Transplantation of Hematopoietic Stem Cells Promotes Functional Improvement Associated with NT-3-MEK-1 Activation in Spinal Cord-Transected Rats

Liu-Lin Xiong††, Fei Liu††, Shi-Kang Deng‡, Jia Liu‡, Qi-Qin Dan†, Piao Zhang‡, Yu Zou†, Qing-Jie Xia†* and Ting-Hua Wang1,2*

1Institute of Neurological Disease, Department of Anesthesiology, Translational Neuroscience Center, West China Hospital, Sichuan University, Chengdu, China, *Institute of Neuroscience, Kunming Medical University, Kunming, China

INTRODUCTION

Spinal cord injury (SCI) is a devastating clinical disorder that usually results severe damage to sensory, motor and autonomic functions distal to the level of the trauma (Hirano et al., 2012; Kamei et al., 2012; Vawda and Fehlings, 2013; Dasari et al., 2014). Current treatments consist of decompressing and stabilizing the injury, preventing secondary complications and rehabilitating...
HSC transplantation needs to be explored. A crucial role for NTFs in the treatment of neurological diseases (Li et al., 2016). Accumulating evidence has demonstrated a microenvironment for cell survival and nerve regeneration in SCI (Mothe and Tator, 2012; Vawda and Fehlings, 2013). Hematopoietic stem cells (HSCs; Aggarwal et al., 2012), a type of multipotent stem cell that can give rise to all types of blood cells and lymphoid lineages, have been used to treat various hematological disorders such as severe combined immunodeficiency, congenital neutropenia (Tyndall et al., 1999; Burt et al., 2008), and malignancies (Mendez et al., 2005). Recently, HSC transplantation (Huang et al., 2010), has also been used as a valuable therapeutic intervention for stroke, traumatic brain injury and multiple sclerosis (MS; Burt et al., 1995), and to study the underlying mechanisms involved in transdifferentiation, neuroprotection through trophic support and cell fusion (Haas et al., 2005), as well as the replacement of lost or damaged cell populations (Nishio et al., 2006; Schwarting et al., 2008; Xu and Onifer, 2009; Mothe and Tator, 2012; Boulland et al., 2013; Vawda and Fehlings, 2013; Nicaise et al., 2015; Singh, 2015). In addition, several previous studies have indicated that transplantation of HSCs from bone marrow or human umbilical cord blood (HUCB) could effectively promote the repair of the spinal cord in animals 1 week post-injury (Koshizuka et al., 2004; Cabanes et al., 2007; Dasari et al., 2008; Deda et al., 2008). Moreover, HSCs have been used in the treatment of SCI patients in clinic, and have shown beneficial effects in reducing deterioration after SCI (Deda et al., 2008; Bryukhovetskiy and Bryukhovetskiy, 2015; Thakkar et al., 2016). Bryukhovetskiy and Bryukhovetskiy (2015) demonstrated the safety and effectiveness of HSCs transplantation in 202 cases of SCI, showing that the administration of HSC can effectively improve the quality of life for SCI patients. However, the concrete mechanisms underlying the effect of HSC transplantation for both morphological remodeling and molecular alternation remain to be understood. Previously, it has been shown that vascular endothelial growth factor (VEGF), and stromal cell-derived factor-1 (SDF1), and its receptor, Cxc chemokine receptor 4 (CXCR4) were involved in the effects caused by HSC transplantation (Deda et al., 2008). However, crucial evidence linking HSCs to improvement in neural behavior in terms of both morphology and at the molecular level is too limited.

Neurotrophic factors (NTFs) play a vital role in nerve regeneration, neovascularization and growth and differentiation of neurons and non-neuronal cells (Lambert et al., 2004; Feng et al., 2005). Persistent delivery of NTFs is crucial for creating a microenvironment for cell survival and nerve regeneration in SCI (Li et al., 2016). Accumulating evidence has demonstrated a crucial role for NTFs in the treatment of neurological diseases after cell transplantation (Issele et al., 2007; Müller et al., 2009; Abbaszadeh et al., 2015), but the role of Neurotrophin-3 (NT-3), an important member of the NTF family, in SCI following with HSC transplantation needs to be explored.

As previous reports have revealed that NT-3 can promote proprioceptive axon regeneration in the injured spinal cord (Li et al., 2016; Liu et al., 2016; Keefe et al., 2017) and that the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase/cAMP-response element binding protein (MEK/ERK/CREB) signaling pathway is crucial for neuroprotection (Lebesgue et al., 2009), the current study was designed to evaluate the role of HSC transplantation in the transected spinal cord, and to explore associated mechanisms involving NT-3-MEK signaling so as to provide translational evidence for the usage of HSCs in future clinical trials. The results revealed that HSC transplantation can improve the neurological function of transected spinal cord injury (SCT) rats by enhancing 5-hydroxytryptamine (5-HT) positive fibers and oligogenesis, suppressing excessive astroglisis, and upregulating NT3-regulated MEK-1 activation in the spinal cord. These findings reveal the mechanism of cell therapy and contribute to a novel therapeutic target for the treatment of SCI.

**MATERIALS AND METHODS**

**Animal Protocol**

Fifty-seven adult male Sprague-Dawley (SD) rats, weighing 220 ± 20 g, were provided by the Center of Experimental Animals, Kunming Medical University. Animals were provided access to pellet chow and water ad libitum and were housed in individual cages in a temperature (21–25°C) and humidity (45%–50%) controlled room with a 12-h light/dark cycle. In addition, following SCT, rats were placed in warm condition (45%–50%) controlled room with a 12-h light/dark cycle. In addition, following SCT, rats were placed in warm condition to keep body temperatures and the bladders were manually massaged three times a day to enhance their function. This study was carried out in accordance with the recommendations of guidelines for laboratory animal care and safety from the United States National Institutes of Health. The protocol was approved by the guidelines of the Institutional Medical Experimental Animal Care Committee of Sichuan University, West China Hospital, China.

The rats were randomly divided into three groups. The sham group (n = 15) received no transection or transplantation; the SCT group (n = 15) had SCT performed and treated with Dulbecco’s Modified Eagle Medium: nutrient mixture F-12 (DMEM/F12; Hyclone, Logan, UT, USA); the transplantation group (n = 24) were subjected to SCT and then injected with an HSC suspension. The assignment of cases is shown in Table 1.

**Transected Spinal Cord Injury Model**

The SCT procedure was established as previously described (Liu et al., 2014). Briefly, rats were anesthetized intraperitoneally with 2% sodium pentobarbital sodium at a dose of 30 mg/kg. A laminectomy of T9–11 was subsequently performed, and the dura was opened with a surgical blade to expose a length of spinal cord about approximately 1.5 cm long. Complete transection of the T10 spinal cord was performed and the intervening tissue was removed. The completeness of the transection was assured by lifting the cut ends with small forceps. Sham animals underwent...
were plated in a 75 cm$^2$ flask. After centrifuging (1000 rpm, 5 min) and re-suspending, cells were manually massaged three times a day until recovery of motility upon introduction to a new environment. Avoid motionlessness when testing.

**Cell Isolation and Identification**

Three SD rats were sacrificed routinely after being anesthetized as described above, and the femurs were removed. Bone marrow was obtained from femoral bones as previously reported (Sasaki et al., 2001; Koda et al., 2005). Briefly, the epiphyses of the femurs were removed, and the femurs were dissected down the midline. The marrow was then extruded using a syringe filled with DMEM/F12 containing 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) to obtain the amount of marrow from the head of the femur. Afterward, the bone marrow was beaten into a single cell suspension with 5 ml DMEM/F12 containing 10% FBS and 10,000 U/L penicillin and 10 mg/L streptomycin. After centrifuging (1000 rpm, 5 min) and re-suspending, cells were plated in a 75 cm$^2$ culture flask at a density of $1 \times 10^6$ cells/ml and incubated for 12 h (37°C, 95% humidity, 5% CO$_2$). The suspended cells were then collected in a flask and transferred to another culture flask for further incubating. Culture media was changed twice per week. When the cells grew to a density of between 4 and $5 \times 10^6$ cells/cm$^2$, they were passaged. Only suspended cells were collected for 2–3 weeks following cultivation due to a lack of additional adherent cells any more. Thus, following the third passage, we were left with pure suspended HSCs. After culturing for 3 days, the HSCs were identified by CD34 enzyme histo-cytochemical staining.

**Cell Marking**

HSCs were labeled by Hoechst 33342 2 h prior to transplantation. Briefly, a fluorescent dye was added after replacing the media, then the cells were washed with phosphate-buffered saline (PBS; no Ca$^{2+}$ or Mg$^{2+}$) after being incubated at 37°C with 5% CO$_2$ for 2 h. After incubation, DMEM/F12 was added and the cells were counted and concentrated at a final concentration of $2.0 \times 10^4$/µl.

**Cell Transplantation**

Each rat was anesthetized and placed in a stereotaxic frame. Six sites around the rostral, scar and caudal area of the transected spinal cord were injected using the following coordinates: two sites 5 mm rostral and two sites 5 mm caudal to the injured site, two sites located in the injured site. At a rate of 600 nl/min, a total of 6 µl of the cell suspension was injected into the spinal cord through a glass micropipette positioned at 60 degrees, with 1 µl of the cell suspension ($2.0 \times 10^4$/µl) injected at each site. After injection, the glass pipette remained in its position for 5 min before being slowly retracted. The control group underwent the same procedure using DMEM/F12. Then, the rat was removed from the device, and the incision was sutured. For suppressing the immunoreaction, cyclosporin-A (10 mg/kg per day) was intraperitoneally used from the third day before transplantation, and kept till the animals were sacrificed.

**Behavioral Assessment**

From week 1 to 24 post-SCT, Basso, Beattie and Bresnahan (BBB) locomotor rating scale values were recorded in an open enclosure (99 cm in diameter, 23 cm deep) with scores graded from 0 points (absence of any hind limb movement) to 21 points (normal mobility Basso et al., 1995). In order to avoid motionlessness upon introduction to a new environment, subjects were acclimated to the observation fields for 3 days prior to surgery for 5 min per day. During testing, each subject was placed in the open field and observed for 4 min (He et al., 2016). The final score was the average of three individual researchers, who were blinded to the experimental treatment.

**Tissue Harvest**

Twenty-four weeks after SCT, rats were anesthetized with 3.6% chloral hydrate (1 ml/100 g, intraperitoneal injection) and transcardially perfused with heparinized physiological saline followed by 4% paraformaldehyde in 0.1 M ice-cold phosphate buffer, pH 7.4. Immediately after perfusion, a length of spinal cord extending from 10 mm rostral to 10 mm caudal to the injured site was collected. Samples for quantitative real-time polymerase chain reaction (qRT-PCR) were harvested and stored in 1.5 ml RNase-free Eppendorf tubes at −80°C. Spinal cords for immunohistochemical staining were post-fixed for 5 h at 4°C. The tissues were stored in 30% sucrose in 0.1 M phosphate buffer, pH 7.4, for 72 h at 4°C. Then the rostral, scar and caudal segments of transected spinal cord from the different groups were embedded in the same paraffin block and sectioned at 5 µm thickness. After routinely deparaffinized and rehydrated, immunohistochemistry was performed on the slices of spinal cord tissue. Some slices were placed under a fluorescent microscope for direct observation of the state of the transplanted cells.

---

**TABLE 1** | The number of animals in each group.

| Group         | BBB evaluation (week 1–24 post SCT) | HSCs survival detection (1 m/3 m/6 m post SCT) | qRT-PCR 6 m post SCT | IHC/IF 6 m post SCT |
|---------------|-------------------------------------|-----------------------------------------------|----------------------|---------------------|
| SCT (n = 15)  | 15                                  | -                                             | 8                    | 7                   |
| HSC (n = 24)  | 15                                  | 3/3/3                                         | 8                    | 7                   |
| sham (n = 15) | 15                                  | -                                             | 8                    | 7                   |

SCT, transected spinal cord injury with culture medium; HSCs, hematopoietic stem cells; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; IHC, immunohistochemistry; IF, immunofluorescent staining; 1 m/3 m/6 m, 1 month/3 months/6 months.
TABLE 2 | Antibodies used in the study.

| Antibodies | Manufacturer | Source | Reactivity | Dilution |
|------------|--------------|--------|------------|----------|
| CD34       | Sigma        | Mouse  | Rat        | 1:100    |
| 5-HT       | Immunostar   | Rabbit | Rat        | 1:20,000 |
| GFAP       | Millipore    | Rabbit | Rat        | 1:50     |
| APC        | Calbiochem   | Mouse  | Rat        | 1:200    |
| NeuN       | Immunostar   | Rabbit | Rat        | 1:500    |

Immunofluorescence Observation of HSC Differentiation

To observe the differentiation of HSCs into neurons and astrocytes in vitro and in vivo, immunofluorescence staining of Tuj1 and aldehyde dehydrogenase 1 family, member L1 (ALDH1L1) was performed, respectively. In brief, the primary antibodies directed against Tuj1 (1:100, Rabbit, ABclonal, College Park, MD, USA) and ALDH1L1 (1:100, Rabbit, ABclonal Biotech), as well as the secondary antibody Dylight 594 (1:100, goat anti-rabbit, Abbkine) were applied progressively as previously described (Liu et al., 2014). A negative control was performed by using PBS in place of the primary antibodies. Slides were then viewed and photographed using a fluorescence microscope (Leica, Germany).

Enzyme Histocytochemical Staining

Immunohistochemistry was used to determine the purity of cultured HSCs (CD34 immunohistochemical staining) and observe the alternations in the oligodendrocyte precursor cells after HSC transplantation into the spinal cord. Oligodendrocytes, neurons and astrocytes were identified using APC, NeuN and GFAP antibodies, respectively. In addition, 5-HT fibers were also identified. Briefly, slices were washed in 0.01 mol/L PBS repeated three times for 5 min each, then incubated at 37.5°C in 3% hydrogen peroxide for 30 min in the dark to block the action of endogenous peroxidases. This was followed by a 5-min immersion in PBS, repeated three times, then immersion in 5% goat serum at 37.5°C for 30 min. Afterwards, sections were incubated overnight at 4°C in the primary antibody solutions shown in Table 2. A negative control was prepared by using PBS in place of the primary antibodies. Next, sections were washed three times with 0.01 mol/L PBS Tween-20 (PBST-20) and incubated with PV-9000 reagent 1 for 30 min at 37.5°C, followed by PV-9000 reagent 2 for 30 min at 37.5°C. Subsequently, sections were in incubated in the chromogenic agent 3,3′-Diaminobenzidine (DAB) for 3–7 min in the dark. The sections were rinsed with water, then counter-stained with hematoxylin. Following dehydration, sealing took place using a transparent and neutral gum, and then positive staining was visualized under an inverted phase contrast microscope imaging system. The cell numbers and sizes were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

Quantitative Real-Time PCR (qRT-PCR)

Twenty-four weeks post-SCT, sections of spinal cord (10-mm in length, containing the injury and the graft) from the HSC, SCT and sham groups were collected and homogenized to determine the level of MEK-1 and NT3 mRNA. Total RNA was isolated with Trizol reagent (Takara Bio Inc., Otsu, Japan) and was reverse transcribed into cDNA. Subsequently, qRT-PCR of DNA was performed using the following primers (TaKaRa Company (Japan)): NT3 (forward) 5′-GTCCATCTTGTATTAT GTGAT-3′, (reverse) 5′-GTGCTCTGGAATTTTCTT-3′; MEK-1 (forward) 5′-GCAATCCGGAACAGATCAT-3′, (reverse) 5′-CAGGAATTCTTCCAGTTTCTT-3′. β-actin was used as the internal control. PCR conditions were as follows: initial denaturation at 95°C for 2 min, denaturation at 95°C for 15 s and amplification at 53°C for 20 s, followed by extension at 60°C for 30 s for a total of 40 cycles. The threshold cycle (Ct) of each sample was recorded, and data were analyzed by normalization to β-actin values using the 2−ΔΔCt method (Livak and Schmittgen, 2001; Liu et al., 2014).

Statistical Analysis

All statistical analyses were performed with SPSS19.0 software (IBM Corporation, NY, USA). Data were analyzed by Student’s t test between two groups. For multiple group comparison, one-way ANOVA with Tukey’s post hoc multiple comparisons was applied. P < 0.05 was considered
RESULTS

Identification and Morphology of HSCs Cultured In Vitro
At 3 days post-culture, HSCs were round in shape with bright edges and exhibited a floating growth status (Figure 1A). They increased greatly in number 7 days later (Figure 1B). CD34+, a specific marker for HSCs, was used to identify the cultured HSCs at 3 days post-culture. The results showed that CD34+ positive staining was expressed in these cells, and that the positive rate was >90% at 3 days post-culture (Figure 1C), which further confirmed the purity of the HSCs. As cultured HSCs would be transplanted into the host, Hoechst 33342 was also used to label the cultured cells, which showed a blue color with nucleus staining (Figure 1D).

Differentiation of the Cultured HSCs In Vitro
In order to detect the differentiation of HSCs into neurons and astrocytes, an immunofluorescence staining of Tuj1 and aldehyde dehydrogenase 1 family, member L1 (ALDH1L1) antibodies, respectively, while the negative control showed no positive staining (G–I). Bar = 25 μm. White arrows represented the positive cells.

Figure 2 | Cultured HSCs differentiate towards neurons and glia cells in vitro. Cultured HSCs differentiated toward neurons (A–C) and astrocytes (D–F), which were identified by immunofluorescent staining of Tuj1 and aldehyde dehydrogenase 1 family, member L1 (ALDH1L1) antibodies, respectively, while the negative control showed no positive staining (G–I). Bar = 25 μm. White arrows represented the positive cells.

Figure 3 | Transplanted HSCs survived and migrated in the host spinal cord. There are no positive HSCs with blue staining marked by Hoechst 33342 in the spinal cord without HSC transplantation. While, HSCs with blue staining in nucleus could be seen at the first month (A), third month (B) and sixth month post transected spinal cord injury (SCT) (C), indicating the survival and migration of the transplanted HSCs in the host spinal cord. (D) Bar chart for quantitative analysis of the survived HSCs in vivo using Image-Pro Plus 6.0 software. Data are presented as the mean ± SD (n = 3, one-way ANOVA). Bar = 50 μm. m, month.

Statistically significant. Data are expressed as mean ± standard deviation (SD).
Fate of HSCs in the Host Spinal Cord

There were no Hoechst-positive cells found in the SCT control group. Comparatively, HSCs with blue staining in the nucleus could be seen in HSC transplanted rats at the first month (Figure 3A), third month (Figure 3B) and sixth month (Figure 3C) post-operation. Implanted HSCs also migrated towards the caudal and rostral ends of the cord, and the number of surviving cells was approximately 90% of the total number of transplanted cells at sixth months post-SCT (Figure 3D).

Differentiation of HSCs Following Transplantation into the Host Spinal Cord

Implanted HSCs with blue staining labeled by Hoechst 33342 could be found in the host spinal cord, which confirmed the survival of HSCs (Figures 4A,D,G). Simultaneously, some HSCs exhibited positive Tuj1 staining (Figures 4B,C) or ALDH1L1 staining (Figures 4E,F). Quantitative analysis showed that 3.1% of the HSCs were positive for Tuj1 and 18.3% were positive for ALDH1L1. These data demonstrate that the transplanted HSCs can develop into astrocytes in vivo, with fewer neuronal differentiations. The negative control showed no positive staining (Figures 4G–I).

Behavioral Assessment

BBB scores from week 1 to 24 showed that SCT resulted in a complete locomotor function deficit, and that locomotor function could be partially restored over time. Comparatively, BBB scores in the HSC group were higher than in the SCT group from week 4 to 24 post-SCT. The behavioral evaluation from the BBB scores demonstrated that HSC transplantation dramatically improves the neurological function of SCT rats ($P < 0.05$, Figure 5).

**FIGURE 4** | Transplanted HSCs could differentiate into neurons and glia like cells in vivo. (A) Positive HSCs with blue staining in nucleus marked by Hoechst 33342 in the spinal cord could be seen after HSC transplantation. (B) A few of HSCs could differentiate into neurons with Tuj1 positive staining (red). (C) The merged picture of Tuj1 and Hoechst 33342, the positive ratio of Tuj1/Hoechst 33342 is about 3.1%. (D) Positive HSCs with Hoechst 33342 blue staining. (E) A few of HSCs could differentiate into astrocytes with ALDH1L1 positive staining (red). (F) The merged picture of ALDH1L1 and Hoechst 33342, which showed the positive ratio is about 18.3%. (G) Hoechst 33342 blue staining in the negative control (no primary antibody). (H) There is no positive red staining in the negative control (no primary antibody). (I) The merged picture. Bar = 50 µm. White arrows represented the positive cells. The positive ratio of Tuj1 (Tuj1/Hoechst 33342) and ALDH1L1 (ALDH1L1/Hoechst 33342) was quantified using Image-Pro Plus 6.0 software ($n = 7$).
Morphological Changes in the Spinal Cord following HSC Transplantation

The spinal cord appeared normal in shape, with a plum shape and normal volume, at both the rostral or caudal sides in the sham group at sixth months post-SCT (Figure 6A), while both the rostral and caudal spinal cord in the SCT group exhibited a narrow shape as compared with the sham group. A decreased volume at the center of the injury was
FIGURE 8 | HSC transplantation decreased the number of astrocytes. SCT greatly increased the number of GFAP+ cells in the rostral, scar and caudal sites of the transected cord (A–D). While HSCs transplantation effectively impressed the number of astrocytes in these segments (E–H). Histogram showed that the number of astrocytes in HSC transplanted group was lower than that of SCT one (I–L). (A,E,I) Scar center; (B,F,J) Posterior Funiculus of rostral scar; (C,G,K) gray matter of rostral scar; (D,H,I) Dorsal horn of caudal scar. Data are presented as the mean ± SD (n = 7). Student’s t test was used to analyze the data. Data in the sham group were not shown. *P < 0.05 vs. SCT. Bar = 50 µm.

most apparent (Figures 6B,C). In contrast, HSC transplantation markedly restored the volume of the spinal cord, and promoted the morphological recovery of the injured spinal cord (Figures 6B,D).

The Effect of HSC Transplantation on Neural Survival
Following SCT, the number and size of neurons in layer VIII and IX of the ventral horn became much fewer and smaller than in the sham and HSC group (Figures 7A–D). However, quantitative analysis showed that the cellular number and size in the HSC group was not significantly greater than in the SCT group (P > 0.05; Figures 7G,H), which indicated that administration of HSCs did not increase the number and size of neurons in the ventral horn (Figures 7E,F).

HSC Transplantation Suppressed Astrocyte Proliferation
To investigate whether astrocytes were changed after HSC transplantation, double staining of Hoechst 33342 and GFAP was performed in the injured center, and in the rostral and caudal regions 6 months post-SCT. SCT induced a marked increase in the number of GFAP+ cells in the injured center, and rostral and caudal ends of the spinal cord (Figures 8A–D,I–L), compared with the sham group.
This finding was confirmed in a previous observation (data not shown). Following HSC transplantation, the number of Hoechst 33342/GFAP-labeled cells effectively decreased in the observed areas (injured center, and rostral and caudal ends of the spinal cord), when compared with the SCT group ($P < 0.05$; Figures 8E–L).

**The Effect of HSC Transplantation on Oligodendrocytes**

Double staining of Hoechst 33342 and APC showed a distinct decrease in the number of oligodendrocytes in SCT rats compared with the sham group from a previous observation (sham data not shown, Figures 9A–D, I–L). On the contrary, in the HSC group, the number of oligodendrocytes was increased conspicuously when compared with the SCT group ($P < 0.05$; Figures 9E–L). The increase was observed in the scar area as well as neighboring spinal tissue, including the rostral and caudal cords (Figures 9E–L).

**Regeneration of 5-HT Fibers after HSC Transplantation**

5-HT fibers exhibited a weak regeneration capacity post-SCT compared with the sham group from a previous observation (data not shown). Moreover, 24 weeks post-SCT, the number of 5-HT-positive fibers had increased compared with the SCT group. Descending 5-HT fibers, known for transducing motor signals from the brain to the end effectors, regenerated approximately 2 cm into the scar area (Figure 10).
HSC Transplantation Promotes Functional Improvement Associated with Nerve Regeneration, Oligogenesis and Astrogliosis Inhibition

In this study, we found that HSCs transplanted into SCT rats could survive and migrate around the injured site and ameliorate behavioral deficits after SCT. We also found that the decrease in the spinal cord volume of HSC-treated rats was smaller than in SCT rats. It has been shown that the compromised transected tissue and the subsequent cavity formation are characteristics of progressive tissue necrosis following initial primary cell destruction in SCT (Park et al., 2012). Therefore, reduction of the structural injury or cavity volume indicates that the transplanted HSCs have a neuroprotective effect after SCT. Moreover, the results of the present study demonstrate the therapeutic effect of HSCs in chronic SCT rats, which differed from findings of previous studies (Deda et al., 2008; Sasaki et al., 2009). The morphological mechanisms for improving the functional deficits involved in promoting nerve regeneration and oligogenesis, together with astrogliosis inhibition, were shown.

In the previous studies involving animals, reactive astrogliosis was shown to promote the regeneration of severed axons, and that this may have occurred through glial scar-associated extracellular matrix proteins (GrandPré et al., 2000; Bradbury et al., 2002; Kamei et al., 2010, 2012). While the issue of whether reactive astrogliosis is beneficial or detrimental remains controversial, it may play a critical and necessary role in the early stages of destructive CNS processes, but may be harmful in latter stages by contributing to an inhibition of axonal regeneration. Previous studies have also shown that in the repair process of central nervous system damage, an excessive increase in astrocytes promotes the secretion of inhibitory molecules and the formation of fibrotic scarring in the injured spinal cord, which then prevents axonal or nerve regeneration (Frisén et al., 1995;
Yu et al., 2000; Shearer and Fawcett, 2001; Fields and Stevens-Graham, 2002. In the current study, we found that the BBB score was improved, astrogliosis was inhibited and 5-HT fibers were increased at 24 weeks post-SCT. These data suggest that the moderate decrease in the number of astrocytes following HSC transplantation may provide an opportunity for nerve regeneration. Our data indirectly show that reactive astrogliosis may be harmful in the latter stages of destructive CNS processes. As our observations lasted 6 months and a decreased number of astrocytes were found, our findings support the hypothesis that HSCs grafts have a protective role in chronic SCI, which is different from previous observations (Deda et al., 2008; Sasaki et al., 2009; Kamei et al., 2010).

Enhanced oligogenesis was also observed after HSC transplantation. It has been suggested that oligogenesis by and survival of endogenous oligodendrocyte progenitor cells (OPCs) can contribute to self-repair after myelin loss (Deda et al., 2008; Park et al., 2012). This finding indicates that oligodendrocytes are important myelin-forming cells responsible for the formation of myelin surrounding axons in the central nervous system. Previous studies have reported that transplanted peripheral blood stem cells mobilized by granulocyte colony-stimulating factor promote hindlimb functional recovery following SCI in mice by suppressing oligodendrocyte apoptosis (Takahashi et al., 2016). Similarly, in the current study, we found that HSC transplantation effectively increased the number of oligodendrocytes and 5-HT fibers in vivo. Therefore, the results revealed that HSC transplantation is very important for promotion of functional improvement after SCT by increasing the number of oligodendrocytes and 5-HT fibers. It has been shown that 5-HT fibers promote surviving nerve axons to extend their lateral branches towards damaged axons, and that oligodendrocytes can repair demyelinated CNS neurons and improve the function of spinal cord nerves (Sasaki et al., 2001; Bjugstad et al., 2005; Garbuzova-Davis et al., 2006; Leu et al., 2010), which further supports their role in the functional recovery of the transected spinal cord.

Although HSCs have been proposed as a potential source of neural cells for repairing brain lesions (Sigurjonssson et al., 2005), we found no significant changes in the number or size of neurons in the transected spinal cords treated with HSC transplantation in our research. The degeneration and atrophy of injured neurons post-SCT were difficult to evaluate (Dobkin and Havton, 2004). Moreover, HSC engraftment exhibited the potential to restore injured spinal cord and promote functional recovery, similar to narrow stromal cells in contusion conditions (Koda et al., 2005). The underlying mechanism was involved in promoting the oligogenesis and nerve regeneration from 5-HT fibers, as well as inhibiting excessive astrogliosis.

**Molecular Mechanism Involved in HSC Transplantation**

Although the roles of HSC transplantation have been preliminarily determined, the source of HSCs is still an obstacle for their wide application. Thus, the molecular mechanism of HSC transplantation must be explored in order to find alternative treatments. Previous studies have reported that VEGF, SDF1, CXCR4, PI3-K/Akt and MAPK signaling may be involved in protection following HSC transplantation (Isele et al., 2007; Deda et al., 2008). Based on the neuroprotective role of NT-3 and the MEK/ERK/CREB signaling pathway (Isele et al., 2007; Lebesgue et al., 2009; Li et al., 2016; Liu et al., 2016; Keefe et al., 2017), we speculate that HSC transplantation may promote functional improvement after SCT by increasing the expression of NT3 and MEK-1 signaling. Our findings show that SCT induced a significant decline in NT3 and MEK-1 mRNA levels, while HSC transplantation was able to restore this decrease. This indicates that upregulation of NT3 and MEK-1 signaling may be a potential molecular mechanism underlying protection following HSC transplantation. Therefore, activation of NT-3 or MEK-1 signaling may present a new alternative strategy for the treatment of SCI in future clinical practice.

**CONCLUSION**

Our study revealed that HSC transplantation promotes functional improvement in SCT rats and found possible mechanisms that are involved in morphological remodeling and the activation of NT-3 and MEK-1 signaling. These findings may contribute to the understanding of the mechanism of SCI and provide a novel treatment strategy for neural repair following SCI.

**AUTHOR CONTRIBUTIONS**

L-LX, FL, Q-JX and T-HW designed the experiments. L-LX, FL, S-KD, JL, Q-QD and YZ performed the experiments. L-LX, PZ and Q-JX analyzed the data. L-LX, FL and T-HW wrote the manuscript. All authors read and approved the final manuscript.

**ACKNOWLEDGMENTS**

This study was supported by the Grant of National Science foundation (No.81471268, No.81601074) and the Program Innovative Research Team in Science and Technology in Yunnan province and IRTSTYN.

**REFERENCES**

Abbaspazadeh, H. A., Tiraahi, T., Noori-Zadeh, A., Delshad, A. R., Sadeghizadeh, M., and Taheri, T. (2015). Human ciliary neurotrophic factor-overexpressing stable bone marrow stromal cells in the treatment of a rat model of traumatic spinal cord injury. *Cytotherapy* 17, 912–921. doi: 10.1016/j.jcyt.2015.03.689
Xiong et al. HSCs Transplantation Improves SCI

Bavaria, J. E., Appoo, J. J., Makaroun, M. S., Verer, J., Yu, Z. F., and Mitchell, R. S. (2007). Endovascular stent grafting versus open surgical repair of descending thoracic aortic aneurysms in low-risk patients: a multicenter comparative trial. J. Thorac. Cardiovasc. Surg. 133, 369–377. doi: 10.1016/j.jtcvs.2006.07.040

Bjugstad, K. B., Redmond, D. E. Jr., Teng, Y. D., Eklow, J. D., Roth, R. H., Blanchard, B. C., et al. (2005). Neural stem cells implanted into MPTP-treated monkeys increase the size of endogenous tyrosine hydroxylase positive cells/found in the striatum: a return to control measures. Cell Transplant. 14, 183–192. doi: 10.3727/00000000578398098

Boulland, J. L., Lambert, F. M., Züchner, M., Strom, and Glover, J. C. (2013). A neonatal mouse spinal cord injury model for assessing post-injury adaptive plasticity and human stem cell integration. PLoS One. 8:e71701. doi: 10.1371/journal.pone.0071701

Bracken, M. B. (2012). Steroids for acute spinal cord injury. Cochrane Database Syst. Rev. 1CD001046. doi: 10.1002/14651858.cd001046.pub2

Burt, R. K., Burns, W., and Hess, A. (1995). Bone marrow transplantation for multiple sclerosis. Bone Marrow Transplant. 16, 1–6.

Burt, R. K., Loh, Y., Pearce, W., Barr, W. G., Craig, R., et al. (2004). Basic advances and new data using real-time quantitative PCR and the 2-\(\Delta\Delta\)CT method. Methods 25, 402–408. doi: 10.1006/meth.2001.1262

Coutts, M., and Keirstead, H. S. (2008). Stem cells for the treatment of spinal cord injury. Exp. Neurol. 209, 368–377. doi: 10.1016/j.expneurol.2007.09.002

Cochrane Database Syst. Rev. 1:CD001046. doi: 10.1002/14651858.cd001046.pub2

Deda, H., Inci, M. C., Kürekci, A. E., Kayihan, K., Ozgün, E., Ustünsoy, G. E., et al. (2015). Effectiveness of repeated transplantations of hematopoietic stem cells in spinal cord injury. World J. Transplant. 5, 110–128. doi: 10.5550/wjt.v5.i3.110

Fields, R. D., and Stevens-Graham, B. (2002). New insights into neuron-glia avenues in therapy of spinal cord injury. Annu. Rev. Med. 53, 169–177. doi: 10.1146/annurev.med.53.091902.104338

Feng, S. Q., Kong, X. H., Guo, S. F., Wang, P., Li, L., Zhong, J. H., et al. (2005). MicroRNA-127 targeting of mitoNEET inhibits neurite outgrowth, induces cell apoptosis and contributes to physiological dysfunction after spinal cord transection. Sci. Rep. 6:35205. doi: 10.1038/srep35205

Feng, S. Q., Kong, X. H., Guo, S. F., Wang, P., Li, L., Zhong, J. H., et al. (2005). MicroRNA-127 targeting of mitoNEET inhibits neurite outgrowth, induces cell apoptosis and contributes to physiological dysfunction after spinal cord transection. Sci. Rep. 6:35205. doi: 10.1038/srep35205

Hiran, K., Wagner, K., Mark, P., Pittermann, E., Gabel, R., Furlani, D., et al. (2012). Erythropoietin attenuates the sequel of ischaemic spinal cord injury with enhanced recruitment of CD34\(^+\) cells in mice. J. Cell. Mol. Med. 16, 1792–1802. doi: 10.1111/j.1528-415X.2011.01898.x

Huang, H. Y., Chen, L., and Sanberg, P. (2010). Cell therapy from bench to bedside translation in CNS neuroregeneration era. Cell Med. 1, 15–46. doi: 10.3390/ijms10030567

Isele, N. B., Lee, H. S., Landshamer, S., Straube, A., Padovan, C. S., Pleasnla, N., et al. (2007). Bone marrow stromal cells mediate protection through stimulation of P13-K/Akt and MAPK signaling in neurons. Neurochem. Int. 50, 243–250. doi: 10.1016/j.neuti.2006.08.007

Kamei, N., Kwon, S. M., Alev, C., Ishikawa, M., Yokoyama, A., Nakashii, K., et al. (2010). Link deletion reinforces the function of bone marrow progenitors in promoting neurovascularization and astroglial following spinal cord injury. Stem Cells 28, 365–375. doi: 10.1002/stem.243

Kamei, N., Kwon, S. M., Kawamota, A., Ji, M., Ishikawa, M., Ochi, M., et al. (2012). Contribution of bone marrow-derived endothelial progenitor cells to neovascularization and astroglial following spinal cord injury. J. Neurosci. Res. 90, 2281–2292. doi: 10.1002/jnr.23113

Keeffe, K. M., Sheikh, I. S., and Smith, G. M. (2017). Targeting neurotrophins to specific populations of neurons: NGF, BDNF, and NT-3 and their relevance for treatment of spinal cord injury. Int. J. Mol. Sci. 18:E548. doi: 10.3390/ijms18030548

Koda, M., Okada, S., Nakayama, T., Koshizuka, S., Kamada, T., Nishiy, Y., et al. (2005). Hematopoietic stem cell and marrow stromal cell for spinal cord injury in mice. Neuroreport 16, 1763–1767. doi: 10.1097/01.wnr.0000183329.05994.d7

Koshizuka, S., Okada, S., Akawa, O., Koda, M., Murasawa, H., Hashimoto, M., et al. (2004). Transplanted hematopoietic stem cells from bone marrow differentiate into neural lineage cells and promote functional recovery after spinal cord injury in mice. J. Neurotrauma. Exp. Neurol. 263, 64–67. doi: 10.1093/jenm/63.1.64

Levesque, D., Chevalerey, V., Zukin, R. S., and Etgen, A. M. (2009). Estradiol rescues neurons from global ischemia-induced cell death: multiple cellular pathways of neuroprotection. Steroids 74, 555–561. doi: 10.1016/j.steroids.2009.01.003

Leu, S., Liu, Y. C., Yuen, C. M., Yen, C. H., Kao, Y. H., Sun, C. K., et al. (2010). Adipose-derived mesenchymal stem cells markedly attenuate brain infarct size and improve neurological function in rats. J. Transl. Med. 8:63. doi: 10.1186/1471-2210-8-63

Li, G., Che, M. T., Zhang, K., Qin, L. N., Zhang, Y. T., Chen, R. Q., et al. (2016). Graft of the NT-3 persistent delivery gelatin sponge scaffold promotes axon regeneration, attenuates inflammation, and indu. Biomaterials 31, 233–248. doi: 10.1016/j.biomaterials.2015.11.059

Liu, Y., Kelamangalath, L., Kim, H., Han, S. B., Tang, X., Zhai, J., et al. (2016). NT-3 promotes proangiogenic axon regeneration when combined with activation of the mTOR intrinsic growth pathway but not with reduction of myelin extrinsic inhibitors. Exp. Neurol. 283, 73–84. doi: 10.1016/j.expneurol.2016.05.021

Liu, R., Zhao, W., Zhao, Q., Liu, S. J., Liu, J., He, M., et al. (2014). Endoplasmic reticulum protein 29 protects cortical neurons from apoptosis and promoting corticospinal tract regeneration to improve neural behavior via caspase3 and Erk signal in rats with spinal cord transection. Mol. Neurobiol. 50, 1035–1048. doi: 10.1007/s12035-014-8861-1

Liva, K. J., and Schmittenegger, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-\(\Delta\Delta\)CT method. Methods 25, 402–408. doi: 10.1006/meth.2001.1262

Mendez, I., Sanchez-Pernaute, R., Cooper, O., Vinuea, A., Ferrari, D., Bjorklund, L., et al. (2005). Cell type analysis of functional fetal dopamine cellsuspension transplants in the striatum and substantianguina of patients with Parkinson’s disease. Brain 128, 1498–1510. doi: 10.1093/brain/awh310

Mothe, A. J., and Tator, C. H. (2012). Advances in stem cell therapy for spinal cord injury. J. Clin. Invest. 122, 3824–3834. doi: 10.1172/JCI64124
Müller, A., Hauk, T. G., Leibinger, M., Marienfeld, R., and Fischer, D. (2009). Exogenous CNTF stimulates axon regeneration of retinal ganglion cells part-tially via endogenous CNTF. Mol. Cell. Neurosci. 41, 233–246. doi: 10.1016/j.mcn.2009.03.002

Niccaise, C., Mitrecic, D., Falnikar, A., and Lepore, A. C. (2015). Transplantation of stem cell-derived astrocytes for the treatment of amyotrophic lateral sc-erosis and spinal cord injury. World J. Stem Cells 7, 380–398. doi: 10.4252/wjsc.v7.i2.380

Nishio, Y., Kodama, T., Someya, Y., Yoshinaga, K., Okada, S., et al. (2006). The use of hemopoietic stem cells derived from human umbilical cord to promote restoration of spinal cord tissue and recovery of hindlimb function in adult rats. J. Neurosurg. Spine 5, 424–433. doi: 10.3171/spi.2006.5.5.424

Park, S. I., Lim, J. Y., Jeong, C. H., Kim, S. M., Jun, J. A., Jeun, S. S., et al. (2012). Human umbilical cord blood-derived mesenchymal stem cell therapy promotes functional recovery of contused rat spinal cord through enhancement of endogenous cell proliferation and oligogenesis. J. Biomed. Biotechnol. 2012:362473. doi: 10.1155/2012/362473

Sasaki, M., Honmou, O., Akiyama, Y., Uede, T., Hashi, K., and Kocsis, J. D. (2001). Transplantation of an acutely isolated bone marrow fraction repairs demyelinated adult rat spinal cord axons. Glia 35, 26–34. doi: 10.1002/glia.1067

Sasaki, H., Ishikawa, M., Tanaka, N., Nakaniishi, K., Kamei, N., Ashara, T., et al. (2009). Administration of human peripheral blood-derived CD133+ cells accelerates functional recovery in a rat spinal cord injury model. Spine 34, 249–254. doi: 10.1097/BRS.0b013e3181913cde

Schwarting, S., Litwak, S., Hao, W., Bähr, M., Weise, J., and Neumann, H. (2008). Hematopoietic stem cells reduce post ischemic inflammation and ameliorate ischemic brain injury. Stroke 39, 2867–2875. doi: 10.1161/STROKEAHA.108.513978

Shearer, M. C., and Fawcett, J. W. (2001). The astrocyte/meningeal cell interfacebarrier to successful nerve regeneration. Cell Tissue Res. 305, 267–273. doi: 10.1007/s004410100384

Sigurjonsson, O. E., Perreault, M. C., Egeland, T., and Glover, J. C. (2005). Adulthuman hematopoietic stem cells produce neurons efficiently in the regenerating chicken embryo spinal cord. Proc. Natl. Acad. Sci. U S A 102, 5227–5232. doi: 10.1073/pnas.0501029102

Singh, S. (2015). Stem cell therapy in spinal trauma: does it have scientific validity? Indian J. Orthop. 49:485. doi: 10.4103/0019-5413.159683

Takahashi, H., Koda, M., Hashimoto, M., Furuya, T., Sakuma, T., Kato, K., et al. (2016). Transplanted peripheral blood stem cells mobilized by granulo-cyte colony-stimulating factor promoted hindlimb functional recovery after spinal cord injury in mice. Cell Transplant. 25, 283–292. doi: 10.3727/096368915x688146

Tederko, P., Krasuski, M., Kiwerski, J., Nyka, L. and Bialoszewski, D. (2009). Strategies for neuroprotection following spinal cord injury. Ortop. Traumatol. Rehabil. 11, 103–110.

Thakkar, U. G., Vanikar, A. V., Trivedi, H. L., Shah, V. R., Dave, S. D., Dixit, S. B., et al. (2016). Infusion of autologous adipose tissue derived neuronal differentiated mesenchymal stem cells and hematopoietic stem cells in post-traumaticparaplegia offers viable therapeutic approach. Adv. Biomed. Res. 5:51. doi: 10.4103/2277-9175.178792

Tyndall, A., Fassas, A., Passweg, J., Ruiz de Elvira, C., Attal, M., Brooks, P., et al. (1999). Autologous hematopoietic stem cell transplants for autoimmune disease-feasibility and transplant-related mortality. Bone Marrow Transplant. 24, 729–734. doi: 10.1038/sj.bmt.1701987

Vawda, R., and Fehlings, M. G. (2013). Mesenchymal cells in the treatment of spinal cord injury: current & future perspectives. Curr. Stem Cell Res. Ther. 8, 25–38. doi: 10.2174/1574889x11308010005

Xu, X. M., and Onifer, S. M. (2009). Transplantation-mediated strategies to promote axonal regeneration following spinal cord injury. Respir. Physiol. Neurobiol. 169, 171–182. doi: 10.1016/j.resp.2009.07.016

Yu, W. R., Westergren, H., Farooque, M., Holtz, A., and Olsson, Y. (2000). Syste-mic hypothermia following compression injury of rat spinal cord an immunohistochemical study on MAP2 with special reference to dendrite changes. Acta Neuropathol. 100, 546–552. doi: 10.1007/s004010000206

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Xiong, Liu, Deng, Liu, Dan, Zhang, Zou, Xia and Wang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.