

Original Article

Intraspinal transplantation of endothelial progenitor cells provides neuroprotection after spinal cord injury in rats

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Abstract: Primary injury of the spinal cord leads to local vascular disruption and parenchymal damage, which contribute to secondary degeneration. Extenuating cell death and enhancing angiogenesis constitute an attractive therapeutic approach for spinal cord injury (SCI). Transplantation of umbilical cord blood derived-endothelial progenitor cells (EPCs) has been shown to promote functional recovery of injured spinal cord. In this study, we examined the neuroprotective effects of bone marrow derived-EPCs in a rat model of spinal cord injury (SCI). We established the rat SCI model by the modified Allen method and immediately transplanted EPCs into the lesion site with an EPC culture medium as a control. Administration of EPCs resulted in increased VEGF-A protein expression, and subsequent angiogenesis, attenuated apoptotic cell death, increased neuronal preservation, and reduced number of astrocytes. Moreover, EPC-treated rats exhibited significant improvements in tissue preservation and behavioral recovery. These data reveal that transplantation of EPCs could effectively provide neuroprotection following SCI and ultimately promote SCI recovery. The results also indicate that administration of EPCs is a promising therapeutic method for SCI.

Keywords: Endothelial progenitor cells, spinal cord injury, neuroprotection

Introduction

Spinal cord injury (SCI) is a neurological condition that causes significant loss of function, with a limited regenerative capacity. Although several cell-based transplantations to ameliorate the microenvironment for SCI have been reported in recent years, no optimal cellular source has yet been defined [1].

Endothelial progenitor cells (EPCs), which mainly exist in bone marrow, are precursors of endothelial cells that can quickly proliferate and differentiate into mature endothelial cells with high proliferation potential [2]. Compared to other source cells, such as neural stem cells, embryonic stem cells and induced pluripotent stem cells, for transplantation after spinal cord injury, transplantation of EPCs have some

absolutely advantages. EPCs mainly exist in bone marrow. Thus, it is convenient to obtain EPCs from each patient. If cell transplantation were widely used to treat spinal cord injured patients, the use of EPCs would not bring ethical issues and immune rejection. Besides, EPCs can secrete a variety of cell growth factors, including vascular endothelial growth factor (VEGF), insulin-like growth factors (IGF), fibroblast growth factor, and interleukin 8 (IL-8) [3]. Therefore, we consider EPCs as an ideal source cell for transplantation to treat SCI. Evidence for a potential therapeutic effect of EPCs has been reported in animal models of acute myocardial infarction [4], hand limb ischemia [5], and diabetes [6].

Although some studies reported that umbilical cord blood derived-EPCs are beneficial for the

functional recovery of SCI [7], the exact mechanism remains unclear. Besides, umbilical cord blood derived-EPCs are restricted to apply in clinical therapy for the immune rejection after transplantation. To understand the neuroprotective effects of EPCs in the recovery of SCI, we transplanted bone marrow derived-EPCs into the lesion site of the rat spinal cord following SCI. Our results indicated that EPCs transplantation provided a neuroprotection effect and promoted angiogenesis in the local injured site, thus promoting the functional recovery.

Materials and methods

Isolation, culture, and characterization of EPCs

EPCs derived from rat bone marrow were cultured as previous described [8]. Bone marrow mononuclear cells were isolated from 4-week-old female Sprague-Dawley rats by density gradient centrifugation, cultured in endothelial cell basal medium-2 (EBM-2; Lonza, Switzerland) with fibronectin coating. The cultured cells were characterized by the EPC-specific marker CD133 and the vascular endothelial growth factor receptor-2 (VEGFR2; Abcam, UK) using immunofluorescence staining, or by double staining with dil-acetylated low-density lipoprotein (Dil-acLDL; Life Technologies, USA), and FITC-Ulex europaeus agglutinin (FITC-UEA; Life Technologies, USA) on day 14. The EPCs were fluorescently labeled with Dil (a dye for labeling and tracking live cells, Invitrogen, UK) at a concentration of 2.5 mg/ml before transplantation.

Rat model of acute traumatic SCI

All animal protocol was approved by the Animal Care and Use Committee of Anhui Medical University in accordance with Guide for the Care and Use of Laboratory Animals. Adult female Sprague-Dawley rats (220-250 g) were purchased from Anhui province experimental animal center (Hefei, China), and were housed in a temperature-controlled, warm room (26°C, 12:12-hour light/dark cycle) with *ad libitum* access to food and water.

The rat model of acute traumatic SCI was developed in accordance with the modified Allen's method as reported by Basso [9]. The surgical procedures were performed aseptically under anesthesia. Rats received intraperitoneal injections of 10% chloral hydrate at a dose of 350

mg/kg. After deep anesthesia, a complete laminectomy was performed at the T10 level and the dorsal aspect of the spinal cord exposed. The rat model of moderate traumatic SCI was created by dropping a rod weight 10 g from a distance of 25 mm. The incision was closed with sutures for each layer after the intraspinal microinjection of EPCs or culture medium, and Subcutaneous injection of penicillin sodium 0.8 mg/g was administered per day post operation until hematuria disappeared. All the models must comply with the criterion that the BBB score of the rat is 0 after injury. The bladder was compressed by manual abdominal pressure three times daily until bladder function was restored.

In vivo 5-bromodeoxyuridine (BrdU) labeling

To examine the cellular proliferation after SCI, BrdU (50 mg/kg; Sigma, MO) was injected intravenously daily for 6 consecutive days, 3 days before operation and 3 days after operation.

Intraspinal microinjection

The rats were randomized into three groups; (1) SCI group: rats were injured and microinjected with 5 μ l fresh EPCs culture medium; and (2) EPCs group: rats were injured and microinjected with 5 μ l single cell suspensions of EPCs with a concentration of 1×10^5 cells/ml. The single cell suspensions of EPCs and the EPC culture medium were prepared before surgical procedure and maintained on ice during use. All the cells and the cell culture medium were injected into the subdural of spinal cord immediately following SCI through a capillary needle (diameter 60 μ m) connected to a Hamilton micro syringe system.

Western blot analysis

Total protein was taken from a 5-mm length of the spinal cord center at the injury site (n=5-6/group) and 20 μ g of protein was loaded, separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride (PVDF) membranes. VEGF was probed with monoclonal mouse IgG anti-VEGF-A antibody (1:500, Santa Cruz, USA). Secondary antibodies labeled with horseradish peroxidase-conjugated (goat anti-mouse IgG, 1:10000) were purchased from Santa Cruz, USA. The protein bands were imaged by an

enhanced chemiluminescence (ECL, Thermo Scientific, USA) detection system. Beta-actin was immunoblotted as a control. Image Pro-Plus (Media Cybernetics, Inc, USA) was used for integrated optical density (OD) analysis.

Histology analysis

Two or 6 weeks (n=6-8/group) post-surgery, rats were deeply anesthetized with chloral hydrate and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Each spinal cord segment was embedded in paraffin, 5- μ m thick sections were prepared for hematoxylin-eosin (HE), and terminal-deoxy-transferase mediated d-UTP nick end labeling (TUNEL) staining. The spinal cord segment for fluorescence immunolabeling was dissected and cryoprotected in 30% sucrose in PBS for 24 h. The spinal cord segment was frozen in tissue-embedding medium (O.C.T. compound, Tissue-Tek, USA) and serially sectioned on a cryostat at a 10- μ m thickness. Sections for fluorescence immunolabeling and HE staining were taken from the epicenter of the lesion site and sections for TUNEL staining were taken 2 mm from the epicenter.

Fluorescence immunolabeling

Tissue sections were washed in PBS and incubated with 10% normal goat serum in PBS with 0.3% Triton X-100 at 25°C for 30 min. The sections were incubated overnight at 4°C with the following primary antibodies: mouse anti-BrdU (1:500, Santa Cruz, USA) for proliferated cells, mouse anti-CD31 (1:100; Abcam, UK) for endothelial cells, mouse anti-NeuN (1:100; Abcam, UK) for neurons, and rabbit anti-GFAP (1:500, Santa Cruz, USA) for astrocytes. After primary antibody incubation, the sections were washed in PBS and incubated with goat anti-mouse/rabbit secondary antibody (1:50; Beyotime, China) at 25°C for 1 h. The sections were rinsed with PBS and incubated with DAPI (1:50; Beyotime, China) for 30 min to counterstain the nuclei. The sections were coverslipped with Mowiol mounting medium. The images were obtained using a fluorescence microscope (Nikon, Japan) and merged by Image-Pro Plus.

TUNEL staining

A TUNEL apoptosis kit (Roche, Switzerland) was used to label apoptotic cells at the lesion site.

TUNEL staining was completed as described in the manufacture's instruction. Briefly, tissue sections were incubated with 0.3% Triton X-100 on ice for 2 min and endogenous peroxides activity was quenched with 0.3% hydrogen peroxide in methanol for 30 min at 25°C. After rinsed with PBS, sections were incubated with TUNEL reaction mixture for 30 min at 37°C, followed by TUNEL POD for 30 min at 25°C. The reaction was visualized with DAB reaction.

HE staining

Tissue sparing at the center of the lesion site was analyzed 6 weeks after SCI. Sections were stained with HE as previously described [10]. Briefly, after deparaffinization, sections were washed in running tap water for 5 min and incubated in hematoxylin (Beyotime, China) for 15 min, incubated in eosin (Beyotime, China) for 3 min, followed by a microscopic observation; sections were processed by dehydration in graded ethanol, cleared with xylene, and finally mounted with mounting medium (Beyotime, China).

Behavioral testing

From 1 to 6 weeks post-surgery, locomotor function recovery was evaluated by two independent observers using the 21 point Basso, Beattie and Bresnahan (BBB) score [9], an open field locomotor test for rats. The BBB scale was used to assess hind-limb locomotor recovery, including movement at all joints of the hind limb, weight-bearing capability, coordinated and proper gait, and full weight support, and appropriate limb, body and tail position. The testing was performed every week after surgery on a blinded basis and the testing time of each session was 5 min per rat. Scores were recorded and averaged across the left and right hind limbs.

Statistical analysis

The results of the expression of VEGF, percentage of endothelial cells, neurons, astrocytes apoptotic cells and tissue preservation and BBB scores were compared between EPCs transplanted group and control group with t-test. All data are presented as mean \pm SEM and analyzed by SPSS 22.0 (IBM, Inc, USA). *P* values <0.05 were considered to be statistically significant.

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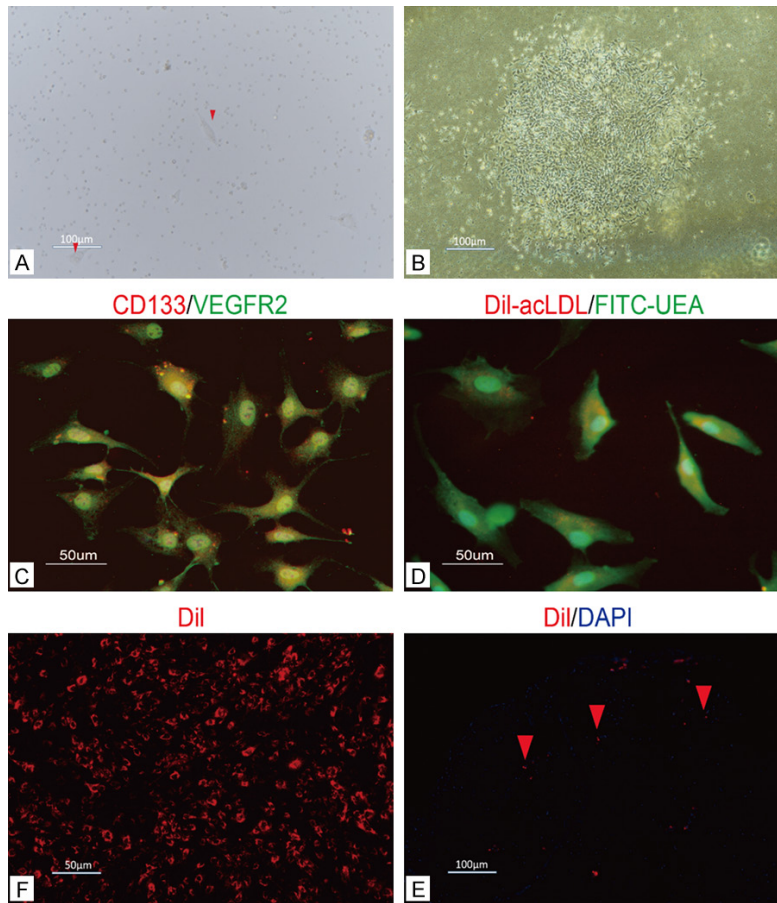


Figure 1. Culture, characterization, and label of EPCs. A. As red spots showing, some of the adherent cells turned into typical cobblestone-like morphology. B. Cells converged into colonies. C. EPCs positively expressed specific surface marker CD133 and VEGFR2. D. Functional experiments were characterized by Dil-acLDL and FITC-UEA. E. Cells were labeled by Dil before transplantation. F. Grafted cells (as red spots showing) labeled by Dil survived in the lesion site.

Results

Characterization of EPCs

The mononuclear cells isolated from bone marrow by density gradient centrifugation were small and spherical. Cultured in EBM2-MV medium for 48 h, numerous adherent cells appeared. After 7 days, some of the adherent cells turned into typical cobblestone-like morphology (**Figure 1A**) and gradually converged to form colonies (**Figure 1B**), indicating EPCs differentiation. Positive expression of EPC surface marker CD133 (an endothelial cell surface marker) and VEGFR2 (a progenitor cell surface antigen, **Figure 1C**) was simultaneously observed by immunofluorescence staining. Dil-acLDL and FITC-UEA (**Figure 1D**) double staining confirmed the functional characterization of

EPCs. Generally, these cells are considered to be EPCs [11].

Survival of the transplanted EPCs

The cells labeled with Dil were red under the fluorescence microscope before transplantation (**Figure 1E**). Two weeks post-injury, the transplanted cells at the lesion site survived and migrated into the spinal cord parenchyma (**Figure 1F**).

Increased VEGF expression in the transplanted EPCs

Rats were sacrificed 1 week and 2 weeks after transplantation, and the expression of VEGF-A protein was evaluated with Western blot analysis using a monoclonal anti-VEGF antibody. This antibody can detect two splice variants of VEGF at 42 kDa and 25 kDa bands (**Figure 2A**). As shown in **Figure 2B**, the expression of VEGF was significantly increased by 2.1-fold ($P < 0.05$) 1 week post injury and by 1.9-fold ($P < 0.05$) 2 weeks post injury in

EPCs transplanted rats compared to culture medium-treated controls. These results indicated that transplantation of EPCs to the injury site increases the expression of VEGF protein in the injured spinal cord of rats.

Promotion of angiogenesis by the transplanted EPCs

To assess the angiogenic response induced by the transplanted EPCs, we performed immunostaining with CD31 2 weeks after SCI. A significantly increase ($P < 0.05$) in vascular density was observed in the EPCs-treated group ($38.32 \pm 1.49\%$) compared to the culture medium control group ($19.02 \pm 0.47\%$). **Figure 2B** demonstrated that EPCs treatment significantly increased the number of endothelial cells compared to the controls. BrdU staining was used

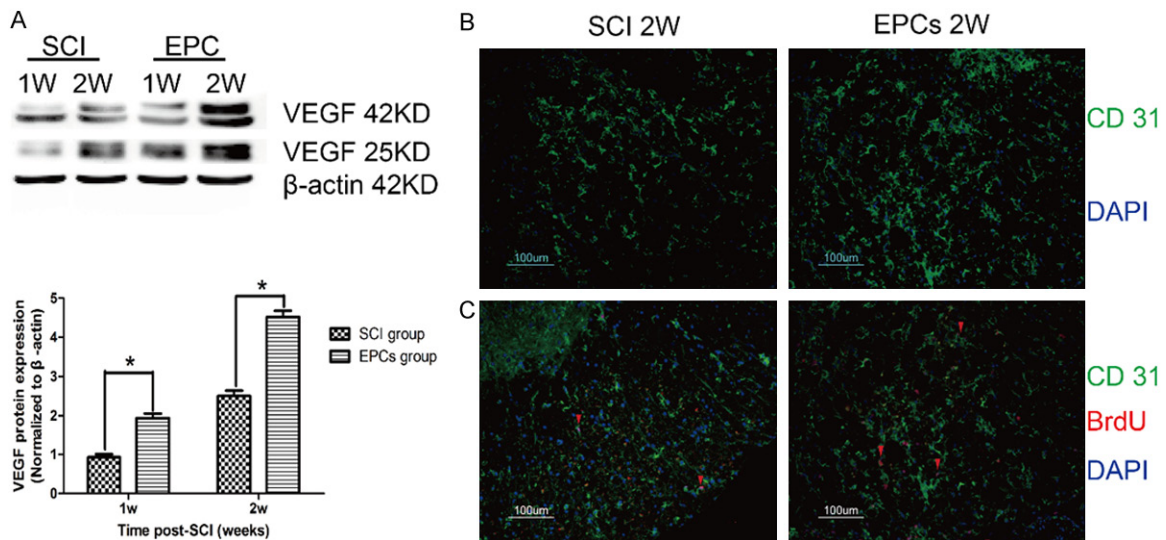


Figure 2. Transplanted EPCs increased VEGF-A protein expression and promoted angiogenic response. A. Expression of VEGF (42 kDa and 25 kDa) was significantly increased by 2.1-fold at 1-week post injury and by 1.9-fold at 2-week post injury in EPCs transplanted rats compared with culture medium treated controls. B. Vascular density was significantly ($P < 0.05$) preserved in EPCs treated rats ($38.32 \pm 1.49\%$) than EPC medium treated rats ($19.02 \pm 0.47\%$). C. The number of new born endothelial cells (as red spots showing) was higher in the EPCs grafted group compared to controls ($16.12 \pm 0.79\%$ versus $7.61 \pm 0.63\%$, $P < 0.05$). * $P < 0.05$.

to quantify the newly developing cells following SCI. The cells co-labeled with CD31 and BrdU were significantly increased in the EPCs treated animals compare to the control animals 2 weeks post-injury ($16.12 \pm 0.79\%$ versus $7.61 \pm 0.63\%$, $P < 0.05$) (**Figure 2C**).

Induction of neuron preservation by EPC transplantation

To directly assess the neuroprotective effects of EPCs following SCI, we quantified neuron sparing in the anterior horn of spinal cord 6 weeks post-injury. Neurons were identified by NeuN, which recognizes mature neuronal cell bodies, in cross-sections of spinal cord tissue. As shown in **Figure 3A**, EPC transplantation resulted in a significant sparing of neurons ($32.04 \pm 3.33\%$) compared to culture medium controls ($19.34 \pm 2.70\%$) ($P < 0.05$).

In or near the lesion site, many astrocytes turn into reactive astrocytes, induced by the local inflammatory reaction, and form a glial scar. To assess the glial reaction following SCI, we used GFAP to identify astrocytes. As shown in **Figure 3B**, the counts of GFAP-positive cells were significantly alleviated by 1.5-fold ($P < 0.05$) 6 weeks post-injury in the EPC-treated animals

compared to the control animals. Thus, SCI may promote astrocytes proliferation and EPCs transplantation may alleviate this proliferation.

Alleviation of post-injury apoptosis by EPC transplantation

Previous studies have shown that apoptotic cell death peaks at 14 days following SCI [12]. Therefore, TUNEL staining was performed to quantify the apoptotic cells in gray matter induced by SCI (**Figure 3C**). Compared with EPC transplanted rats ($18.67 \pm 1.86\%$), the number of brown TUNEL-stained nuclei in gray matter in culture medium transplanted rats ($38.50 \pm 5.01\%$) was significantly increased ($P < 0.05$). Therefore, EPC transplantation may decrease apoptotic cells and increase neuronal preservation.

Enhancement of tissue preservation by EPC transplantation

A greater extent of spared tissue of the spinal cord in the injury epicenter was observed in the EPCs-treated rats 6 weeks after SCI compared to the culture-treated rats (**Figure 4A**). Measurements of the residual tissue in the cross-section area were expressed as a percent of the total cross-section area of the section. The

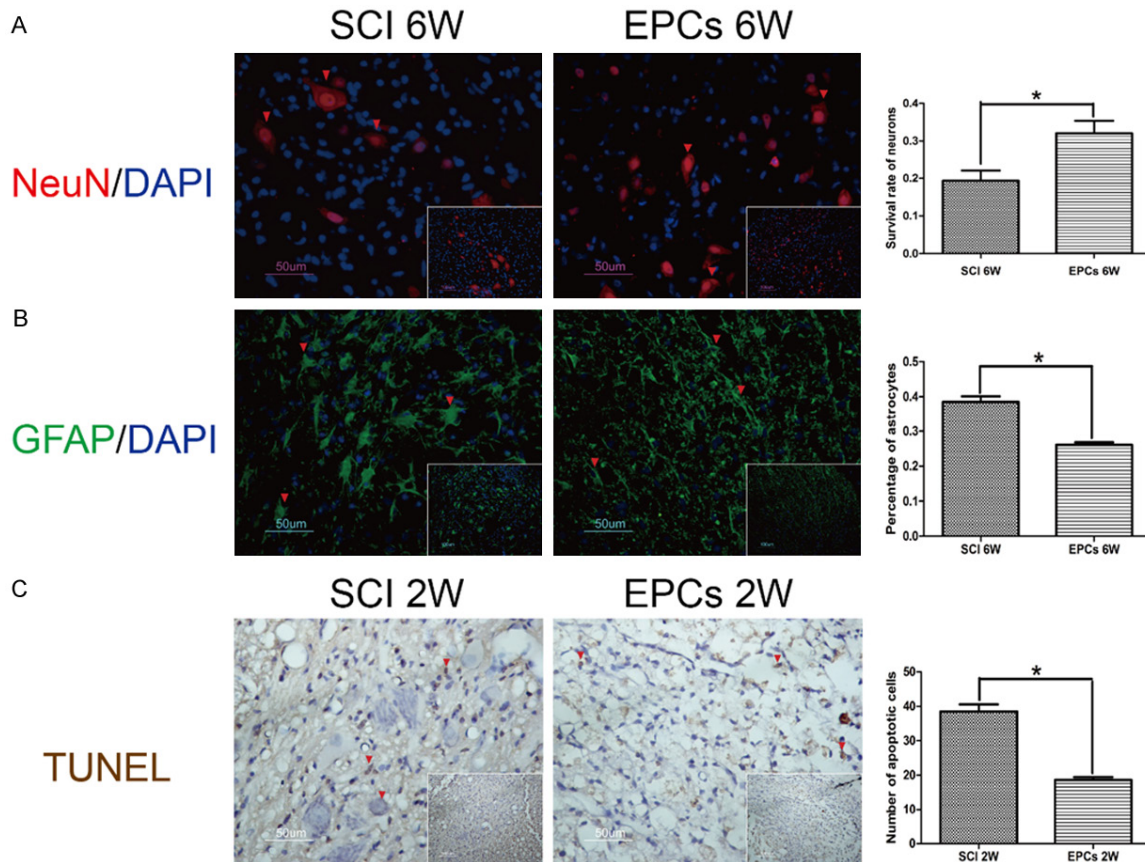


Figure 3. EPCs transplantation provided neuroprotection. (A) More neurons (as red spots showing) in spinal anterior horn were preserved ($32.04 \pm 3.33\%$ versus $19.34 \pm 2.70\%$, $P < 0.05$) and (B) less astrocytes (as red spots showing) were proliferated in EPS transplanted group compared to controls. (C) EPCs transplantation decreased apoptotic cells (as red spots showing) ($18.67 \pm 1.86\%$ versus $38.50 \pm 5.01\%$, $P < 0.05$). * $P < 0.05$.

percentage of remaining tissue was $73 \pm 4.59\%$ in EPCs-treated rats and $41 \pm 3.27\%$ in control rats at the injury epicenter ($P < 0.05$).

Promotion of motor functional recovery by EPCs transplantation

We evaluated the motor function recovery from 1 to 6 weeks after surgery by using BBB scores. The hind limbs of injured rats were completely paralyzed and most scores were 0 immediately after SCI. Hind-limb performance gradually improved in both the SCI and EPCs groups. However, the score in EPCs-treated group was significantly higher than that of the SCI group at 6 weeks (**Figure 4B**, $P < 0.05$). The average score in the SCI group was 6.1 ± 0.35 , indicating non-functional movement of the three joints. In contrast, the EPCs-treated rats reached an average score of 7.7 ± 0.42 , indicating the ability of the hind legs to make sweeping movements.

Discussion

Studies of EPCs in SCI have been reported in recent years, Sasaki firstly reported that transplantation of umbilical cord blood derived-CD133+ cells enhanced angiogenesis and axonal regeneration, and reduced cavity formation in a rat SCI model [13]. In Kamei' study, they found that bone marrow-derived EPCs could promote neovascularization and astrogliosis in the acute phase (day 3) following SCI [14] and they also found that it promotes astrogliosis through Jagged1-dependent Notch signaling. Recently, Kamei [7] demonstrated that only CD133+ mononuclear cells (MNCs) have the effects in promoting angiogenesis and astrogliosis, except for CD133- MNCs.

In our study, we hypothesized that bone marrow derived-EPCs provide neuroprotective effects following SCI. Our results demonstrated that bone marrow derived-EPCs could survive in the

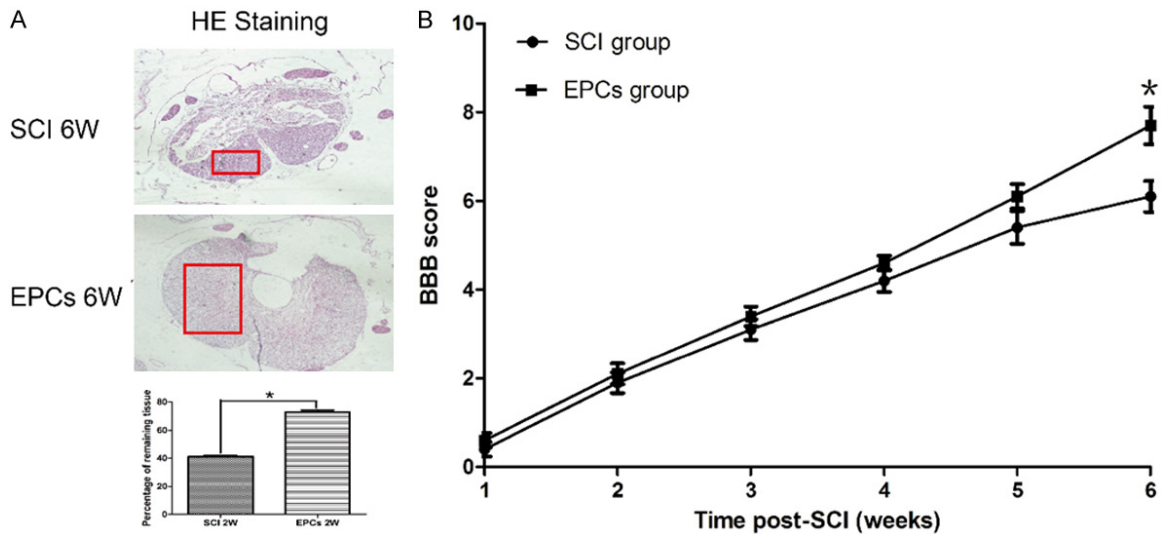


Figure 4. Increased tissue preservation and improved functional recovery with EPCs transplantation. A. Greater tissue was preserved in both the lesion epicenter and 2 mm caudal to epicenter in EPCs transplanted rats compared to controls. The percentage of remaining tissue was $73 \pm 4.59\%$ in EPCs-treated rats and $41 \pm 3.27\%$ in control rats at the injury epicenter ($P < 0.05$). The area in red border showing the preserved tissue. B. EPC transplantation significantly ($P < 0.05$) promoted functional recovery 6 weeks post-injury. The average score in the SCI group was 6.1 ± 0.35 . In contrast, the EPCs-treated rats reached an average score of 7.7 ± 0.42 . * $P < 0.05$.

lesion site following SCI after grafting. Furthermore, we showed that transplanted EPCs issued greater beneficial effects in neurological function recovery compared to the EPC culture medium. Specifically, administration of EPCs resulted in an increase in VEGF-A protein expression, produced an angiogenic effect, attenuated apoptotic cell death, increased neuronal preservation, and reduced astrocytes. Lastly, EPCs treated rats exhibited significant improvements in tissue preservation and behavioral outcomes.

Comparing to previous studies, our study has some new findings. Firstly, EPC is a term that has been applied to multiple cell types that play roles in the regeneration of the endothelial lining of blood vessels [15]. There is still no criterion for the characterization of EPC so far. As most researchers reported [16], the EPCs we used were characterized by double staining with CD133 and VEGFR2 using immunofluorescence staining, and double staining with Dil-acLDL and FITC-UEA. Besides, the EPCs that Sasaki and Kamei transplanted were separated from umbilical cord blood. Because of immune rejection, it is difficult to be applied in patients suffering from SCI. Therefore, we separated EPCs from bone marrow, it is convenient to obtain cells from patients' bone marrow if

EPCs play an important role in the recovery of SCI. Secondly, in our study, we found that transplantation of EPCs promote angiogenesis, as Kamei [14] reported. In addition, we firstly demonstrated that the expression of VEGF-A protein was increased in EPCs transplanted rats following SCI. As Liu reported that ZFP-VEGF treatment attenuates apoptosis post SCI [17], we detected the apoptotic cells by TUNEL staining and we found that EPCs alleviate post-injury apoptosis in gray matter of spinal cord. Lastly, we demonstrated that EPCs could enhance neuron preservation and reduce the number of astrocytes in long-term period, which others never reported. More neuron preserved and less astrocytes proliferated is beneficial for the function recovery from SCI.

Administration of VEGF increases the number of neuron cells in injured spinal cord, therefore [18], we hypothesized that transplantation of EPCs could also increase neuron preservation through increasing the expression of VEGF-A protein post-injury. In our followed study, the inhibitor of VEGF-A may be used to explore the exactly mechanism of EPCs in SCI. In previous studies, the number of astrocytes was increased in EPCs transplanted rats in acute phase; however, we report that EPCs reduce the number of astrocytes in long-term period. In

acute phase of SCI, astrocytes in the lesion site proliferate and turn into reactive astrocytes and form astrocytic scars. These scars isolate the neural tissue from inflammatory cells and reduce inflammation in local site, but in long-term period, the scar prevents the recovery from SCI. We found that EPCs could reduce the astrocytes at 6 weeks post injury, the mechanism is still unclear.

There are also some shortcomings in this experiment. Overall, the number of rats in each was small. The number of rats for western blotting was 5-6 for each group. What I reported 6-8/group were used in two or six weeks post-injury in Histology analysis is mean that there are 6-8 rats for each group in two and six weeks, respectively. Two weeks after surgery, 3 or 4 rats in each group were prepared for paraffin section and the other were prepared for frozen section, same as 6 weeks post-injury. We prepared 6 rats for each group for each kind of section in the beginning, but there always 2 or 3 rats died in the experimental period, thus we often got 6-8 rats for each group at last. The change of functional recovery is also very modest that the BBB scores were significantly different at 6 weeks post injury and the EPCs-treated rats only reached an average score of 7.7 ± 0.42 . Actually, spinal cord is severely damaged during primary injury and second injury. It is difficult to get an ideal recovery from such a terrible condition. This is a preliminary experiment to shed light of the effects of EPCs transplantation in SCI. More experiments should be done to explore that whether EPCs is an ideal source cell for cell transplantation to treat rat spinal cord injury.

Conclusion

The present study showed that grafted EPCs provide neuroprotective effects on SCI. These findings may reveal new cell-based transplantation therapeutic strategies for SCI, and more studies should be undertaken to explore the optimum strategies to treat SCI.

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Disclosure of conflict of interest

None.

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