A Preventive Injection of Endothelial Progenitor Cells Prolongs Lifespan in Stroke-prone Spontaneously Hypertensive Rats

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Running title: EPCs and lifespan in SHR-SP

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Abstract

There is a pressing need for new approaches to preventing stroke. Endothelial progenitor cells (EPCs) promote vascular repair and revascularization in the ischemic brain. This study sought to evaluate whether preventive delivery of EPCs could prevent or protect against stroke. Stroke-prone spontaneously hypertensive rats (SHR-SP) received a single injection of EPCs, and their survival time was monitored. In addition, at 28 and/or 42 days after a single injection of EPCs, SHR-SP and mice were subjected to cerebral ischemia, and cerebral ischemic injury, local angiogenesis and *in vivo* EPC integration were determined. Other experiments examined the effects of EPC conditioned media, and the distribution of donor EPCs taken from green fluorescent protein transgenic mice. It was found that EPC-pretreated SHR-SP showed longer lifespans than untreated controls. A single preventive injection of EPCs could produce persistent protective effects against cerebral ischemic injury (lasting at least 42 days), and promote local angiogenesis in the ischemic brain, in two types of animals (SHR-SP and normotensive mice). EPCs of donor origin could be detected in the recipient peripheral blood, and integrated into the recipient ischemic brains. Furthermore, it was suggested that mouse EPCs might exert paracrine effects on cerebral ischemic injury in addition to their direct angiogenic effects. In conclusion, a single preventive injection of EPCs prolonged the lifespan of SHR-SP, and protected against cerebral ischemic injury for at least seven weeks. It is implied that EPC injection might be a promising candidate for a preventive role in patients at high risk for stroke.

Key Words: stroke prevention, Stroke-prone spontaneously hypertensive rats, lifespan, endothelial progenitor cells, cell transplantation

Introduction

Ischemic stroke is a devastating disease, and a major cause of mortality and morbidity worldwide [1]. To date, treatment options for stroke are limited to the thrombolytic drug tissue plasminogen activator (tPA), but only about 3% of the population suffering an ischemic stroke benefit from tPA, largely due to the drug's narrow therapeutic window [2]. Despite the advent of treatment with tPA and the promise of other acute therapies, effective prevention remains the best approach for reducing the burden of stroke [2,3]. Various risk-reduction measures, such as the use of antihypertensives, warfarin, platelet antiaggregants, statins, and so on, have been employed for primary and secondary prevention of stroke. Nevertheless, stroke remains a major healthcare problem [3]. Accordingly, it is of great clinical importance to develop new strategies for acute stroke prevention.

Endothelial progenitor cells (EPCs) are a circulating bone marrow-derived cell population that participate in both vasculogenesis and vascular homeostasis, and have been used to improve functional recovery in damaged organs, including the brain, after ischemic injury [2,4-7]. Recent reports suggest that EPCs may serve as a new marker of stroke outcomes [2,5-7]. Therefore, it is reasonable to speculate that EPC transplantation before the onset of cerebral ischemia might be helpful for stroke prevention. However, transplantation of EPCs just before or after cerebral ischemia onset has limited clinical feasibility, because the onset of stroke is usually unpredictable, and the isolation and enrichment of autologous EPCs would take several days [4,6,8].

A recent clinical study showed that a single intracerebral transplantation of modified bone marrow-derived mesenchymal stem cells produced a persistent and significant improvement in neurological function 12 months after transplantation, in patients with stable chronic stroke [9]. In addition, it has been demonstrated that in mice, donor bone marrow cells can be detected among recipient bone marrow cells 28 days after bone marrow transplantation [10]. Furthermore, it was found that in aged stroke-prone spontaneously hypertensive rats (SHR-SP), transplantation of bone marrow cells showed no effect on blood pressure, but could reduce ischemic brain damage induced 30 days after transplantation [11]. An analysis of the peripheral blood of the recipient 28 days after transplantation found that 5% of the circulating blood cells were of donor origin [11]. These findings suggest that an injection of bone

marrow-derived EPCs may have the potential to produce a persistent (28 days or longer) preventive effect on cerebral ischemic injury in animals.

The objective of this study was to evaluate whether the preventive delivery of EPCs before ischemia onset is a viable option for stroke prevention. To address this issue, two sets of experiments were performed in the present work: 1) a test of whether a single injection of EPCs can prolong the lifespan of SHR-SP, a widely used animal model for studying the pathogenesis of stroke, in which stroke develops spontaneously with a high incidence (more than 80%) in males [12,13]. 2) investigations of possible mechanisms underlying EPCs' effects on the lifespan of SHR-SP. We demonstrated that a single preventive injection of EPCs could indeed prolong the lifespan of SHR-SP, and that the effect can at least partly be attributed to the EPCs' persistent protective effects against cerebral ischemic injury, which can also be observed in mice.

Materials and methods

Animals

Male C57BL/6 mice (10–12 weeks, 20–25 g) and SD rats (8–9 weeks, 230–260 g**)** were purchased from Sino-British SIPPR/BK Lab Animal Ltd., Shanghai, China. Male stroke-prone spontaneously hypertensive rats (SHR-SP) were provided by the animal center of the Second Military Medical University, Shanghai, China. Male C57BL/6-Tg (CAG-EGFP) green fluorescent protein (GFP) transgenic mice (10–12 weeks) were purchased from Shanghai Model Organisms Center, Inc., Shanghai, China. All animals received humane care, and the experimental procedures complied with the institutional animal care guidelines. All the experiments were performed in a random and blind fashion.

EPC isolation, enrichment and characterization

EPCs from bone marrow of C57BL/6 mice and SD rats were isolated, cultured, and characterized using previously described techniques [4,7,8,14]. Briefly, bone marrow-derived mononuclear cells were isolated from mouse and rat tibia and femur, seeded in 6-well cell culture plates coated with rat vitronectin (Sigma) at a density of 5×10^6 cells/well, and cultured in endothelial growth medium-2 (EGM-2, Lonza). After 4 days of culture, nonadherent cells were removed, and the adherent cells were further cultivated for 3 days. The EPCs were characterized using flow cytometry and

Dil-ac-LDL/lectin/Hoechst triple staining, using a previously described technique [4,8,14].

After 7-day culture, the attached cells were labeled with Dil-acLDL (10 μg/ml; Invitrogen) at 37°C for 4 hours, washed 3 times in phosphate buffer solution (PBS), and immersed in 2% paraformaldehyde for 10 minutes. The cells were then incubated with fluorescein isothiocyanate (FITC)-labeled *Ulexeuropaeus* agglutinin (lectin, 10 μg/ml; Sigma-Aldrich) for 1 hour. After nuclei staining with a Hoechst stain (5 μg/ml; Sigma-Aldrich), the cells were viewed under an inverted fluorescent microscope (Leica). Pictures were taken of high-power fields (magnification, ×200). Cells demonstrating triple-positive fluorescence of Dil-acLDL, lectin, and the Hoechst dye were identified as EPCs (Supplementary Figure S1) [4,8,14].

The phenotype of the mouse and rat EPCs was further examined by flow cytometry. Mouse EPCs were placed in polypropylene tubes in 100 μl PBS with 1% albumin. They were then stained with FITC-conjugated Sca-1 antibodies (BD Bioscience) and PE-conjugated Flk-1 antibodies (BD Bioscience) for 1 hour at 4℃and washed 3 times in PBS with 1% albumin. Quantifications of $Sca\text{-}1^+/Flk\text{-}1^+$ cells were performed with a BD Flow cytometer. In addition, rat EPCs were placed in polypropylene tubes in 100 μl PBS with 1% albumin. They were then stained with FITC-conjugated Flk-1 antibodies (abcam) and PE-conjugated CD34 antibodies (Santa Cruz) for 1 hour at 4℃and washed 3 times in PBS with 1% albumin. Quantifications of $CD34^+/Flk\text{-}1^+$ cells were performed with a BD Flow Cytometer. A non-stained sample was used to set up a threshold, and the isotype specific conjugated anti-IgG was used as a negative control [4,8,14].

In vitro EPC function assays

Migration assay

A number of 5×10^4 cells were applied into upper Boyden's chamber with M199. The lower chambers were loaded with M199 supplemented 50ng/ml VEGF. EPCs were allowed to migrate for 24 hours, fixed and stained Hochest 33258 (Sigma, America). The number of cells on the lower side of the membrane was counted at magnification ×100, and the mean value of 5 different areas was determined for each sample [7,8].

Tube formation assay

Matrigel-Matrix (BD Biosciences) was placed in the well of a 96-well cell culture plate and a number of 5×10^4 EPCs were plated in each well with EGM-2. After 18 hours of incubation, images of tube morphology was taken and tube number was counted at random under 5 high-power fields (magnifications ×100) per sample [7,8].

Adhesion assay

In adhesion assays, 1×10^4 cells were plated in 96-well plates coated with 1μg/mL mouse vitronectin. After two hours of incubation, non-adherent cells were washed away and adherent cells were fixed with 2% paraformaldehyde. Nuclei were stained with Hoechst33528 (5×10^{-6} mol/L, 10 min, Molecular Probes). A number of adherent cells were counted at random under 5 high-power fields (magnifications ×100) per sample, and the mean value of the four wells was determined for each sample [7,8].

EPC transplantation and survival time in SHR-SP

Male SHR-SP developed strokes at an average age of about 10 months (300 days) in our laboratory [12,13]. To determine the effects of EPC transplantation on the lifespan of SHR-SP at different degrees of stroke risk, two groups of animals of different ages were used: 300-day-old (at a high risk of stroke) and 180-day-old (at a relatively low risk of stroke) (Figure 1A).

Because EPC functions are significantly impaired in SHR-SP compared to SD rats (as demonstrated in Supplementary Figure S2), we used EPCs obtained from SD rats for transplantation into SHR-SP. Rat EPCs (4×10^6 cells) in 500 μl PBS were systemically injected into SHR-SP via the tail vein, at the age of 180 days (n = 14 per group) or 300 days (n = 9–10 per group), and control animals matched for both age and blood pressure received equal volumes of vehicle [6,15]. Survival time was monitored for 240 days after the injection of EPCs (rats that died of causes other than stroke were discarded) (Figure 1A).

EPC transplantation and animal stroke model in SHR-SP and mice

SD Rat EPCs (4×10^6 cells) in 500 μl phosphate-buffered saline (PBS) or mouse EPCs (1×10^6 cells) in 200 μl PBS were systemically injected into SHR-SP (14-16 weeks) or mice via the tail vein, while control animals received equal volumes of vehicle [6,7,15].

At 28 or 42 days after a single injection of EPCs or vehicle, SHR-SP (Figure 2A; n

= 10 per group) and mice (Figure 4A; n = 10 per group) were subjected to permanent focal cerebral ischemia using a previously reported method [16,17]. Briefly, the animals were anesthetized by intraperitoneal injection of 0.1 ml of 3.5% chloral hydrate per 10 g body weight (mice) or 0.1 ml of 15% chloral hydrate per 100 g body weight (SHRSP). A skin incision was made on the left side, between the ear and the orbit. After the temporalis muscle was split, a burr hole was drilled at the junction of the zygomatic arch and the squamous bone, through which the stem of the left middle cerebral artery (MCA) was exposed and occluded by electrocoagulation. Behavioral tests including Body Asymmetry Test and Beam Test were performed 1 and 3 days after MCA occlusion. The animals were then euthanized, and the brains were stained with 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, USA) to determine the infarct volume, as previously reported [16,17].

After 3 days of cerebral ischemia, the SHR-SP (Figure 3A, n = 5–6 per group) and mice (Figure 5A, n = 8 per group) were euthanized, and the ischemic brains were fixed by transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde, before being embedded in paraffin [18]. A series of 6-μm-thick sections were cut from the block. Every 10th coronal section, for a total of three sections, was used for immunohistochemical staining. Immunostaining was performed against CD31 antibodies (BD Biosciences) to detect angiogenesis in the ischemic brain [19].

The systolic blood pressure in each animal was measured by a noninvasive tail-cuff method (ALC-NIBP non-invasive blood pressure system, Shanghai Alcott biotech Co., Ltd., China) 42 days after an injection of EPCs or vehicle [8], but no significant difference in blood pressure levels was found between the groups (data not shown).

EPC transplantation and *in vivo* EPC integration in SHR-SP and mice

On day 5 of culturing, EPCs were labeled with 5-Bromo-2′-Deoxyuridine (BrdU, Thermo Fisher SCIENTIFIC), as described previously [4]. Briefly, BrdU-labeling reagent was diluted to 1:100 in EGM-2, filtered through a 0.2-μm filter, and warmed to 37°C. Next, 2 ml of BrdU/EGM-2 was added to cells in a 6-well plate, and new media were added daily until day 7. On day 7, the wells were washed 3 times with PBS, followed by trypsinization to resuspend the cells. As showed in Figures 3A and 5A, SD rat EPCs (4×10^6 cells) and mouse EPCs (1×10^6 cells) were then transplanted into

SHR-SP and mice, respectively, via the tail vein. At 28 or 42 days after a single injection of EPCs or vehicle, SHR-SP and mice were subjected to permanent focal cerebral ischemia [16,17].

After 3 days of cerebral ischemia, the SHR-SP and mice were euthanized and the ischemic brains were fixed by transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde, before being embedded in paraffin. A series of 6-μm-thick sections were cut from the block. Every 10th coronal section, for a total of three sections, was used for immunohistochemical staining. The slides were stained with anti-CD31 antibodies (BD Biosciences), followed by BrdU antibody (Santa Cruz Bio-technology Inc.) incubation. The secondary antibodies were AlexaFluor 488 (Abcam) or Cy3 (Abcam). The nucleus was counterstained with DAPI (4',6-diamidino-2-phenylindole, Cell Signaling Technology) [4,18,19].

In addition, EPCs from GFP transgenic mice (GFP-EPCs) were also used for cell tracking in ischemic brain to detect *in vivo* EPC integration in mice. GFP-EPCs (1×10^6 cells) in 200 μl PBS were transplanted into mice via the tail vein, as previously reported [6,15]. Twenty-eight days later, the mice were subjected to permanent focal cerebral ischemia [16,17]. After 3 days of cerebral ischemia, the mice were euthanized, and the ischemic brains were harvested. The cell nuclei were counterstained with DAPI (Cell Signaling Technology), and the GFP-EPCs were detected in frozen ischemic brain sections [6].

EPC transplantation and cell tracking in peripheral blood in mice

Bone marrow-derived EPCs from green fluorescent protein (GFP) transgenic mice (GFP-EPCs) were used for cell tracking in peripheral blood in mice (Supplementary Figures S3A and S4A). GFP-EPCs (1×10^6 cells) in 200 μl PBS were transplanted into mice via the tail vein, as previously reported [6,15]. At 21 and 28 days after a single injection of GFP-EPCs, peripheral blood mononuclear cells (PB-MNCs) were isolated according to a previously described technique [8,17]. Briefly, peripheral blood was obtained by cardiac puncture after the mice were anesthetized. PB-MNCs were isolated by Histopaque-1083 (Sigma) density gradient centrifugation at 400 g for 30 min. The mononuclear fraction was collected, and washed in PBS (pH 7.4). Red blood cells were lysed with ammonium chloride solution (Stemcell Technologies), and then PB-MNCs were washed 3 times in PBS and immersed in 2% paraformaldehyde for 15 minutes. After washing and centrifugation, the PB-MNCs

were re-suspended in 2 ml PBS, and the cell nucleus was stained with DAPI (Cell Signaling Technology). Then, cells were viewed on PB-MNCs smear under an inverted fluorescent microscope (Leica).

Treatment of cerebral ischemic injury with EPC conditioned media in mice

Bone marrow-derived mononuclear cells from mice were plated on a vitronectin-coated (Sigma-Aldrich) 6-well plate at 5×10^6 cells/well in EGM-2 at 37°C with 5% CO_2. After 4 days of culture, nonadherent cells were removed, and fresh EGM-2 was substituted daily. At day 6 of culture, the growing EPCs were washed twice with endothelial basal medium-2 (EBM-2, Lonza) without growth factors and serum. Fresh EBM-2 (1.5 mL/well) was added then to obtain conditioned media (CM) that was collected 24 hours later. Finally, 4 ml of CM was concentrated for 40 min using a 10 kDa filter unit (Millipore, Ireland), yielding a volume of approximately 200 μl. As showed in Figure 6A (n = 8 per group), the concentrated CM (200 μl) was injected into mice via the tail vein just after cerebral ischemia. The control mice received equal volumes of vehicle (EBM-2) [4,20,21]. After 24 hours of cerebral ischemia, behavioral tests including Body Asymmetry Test and Beam Test were performed, and the cerebral infarct volumes were determined as previously reported [16,17].

In addition, to investigate the potential paracrine factors of EPCs in mice and rats, the secreted platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) levels in the concentrated CM were assessed by Western blot analysis (Figure 7A) [4,20,21]. Protein concentrations were determined using the BCA protein assay kit (Pierce, Thermo), and samples containing equal amounts of protein were subjected to 8% SDS/PAGE. Gels were transferred to nitrocellulose membranes and incubated with rabbit anti-PDGF polyclonal antibody (abcam Inc.), rabbit anti-VEGF polyclonal antibody (abcam Inc.) and rabbit anti-HGF polyclonal antibody (abcam Inc.). Secondary antibody was IR Dye 800 conjugated anti-rabbit IgG (1:2000, Rockland). Bands were visualized using Odyssey Imager with Odyssey 1.1 software (Li-Cor) and quantified using NIH image J software.

Statistical analysis

Data are expressed as mean ± SEM. Statistical differences between two groups were

analyzed with Student's *t*-test. For survival time analysis, Kaplan-Meier analysis was used, followed by a log-rank test. A value of $P<0.05$ was considered statistically significant.

Results

Characterization of bone marrow–derived EPCs

Cultured bone marrow-derived EPCs (day 7) from mice (Supplementary Figure S1A) and SD rats (Supplementary Figure S1B) were identified by Dil-ac-LDL/lectin/Hoechst triple staining. In addition, the phenotype of EPCs (day 7) was examined by flow cytometry. To characterize 7-day cultured mouse EPCs, the stem cell marker (Sca-1) and endothelial cell marker (Flk-1) were examined, and the percentage of Sca-1^+/Flk-1^+ cells were 9.99 ± 0.49 % (n=5 per group). To characterize 7-day cultured rat EPCs, the stem cell markers (CD34) and endothelial cell markers (Flk-1) were examined, and the percentage of $CD34^+$/Flk-1^+ cells were 9.31 ± 0.06 % (n=5 per group).

Preventive injection of EPCs prolonged survival time in SHR-SP

SHR-SP, in which strokes occurred spontaneously [12,13], were used to determine whether a single injection of EPCs could prolong their lives (Figure 1A). As noted in the Methods section, we used EPCs from SD rats instead of SHR-SP for transplantation into SHR-SP.

When EPCs were injected into SHR-SP at the age of 300 days, the survival time was significantly prolonged in comparison to non-pretreated control rats ($p < 0.01$). The median survival times were 366 days for the controls, and 489 days for the EPC-pretreated SHR-SP (Figure 1B).

A prolongation of lifespan was also observed in SHR-SP when EPCs were administered at the age of 180 days. By the age of 340 days, the percentage of surviving EPC-pretreated SHR-SP was significantly larger than the percentage of surviving non-pretreated controls (85.7% *vs.* 42.9%, $p < 0.05$) (Figure 1C). Thereafter, the degree of lifespan prolongation produced by EPCs injection was gradually reduced, and at the age of 420 days the two groups of SHR-SP showed the same survival percentage (14.3%). The median survival time was 337 days for the controls and 367 days for the EPC-pretreated SHR-SP (Figure 1D).

These results indicate that a single injection of EPCs produced a more pronounced prolongation of lifespan in high-risk 300-day-old SHR-SP than in relatively low-risk 180-day-old animals (the increases in median survival time were respectively 123 and 30 days). In 180-day-old SHR-SP, a stronger effect on lifespan may require either increasing the number of EPCs injected or adding a second injection of EPCs at a later age.

Preventive injection of EPCs protected against cerebral ischemic injury and promoted angiogenesis in the ischemic brain in SHR-SP

To investigate the possible mechanisms underlying the EPC-mediated prolongation of survival time in SHR-SP, we sought to determine whether a single preventive injection of EPCs could provide persistent protection against cerebral ischemic injury, and enhance local angiogenesis in the ischemic brain, in SHR-SP. At 42 days after a preventive injection of EPCs, SHR-SP were subjected to permanent focal cerebral ischemia (Figure 2A). In the EPC-pretreated SHR-SP, the infarct volumes were significantly reduced (-25%, $p < 0.01$) and neurobehavioral outcomes were markedly improved, compared to the non-pretreated controls (Figure 2B).

EPCs can promote angiogenesis by secreting various angiogenic growth factors [6]. Therefore, local angiogenesis in the ischemic brain was also assessed (Figure 3A). In the EPCs-pretreated SHR-SP, the capillary density was significantly higher than in the non-pretreated controls (+78%, $p < 0.01$) (Figure 3B).

To verify that BrdU-labeled rat EPCs were incorporated into the ischemic brains, slides were stained for the EC-specific marker CD31, followed by BrdU staining. As shown in Figure 3C, some BrdU-positive cells (red fluorescence) were integrated into CD31-positive microvessels (green fluorescence) in the ischemic brains of SHR-SP that received EPC transplantation. Some BrdU-positive cells were also found near the microvessels. Previous studies have shown that cells of donor origin can be detected in the peripheral blood or among the bone marrow cells of recipients 28 days after bone marrow transplantation in SHR-SP or mice [10,11], and that transplanted bone marrow-derived EPCs can participate in wound angiogenesis in mice [4]. The present findings suggest that cells of donor origin could integrate into the recipient ischemic brain and participate in angiogenesis in SHR-SP.

These results indicate that in SHR-SP, a preventive injection of EPCs can promote angiogenesis in the ischemic brain, and protect against cerebral ischemic injury for a

period of at least 42 days after cell delivery.

Preventive injection of EPCs protected against cerebral ischemic injury and promoted angiogenesis in the ischemic brains of mice

Wild-type mice were used to investigate whether the positive effects of a preventive EPC injection could be observed in other types of animals than SHR-SP. At 28 and 42 days after a preventive injection of EPCs, mice were subjected to permanent focal cerebral ischemia (Figure 4A). In EPC-pretreated mice, the infarct volumes were significantly reduced (-38% and -26% respectively, $p < 0.01$), and neurobehavioral outcomes were markedly improved, compared to non-pretreated controls (Figure 4B and 4C).

Local angiogenesis was also assessed in the ischemic mouse brains, 28 days after a preventive injection of EPCs (Figure 5A). In the EPC-pretreated mice, capillary density was significantly higher than in non-pretreated controls (+35%, $p < 0.05$) (Figure 5B). In addition, double immunostaining (Figure 5C) showed that some BrdU-positive cells (red fluorescence) were integrated into CD31-positive microvessels (green fluorescence) in the ischemic brains of mice that received EPC transplantation. BrdU-positive cells were also found near the microvessels. Moreover, EPCs from GFP transgenic mice were also used for cell tracking, to detect the *in vivo* integration of EPCs in mice. It was found that intravenously delivered GFP-EPCs (green fluorescence) could home into the ischemic brain 28 days after injection (Figure 5D). Intravenously delivered GFP-EPCs (green fluorescence) could also be detected in the peripheral blood of the recipients 21 or 28 days after cell injection in mice (Supplementary Figures S3 and S4). This finding suggests that in mice, cells of donor origin could integrate into the recipient ischemic brain and participate in angiogenesis.

These results in mice provide further support for the conclusion that a single preventive injection of EPCs can promote angiogenesis in the ischemic brain and protect against cerebral ischemic injury for at least 28 days.

Preventive injection of EPCs improved neurobehavioral outcomes after 1 day of cerebral ischemia in SHR-SP and mice

It has been demonstrated that an angiogenic reaction starts between 24 and 48 hours after cerebral infarction [22]. To determine whether preventively-injected EPCs exert non-angiogenic effects (such as paracrine effects) on cerebral ischemic injury in

addition to their direct angiogenic effects, we assessed neurobehavioral outcomes 24 hours after cerebral ischemia in both SHR-SP and mice. At 42 days after a preventive injection of EPCs, SHR-SP were subjected to permanent focal cerebral ischemia (Figure 2A). After 1 day of ischemia, the neurobehavioral outcomes were significantly better in EPC-pretreated SHR-SP than in un-pretreated controls (Figure 2B). In addition, 28 or 42 days after a preventive injection of EPCs, mice were subjected to permanent focal cerebral ischemia (Figure 4A). After 1 day of ischemia, the neurobehavioral outcomes were markedly better in EPCs-pretreated mice than in un-pretreated controls (Figure 4B and 4C).

These results indicate that preventively-injected EPCs might exert non-angiogenic effects on cerebral ischemic injury in addition to their direct angiogenic effects, over a period of at least 42 days after cell delivery, in both SHR-SP and mice.

EPC conditioned media reduced cerebral ischemic injury in mice

To study the paracrine effect of EPCs on cerebral ischemic injury, conditioned media (CM) from mouse EPCs was collected and injected into mice via the tail vein (Figure 6A). In CM-treated mice, the infarct volumes were significantly reduced (-23%, $p < 0.01$) and the neurobehavioral outcomes were markedly improved, compared to non-CM-treated controls (Figure 6B). Previous studies have shown that EPC-conditioned media can accelerate diabetic wound healing [4], and that an intravenous injection of EPC-conditioned media can enhance neurorepair responses after cerebral ischemia in mice [20]. In addition, previous studies [20,21] and the present work (Figure 7) found that mouse and rat EPC-conditioned media contains paracrine growth factors, including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), etc. Accordingly, our present findings suggest that EPCs might exert paracrine effects on cerebral ischemic injury in additional to their direct angiogenic effects.

Discussion

The present work showed that a single preventive delivery of EPCs could significantly prolong the lifespan of SHR-SP. This unexpected result suggests the potential value of preventive EPC delivery as a strategy for stroke prevention.

EPCs play an important role in tissue repair and regeneration in the ischemic brain

[4-8]. We found that a single preventive injection of EPCs could produce persistent protective effects against cerebral ischemic injury (lasting at least 42 days), and promote local angiogenesis in the ischemic brain, in two types of animals (SHR-SP and normotensive mice). Of course, while increased angiogenesis is indeed seen, this is not necessarily the cause of EPC injection-induced protection, which remains to be clarified by further proof. It has previously been shown that 28 days after bone marrow transplantation in mice or SHR-SP, cells of donor origin can be detected among recipient bone marrow cells or in peripheral blood [10,11]. Previous studies also showed that EPC-conditioned media can accelerate diabetic wound healing [4], promote angiogenesis, and improve neurological outcomes after cerebral ischemia in mice [20,21]. The present study found that 28 days after bone marrow-derived EPC transplantation, cells of donor origin could be detected in the recipient ischemic brains and peripheral blood. Moreover, our findings suggest that EPCs might exert paracrine effects on cerebral ischemic injury repair processes in addition to their direct angiogenic effects. Therefore, the persistent protective effects of EPCs against cerebral ischemic injury, as observed in the present study, may partly contribute to the lifespan prolongation produced by a preventive delivery of EPCs in SHR-SP.

It has been demonstrated that in aged SHR-SP, bone marrow cell transplantation can promote the response to ischemic injury potentially at the level of endothelial/vascular activation [11]. Thus, a preventive delivery of bone marrow-derived EPCs might also have the potential to prolong the lifespan by promoting endothelial/vascular activation and postponing stroke onset in SHR-SP, which remains to be tested by further studies.

Two other important new findings of the present work are: 1) preventive EPC delivery is more effective in lifespan prolongation in relatively old (high-risk) SHR-SP than in young (low-risk) animals; 2) in relatively young SHR-SP, a single delivery of EPCs may be not enough, and one more injection of EPCs at a later age, or increasing the quantity of EPCs in a single injection, may be needed to maintain a more persistent effect on lifespan prolongation. These factors should be taken into account when contemplating an EPC-based clinical stroke prevention approach.

Preventive EPC delivery has some obvious advantages compared with other measures for stroke prevention [3]: 1) It is a long-acting preventive approach, and a single injection lasts for lifetime in high-risk SHR-SP (Figure 1B). 2) It is potentially a broad-spectrum preventive approach, and might be applicable to patients with

different risk factors of stroke, such as hypertension, aging etc. 3) Given that EPCs are often dysfunctional in patients at risk for cerebrovascular or cardiovascular diseases [4-8], *in vitro* pharmacological or genetic interventions could be used to restore impaired functions of autologous EPCs and thus promote their stroke-preventive effects.

In addition, since EPCs play an important role in vascular repair and revascularization in various ischemic organs besides brain tissue [4-8], the preventive delivery of EPCs might also serve as a potential strategy to prevent other ischemic diseases beside stroke, such as myocardial infarction, limbic ischemic injury, etc. This suggestion remains to be tested by further studies.

In conclusion, a single preventive injection of EPCs could prolong the lifespan of SHR-SP, an effect that might at least partly be attributed to the persistent protective effects of EPCs against cerebral ischemic injury. It is implied that a preventive delivery of EPCs, which is clinically feasible, might have the potential to serve as a long-acting strategy for stroke prevention, and might be especially beneficial to the patients at high risk for stroke.

Clinical perspectives

- There is a pressing need for new approaches to preventing stroke. This study sought to evaluate whether preventive delivery of EPCs could prevent or protect against stroke.
- It was demonstrated that a single preventive injection of EPCs prolonged the lifespan of Stroke-prone spontaneously hypertensive rats, and protected against cerebral ischemic injury for at least seven weeks.
- It is implied that a preventive delivery of EPCs, which is clinically feasible, might be a promising candidate for a preventive role in patients at high risk for stroke.

Author contribution

H.H.X. conceived and designed the experiments. C.P., X.H.D., J.L.L., Y.L.T., C.F.X., L.P.W. and C.L.L. performed the experiments. H.H.X., C.Z., X.T. and A.F.C. analyzed and interpreted the data. D.F.S. provided intellectual contribution and useful discussion. H.H.X. and C.P. wrote the manuscript.

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Competing interests

The authors declare that there are no competing interests associated with the manuscript.

References

1. Lopez, A.D., Mathers. C.D., Ezzati, M., Jamison, D.T., Murray, C.J.L. (2006) Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *Lancet* **367**, 1747–1757.
2. Glover, L.E., Tajiri, N., Weinbren, N.L., Ishikawa, H., Shinozuka, K., Kaneko, Y. et al. (2012) A Step-up Approach for Cell Therapy in Stroke: Translational Hurdles of Bone Marrow-Derived Stem Cells. *Transl. Stroke. Res* **3**, 90-98.
3. Meschia, J.F., Bushnell, C., Boden-Albala, B., Braun, L.T., Bravata, D.M., Chaturvedi, S. et al. (2014) Guidelines for the primary prevention of stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke* **45**, 3754-3832.
4. Marrotte, E., Chen, D.D., Hakim, J.S., Chen, A.F. (2010) Manganese superoxide dismutase expression in endothelial progenitor cell accelerates wound healing in diabetic mice. *J. Clin. Invest* **120**, 4207-4219.
5. Khakoo, A.Y., Finkel, T. (2005) Endothelial progenitor cells. *Annu. Rev. Med* **56**, 79-101.
6. Fan, Y., Shen, F., Frenzel, T., Zhu, W., Ye, J., Liu, J. et al. (2010) Endothelial progenitor cell transplantation improves long-term stroke outcome in mice. *Ann. Neurol* **67**, 488-497.
7. Xin, B., Liu, C.L., Yang, H., Peng, C., Dong, X.H., Zhang, C. et al. (2016) Prolonged fasting improves endothelial progenitor cell-mediated ischemic angiogenesis in mice. *Cell. Physiol. Biochem* **40**, 693-706.

8. Xie, H.H., Zhou, S., Chen, D., Channon, K.M., Su, D.F., Chen, A.F. (2010) GTP cyclohydrolase I/BH4 pathway protects EPCs via suppressing oxidative stress and thrombospondin-1 in salt-sensitive hypertension. *Hypertension* **56**, 1137-1144.

9. Steinberg, G.K., Kondziolka, D., Wechsler, L.R., Lunsford, L.D., Coburn, M.L., Billigen, J.B. et al. (2016) Clinical Outcomes of Transplanted Modified Bone Marrow-Derived Mesenchymal Stem Cells in Stroke: A Phase 1/2a Study. *Stroke* **47**, 1817-1824.

10. Rao, S.S., Peters, S.O., Crittenden, R.B., Stewart, F.M., Ramshaw, H.S., Quesenberry, P.J. (1997) Stem cell transplantation in the normal nonmyeloablated host: relationship between cell dose, schedule, and engraftment. *Exp. Hematol* **25**, 114-121.

11. Taguchi, A., Zhu, P., Cao, F., Kikuchi-Taura, A., Kasahara, Y., Stern, DM. et al. (2011) Reduced ischemic brain injury by partial rejuvenation of bone marrow cells in aged rats. *J. Cereb. Blood. Flow. Metab* **31**, 855-867.

12. Yamori, Y., Horie, R., Handa, H., Sato, M., Fukase, M. (1976) Pathogenic similarity of strokes in stroke-prone spontaneously hypertensive rats and humans. *Stroke* **7**, 46–53.

13. Guo, J.M., Liu, A.J., Zang, P., Dong, W.Z., Ying, L., Wang, W. et al. (2013) ALDH2 protects against stroke by clearing 4-HNE. *Cell. Res* **23**, 915-930.

14. Chen, D.D., Dong, Y.G., Yuan, H., Chen, A.F. (2012) Endothelin 1 activation of endothelin A receptor/NADPH oxidase pathway and diminished antioxidants critically contribute to endothelial progenitor cell reduction and dysfunction in salt-sensitive hypertention. *Hypertension* **59**, 1037-1043.

15. Zhang, Z.G., Zhang, L., Jiang, Q., Chopp, M. (2002) Bone marrow-derived endothelial progenitor cells participate in cerebral neovascularization after focal cerebral ischemia in the adult mouse. *Circ. Res* **90**, 284-288.

16. Zhu, W., Fan, Y., Frenzel, T., Gasmi, M., Bartus, R.T., Young, W.L. et al. (2008) Insulin growth factor-1 gene transfer enhances neurovascular remodeling and improves long-term stroke outcome in mice. *Stroke* **39**, 1254-1261.

17. Dong, X.H., Sun, X., Jiang, G.J., Chen, A.F., Xie, H.H. (2015) Dietary intake of sugar substitutes aggravates cerebral ischemic injury and impairs endothelial progenitor cells in mice. *Stroke* **46**, 1714-18.

18. Chen, J., Zhang, C., Jiang, H., Li, Y., Zhang, L., Robin, A. et al. (2005) Atorvastatin induction of VEGF and BDNF promotes brain plasticity after stroke in mice. *J. Cereb. Blood. Flow. Metab* **25**, 281–290.

19. Li, Y., Jiang, N., Powers, C., Chopp, M. (1998) Neuronal damage and plasticity identified by microtubule-associated protein 2, growth-associated protein 43, and cyclin D1 immunoreactivity after focal cerebral ischemia in rats. *Stroke* **29**, 1972–1980.

20. Rosell, A., Morancho, A., Navarro-Sobrino, M., Martínez-Saez, E., Hernández-Guillamon, M., Lope-Piedrafita, S. et al. (2013) Factors secreted by endothelial progenitor cells enhance neurorepair responses after cerebral ischemia in mice. *PLoS. One* **8**, e73244.

21. Dong, X.H., Peng, C., Zhang, Y.Y., Tao, Y.L., Tao, X., Zhang, C. et al. (2017) Chronic exposure to subtherapeutic antibiotics aggravates ischemic stroke outcome in mice. *EBiomedicine* **24**, 116-126

22. Marti, H.J., Bernaudin, M., Bellail, A., Schoch, H., Euler, M., Petit, E. et al. (2000) Hypoxia-induced vascular endothelial growth factor expression precedes neovascularization after cerebral ischemia. *Am. J. Pathol* **156**, 965-976.

Figure legends

Figure 1. Effects of a preventive EPCs delivery on the survival time in SHR-SP.

A, Experimental protocols: SHR-SP were randomly divided into 4 groups. Animals received a single injection of EPCs or vehicle at the age of 300 days (n=9-10 per group) or 180 days (n=14 per group), and their survival time was monitored for 240 days. B, SHR-SP received a single injection of EPCs at the age of 300 days. $^{**}p<0.01$ *vs.* SHR-SP/Vehicle. C and D, SHR-SP received a single injection of EPCs at the age of 180 days.$^{*}p<0.05$ *vs.* SHR-SP/Vehicle. SHR-SP, stroke-prone spontaneously hypertensive rats.

Figure 2. A preventive delivery of EPCs protected against cerebral ischemic injury in SHR-SP.

A, Surgical protocols: SHR-SP were randomly allocated to 2 groups (n=10 per group). Animals received a single injection of EPCs or vehicle, and 42 days later, SHR-SP were subjected to permanent left middle cerebral artery occlusion by electrocoagulation. Neurobehavioral outcome assessment was performed at 1 and 3 days after cerebral ischemia, and then the infarct volumes were determined. B, the cerebral infarct volumes were reduced and the corresponding neurobehavioral outcomes (including Bean Test and Body Asymmetry Test) were improved at 42 days after a single injection of EPCs in SHR-SP. $^{**}p<0.01$ *vs.* SHR-SP/Vehicle.

Figure 3. A preventive delivery of EPCs promoted angiogenesis in ischemic brain in SHR-SP.

A, Surgical protocols: SHR-SP were randomly allocated to 2 groups. Animals received a single injection of EPCs (n=5) or vehicle (n=6), and 42 days later, SHR-SP were subjected to permanent left middle cerebral artery occlusion by electrocoagulation. After 3 days of ischemia, we determined the local angiogenesis in the ischemic brain in SHR-SP. B, The local angiogenesis in the ischemic brain were increased at 42 days after a single injection of EPCs in SHR-SP. CD31 immunostaining shows microvessels in ischemic brain of SHR-SP pretreated with vehicle or EPCs. The bar graph shows that the number of microvessels in EPCs-pretreated SHR-SP was significantly increased compared with control. $^{**}P<0.01$ *vs.* SHR-SP/Vehicle. Scale bar: 100 μm (left); 50 μm (right). C, To ascertain

that BrdU-labeled EPCs were incorporated into the ischemic brain, slides were stained for CD31, followed by BrdU staining. Typical photographs (taken from the ischemic boundary area) indicate that some BrdU-positive cells (red) were incorporated into CD31-positive microvessels (green). Some BrdU-positive cells were found surrounding the microvessels. The nucleus was counterstained with DAPI (blue). Scale bar: 35 μm. SHR-SP, stroke-prone spontaneously hypertensive rats.

Figure 4. A preventive delivery of EPCs protected against cerebral ischemic injury in mice.

A, Surgical protocols: male C57BL/6 mice were randomly allocated to 4 groups (n=10 per group). Animals received a single injection of EPCs or vehicle, and 28 or 42 days later, mice were subjected to permanent left middle cerebral artery occlusion by electrocoagulation. Neurobehavioral outcome assessment was performed at 1 and 3 days after cerebral ischemia, and then the infarct volumes were determined. B and C, cerebral infarct volumes were reduced and the corresponding neurobehavioral outcomes (including Bean Test and Body Asymmetry Test) were improved at 28 days (B) and 42 days (C) after a single injection of EPCs in mice. *p<0.05, **p<0.01 *vs.* Mice/Vehicle.

Figure 5. A preventive delivery of EPCs promoted angiogenesis in ischemic brain in mice.

A, Surgical protocols: male C57BL/6 mice were randomly allocated to 2 groups (n=8 per group). Animals received a single injection of EPCs or vehicle, and 28 days later, mice were subjected to permanent left middle cerebral artery occlusion by electrocoagulation. After 3 days of ischemia, we determined the local angiogenesis in the ischemic brain in mice. B, The local angiogenesis in the ischemic brain was increased at 28 days after a single injection of EPCs in mice. CD31 immunostaining shows microvessels in ischemic brain of mice pretreated with vehicle or EPCs. The bar graph shows that the number of microvessels in EPCs-pretreated mice was significantly increased compared with control. *P<0.05 *vs.* Mice/Vehicle. Scale bar: 100 μm (left); 50 μm (right). C, To ascertain that BrdU-labeled EPCs were incorporated into the ischemic brain, slides were stained for CD31, followed by BrdU staining. Typical photographs (taken from the ischemic boundary area) indicate that some BrdU-positive cells (red) were incorporated into CD31-positive microvessels

(green). Some BrdU-positive cells were found surrounding the microvessels. The nucleus was counterstained with DAPI (blue). Scale bar: 35μm. D, EPCs from GFP transgenic mice (GFP-EPCs) were also used for cell tracking in ischemic brain to detect the *in vivo* EPC integration in mice. Photomicrographs (taken from the ischemic boundary area) show that intravenously delivered GFP-EPCs (green) could home into ischemic brain at 28 days after cells injection in mice. The nucleus was counterstained with DAPI (blue). Inserted boxes show increased magnification of cells. Scale bar: 50μm.

Figure 6. EPC-conditioned media reduced cerebral ischemic injury in mice.

A, Surgical protocols: male C57BL/6 mice were randomly allocated to 2 groups (n=8 per group). Animals received a single injection of EPC-conditioned media (CM) or vehicle (EBM-2) just after permanent left middle cerebral artery occlusion by electrocoagulation. After 1 day of ischemia, we determined the cerebral infarct volumes and neurobehavioral outcomes in mice. B, Conditioned media of mouse EPCs reduced the infarct volumes and improved the corresponding neurobehavioral outcomes (including Bean Test and Body Asymmetry Test). $^{**}p<0.01$ vs. Mice/Vehicle.

Figure 7. The secreted PDGF, VEGF, and HGF levels in EPC-conditioned culture media (CM) in mice and rats.

A. Experimental protocols: mouse and rat EPCs were isolated and cultured, and the EPC-conditioned culture media was collected and concentrated. Secreted PDGF, VEGF, and HGF levels in the CM were assessed by western blot analysis. B. Secreted PDGF, VEGF, and HGF levels in the CM. EPCs, endothelial progenitor cells; CM, conditioned culture media; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor.

Figure 1

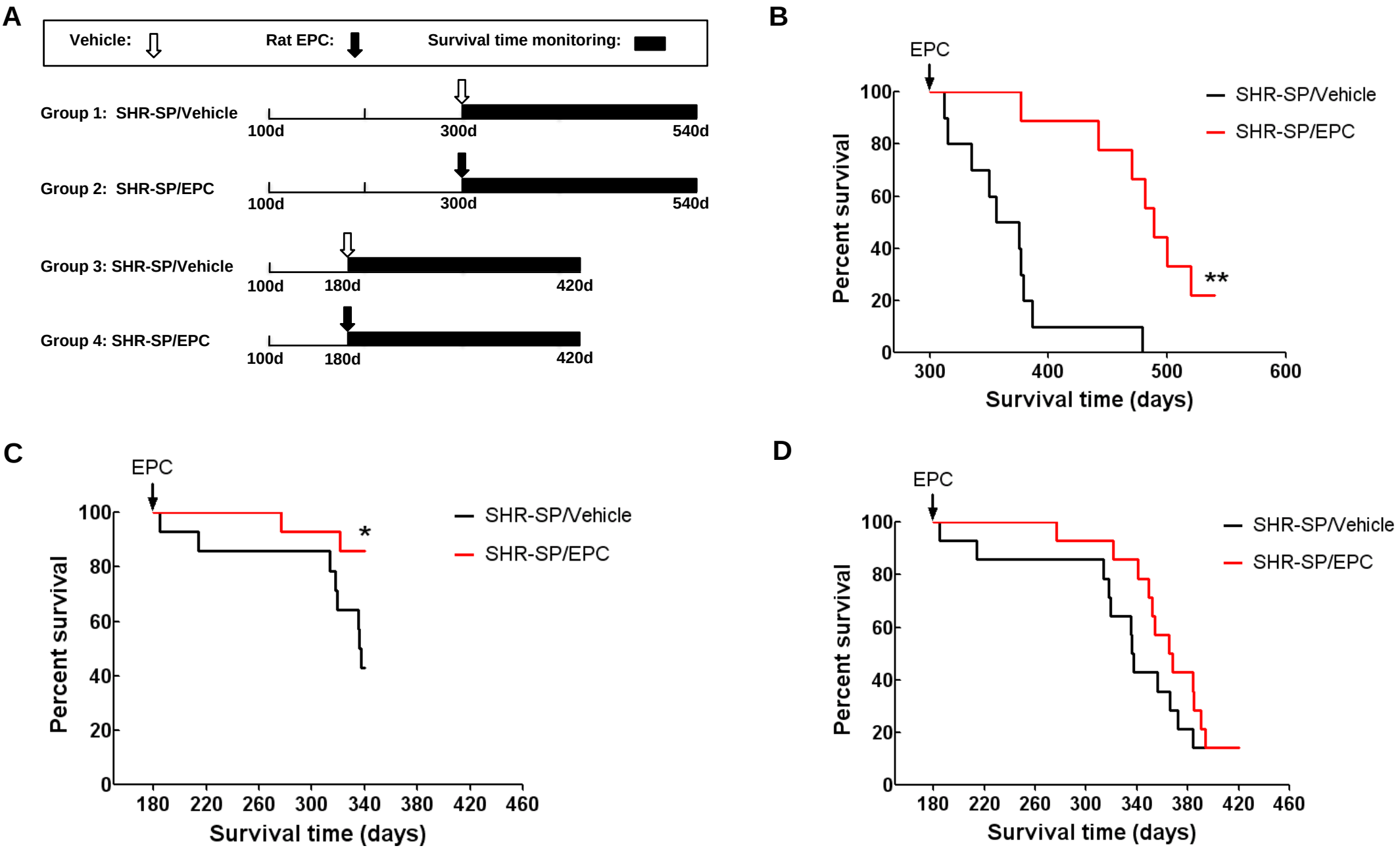

A
Vehicle:
Rat EPC:
Survival time monitoring:
Group 1: SHR-SP/Vehicle
100d
300d
540d
Group 2: SHR-SP/EPC
100d
300d
540d
Group 3: SHR-SP/Vehicle
100d
180d
420d
Group 4: SHR-SP/EPC
100d
180d
420d
B
EPC
SHR-SP/Vehicle
SHR-SP/EPC
**
Percent survival
Survival time (days)
C
EPC
SHR-SP/Vehicle
SHR-SP/EPC
*
Percent survival
Survival time (days)
D
EPC
SHR-SP/Vehicle
SHR-SP/EPC
Percent survival
Survival time (days)

Figure 2

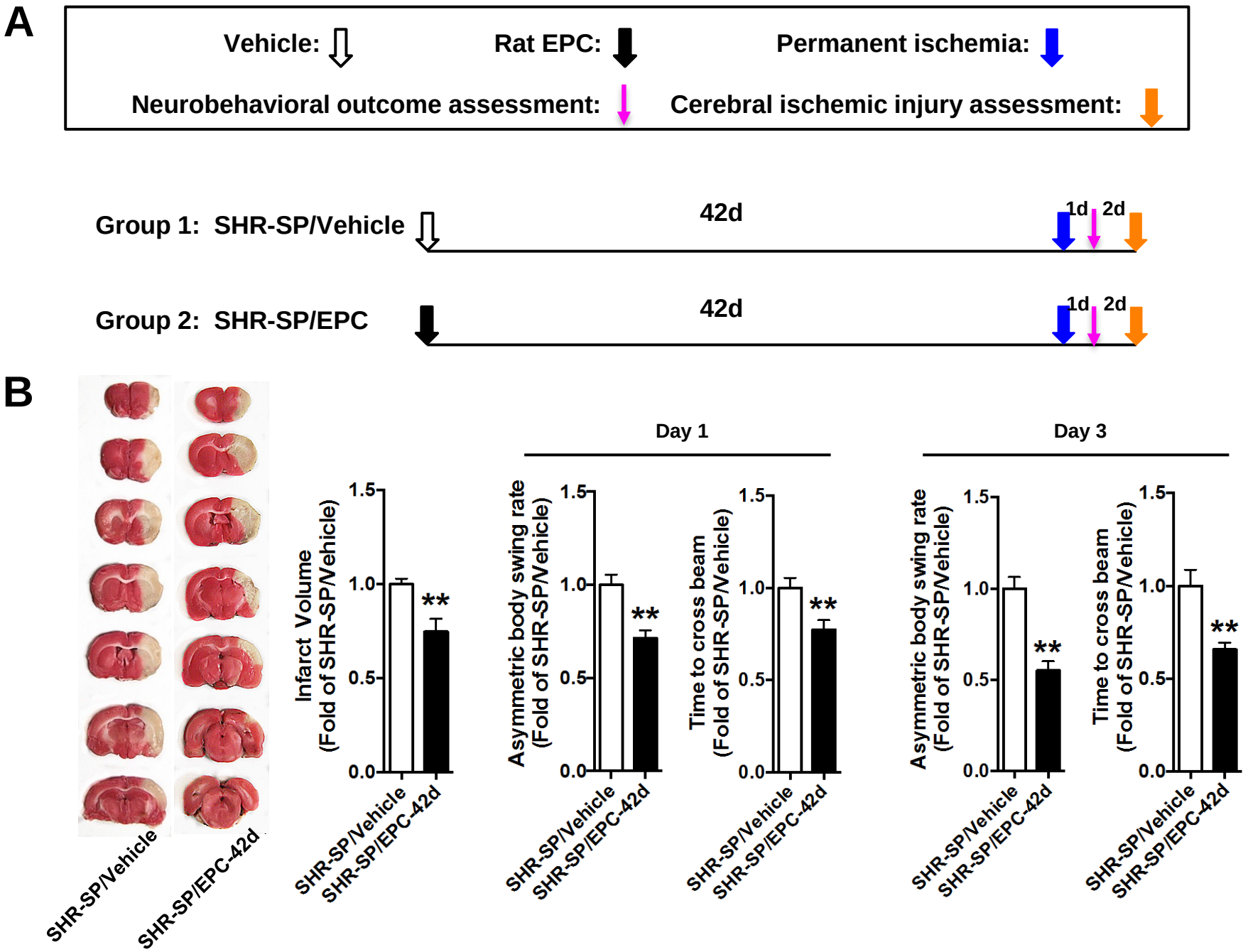

A
Vehicle:
Rat EPC:
Permanent ischemia:
Neurobehavioral outcome assessment:
Cerebral ischemic injury assessment:
Group 1: SHR-SP/Vehicle
42d
1d
2d
Group 2: SHR-SP/EPC
42d
1d
2d
B
SHR-SP/Vehicle
SHR-SP/EPC-42d
Infarct Volume (Fold of SHR-SP/Vehicle)
Day 1
Asymmetric body swing rate (Fold of SHR-SP/Vehicle)
Time to cross beam (Fold of SHR-SP/Vehicle)
Day 3
Asymmetric body swing rate (Fold of SHR-SP/Vehicle)
Time to cross beam (Fold of SHR-SP/Vehicle)
1.5
1.0
0.5
0.0
**
SHR-SP/Vehicle
SHR-SP/EPC-42d

Figure 3

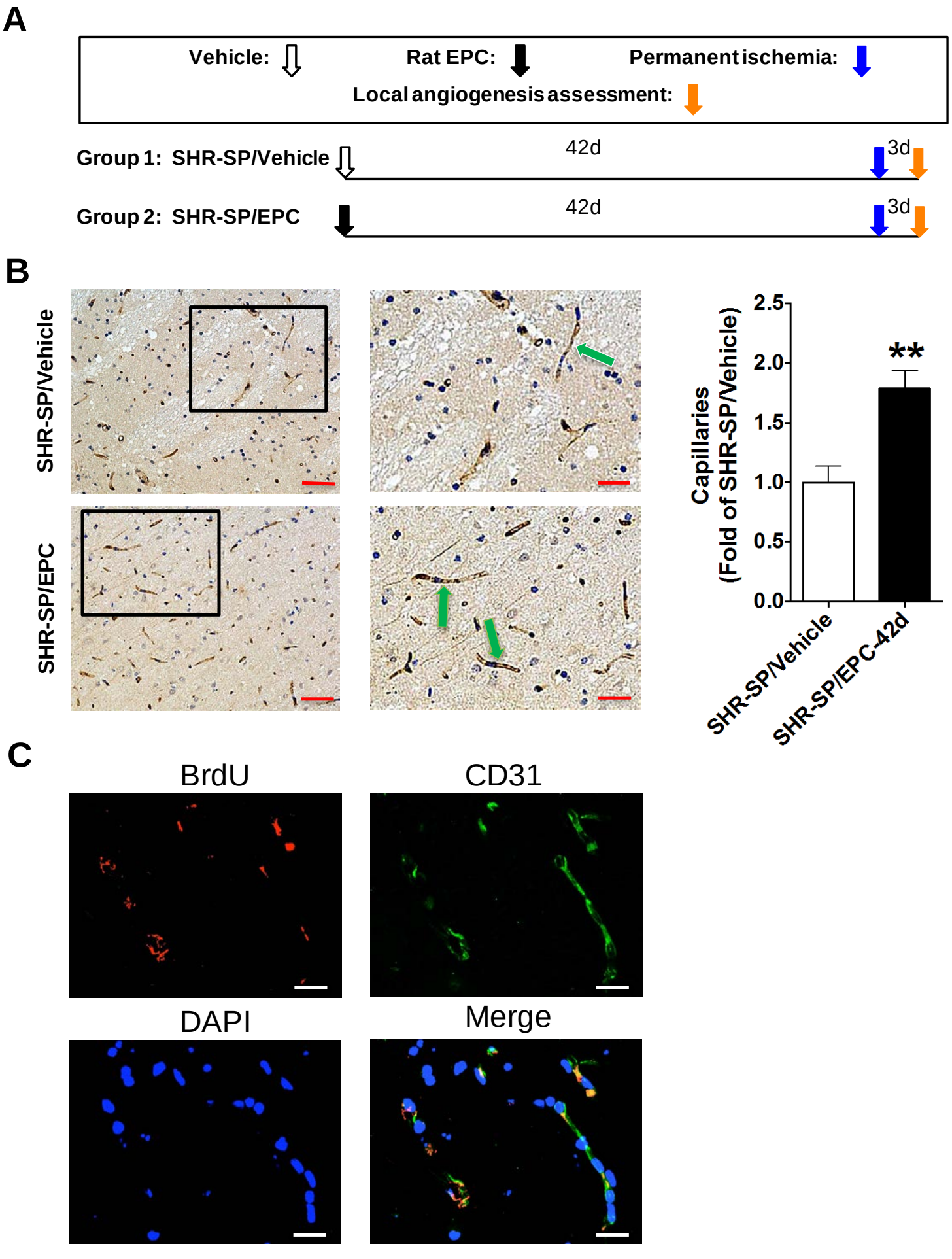
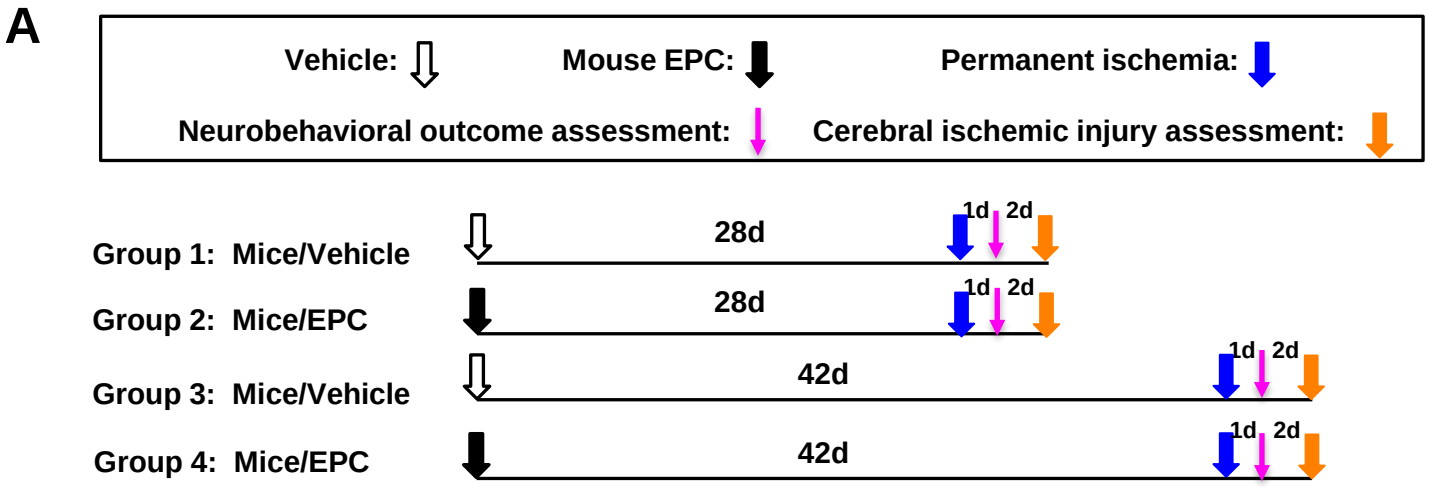
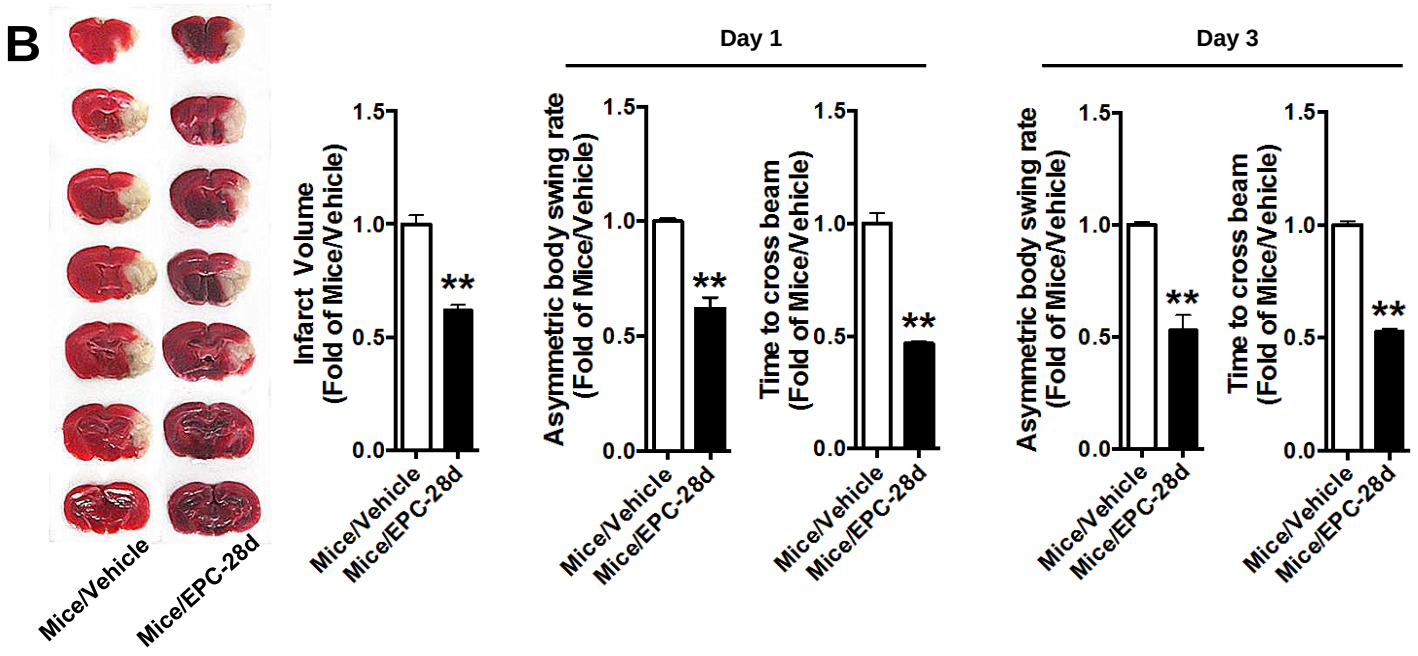

A
Vehicle:
Rat EPC:
Permanent ischemia:
Local angiogenesis assessment:
Group 1: SHR-SP/Vehicle
42d
3d
Group 2: SHR-SP/EPC
42d
3d
B
SHR-SP/Vehicle
SHR-SP/EPC
Capillaries
(Fold of SHR-SP/Vehicle)
2.5
2.0
1.5
1.0
0.5
0.0
**
SHR-SP/Vehicle
SHR-SP/EPC-42d
C
BrdU
CD31
DAPI
Merge

Figure 4


A
Vehicle:
Mouse EPC:
Permanent ischemia:
Neurobehavioral outcome assessment:
Cerebral ischemic injury assessment:
Group 1: Mice/Vehicle
Group 2: Mice/EPC
Group 3: Mice/Vehicle
Group 4: Mice/EPC
28d
28d
42d
42d
1d
2d


B
Mice/Vehicle
Mice/EPC-28d
Day 1
Day 3
Infarct Volume (Fold of Mice/Vehicle)
Asymmetric body swing rate (Fold of Mice/Vehicle)
Time to cross beam (Fold of Mice/Vehicle)
1.5
1.0
0.5
0.0
**

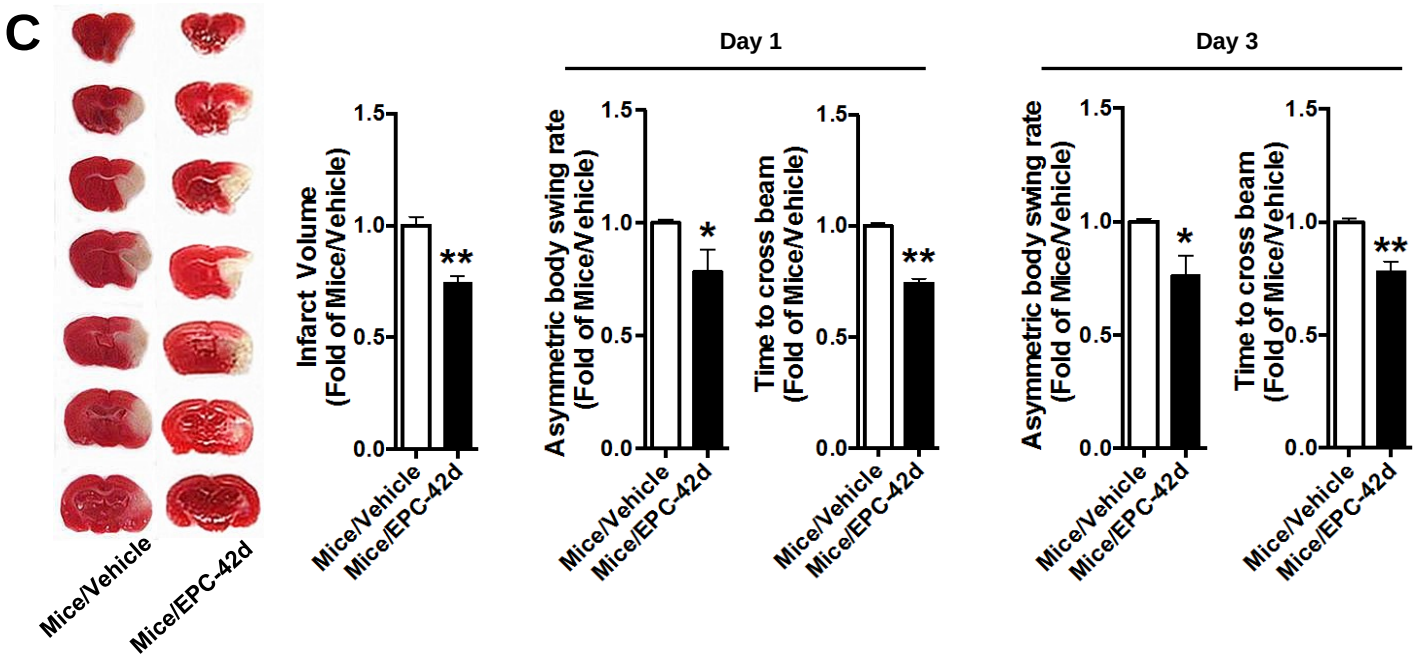

C
Mice/Vehicle
Mice/EPC-42d
Day 1
Day 3
Infarct Volume (Fold of Mice/Vehicle)
Asymmetric body swing rate (Fold of Mice/Vehicle)
Time to cross beam (Fold of Mice/Vehicle)
1.5
1.0
0.5
0.0
*
**

Figure 5

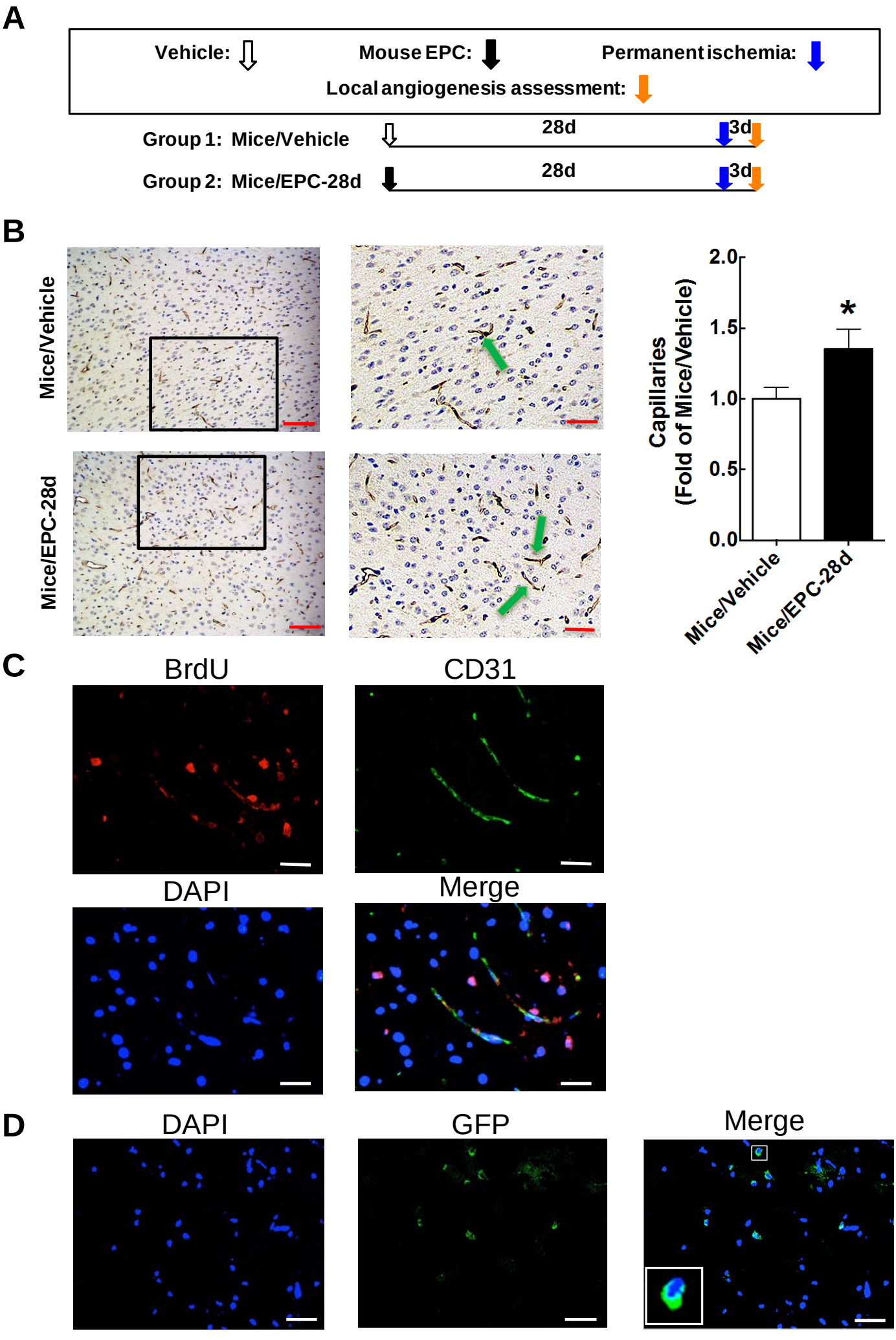

A
Vehicle:
Mouse EPC:
Permanent ischemia:
Local angiogenesis assessment:
Group 1: Mice/Vehicle
28d
3d
Group 2: Mice/EPC-28d
28d
3d
B
Mice/Vehicle
Mice/EPC-28d
Capillaries (Fold of Mice/Vehicle)
2.0
1.5
1.0
0.5
0.0
*
Mice/Vehicle
Mice/EPC-28d
C
BrdU
CD31
DAPI
Merge
D
DAPI
GFP
Merge

Figure 6

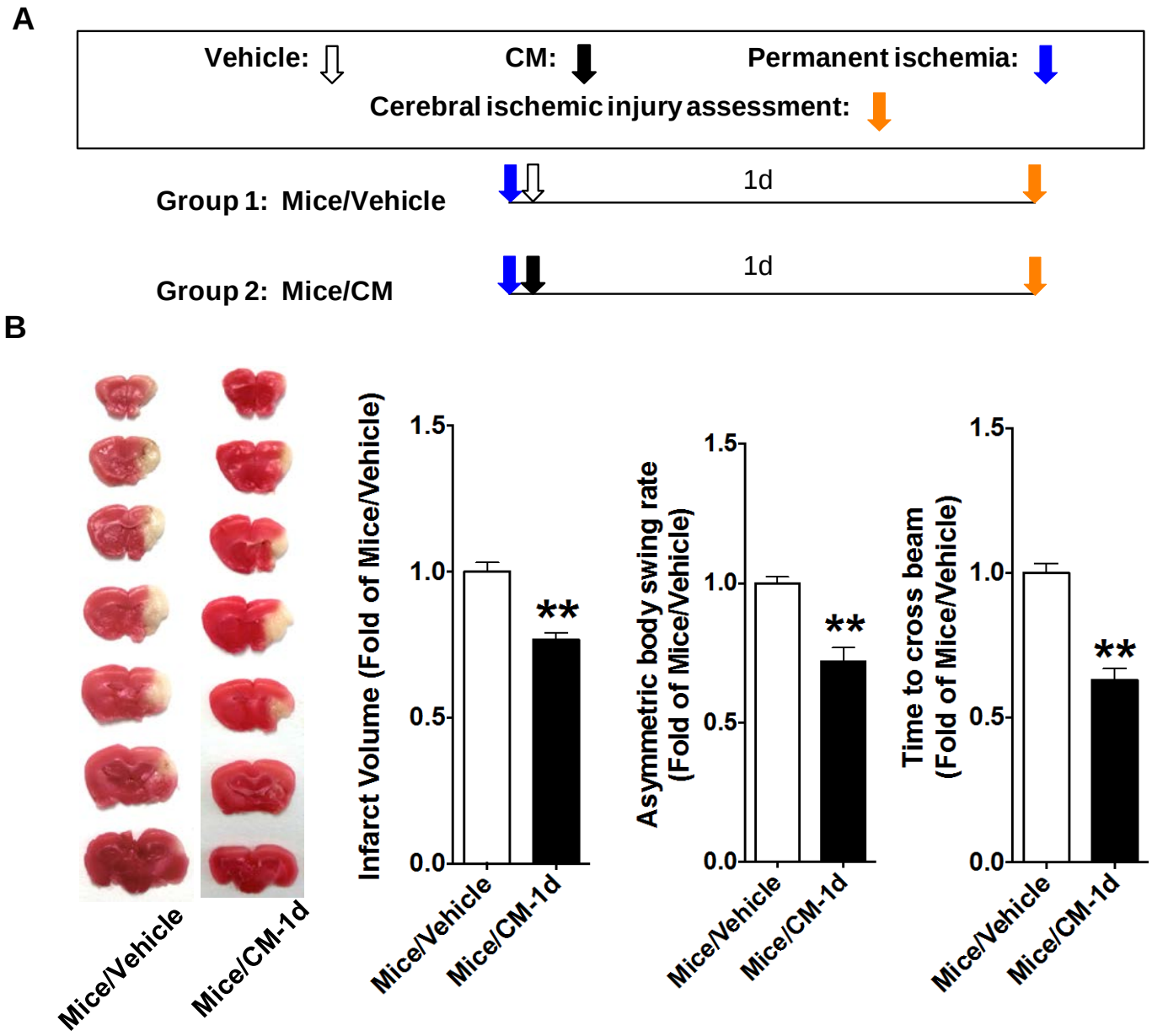
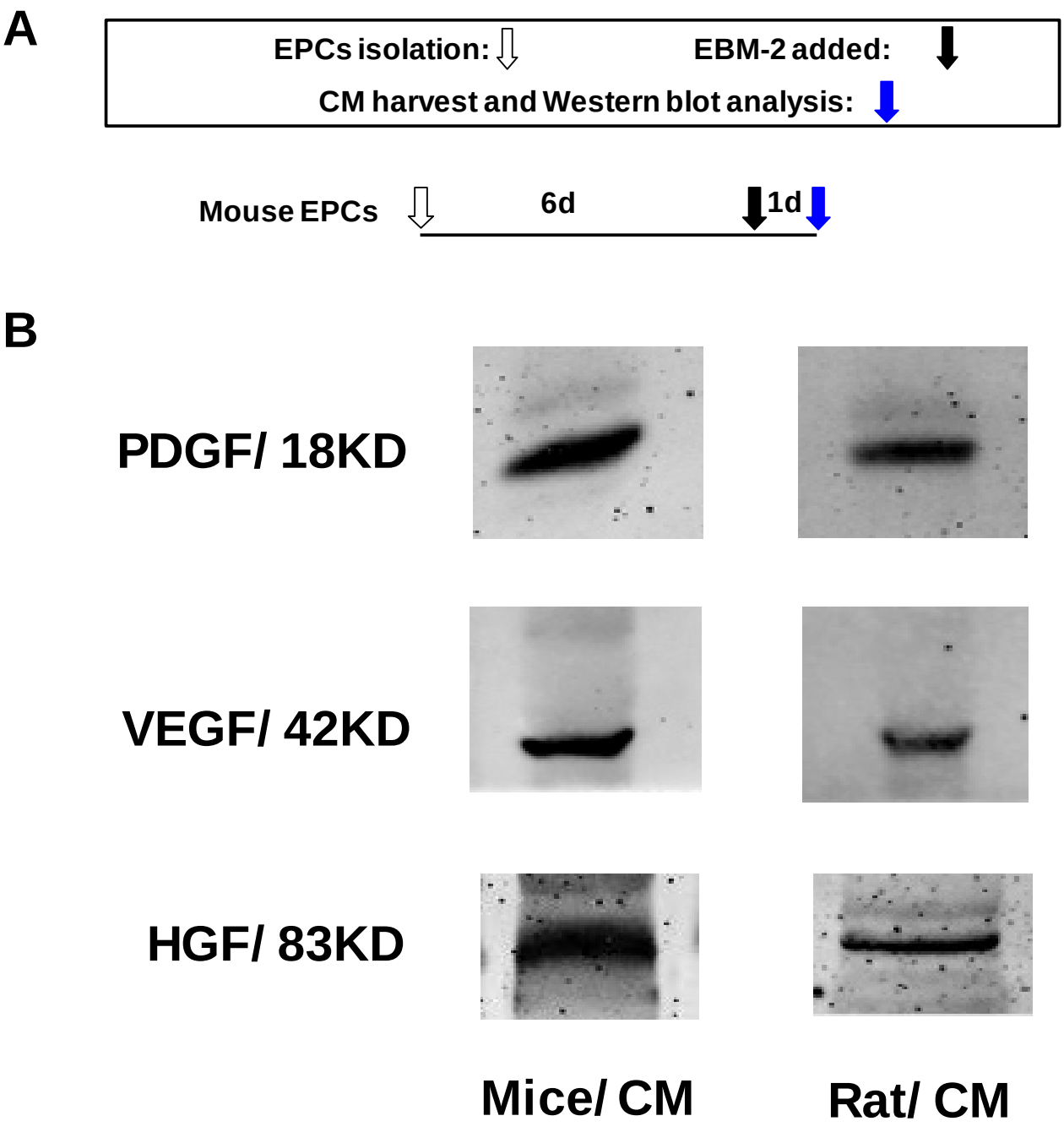

A
Vehicle:
CM:
Permanent ischemia:
Cerebral ischemic injury assessment:
Group 1: Mice/Vehicle
1d
Group 2: Mice/CM
1d
B
Mice/Vehicle
Mice/CM-1d
Infarct Volume (Fold of Mice/Vehicle)
1.5
1.0
0.5
0.0
**
Mice/Vehicle
Mice/CM-1d
Asymmetric body swing rate (Fold of Mice/Vehicle)
1.5
1.0
0.5
0.0
**
Mice/Vehicle
Mice/CM-1d
Time to cross beam (Fold of Mice/Vehicle)
1.5
1.0
0.5
0.0
**
Mice/Vehicle
Mice/CM-1d

Figure 7


A
EPCs isolation:
EBM-2 added:
CM harvest and Western blot analysis:
Mouse EPCs
6d
1d
B
PDGF/ 18KD
VEGF/ 42KD
HGF/ 83KD
Mice/ CM
Rat/ CM

A Preventive Transplantation of Endothelial Progenitor Cells Prolongs Lifespan in Stroke-prone Spontaneously Hypertensive Rats

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SUPPLEMENTARY MATERIAL

Supplementary Figure legends

Supplementary Figure S1. Characterization of mouse (A) and SD rat (B) bone marrow derived EPCs.

After 7 days of culture, cells were stained with Dil-ac-LDL (red) and lectin (green). The nuclei were counterstained with Hoechst33258 (blue). Scale bar: 50 μm.

Supplementary Figure S2. EPC functions were impaired in SHR-SP compared to normotensive SD rats.

A, Migration assay of BM-EPCs. ***P*<0.01 *vs.* SD. n=6. Scale bar: 100 μm. B, Adhesion assay of BM-EPCs. ***P*<0.01 *vs.* SD. n=6. Scale bar: 100 μm. C, Tube formation assay of BM-EPCs. ***P*<0.01 *vs.* SD. n=5. Scale bar: 100 μm. SD, normotensive SD rats. SHR-SP, stroke-prone spontaneously hypertensive rats.

Supplementary Figure S3. Intravenously delivered EPCs could be detected in recipient peripheral blood at 21 days after a single injection of EPCs in mice.

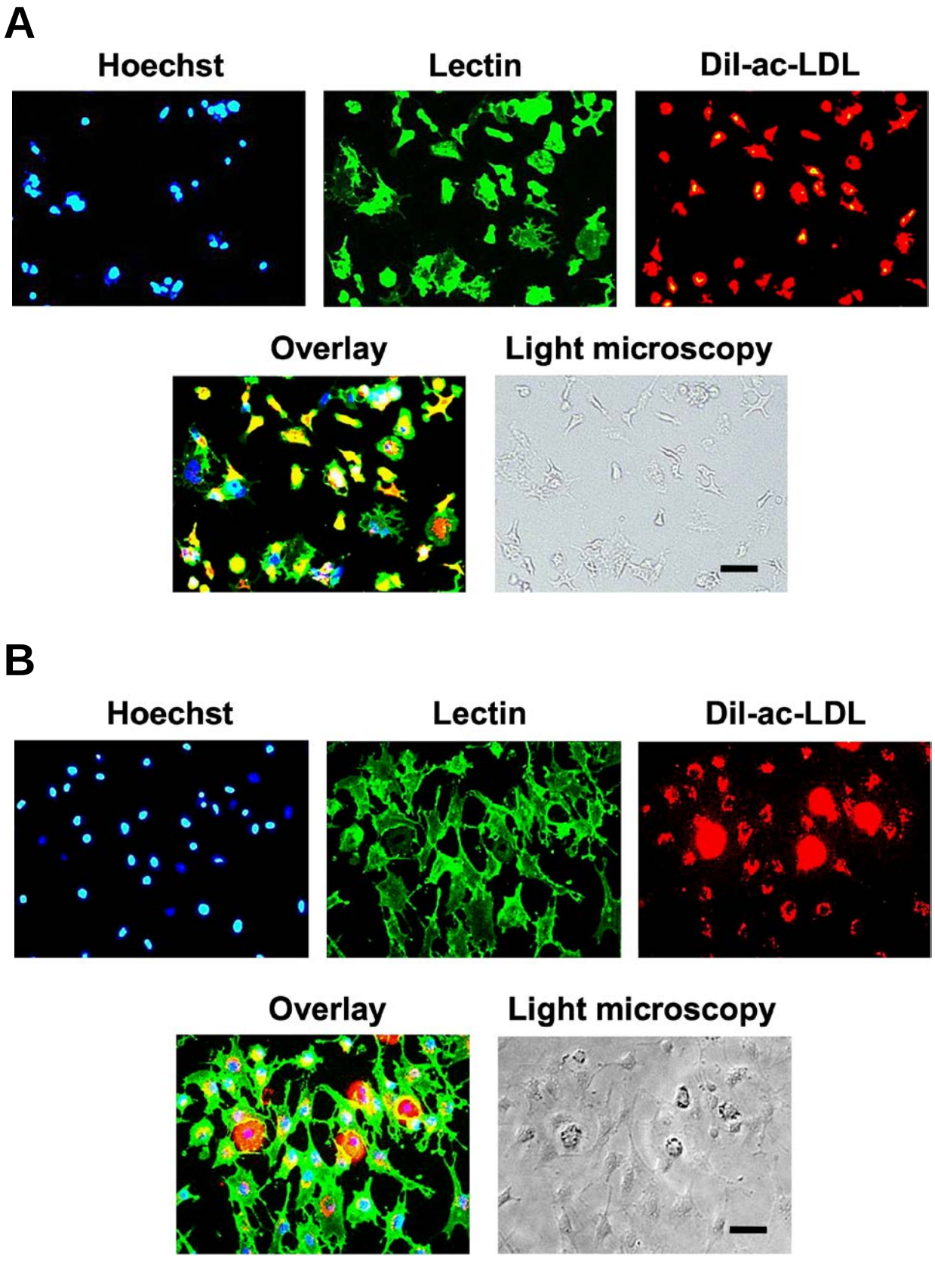
A, Surgical protocols: male C57BL/6 mice received a single injection of EPCs from green fluorescent protein (GFP) transgenic mice (GFP-EPCs) via the tail vein, and 21 days later, peripheral blood mononuclear cells (PB-MNCs) were isolated, and cells were viewed on PB-MNCs smear. B, All the GFP-EPCs found on a PB-MNCs smear. It was found that intravenously delivered GFP-EPCs (green fluorescence) could be detected in recipient peripheral blood at 21 days after cells injection in mice. The cell nucleus was stained with DAPI (blue fluorescence). Boxed regions (Merge) are shown at higher magnification to the right. Inserted boxes show increased magnification of cells (right). Scale bar: 100 μm (GFP, DAPI, Merge); 50 μm (right). PB-MNCs, peripheral blood mononuclear cells.

Supplementary Figure S4. Intravenously delivered EPCs could be detected in recipient peripheral blood at 28 days after a single injection of EPCs in mice.

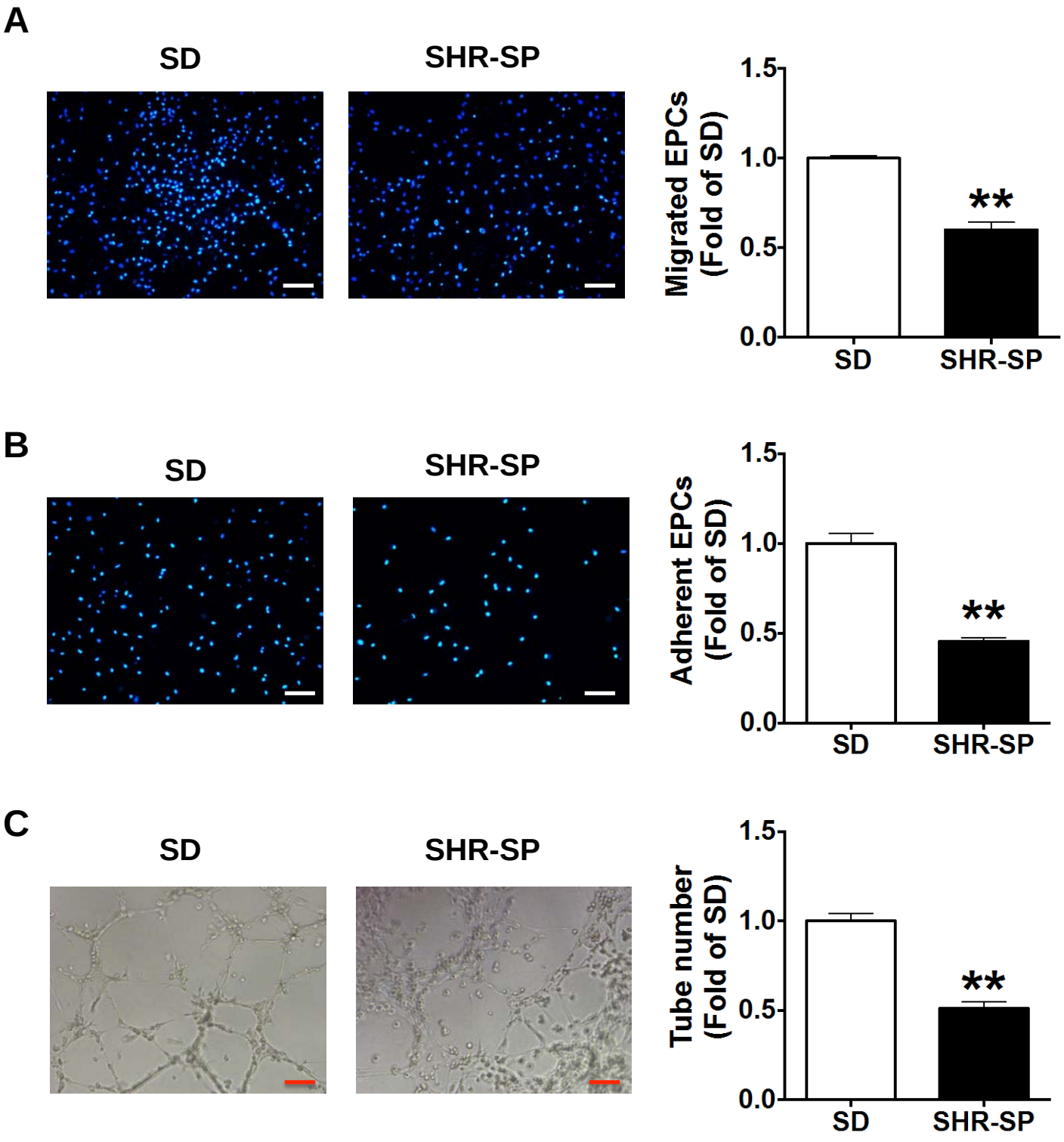
A, Surgical protocols: male C57BL/6 mice received a single injection of EPCs from green fluorescent protein (GFP) transgenic mice (GFP-EPCs) via the tail vein, and 28

days later, peripheral blood mononuclear cells (PB-MNCs) were isolated, and cells were viewed on PB-MNCs smear. B, All the GFP-EPCs found on a PB-MNCs smear. It was found that intravenously delivered GFP-EPCs (green fluorescence) could be detected in recipient peripheral blood at 28 days after cells injection in mice. The cell nucleus was stained with DAPI (blue fluorescence). Boxed regions (Merge) are shown at higher magnification to the right. Inserted boxes show increased magnification of cells (right). Scale bar: 100 μm (GFP, DAPI, Merge); 50 μm (right). PB-MNCs, peripheral blood mononuclear cells.

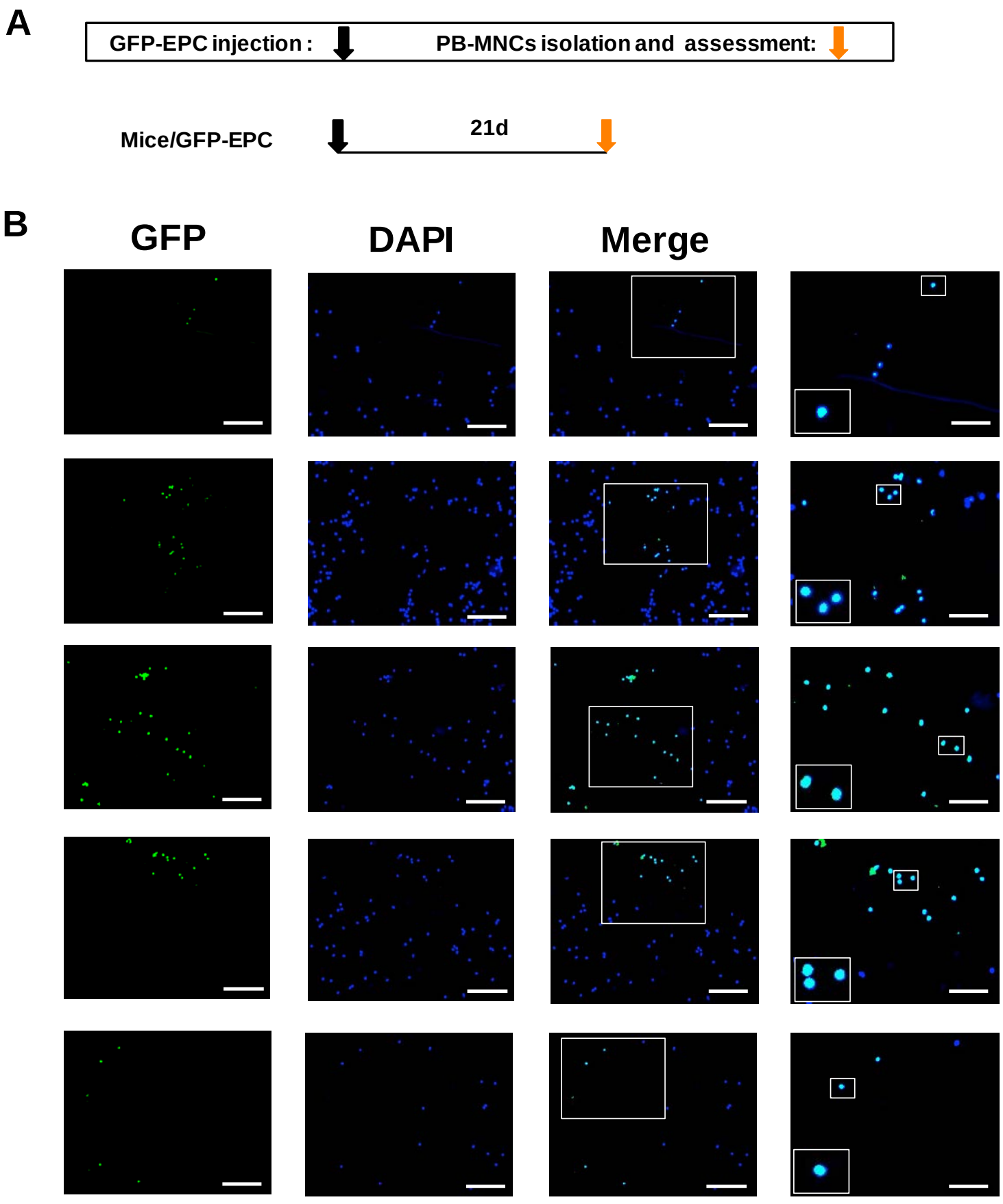
Supplementary Figure S1



Supplementary Figure S2



Supplementary Figure S3



Supplementary Figure S4