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# Neural Stem Cell Transplantation Improves Locomotor Function in Spinal Cord Transection Rats Associated with Nerve Regeneration and IGF-1 R Expression

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## Abstract

Transplantation of neural stem cells (NSCs) is a potential strategy for the treatment of spinal cord transection (SCT). Here we investigated whether transplanted NSCs would improve motor function of rats with SCT and explored the underlying mechanism. First, the rats were divided into sham, SCT, and NSC groups. Rats in the SCT and NSC groups were all subjected to SCT in T10, and were administered with media and NSC transplantation into the lesion site, respectively. Immunohistochemistry was used to label Nestin-, TUNEL-, and NeuN-positive cells and reveal the expression and location of type I insulin-like growth factor receptor (IGF-1 R). Locomotor function of hind limbs was assessed by Basso, Beattie, Bresnahan (BBB) score and inclined plane test. The conduction velocity and amplitude of spinal nerve fibers were measured by electrophysiology and the anatomical changes were measured using magnetic resonance imaging. Moreover, expression of IGF-1 R was determined by real-time polymerase chain reaction and Western blotting. The results showed that NSCs could survive and differentiate into neurons in vitro and in vivo. SCT-induced deficits were reduced by NSC transplantation, including increase in NeuN-positive cells and decrease in apoptotic cells. Moreover, neurophysiological profiles indicated that the latent period was decreased and the peak-to-peak amplitude of spinal nerve fibers conduction was increased in transplanted rats, while morphological measures indicated that fractional anisotropy and the number of nerve fibers in the site of spinal cord injury were increased after NSC transplantation. In addition, mRNA and protein level of IGF-1 R were increased in the rostral segment in the NSC group, especially in neurons. Therefore, we concluded that NSC transplantation promotes motor function improvement of SCT, which might be associated with activated IGF-1 R, especially in the rostral site. All of the above suggests that this approach has potential for clinical treatment of spinal cord injury.

## Keywords

spinal cord transection, neural stem cells, transplantation, insulin-like growth factor 1 receptor, motor function

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## Introduction

Spinal cord injury (SCI) is a severe condition that can lead to serious physical and psychological dysfunction in patients, and which creates significant human health and economic burdens for society[1]. At present, treatments for SCI are focused on enhancing nerve regeneration and synaptic remodeling to restore neurological function, and therapies based on transplantation of neural stem cells (NSCs) have shown promise in experimental research involving central nervous system (CNS) diseases in recent years[1,2].

It is known that NSCs have the capacity to self-renew, proliferate, and differentiate[3]. The major function of NSCs is to replace apoptotic and dead cells and/or facilitate recovery after injury, as reserve cells in the CNS. A number of studies have shown that NSCs can differentiate into neurons, astrocytes, and oligodendroglia, and promote synaptic growth by producing growth factors[4–8]. However, the underlying mechanism of NSC transplantation after SCI remains unclear.

Neurotrophic factors, a group of polypeptide substances, regulate cell growth and other cellular functions by binding with their specific receptors[9,10]. Insulin-like growth factor (IGF), a growth factor, has a vital catalytic role in the differentiation and proliferation of cells and in the development of organisms. It was shown that the increase of IGF and its receptor IGF-1 R would enhance recovery from injury after SCI[11]. Meanwhile, IGF-1 R is considered essential for axonal regeneration of neurons in the adult CNS[12]. In addition, a recent study has shown that extracellular vimentin interacts with IGF-1 R to promote axonal growth[13]. These studies suggest that IGF-1 R is a crucial functional molecule in the process of SCI repair.

In this study, we investigated the effect of NSC transplantation on the repair of motor function in spinal cord transection (SCT) rats and explored whether the underlying mechanism was related to change in levels of IGF-1 R.

## Materials and Methods

### Isolation, Culture, and Identification of NSCs

NSCs have been found in many parts of the body, including cerebral cortex, subependymal layer, striatum, hippocampus, and midbrain[14,15]. In this study, we extracted NSCs from the hippocampus of neonatal Sprague-Dawley (SD) rats (in 24 h after birth) for the treatment of SCT[16]. The brains were harvested and washed in D-Hanks' solution. The hippocampi were isolated and were cut into fragments of about 1 $mm^3$. These fragments were triturated and the cells were suspended in a serum-free medium consisting of DMEM/F12 (1:1, Gibco Laboratories, Grand Island, NY, USA), B27 (Gibco Laboratories), and bFGF (20 ng/ml, Gibco Laboratories). The 500 µl cell suspensions at a density of $5\times10^5$ cells/ml were then incubated in 24-well plates for neurosphere formation at 37°C with 5% $CO_2$. The NSCs were randomly separated into three groups for subsequent experiments. First, the NSCs in the first group were labeled with anti-Nestin primary antibody by immunoenzymatic histochemistry (see below). Second, the NSCs of the second group were used for identification of NSC differentiation in vitro. They were transferred into 24-well plates covered with polylysine to incubate for 7 days in the DMEM/F12 medium containing 10% fetal bovine serum, anti-NeuN primary antibody was used to recognize the NSC differentiation by immunoenzymatic histochemistry (see below). Finally, the NSCs in the last group were transfected with GFP to emit green fluorescence, and then transplanted into the lesion sites to confirm the survival and differentiation of NSCs *in vivo*. Note that the transfection method is shown in the Supplementary Materials, and the transfection efficiency is shown in Supplementary Fig. 1.

### Animal Grouping and Surgery Procedure

Adult SD rats, weighing 180–220 g, were purchased from the Experimental Animal Center of Kunming Medical University and used for this study. All surgical and sampling procedures were done after anesthesia achieved by intraperitoneal (IP) injection of 3.6% chloral hydrate solution (1 ml/100 g). The animal grouping and numbers in each procedure are summarized in Table 1. Ethical approval to report this case series was obtained from the Ethical Committee of Kunming Medical University (reference number: kmmu 2018016), and all procedures in this study were conducted in accordance with the approved protocols from the Ethical Committee of Kunming Medical University (reference number: kmmu 2018016).

Completed SCI was performed as described previously[17,18]. Briefly, a laminectomy was performed at thoracic vertebra level 9–11 (T9–11) to expose the T10 spinal segment. Then, the whole spinal cord was gently lifted up by hooked microtweezers, and was then completely transected with surgical scissors. Afterwards, the spinal cord was returned to its original position. To reconfirm that the spinal cord was completely transected, microtweezers were used to pick up the rostral segment of the spinal cord and it was observed that no nerve fibers were connected to the caudal segment of spinal cord. The rats in the sham group underwent the same procedure, in which the spinal cord was exposed at vertebral levels T9–T11 without any injury to the spinal cord. After the operation, all the rats received daily injections of benzyl-penicillin (dosage 40 IU) for 3 days. Manual bladder evacuation was administrated three times daily.

### NSC Transplantation

In our study, NSC transplantation included two parts. In part one, the NSCs carrying GFP were transplanted for confirmation of the survival and differentiation of transplanted NSCs in vivo as described in the "Isolation, Culture, and Identification of NSCs" section. After NSC transplantation for

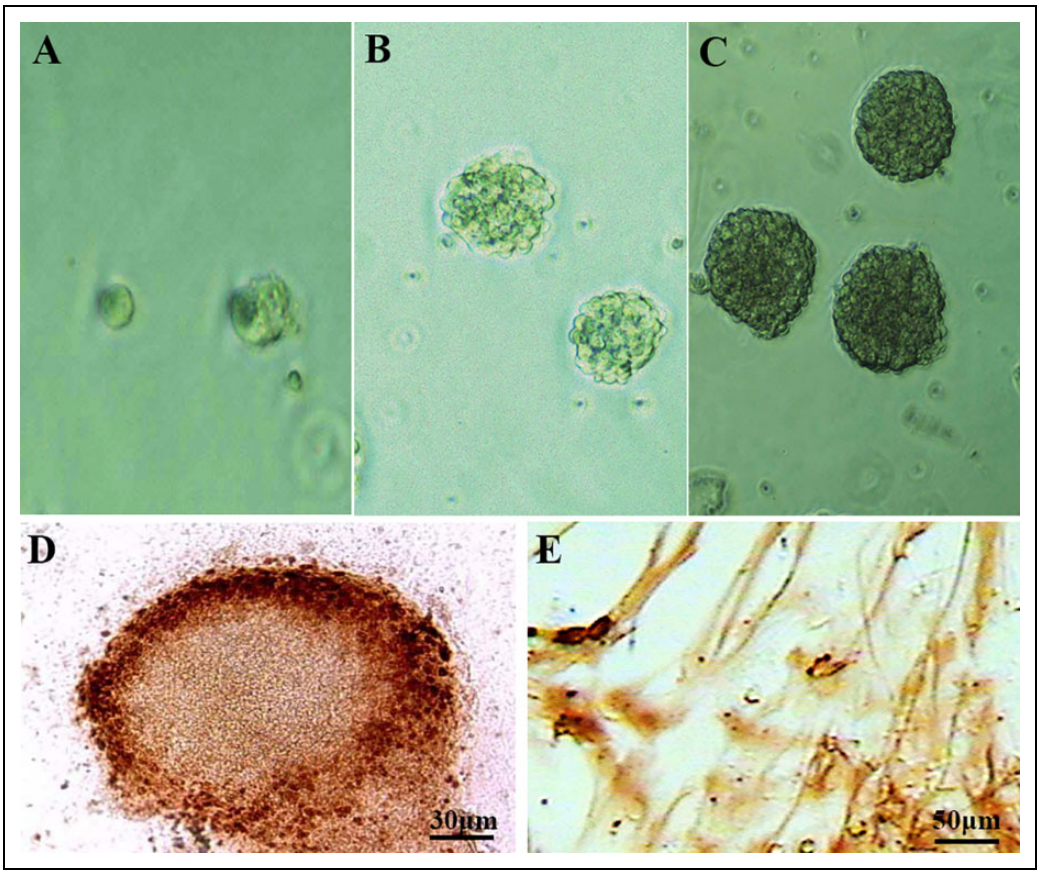


**Figure 1.** Characterization of NSCs in vitro. (A) NSCs were small, round, and non-fluorescent after isolation from rat hippocampus and (B) formed neurospheres in 3 days of culture, (C) and became tighter and larger after 5 days. (D) nestin+ neurosphere formation. (E) NSCs differentiated into neurons. Scale bars are 30 μm in (A, B, C, D) and 50 μm in (E). NSC, neural stem cell.

**Table 1.** Animal Numbers in Each Procedure.

| Group | BBB (7d, 14d and 21 d) | IHC (14 d) | PCR (1d, 3d, 7d, 14d) /WB (14d) |
|---|---|---|---|
| Sham | 6 | 6 | 20 |
| SCT | 6 | 6 | 20 |
| NSC | 6 | 6 | 20 |

14 days, the tissues in the transplanted area were harvested. Here the cells containing GFP were the surviving transplanted NSCs. In order to evaluate the differentiation of NSCs into neurons, the tissues were incubated with primary antibody NeuN (1:50, rabbit, ZSGB, Beijing, China) and goat anti-rabbit secondary antibody, Alexa fluor 594 (1:200, anti-rabbit, ZSGB) (see below for details about immunofluorescence histochemistry).

In part two, the transplanted NSCs, without the GFP, were used to assess their functional efficacy when transplanted into the SCI. Based on previous studies, NSCs at passage two were used because of their high purity and cell viability, and were transplanted on the 7th day after SCT[6,19]. Before transplantation, NSCs were trypsinized with 0.125% trypsin for 5 min, and gently titrated into medium containing serum to inactivate the trypsin. The cell suspension was collected by gently pelleting the cells and followed by a low-speed centrifugation (900*g*). Then, the NSCs were washed three times. After that, they were resuspended with DMEM/F-12 to acquire a cell suspension at a final concentration of $1\times10^7$ cells/ml. Trypan blue was used to assess their viability. The suspension was kept on ice and gently triturated before each injection to keep the suspension dispersed and free of cell clumps. In the NSC group, 5 μl suspension (10,000 NSCs/μl) was separately injected in caudal and rostral segments from the lesion site 2 mm from the SCI with a 22 gauge, 10 μl Hamilton syringe and delivered at a rate of 0.5 μl/min. The needle was left in for 10 min after injection. SCT animals received only 5 μl injections of the media in the same location and by the same procedure. All transplanted rats were received a daily IP injections of cyclosporine A (1.5 mg into 0.3 ml per rat; BioVision, San Francisco, CA, USA) starting 3 days before transplantation and continuing to the end of the experiments in order to suppress the immunologic rejection of the transplants.

### Inclined Plane Test

The inclined plane test was used to detect the ability of the animals to maintain their body position on an inclined board. Each rat was placed on the inclined plane (length 30 cm, width 20 cm), which was tilted slowly, and the maximum angle at which the rats could hold their position for 5 s was considered as the final incline. This experiment was assessed by two independent reviewers, who were blinded to the group identity of the rats, and was repeated three times with an interval of more than 1 h between the two trials. Finally, the average data of the three trials was obtained. All animals were assessed 4 weeks after SCT[20,21].

### Basso, Beattie, Bresnahan Scores

Hind-limb locomotor function of rats in each group was evaluated by the Basso, Beattie, Bresnahan (BBB) score on days 7, 14, and 21 after transplantation[22]. Animals were allowed to walk around freely in an open-field (45 cm×60 cm rectangular tray) for 5 min and the movement of hind limbs was closely observed. According to the scoring system, the evaluation included frequency and quality of hind-limb movement. Three evaluators, who were blinded to the group identity of the rats, performed the evaluation. The average of the three scores from the three observers was calculated. All behavioral evaluations were performed daily at 8–9 am. Moreover, the bladders were evacuated before testing, as spontaneous bladder contraction often affects hind-limb activity.

### Electrophysiology

Two weeks after cell transplantation, motor evoked potentials (MEPs) of the animals ($n = 6$ for each group) were recorded to assess motor nerve conduction by MP150 Data Acquisition Analysis System (BIOPAC, Goleta, CA, USA)[23]. Briefly, after injections of the intravenous anesthetic 3.6% chloral hydrate (1 ml/100 g), the sciatic nerve and sensorimotor cortex at 2 mm lateral to the midline and 2 mm posterior to the bregma were exposed. The stimulating electrode and recording electrode were connected to the sensorimotor cortex and sciatic nerve, respectively. In addition, the ground wire was attached to the rat's tail. MEPs were elicited by electrical stimulation of the sensorimotor cortex. Single pulse stimulation of 50 ms duration was employed. A voltage was adjusted to produce the maximum amplitude of MEPs. Under normal conditions, it was appropriate to adjust the voltage to 5 V. After that, the amplitude and latency of MEPs were obtained.

### Magnetic Resonance Imaging (MRI) Acquisition and Data Processing

A T2-weighted scan was employed to detect the connection of the caudal and rostral segments in the lesion site at 2 weeks post-transplantation. All the rats were anesthetized with 2% isoflurane in air/$O_2$ (4/1) and detected with MRI on a 7.0-T/30 cm magnetic resonance system (Bruker, Pharma Scan, Karlsruhe, Germany). The T2-weighted scan was obtained by a rapid acquisition of relaxation enhancement pulse sequence with a repetition time (TR) of 2000 millisecond (msec), an effective echo time (TE) of 32.2 msec, 8 echoes per image, two averages, 30 coronal sections at 1.5 mm thickness, providing a field of view of 3.0×2.5 cm, and an in-plane resolution of 117 μm×98 μm. For DTI data, conventional pulsed gradient spin echo acquisition with a diffusion-weighted spin echo planar imaging (DW–EPI) sequence was employed. Then, the fractional anisotropy (FA), number, and length of nerve fibers in the site of SCI were measured using TrackVis software[24].

### Tissue Collection of RT-PCR and Western Blot

To investigate the mRNA and protein expression of IGF-1 R, the SD rats were perfused with normal saline through the heart until their livers became white and then a 1 cm section of spinal cord from the rostral and the caudal segments close to the transection site was obtained from rats on days 1, 3, 7, and 14 post-transplantation. All of these tissues were stored in an ultrafreezer at –80°C.

### Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The obtained samples of spinal cord from the rostral and the caudal segments closed to the transection were used for RT-PCR test. Total RNA was extracted with TRIZOL reagent (Gibco Life Technologies, Rockville, MD, USA) in accordance with the manufacturer's protocol and eluted in 20 μl of RNase-free water. RNase-free DNase I was used to remove DNA contamination. The obtained RNA sample was kept on ice and the concentration was measured in a Nanodrop spectrophotometer (ND-1000). Then an equal amount of RNA (4 μg) was used for cDNA synthesis by Revert AidTM First Strand cDNA Synthesis Kit (Fermentas Company, Waltham, MA, USA). After that, RT-PCR for IGF-1 R mRNA was performed with the PCR Master Mix Kit (Fermentas Company) and β-actin was used as a reference. In the reaction system of RT-PCR, the primers were synthesized by TaKaRa Company (Beijing, China) as follows: IGF-1 R (227 bp), forward: 5′ AAACGCTGACCTCTGTTACCT 3′ and reverse: 5′ CGTAGCCAGTCACCACCTC 3′; β-actin (256 bp), forward: 5′ GTAAAGACCTCTATGCCAACA 3′ and reverse: 5′ GGACTCATCGTACTCCTGCT 3′. For cDNA amplification, the reaction conditions were as follows: 30 cycles, and each cycle consisted of denaturation at 94°C for 20 s, annealing at 55°C for 10 s, and extension at 72°C for 30 s. Then, the RT-PCR products were electrophoresed in 1% agarose gel stained with ethidium bromide and visualized using an ultraviolet gel imager (Bio-Rad Laboratories, Inc., Richmond, CA, USA). Image analysis was done by Quantity One software (Bio-Rad Laboratories).

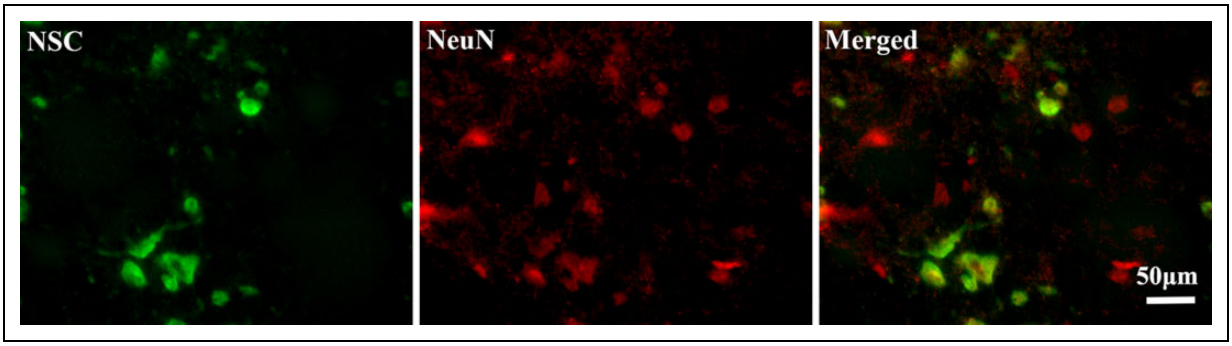


**Fig 2.** Survival and differentiation of NSC in vivo. Cells emitting green fluorescence were found in spinal cord tissue, confirming these are transplanted NSCs. The differentiation of NSCs into neurons exhibiting NeuN marker and emitting red fluorescence was indentified in host spinal cord.

The gray scale of each objective band was detected and expressed by relative intensity to the level of β-actin.

### *Western Blot*

The tissue samples from the rostral cord and the caudal cord segments that were obtained at 14 days after cell transplantation were dissociated with RIPA lysate and ground with pestles on ice. Then the dissociated mixtures were centrifuged at 12,000 rpm for 30 min. The supernatant was obtained and stored at –80°C for later use. Protein concentration was assayed with BCA kit (Sigma, St. Louis, MO, USA). After protein concentration determination, a 20 μl aliquot of the samples was loaded into each lane and electrophoresed on 10% SDS-polyacrylamide gel (SDS-PAGE) at a constant voltage of 100 V for 1.5 h. Proteins were transferred from the gel to a PVDF membrane at 20 V for 18 min. The membrane was blocked at room temperature for 1 h with 1×TBS (Tris-buffered saline: 50 mM Tris, 150 mM NaCl, pH 7.6) containing 5% skim milk. Then the membrane was incubated with the primary antibody of IGF-1 R (1:1,000, Rabbit, Santa Cruz Biotechnology, Santa Cruz, CA, USA) on the shaker at 4°C overnight. After washing three times for 10 min/time, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG (1:5,000; Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature, and then washed as described above. The membranes were photographed by Molecular Imager ChemiDoc$^{TM}$ XSR+ Imaging System (Bio-Rad Laboratories) and the band intensity was quantified by ImageJ software (NIH, Bethesda, MD, USA). The relative quantification of IGF-1 R protein was achieved with β-actin (1:500, Santa Cruz Biotechnology) as an internal control.

### *Immunohistochemistry and TdT-Mediated dUTP Nick End Labeling (TUNEL) Assay*

Immunoenzymatic histochemistry was performed to identify the NSCs and neurons (see Fig. 1D and E). Briefly, the cultured NSCs and neurons were rinsed with 0.01 M PBS and soaked in PBS containing 3% $H_2O_2$ for 30 min at room temperature. Subsequently, they were immersed in PBS containing 5% goat serum and 0.3% TritonX-100 solution at 37°C for 30 min, and then incubated at 4°C for 24 h with anti-Nestin primary antibody (1:200, rabbit, Millipore Bioscience Research Reagents, Temecula, CA, USA) and anti-NeuN primary antibody (1:50, mouse, ZSGB), respectively. After washing with 0.01 M PBS, the NSCs were incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody (1:100 dilution, Zhongshan Golden Bridge Biotechnology, Beijing, China) and neurons were incubated with HRP-labeled goat anti-mouse secondary antibody (1:100 dilution, Zhongshan Golden Bridge Biotechnology) for 1 h at 37°C, respectively. After washing three times, positive immunoreactivity was visualized as brown staining using diaminobenzidine (DAB) as substrate for 5 min.

Immunofluorescence histochemistry was used to quantify TUNEL- and NeuN-positive cells and show the expression and localization of IGF-1 R in this study (see Fig. 2, 3, 7). After anesthesia with 3.6% chloral hydrate (1 ml/100 g), rats at 14 days post-transplantation (21 day after injury, 21 DPI) were perfused with 200 ml of normal saline for 5 min, followed by 200 ml of 4% paraformaldehyde solution for 10 min. Referring to previous research, a 1.0 cm long spinal cord segment was harvested from the center of the lesion site as the center[25], postfixed for 6–12 h, and then immersed overnight in 0.1 M PBS containing 30% sucrose, until the specimen sank to the bottom of the bottle. Because the distance from the dorsal side of the spinal cord to the center of corticospinal tract was about 700 μm[26], 20 sections (with a thickness was 35 μm) from the dorsal side were first cut coronally to get access to the corticospinal tract, and then sections of 10 μm thickness were coronally cut in a freezing microtome (Leica CM1900, Watzlar, Hesse-Darmstadt, Germany). Next, the sections were rinsed three times for 5 min each with 0.01 M PBS, and immersed in 0.01 M PBS containing 5% goat serum and 0.3% TritonX-100 solution at 37°C for 30 min. First, for TUNEL and NeuN double staining, the sections were subsequently incubated at 4°C

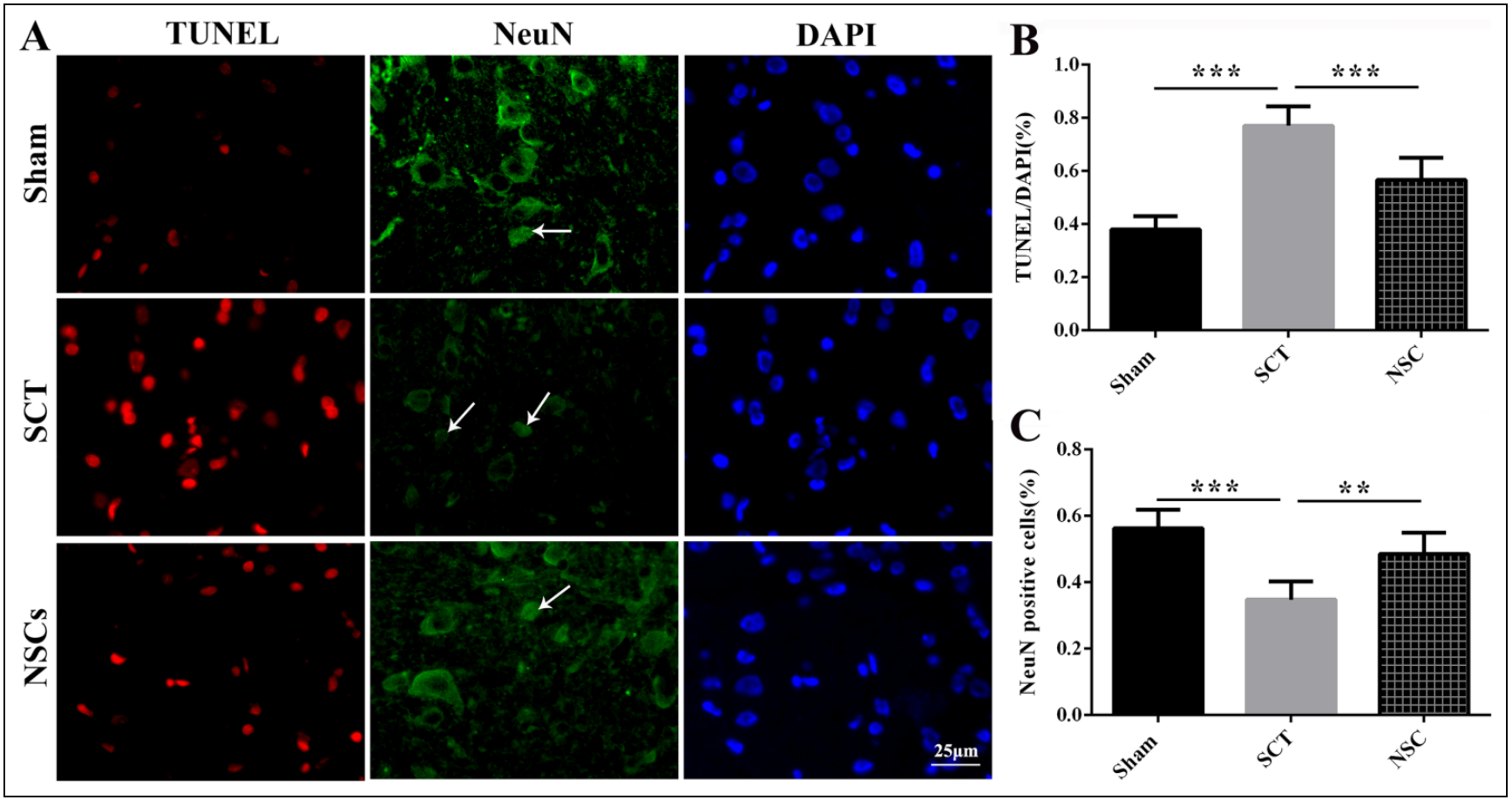


**Figure 3.** The effect of NSC transplantation. (A, B) TUNEL staining showed that neuronal apoptosis increased significantly after SCT, whereas transplantation of NSC had a reverse effect on neuronal apoptosis. Data represented as mean ± standard deviation (SD) (***$p < 0.001$) with $n=6$ using one-way ANOVA with LSD(L) post-test comparison in Fig 3. B, in which F(2,15) is 46.919. (A, C) The number of neurons labeled by NeuN in host spinal cord significantly decreased following SCT when compared with sham group. However, transplantation of NSC increased the neuron number. Data represented as mean ± SD (**$p < 0.01$, ***$p < 0.001$) with $n=6$ using one-way ANOVA with LSD(L) post-test comparison in Fig 3. C, in which F(2,15) is 20.657.

overnight with 2% goat serum containing primary antibody NeuN (1:50, mouse, ZSGB). Then they were washed three times (5 min each time) in 0.01 M PBS containing 0.1% Tween-20 (PBST), and incubated with the TUNEL reaction mixture (consisting of 50 μl TdT and 450 μl fluorescein-labeled dUTP solution) and goat anti-mouse secondary antibody, Alexa fluor 488 (1:100, anti-mouse, Invitrogen, Waltham, MA, USA) at 37°C for 1 h. Second, for NeuN and IGF-1 R double staining, the sections were incubated at 4°C overnight with primary antibody NeuN (1:50, mouse, ZSGB) and IGF-1 R (1:250, rabbit, Santa Cruz). Similarly, they were then washed three times (5 min each time) in 0.01 M PBS containing 0.1% Tween-20 (PBST), and incubated with goat anti-mouse secondary antibody, Alexa fluor 488 (1:100, anti-mouse, Invitrogen) and goat anti-rabbit secondary antibody, Alexa fluor 594 (1:200, anti-rabbit, ZSGB) at 37°C for 60 min. After that, the sections were again rinsed three times, each for 5 min in 0.01 M PBST. Finally, the nucleus was stained with DAPI. Negative controls were performed by replacing the primary antibody with 2% goat serum to ascertain the specificity of staining. Immunoreactive products were observed and photographed with a light microscope (Leica, DM4000B, Wetzlar, Germany) coupled with a computer-assisted video camera. After image capture, five fields at 200× magnification were selected from each rat to measure TUNEL- and NeuN-positive cells by Image J software (NIH), and the mean value across the five fields was calculated.

### Statistical Analysis

Data are expressed as the mean ± standard deviation. Analysis of variance was performed using SPSS 19.0 statistical software. The data were subjected to statistical analyses using one-way analysis of variance (ANOVA) or two-way ANOVA Fischer Least Significant Difference (LSD) post hoc tests were conducted when appropriate. An alpha level of $p<0.05$ was considered as statistically significant.

## Results

### Survival, Proliferation and Differentiation of NSCs in vitro

At 12 h in primary culture, the cells were small, round, spherical (5 ~ 10μm in diameter), lightproof and sprinkled throughout the nutrient fluid (Fig. 1A). After 2–3 days in culture, the cells began to form neurospheres and showed an increase in number and size (Fig. 1B). At 5 days, the cells had increased in number and became larger in size (Fig. 1C). The cells that were Nestin-positive were confirmed as NSCs (Fig. 1D), indicating the NSCs were successfully isolated and had the ability to proliferate. In addition, the NSCs were NeuN-positive cells (Fig. 1E), which showed that NSC exhibited the capacity to differentiate into neuron in vitro.

### Survival and Differentiation of NSCs in vivo

Cells emitting green fluorescence were found in spinal cord tissue, confirming that they were transplanted NSCs (Fig. 2) and suggesting that the grafted cells could survive and migrate around the injured site. Moreover, part of the NSCs exhibited NeuN marker emitting red fluorescence (Fig. 2), indicating that the transplanted NSCs could differentiate into neurons in host spinal cord.

### Neuronal Apoptosis and Survival in Host Spinal Cord

TUNEL-positive cells were observed in the sham group, SCT group, and NSC group (Fig. 3A), indicating that apoptosis of neurons in spinal cord was significantly increased in SCT group, compared with that in the sham group (Fig. 3B). However, after NSC transplantation, the apoptosis of spinal cord neurons significantly decreased (Fig. 3B). NeuN-positive cells were observed in the sham group, SCT group, and NSC group (Fig. 3A) and the number of NeuN-positive cells was significantly decreased in SCT group compared with that of the sham group (Fig. 3C), while the NeuN-positive cells in NSC group was significantly increased compared with that of the SCT group (Fig. 3C).

### Evaluation of Motor Function

In the inclined plane test, we found that the SCT rats tolerated a smaller angle of the inclined plane than did the rats in the sham group (Fig. 4A). In addition, at 2 weeks following neural stem cell transplants, the angle of the inclined plane in the NSC group was significantly increased (Fig. 4A), indicating that the motor ability of rats in the NSC group was significantly improved.

In addition, the BBB score was assessed for evaluating motor function. Before SCT or sham operation, all rats received a score of 21 on the BBB scale and showed normal spinal cord function. However, BBB scores of the SCT and NSC groups significantly decreased after surgery, compared with that of the sham group (Fig. 4B). Moreover, the SCT rats showed adiaphorous tail and urinary dysfunction, as well as a defecation disorder. In addition, locomotor function of hind limb exhibited a slight recovery after 14 days in SCT rats, but it was still lower than that of the sham group (Fig. 4B). Despite of the remnants in urinary bladder dysfunction, the urinary function gradually recovered after 14 days in SCT rats. Nevertheless, compared with SCT rats, the NSC-treated rats showed a prominent increase in the BBB scores at 14 days after transplantation, suggesting a significant improvement of neurological function (Fig. 4B).

### Electrophysiological Analysis

In the MEP experiment, the latency period of motor stimulation signal in the SCT group was significantly longer than that of the sham group (Fig. 4C, D). However, after cell transplantation, the latency of signal was significantly

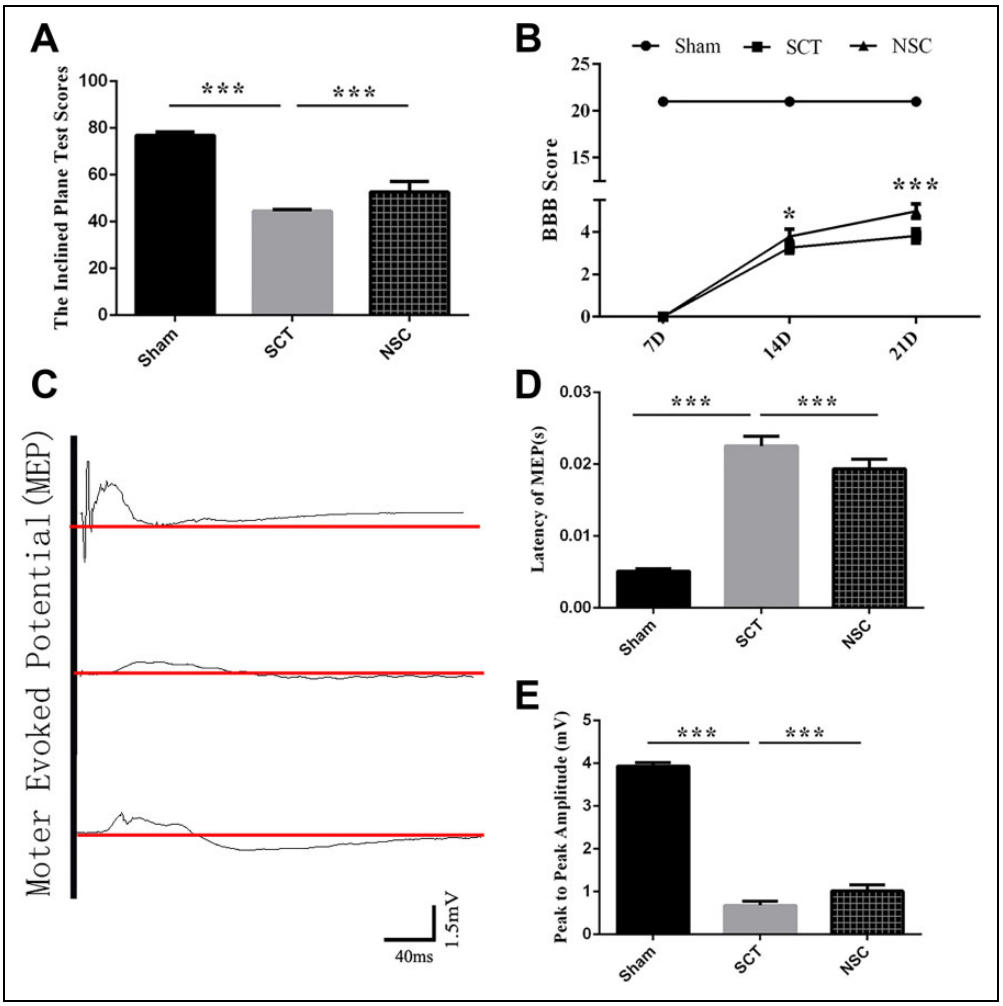


**Figure 4.** The behavior change of rats after NSC transplantation. (A) The inclined plane test was assessed 3 weeks after SCT. Data represented as mean ± SD (***$p < 0.001$) with $n=6$ using one-way ANOVA with LSD(L) post-test comparison in Fig 4. A, in which F(2,15) is 224.917. (B) The BBB score of rats that received NSC transplantation was significantly increased at 21 days after injury. Data represented as mean ± SD (*$p < 0.05$, ***$p < 0.001$) with $n=6$ using two-way ANOVA in Fig 4. B, in which F(2,15) is 9726.604 in D14 and 7249.571 in D21. (C) Motor evoked potentials were recorded to assess motor nerve conduction. (D) The latent period of spinal nerve fiber conduction was observed in three groups of rats. Data represented as mean ± SD (***$p < 0.001$) with $n=6$ using one-way ANOVA with LSD(L) post-test comparison in Fig 4. D, in which F(2,15) is 416.868. (E) Peak-to-peak amplitude of spinal nerve fibers conduction in rats of three groups. Data represented as mean ± SD (***$p < 0.001$) with $n=6$ using one-way ANOVA with LSD(L) post-test comparison in Fig 4.E, in which F(2,15) is 1593.118.

shorter than that of the SCT group (Fig. 4C, D). Moreover, the peak-to-peak amplitude of the SCT group was significantly lower than that of the sham group (Fig. 4C, E), while the peak-to-peak amplitude in the NSC group was higher than that of the SCT group (Fig. 4C, E). These results indicated that NSC transplantation was conducive to recovery in spinal cord-injured SD rats.

## *T2-weighted and DTI Analysis*

In the MRI experiment (Fig. 5A–F), the length and number of nerve fibers and FA in the SCT group were significantly lower than those in the sham group (Fig. 5G-I). However, after cell transplantation, those parameters were significantly higher in the NSC group than that in the SCT group, but still poorer than that in the sham group (Fig. 5G–I), which indicated that NSC transplantation was beneficial to the repair and regeneration of nerve fibers in certain degree.

## *mRNA Levels of IGF-1 R in Spinal Cord after Injection of NSCs*

In the SCT group, the mRNA expression of IGF-1 R in rostral cord did not show significant changes compared with that of the sham group. However, the mRNA level of IGF-1 R in NSC group was significantly increased at 3, 7, and

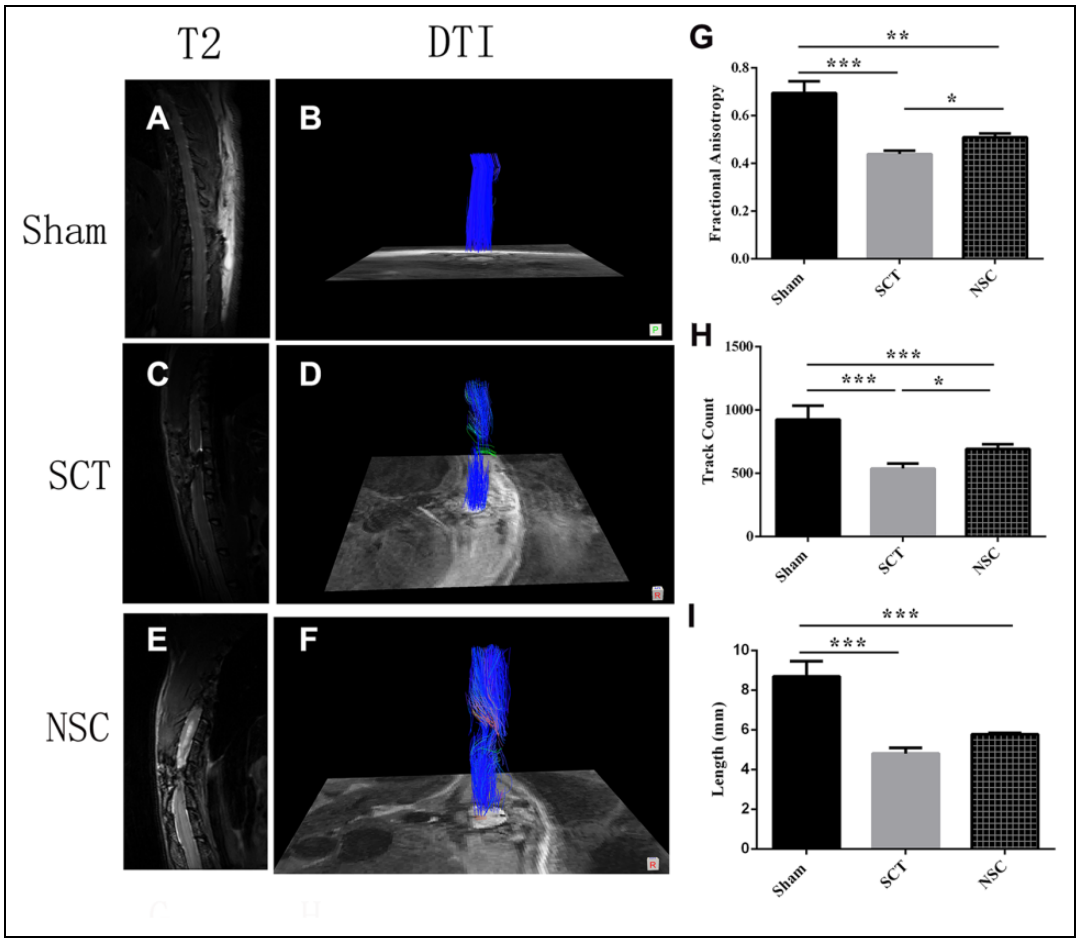


**Figure 5.** T2-weight and DTI drawing in sham group, SCT group and NSC group. (A, C, E) The structural drawing of rats in the three groups. (B, D, F) The DTI drawing of rats in different groups. The fractional anisotropy (G, $F(2,6)=52.283$), the length (I, $F(2,6)=22.209$) and number (H, $F(2,6)=52.663$) of nerve fibers in the site of spinal cord injury in different groups. Data represented as mean ± SD (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) with $n=3$ using one-way ANOVA with LSD(L) post-test comparison in Fig 5.G, H, I.

14 days after transplantation (Fig. 6A). Compared with the SCT group, the mRNA expression of IGF-1 R in NSC group significantly increased at 14 days after transplantation (Fig. 6A).

In the caudal cord, the mRNA level of IGF-1 R in NSC group decreased significantly at 7 days and 14 days after transplantation compared with that of the sham group (Fig. 6B). Meanwhile, when compared with SCT group, the content of IGF-1 R mRNA in NSC group was also decreased significantly at 7 and 14 days after transplantation (Fig. 6B). No significant differences were found between the other groups (Fig. 6A, B).

## *Change of IGF-1 R Protein Level in Spinal Cord after Injection of NSCs*

Compared with sham group, the protein levels of IGF-1 R in rostral cord from the NSC group were significantly increased at 14 days after transplantation (Fig. 6C, E), which was similar to that of measured mRNA levels. In addition, compared with SCT group, the protein expression of IGF-1 R in rostral cord in the NSC group also increased significantly at 14 days after transplantation (Fig. 6C, E). The protein level of IGF-1 R in the NSC group was significantly reduced, compared with that of SCT group at 14 days after transplantation (Fig. 6D, F).

## *Localization and Expression of IGF-1 R in the Rostral Segment of Spinal Cord*

Due to its increased mRNA and protein levels as measured by RT-PCR and Western blotting in the rostral, but not caudal segments, the localization and expression of IGF-1 R in the rostral segment was determined by immunohistochemistry. IGF-1 R protein was mainly distributed in cytoplasm and cell membrane of neurons. The number of IGF-1 R-positive cells in the rostral segment of spinal cord from the NSC group was significantly increased compared with that of the SCT group (Fig. 7).

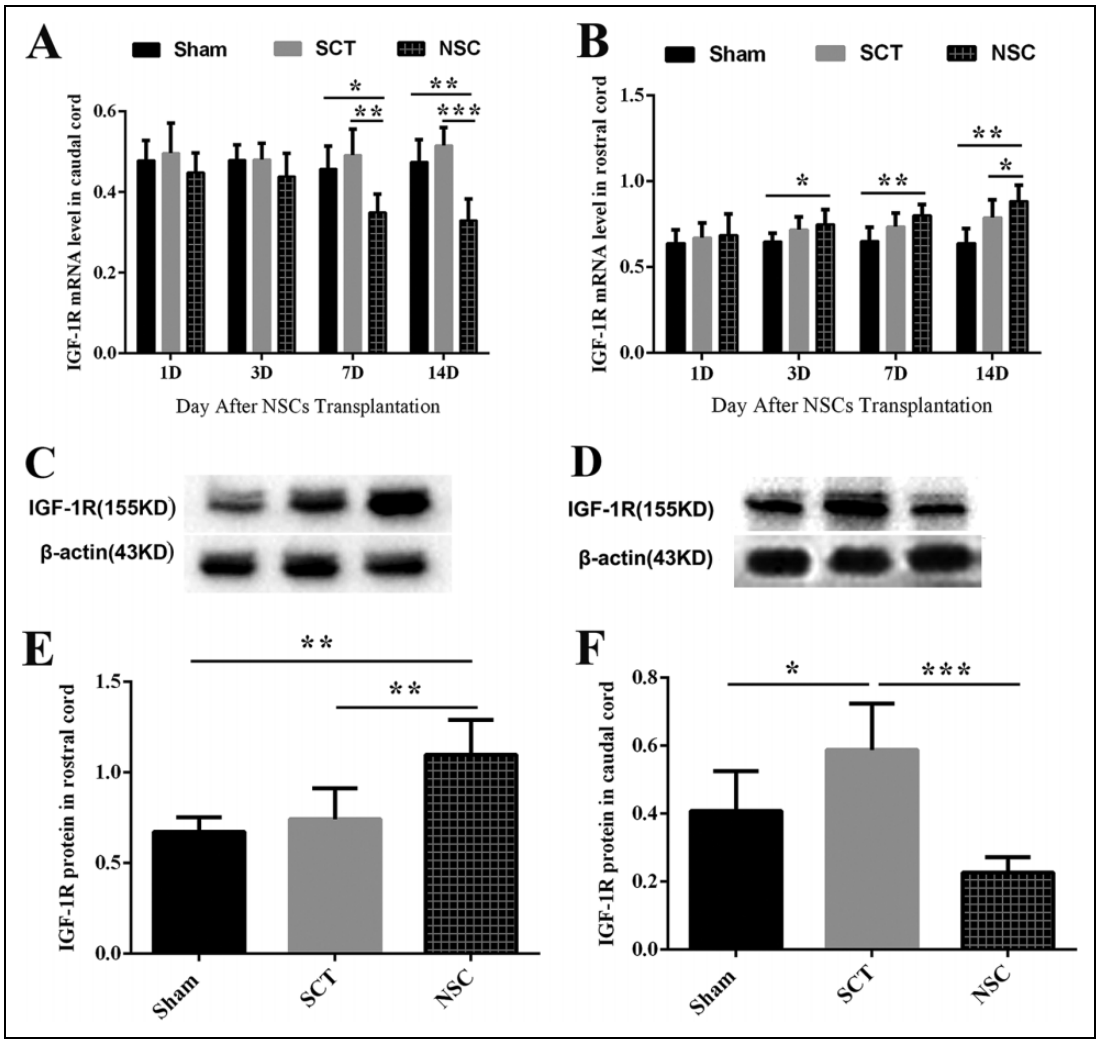


**Figure 6.** The expression of IGF-1 R in spinal cord. The expression of IGF-1 R in spinal cord was examined by RT-PCR and WB. The mRNA expression of IGF-1 R (A) in rostral cord and (B) in caudal cord was examined by RT-PCR. (A) F(2,12)=0.279 in 1D, 2.548 in 3D, 5.060 in 7D and 8.461 in 14D; (B) F(2,12)=0.861 in 1D, 1.375 in 3D, 8.658 in 7D and 18.439 in 14D. The protein expression of IGF-1 R (C, E) in rostral cord and (D, F) in caudal cord was examined by Western blot. (E) F(2,12)=10.724; (F) F(2,12)=14.190. Data represented as mean ± SD (*$p$ < 0.05, **$p$ < 0.01, ***$p$ < 0.001) with $n$=5 using one-way ANOVA with LSD(L) post-test comparison in Fig 6. A, B, E, F.

## Discussion

In this study, transplanted NSCs survived and differentiated into neurons in vitro and in vivo. Moreover, NSC transplantation promoted the recovery of motor function and increased the expression of IGF-1 R in the rostral cord segment. These results suggest that the NSC transplantation that promoted the preservation of motor function, nerve regeneration, and associated mechanisms is related to the expression of IGF-1 R in SCT.

The damage to nerve function caused by SCI is difficult to overcome. Nowadays, replacement of injured cells is a promising strategy, but the best source of cells, including those types which survive and can differentiate into specific neurons at the injured site to replace the injured cells, needs to be determined. NSCs have been found in many parts of the body, including the cerebral cortex, subependymal layer, striatum, hippocampus, and midbrain[14,15]. Moreover, NSCs have the potential for self-proliferation and multi-differentiation, so they could proliferate and differentiate into neurons to form a "cell bridge" in vivo[27,28]. In addition, many studies have shown that delivering NSCs in situ or by circulatory injection can effectively treat traumatic brain injury (TBI) and SCI[6,29]. Therefore, in this study, we extracted NSCs from the hippocampus of neonatal SD rats for the treatment of SCT. We found that NSCs could differentiate into neurons both in vitro and in vivo, suggesting cultured NSCs possess the characteristic of stem cells that could be used for transplantation.

It is well known that NSCs can differentiate into many cells, such as neuron, astrocyte, and microglia. So, what

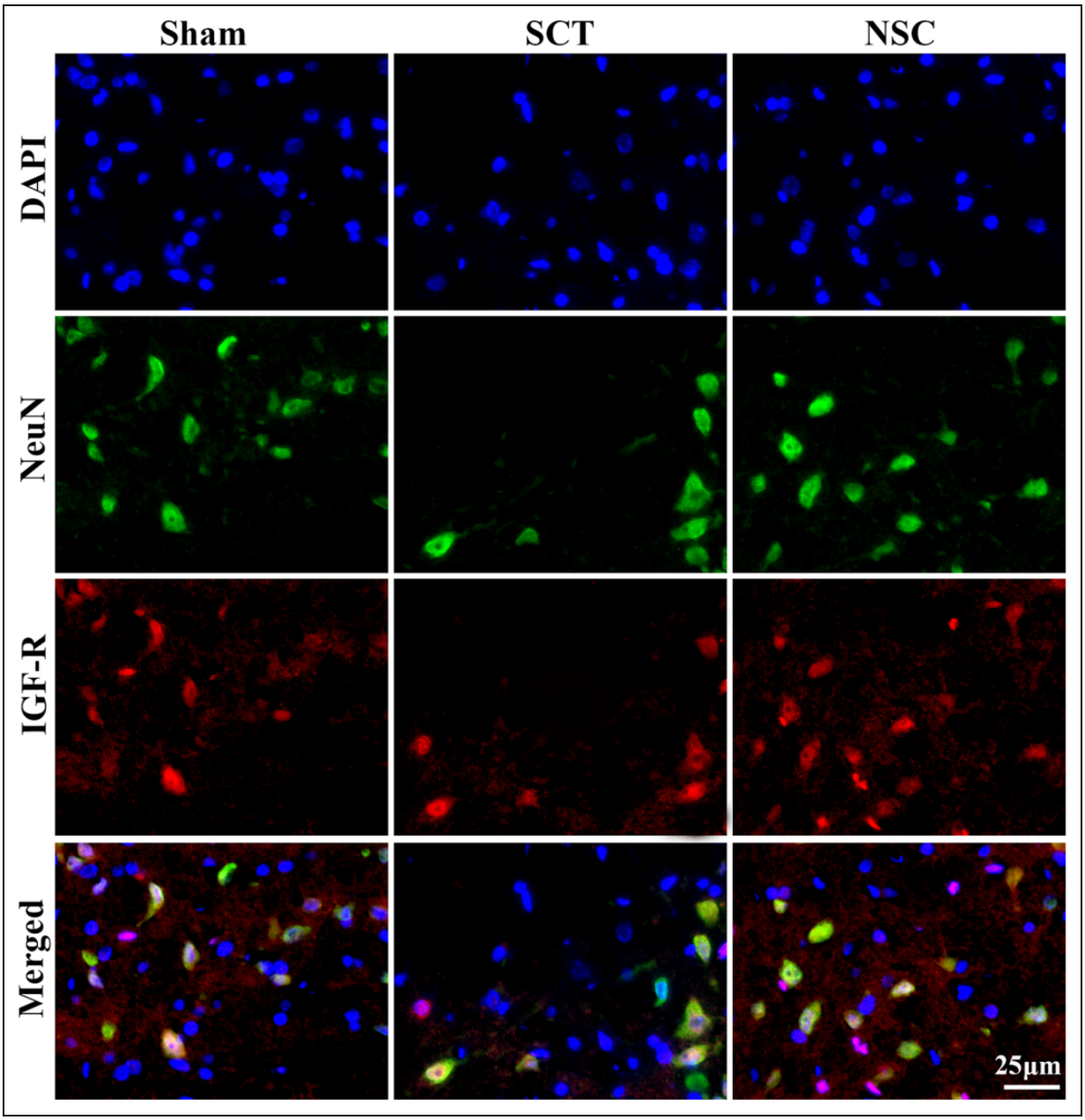


**Figure 7.** Localization and expression of IGF-1 R in the rostral segment of spinal cord. The expression of IGF-1 R in the rostral segment of spinal cord was examined by immunohistochemistry. In this figure, from top to bottom, the staining for DAPI, NeuN, IGF-1 R and merged pictures were exhibited, respectively, while from left to right, were the sham-, SCT -, and NSC- in turn, respectively.

elements play a role in SCI treatment? Riess and colleagues found that transplanted NSCs could survive and differentiate into neurons and astrocytes but not oligodendrocytes at the injury site[30]. The astrocytes from NSCs usually limited the transplantation treatment effectiveness[31], impeded neural regeneration[32], and formed a scar by 7 days after injury[33]. However, many studies have shown that NSC transplantation after SCI could improve and stabilize spinal cord function for a long time[34–37], suggesting that neurons differentiated from NSCs at the site of injury played a key role in nerve function. Furthermore, Tang and colleagues found that following co-transplantation of NSCs with olfactory ensheathing cells, the number of neurons that differentiated from NSCs was significantly greater than in those animals receiving transplants of NSCs only[38], and that more of these differentiated into neurons and promoted recovery of motor function recovery in rats given intracerebral hemorrhage[38]. This suggests that neuronal differentiation underlies the ameliorative effects observed in this study. Similarly, Cowley and colleagues showed that neurons could promote the repair of SCI[39], and others have shown that NSCs can differentiate into neurons and resist cell death[40]. Zhang and

colleagues[41] showed that NSC transplantation significantly improved neurological function in cerebral ischemia and reduced the number of TUNEL- and Bax-positive cells in the penumbra, while increasing the number of Bcl-2-positive cells in the penumbra at 7 days after transplantation, indicating that NSCs could play a complementary role in inhibiting apoptosis in the injured spinal cord.

We found that neurons which were derived from NSCs acted as a bridge, connecting axons in the damaged area. Morito and colleagues also found that grafted NSCs efficiently differentiated into neurons and contributed to polysynaptic reconnection after SCI in aged mice[42]. Collectively, these findings suggest that the transplanted NSCs were able to generate neurons, reduce cell apoptosis or death, and promote the recovery of injury, supporting the findings of the present study.

Previous studies also showed that NSC transplantation could help the recovery of motor function after SCI in rats, and that BDNF and NT-3 were significantly increased, while caspase-3 was significantly decreased at the injury site[43]. Nemati and colleagues found that NSCs could also help restore spinal cord motor function after SCI in the T9–T10 regions of the rhesus monkey[44]. Furthermore, several studies have shown that NSC transplantation contributes to the recovery of the CNS after injury[45–47]. Therefore, NSC transplantation may be an effective strategy for the treatment of SCI. Our study showed that transplanted NSCs contributed to the gradual recovery of motor function, which was confirmed by BBB scores, inclined plane test, and MEP analysis. As was done in our study, Wang visualized and quantified the extent of SCI using MRI[24], showing that DTI was a good predictor for SCI in rats. DTI tractography images, especially the number and length of nerve fibers, can visually determine the extent of injury of the spinal cord tract. In addition, the FA value from DTI data appeared to mirror locomotor outcome and could be used to assess the severity of the injury. In this study, we found that the number of nerve fibers and FA were significantly higher than those in the SCT group after transplantation, indicating that NSC transplantation was beneficial to the repair and regeneration of nerve fibers. However, there was some residual connection of nerve fibers in the SCT group. This is likely due to remaining endogenous NSCs in the spinal cord itself. The SCI in this study did not eliminate endogenous NSCs; on the contrary, it probably caused the activation of endogenous NSCs to differentiate into neurons, leading to the reconnection of the transected spinal cord[48,49]. Due to the activation of endogenous NSCs and the addition of NSCs, the number and density of nerve fiber connection was increased. Therefore, the NSC group had improved spinal cord function, relative to the SCT group. Of course, the recovery of spinal cord function might not only be due to the increase in the number of neurons, but also the secretion of neurotrophic factors by NSCs.

IGF-1 R, as the functional receptor of IGF-1, is the key to restoring the function of IGF-1. In a large number of studies, activation of IGF-1 R proved helpful in the regeneration and repair of motor neurons[50], and reduced nerve degeneration[51]. In one study, it was shown that increasing the concentration of IGF-1 in the cerebrospinal fluid, along with treadmill training, could markedly attenuate reactive nitrogen or oxygen species, which are detrimental to NSC survival after transplantation. Moreover, Hwang and colleagues found that mice receiving IGF-1R$^{+/-}$ NSC grafts showed reduced locomotor recovery compared with those receiving wild-type (IGF-1R$^{+/+}$) NSCs, illustrating that IGF-1 R plays an important role locomotor recovery[52]. In our study, we found the level of IGF-1 R in the rostral segment was increased significantly after transplanting NSCs in the site of the SCT. Comparatively, it decreased after NSC transplantation in the caudal segment of injured spinal cord. These results suggest that NSCs contribute to the improvement of motor function after SCI, and we hypothesize that this recovery is associated with IGF-1 R, especially in the rostral segment, which maintained the activity of cells and promoted the integration of the original cells and the transplanted NSCs.

Why was different change produced in the rostral and caudal segment of spinal cord? There are two special features of the rostral segments that may account for the preferential recovery observed. First, adhesion of the arachnoid membrane might be caused by the transverse injury, and the caudal segment could not be nourished by the cerebrospinal fluid, which could provide more trophic support for the rostral regions. Cerebrospinal fluid was once thought to be a simple plasma super filtrate, but now it is known that the cerebrospinal fluid contains a wealth of peptides, growth factors (including IGF), and hormones, which could help the maintenance of NSCs[53]. Moreover, IGF-1 R could promote proliferation and differentiation of NSCs. Second, cortical stimulation is more likely to be obtained and received in the rostral regions. Moreover, our previous studies have shown more survival and differentiation of NSCs in the rostral spinal cord than in the caudal segments[54]. Hence, NSC survival and the IGF-1 R level were higher in the rostral segments than in the caudal segments. Other studies showed that NSC transplantation could fill the lesion cavity and promote axonal extension, but most of the grafts did not produce a continuous bridge between the rostral and caudal segments of the nerve tissue[55]. The limitations of NSCs to establish a bridge between the rostral and caudal segments also may lead to differential recovery in each of the now-segregated caudal and rostral environments. Therefore, we surmised that NSCs could enhance the repair of the SCI by providing different types of support in these two disparate environments.

In summary, our results suggest that NSC transplantation promotes recovery of motor function through differentiation of NSCs into neurons and enhances the expression of IGF-1 R in region of the SCT. These findings could provide new strategies and theoretical explanation for treatments of SCI.

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## Author Contributions

Xiao-Ming Zhao, Xiu-Ying He, Zi-Bin Zhang, and Ting-Hua Wang contributed equally to this work.

## Ethical Approval

All animal procedures used in this study were approved by the Ethics Committee of Kunming Medical University, Yunnan Province, China (reference number: kmmu 2018016).

## Statement of Human and Animal Rights

All of the experimental procedures involving animals were conducted in accordance with Institutional Animal Care guidelines of Kunming Medical University, China and approved by the Administration Committee of Experimental Animals, Yunnan Province, China.

## Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

## Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## Supplemental Material

Supplemental material for this article is available online.

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