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Endothelial progenitor cell transplantation improves long-term outcome in mice

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Abstract

Endothelial progenitor cells (EPCs) have been implicated in playing an important role in vascular repair and revascularization in ischemic organs including brain tissue. However, the cause of EPC migration and the function of EPC playing following post-ischemia are unclear. Here, we reported EPC therapy in a mouse model of transient middle cerebral artery occlusion (tMCAO) to explore the roles of EPC following ischemic brain injury.

Human EPCs were cultured, characterized, and confirmed with flow cytometry. Ex vivo expanded EPCs (1×10^6) were injected via jugular vein after 1 hour of tMCAO. Histological and behavioral analyses were performed from day 1 to 28 days after tMCAO.

EPCs were detected in ischemic brain region 24 hours after MCAO. EPC transplantation significantly reduced ischemic infarct volume at 3 days following MCAO compared to the control ($p < 0.05$). CXCR4 was expressed on majority of EPCs and SDF-1-induced EPC migration was blocked by AMD3100 *in vitro*. SDF-1 was up-regulated in ischemic brain and AMD3100 could reduce EPCs migration to the ischemic region *in vivo*, suggesting that SDF-1/CXCR4 was involved in EPC-mediated neuroprotection. Compared to the control, EPC therapy reduced mouse cortex atrophy 4 weeks after tMCAO, which was accompanied by improved neurobehavioral outcomes ($p < 0.05$). In addition, EPC injection potently increased angiogenesis in the peri-infarction area ($p < 0.05$).

We conclude that systemic delivery of EPC protect against cerebral ischemic injury, promote neurovascular repair, and improve long-term neurobehavioral outcomes. Our data suggests that SDF-1/CXCR4 plays a critical role in EPC-mediated neuroprotection.

Keywords

angiogenesis; EPCs; ischemia; mice; neuroprotection

INTRODUCTION

Circulating endothelial progenitor cells (EPCs) are regarded as the cells expressing both human stem cell marker CD34 or CD133, and endothelial cell markers CD31, KDR, vWF, VE-Cadherin, or Tie2, contributing to be a cell reservoir to maintain the vascular homeostasis by monitoring and repairing dysfunctional endothelium.^{1, 2} Since being identified from human peripheral blood,³ EPCs have been widely studied with growing interest because of its potential for prognostic and therapeutic application. The discovery of EPCs has broken the dogma that neovascularization only occurred during embryonic development given that injection of EPCs into ischemic limb initiated the *de novo* new vascularization.³ Long-term studies indicate that the EPC number/function in peripheral blood is an independent risk marker for future cardiovascular events.⁴ Evidence from animal models and human clinical studies indicate that transplantation of circulating EPCs improves function recovery of heart or limbs after ischemic injury.^{5–9}

Ischemic stroke is a leading cause of mortality and morbidity in the world, there is still no applicable therapies currently available despite some promising advances in basic research.^{10, 11} Based on successful experience of cell therapy in cardiovascular diseases, EPC transplantation therefore is a promising option for the treatment of cerebral ischemia. Recent reports showed that patients with higher number of EPC after stroke have better outcomes than those have less EPCs,^{12–14} suggesting that EPC may serve as a new marker for stroke outcomes. Studies also showed that EPCs participate in cerebral neovascularization after focal cerebral ischemia¹⁵ and EPCs isolated from bone marrow attenuated ischemic injury in rats.¹⁶ However, it is unclear whether neovascularization mediated by EPCs improves long-term neurobehavioral outcome during ischemic stroke.

In the present studies, we administrate human EPCs intravenously to adult nude mouse after tMCAO to explore whether EPCs are able to home to ischemic perifocal region, and whether EPC therapy results in improvement of neurobehavioral outcomes. Furthermore, we investigate whether EPC-homing is mediated by SDF-1/CXCR4 signaling.

MATERIALS AND METHODS

EPCs isolation and enrichment

Studies involving human were IRB approved and procedures for the use of laboratory animals were approved by the Institutional Animal Care and Use Committee (UCSF). The methods of EPC isolation were described in our previous study.¹⁷ The isolated cells were resuspended in the medium EBM-2 MV and were plated at the density of 5×10^5 cells/cm² in 6-well plates coated with human fibronectin (Sigma, St Louis, MO). After 5 days incubation at 37°C with 5% CO₂, the cells were collected for characterization or systemic administration in nude mice with tMCAO.

Immunocytology and Immunohistochemistry

Mononuclear cells were plated on fibronectin-coated chamber slides. After 5 days of culture, putative EPCs were extensively washed with PBS and stained with 2.5 µg/ml of DiI-Ac-LDL (Cell Systems) at 37°C for 30 minutes, following FITC-UEA staining (Sigma). For characterization, EPCs were incubated in primary antibodies: vWF (1:500 Chemicon,

Temecula, CA), VE-Cadherin (1:500; Santa Cruz Biotechnology Inc.), KDR (1:200; Santa Cruz Biotech) and Tie2 (1:200; Santa Cruz Biotech). Second antibodies conjugated with fluorescence were applied (Molecular Probes). Iso-type-specific antibodies (BD Biosciences, San Jose, CA) were used as negative controls. For brain sections, the primary antibodies (SDF-1, 1:100, Upstate; CD31, 1:200, BD bioscience, doublecortin (DCX), 1:200, Cell signaling; CD34, 1:200, BD Pharmingen) were applied overnight at 4°C. The second antibodies were Alexa Fluor 594-conjugated or Alexa Fluor 488-conjugated IgG or Cy3-conjugated GFAP. The peroxidase-conjugated streptavidin and subtract were used for detection. Negative controls were performed by omitting the primary antibodies during the immunostaining.

Flow cytometry analysis

EPC markers were further analyzed by using a flow cytometry (FACScalibur, BD Biosciences). EPCs were incubated with fluorescence conjugated mouse anti-human monoclonal antibodies in the dark for 20 minutes (CD34-PE, KDR-APC, VE-Cadherin-FITC, CD31-PE, CD11b-APC, CD14-PerCP; CXCR4-APC, BD Biosciences). Finally, the cells were washed 3 times with FACS buffer, fixed with 2% PFA and resuspended in FACS buffer. Human IgG was used as negative controls. 5000 events were analyzed for each sample.

Transient Middle cerebral artery occlusion (tMCAO) models

Adult nude CD-1 mice (Charles River) weighing 30–35 grams were subjected to tMCAO. MCA was occluded using 10-0 nylon suture (Ethicon, NJ, USA) for one hour and left common carotid artery was occluded. Reperfusion was performed by removing the suture.

EPCs (1×10^6) were systemically injected into these nude mice through the left jugular vein one hour after tMCAO. Control animals received the same amount of human brain endothelial cells (HBECs). DiI-labeled EPCs and HBECs were used for the cell tracking in ischemic brain. All three groups were sacrificed following 3 days or 4 weeks of reperfusion.

Behavioral tests

Animals underwent three behavioral tests before tMCAO, and 1, 7, 14 and 28 days following tMCAO. Mice were trained for 3 days before tMCAO with 3 consecutive trials to generate stable baseline values, 1) beam walk test: mice were trained to traverse a series of elevated, 7 mm in diameter beam to reach an enclosed escape platform. Mice were placed on one end of the beam and the latency to traverse the central 80% of the beam towards the enclosed escape platform was recorded. Animal that does not cross within 60 seconds is allocated a maximum value of 60 seconds for statistical analysis. Motor test data were analyzed as mean latency to cross the beam (3 trials) compared with the internal baseline values; 2) rotor-rod test: the task requires the mouse to balance on a rotating rod. Performance can be evaluated in trials of fixed speed. After a 1 minute adaptation period on the rod at rest, the rod was continuously accelerated by 40 rpm over 4 minutes and the length of time mice remain on the rod (fall latency) was recorded. Motor test data were analyzed as percentage of mean duration (3 trials) on the rotor-rod compared with the internal baseline values; 3) corner test: The apparatus to perform the test was placed in the home cage and consists of two boards ($30 \times 30 \text{ cm}^2$) attached at their edges at a 30° angle with a small opening along the joint between the two boards to encourage entry into the corner. The mouse was placed between the two angled boards facing the corner and half way to the corner. When entering deep into the corner both sides of the vibrissae were stimulated together. The mouse then rears forward and upward, then turns back to face the open end. The turns in one versus the other direction were recorded from 10 trials for each

test. Turning movements that were not part of a rearing movement was not scored. Data are presented as turns to the left per test.

Cerebral Infarction volume and atrophy volume determination

Serial coronal sections, 20 μm in thickness and 200 μm in interval from the frontal pole, were used and Cresyl violet staining was used to identify the infarct area. Infarct volume (mm^3) was measured using the NIH image J. Brain atrophy volume (mm^3) following 4 weeks was calculated by subtraction of the volume of hemisphere with tMCAO from the volume of contralateral hemisphere.

Capillary density and cell assessment

Capillary density was determined as described.¹⁸ Capillary with a diameter between 3 and 8 μm were counted. The number of capillaries was calculated as the mean of the vascular counts obtained from three pictures. The number of cells was calculated in the same manner through a 20 $\times$ objective len. Vessel counting was conducted blinded to treatment allocation.

Statistical analysis

Data are presented as mean $\pm$ deviation. Parametric data from the EPC, HBEC and saline-treated groups were assessed using a one-way ANOVA followed by Fisher's PLSD test. A $p < 0.05$ was considered statistical significance.

RESULTS

EPC expansion and characterization

The putative EPCs after 5 day-culture of MNC from adult human blood in the medium EBM-2 as previously described,¹⁷ which attached on the plate and gave rise to a spindle-like shape (Fig 1A). Double staining of FITC-UEA and DiI-acLDL showed that more than 95% of these EPCs were able to uptake acLDL and bind EC-specific lectin UEA (1B). Based on immunostaining, the cells expressed EC markers KDR, VE-Cadherin, vWF and Tie-2 (1C). The phenotype of EPCs was further examined by Flow cytometry analysis, confirmed majority of the cells expressed KDR, VE-Cadherin, CD31, CD11b, and CD14 (1D).

EPCs homed to ischemic brain and protected against the acute ischemic injury

EPCs or HBECs were labeled with DiI to track the transplanted EPCs. DiI-EPCs were found around the microvessels in ischemic boundary zone between microvessel walls and brain tissue following 24 hours tMCAO. Majority of DiI-EPCs was detected in ischemia boundary zone, while a few DiI-HBECs were found in ischemic brain following 72 hours tMCAO (Fig 2A). Quantitative analysis demonstrated that the number of DiI-EPCs in ischemic areas was higher than the DiI-HBECs ($30 \pm 6/\text{field}$ vs. $10 \pm 3/\text{field}$, $p < 0.01$, 2C), suggesting that systemically administrated EPCs homed into ischemic region driven by some chemotactical mechanism not leakage from blood.

To evaluate if transplanted EPCs protected against brain injury after ischemia, 1×10^6 EPCs or equal volume of saline were systemically injected into mice. We found that infarct volume in EPC-treated group ($13.9 \pm 3.3 \text{ mm}^3$) was significantly reduced compared to the saline group ($27.5 \pm 5.7 \text{ mm}^3$, $p < 0.05$; Fig 3), suggesting systemically delivered EPCs not only homed into ischemic brain, but were able to protect against ischemic injury.

SDF-1/CXCR4 system was involved in EPC-mediated neuroprotection

To clarify whether SDF-1/CXCR4 involved in EPC-mediated neuroprotection, we first examine CXCR4 expression on EPCs and investigate whether SDF-1 induces EPC

migration *in vitro*. Immunostaining detected CXCR4 expression in the majority of the cultured EPCs but not on HBECs (Fig 4A). Cell sorting showed that ~80% of EPCs was expressing CXCR4 (4B). When SDF-1 was applied into medium in the lower chamber of the Boyden migration system, a large number of EPCs migrated across the porous filter in a dose-dependent manner (4C). SDF-1-induced EPC migration was attenuated when they were pretreated with AMD3100, suggesting that SDF-1 and its receptor CXCR4 mediated EPC migration.

We further tested whether SDF-1 expression was up-regulated in brain subjected to 1 hour of tMCAO following 1, 3 and 7 days of reperfusion. Western blot showed that SDF-1 expression in ischemic brains increased at 24 hours after tMCAO compared with the control, such high expression was maintained to 7 days ($p < 0.05$, Fig 5A). In addition, immunostaining showed that SDF-1-positive cells were distributed in the ischemic perifocal region but not in contralateral hemisphere. Based on double staining and cell morphology, SDF-1 was expressed in endothelial cells, neurons and astrocytes (5B).

To examine whether blocking CXCR4 inhibits neuroprotection. Equal number of AMD3100-pretreated or non-pretreated EPCs was applied. Mice were treated with AMD3100-pretreated EPCs showed significantly increased infarct volume than the mice treated with non-pretreated EPCs ($22.8 \pm 4.9 \text{ mm}^3$ vs. $13.9 \pm 3.3 \text{ mm}^3$; $p < 0.05$; Fig 6), suggesting that EPC-mediated neuroprotective effect is abolished by AMD3100.

EPCs reduced brain cortex atrophy and improved long-term neurobehavioral outcome after tMCAO

We further evaluate the potential long-term neuroprotection of EPCs against ischemia-induced brain injury. We found that the overall atrophy volume of hemisphere in the EPC-treated mice ($18 \pm 4 \text{ mm}^3$) was significantly reduced compared to the saline ($31 \pm 8 \text{ mm}^3$) or HBEC-treated mice ($33 \pm 8 \text{ mm}^3$, $p < 0.05$) but there was no difference between the HBEC- and saline-treated mice ($p > 0.05$, Fig 7A). We then examined neurobehavioral functions at 1, 7, 14, and 28 days after tMCAO. Beam walk showed that there was no difference in performance after 24 hours of tMCAO among groups. As expected, tMCAO mice with EPC treatment showed significant improvements in performance after 7 days of tMCAO, as compared to the controls ($p < 0.05$, 7B). Rotor-rod test showed that there was no difference among groups after 1 and 7 days of tMCAO. However, a significant difference in motor learning was observed between EPC-treated and control mice after 14 days of tMCAO. HBEC-treated mice showed transitory improvement at 14 days of tMCAO, but reversed at 28 days of tMCAO ($p < 0.05$, 7B). Corner test demonstrated that EPC-treated mice reduced preferential turning compared to the control at 7, 14 and 28 days after tMCAO.

EPCs transplantation promoted angiogenesis in ischemic brain

EPCs were able to secrete various angiogenic growth factors to promote angiogenesis.¹⁹ Therefore, we quantitatively evaluated the angiogenesis by counting the capillaries in ischemic boundary zone following tMCAO. We found that mice treated with EPCs had much higher capillary density ($261 \pm 53/\text{field}$) than that of the mice treated with saline ($115 \pm 25/\text{field}$) or HBEC ($118 \pm 28/\text{field}$, $p < 0.01$, Fig 8A). Double immunostaining showed that many BrdU-positive cells were co-localized with CD31-positive cells in the mouse brain that received EPC-transplantation (8B). In addition, it is quite interesting that when anti-human CD34 antibody was used to track the transplanted EPCs, we found several human CD34-positive microvessels in ischemic brains (8C). These results provided the evidence that transplanted EPC induced microvessel formation not only by promoting angiogenesis, but also by initiating the *de nova* neovascularization in ischemic brain.

DISCUSSION

In the present studies, we transplanted hEPCs intravenously into nude mice with tMCAO and found that the delivered hEPCs were able to home to ischemic brain as early as 24 hours after delivery, which not only protected against ischemic brain injury at acute stage, but also promoted brain regeneration by promoting angiogenesis and initiating *de nova* revascularization, eventually leading to improved neurobehavioral outcome. Our data also showed that blocking CXCR4 on EPCs abolished the EPCs-induced neuroprotection, suggesting that SDF-1/CXCR4 played crucial roles in EPCs-mediated therapeutic effects. Our findings provided evidence that EPC-based cell therapy is a novel strategy for ischemic stroke therapy.

We isolated and enriched EPCs by cultivation of MNCs collected from human peripheral blood. It has shown that culture-expanded EPCs produce much better neovascularization than fresh-isolated CD34-positive cells after translation.^{20, 21} The advantage of MNC cultivation for cell therapy is that it gives rise to both EPCs and angiogenic cells as well derived from different cell lineage origins. In general, EPCs, especially *ex vivo* cultured ones represent heterogeneous groups without a straightforward definitive marker. Only limited cells adhering early (within 48 hours) in culture were “true EPCs”, which stayed quiescent at early stage of culture but quickly proliferated and differentiated to late EPCs or outgrowth ECs after 2 weeks of culture.²² Interestingly, we found a few human CD34-positive microvessels in mouse brains after 4 weeks of EPC transplantation, which could be formed from such real EPCs. Nevertheless, it is unnecessary to identify and purify the genuine EPCs for the purpose of therapeutic strategy design.

Previous reports showed that systemically injected EPCs were able to home to local injured tissue and promote vascular remodeling and revascularization, improving tissue repair and regeneration.²³ Animal studies show that CD34-positive cells or EPCs derived from bone marrow reduce ischemic brain injury.^{16, 24, 25} We observed that intravenously transplanted-EPCs homed into ischemic zone as early as one day of tMCAO, which not only significantly reduced brain infarct volume at the acute stage, but most importantly, significantly decreased cortical atrophy and improved long-term neurobehavioral outcomes. When homing to ischemic zone, the transplanted EPCs secreted various growth factors such as IGF-1, VEGF, GM-CSF, G-CSF, SDF-1, NO and etc.^{14, 19} The therapeutic effect at early stage of ischemia may result from the neuroprotective growth factors, inhibiting cell apoptosis or promoting the neuronal survival. On the other hand, these EPC secreted growth factors could induce local angiogenesis, recruit endogenous progenitor cells for neovascularization and neurogenesis.^{26–28} In addition, transplanted EPCs also induce *de nova* vessel formation, as was shown in our studies that human CD31-positive microvessels were found in the ischemic brain with EPC transplantation. Therefore, the increased blood vessels, both from angiogenesis and from *de nova* neovascularization, may contribute to the long-term protective effects of EPCs against brain ischemia, which enhanced the local blood flow, improved brain cell survival and promoted brain regeneration. Besides, implanted peripheral blood CD34-positive cells may differentiate into glial (GFAP) and neurons (Nestin, MAP-2 or NeuN-positive cells), thereby enhancing neuroplastic effects in the ischemic brain.²⁵

The interaction of gradient SDF-1 with its unique receptor CXCR4 plays a primary role in governing stem cell homing and engraftment in the bone marrow.²⁹ SDF-1 has been reported to mediate bone marrow cell homing to brain ischemic region.³⁰ However, other studies demonstrated that blockage of CXCR4 with AMD3100 did not inhibit BM cells homing to the recipient mice after transplantation, whereas blockage of both integrin $\alpha 4$ /VCAM-1 and SDF-1/CXCR4 did.³¹ In addition, overexpressing SDF-1 by gene transfer in

the heart after myocardial infarction increased BM cell recruitment but did not enhance recruitment into heart without infarction,³² suggesting a more complicated mechanism is involved in SDF-1 function in stem cell homing. To investigate if SDF-1/CXCR4 system was involved in EPC-mediated neuroprotection, we analyzed SDF-1 expression in ischemic brain and CXCR4 expression on EPCs. Consistent with others' reports, we found that SDF-1 expression was increased in ischemic brains started 24 hours of tMCAO, by neurons, astrocytes, endothelial cells as expected. While most of EPCs enriched expressing CXCR4. SDF-1 was capable to induce EPCs migration which was robustly inhibited when pretreated EPCs with AMD3100. *In vivo* studies also showed that EPC treatment could protect against ischemic brain injury but AMD3100-pretreated EPCs could not. Besides, it has also been reported that pre-incubation of transplanted-EPCs with CXCR4 reduced EPC incorporation and CXCR4^{+/−} mice showed impaired capacity to enhance recovery of ischemic blood flow in nude mice.^{33, 34} Taken together, it is suggested that SDF-1/CXCR4 played a critical role in EPC migration and EPC-mediated neuronal protection after ischemia. SDF-1 is one of the factors regulated by hypoxia-inducible factor (HIF)-1.^{33, 35} Ischemia and the subsequent alteration of HIF-1 lead to increase SDF-1 expression, which cooperated with adhesion molecular such as VECAM-1, ICAM-136 and ultimately triggered recruitment of CXCR4-positive EPCs and homing into local brain with injury, either secreting various growth factors to drive angiogenesis/neovascularization or incorporating into newly formed vessels, accelerating the neovascularization.

We used *h*EPCs instead of mouse EPCs to treat mouse brain ischemia because *h*EPCs is relative easy to be obtained and potential for clinical application, and brain tissue is less immuno-response. However, how to optimize the dose and time of EPCs injection for clinical application needs to be further studied.

Abbreviations

BrdU	5-bromo-2-deoxyuridine-5-monophosphate
EPCs	endothelial progenitor cells
HBEC	human brain endothelial cells
HSC	human stem cell
MCAO	middle cerebral artery occlusion
MNCs	mononuclear cells
SDF-1	stromal derived factor-1

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Fig 1. EPC culture and identification

A. Mononuclear cells isolated from human peripheral blood were plated in fibronectin-coated wells at 5×10^5 cells /cm² (a). After 5 days culture, the spindle-like attached cells that supposed to be EPCs (b) were collected for use (Bar=50 μ m). **B.** The putative EPCs were able to take up DiI-labeled acetylated low density lipoprotein (Ac-LDL, a) and bind the endothelial specific lectin FITC-UEA-1 (b), which were colocalized in more than 95% cells (c) (Bar=50 μ m). **C.** Immunofluorescent staining showed the putative EPCs were positive for endothelial cell markers KDR (a), VE-Cadherin (b), vWF (c) and Tie-2 (d) (Bar=50 μ m). **D.** The cultured cells were further confirmed by flow cytometry analysis after being labeled with different markers (d, e, f, j, k, l) and fluorescent labeled IgG with the same isotypes for negative control (a, b, c, g, h, i). The results showed that ~2% cells expressed the hematopoietic cell marker CD34-PE (d) and the majority of cells expressed endothelial markers KDR (66–88%, d and e), VE-Cadherin (78%, f), CD-31 (94%, j) and monocyte markers CD11b (78%, k) and CD14 (82%, l).

Fig 2. Intravenously delivered EPCs home to ischemic brain

A. Photomicrographs show DiI labeled EPCs migrated across microvessel walls at 24 hours after injection (b, c) and into ischemic area after 72 hours of injection (e). Inserted boxes in each picture show the large magnification of cells. In the control group, DiI labeled HBEC did not show at 24 hours after injection (a) and few cells were observed in the ischemic zone (d). DAPI was used for the counterstaining. **B.** The figure shows the ischemic area and the ischemic boundary area. **C.** The bar graph shows that a great number of DiI-EPC homed into ischemic brains compared with DiI-HBEC 72 hours after injection i.v. Data are mean $\pm$ SD, n=6 in each group. *, p<0.01, the DiI-EPC number vs. DiI-HBEC number in ischemic brain. Bar=50 μ m.

Fig 3. EPC transplantation decreased acute ischemic infarction

A. Nissl's staining indicates that EPCs-treated mice showed much smaller infarct area than saline-treated group. The mice were subjected to 1 hour of MCAO followed with reperfusion and EPC transplantation or equal volume of saline as a control through left jugular vein. After 3 days of reperfusion, the ischemic brains were collected and brain sections were prepared. **B.** Bar graph of quantitative analysis showed that systemic administration of EPC significantly reduced the infarct volume 3 days after tMCAO compared with control group. Data are mean $\pm$ SD, n=6 in each group. *, p<0.05, EPC treated mice vs. saline treated mice.

Fig 4. EPC expressed CXCR4 and SDF-1 induced EPC migration *in vitro*

A. The photomicrographs show the expression of CXCR4, the receptor of SDF-1 (Red) on adult endothelial cells HBECs (a) and EPCs (b), with the counterstaining of DAPI (blue). Few adult endothelial cells expressed CXCR4 while the majority of EPCs did. Bar=50 μ m. **B.** the scatter graph showed CXCR4 expression on EPCs detected with the flowcytometry. (a), control staining with IgG with the same isotope as CXCR4 antibody. (b), more than 80% of cultured EPCs expressed CXCR4. **C.** Bar graphs show that SDF-1 induced EPC migration assayed with the Boyden chamber system. After 18 hours of SDF-1 stimulation, cells cross the porous filter were counted and data are presented as fold increase in migrating cells relative to the control medium. (a) a large number of EPCs migrated across the porous filter compared with the control, while pretreatment with 5ug/ml of AMD3100 significantly inhibited SDF-1-induced EPC migration (* $P < 0.01$, SDF-1 treated group vs. PBS treated control; ** $P < 0.01$, AMD3100 treated EPC vs. non-AMD 3100 treated group). (b) the bar graph shows that even 1ng/ml of SDF-1 significantly increased EPC migration (* $P < 0.05$, SDF-1-treated groups vs. PBS control group); (c) the bar graph shows as low as 1ug/ml of AMD3100 significantly inhibited the SDF-1-induced EPC migration (* $P < 0.05$, AMD3100-treated groups vs. non-treated group). All the results are expressed as mean $\pm$ SD and each panel represents the results of at least three independent experiments performed in triplicate.

Fig 5. SDF-1 expression was up-regulated in mouse brain subjected to tMCAO following reperfusion

A. The western blot results show SDF-1 expression in ischemic brains, with β -actin as an internal control (upper panel). SDF-1 expression significantly increased at 1 day, 3 days and 7 days, with a peak at 1 day after tMCAO (lower panel). Data are mean $\pm$ SD, n= 6. * P<0.05, SDF-1 expression in the brains of tMCAO mice vs. saline treated mice. **B.** The photomicrographs show the immunofluorescent staining of SDF-1 (green) in (a) non-ischemic brains and (b) brains subjected to 1 hour of tMCAO with 24 hours of reperfusion. Double staining (c) of SDF-1 (green) with CD31 (red, upper panel) or GFAP (red, middle panel) showed that SDF-1 was expressed in endothelial cells and astrocytes. Bar=50 μ m.

Based on the cell morphology, SDF-1 was also expressed in neurons (c, low panel). **Bar=20**
 μm .

Fig 6. AMD3100-treated EPCs abolished cerebral protection against ischemic injury

A. Nissl's staining showed that the infarct area of mice with AMD3100 treated-EPC was much bigger than that treated with EPCs. The mice were subjected to 1 hour of MCAO followed with reperfusion and transplantation of EPCs or 5ug/ml of AMD3100-treated EPCs through left jugular vein. After 3 days of reperfusion, the ischemic brains were collected and brain sections were prepared. **B.** Bar graph showed that the brain infarction volume of mice with AMD3100-treated EPCs significantly increased compared with the non-treated EPC group. Data are mean±SD, n= 6. *P<0.05, AMD3100-treated EPC group vs. non-treated-EPC group.

Fig 7. EPC transplantation reduced mouse cortical atrophy and improved neurobehavioral outcomes

A. EPC-treated mice showed reduced cortical atrophy after tMCAO/reperfusion. The mice were subjected to 1 hour of MCAO followed with reperfusion and EPC transplantation. Saline and HBEC were used as controls. The ischemic brains were collected 4 weeks after tMCAO. (a). Nissl's staining showed much smaller atrophy in the brains of EPC-treated group than that of saline or HBEC-treated group. (b) Quantitative analysis showed that systemic administration of EPC significantly reduced the brain atrophy 4 weeks after tMCAO compared with saline or HBEC-treated group (b). Data are mean $\pm$ SD, n=6 in each group. *, p<0.05, EPC treated mice vs. saline or HBEC treated mice. **B.** EPC-treated mice showed improved neurobehavioral outcome. All mice were trained for 3 days before tMCAO with 3 consecutive trials to generate stable baseline values. Neurobehavioral functions of mice were tested at 1 day, 7 days, 14 days, and 28 days after tMCAO. Line graphs show that EPC-treated mice showed significantly improved performance in crossing the beam walk (a, beam test), staying on the rotor-rod (b, rotor-rod test), and turn preferentially towards non-impaired ipsilateral side (c, corner test) compared with mice treated with saline or HBEC but there was no significant difference between saline and HBEC-treated mice 4 weeks after tMCAO. Data are mean $\pm$ SD, n=6 in each group. *, p<0.05, EPC treated mice vs. saline or HBEC-treated mice.

Fig 8. Intravenously delivered EPCs promoted local angiogenesis in ischemic brain

A. CD31 immunostaining shows microvessels in mouse brain treated with saline (a), HBEC (b) and EPC (d) 4 weeks after 1 hour of tMCAO. The figure (c) shows the ischemic area and the ischemic boundary area. The bar graph (e) shows that the number of microvessels in EPC-treated mice was significantly increased compared with that in saline or HBEC-treated mice. Data are mean $\pm$ SD, n=6 in each group. *, p<0.05, the microvessel number in EPC-treated mice vs. that of HBEC or saline-treated mice. Bar=100 μ m. **B.** Photomicrographs show CD31 (red, a, b, c) and BrdU (green, d, e, f) double staining in ischemic brain of saline (a, d, g), HBEC (b, e, h) and EPC (c, f, i)-treated mice. In EPC treated group, increased BrdU (+) cells were localized in endothelial cells (yellow, g, h, i), suggesting EPC

transplantation promoted new vessel formation in ischemic brain. Bar=50 μ m. Inserted boxes in each picture show the large magnification of cells. **C.** Photomicrographs human CD34-positive microvessels (arrow heads) in ischemic boundary areas of mouse brain. 4 weeks after tMCAO, anti-human CD34 antibody was used to track the transplanted human EPCs in mouse brain. The human CD34-positive microvessels were not observed in saline control group (a) or the adult HBEC-treated group (b), but formed in EPC transplanted mouse brains (c). Bar=100 μ m.