Online Supplementary:

Endothelial progenitor cell transplantation alleviated ischemic brain injury via inhibiting C3/C3aR pathway

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Material and Methods

Brain infarct volume and atrophy volume measurement

Brain tissue was collected at 3 and 14 days after cerebral ischemia and prepared for the following experiments as described previously [1]. Briefly, 30 μm sections of brain tissue were cut from the anterior commissure to the hippocampus. A total of 20 coronal sections of each brain were mounted on the slides, stained with 0.1% cresyl violet, and used for brain infarct or atrophy volume measurement. The distance between two adjacent sections was 300 μm. The brain area was measured using the ImageJ software (National Institutes of Health, Bethesda, MD). The area of infarct and atrophy were calculated by subtracting the area of normal brain in the ischemic hemisphere from the area of contralateral hemisphere. The total brain infarct and atrophy volume were further determined according to the formula V＝ $\sum_1^n \left[\left(S_n + \sqrt{S_n * S_{n+1}} + S_{n+1}\right) * \frac{h}{3}\right]$, in which, *h* was the distance between two adjacent sections, and S_n and S_{n+1} were infarct or atrophy area of two adjacent sections, respectively [1, 2]. The remnant sections of brain tissue were collected and used for immunohistochemistry.

Real-time PCR

Total RNA was extracted from ischemic brain using TRIzol reagent (Invitrogen) and transcribed to cDNA using ZymoScript II First Strand cDNA Synthesis Kit (Abconal, Shanghai, China) according to the manufacturer's recommendations. The sequence of each primer used in the whole study was summarized in **Suppl. Table 1**.

Western blot analysis

Equal amounts of proteins were loaded onto a 10 % resolving gel for electrophoresis and were transferred onto polyvinylidene fluoride membranes (Immobilon-P, Billerica, MA). Then the membranes were blocked with 5% non-fat milk for 1 hour at temperature and incubated with primary antibodies C3 (1:1000 dilution, Abcam), C3aR (1:1000 dilution, Santa Cruz Biotechnology, Dallas, TX) and β-actin (1:1000 dilution, Abcam) at 4 °C overnight. After three times washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse immunoglobulin G for 1 hour at room temperature, and then reacted with an enhanced chemiluminescent substrate (Thermo Scientific, Waltham, UK). The results of chemiluminescence were semi-quantified using the ImageJ software (National Institutes of Health).

Immunohistochemistry

Paraformaldehyde-fixed brain sections were washed with PBS for three times and then incubated with 0.3% Triton-X 100 for 10 minutes at room temperature. After blocking with 10% donkey serum for 1 hour at room temperature, the brain sections were incubated with primary antibodies at 4℃ for 16 hours. The primary antibodies included GFAP (1:500 dilution, Millipore, Bedford, MA), Iba1 (1:200 dilution, WAKO, Osaka, Japan), MAP2 (1:200 dilution, Millipore), CD31 (1:200 dilution, R&D, Minneapolis, MN) and C3 (1:100 dilution, Abcam, Cambridge, MA). After three times washing, the brain sections were

incubated with fluorescence-conjugated secondary antibodies for 1 hour at room temperature. Then the brain sections were incubated with 4', 6-diamidino-2-phenylindole (DAPI, Life Technologies, Mulgrave, VIC, Australia) for 5 minutes at room temperature. The brain sections were covered and sealed with mounting medium (Vector Labs, Burlingame, CA) for further study.

Cell counting

For analysis of C3 and GFAP immunostaining, 4 fields were randomly selected in the peri-infarct regions at ×40 objective. For analysis of C3 and GFAP immunostaining, 4 fields were randomly selected in the peri-infarct regions at ×40 objective. Images were collected using a confocal microscope (Leica, Solms, Germany) under the same conditions. The number of $C3^+$, $GFAP^+$ and $C3^+$/GFAP cells were counted, respectively. Four serial sections (1.10 mm to -0.70 mm from the bregma) were selected from each animal. The distance from each section was 300 μm.

Results

EPC transplantation reduced pro-inflammatory (M1) and enhanced anti-inflammatory (M2) markers of microglia in ischemic brain

We detected more microglia in EPC treated group. To clarify the phenotype of these microglia, we examined pro-inflammatory (M1) and anti-inflammatory (M2) markers of microglia in ischemic brain using real-time PCR. The results showed that the expression of pro-inflammatory marker iNOS was increased (3.2 folds vs. the sham) and CD16 and CD86 expression were reduced (0.23 and 0.31 folds) at 3 days after ischemic stroke. The expression of anti-inflammatory marker Arginase was increased (**Suppl. Fig. 2A**, 25 folds, $p<0.05$). EPC transplantation did not affect iNOS, CD16 and CD86 expression but enhanced Arginase and

CD206 expression (**Suppl. Fig. 2A,** 1.76 and 168 folds, $p<0.05$). At 14 days after ischemic stroke, the expression of iNOS, CD86 and CD206 were increased (3.5, 1.8 and 2.7 folds vs. the sham) but Arginase expression was reduced (**Suppl. Fig. 2B**, 0.41 folds, $p<0.05$). EPC transplantation reduced iNOS (1.7 folds) and CD16 (0.6 folds) and enhanced Arginase expression (0.76 folds) (**Suppl. Fig. 2B**, $p<0.05$). These results suggested that EPC transplantation mainly increased anti-inflammatory phenotype of microglia in the ischemic brain.

EPC transplantation reduced Cpb1 and TWIST1 expression in the brain after ischemic stroke

In order to elucidate the mechanism of EPC transplantation on reducing C3 activation after cerebral ischemia, we detected the expression of TWIST1 and Cpb1, which were reported to be involved in C3 synthesis and activity in tumor cells and macrophages [3, 4]. Our results showed that Cpb1 and TWIST1 mRNA were increased (≈2 folds vs. the control) at 3 and 14 days after cerebral ischemia (**Suppl. Fig. 3,** $p<0.05$). EPC transplantation did not affect TWIST1 expression but reduced Cpb1 expression at 3 days after tMCAO. Conversely, at 14 days after tMCAO, EPC transplantation did not affect Cpb1 expression but reduced TWIST1 expression (**Suppl. Fig. 3**, $p<0.05$). These results suggested that EPC transplantation could affect Cpb1 and TWIST1 expression at different stages of ischemic stroke to reduce C3 activation in ischemic brain.

References

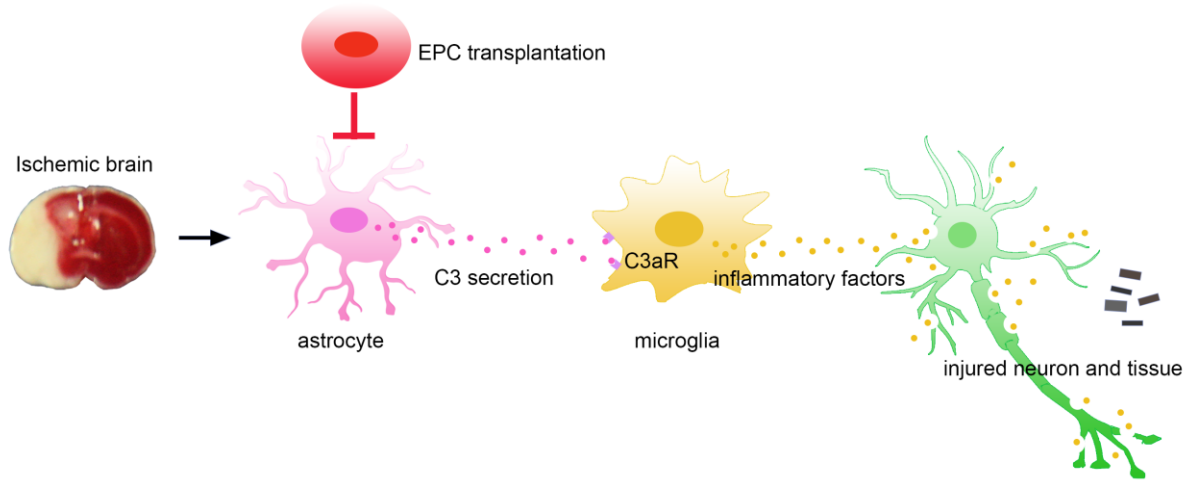
1. Li Y, Huang J, He X, et al. Postacute stromal cell-derived factor-1alpha expression promotes neurovascular recovery in ischemic mice. *Stroke* 2014; 45: 1822-1829.
2. Huang J, Li Y, Tang Y, et al. CXCR4 antagonist AMD3100 protects blood-brain barrier

integrity and reduces inflammatory response after focal ischemia in mice. *Stroke* 2013; 44: 190-197.

3. Napier BA, Brubaker SW, Sweeney TE, et al. Complement pathway amplifies caspase-11-dependent cell death and endotoxin-induced sepsis severity. *J Exp Med* 2016; 213: 2365-2382.

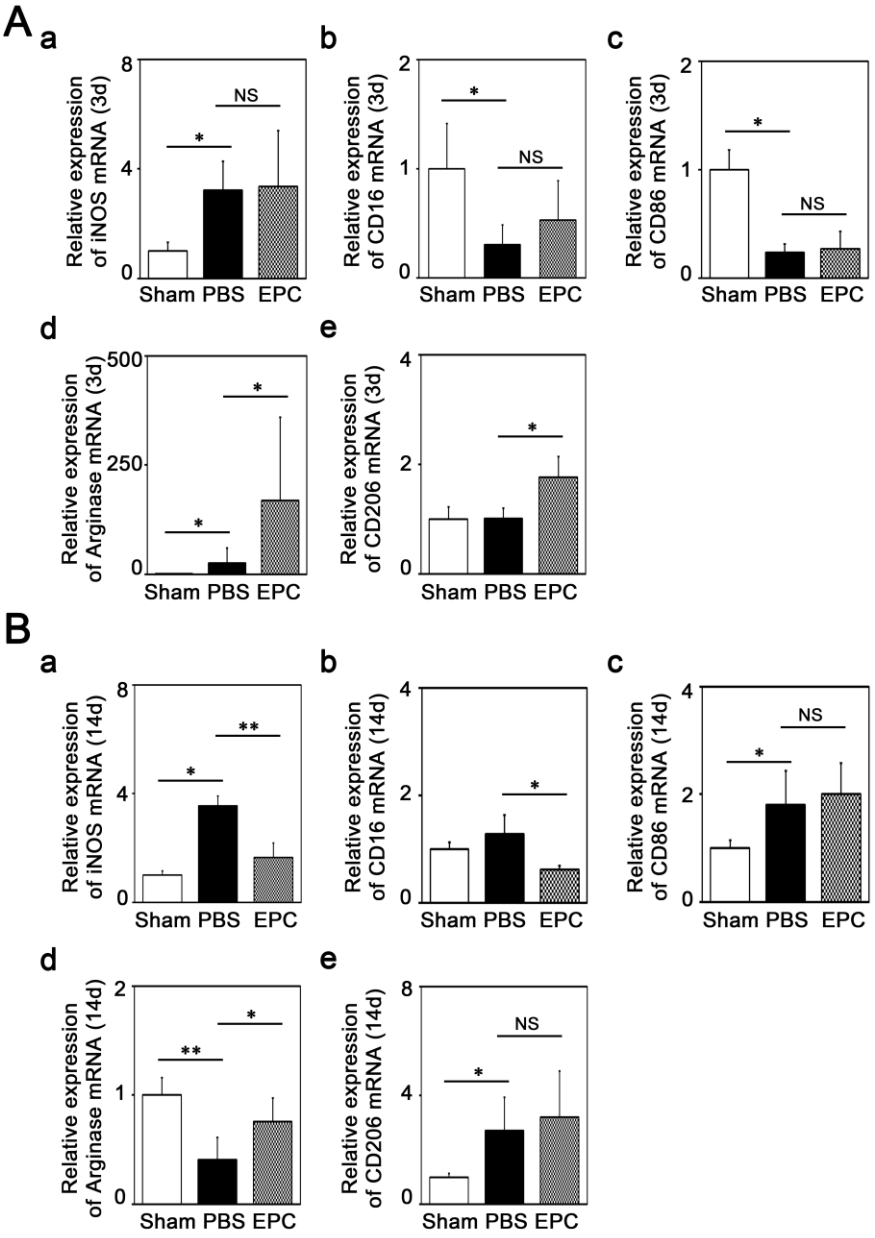
4. Cho MS, Rupaimoole R, Choi HJ, et al. Complement Component 3 Is Regulated by TWIST1 and Mediates Epithelial-Mesenchymal Transition. *J Immunol* 2016; 196: 1412-1418.

Figures



Suppl. Fig. 1

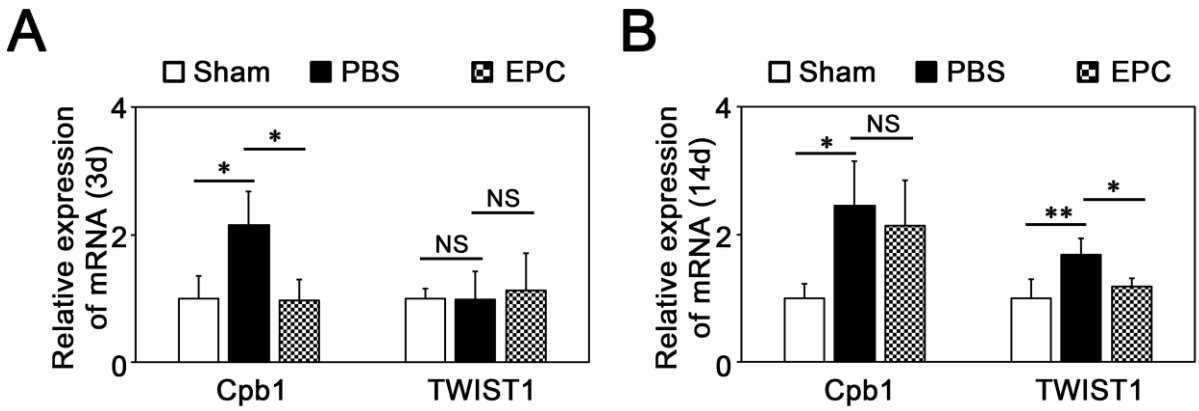
After cerebral ischemia, astrocytes were activated and secreted C3, which bind with C3aR on microglia and induced microglia to release inflammatory factors, contributing to tissue injury and neurological function deficit. EPC transplantation attenuated ischemia-induced astrocyte proliferation and astrocyte-derived C3 release, reducing the subsequent inflammatory response and tissue injury, contributing to improved neurological function recovery.



Suppl. Fig. 2

EPC transplantation reduced pro-inflammatory (M1) and enhanced anti-inflammatory (M2) markers of microglia in the brain

(A-B) Quantifications of the expressions of iNOS (a), CD16 (b), CD86 (c), Arginase (d), CD206 (e) mRNA in the EPC and PBS groups at 3 and 14 days after tMCAO, respectively. The data in Sham group was normalized to 1. Data are represented as mean±SD. n=3-4 per group. *, $p<0.05$, **, $p<0.01$.



Suppl. Fig. 3

EPC transplantation reduced Cpb1 and TWIST1 expression in ischemic brain

(A-B) Quantifications of the expressions of Cpb1 and TWIST1 mRNA in the EPC and PBS groups at 3 and 14 days after tMCAO, respectively. The data in Sham group was normalized to 1. Data are represented as mean±SD. n=3-4 per group. *, $p<0.05$, **, $p<0.01$.

Suppl. Table 1. Real-time PCR primers

Gene	Animal	Forward primers （5'～3'）	Reverse primers （5'～3'）	Amplicons size bp
C3	mouse	GCTGTGGACAAGGGAGTGTTT	GCACTCAAGATCTGCTCTCTGTT	198
C3aR	mouse	TGCTCAGCAACTCGTCCAA	CCATGGCTCAGTCAAGCACAC	129
Cpb1	mouse	TACACATGGGCTAAGGACCGA	TTCACACCAGCCAGCATCAA	108
TWIST 1	mouse	GTCCGCAGTCTTACGAGGAG	GCTTGAGGGTCTGAATCTTGCT	156
TNF-α	mouse	GGAACACGTCGTGGGATAATG	GGCAGACTTTGGATGCTTCTT	213
IL-1β	mouse	GCAACTGTTCTTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT	89
IL-6	mouse	TCTATACCACTTCACAAGTCGGA	GAATTGCCATTGCACAACTCTTT	88
iNOS	mouse	CAGAGGACCCAGAGACAAGC	TGCTGAAACATTTCCTGTGC	300
CD16	mouse	CTGTCCAAGACCCAGCAACT	TGTCTTGAGGAGCCTGGTG	189
CD86	mouse	GAACTTACGGAAGCACCCAC	AAACAGCATCTGAGATCAGCAA	94

CD206	mouse	GGCTGATTACGAGCA GTGGAA	TCCAGGTGAACCCC TCTGAA	84
Arginase	mouse	CTAACCCAGAGAGAG CATGA	CGAGGCTGTCCTTT TGAGAA	78
GAPDH	mouse	GGTTGTCTCCTGCGAC TT CA	TGGTCCAGGGTTTC TTACTCC	183