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Original Article

Enhanced penetration of exogenous EPCs into brains of APP/PS1 transgenic mice

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Abstract: The aim of this study was to investigate the repair function of exogenous Endothelial progenitor cells (EPCs) for brain microvascular damage of the APP/PS1 transgenic mouse model of Alzheimer's disease (AD). This study used a density-gradient centrifugation method to isolate mononuclear cells (MNCs) from mouse bone marrow, which were subsequently seeded and cultured. Cells were characterized by morphology and detection of the surface markers CD34 and CD133 at different time points by immunofluorescence (IF) and flow cytometry (FCM). Then, EPCs were transfected with GFP adenoviral vectors (GFP-EPCs). Wild-type (WT) and APP/PS1 transgenic mice both received GFP-EPCs injection through the tail vein, and using a PBS buffer injection as the control. Seven days later, the animals' brain tissue was isolated. Expression of GFP was detected by quantitative polymerase chain reaction (qPCR) and western-blot (WB), while the fluorescence of GFP within the brains of mice was observed under a fluorescence microscope. Higher mRNA and protein expression of GFP, accompanied with increased green fluorescence, were detected in the brain of GFP-EPCs-injected APP/PS1 mice, as compared with GFP-EPCs-injected WT mice. The results show that the APP/PS1 transgenic mouse model of AD exhibited enhanced penetration of exogenous EPCs into brains than the WT mice.

Keywords: APP/PS1 transgenic mouse model of AD, endothelial progenitor cells, penetration

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder and the most common cause of dementia among elderly people [1]. One of the main pathological features of AD is the extensive deposition of amyloid-β (Aβ) peptide in certain regions of the brain, such as the entorhinal cortex, hippocampus and basal forebrain, which forms senile plaques in the cortex and hippocampus [2]. More than 4 million people in the USA and 12 million worldwide suffer from AD [3]. During the early and middle stages, many AD patients experience cerebral amyloid angiopathy (CAA), accompanied by capillary changes, including a decrease of capillary density of specific sites, destruction of the blood brain barrier (BBB), deformation of endothelial cells, and so on [4]. Meanwhile, the gradual deposition of Aβ42 in cerebrovascular wall has been shown to be the main cause of the brain's microvascular damage [2].

Endothelial progenitor cells (EPCs) originating from the bone marrow (BM) [5] contribute to ischemic tissue regeneration via vascular repair and angiogenesis [6, 7]. In the case of ischemia, EPCs can be mobilized from the bone marrow to the ischemic area driven by the gradient of cytokine/chemokine released by ischemic tissue, and then participate in the local formation of new blood vessels directly through their structural role of differentiating into mature endothelial cells and indirectly through their paracrine effect and secreting angiogenic factors [7, 8]. Until recently, it was understood that neovascularization, or the formation of new blood vessels, mainly resulted from the proliferation and migration of pre-existing endothelial cells, a process referred to as angiogenesis [9]. Therefore, autologous mobilization or

transplantation of EPCs provides a new target for the treatment of ischemic diseases [10].

Surface markers CD34 and CD133, as well as vascular endothelial growth factor receptor 2 (VEGFR2) were widely used for isolation and identification of EPCs [8]. Patients with acute cerebral infarction can lead to increases of brain blood CD34+/CD133+ cells levels, and were the first to point out that EPCs may play an important role in the recovery process of stroke disease [11]. It has been shown that EPCs, isolated from mononuclear cells (MNCs), could incorporate into the foci of myocardial neovascularization [12], and the intracoronary infusion of BM-derived progenitors in patients with acute myocardial infarction was associated with significant benefits in post-infarction remodeling [13]. Studies in animal models also indicated that transplanting EPCs can improve functional recovery of limb ischemia, myocardial ischemia [14], and ischemic stroke [15, 16].

A recent study also indicates that EPCs have a repair effect not only on blood vessels, but also directly or indirectly contribute to the growth and repair of the central nerve system [16]. In AD patients, the brain microvascular system and the nervous system are in a constant state of accelerated damage that exceeds the normal repair process [4], which eventually leads to irreversible damage of AD brains. Therefore, a hypothesis is put forward to consider whether exogenous EPCs can penetrate into the brain of APP/PS1 transgenic mouse model of AD and enhance the body's internal repair function for damaged capillaries and nervous system and ultimately delay the incidence of AD.

Materials and methods

Cell stage

Isolation of mononuclear cells from mouse bone marrow: All animal procedures were approved by the laboratory animal ethics committee at the Tongji hospital, Huazhong University of Science and technology and conformed to the national guidelines for care and use of laboratory animals. Isolation and culture of MNCs were described previously [17]. Bones from 4-week-old C57/BL6 male mice were repeatedly washed with PBS until the washing fluid of the bone marrow cavity became clear. The washing fluid was filtered into a single-cell suspension with a 100-μm mesh. The mixture was then centrifuged at 1,400 rpm for 10 min to separate the cells. The supernatant liquid was removed and a red blood cell lysis buffer was then added to the cells. Next, the mixture was centrifuged at 1,400 rpm for 10 min a second time. The cells were washed two times with PBS, resuspended with EGM-2MV (Lonza, Endothelial cell basal medium-2, plus FBS, VEGF, R3-IGF-1, rhEGF, rhFGF-B, GA-1000, hydrocortisone and ascorbic acid) and seeded in either fibronectin-coated plates or culture bottles to a cell density of $10^6/cm^2$. Four days later, non-adherent cells were washed off with PBS, and fresh media were added to the cultures every 3 days.

Cells morphology: Cultured cells were observed by an inverted microscope at two different time points: 3 days and 7 days, during which time the cell morphology of pictures were taken and analyzed.

Immunofluorescence (IF): Differentiated EPCs were analyzed for the markers of endothelial cells. Differentiated EPCs were incubated with either anti mouse CD133 (Millipore, Cat #: MAB4310) or rat CD34 (Abcam, Cat #: 2150-1) for 60 min at room temperature. Positive staining was detected using Dylight594 or FITC conjugated secondary antibodies using a fluorescent microscope.

Flow cytometry (FCM) detection of surface markers on EPCs: Adherent cells were digested into a single-cell suspension, blocked for 30 min and then incubated with antibodies against either mouse CD133 (Millipore, Cat #: MAB4310) or mouse CD34 (Abcam, Cat #: 2150-1) for 30 min at 4°C. One tube was not incubated with any antibodies as a negative control. After washing twice with PBS, the cells were incubated with a FITC goat anti-rabbit secondary antibody (Abbkine, Cat #: A22120) and a Dylight594 labeled goat anti-rat secondary antibody (Abbkine, Cat #: 23440) for 30 min at 4°C, washed twice with PBS, and then fixed with 4% paraformaldehyde. EPC surface markers were analyzed by FCM.

Transfected EPCs: Isolated and identified EPCs were seeded in 6-well plate and incubated with a change of medium every 3 or 4 days for 7 days until reaching about 75-80% confluence, and transfected with GFP-vectors (MOI=10). The green fluorescence was observed after 24 h.

Animal stage

Experimental animals and grouping: APP/PS1 transgenic male mice at 7 to 8 months old and weighing 26-28 g (Nanjing biomedical research institute, Nanjing, China) were used as the experimental mice. The C57/BL6 male mice at the same conditions (Tongji hospital, Huazhong University of Science and Technology) were used as controls. These mice were randomly divided into a GFP-EPCs transplantation group and a PBS group, each with six mice. All mice were housed in large spacious cages, and supplied with food and water ad libitum. The animal room was well ventilated and had a regular 12-hour/12-hour light/dark cycle throughout the experimental period. All protocols involving animal care and handling were approved by the National Institute of Health Guide for the Care and Use of Laboratory Animals.

GFP-EPCs injection: Cells at 75-80% confluence were trypsinized and resuspended in complete medium at 2.0 x 10^6 cells/ml. The mice were put in a fixed frame and repeatedly wiped with 75% alcohol until the mice tail vein was straight. The prepared cells were then slowly injected into the tail using an insulin syringe. After the syringe was pulled out, the needle position was pressed to avoid the loss of cells.

Brain tissue processing: The animals were killed by cervical dislocation at 1 week post-transplantation. Half of the brains was removed and post-fixed in glutaraldehyde. The other half was snap-frozen in liquid nitrogen. Frozen tissues were sectioned at 5 mm thickness using a cryostat and stored in a freezer at -80°C.

Observation using fluorescence microscope: Freshly extracted brain tissue was embedded with optimum cutting temperature compound (OCT Compound, Sakura, Japan) and sectioned at 5 mm thickness using a cryostat. The sections were then evaluated using a fluorescence microscope (Olympus, Tokyo, Japan) to search for the presence of GFP-EPCs that had survived. Finally, the distribution of EPCs in the hippocampus was analyzed.

Western-blot (WB): The details of western blot analysis were described previously [18]. Brain samples were minced into small pieces and homogenized in a lysis buffer. The homogenate was centrifuged at 12000 rpm for 30 min and the supernatant was divided into aliquots and frozen at -80°C. Protein samples were separated by gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked for 1.0 hour in 5% nonfat milk and then incubated overnight at 4°C in specific primary antibodies: mouse anti-GFP (1:5000; Millipore, Cat #: MAB3580) and mouse beta-actin (1:1000; SANTA CRUZ, Cat #: sc-47778). The procedures were replicated three times. The band density was quantitated by software (Bio-Rad ChemiDocTM MP).

mRNA extraction and real-time PCR analysis: Total RNA was extracted from each tissue using an RNA extraction kit (Magen). Tissues were pulverized in liquid nitrogen using a Cryo Press and subjected to RNA extraction. Single-strand cDNA was synthesized from 1 μg of total RNA using a reverse transcription kit (Thermo). Real-time RT-PCR reactions were carried out in a SYBR Green PCR master mix (TOYOBO) and the quantitative analyses of GFP mRNA were performed by the StepOne Quantitative real-time PCR System (Applied Biosystems, StepOne Plus, CA, USA). Quantitative data were normalized to the expression level of GAPDH. Real-time PCR conditions were 95°C for 1 min followed by 40 cycles of 95°C for 15 s and 60°C for 15 s. For quantitative real-time PCR, the GFP primers were sense 5'-CTCGTGACCACCCTGACCT-3' and antisense 5'-GATGCCGTTCTTCTGCTTG-3'. The GAPDH primers were sense 5'-AAT GGT GAA GGT CGG TGT G-3' and antisense 5'-GTG GAG TCA TAC TGG AAC ATG TAG-3'.

Statistical analysis

Data were presented as group means ± standard error of the mean (SEM). A student t-test was used to evaluate the differences between the experimental groups. A *p*-value of less than 0.05 was considered statistically significant. The Sigma-Stat software (SPSS Science, Chicago, IL, USA) was used for all statistical analyses.

Results

The morphology of mouse bone marrow MNCs induced by EGM-2MV medium

The MNCs cultured in EGM-2MV showed a fusiform or cobblestone shape (**Figure 1A**) at day 7.

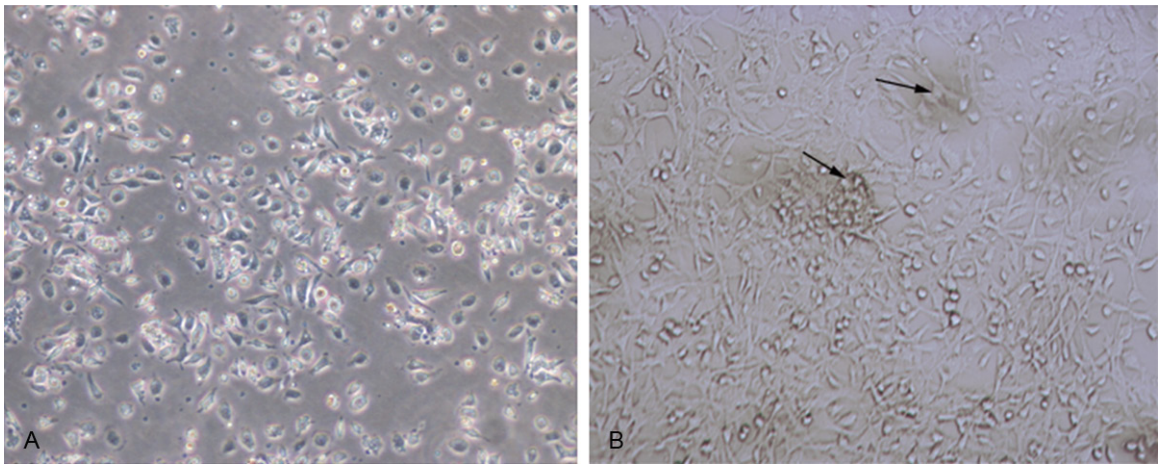

Figure 1. The phenotypes of the MNCs. A. Mouse MNCs from bone marrow showed a fusiform shape or a cobblestone shape at day 7 (magnified 100 X). B. The cells gradually formed endothelial cells-like sprouts (magnified 100 X).

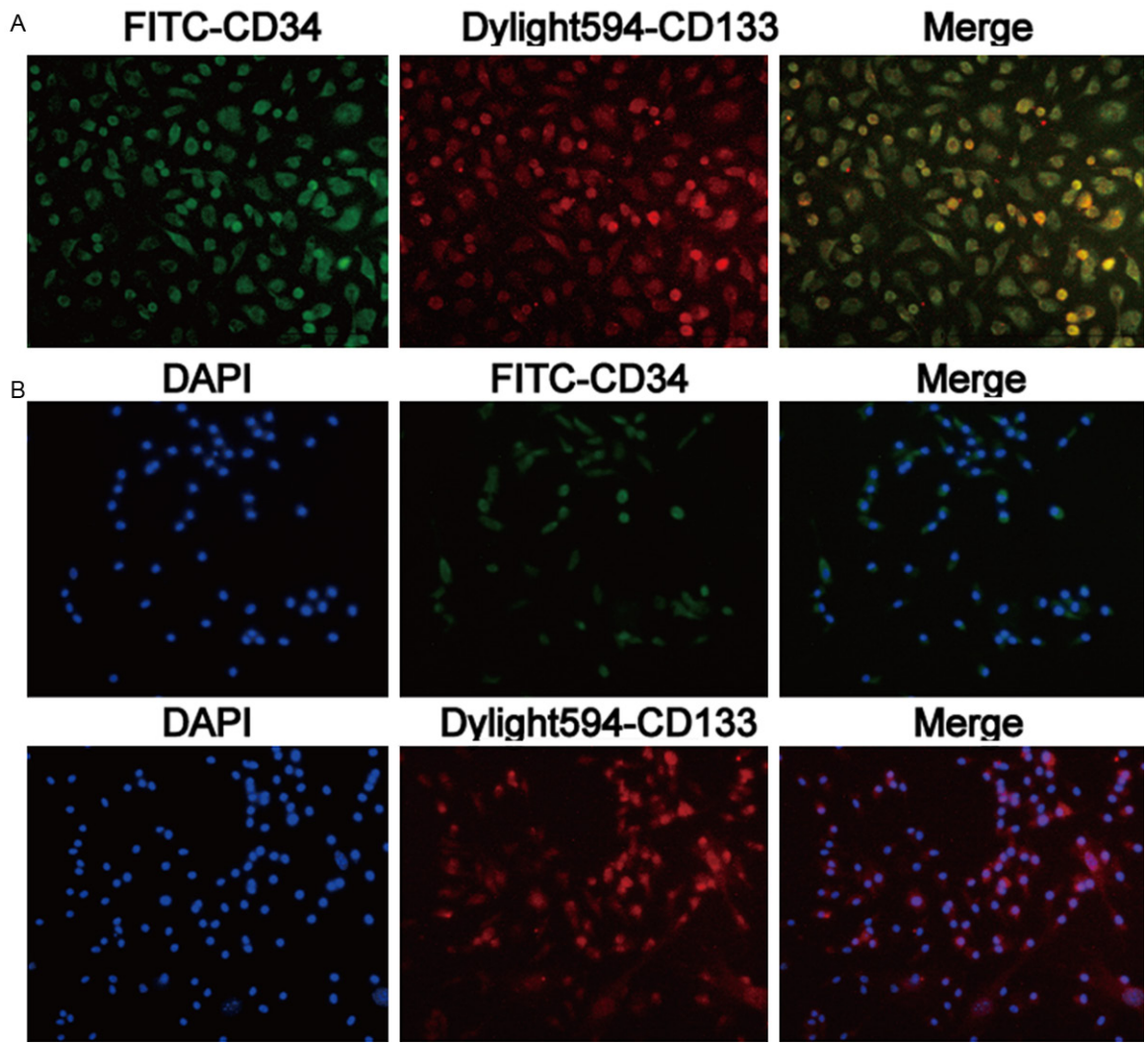

Figure 2. Identification of EPC biomarkers by immunofluorescence. A. Mouse MNCs from bone marrow double labeling by FITC-CD34+ cells and Dylight594-CD133+ cells (magnified 200 X). B. The cells labeled by FITC and Dylight594, separately (magnified 200 X).

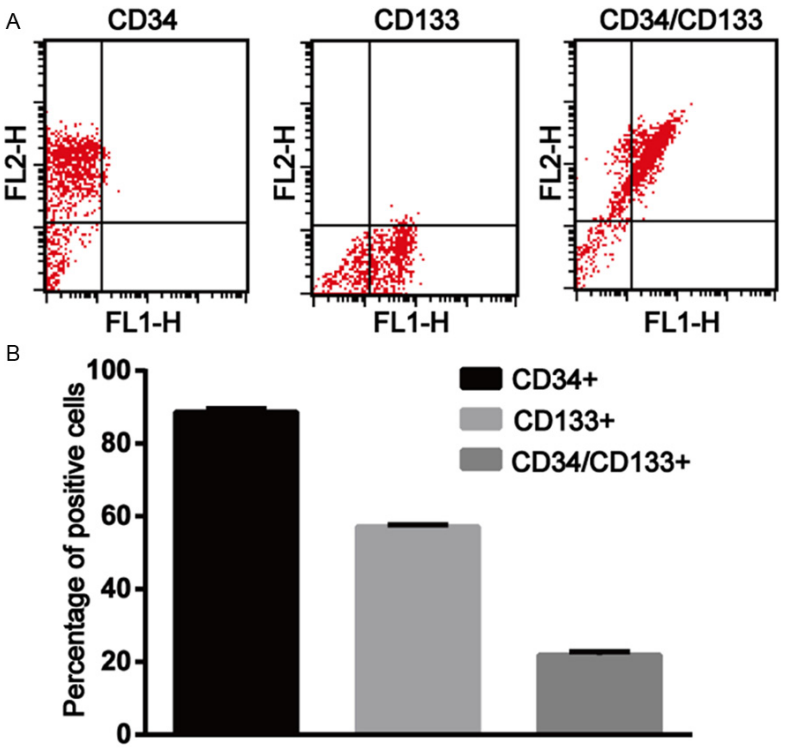

Figure 3. Flow cytometric analysis of surface markers of EPCs differentiated from mouse bone marrow MNCs. Data are presented as the mean ± SD, n=3 (*p< 0.05). A. The expression of CD133 and CD34 were separately detected using FL1-H and FL2-H, respectively. Control represents negative control with primary antibody incubation. B. The percentage of positive cells, corresponding data in A.

The stem/progenitor cell qualities of the EPCs were evidenced by their proliferative capacity to form endothelial colonies [19] (**Figure 1B**).

Identification of EPC biomarkers in mouse bone marrow-derived MNCs by immunofluorescence

We were able to detect the expression of CD34 (**Figure 2A** green fluorescence), CD133 (**Figure 2A** red fluorescence), and CD34/CD133 under a fluorescence microscope (**Figure 2A** yellow fluorescence). EPCs were qualified as adherent cells if stained double positive for CD34 and CD133 (**Figure 2**).

Distinguish EPCs biomarkers in mouse bone marrow-derived MNCs by flow cytometry

Flow cytometry (FCM) was performed to identify the cellular population mobilized by CD34 and CD133. We were able to detect the expression of CD34 (88.04±1.95745%), CD133 (56.60±1.13137%), and CD34/CD133 (21.82 ±1.00015%) on day 11 (**Figure 3**).

Transfection of EPCs by GFP-adenoviral vector

Transfection of EPCs with GFP-adenoviral vector was confirmed by the presence of strong green fluorescence under a fluorescence microscope (**Figure 4**, GFP-EPCs), as compared with the absence of fluorescence in the control EPCs transfected with PBS. (**Figure 4**, Control).

Distribution of GFP-EPCs in the brains after transplantation

One week after GFP-EPCs injection through the mice tail veins, a scattered presence of green fluorescent positive EPCs within the brains of APP/PS1 mice, with an accent at the peripheral hippocampal area were identified under a microscope (**Figure 5D-F**). There was no green fluorescence detected within the brain of WT mice injected with PBS (**Figure 5A-C**) and GFP-EPCs (**Figure 5G-I**).

Expression of GFP in the brains increased after transplantation

We found that the expression of GFP protein in brains of APP/PS1 mice increased after GFP-EPCs transplantation when compared to the control group (**Figure 6A**, **6B**). The mRNA levels in GFP-EPCs group were higher ($p<0.01$) than the negative control group after transplantation (**Figure 6C**).

Discussion

In this study, we employed a transgenic mouse model of AD to test a hypothesis concerning the repair function of exogenous EPCs for brain microvascular damage of APP/PS1 transgenic mouse model of AD. The results showed that exogenous EPCs could distribute in different areas of the brains 7-days after being injected

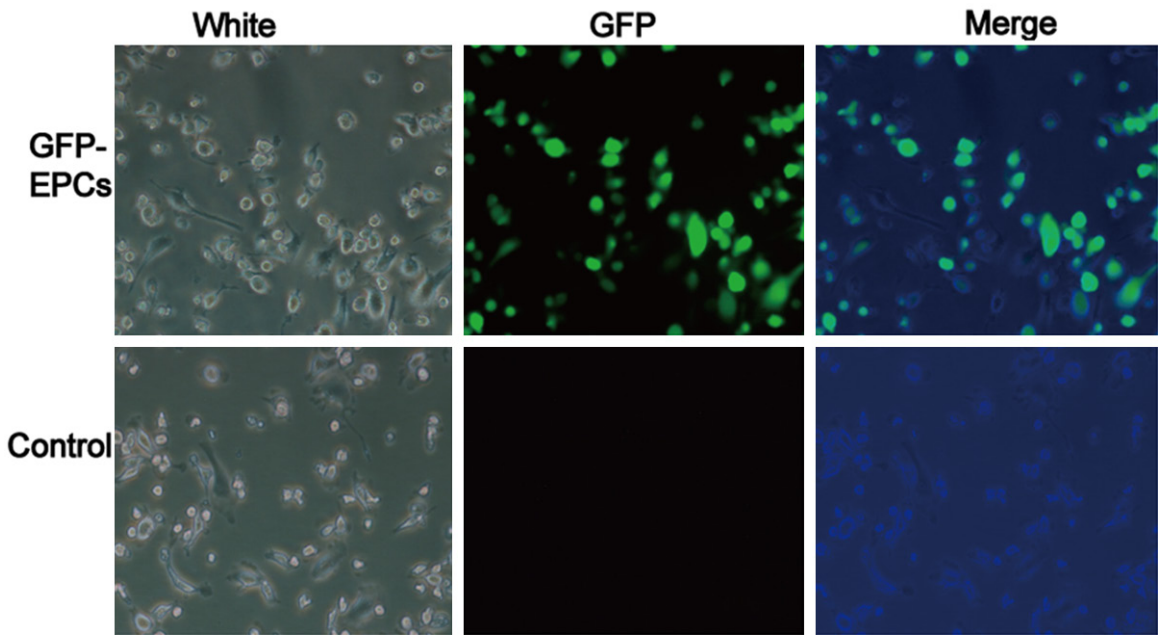

Fig ure 4. The EPCs were transfected with GFP-vector and negative control, respectively. The GFP-vector group and negative control group was observed by inverted microscope (White). The two groups were observed by fluorescence microscope (GFP).

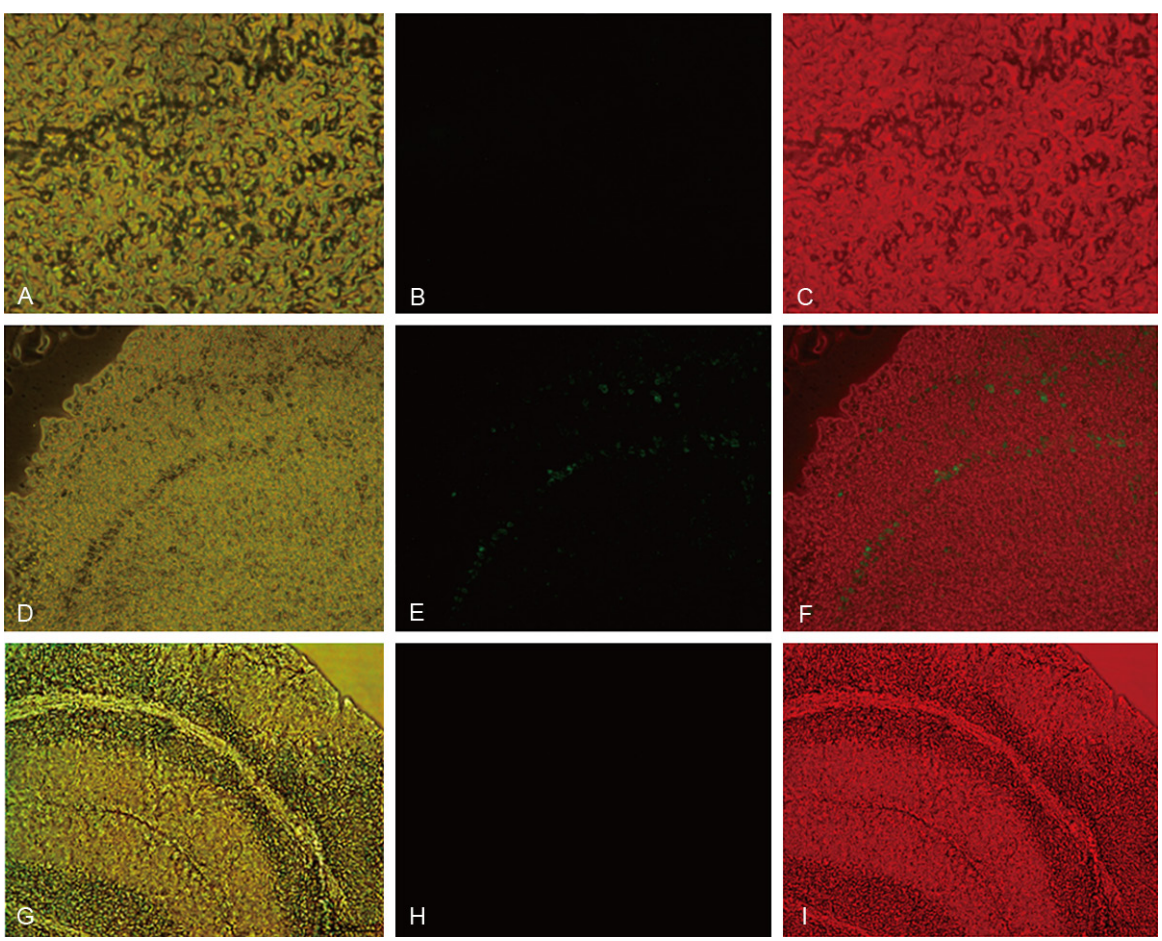

Figure 5. Distribution of GFP-EPCs in the brains after transplantation. D, G were the brain tissue of APP/PS1 and WT with GFP-EPCs groups, and A was the negative control group by inverted microscope, respectively. B, E and H were the three groups by fluorescence microscope. C, F and I were the results of merged, A and B, D and E, G and H, respectively.

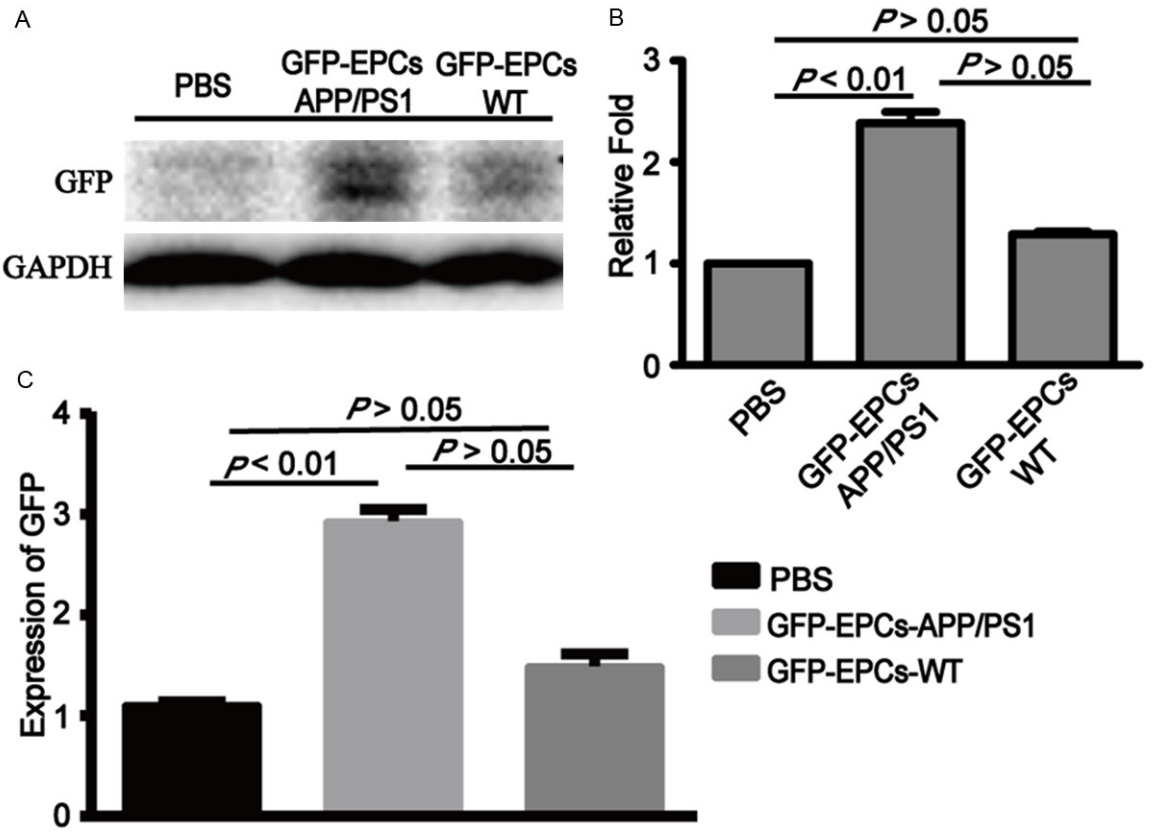

Figure 6. A, B. Expression of GFP protein was detected by the method of Western-blot detect and its relative fold. C. Expression of GFP mRNA was detected by the method of qPCR. Data are presented as means ± SD; n = 3. This is representative of three separate experiments. *$P < 0.01$ compared with controls.

into the mouse tail vein. The EPCs-injected APP/PS1 mice showed a significantly increased expression of GFP protein and mRNA when compared to the control groups. We analyze the potential reasons for this enhanced penetration of Exogenous EPCs into the brains of APP/PS1 transgenic mice as follows.

The BBB is a highly specialized region of the vascular tree and consists of a tight sealed barrier between circulating blood and the central neurons system (CNS). This arrangement preserves the integrity of the nervous system by limiting the passage of harmful substances and inflammatory cells into the brain [20]. The endothelial cells that form the BBB are connected by tight and adherens junctions, and confer the low paracellular permeability of the BBB [21]. Alzheimer's disease decreases the levels of many tight junction (TJ) proteins, their adaptor molecules and adherens junction proteins [22], leading to the breakdown of BBB. Endothelial cell-pericyte interactions are crucial for the formation [23] and maintenance of the BBB [24]. Pericyte deficiency can also lead to a reduced expression of certain tight junction proteins. Both processes can lead to extravasation of multiple small and large circulating macromolecules into the brain [25]. The TJ between endothelial cells (ECs) is a metabolic and physical barrier that controls the transport of macromolecules through the BBB, which plays a key role in brain homeostasis [26]. In AD, BBB integrity is compromised as a consequence of the breakdown of EC-cell junctions [27]. Therefore, we could suggest that the

breakdown of BBB in APP/PS1 mice enhances the penetration of exogenous EPCs into the brains.

A meta-analysis of BBB permeability in 1,953 individuals showed that neurologically healthy humans had an age-dependent increase in vascular permeability. Moreover, patients with Alzheimer's disease-type dementia had an even greater age-dependent increase in vascular permeability [28]. Reduced cerebrospinal fluid (CSF) observed in aging and in AD patients impaired the Aβ clearance and enhanced its accumulation and aggregation in the CNS [29]. According to this study, the vascular permeability of AD models was markedly increased, perhaps suggesting more exogenous EPCs were present in the brains of APP/PS1 mice.

The development of amyloid plaques is a well-characterized feature of AD, and transgenic mouse models have become indispensable tools for the study of AD. Considerable research shows that cognitive deficits in APP-overexpressing mice are correlated with the degree of Aβ aggregation in the brain [30]. Amyloid deposition is detectable in hippocampus and neocortex of APP/PS1 double-transgenic mice [18]. Our results showed that exogenous EPCs were detected in the hippocampus, which is consistent with the deposition of Aβ according to previous studies. Indeed, the soluble oligomeric Aβ species are believed to be the major toxic forms and are the key mediators of cognitive dysfunction in AD [31, 32]. However, Alzheimer's pathology is not limited to neurons, as one of the earlier manifestations of the disease is abnormal cerebral micro-vessels [33]. This cerebral microvascular pathology may promote other Aβ mediated pathologies including BBB disruption, or even affect neuronal damage directly [34]. Furthermore, BBB dysfunction induced by Aβ subsequently impairs vascular clearance of brain Aβ and increases the influx of peripheral Aβ into the brain, thereby accelerating the accumulation of senile plaque formation and subsequently inducing cognitive differences [35].

The involvement of the receptor for advanced glycation end-products (RAGE) appears to be very important in the development of AD pathology. RAGE is not only the pivotal transporter that mediates the influx of Aβ into the brain parenchyma and, consequently, in an unbalanced situation enhances Aβ accumulation, but RAGE also induces Aβ cytotoxicity that leads to neuron injury. RAGE-Aβ interaction at the BBB activates results in brain endothelium injury and ultimately in loss of BBB function [36]. Therefore, from our study, we might speculate that the exogenous EPCs could cross the brains of APP/PS1 mice easier as a result of the dysfunctional BBB.

Evidences have been confirmed that AD are also characterized by microvascular ischemia, and the increased incidence of morbidity and mortality from microvessel disorders may be ascribed to an impaired ability to form neovascular repair vessels and neurons during the pathological process of AD [2]. Meanwhile, neovascularization plays an important role in the response to hypoxia and is a fundamental process for recovery from ischemic damage. In the brain and other organs, tissue ischemia is a potent stimulus for EPCs mobilization, a response that releases a variety of factors including vascular endothelial growth factor (VEGF), stromal cell derived factor 1 (SDF-1) and others. Under concentration gradient action, EPCs could migrate to the ischemic tissues to compensatory angiogenesis and/or endothelial repair in the injured tissue [7]. VEGF is an endothelial cell-specific growth factor and a regulator of physiological and pathological angiogenesis. It is able to increase the permeability of capillary vessels to different macromolecules. VEGF has long been known to stimulate EPCs proliferation, migration, tubulogenesis and homing both in vitro and in vivo. In particular, VEGF released in circulation upon an ischemic insult redirects circulating EPCs to either the infarcted myocardium or the obstructed arteries, depending on the injury site [37]. Therefore, these results suggest that for our study that the predominant cause of EPC integration into the most severe ischemic brain area may be the induced effect and/or the elevated levels of VEGF in the ischemic brain area of APP/PS1 mice.

A growing body of evidence indicates that the cerebral vasculature is an important target of Aβ and that vascular dysfunction significantly contributes to neuronal damage and dementia [38, 39]. In addition, decreased endothelium-derived nitric oxide (NO) bioavailability and vas-

cular dysfunction have been demonstrated in AD [40, 41]. NO production at the endothelial cell level involves the activity of the enzyme endothelial nitric oxide synthase (eNOS, NOS III), which is constitutively expressed and produces NO in a calcium-dependent manner [42]. In models of chronic brain hypoperfusion, in vivo administration of Aβ has been shown to increase eNOS expression and yet, paradoxically, decrease endothelium-derived NO formation [39]. This finding suggests that Aβ could affect the activity of this enzyme. eNOS post-translational modification can profoundly affect its activity and therefore influence NO production. Also, Aβ reduces the production of NO through blocking eNOS phosphorylation at serine 1179 and eNOS-dependent NO production. Altered eNOS-dependent NO production results in endothelial dysfunction, which is associated with BBB penetration and has been shown to play a pathogenic role in AD [43, 44]. Therefore, we may also conclusion that because of the BBB damage caused by endothelial dysfunction, the brains of APP/PS1 mice show more exogenous EPCs than the controls.

Our study has some limitations. First, in this study, the effects of EPCs permeating into the brain tissue have not been studied. But, as the next step, we will research the repair function of EPCs for the microvascular and cerebral nervous system and observe how it strengthens the immune system function. Because of the enhanced effects of VEGF for concentration, mobilization and differentiation of EPCs, we project to structure a supporter that responds to the stimulation of tissue hypoxia and specificity only starting downstream gene expression in endothelial cells, can be fixed in vascular contraction, injury of ischemia expression of VEGF, and make VEGF concentration gradient, as a result, accelerate the EPCs mobilization of oxygen to the damaged area. Finally, we could further research the effects of EPCs for AD.

In summary, these observations may suggest new possibilities for a potentially effective therapy method for AD. In our study, the exogenous EPCs increased across the brains of APP/PS1 mice compared to the controls; however, the concrete mechanisms are still unclear. Further investigations are needed to confirm these preliminary findings and explore the various mechanisms in detail.

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Disclosure of conflict of interest

None.

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