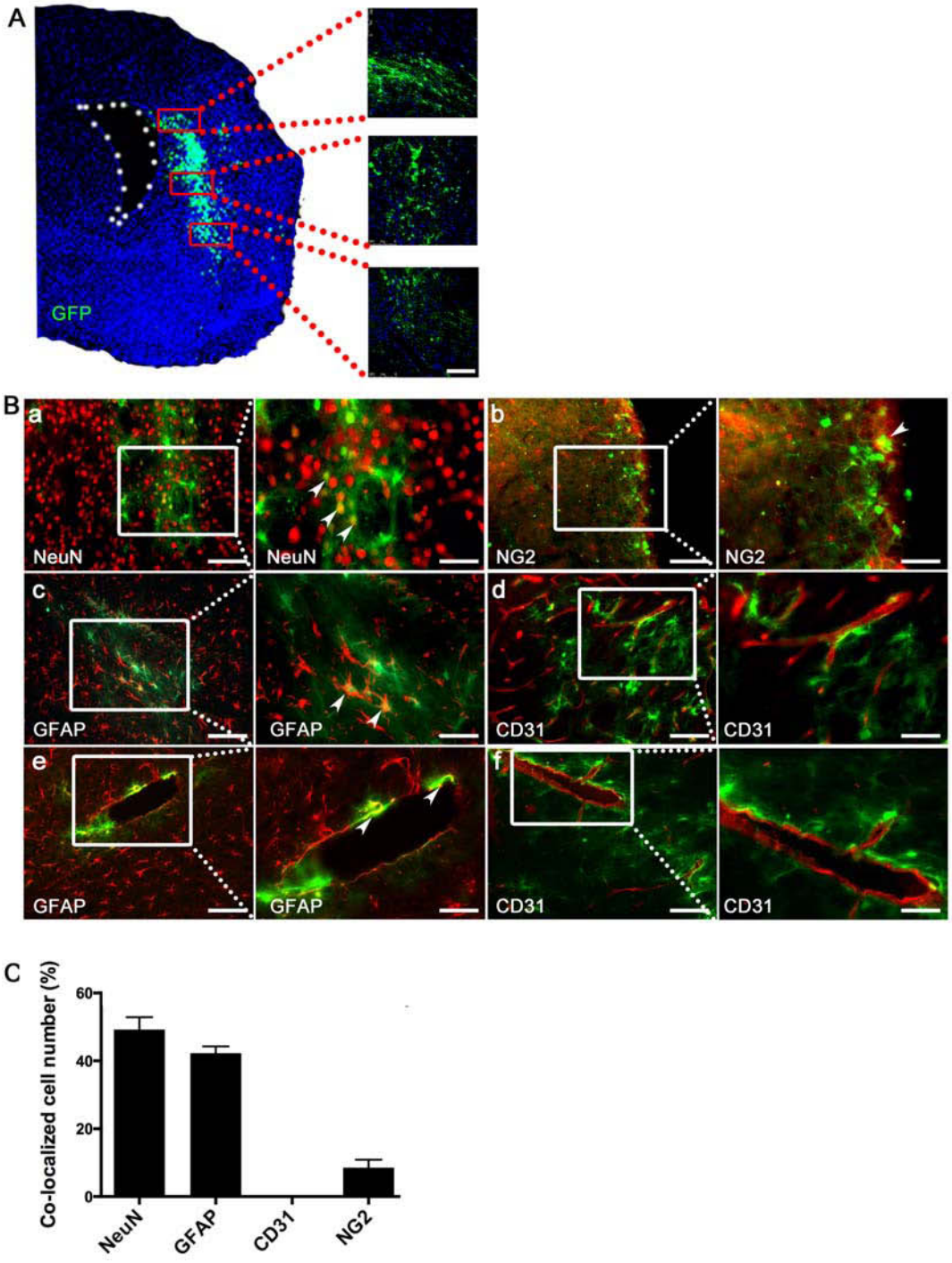

Fig. 4. HRE-GFP lentiviral vector was mainly expressed in neurons and astrocytes. (A) Virus-transduced (GFP$^+$) brain sections counterstained with DAPI (blue) at 5 weeks after tMCAO. Three typical fields were chosen to show the morphology of infected GFP$^+$ cells (scale bar, 100 μm). (B) Lentivirus-mediated gene expression (green) was mainly in neurons (NeuN/GFP double-staining in a) and astrocytes (GFAP/GFP double-staining in c), only a few oligodendrocyte progenitor cells showed expression (NG2/GFP double-staining in b), but no endothelial cells did so (CD31/GFP double-staining in d). (e–f) GFP$^+$ cells surrounding blood vessels were mainly astrocytes (scale bars, 100 μm in a–f, 50 μm in magnified images). (C) Statistical data of co-localization as percentage of NeuN/GFP, GFAP/GFP, CD31/GFP, and NG2/GFP double-positive cells (n = 6/group; mean ± SD).

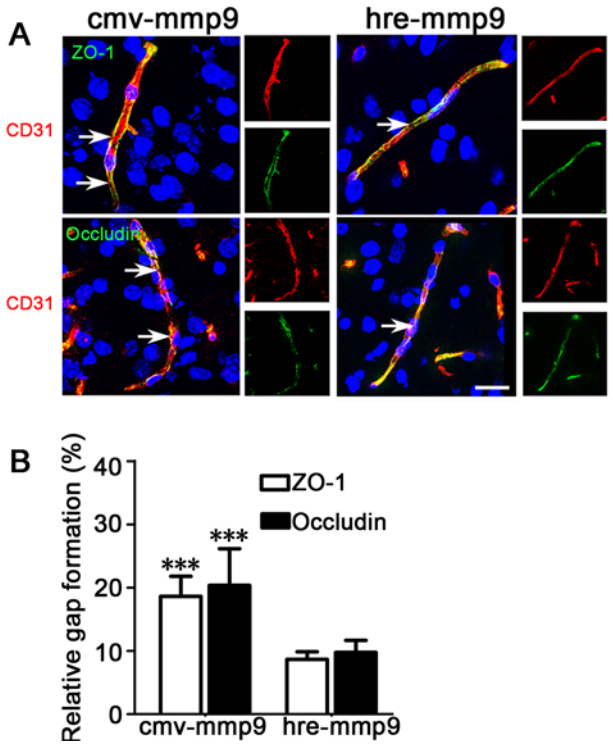

Fig. 5. HRE-MMP-9 did not severely damage tight junctions of the BBB. (A) Images of ZO-1/CD31 and occludin/CD31 double-immunostaining in the CMV-MMP-9 and HRE-MMP-9 treated mice (scale bar, 25 μm). (B) Quantification of percentage gap length for ZO-1 and occludin (*n* = 3/group; ****P* <0.001, CMV-MMP-9 *vs* HRE-MMP-9).

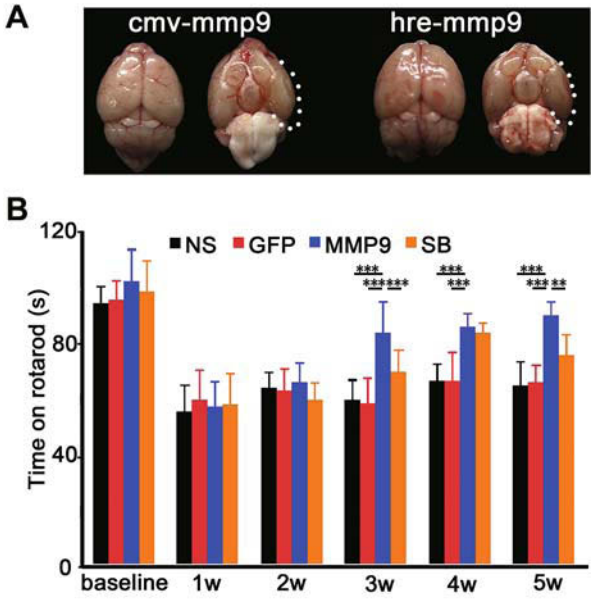

Fig. 6. Hypoxia-controlled MMP-9 promoted behavioral recovery. (A) Images showing atrophy 3 weeks after tMCAO in mouse brains injected with CMV-MMP-9 or HRE-MMP-9. (B) Bar graphs of rotarod assessment 1–5 weeks after tMCAO in the normal saline, Lenti-HRE-GFP, Lenti-HRE-MMP-9, and SB-3CT-treated groups (*n* = 14–17/group; mean ± SD; ***P* < 0.01, ****P* <0.001 among each group).

neuroinflammation were not the most prominent factors and the brain environment tended to facilitate tissue recovery and repair. However, if MMP-9 is hyperexpressed in the sub-acute phase of ischemic stroke, it may still cause tissue damage and finally worsen the therapeutic effects. Therefore, it is important and necessary to control MMP-9 expression when gene transduction is used.

After preliminary tests, nine copies of HRE was the optimal copy number in response to hypoxia[20, 40]. The SV40 promoter is a eukaryotic promoter that is weaker than the CMV promoter[41]. Only when HRE was conjoined with a relatively weak promoter like SV40, was the characteristic of hypoxic regulation revealed. Furthermore, 9HRE-SV40-VEGF has been successfully used in the treatment of cardiac ischemia and attenuates VEGF-induced brain hemorrhage[22]. Therefore, in this study, we selected nine copies of HRE and the SV40 promoter fragment to control MMP-9 expression. Our results showed that MMP-9 was successfully hyperexpressed in the HRE-MMP-9 group during the sub-acute phase of brain ischemia without affecting the endogenous MMP-2 level, eliminating the possibility that MMP-9 elevation induced changes of endogenous MMPs. We used SB-3CT to inhibit MMP-9 activity, but it is also an inhibitor of MMP-2. Our result showed that MMP-2 activity remained unchanged after SB-3CT treatment, possibly because of the hyperexpression of MMP-9 in the SB-3CT group. Based on the interaction model of enzyme-substrate, limited quantities of SB-3CT would preferentially inhibit hyperexpressed exogenous MMP-9. Moreover, our results showed that the HRE-MMP-9 system was a better candidate for MMP-9 expression than the non-HRE controlled CMV-MMP-9 as indicated by both the tissue MMP-9 zymogram and the brain GFP expression level, consistent with previous studies showing that the HIF-HRE system allows the expression of targeted genes to be controlled by local levels of hypoxia. Although other regulatory systems may exist, our purpose was to make exogenous MMP-9 expression controllable by hypoxia.

Furthermore, we injected HRE-MMP-9 into the peri-infarct area at 7 days after tMCAO, and found that lentivirus-mediated HRE-MMP-9 was mainly located in neurons and astrocytes, consistent with the finding that the MMP-9 expression level increases in the peri-infarct area at 7–14 days after ischemic stroke, and is mainly localized in neurons and astrocytes[15]. It was noteworthy that a few GFP-positive cells were also NG2-positive, meaning that lentivirus infected some oligodendrocyte progenitor cells (they myelinate axons)[42]. Further investigation is needed to determine whether these NG2-positive cells also play roles during the sub-acute phase of ischemia. Meanwhile, other cell types such as pericytes and microglia also deserve investigation.

Since MMP-9 also degrades tight-junction-related proteins, we explored whether HRE-MMP-9 elevation provoked severe protein damage. Two typical tight-junction proteins were selected – ZO-1 and occludin. We found that gap formation was 10% higher in CMV-MMP-9 mice than that in the HRE-MMP-9 group, indicating that after brain injection, the HRE-MMP-9 system did not worsen gap formation. This may be associated with the low-dose and slow-release model of HRE. Since the BBB-related index is closely associated with stroke outcome, further measurements such as functional leakage assays (like Evan's Blue or IgG staining) are needed[31]. Furthermore, we assessed brain atrophy in both groups at 3 weeks after tMCAO, and found that the HRE-MMP-9 group had less brain tissue loss than the CMV-MMP-9 group, suggesting that the HRE system did not aggravate brain atrophy.

Finally, we showed that MMP-9 hyperexpression promoted behavioral recovery. The rotarod test showed that mice stayed on the rod for longer in the sub-acute phase of stroke in the HRE-MMP-9 group compared to the control groups. Other reports also showed that a sustained deficit in neurobehavioral tests existed for up to 5 weeks in adult ischemic mice[43]. This provides preliminary evidence that HRE-MMP-9 gene therapy is effective in the sub-acute phase of ischemic stroke treatment. However, how MMP-9 affects brain atrophy and behavioral recovery in the sub-acute phase after stroke needs further investigation. Our results suggest that hypoxia-controlled MMP-9 is a useful approach to enhancing its beneficial role by attenuating BBB damage and reducing brain atrophy during the sub-acute phase of stroke.

Taken together, our findings suggest that, compared with uncontrolled MMP-9 delivery, HRE-controlled delivery of MMP-9 gene in the sub-acute phase after stroke may provide a promising strategy for the treatment of cerebral ischemia.

ELECTRONIC SUPPLEMENTARY MATERIAL

Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s12264-015-1533-1.

ACKNOWLEDGEMENTS

This work was supported by the grants from the National Natural Science Foundation of China (U1232205), the National Basic Research Development Program (973 Program) of China (2011CB504405), the Science and Technology Commission of Shanghai Municipality, China (13140903500 and 13ZR1422600), Shanghai Jiao Tong University Foundation for Technological Innovation in Major Projects (12X190030021) and the KC Wong Foundation.

Received date: 2015-02-06; Accepted date: 2015-04-17

REFERENCES

[1] Durukan A, Tatlisumak T. Acute ischemic stroke: overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia. Pharmacol Biochem Behav 2007, 87: 179–197.

[2] Tang Y, Cai B, Yuan F, He X, Lin X, Wang J*, et al.* Melatonin pretreatment improves the survival and function of transplanted mesenchymal stem cells after focal cerebral ischemia. Cell Transplant 2014, 23: 1279–1291.

[3] Lu H, Wang Y, He X, Yuan F, Lin X, Xie B*, et al.* Netrin-1 hyperexpression in mouse brain promotes angiogenesis and long-term neurological recovery after transient focal ischemia. Stroke 2012, 43: 838–843.

[4] Liu Y, Tang G, Li Y, Wang Y, Chen X, Gu X*, et al.* Metformin attenuates blood-brain barrier disruption in mice following middle cerebral artery occlusion. J Neuroinflammation 2014, 11: 177.

[5] Clark KR, Johnson PR. Gene delivery of vaccines for infectious disease. Curr Opin Mol Ther 2001, 3: 375–384.

[6] Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. Nat Rev Genet 2003, 4: 346–358.

[7] Mott JD, Werb Z. Regulation of matrix biology by matrix metalloproteinases. Curr Opin Cell Biol 2004, 16: 558–564.

[8] Rosenberg GA. Matrix metalloproteinases and their multiple roles in neurodegenerative diseases. Lancet Neurol 2009, 8: 205–216.

[9] Planas AM, Sole S, Justicia C. Expression and activation of matrix metalloproteinase-2 and -9 in rat brain after transient focal cerebral ischemia. Neurobiol Dis 2001, 8: 834–846.

[10] Gu Z, Kaul M, Yan B, Kridel SJ, Cui J, Strongin A*, et al.* S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. Science 2002, 297: 1186–1190.

[11] Huang PH, Chen YH, Wang CH, Chen JS, Tsai HY, Lin FY*, et al.* Matrix metalloproteinase-9 is essential for ischemia-induced neovascularization by modulating bone marrow-derived endothelial progenitor cells. Arterioscler Thromb Vasc Biol 2009, 29: 1179–1184.

[12] Ferguson TA, Muir D. MMP-2 and MMP-9 increase the neurite-promoting potential of schwann cell basal laminae and are upregulated in degenerated nerve. Mol Cell Neurosci 2000, 16: 157–167.

[13] Larsen PH, Wells JE, Stallcup WB, Opdenakker G, Yong VW. Matrix metalloproteinase-9 facilitates remyelination in part by processing the inhibitory NG2 proteoglycan. J Neurosci 2003, 23: 11127–11135.

[14] Liu XS, Zhang ZG, Zhang RL, Gregg S, Morris DC, Wang Y*, et al.* Stroke induces gene profile changes associated with neurogenesis and angiogenesis in adult subventricular zone progenitor cells. J Cereb Blood Flow Metab 2007, 27: 564–574.

[15] Zhao BQ, Wang S, Kim HY, Storrie H, Rosen BR, Mooney DJ*, et al.* Role of matrix metalloproteinases in delayed cortical responses after stroke. Nat Med 2006, 12: 441–445.

[16] Yong VW, Power C, Forsyth P, Edwards DR. Metalloproteinases in biology and pathology of the nervous system. Nat Rev Neurosci 2001, 2: 502–511.

[17] Yong VW, Krekoski CA, Forsyth PA, Bell R, Edwards DR. Matrix metalloproteinases and diseases of the CNS. Trends Neurosci 1998, 21: 75–80.

[18] Morancho A, Hernandez-Guillamon M, Boada C, Barcelo V, Giralt D, Ortega L*, et al.* Cerebral ischaemia and matrix metalloproteinase-9 modulate the angiogenic function of early and late outgrowth endothelial progenitor cells. J Cell Mol Med 2013, 17: 1543–1553.

[19] Tu XK, Yang WZ, Liang RS, Shi SS, Chen JP, Chen CM*, et al.* Effect of baicalin on matrix metalloproteinase-9 expression and blood-brain barrier permeability following focal cerebral ischemia in rats. Neurochem Res 2011, 36: 2022–2028.

[20] Su H, Arakawa-Hoyt J, Kan YW. Adeno-associated viral vector-mediated hypoxia response element-regulated gene expression in mouse ischemic heart model. Proc Natl Acad Sci U S A 2002, 99: 9480–9485.

[21] Dong H, Wang Q, Zhang Y, Jiang B, Xu X, Zhang Z. Angiogenesis induced by hVEGF165 gene controlled by hypoxic response elements in rabbit ischemia myocardium. Exp Biol Med (Maywood) 2009, 234: 1417–1424.

[22] Su H, Joho S, Huang Y, Barcena A, Arakawa-Hoyt J, Grossman W*, et al.* Adeno-associated viral vector delivers cardiac-specific and hypoxia-inducible VEGF expression in ischemic mouse hearts. Proc Natl Acad Sci U S A 2004, 101: 16280–16285.

[23] Tiscornia G, Singer O, Verma IM. Production and purification of lentiviral vectors. Nat Protoc 2006, 1: 241–245.

[24] He X, Li Y, Lu H, Zhang Z, Wang Y, Yang GY. Netrin-1 overexpression promotes white matter repairing and remodeling after focal cerebral ischemia in mice. J Cereb Blood Flow Metab 2013, 33: 1921–1927.

[25] Gu Z, Cui J, Brown S, Fridman R, Mobashery S, Strongin AY*, et al.* A highly specific inhibitor of matrix metalloproteinase-9 rescues laminin from proteolysis and neurons from apoptosis in transient focal cerebral ischemia. J Neurosci 2005, 25: 6401–6408.

[26] McColl BW, Rothwell NJ, Allan SM. Systemic inflammation alters the kinetics of cerebrovascular tight junction disruption after experimental stroke in mice. J Neurosci 2008, 28: 9451–9462.

[27] Liu H, Shubayev VI. Matrix metalloproteinase-9 controls proliferation of NG2+ progenitor cells immediately after spinal cord injury. Exp Neurol 2011, 231: 236–246.

[28] Gyenge M, Amagase K, Kunimi S, Matsuoka R, Takeuchi K. Roles of pro-angiogenic and anti-angiogenic factors as well as matrix metalloproteinases in healing of NSAID-induced small intestinal ulcers in rats. Life Sci 2013, 93: 441–447.

[29] Chen C, Lin X, Wang J, Tang G, Mu Z, Chen X*, et al.* Effect of HMGB1 on the paracrine action of EPC promotes post-ischemic neovascularization in mice. Stem Cells 2014, 32: 2679–2689.

[30] Huang J, Li Y, Tang Y, Tang G, Yang GY, Wang Y. CXCR4 antagonist AMD3100 protects blood-brain barrier integrity and reduces inflammatory response after focal ischemia in mice. Stroke 2013, 44: 190–197.

[31] Tang G, Liu Y, Zhang Z, Lu Y, Wang Y, Huang J*, et al.* Mesenchymal stem cells maintain blood-brain barrier integrity by inhibiting aquaporin-4 upregulation after cerebral ischemia. Stem Cells 2014, 32: 3150–3162.

[32] Li Y, Huang J, He X, Tang G, Tang YH, Liu Y*, et al.* Postacute stromal cell-derived factor-1alpha expression promotes neurovascular recovery in ischemic mice. Stroke 2014, 45: 1822–1829.

[33] Li Y, Chopp M, Chen J, Wang L, Gautam SC, Xu YX*, et al.* Intrastriatal transplantation of bone marrow nonhematopoietic cells improves functional recovery after stroke in adult mice.

J Cereb Blood Flow Metab 2000, 20: 1311–1319.

[34] Yagi K, Kitazato KT, Uno M, Tada Y, Kinouchi T, Shimada K*, et al.* Edaravone, a free radical scavenger, inhibits MMP-9-related brain hemorrhage in rats treated with tissue plasminogen activator. Stroke 2009, 40: 626–631.

[35] Fan Y, Shen F, Frenzel T, Zhu W, Ye J, Liu J*, et al*. Endothelial progenitor cell transplantation improves long-term stroke outcome in mice. Ann Neurol 2010, 67: 488–497.

[36] Montaner J, Alvarez-Sabin J, Molina C, Angles A, Abilleira S, Arenillas J*, et al.* Matrix metalloproteinase expression after human cardioembolic stroke: temporal profile and relation to neurological impairment. Stroke 2001, 32: 1759–1766.

[37] Turner NA, Aley PK, Hall KT, Warburton P, Galloway S, Midgley L*, et al*. Simvastatin inhibits TNFalpha-induced invasion of human cardiac myofibroblasts via both MMP-9-dependent and -independent mechanisms. J Mol Cell Cardiol 2007, 43: 168–176.

[38] Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Curr Opin Cell Biol 1998, 10: 602–608.

[39] Lee S, Jilani SM, Nikolova GV, Carpizo D, Iruela-Arispe ML. Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. J Cell Biol 2005, 169: 681–691.

[40] Ruan H, Su H, Hu L, Lamborn KR, Kan YW, Deen DF. A hypoxia-regulated adeno-associated virus vector for cancer-specific gene therapy. Neoplasia 2001, 3: 255–263.

[41] Xu ZL, Mizuguchi H, Ishii-Watabe A, Uchida E, Mayumi T, Hayakawa T. Optimization of transcriptional regulatory elements for constructing plasmid vectors. Gene 2001, 272: 149–156.

[42] Dawson MR, Polito A, Levine JM, Reynolds R. NG2-expressing glial progenitor cells: an abundant and widespread population of cycling cells in the adult rat CNS. Mol Cell Neurosci 2003, 24: 476–488.

[43] Li J, Siegel M, Yuan M, Zeng Z, Finnucan L, Persky R*, et al.* Estrogen enhances neurogenesis and behavioral recovery after stroke. J Cereb Blood Flow Metab 2011, 31: 413–425.