Spinal Cord (2007) 45, 15–24

www.nature.com/sc

Original Article

Cotransplant of neural stem cells and NT-3 gene modified Schwann cells promote the recovery of transected spinal cord injury

J-S Guo[1,2], Y-S Zeng*[,1], H-B Li[1], W-L Huang[3], R-Y Liu[3], X-B Li[1], Y Ding[1], L-Z Wu[3] and D-Z Cai[4]

[1]*Division of Neuroscience, Department of Histology and Embryology, Zhongshan Medical College, Sun Yat-sen University, Guangzhou, China;* [2]*Department of Histology and Embryology, Southern Medical University, Guangzhou, China;* [3]*Cancer Center of Sun Yat-sen University, Guangzhou, China;* [4]*Department of Orthopaedics, 3rd Affiliated Hospital, Sun Yet-sen University, Guangzhou, China*

Study design: An animal model of transected spinal cord injury (SCI) was used to test the hypothesis that cografted neural stem cells (NSCs) and NT-3-SCs promote morphologic and functional recoveries of injured spinal cord.
Objective: To explore whether cotransplant of NSCs and NT-3-SCs could promote the injured spinal cord repair.
Setting: Zhongshan Medical College, Sun Yat-sen University, PR China.
Methods: Female Sprague–Dawley (SD) rats weighing on 200–220 g were used to prepare SCI models. The spinal cord was transected between T_9 and T_{10}, then NSCs, SCs + NSCs, LacZ-SCs + NSCs, or NT-3-SCs + NSCs were grafted into the transected site.
Results: (1) Part of NSCs could differentiate to neuron-like cells in the transected site and the percentage of differentiation was NT-3-SCs + NSCs group > SCs + NSCs group > NSCs group. (2) In the grafted groups, there were 5-HT, CGRP, and SP positive nerve fibres within the transected site. Some fluorogold (FG)-labeled cells were found in the spinal cord rostral to the transected site, the red nuclei and the inner pyramidal layer of sensorimotor cortex. (3) The cells grafted could enhance the injured neurons survival in inner pyramidal layer of sensorimotor cortex, red nuclei of midbrain, and Clark's nuclei of spinal cord's L1 segment, could decrease the latency and increase the amplitude of cortical somatosensory evoked potential (CSEP) and cortical motor evoked potential (CMEP), and could promote partly structural and functional recovery of the SCI rats.
Conclusion: These results demonstrate that cografted NT-3-SCs and NSCs is a potential therapy for SCI.
Sponsorship: This research was supported by Chinese National Key Project for Basic Research (G1999054009), Chinese National Natural Science Foundation (30270700) and Social Developmental Foundation of Guangdong Province (2003C33808) to YS Zeng; Natural Science Foundation of Guangdong Province (04300468) and Medical Science Research Grant of Guangdong Province (A2004081) to JS Guo.
Spinal Cord (2007) **45,** 15–24. doi:10.1038/sj.sc.3101943; published online 13 June 2006

Keywords: neural stem cells; Schwann cells; neurotrophin-3; transplantation; spinal cord injury; regeneration

Introduction

Neural stem cells (NSCs) that can be maintained *in vitro* in an actively proliferating state and able to differentiate into mature neurons and glia are attractive candidates for use as transplantation in repair of the damaged CNS.[1] For therapy of spinal cord injury (SCI), transplantation of NSCs has been an increasing attractive strategy. Our preliminary observation has showed that engrafted NSCs promoted partly structural and functional recovery of the completely transected spinal cord of adult rats. However, the curative value is limited and we found NSCs differentiated into more neuroglial cells, and less neurons.[2,3] Permanent functional deficit with SCI is in part due to severe nerve cell death. Therefore, increasing the rate of differentiation of

*Correspondence: Y-S Zeng, Department of Histology and Embryology, Zhongshan Medical College, Sun Yat-sen University, 74# Zhongshan Road 2, Guangzhou 510080, P.R. China

transplanted NSCs into neurons is important to compensate the loss of nerve cells.

The potential of engrafted Schwann cells (SCs) to promote axonal regeneration in the injured spinal cord is now well established.[4–6] It was demonstrated that SCs could synthesize many kinds of neurotrophic factors, cell adhesion, and extracellular matrix molecules. All of these factors are prerequisites for the survival and axonal regeneration of damaged neurons.[7–9]

Neurotropin-3 (NT-3) is one of the important neurotrophic factors. It can prevent atrophy of mature CNS neurons, and promote corticospinal tract axonal regeneration and recovery of hindlimb's function in spinal cord injured animal[10–15] and Hapner *et al*[16] had indicated that NT-3 could upregulate the TrkC expression of NSCs and promote for them to differentiate into neurons.

We have obtained NT-3 gene modified SCs (which were named as NT-3-SCs in this paper) and have demonstrated it could overexpress NT-3 in our previous studies.[17,18] Based on the above data, we hypothesize that NT-3-SCs would promote NSCs to differentiate into neurons. And we hope that the cografted NSCs and NT-3-SCs could promote the recovery of morphology and function in the transected spinal cord rats.

Materials and methods

Cell isolation and culture for transplantation

Neural stem cells NSCs culture was following the procedure described by previous publication.[2] Briefly, hippocampal tissue from neonatal Sprague–Dawley (SD) rats was dissected and cut into small pieces in D-Hank's balanced salt solution (HBSS). These pieces were digested with 0.25% trypsin (Sigma) for 10 min at 37°C. The cell suspension was sieved through a 75 μm steel mesh and then centrifuged at 1000 r.p.m. for 5 min and the pellet was then resuspended in 0.5 ml of HBSS for cell counting. The final volume was adjusted to a cell density of 1×10^5 viable cells/ml of culture medium containing Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1 Gibco), B27 supplement (2 ml/100 ml, Gibco) and bFGF (20 ng/ml, Sigma). The cells were planted onto 75 ml culture flasks. The medium was replaced half every 3 days. Typically, the cells grew in suspending neurospheres which were passaged by mechanical dissociation approximately once each week and reseeded at approximately 1×10^5 cells/ml. In this study, the second passaged neurospheres were used for transplantation. The neurospheres were identified as nestin-positive by immunocytochemistry.[2,18] In order to enable the transplanted NSCs be detected in host tissue, cultured NSCs neurospheres were labeled with blue nuclear fluorescence Hoechst33342 which was added to the culture medium at 10 μg/1 ml for 2 h before the preparation of cells for transplantation.

Schwann cells SCs culture was following the procedure as described in previous studies.[2] Briefly, sciatic nerves and brachial plexus nerves removed from neonatal SD rats were digested in 0.25% trypsin (Sigma) for 15 min and 0.16% collagenase (Sigma) for 20 min at 37°C. Following digestion, the cells were centrifuged at 1000 r.p.m. for 5 min, then resuspended in DMEM/F12 with 10% FBS and plated on poly-L-lysine (0.01%, Sigma) coated culture flasks at a density of 1×10^4 cells/ml. On the following day, cells were treated with cytosine arabinoside (1×10^{-5} M; Sigma) for 24–48 h to rapidly remove fibroblasts. Then the medium was replaced every 3–4 days, passaged every week and reseeded at approximately 1×10^4 cells/ml. The second passaged SCs were used for genetically modify and transplantation. Based on the bipolar morphology and S-100 (one of the SCs markers) immunocytochemistry, the purity of SCs is about 90–95%.[2,18]

SCs genetically modified by AdvNT-3 and AdvLacZ

The secondary cultured SCs were genetically modified to secrete NT-3 or β-galactosidase mediated by recombinant adenovirus containing the NT-3 gene or LacZ gene (AdvNT-3, AdvLacZ), which were obtained from Dr Huang WL at Cancer Center of Sun Yet-sen University. The procedure was described in our previous study.[16] Briefly, having been washed two times by RPMI Medium 1640 (Gibco), the SCs were cultured in RPMI Medium 1640 contained glutamine, arginine and AdvNT-3 or AdvLacZ for 3 h. Then, the medium was replaced by DMEM/F12 with 10% FBS and lasting 48 h. The percentage of SCs genetically modified was about 90–95% based on staining of X-gal histochemistry and NT-3 immunocytochemistry. The SCs genetically modified by AdvNT-3 and AdvLacZ were named as NT-3-SCs and LacZ-SCs in the present study. Detected by ELISA, it showed that the concentration of NT-3 within the culture medium of NT-3-SCs was significantly higher than that of normal SCs. And it indicated that the NT-3 genetically modified SCs have the ability to overexpress NT-3.[17]

Co-grafts of NT-3-SCs and NSCs into the transected site of the spinal cord

preparing cells for transplantation For transplantation, the Hoechst33342 labeled neurospheres were trypsinized after rinsed twice by HBSS, then triturated using a fire-polished glass pipette and washed three times by centrifugation and resuspension in HBSS. The cells were resuspended in 1 ml of DMEM/F12 medium for cell counting and the final volume was adjusted to a cell density of 0.8×10^6 viable cells/μl in DMEM/F12 medium.

The Cultures of SCs, NT-3-SCs and LacZ-SCs were trypsinized, and cells were washed twice by HBSS, collected by centrifugation at 1000 r.p.m. for 5 min and resuspended in 1 ml of DMEM/F12 medium to check the cell viability. The SCs, NT-3-SCs and LacZ-SCs were centrifuged a second time and resuspended at the density of 0.8×10^6 viable cells/μl.

Surgical procedures A total of 60 adult female Sprague–Dawley rats (200–220 g, supplied by the experimental animal center of Sun Yat-sen University) were subjects of this study. The rats were divided into six groups as follow, normal control group (the rats did not receive any surgery and cell transplant), SCI control group (a scaffold with medium without any cell was transplanted into the transected spinal cord), NSCs grafted group (a scaffold with NSCs was transplanted into the transected spinal cord), SCs + NSCs cografted group (a scaffold with SCs and NSCs was transplanted into the transected spinal cord), LacZ-SCs + NSCs cografted group (a scaffold with LacZ-SCs and NSCs was transplanted into the transected spinal cord), NT-3-SCs + NSCs cografted group (a scaffold with NT-3-SCs and NSCs was transplanted into the transected spinal cord). Each group had 10 rats.

To prepare the SCI model, the animals were anesthetized with 1% pentobarbital (30 mg/kg, i.p.). A laminectomy was carried out to expose the T_9 and T_{10} spinal segment. Spinal cord (2 mm) was transected and cut away between T_9 and T_{10} segment. A scaffold made of type I collagen (gift from Laboratory of Orthopaedics, Harvard Medical School, Harvard University), which size is cut to about $2 \times 2 \times 2\,mm^3$, was filled into the cavity as a carrier of the grafted cells. For cells transplantation in different groups, 5 μl NSCs suspension, 5 μl NSCs suspension + 5 μl SCs suspension, 5 μl NSCs suspension + 5 μl LacZ-SCs suspension, or 5 μl NSCs suspension + 5 μl NT-3-SCs suspension were microinjected into the center of the scaffold respectively. In the SCI control group, only 5 μl DMEM/F12 without any cell was injected into the scaffold.

After closed surgical incisions, the rats returned to their cages and then received extensive care including intraperitoneal injection of penicillin (50 000 U/kg/day) for 3 days and manual emiction two times daily until the reflex of bladder emptying was established. All procedures were approved in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals.

Functional analysis
At 60 days after transplantation, the hindlimb locomotive function in the cells grafted rats and the SCI control rats was analyzed using the BBB locomotor rating scale as described by Basso *et al*[19] and inclined-grid climbing test as described by Ramon-Cueto *et al*.[20]

Retrograde labeling of the neurons at sensorimotor cortex and red nuclei
Fluorogold (FG, 5%) was used to label the neurons in sensorimotor cortex (SMC) and red nuclei (RN) that had regenerated their axons across the transected site of spinal cord. After functional analysis, the rats were anesthetized. The second surgical procedure was performed to expose the spinal cord caudal to the transected site. FG (0.5 μl) was bilaterally injected into the spinal cord 3 mm caudal to the transected site and 0.5 mm beside from the spinal med-line using microsyringe.

Electro-physiologic analysis
At 7 days after FG injected, the rats were anesthetized and stereotaxically fixed. Following the exposure of sciatic nerves and SMC, the electrodes of BL-420E Data Acquisitionant Analysis System for Life Science (Taimeng) were connected to the sciatic nerve and SMC. Abided by the description of the system, the latency and amplitude of cortical somatosensory evoked potential (CSEP) and cortical motor evoked potential (CMEP) were detected.

Killing and cryosection
After the electro-physiologic analysis was performed, all rats were perfused transcardially with normal saline contained 0.002% $NaNO_2$ and 0.002% Heparin, followed by fixative containing 4% paraformaldehyde in 0.01 M PBS (pH 7.4). The spinal cord and brain were dissected and postfixed, then placed in 30% phosphate-buffered sucrose at 4°C more than 48 h. Spinal cord's T_8–T_{12} successive segments, L_1 segment and brain were cryosected by proposal as follows: spinal cord's T_8–T_{12} successive segments were cut longitudinally, L_1 segment was cut coronarily. According to anatomical atlas, part of the brain, which contained RN or SMC was cut coronarily. The thickness of all slices was 30 μm.

Fluoromicroscopic observing
By fluoromicroscope, the distribution of NSCs labeled with Hoechst33342 within host spinal cord was observed in the slices of T_8–T_{12} spinal cord segments and the FG labeled neurons were detected in the SMC, RN, and spinal cord.

X-gal histochemical staining
To identify the gene expression of the genenatical modified SCs, some slices of T_8–T_{12} spinal cord segments in LacZ-SCs + NSCs group were selected to perform X-gal histochemical staining which has been described in detail elsewhere.[21] Briefly, slices were rinsed three times (5 min each time) with 0.01 M PBS (pH 7.4) followed by incubation in X-gal reagent (Molecular Probes, 1 mg/ml final concentration) with X-gal mixer (35 mM $K_3Fe(CN)_6$, 35 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ in PBS) at 37°C overnight. Before coverslipped, the slices were counterstained with neutral red.

Tissue processes for immunohistochemistry
Part spinal cord slices of T_8–T_{12} segments in the cells grafted groups were performed SABC-BAD immunohistochemistry. Briefly, the slices were rinsed with 0.3% Triton X-100 for 20 min and 10% goat serum for 30 min then incubated with appropriate primary antibodies as

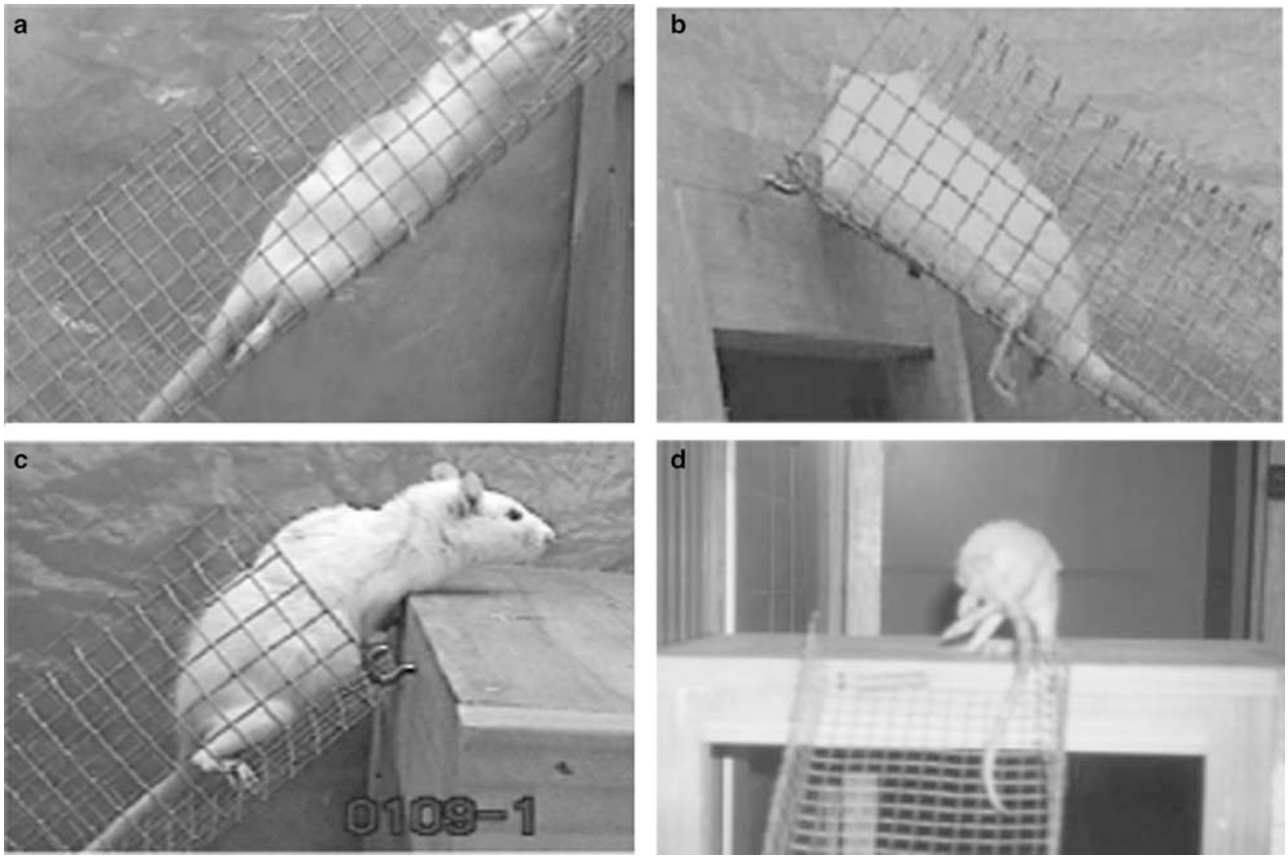

Figure 1 The SCI rats climbed onto the inclined grid 60 days after surgical procedures. (**a** and **b**) An example of SCI control rat was climbing with the forelimbs. The hindlimbs were drawing (**a**) or falling (**b**). (**c**) An example of NSCs grafted rat was climbing with the hindlimbs stepping grid. (**d**) An example of NT3-SCs + NSCs grafted rat skipping with the hindlimbs when it climbing onto the inclined grid

Table 1 The BBB score of the locomotor function of the hindlimbs ($x \pm s$)

Groups	*BBB score*
SCI control group	0.54 ± 0.32^{a}
NSCs grafted group	3.63 ± 1.71^{b}
SCs + NSCs cografted group	7.03 ± 2.35^{c}
LacZ-SCs + NSCs cografted group	6.87 ± 3.06^{d}
NT-3-SCs + NSCs cografted group	10.76 ± 3.43^{e}

t-test: a *versus* b, a *versus* c, a *versus* d, a *versus* e, b *versus* c, b *versus* d, b *versus* e $P<0.01$; c *versus* e, d *versus* e $P<0.05$; c *versus* d $P<0.05$

follows: anti-Neurotrophin-3 (NT-3, Zhongshan Biotech, at 1:200 dilution), anti-5-hydroxytrptamine (5–TH, Boster, at 1:200 dilution), anti-Calcitonin Gene Related Peptide (CGRP, Boster, at 1:200 dilution) and anti-Substance P (SP, Boster, at 1:200 dilution). Incubation with primary antibodies were performed at the dilutions mentioned above overnight at 4°C, and with the biotin secondary antibodies for 30 min, the strept actividin biotin complex (SABC, Boster) for 30 min, and a solution of 0.05% DAB, 0.01% H_2O_2, 0.05 M Tris-HCl for 5 min. Between each steps, the slices were rinsed three times (5 min each time) with 0.01 M PBS (pH 7.4).

Tissue processes for immunofluorochemistry

Other spinal cord slices of T_8–T_{12} segments in the cells grafted groups were performed SABC-Cy3 immunofluorochemistry. Briefly, followed by 0.3% Triton X-100 and 10% goat serum, the slices were incubated with appropriate primary antibodies overnight at 4°C, the primary antibodies are anti-neurofilament (NF, Boster, at 1:200 dilution) and anti-Glial fibrillary acidic protein (GFAP, Boster, at 1:200 dilution). Then incubated with biotin-secondary antibodies for 30 min, SABC-Cy3 (Boster) for 30 min.

Nissl staining and neuron counting

The serial brain slices contained SMC or RN which were selected one per six in serial and 50 serial spinal cord slices taken from L_1 segment of each rat were stained with neutral red. The neuronal densities of RN and the inner pyramidal layer of SMC were counted under grid-equipped microscopy based on stereologic principle. The whole neuronal number of Clarke's nuclei (CN) in 50

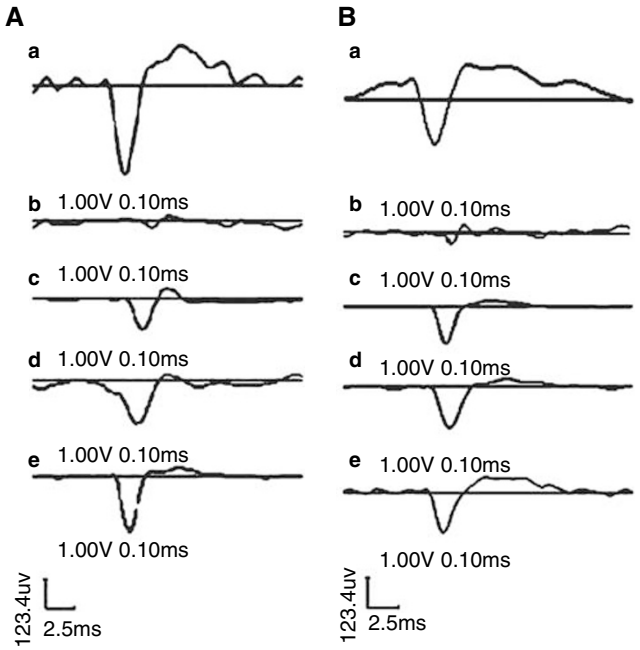

Figure 2 The cortical motor evoked potential (**A**) and cortical somatosensory evoked potential (**B**) of rats 67 days after surgical procedures. (**a**) Normal control group. (**b**) SCI control group. (**c**) NSCs grafted group. (**d**) SCs + NSCs cografted group. (**e**) NT3-SCs + NSCs cografted group

Table 2 The latency and amplitude of CSEP ($x \pm s$)

Groups	*Latency (ms)*	*Amplitude (μV)*
Normal control group	2.63 ± 0.46^{a}	365.78 ± 82.94^{a}
SCI control group	3.48 ± 1.08^{b}	43.54 ± 12.47^{b}
NSCs grafted group	3.11 ± 0.75^{c}	133.28 ± 24.38^{c}
SCs + NSCs cografted group	2.97 ± 0.85^{d}	165.57 ± 43.82^{d}
LacZ-SCs + NSCs cografted group	2.91 ± 0.56^{e}	159.81 ± 43.27^{e}
NT-3-SCs + NSCs cografted group	2.76 ± 0.73^{f}	201.19 ± 73.25^{f}

t-test: Latency: a *versus* b, a *versus* c, b *versus* d, b *versus* e, b *versus* f $P<0.01$; a *versus* d, a *versus* e, b *versus* c, c *versus* f $P<0.05$; a *versus* f, c *versus* d, c *versus* e, d *versus* e, d *versus* f, e *versus* f $P<0.05$. Ampitude: a *versus* b, a *versus* c, a *versus* d, a *versus* e, a *versus* f, b *versus* c, b *versus* d, b *versus* e, b *versus* f, c *versus* f, d *versus* f, e *versus* f $P<0.01$; c *versus* d, c *versus* e $P<0.05$; d *versus* e $P<0.05$

serial spinal cord slices of L_1 segment of each rat was counted.

In order to detected the percentage of the neuron-like cells derived from NSCs, the number of Hoechst33342 labeled cells and those of double-labeled by Cy-3 were counted in the NF immunofluorochemistric labeled slices as following design: five slices in every rat, and four microscopic visual fields in every slice. It is to say,

Table 3 The latency and amplitude of CMEP ($x \pm s$)

Groups	*Latency (ms)*	*Amplitude (μV)*
Normal control group	2.85 ± 0.91^{a}	274.38 ± 59.45^{a}
SCI control group	3.54 ± 0.72^{b}	28.34 ± 13.46^{b}
NSCs grafted group	3.34 ± 0.82^{c}	109.19 ± 38.51^{c}
SCs + NSCs cografted group	3.18 ± 0.57^{d}	178.06 ± 60.29^{d}
LacZ-SCs + NSCs cografted group	3.15 ± 0.75^{e}	181.13 ± 82.45^{e}
NT-3-SCs + NSCs cografted group	3.10 ± 0.96^{f}	217.63 ± 49.23^{f}

t-test: latency: a *versus* b, a *versus* c, b *versus* f $P<0.01$; a *versus* d, a *versus* e, a *versus* f, b *versus* c, b *versus* d, b *versus* e, c *versus* f $P<0.05$; c *versus* d, c *versus* e, d *versus* e, d *versus* f, e *versus* f $P>0.05$. Amplitude: a *versus* b, a *versus* c, a *versus* d, a *versus* e, a *versus* f, b *versus* c, b *versus* d, b *versus* e, b *versus* f, c *versus* f, d *versus* f, e *versus* f, c *versus* d, c *versus* e $P<0.01$; d *versus* e $P>0.05$

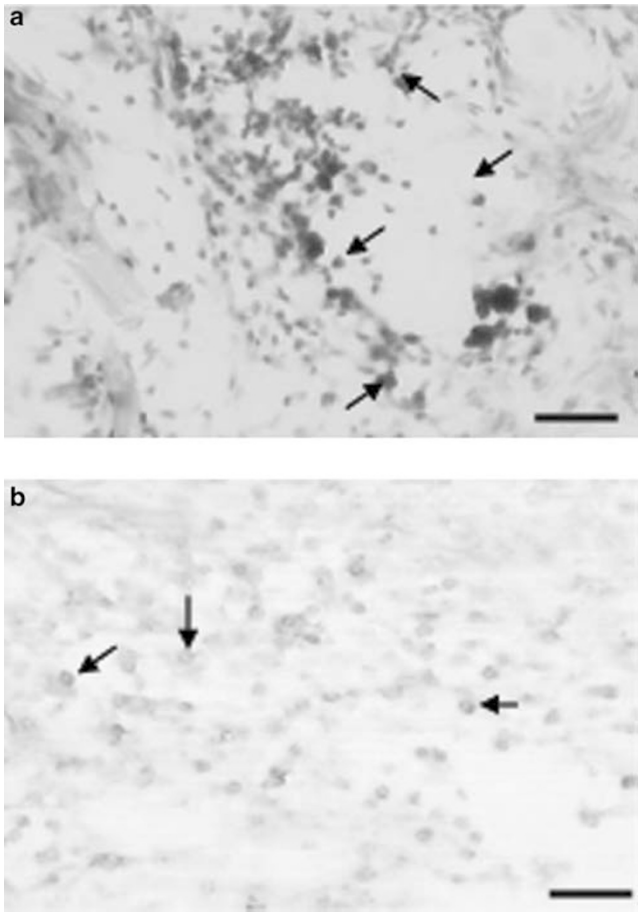

Figure 3 The survival and xenogene expression of SCs genetically modified within transected and grafted site 67 days after transplantation. (**a**) Showed the *β*-gal positive SCs (arrows) stained by X-gal histochemistry in LacZ-SCs + NSCs grafted group. (**b**) Showed the NT-3 positive SCs (arrows) stained by immunohistochemistry using DAB as chromagen in NT-3-SCs + NSCs grafted group. These pictures were taken from within the grafts. Scale bars = 50 *μ*m

there are 20 visual fields in every rat were selected to count the total number of simple-labeled or double-labeled cells. The counting was performed within the host tissue just nearby the transected site. The distance to the injury and graft is about 100 μm.

Differences in all data between groups were evaluated statistically using one-way ANOVA and *post hoc* Student's *t*-test.

Results

Functional recovery

Typically, the spinal cord transected rats showed complete paralysis. When climbed onto the inclined grid, their hindlimbs drawing by the forelimbs movement, and falling frequently whilst movement stopped. However, the rats received NSCs transplant could step their hindlimbs onto the grid occasionally, while the rats transplanted NT-3-SCs and NSCs had more chance to step their hindlimbs onto grid, and fortunately, we had captured one of these rats skipping with hindlimbs (Figure 1). And the scores of the locomotor function of the hindlimbs obtained by BBB method showed significant difference between groups (Table 1).

Electro-physiology

The CSEP and CMEP of the rats in the SCI control group were very weak. Its' latencies were prolonged and amplitudes were declined significantly compare with the normal control group. But in the cells grafted groups, the CSEP and CMEP recovered in different degree compare with the SCI control group (Figure 2 and Tables 2 and 3).

In vivo expression of exogenous gene

In the LacZ-SCs + NSCs cografted group, there were many β-gal positive cells distributing within and nearby

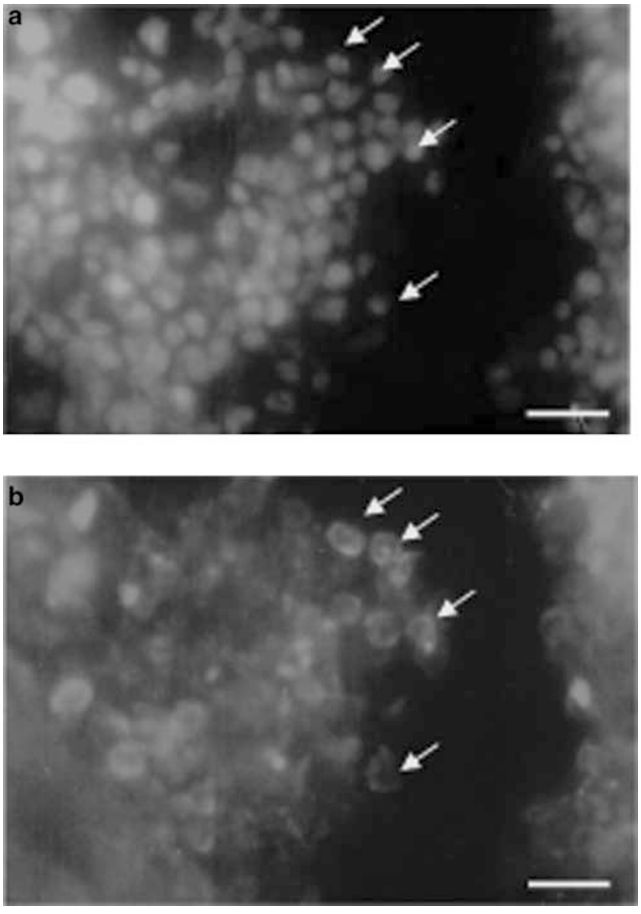

Figure 4 GFAP-positive cells derived from NSCs 67 days after transplantation, showed by different fluoromicroscopic excitation waves in the same visual field. (**a**) Hoechst33342 labeled nucleus is blue (arrows). (**b**) Cytoplasm stained by GFAP is red (arrows). These pictures were taken from within the graft nearby the host. Scale bars = 100 μm (see online for colour figure)

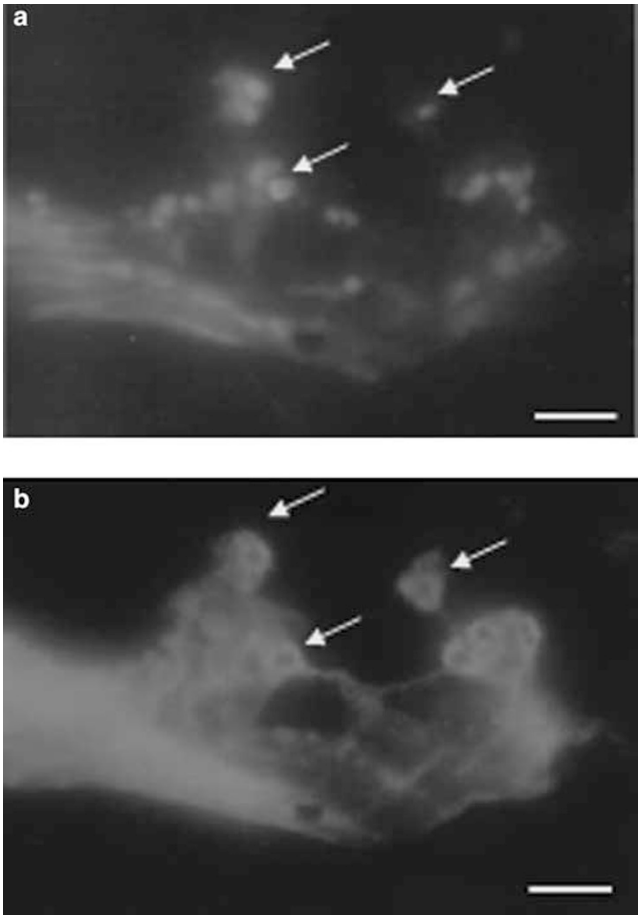

Figure 5 NF-positive cells derived from NSCs 67 days after transplantation, showed by different fluoromicroscopic excitation waves in the same visual field. (**a**) Hoechst33342 labeled nucleus is blue (arrows). (**b**) Cytoplasm stained by NF is red (arrows). These pictures were taken from within the graft nearby the host. Scale bars = 100 μm (see online for colour figure)

the grafted site showed by X-gal histochemical staining (Figure 3a). In the NT-3-SCs + NSCs cografted group, it could be observed lots of NT-3 positive cells within or nearby the grafted site (Figure 3b). As endogenous β-gal activity was not seen when the X-gal histochemical reaction was less than 4 h,[21,22] short incubation times in this study allowed us to exactly distinguish exogenous gene expression from nonspecific endogenous galactosidase activities.

Differentiation of grafted NSCs in host spinal cord

Part of Hoechst33342 labeled NSCs in spinal cord could be double-labeled by GFAP or NF immunofluorochemistry (Figures 4 and 5). As GFAP and NF are special proteins (maker) of neuroglial cell and neuron respectively, it indicated NSCs could differentiate into neuroglial cells or neurons in injured spinal cord. In the present study, functions of the GFAP-positive and NF-positive cells were not detected, so we just named them as neuroglia-like cells and neuron-like cells. In order to explore SCs and NT-3-SCs whether or no could promote NSCs differentiate into neuron-like cells, the percentages of NF-positive cells in the Hoechst33342 labeled cells were detected in all cells grafted groups, and the results were as followed Table 4.

Survival of injured neurons

As spinal cord contains not only a populations of neuronal bodies but also quantity of ascending and descending nerve fibers, spinal cord transection cause all the nerve fibers to be cut through transected site. Therefore, neurons possessing the nerve fibers were injured. In the present study, we chose some representative areas to explore the survival of injured neurons: SMC represent the pyramidal system, RN represent extrapyramidal system, and CN of L1 spinal segment represent afferent system. As to SMC and RN, neuronal densities were counted. But CN's domain was very narrow, and CN neurons in one slice were quite few. In order to obtain valuable results, the whole neuronal number of CN in 50 serial slices of L1 spinal cord segment of each rat was counted. The counted results stated in followed Table 5.

Axonal regeneration

In the SCI control group, no FG labeled neuron could be found within SMC, RN, and the spinal cord rostral to the transected site. However, few of them could be found in the cells grafted groups (Figure 6). However, the FG-labeled neurons was very few, and not very consistently in different cases. Generally, we could observe one or two FG-labeled neuron(s) in some

Table 4 The percentage (%) of NF-positive cells in the Hoechst33342 labeled cells

Groups	*Number of visual fields be counted*	*Number of Hoechst33342 labeled cells*	*Number of NF-positive cells*	*Percentage of NF-positive cells*
NSCs grafted group	200	421.26 ± 54.28	49.58 ± 10.27	12.07 ± 2.35^{a}
SCs + NSCs cografted group	200	489.39 ± 69.31	78.25 ± 13.97	16.15 ± 3.28^{b}
LacZ-SCs + NSCs cografted group	200	528.91 ± 48.27	81.81 ± 16.32	15.76 ± 3.07^{c}
NT-3-SCs + NSCs cografted group	200	475.37 ± 91.82	96.94 ± 15.64	20.34 ± 4.16^{d}

t-test: a *versus* b, a *versus* c, a *versus* d, b *versus* d, c *versus* d $P<0.01$; b *versus* c $P>0.05$

Table 5 The surviving neurons of inner pyramidal layer, RN and CN ($x \pm s$)

Groups	*RN (neurons/mm^2)*	*Inner pyramidal layer (neurons/mm^2)*	*CN (neurons/50 slices)*
Normal control group	295.32 ± 85.23^{a}	791.84 ± 111.08^{a}	213.54 ± 9.35^{a}
SCI control group	131.69 ± 32.31^{b}	412.69 ± 98.39^{b}	36.76 ± 6.62^{b}
NSCs grafted group	151.06 ± 65.12^{c}	522.73 ± 149.55^{c}	52.82 ± 5.19^{c}
SCs + NSCs cografted group	196.87 ± 59.64^{d}	576.24 ± 85.34^{d}	81.58 ± 8.74^{d}
LacZ-SCs + NSCs cografted group	201.16 ± 75.86^{e}	561.71 ± 64.28^{e}	85.13 ± 7.65^{e}
NT-3-SCs + NSCs cografted group	238.25 ± 64.54^{f}	643.58 ± 79.83^{f}	139.68 ± 8.39^{f}

t-test: RN: a *versus* b, a *versus* c, a *versus* d, a *versus* e, a *versus* f, b *versus* d, b *versus* e, b *versus* f, c *versus* f $P<0.01$; b *versus* c, c *versus* d, c *versus* e, d *versus* f, e *versus* f $P<0.05$; d *versus* e $P<0.05$. Inner pyramidal layer: a *versus* b, a *versus* c, a *versus* d, a *versus* e, b *versus* d, b *versus* e, b *versus* f, c *versus* f $P<0.01$; a *versus* f, b *versus* c, c *versus* d, c *versus* e, d *versus* f, e *versus* f $P<0.05$; d *versus* e $P<0.05$. CN: a *versus* b, a *versus* c, a *versus* d, a *versus* e, a *versus* f, b *versus* d, b *versus* e, b *versus* f, c *versus* f $P<0.01$; b *versus* c, c *versus* d, c *versus* e, d *versus* f, e *versus* f $P<0.05$; d *versus* e $P<0.05$

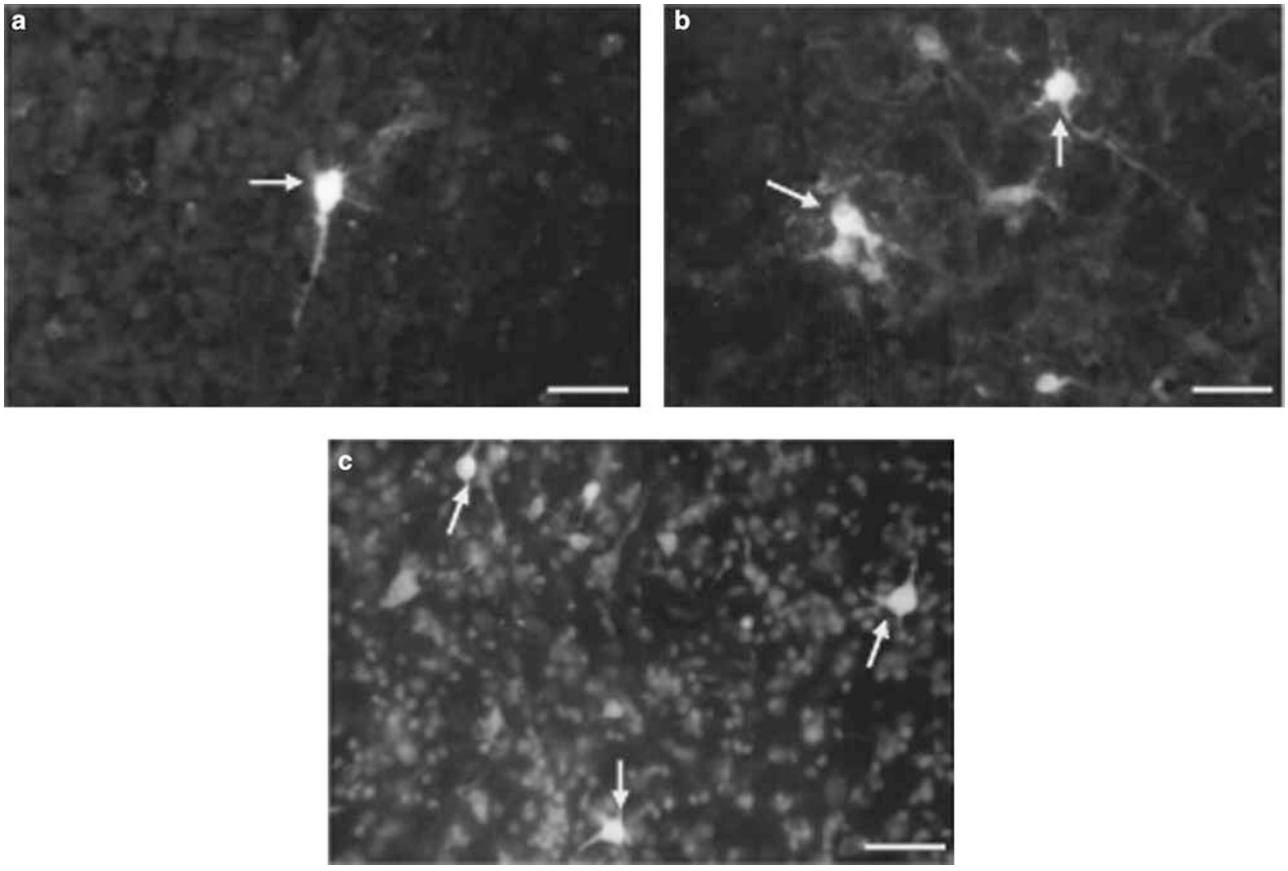

Figure 6 Flourogold labeled neurons (arrows) of the inner pyramidal layer of sensorimotor cortex (**a**), the red nucleus (**b**) and the rostral spinal cord nearby the transected site (**c**) 67 days after surgical procedures in the cells grafted groups. Scale bars = 50 μm

SMC sections, and there was no obvious difference among the cells grafted groups. In RN sections, there had up to eight FG-labeled neurons per section in the NT-3-SCs and NSCs cografted group, but no more than three labeled neurons per section in other cells grafted groups. In the spinal cord rostral to the transected site, the FG labeled neurons were mainly located within 1–2 mm rostral to the transected site, and about up to 20 labeled neurons per section in NT-3-SCs and NSCs cografted group, but no more than 12 labeled neurons per section in other cells grafted groups. In our experiment, the distance of the FG injected site from the transected site was carefully measured and the transection area was checked to make sure that no FG diffused into. The FG retrograde tracing labeled neurons indicated their transected nerve fibers had regenerated through the transected area. Furthermore, in the cells grafted groups but not in the SCI control group, 5-HT, CGRP, and SP positive nerve fibers could frequently be observed within the transected site showed by immunohistochemistry (Figure 7). As 5-HT positive fibers and CGRP positive fibers belong to motor fibers and sensory fibers, respectively, and SP positive fibers come from the neurons of dorsal root ganglion or propriospinal neurons, it indicated a few of motor, sensory and propriospinal nerve fibers could regenerate through the spinal cord transected area.

Discussion

Our previous study has showed that engrafted NSCs could promote the morphologic and functional recovery of the spinal cord transected rats. However, the percentage of the neuron-like cells derived from NSCs is very low and the curative value is limited.[3] In the present study, the overall goal is to determine whether cografted SCs and NT-3-SCs could promote NSCs differentiating into neuron-like cells and enhanced the curative value for SCI.

The inability to replace damaged or dead neurons was considered as one of main reasons severely hindered the development of therapies for CNS injury. The successful engraftment of fetal tissue into the injured CNS suggesting neuronal replacement is a valuable strategy for CNS repair.[23] However, wider clinic use of fetal tissue is manifold problematic, not only from difficult tissue availability but also political and ethical issues. Moreover, as many as 10–15 fetuses may be necessary for the fully efficient transplantation in single patient.[24] The solution to these problems could lie in to find other

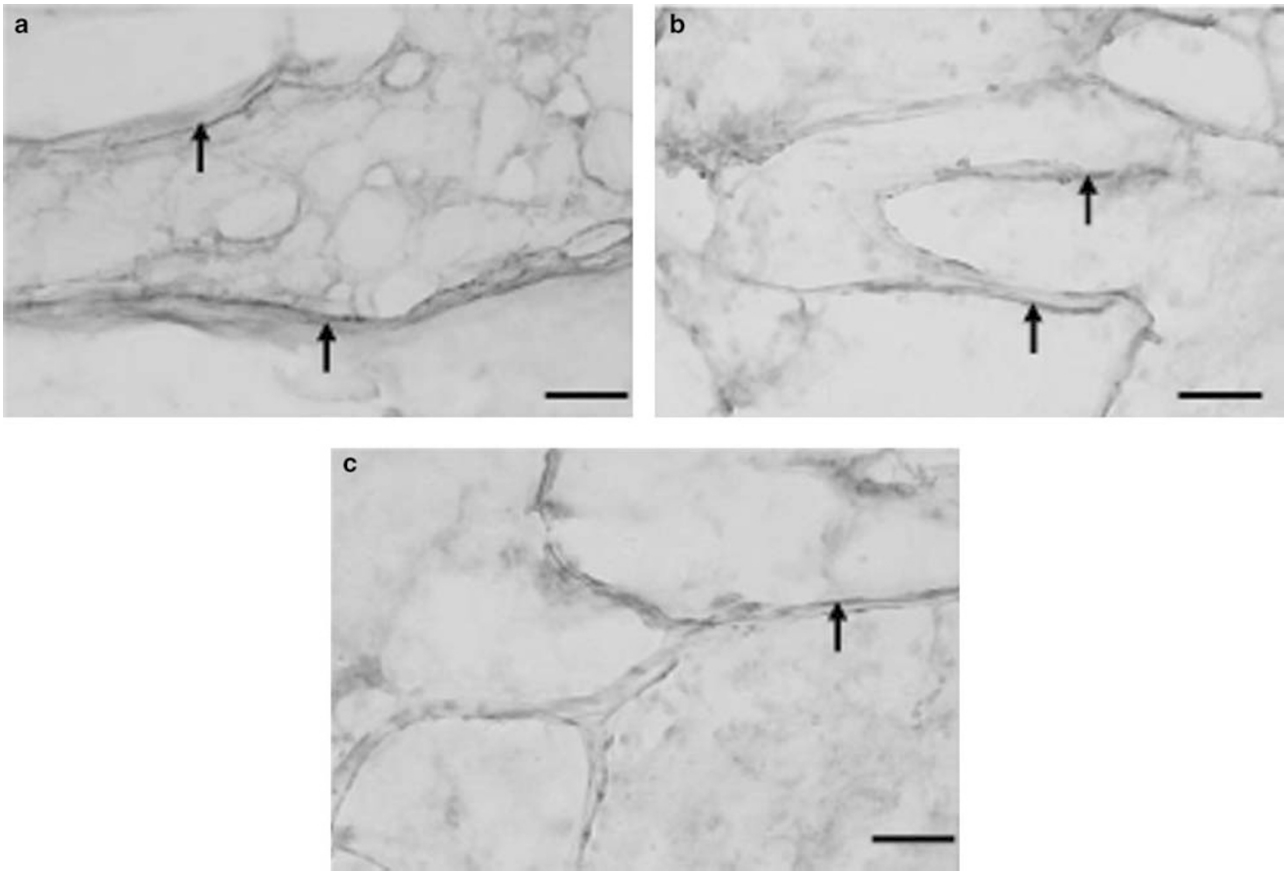

Figure 7 The 5-HT (**a**), CGRP (**b**) and SP (**c**) positive fibers within the transected site of spinal cord 67 days after surgical procedures in the cells grafted groups, stained by immunohistochemistry using DAB as chromagen. Scale bars = 50 μm

tissue or cells to replace the fatal tissue. NSCs are good candidate, which can be clonally expanded in tissue culture and providing a renewable supply of material for transplantation. We hypothesized that NSCs-derived neuron-like cells played main role to promote partly structural and functional recovery of injured spinal cord in the NSCs grafted group. Nevertheless, the percentage of the neuron-like cells in the NSCs grafted group is very low (about 12%, in Table 4), structural and functional recovery of the injured spinal cord are limited. How to increase the percentage of the neuron-like cells, and enhance the recovery of injured spinal cord? In the present study, NT-3-SCs were utilized to cotransplant with NSCs.

Previous studies indicated that differentiation of NSCs was regulated by many factors including neurotrophic factors.[25,26] SCs may synthesize many kinds of neurotrophic factors, cell adhesion, and extracellular matrix molecules. Our previous studies showed that SCs could promote NSCs differentiating into neuron-like cells in the injured spinal cord.[2] After be modified by NT-3 gene, NT-3-SCs could overexpress NT-3.[17] NT-3 could upregulate the TrkC expression in NSCs and promote NSCs differentiating into neurons *in vitro*.[16,18] In the present study, when cografted with SCs or NT-3-SCs, the percentage of NSCs-derived neuron-like cells was increased from 12.07 to 16.15% and 20.34%, respectively. It illuminated that NT-3-SCs could exert an influence on NSCs' differentiation not only by SCs' natural characters but also its' overexpressed NT-3.

Our previous studies have showed that NSCs implantation or SCs implantation could respectively promote the survival and axonal regeneration of damaged neurons and promote partly structural and functional recovery of completely transected spinal cord of rat.[3,27] In the present study, we found the curative value of NSCs and SCs cotransplant is better than that of NSCs single transplant, and NSCs and NT-3-SCs cotransplant is better than NSCs and SCs cotransplant. It means that NT-3-SCs secreted various kinds of neurotrophic factor as SCs' peculiarity and its' overexpressed NT-3 play very important roles to enhance repair value of NSCs in SCI. And we hypothesized NSCs and NT-3-SCs cotransplant is a potential strategy for spinal cord repair.

Acknowledgements

This study was supported by Chinese National Key Project for Basic Research G1999054009, Chinese National Natural Science Foundation 30270700, Social Developmental Foundation of Guangdong Province 2003C33808 to YS Zeng, and

Guangdong Nature Science Foundation 04300468 and Medical Science Research Grant of Guangdong Province (A2004081) to JS Guo.

References

1 Pluchino S *et al*. Neural stem cells and their therapeutic tool in neurological disorders. *Brain Res Rev* 2005; **48:** 211–219.
2 Zeng YS *et al*. Co-transplantation of Schwann cells promotes the survival and differentiation of neural stem cells transplanted into the injured spinal cord. *Dev Neurosci* 2005; **27:** 20–26.
3 Guo JS *et al*. The transplanted neural stem cells promote recovery of structure and function in spinal cord transected rats. *Acta Anatomica Sinica (Chinese)* 2003; **34:** 113–117.
4 Pearse DD *et al*. cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury. *Nat Med* 2004; **10:** 610–616.
5 Keyvan-Fouladi N, Raisman G, Li Y. Delayed repair of corticospinal tract lesions as an assay for the effectiveness of transplantation of Schwann cells. *Glia* 2005; **51:** 306–311.
6 Karim Fouad K *et al*. Combining Schwann cell bridges and olfactory-ensheathing glia grafts with chondroitinase promotes locomotor recovery after complete transection of the spinal cord. *J Neurosci* 2005; **25:** 1169–1178.
7 Bixby JL, Lilien J, Reichardt LF. Identification of the major proteins that promote neuronal process outgrowth on Schwann cells *in vitro*. *J Cell Biol* 1988; **107:** 353–361.
8 Bolin LM *et al*. Interlieukin-6 production by Schwann cells and induction in sciatic nerve injury. *J Neurochem* 1995; **64:** 850–858.
9 Bampton ETW, Taylor JSH. Effects of Schwann cell secreted factors on PC12 cell neuritogenesis and survival. *J Neurobiol* 2005; **63:** 29–48.
10 Bregman BS *et al*. Transplants and neurotrophic factors prevent atrophy of mature CNS neurons after spinal cord injury. *Exp Neurol* 1998; **149:** 13–27.
11 Bamber NI *et al*. Neurotrophins BDNF and NT-3 promote axonal re-entry into the distal host spinal cord through Schwann cell-seed mini-channels. *Eur J Neurosci* 2001; **13:** 257–268.
12 Bradbury EJ *et al*. NT-3, but not BDNF prevents atrophy and death of axotomized spinal cord projection neurons. *Eur J Neurosci* 1998; **10:** 3058–3068.
13 Grill R *et al*. Cellular delivery of neurotrophin-3 promotes corticospinal axonal growth and partial functional recovery after spinal cord injury. *J Neurosci* 1997; **17:** 5560–5572.
14 Novikoval LN, Novikov LN, Kellerth JO. Survival effects of BDNF and NT-3 on axotomized rubrospinal neurons depend on the temporal pattern of neurotrophin administration. *Eur J Neurosci* 2000; **12:** 776–780.
15 Zhang Y *et al*. NT-3 delivered by an adenoviral vector induces injured dorsal root axons to regenerate into the spinal cord of adult rats. *J Neurosci Res* 1998; **54:** 554–562.
16 Hapner SJ *et al*. Neural differentiation promoted by truncated trkC receptors in collaboration with p75(NTR). *Dev Biol* 1998; **201:** 90–100.
17 Guo JS *et al*. Neurotrophin-3 genetically modified Schwann cells using adenoviral vectors. *J Sun Yet-sen Univ (Med Sci) (Chinese)* 2004; **25:** 304–307.
18 Guo JS *et al*. NT-3 genetically modified Schwann cells promote neural stem cells differentiate into neuron-like cells. *Chin J Pathophysiol (Chinese)* 2005; **21:** 26–30.
19 Basso DM, Beattle MS, Bresnahan JC. A sensitive and reliable locomotor rating scale for open field testing in rats. *J Neurotrauma* 1995; **12:** 1–21.
20 Ramon-Cueto A, Cordero MI, Santos-Benito FF. Functional recovery of paraplegic rats and motor axon regeneration in their spinal cords by olfactory ensheathing glia. *Neuron* 2000; **25:** 425–435.
21 Liu Y *et al*. Application of recombinant adenovirus for *in vivo* gene delivery to spinal cord. *Brain Res* 1997; **768:** 19–29.
22 Lal B *et al*. Development of endogenous beta-galactosidase and autofluorescence in rat brain microvessels: implications for cell tracking and gene transfer studies. *J Histochem* 1994; **42:** 953–956.
23 Pincus DW *et al*. Neural stem and progenitor cells: A strategy for gene therapy and brain repair. *Neurosurg* 1998; **42:** 858–868.
24 Bjorklund A. Better cells for brain repair. *Nature* 1993; **362:** 414–415.
25 Abejon CV *et al*. Function of basic fibroblast growth factor and neurotrophins in the differentiation of hippocampal neurons. *Neuron* 1995; **15:** 105–114.
26 Shetty AS, Turner DA. *In vitro* survival and differentiation of neurons derived from epidermal growth factor-responsive postnatal hippocampal stem cells: inducing effects of brain-derived neurotropnic factor. *J Neurobio* 1998; **35:** 395–425.
27 Wu LZ *et al*. Transplanted Schwann cells promote the neural survival of cerebral cortex and red nucleus after rat spinal cord transection. *J Sun Yet-sen Univ (Med Sci) (Chinese)* 2004; **25:** 10–14.