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Supplemental Information

Hypoxia Response Element-Regulated MMP-9 Promotes Neurological Recovery via Glial Scar Degradation and Angiogenesis in Delayed Stroke

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Supplemental figures and legends

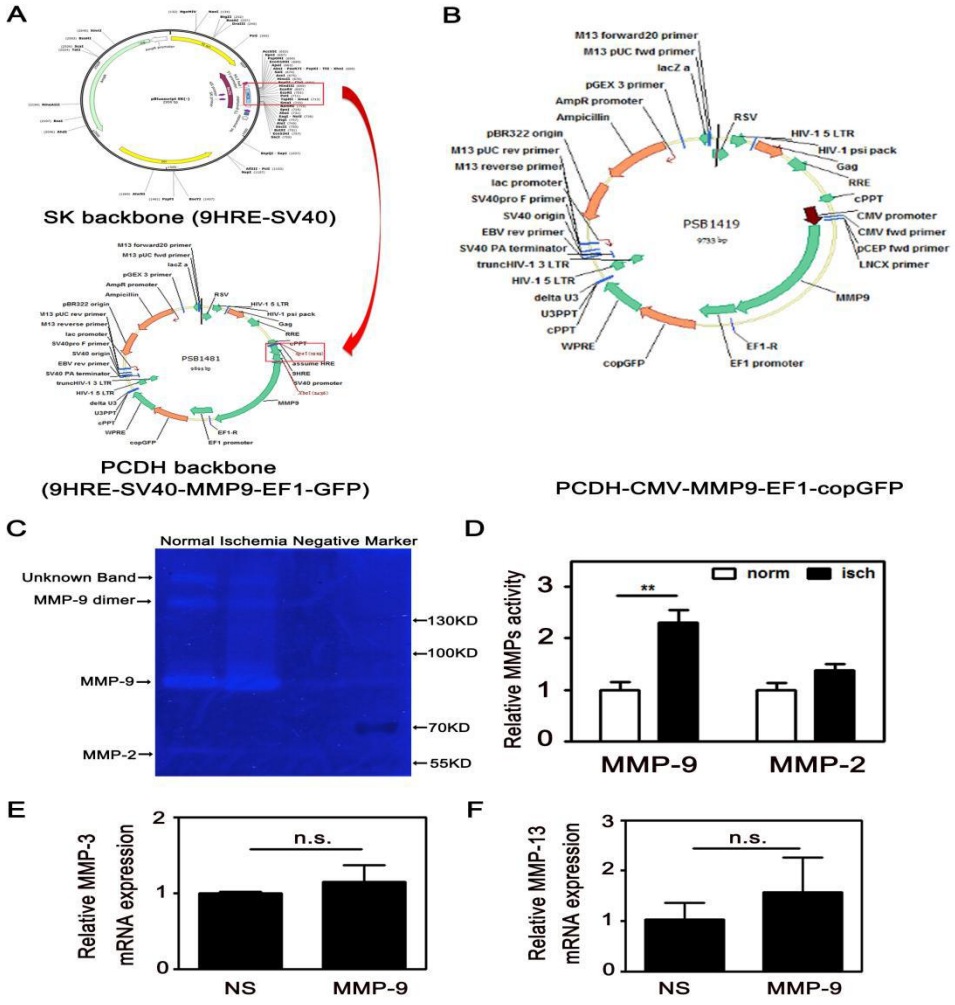

Figure S1. The 9HRE-MMP-9 vector was successfully constructed and controlled by hypoxia *in vivo*.

A) The construction of 9HRE-SV40-MMP9-EF1-GFP plasmid: SK backbone plasmid (upper) was cut by DNAase from the site of HindIII and SmaI; then, the sequence (including 9HRE-SV40) was inserted into the multiple cloning site (MCS) of the pCDH backbone (lower plasmid).

B) The plasmid structure of CMV-MMP9-EF1-GFP: PCDH backbone.

C-D) Representative image of zymography (C) and the quantifications (D) showing MMP-9 and MMP-2 activity in normal and ischemic brains, with sample buffer as a negative control (5 weeks after tMCAO). The normal mice (sham group) were also injected with HRE-MMP-9. N=5 per group. Data are the mean ±SD. * $p<0.05$.

E) Normalized quantifications showing MMP-3 and MMP-13 mRNA in normal and ischemic brains.

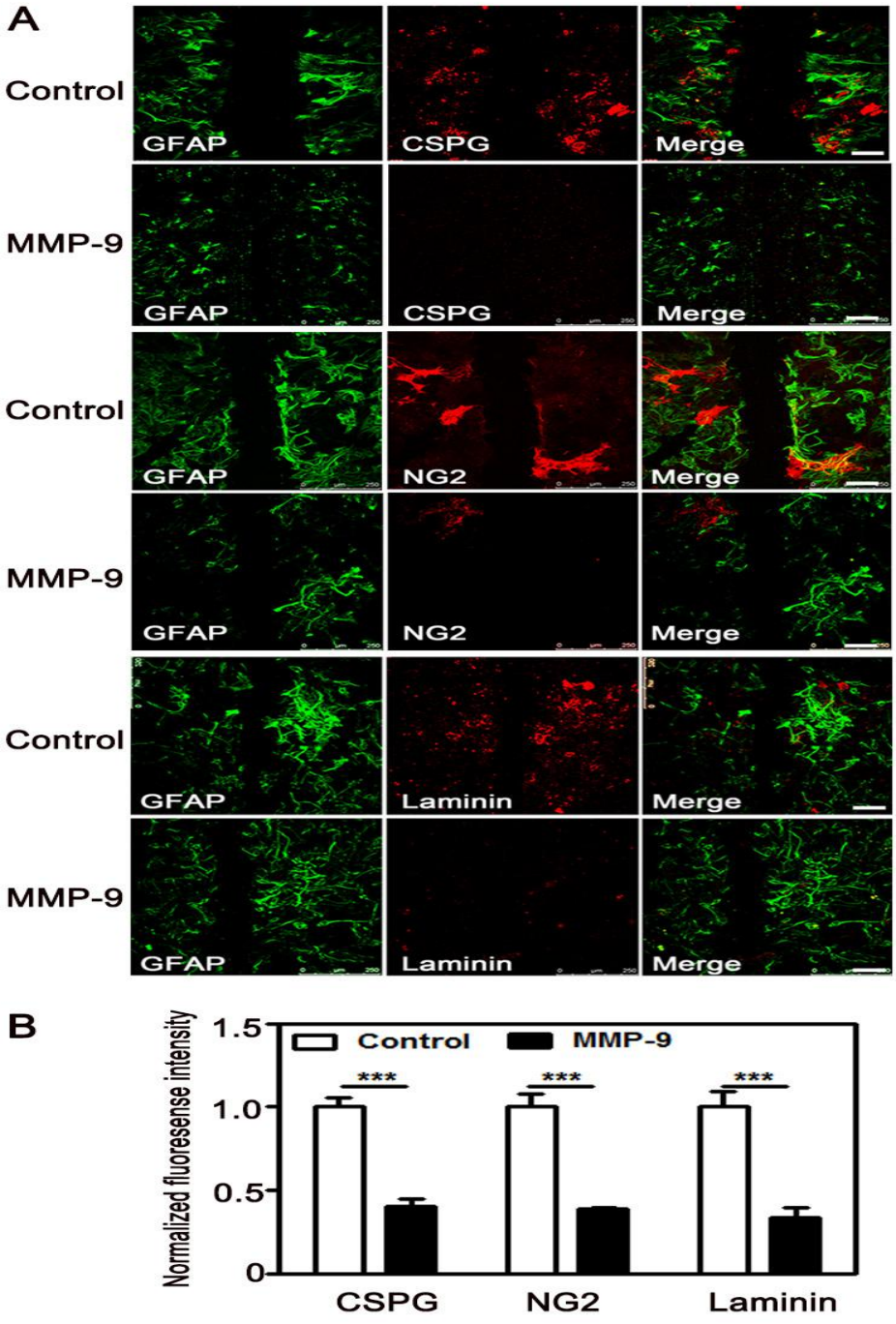

Figure S2. MMP-9 degraded extracellular matrix *in vitro.*

A) Representative image of CSPG, NG2 and laminin (red) double stained with GFAP (green) in the MMP-9 protein group and control group. Scale bar=100 μm.

B) Quantifications of relative immunofluorescence intensities for CSPG, NG2 and laminin (N=3). Data are the mean ±SD. *** $p<0.001$.

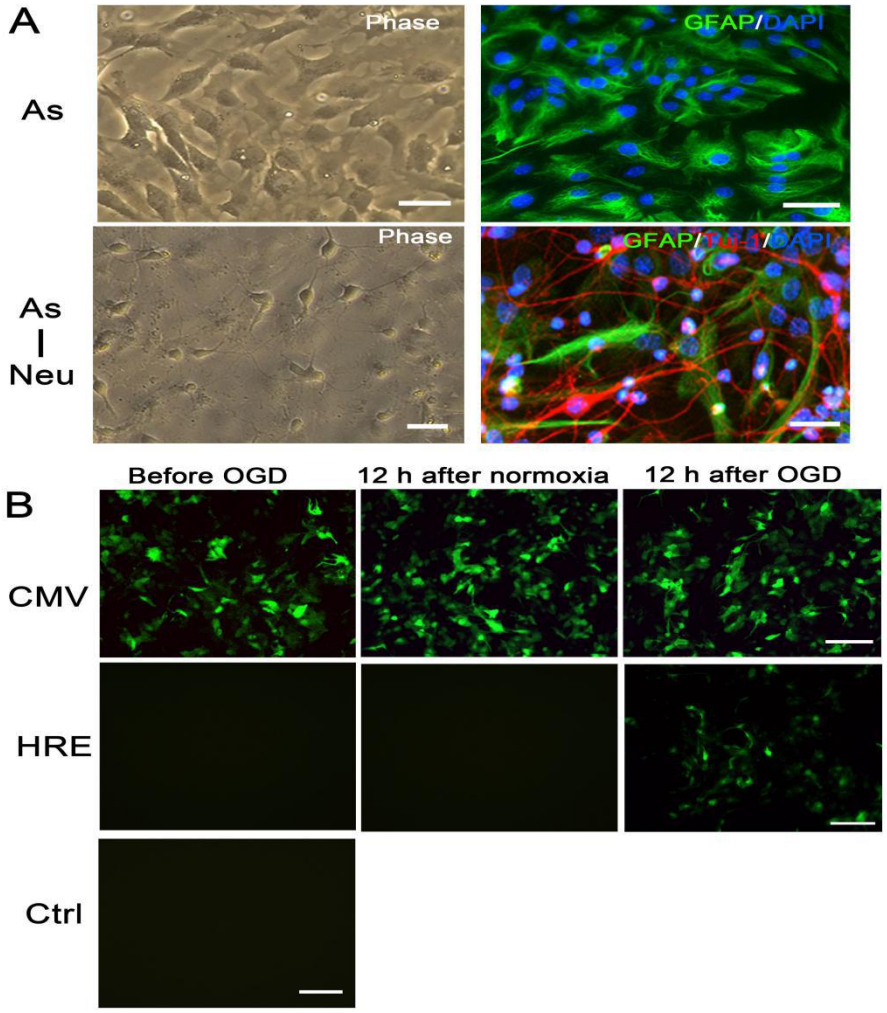

Figure S3. Identification of cell types and HRE vector function *in vitro.*

A) Identification of cultured astrocytes (As) and astrocyte-neurons (As-Neu). Upper panel: Bright field showing astrocyte morphology. Immunofluorescence of GFAP/DAPI in pure astrocytes cultured for 6 days (GFAP, green; DAPI, blue). Lower panel: Bright field showing the morphology of astrocytes and neurons. Immunofluorescence of GFAP/Tuj-1/DAPI in astrocyte-neuron co-culture for 6 days (GFAP, green; Tuj-1, red; DAPI, blue). Scale bar=50 μm.

B) HRE regulated GFP expression under oxygen glucose deprivation (OGD) in vitro. Lenti-CMV-GFP transduced astrocyte-neuron photographed before OGD, 12 hours after normoxic culture, and 12 hours after OGD culture (upper panel). Lenti-HRE-GFP-transduced cells were also photographed before OGD, 12 hours after normoxic culture, and 12 hours after OGD culture (middle panel). Cells without the addition of any viral vector were considered as negative controls (lower panel). Scale bar= 100 mm.

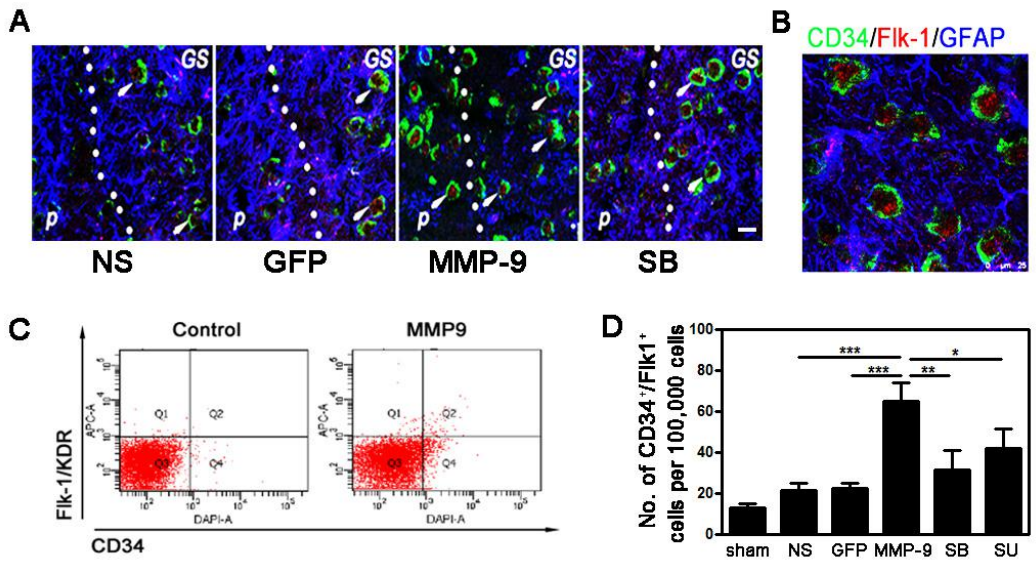

Figure S4. HRE-MMP-9 promoted EPC migration and increased its numbers.

A-B) Brain slices were triple-stained by CD34/Flk-1/GFAP (green, red and blue, respectively) for endogenous EPCs and astrocytes in the peri-infarct area of 4 groups 5 weeks after tMCAO, and the magnified photograph is shown in **B** (N=3 parallel samples). P=peri-infarct; GS=glial scar. Scale bar=20 μm.

C) Flow cytometry analysis of brain tissue (negative control and MMP-9 group).

D) Quantifications of the EPC number in brain for each group 5 weeks after tMCAO (N=6 mice per group).

Data are the mean ±SD, *** $p<0.001$. NS=normal saline; GFP=Lenti-HRE-GFP; MMP-9=Lenti-HRE-MMP-9; SB=Lenti-HRE-MMP9 plus SB-3CT; SU=Lenti-HRE-MMP9 plus SU 5416.

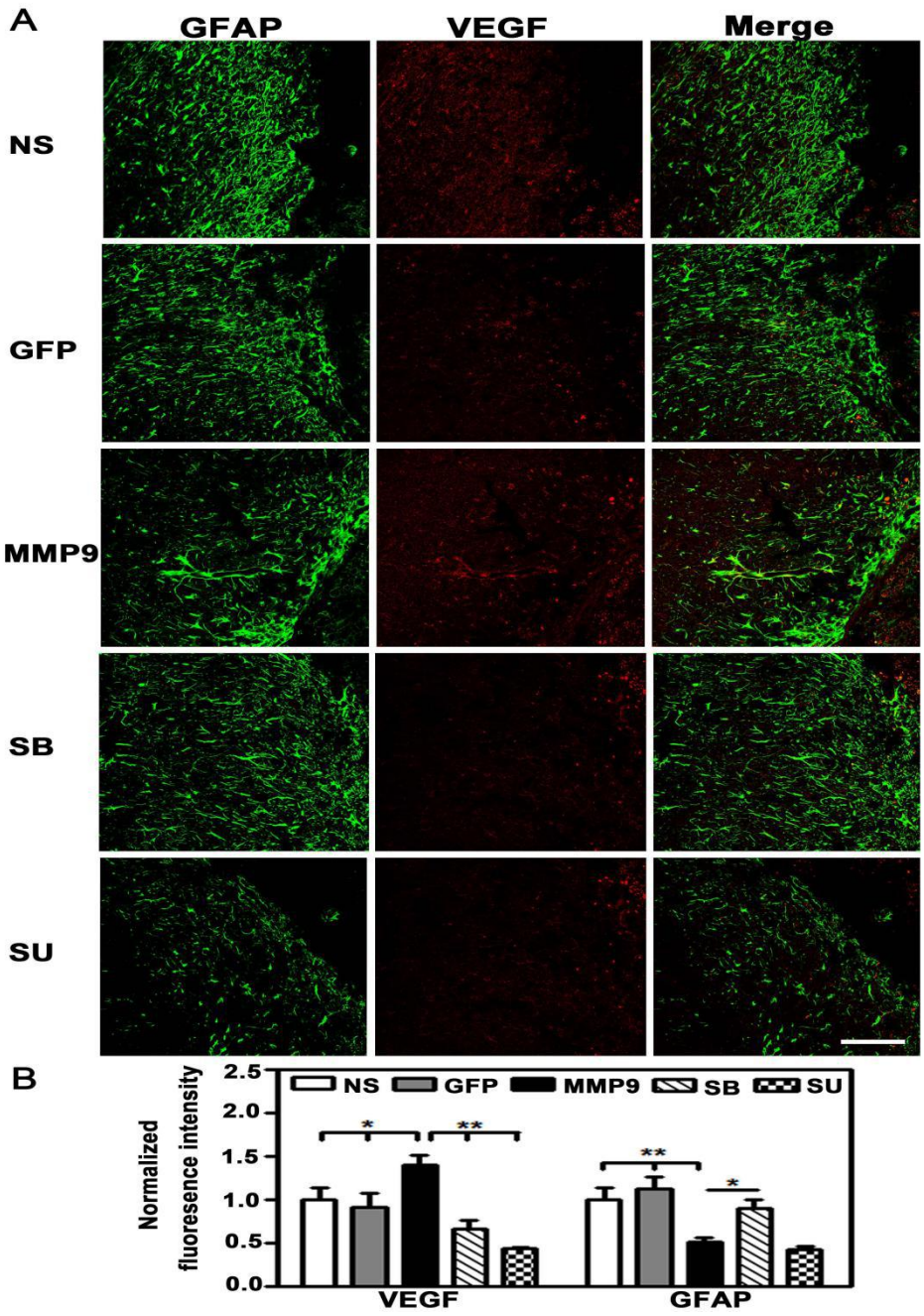

Figure S5. MMP-9 enhanced VEGF expression in scar areas.

A) Representative images of GFAP and VEGF double staining at 3 weeks after tMCAO. B) Quantifications of fluorescence intensities for VEGF and GFAP in the peri-infarct area of NS-, GFP-, MMP-9-, SB-3CT- and SU5416-treated mice (fluorescence intensity in each group was normalized to the NS group). Scale bar=50 μm. N=6 mice per group. Data are the mean ±SD. * $p<0.05$, ** $p<0.01$.

Supplemental method

Gelatin zymography

The method of protein extraction was the same as that in western blot. First, 0.1% gelatin (Sangon Biotech, Shanghai, China) was added in the resolving buffer. Proteins were loaded with a zymogram sample buffer (Bio-Rad). After samples without denaturing were electrophoresed in SDS-PAGE for approximately 2 hours, gels were removed into the renaturing buffer (2.5% Triton X-100, 50 mM Tris-HCl, 5 mM $CaCl_2$, pH=7.6) to incubate for 15 minutes each time and washed 4 times with gentle agitation. The renaturing buffer was decanted and replaced with developing buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 0.02% Brij-35, pH=7.6) for 30 minutes with gentle agitation at room temperature. After adding fresh developing buffer and incubation for 42 hours at 37℃, gels were stained with Coomassie Blue (0.05% Coomassie Brilliant Blue, 30% methanol, 10% acetic acid) for 3 hours and then destained with 30% methanol containing 10% acetic acid for proper color contrast. The final bands were quantified by GelPro-32 densitometer software (Media Cybernetics, Bethesda, MD) by a blinded operator.

Flow cytometry for EPC assay

Cold normal saline was perfused through the heart to deplete EPCs that stayed in the brain vessels. Then, the brain was quickly removed and the cerebellum and meninges were detached. The peri-infarct zone was separated, minced and immersed in 2 ml of DMEM (HyClone, Logan, UT, USA) containing 1 mg/ml collagenase IV (Sigma, St. Louis, MO) and 1 mg/ml deoxyribonuclease I (Sigma, St. Louis, MO) in a 37℃ water bath for 45 minutes of digestion. A cell suspension was obtained by filtering through a 70-mm nylon cell strainer (BD Biosciences, San Jose, CA) to remove large tissue blocks. After centrifugation at 325 g for 5 minutes, the cell pellet was re-suspended in 100 ml of PBS. Then, cells were incubated with antibodies as follows: CD34-FITC (BD Biosciences, San Jose, CA), CD309-APC (Miltenyi Biotec, Bergisch Gladbach, Germany). Samples were incubated on ice for 30 minutes in the dark. Negative controls without antibodies were used in parallel. At least 100,000 cell events in the brain were recorded by FACScalibur (BD Biosciences, San Jose, CA) by a blinded operator.