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Transfusion of CXCR4-Primed Endothelial Progenitor Cells Reduces Cerebral Ischemic Damage and Promotes Repair in db/db Diabetic Mice

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Abstract

This study investigated the role of stromal cell-derived factor-1α (SDF-1α)/CXC chemokine receptor 4 (CXCR4) axis i and explored the efficacy of CXCR4 primed EPCs in treating ischemic stroke in diabetes. The db/db diabetic and db/+ plasma SDF-1α and circulating CD34+CXCR4+ cells were measured. Brain SDF-1α and CXCR4 expression were qua artery occlusion (MCAO). In *in vitro* study, EPCs were transfected with adenovirus carrying null (Ad-null) or CXCR4 (A treatment for 4 days. For pathway block experiments, cells were pre-incubated with PI3K inhibitor or nitric oxide synth expression, function and apoptosis of EPCs were determined. The p-Akt/Akt and p-eNOS/eNOS expression in EPCs v transfected with Ad-null or Ad-CXCR4 were infused into mice via tail vein. On day 2 and 7, the cerebral blood flow, ne microvascular density, angiogenesis and neurogenesis were determined. We found: 1) The levels of plasma SDF-1α a decreased in db/db mice; 2) The basal level of SDF-1α and MCAO-induced up-regulation of SDF-1α/CXCR4 axis wer Ad-CXCR4 transfection increased CXCR4 expression in EPCs and enhanced EPC colonic forming capacity; 4) Ad-CX HG-induced dysfunction (migration and tube formation) and apoptosis via activation of PI3K/Akt/eNOS signal pathway efficacy of EPC infusion in attenuating infarct volume and promoting angiogenesis and neurogenesis. Our data sugges therapeutic effects for ischemia stroke in diabetes than unmodified EPCs do.

Figures

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¶ These authors also contributed equally to this work.

Introduction

Diabetes is a risk factor for stroke, which are the nation's second leading cause of death and the leading cause of lon cerebral damage is exacerbated and the outcome is poor. The responsible mechanisms might include microvascular r impaired angiogenesis. Endothelial progenitor cells (EPCs) are believed to play an important role in maintaining endot participate in angiogenesis which represents an important endogenous tissue repair mechanism [1], [2]. Accumulating reduced in number and impaired in function in diabetic patients and animals [3]–[5]. Studies on ischemic brain, heart a able to reduce tissue injury, promotes angiogenic repair and functional recovery [3], [6], [7]. These positive results pro ischemic stroke in diabetes.

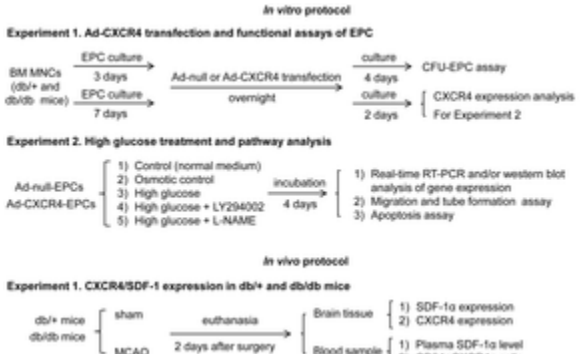
The stromal cell-derived factor-1α (SDF-1α)/CXC chemokine receptor 4 (CXCR4) axis is believed to play an importan tissue [8]–[10] and triggers many intracellular proliferation and anti-apoptosis signals, such as mitogen-activated prote 3-kinase (PI3K) and the serine/threonine kinase Akt [11]. Therefore, it is a potential target for promoting repair in wou ischemic heart and limbs have shown that a combination of SDF-1α/CXCR4 over-expression and stem cell transfusion ischemic diseases. SDF-1α pretreatment increases the therapeutic potential of EPC transfusion in a mouse model of CXCR4 in mesenchymal stem cells enhances *in vivo* engraftment into the ischemic heart and subsequently improves f myoangiogenesis [13]. When compared to low-CXCR4-expressing EPCs, administration of high-CXCR4-expressing E promotes blood flow recovery in ischemic hindlimbs [14]. However, there is little information on EPCs-based therapy f

In this study, we investigated whether the SDF-1α/CXCR4 signal pathway is dysregulated in the brain of db/db diabeti role of CXCR4/PI3K/Akt/eNOS signaling pathway and high glucose (HG) in EPC function and survival. Furthermore, w Ad-CXCR4 primed EPCs is more effective on treating ischemic stroke in db/db mice.

Material and Methods

Animal Experimental Design

Adult male db/db diabetic mice (C57BL6/J) and their age matched (8–10 weeks) controls (db/+) were used for the st Maine). The general characteristics of db/+ and db/db mice are summarized in Table 1. The db/db mice possess an ir receptor and subsequently develop obesity, hyperglycemia and insulin resistance resembling adult-onset diabetes mel used mouse model for type 2 diabetes [15]. The level of fasting plasma glucose was measured after 16 hours fasting Monitor (Roche Diagnostic, Indianapolis, IN). All experimental protocols (Figure 1) were approved by the Laboratory A Wright State University and Guangdong Medical College in accordance to the Guide for the Care and Use of Laborato Health.



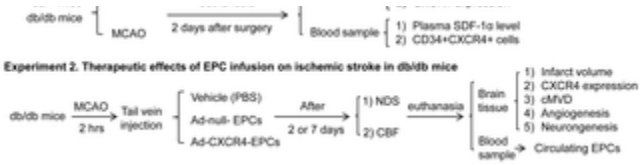


Figure 1. Experimental protocols.
The flow diagrams briefly describe the *in vitro* and *in vivo* protocols.
doi:10.1371/journal.pone.0050105.g001

Variants	db/+ (n = 34)	db/db (n = 90)
Age (W)	8.2±0.5	8.5±0.4
B.W. (g)	27.9±0.6	44.8±1.2**
Blood glucose (mg/dl)	123.6±12.5	421.5±10.4**

Data are means ± SE.
*$P<0.05$, **$P<0.01$, compared with db/+. B.W.: Body weight.
doi:10.1371/journal.pone.0050105.t001

Table 1. General Characteristics of db/+ and db/db Mice.
doi:10.1371/journal.pone.0050105.t001

Protocol one.

For exploring whether SDF-1α/CXCR4 axis is dysregulated in the brain of diabetes (at basal and after ischemic stroke randomly assigned to middle cerebral artery occlusion (MCAO) or sham surgery group. MCAO surgery was performe we previously described [3], [16]. Mice were euthanized 48 hours after surgery, and the brain tissues were immediate contralateral side hemispheres were dissected for analysis of SDF-1α and CXCR4 expression. For real-time RT-PCR into 1 ml tubes containing 0.5 ml RNAlater (Qiagen, CA) and cut into small pieces (<0.5 cm^3). After overnight, tissues western blot analysis, tissues were immediately harvested into tubes and put on dry ice before they were transferred collected for analysis the level of plasma SDF-1α and circulating CD34+CXCR4+ cells.

Protocol two.

For determining the therapeutic efficacy of Ad-CXCR4 primed EPCs on ischemic stroke in diabetes, bone marrow (BM EPCs were cultured for 7 days and then transfected with adenovirus (Ad) carrying null (Ad-null-EPCs) or CXCR4 gene transfection, EPCs were continuously cultured for another 2 days to expansion. After that, the cells were harvested, c were subjected to MCAO surgery (under anesthesia by inhaling 2.5% isoflurane) and randomly assigned to different tr solutions, PBS), Ad-null-EPCs and Ad-CXCR4-EPCs. Pain and discomfort were minimized by an initial injection of Bup another two injections every 12 hours. Mice were injected via the tail vein with EPCs (2×10^5 cells/100 μl in PBS) or the [3], [17]. To label the new generated cells, mice were injected with bromodeoxyuridine (BrdU, 65 μg/g/day, i.p.) immed experimental endpoint [18]. Neurologic motor function was determined and cerebral blood flow (CBF) was measured (right before mice (n = 12/group/time point) were euthanized on day 2 or 7. Blood samples were taken from the heart f deep anesthesia (pentobarbital, 150 mg/kg body weight) [3]. For real-time RT-PCR analysis of CXCR4 expression (n ischemic hemisphere were immediately harvested as described in Protocol one. For histological analysis (n = 6/group/ 4% paraformaldehyde (PFA). Then, brain tissues were fixed in 4% PFA plus 30% sucrose for 3 days. Fixed brains we (20 μm) and divided into four wells for Fluoro-Jade staining analysis of infarct volume, and immunohistological analysis angiogenesis and neurongenesis.

Enzyme-linked Immunosorbent Assay (ELISA) for SDF-1α

The plasma level of SDF-1α was measured by ELISA methods [19]. Briefly, mouse plasma was collected and detecte systems, MN). Absorbance was read at 450 nm.

Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The levels of SDF-1α and CXCR4 of the brain tissues were determined using real-time RT-PCR methods [20]. Brain t kit (Qiagen, CA) and reverse-transcripted with the high capacity cDNA archive kit (Qiagen). The real-time PCR was ru primer sequences: CXCR4 (5′-TTT CAG CCA GCA GTT TCC TT-3′ and 5′-TCA GTG GCT GAC CTC CTC TT-3′); SD GTC GTG-3′ and 5′-AGA GCT GGG CTC CTA CTG TGC GGC CGC GGG-3′). β-actin was chosen for housekeepin expression.

Bone Marrow EPC Culture and Characterization

EPCs were generated from BM mononuclear cells (MNCs) as we previously reported [3], [21]. In brief, BM was flush were isolated by using density gradient centrifuge method. BM MNCs isolated from db/+ and db/db mice were counte coated 24-well plates (BD Bioscience, San Jose, CA, USA) and then grown in endothelial cell basal medium-2 (EBM-2 growth cytokine cocktail (Lonza, Walkersville, MD, USA). After 3 days in culture, non-adherent cells were removed by was changed every 2 days. EPCs were characterized by double staining with Di-LDL and BS-Lectin, and flow cytome (CD34 and VEGFR2) on day 7.

Ad-CXCR4 Preparation and Transfection

The Ad-CXCR4 was kindly provided by Dr. Yigang Wang in the Department of Pathology and Experimental Medicine a cDNA (MGC-36266) was purchased from ATCC (American Type Culture Collection) and sub-cloned in the BglII and H by polymerase chain reaction (PCR) technology. The identity of the gene confirmed by sequencing was subsequently shuttle vector pAdTrack-CMV which contains the enhanced green fluorescence protein (EGFP) expression cassette. F and green fluorescent protein (GFP) under cytomegalovirus (CMV) promoter. EPCs were transfected with Ad-null or A Briefly, EPCs cultured in six-well plates with 75% confluence were incubated with 1×10^7 infectious units of Ad-null or A viruses were removed and the medium was replaced with fresh medium with FCS in the following day. Cells were con confluence for harvest. CXCR4 expression in EPCs was confirmed by real-time RT-PCR and western blot. The percen flow cytometer (Accuri C6 flow cytometer, Inc. Ann Arbor, MI) after staining EPCs with anti-CXCR4 (CXCR4-PE, eBio **=** events of CXCR4+ EPCs/total events of EPCs×100%.

Colony Forming Unit Counts of EPCs

EPCs from db/+ and db/db mice were cultured in EBM-2 medium and seeded in six-well plates precoated with fibrone were removed by washing with PBS and the adherent cells were transfected with Ad-null or Ad-CXCR4 (1×10^7 infecti transfection), the numbers of colony formation unit (CFU) were counted by visual inspection with an inverted microsco defined as a central core of round cells with elongated sprouting cells at the periphery, as previously reported [22].

High Glucose Experiments on EPCs

The HG (25 mmol/L) medium which corresponds to 350–450 mg/dl of plasma glucose levels in diabetic patients was u mM) was used for osmotic control as previous reports [24]–[25]. Ad-null-EPCs or Ad-CXCR4-EPCs were cultured in I supplemented with SDF-1α (100 ng/ml) for 4 days before functional assays. The medium were changed every two da level of the culture supernatant was daily monitored by an oxidase-based colorimetric method [27] during the HG expe pre-incubated with PI3K inhibitor (LY294002, 20 μM, Cell Signaling) or NOS inhibitor (N^G-nitro-arginine methyl ester, L MA) for two hours [28].

EPC Migration and Tube Formation Assays

EPC migration and tube formation were evaluated by using Boyden chamber (Chemicon, Rosemont, IL) and tube form previously described [3]. For migration, EPCs (2×10^4 cells) were placed into upper compartment of the Boyden cham ng/ml vascular endothelial growth factor (VEGF) and 100 ng/ml stromal cell-derived factor-1 (SDF-1) in the lower com migrated across the membrane were counted under an inverted light microscope, quantified and averaged by examini (magnification,×200). For tube formation, ECMatrix' solution was thawed on ice overnight, mixed with 10×ECMatrix' di plate at 37°C for one hour to allow the matrix solution to solidify. EPCs were re-plated (1×10^4 cells/well) on top of the hours at 37°C. Tube formation was evaluated with an inverted light microscope and defined as a tube structure exhibit independent fields were assessed for each well, and the average number of tubes per field (magnification, ×200) was formation assays, EPCs were cultured in the basal EPC medium as previously described [30].

EPC Apoptosis Assay

After 4 days' culture in HG medium, EPCs were harvested for apoptosis analysis by using Alexa Fluor 488 annexin V/ invitrogen, Carlsbad, CA). Briefly, cells were resuspended in annexin-binding buffer, and then incubated with annexin V temperature (RT). The apoptotic EPCs were recognized as PI−/Annexin V+ cells. The percentage of apoptosis was a

Western Blot Analysis

Gene expression of SDF-1α, CXCR4, eNOS, Akt, p-eNOS or p-Akt of the brain tissue or EPCs was determined [28], (Roche Diagnostic) containing protease inhibitor. The proteins were subjected to SDS-PAGE electrophoresis and trans membranes were blocked by incubating with 5% dry milk and Tris-buffered saline for one hour, and then incubated wit systems), CXCR4 (1:100, AnaSpec Inc. CA), Akt (1:1000, Cell Signaling Technology), eNOS (1:1000, Cell Signaling T Technology), or p-eNOS (1:1000) at 4°C overnight. β-actin (1:4000, Sigma, MO) was used to normalize protein loadir were incubated with horseradish peroxidase (HRP) conjugated IgG (1:40000, Jackson Lab) for one hour at RT. Blots chemiluminescence developing solutions and quantified.

Flow Cytometry Analysis of Circulating EPCs and CD34+CXCR4+ Cells

The level of circulating EPCs was determined by flow cytometry as a previous study [3]. Briefly, circulating MNCs wer and stained with anti-mouse CD34-PE (AbD Serotec, Raleigh, NC) and VEGFR2-PE-Cy7 (BD, Bioscience) antibodies CD34+CXCR4+ cells, circulating MNCs were stained with CD34-FITC (AbD Serotec) and CXCR4-PE (eBioscience, S circulating EPCs and CD34+CXCR4+ cells were expressed as cells/ml blood. Isotype (IgG) antibodies were used as calibration.

Functional Evaluation of Neurological Deficits

The neurological deficit scores were evaluated on day 2 or 7 after EPC treatment for functional determination of thera scale method was previously described [16], [32]. The five points are: 0, normal motor function; 1, flexion of contralat animal by the tail; 2, circling to the contralateral side but normal posture at rest; 3, leaning to the contralateral side at neurologic behavior of mice was scored by an investigator who was unaware of animal grouping.

Measurement of Cerebral Blood Flow

On day 2 or 7 following EPC transfusion, the relative CBF in the peri-infarct area was determined as described previo mouse was anesthetized with 2.5% isoflurane and placed on a stereotaxic apparatus. An incision was made in the sca the peri-infarct site of ischemic ipsilateral area (2 mm posterior, 6 mm lateral to bregma) and contralateral site (2 mm serves as basal normal level for calibration) were sequentially determined using a laser Doppler flowmeter (PF2B, Pe diameter 0.5 mm). To minimize variability, the CBF was recorded at each site for at least 5 minutes. The averaged vol CBF for each site. The relative CBF was calculated using the formula: relative CBF = CBF of ipsilateral side/CBF of c

performed CBF measurements was unaware of the information of animal grouping.

Measurement of Infarct Volume and Cerebral Microvascular Density

As we previously described [3], [16], cerebral ischemic damage and the cMVD in peri-infarct area were revealed by s Fluoro-Jade (0.001%, Histo-chem, Jefferson, AR, USA) and CD31 (1:50, Invitrogen), respectively. Infarct volume and software (NIH).

Analysis of Angiogenesis and Neurongenesis

Angiogenesis and neurongenesis in peri-infarct area were determined by using double immunofluorescence staining w 31 (endothelial cells, ECs), neuronal nuclei (NeuN), or glial fibrillary acidic protein (GFAP) [18]. Specifically, brain coro (1:50, Abcam, MA, USA), followed by incubation with cell-specific antibodies: CD31 (1:50, BD Biosciences), GFAP (1 overnight at 4°C. Next, brain sections were reacted with FITC (for BrdU) or Cy3 (for cell specific markers) conjugated 30 min at RT in the dark. The labeled ECs (BrdU+CD31+), neurons (BrdU+NeuN+) and glial cells (BrdU+GFAP+) in th counted under 6 random fields (200×). The average of five sections from rostral to caudal represented the data for ea counted by an investigator who was unaware of animal grouping.

Statistical Analysis

All data, excepting neurologic deficit scores, are presented as mean ± SE. The neurologic deficit scores were expres scores among different groups were compared by the Kruskal–Wallis test. When the Kruskal–Wallis test showed a sig were applied. For the rest measurements, comparisons for two groups were performed by the student's t test. Multip two-way ANOVA. For all tests, a *P*-value <0.05 was considered significant.

Results

Baseline Characterization of Animals

The characterizations of blood glucose, age and body weight in db/db and db/+ mice used in this study are presented db/db mice had higher plasma glucose and body weight as compared with age-matched db/+ control mice. In protoco surgery (blood flow <75% of baseline) and randomized to vehicle, Ad-null-EPC or Ad-CXCR4-EPC infusion groups. Th blood glucose among different treatment groups (Table 2).

Groups	B.W. (g)	Blood glucose (mg/dl)
Vehicle, 2 day	48.2±2.1	428.5±10.4
Vehicle, 7 day	47.9±2.2	425.8±11.2
Ad-null-EPCs, 2 day	43.6±1.5	421.6±12.4
Ad-null-EPCs, 7 day	42.2±1.5	418.4±11.8
Ad-CXCR4-EPCs, 2 day	46.9±1.6	424.8±12.2
Ad-CXCR4-EPCs, 7 day	46.6±1.1	421.5±12.4

Data are means ± SE. n = 6/group. B.W.: Body weight.
doi:10.1371/journal.pone.0050105.t002

Table 2. Baseline Characteristics of db/db Mice in Different Groups.
doi:10.1371/journal.pone.0050105.t002

The Levels of Plasma SDF-1α and Circulating CD34+CXCR4+ Cells are Reduced in

The level of plasma SDF-1α was significantly lower in db/db mice (1.3±0.14 and 1.8±0.15 pg/ml, *P*<0.05, db/db *vs.* dl CD34+CXCR4+ cells was reduced in db/db mice (260±14 and 712±42 cells/ml, *P*<0.01, db/db *vs.* db/+ mice, n = 9/g

The Expression of SDF-1α/CXCR4 Axis is Dysregulated in the Brain of db/db Mice at Stroke

At basal, the db/db diabetic mice had less expression of SDF-1α in the brain tissue at both mRNA and protein levels (difference in CXCR4 expression (*P*>0.05; Figure 2B and D). The levels of brain SDF-1α and CXCR4 in the ischemic ip up-regulated in both db/db and db/+ mice 48 hours following MCAO (*P*<0.05 or 0.01). However, the up-regulations of (*P*<0.05; Figure 2). The levels of brain SDF-1α and CXCR4 in the contralateral hemisphere was unaffected (data not

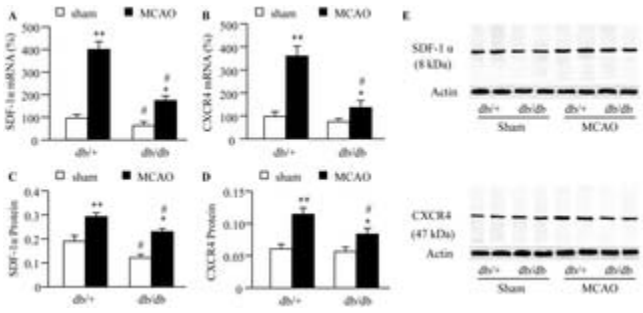


Figure 2. SDF-1α/CXCR4 expression in the brain of db/db mice at basal and in response to ischemia.
(A) SDF-1α mRNA expression. (B) CXCR4 mRNA expression. (C) SDF-1α protein expression. (D) CXCR4 protein bands of SDF-1α and CXCR4. **P*<0.05, ***P*<0.01 *vs.* sham; # *P*<0.05 *vs.* db/+, n = 5/group in mRNA analysis, n = doi:10.1371/journal.pone.0050105.g002

EPC Characterization and CXCR4 Expression in EPCs

BM derived EPCs were defined as cells up-taking Di-LDL and binding with Bs-Lectin, as well as cells expressing CD3 (Figure 3A and B). At the end of EPC culture (7 days), the percentage of CD34+VEGFR2+ EPCs was 89±3.5% (n = EPCs did not cause any change of the percentage of CXCR4+ EPCs (Day 0 *vs.* Day 7; *P*>0.05). The CXCR4+ EPCs

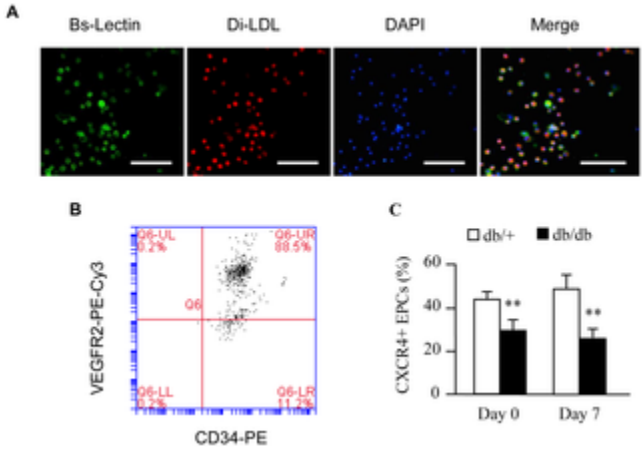


Figure 3. Characterization of bone marrow derived EPCs.

(A) Representative pictures showing cultured EPCs by double staining analysis. Red: Di-LDL up-taking; Green: Bs Yellow: Di-LDL and Bs-Lectin positive cells defined as EPCs. Scale bar: 75 μm. (B) Representative flow plot show expression in EPCs. At the end of EPC culture, cells were stained with CD34 and VEGFR2, and analyzed by flow CD34+VEGFR+ cells. (C) Summarized data of CXCR4 expressing EPCs after day 0 and 7 days' culture. **P<0.0 doi:10.1371/journal.pone.0050105.g003

Ad-CXCR4 Transfection Increases CXCR4 Expression and Colony Forming Capacity

Real-time PCR and western blot analyses showed that Ad-CXCR4 transfection up-regulated CXCR4 expression in EF protein levels (P<0.01; Figure 4A and B). Flow cytometric result showed that Ad-CXCR4 transfection significantly incr (P<0.01; Figure 4C). The number of CFUs was decreased in EPCs from db/db mice (P<0.05 or 0.01; Figure 4D). Ad- from both db/+ and db/db mice (P<0.01; Figure 4D).

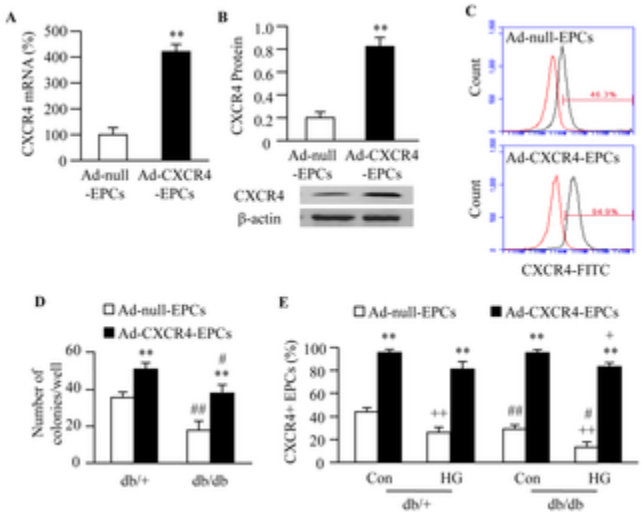


Figure 4. CXCR4 expression and colony forming capacity of EPCs.
CXCR4 mRNA expression (A) and protein expression (B) in EPCs after Ad-CXCR4 transfection. (C) Representativ showing the percentage of CXCR4+ EPCs in Ad-null-EPCs and Ad-CXCR4-EPCs. The red line is the IgG isotype forming units of EPCs. (E) Summarized data showing the expression of CXCR4 in EPCs from both db/+ and db/db treatment. **P<0.01 *vs.* Ad-null-EPCs; ^{+}P<0.05, ^{++}P<0.01 *vs.* Con; $^{\#}P$<0.05, $^{\#\#}P$<0.01 *vs.* db/+, n = 6/group. Con: medium; Ad-null-EPCs: EPCs transfected with Ad-null; Ad-CXCR4-EPCs: EPCs transfected with Ad-CXCR4. doi:10.1371/journal.pone.0050105.g004

Ad-CXCR4 Transfection Protects EPCs from HG-induced Dysfunction and Apoptosis PI3K/Akt/eNOS Signaling Pathway

HG incubation for 4 days significantly decreased the expression of CXCR4 in EPCs from both db/+ and db/db mice (P increased the expression of CXCR4 in EPCs from both db/+ and db/db mice (P<0.01; Figure 4E). HG incubation also formation, P<0.01; Figure 5A–B) and induced EPC apoptosis (P<0.01; Figure 5C). Ad-CXCR4 transfection prevented Figure 5A–B) and apoptosis (P<0.01; Figure 5C). Meanwhile, the expression of p-Akt/p-eNOS in EPCs was measure induced down-regulation of p-Akt/p-eNOS, whereas did not affect the expression of Akt/eNOS in EPCs. Ad-CXCR4 tr down-regulation of p-Akt and p-eNOS and increased the expression of Akt and eNOS in EPCs (Figure 6). Pre-incubat the effects of Ad-CXCR4 transfection on EPC function and apoptosis (P<0.05 or 0.01), Whereas, NOS inhibitor (L-NA (P<0.01; Figure 5).

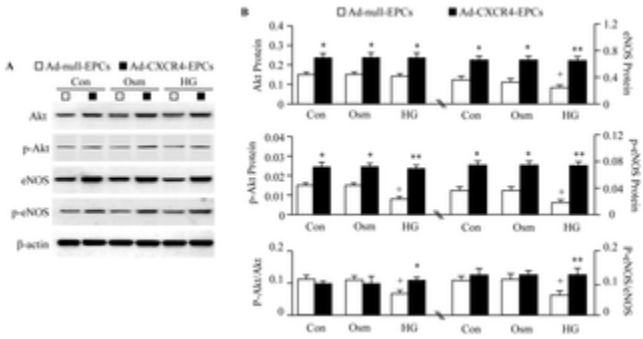


Figure 5. Ad-CXCR4 transfection protects down-regulation of Akt/eNOS activation in EPCs induced by HG
(A) Representative western blot bands showing Akt/eNOS and p-Akt/p-eNOS expression in different treatment gro Akt and p-Akt, and 140 kDa for eNOS and p-eNOS. (B) Summarized data on Akt/eNOS and p-Akt/p-eNOS expres *P<0.05, **P<0.01 *vs.* Ad-null-EPCs; ^{++}P<0.01 *vs.* Con or Osm. Con: control (basal medium); Osm: osmotic contr phosphorylated Akt; p-eNOS: phosphorylated eNOS; PI3K: phosphatidylinositol-3-kinase; NOS: nitric oxide syntha Ad-null; Ad-CXCR4-EPCs: EPCs transfected with Ad-CXCR4.
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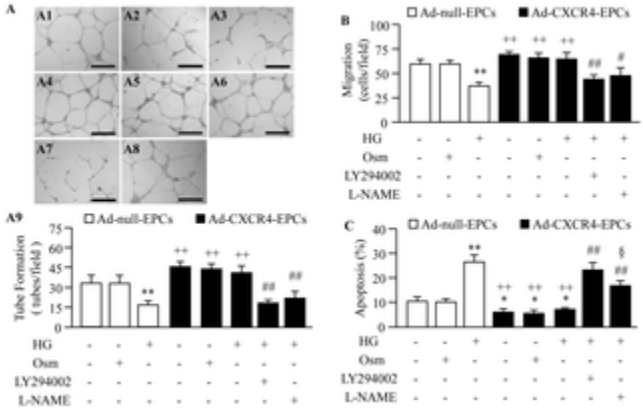


Figure 6. Ad-CXCR4 transfection protects EPCs from HG-induced dysfunction and apoptosis via activatin
Representative tube formation pictures (A1–A8) and summarized data (A9) in different treatment groups. A1: Ad-n A3: Ad-null-EPCs+HG; A4: Ad-CXCR4-EPCs+Con; A5: Ad-CXCR4-EPCs+Osm; A6: Ad-CXCR4-EPCs+HG; A7: A Ad-CXCR4-EPCs+HG+L-NAME. Scale bar: 600 μm. Summarized data on migration ability (B) and the percentage groups. *P<0.05, **P<0.01 *vs.* Ad-null-EPCs or Ad-null-EPCs+Osm; ^{++}P<0.01 *vs.* HG+Ad-null-EPCs; $^{\#}P$<0.05, $^{\#\#}$ *vs.* HG+Ad-CXCR4-EPCs+LY294002, n = 6/group. Con: control (basal medium); Osm: osmotic control; HG: high g transfected with Ad-null; Ad-CXCR4-EPCs: EPCs transfected with Ad-CXCR4.
doi:10.1371/journal.pone.0050105.g006

Infusion of Ad-CXCR4 Primed EPCs Enhances the Efficacy in Increasing the Level of Expression in the Brain

The db/db mice were treated with EPCs two hours after MCAO surgery. Infusion of Ad-null-EPCs was able to increas (P<0.01; Figure 7A). Infusion of Ad-CXCR4 primed EPCs further increased the level of circulating EPCs at these time of Ad-null-EPCs increased CXCR4 expression in the brain of ischemic side on day 7 (P<0.05) with no significant chan infusion of Ad-CXCR4 primed EPCs was more effective to increase CXCR4 expression in the ischemic hemisphere or

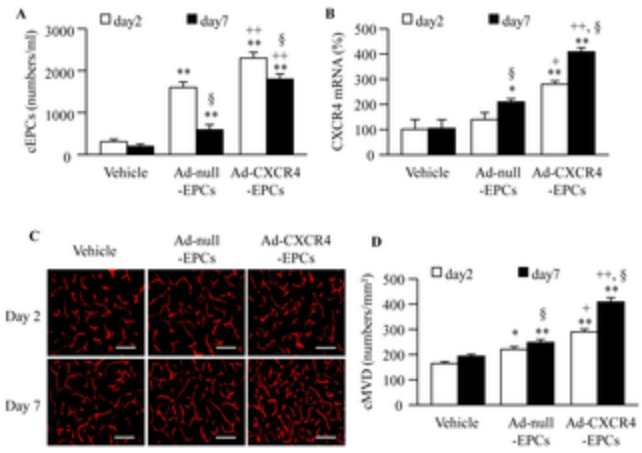


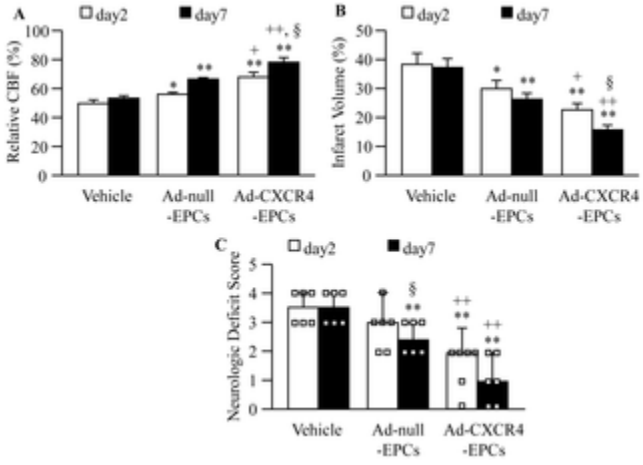
Figure 7. Effects of Ad-CXCR4-EPC infusion on cEPCs, brain CXCR4 expression and cMVD in db/db mice. (A) The level of cEPCs in each therapeutic group. (B) The CXCR4 expression in the brain of db/db mice in each th pictures of cMVD (CD31 immunostaining) in the peri-infarct area. Scale bar: 50 μm. (D) The level of cMVD in the b therapeutic group. **P*<0.05, ***P*<0.01 *vs.* vehicle; $^{+}$*P*<0.05, $^{++}$*P*<0.01 *vs.* Ad-null-EPCs; §*P*<0.05 *vs.* day 2, n = 6/g progenitor cells, cMVD: cerebral microvascular density; Ad-null-EPCs: EPCs transfected with Ad-null; Ad-CXCR4- doi:10.1371/journal.pone.0050105.g007

Infusion of Ad-CXCR4 Primed EPCs Enhances the Efficacy in Increasing cMVD in the Damage

Infusion of Ad-null-EPCs was able to increase the cMVD in peri-infarct area in db/db mice (Day 2, *P*<0.05; Day 7, *P*< transfusion of Ad-CXCR4 primed EPCs could enhance the efficacy (*P*<0.01; Figure 7C and D).

Infusion of Ad-CXCR4 Primed EPCs Enhances the Efficacy in Increasing Relative CB Injury and Neurologic Deficit Score

In agreement with the findings in cMVD, we also found that Ad-null-EPC transfusion improved the relative CBF of peri- Figure 8A) and transfusion of Ad-CXCR4 primed EPCs was more effective (Day 2, *P*<0.05; Day 7, *P*<0.01; Figure 8A (Day 2, *P*<0.05; Day 7, *P*<0.01; Figure 8B) after Ad-null-EPC infusion, and was able to be further decreased after the both day 2 and day 7 (*P*<0.01; Figure 8B). To evaluate the neurologic motor function, we measured neurologic deficit EPC infusion. We found that the neurologic deficit score was reduced in Ad-null-EPC group on day 7 (*P*<0.01; Figure improved neurologic motor function as early as on day 2 and had better efficacy on day 7 (*P*<0.01; Figure 8C).



-EPCs -EPCs

Figure 8. Effects of Ad-CXCR4-EPC infusion on CBF, infarct volume and neurologic deficit score in db/db (A) The relative CBF in peri-infarct area in each therapeutic group. (B) The infarct volume in each therapeutic grou therapeutic group. **P*<0.05, ***P*<0.01 *vs.* vehicle; ^{+}P<0.05, ^{++}P<0.01 *vs.* Ad-null-EPCs; $^{\S}P$<0.05 *vs.* day 2, n = 6/ Ad-null-EPCs: EPCs transfected with Ad-null; Ad-CXCR4-EPCs: EPCs transfected with Ad-CXCR4.
doi:10.1371/journal.pone.0050105.g008

Infusion of Ad-CXCR4 Primed EPCs Enhances the Efficacy in Promoting Angiogenesi

Figure 9A shows representative pictures of angiogenesis (BrdU+CD31+), glial (BrdU+GFAP+) and neuronal (BrdU+Ne Data showed that Ad-null-EPC transfusion increased angiogenesis and neurogenesis on day 7 (*P*<0.05 or 0.01; Figur Moreover, transfusion of Ad-CXCR4 primed EPCs promoted angiogenesis as early as day 2 (*P*<0.01), and had bette neurogenesis on day 7 (*P*<0.01 or 0.05; Figure 9B–D).

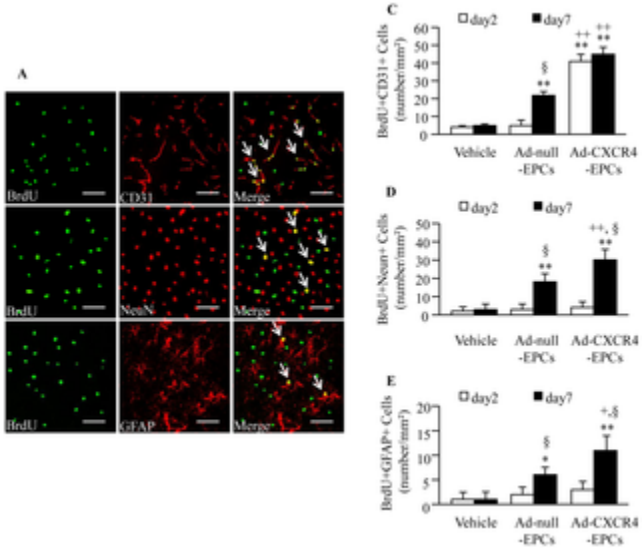


Figure 9. Infusion of Ad-CXCR4-EPCs increases angiogenesis and neurogenesis in db/db mice following is (A) Representative pictures of angiogenesis (BrdU+CD31+), neurogenesis (BrdU+NeuN+) and glia cell genesis (Br after Ad-CXCR4-EPC treatment. Scale bar: 50 μm. Histogram showing the number of BrdU+CD31+ (B), BrdU+Ne peri-infarct area on day 2 and 7 in different therapeutic groups. **P*<0.05, ***P*<0.01 *vs.* vehicle; ^{+}P<0.05, ^{++}P<0.01 6/group. NeuN: neuronal nuclei; GFAP: glial fibrillary acidic protein; Ad-null-EPCs: EPCs transfected with Ad-null; A Ad-CXCR4.
doi:10.1371/journal.pone.0050105.g009

Discussion

There are three major findings in this present study. Firstly, we found that the expression of SDF-1α/CXCR4 axis is dy

at basal and in response to ischemic stroke. Secondly, we illustrated that Ad-CXCR4 primed EPCs display resistance through activation of CXCR4 downstream PI3K/Akt/eNOS signal pathway. Thirdly, we demonstrated that infusion of A efficacy in reducing ischemic injury as well as promoting recovery.

The SDF-1α regulates tissue/organ homeostasis through the CXCR4 receptor expressed in hematopoietic progenitors SDF-1α expression is reduced at basal and that ischemia-induced up-regulation of brain SDF-1α and CXCR4 are less first evidence showing the dysregulation of SDF-1α/CXCR4 axis in the brain of an animal model with diabetes. A previ SDF-1α and CXCR4 is up-regulated in the tunica media of the thoracic aortas in streptozotocin-induced type-1 diabete diabetic models have hyperglycemia, we tentatively attribute this inconsistence to different animal models and/or tissu are supported by other previous reports showing that the SDF-1α/CXCR4 axis is down-regulated in the wounds of db/ model used in our study has hyperglycemia, obesity and dyslipidemia and insulin resistance [15]. Therefore, it deserve besides hyperglycemia can also lead to the impairment of SDF-1α/CXCR4. Our *in vitro* data showed that HG down-re suggesting that hyperglycemia per se can impair the SDF-1α/CXCR4 axis [30], [37]. Furthermore, we found that HG i function, and induced EPC apoptosis. Although diabetes is a stage of severe inflammation and oxidative stress, and th mimic the situation in *in vivo*, our results are supported by the reports from others [30], [23] showing that HG induced data are also in agreement with our previous findings showing lower level of cEPCs and less cerebral microvascular d hyperglycemia should be one of the mechanisms for EPC dysfunction in diabetes. On the other hand, evidence sugges vascular progenitors from the bone marrow [38], [39]. The db/db mice possess an inactivating gene mutation in leptin current findings in db/db mice.

In addition, we found that the levels of plasma SDF-1α and circulating CD34+CXCR4+ cells are reduced in db/db mice diabetic patients [40]. Since the SDF-1α/CXCR4 interaction triggers several intracellular signals including MAPKs, PI3 modulate cell migration, proliferation and apoptosis [11], we investigated the implication of SDF-1α/CXCR4 axis in EP(expected, we found that Ad-CXCR4 transfection protects EPCs from HG-induced dysfunction and apoptosis. The und CXCR4 downstream PI3K/Akt/eNOS signal pathway since PI3K or eNOS inhibitor abolishes or partially blocks these p agreement with previous observations showing that SDF-1α/CXCR4 interaction mediates EPC migration via Akt and e data suggest that the SDF-1α/CXCR4 axis is impaired at multiple sites (brain and EPCs) in diabetes, which might hav repair (enlarged injury and delayed repair); targeting on the dysfunction of SDF-1α/CXCR4 axis could offer a new ave

EPCs have been found to differentiate into ECs and contribute to angiogenic repair [42], [43]. A recent report demons infarct volume in ischemic stroke mice [7]. Yang et al [44] also demonstrated that CD34+ cells could represent a funct beneficial therapeutic effects in myocardial infarction. In patients with diabetes and db/db diabetic mice, circulating EP dysfunctional [3]–[5]. Our previous study demonstrates that transfusion of EPCs from non-diabetic sources has benefi reports by others also suggest that EPCs be useful for therapeutic purposes in diabetes [21], [45]. Because of the me hyperlipemia, etc) changes in diabetes, the efficacy of EPC-based therapy may be limited. This evidence provides a g treating ischemic stroke in diabetes. On the other hand, this evidence suggests the control of metabolic factors in diab efficacy of EPC-based therapy. Here, we conducted *in vivo* studies to evaluate whether Ad-CXCR4 primed EPCs cou treating ischemic stroke in the db/db diabetic mice. In agreement with previous reports showing transfusion of CXCR4 cells has better efficacy than CXCR4 low-expressing cells in treating hindlimb ischemia and myocardial infarction [14], EPCs have better effects over EPCs in alleviating cerebral damage (decreasing the infarct volume, improving neurolog (increasing cMVD, angiogenesis and neurogenesis). Angiogenesis is a vital component of tissue repair processes. EP angiogenesis which represents an important endogenous tissue repair mechanism. The underlying mechanisms of EP(demonstrated. One is that EPCs physically participate in angiogenesis by incorporating and differentiating into mature angiogenic factors promoting the proliferation and survival of resident ECs [6]. In this study, we measured newly gene angiogenesis as commonly used by others [18], [47]. We found that the level of CD31+BrdU+ cells was increased in t more seen after infusion of CXCR4 primed EPCs. Our data demonstrate that CXCR4 over-expressing EPC further inc stroke.

The EPCs are thought to be a mixture of progenitor cells and mononuclear cells. At present, isolation of pure populati characterization of the different types of EPC is currently an open issue with debate [48]. However, the generally acce expression of surface markers including CD34, CD133 and KDR [49]. In this study, we cultured EPCs for 7 days and (We found the percentage of CD34+VEGFR2+ cells was about 88.5%, suggesting most of them are EPCs. Moreover, also important to obtain the high purity EPCs. We isolated BM MNCs by gradient density separation method. BM MN(24-well plates and grown in endothelial cell basal medium-2 containing EPC growth cytokine cocktails in favor of the p

lines [21], [50]. After 3 days of culture, non-adherent cells were removed by washing with PBS to avoid contamination the observed effects are attributed to the transfused EPCs, rather than the CD45+ mononuclear cells.

Our *in vitro* EPC culture and *in vivo* animal studies are in a good agreement for supporting the beneficial effects of Ad stroke. Firstly, Ad-CXCR4 transfection protects EPCs from HG induced apoptosis resulting in increased level of circul prevents EPCs from HG-induced dysfunction (migration and tube formation) and leads to the promotion of angiogenes increases angiogenesis in peri-infarct area as early as day 2, whereas transfusion of EPCs shows this effect on day 7 cMVD and relative CBF on day 2 in EPC treatment group. Although the underline mechanism is unclear, we tentatively which secrete angiogenic factors promoting the proliferation and survival of resident ECs. Another major finding of our more effective than non primed EPCs in promoting cerebral repair processes. This is evidenced by increased angioge the Ad-CXCR4 primed EPC treatment group.

In summary, the present study demonstrates that transfusion of Ad-CXCR4 primed EPCs may be a novel approach fo stroke in diabetes. Over-expression of CXCR4 in EPCs prevents the deleterious effects of HG on EPC function and a which could be the underlying mechanism for the beneficial effects of Ad-CXCR4 primed EPC transfusion. Here, we w determine the level of EPC incorporation into endothelium, the level of local SDF-1α after EPC transfusion, and the pa deserve future investigation.

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Author Contributions

Conceived and designed the experiments: Ji Chen Jianying Chen BZ YC. Performed the experiments: Ji Chen Jianying data: Ji Chen Jianying Chen SC CZ XX LZ AD YZ. Contributed reagents/materials/analysis tools: MM. Wrote the pap manuscript: Ji Chen MM YC.

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