Research Report

Transplantation of artificial neural construct partly improved spinal tissue repair and functional recovery in rats with spinal cord transection

Bao-Ling Du, Yi Xiong, Chen-Guang Zeng, Liu-Min He, Wei Zhang, Da-Ping Quan, Jin-Lang Wu, Yan Li, Yuan-Shan Zeng

Division of Neuroscience, Department of Histology and Embryology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China
Key Laboratory for Stem Cells and Tissue Engineering (Sun Yat-sen University), Ministry of Education, Guangzhou, China
Institute of Spinal Cord Injury, Sun Yat-sen University, Guangzhou, China
School of Chemistry and Chemical Engineering, Sun Yat-sen University, Guangzhou, China
Department of Electron Microscope, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China

ARTICLE INFO

Article history:
Accepted 10 May 2011
Available online 16 May 2011

Keywords:
Transected spinal cord
PLGA
Neurotrophin-3
TrkC
Neural stem cell
Artificial neural construct
Transplantation

ABSTRACT

Delivery of cellular and/or trophic factors to the site of injury may promote neural repair or axonal regeneration and return of function after spinal cord injury. Engineered scaffolds provide a platform to deliver therapeutic cells and neurotrophic molecules. To explore therapeutic potential of engineered neural tissue, we generated an artificial neural construct in vitro, and transplanted this construct into a completely transected spinal cord of adult rats. Two months later, behavioral analysis showed that the locomotion recovery was significantly improved compared with control animals. Immunoreactivity against microtubule associated protein 2 (Map2) and postsynaptic density 95 (PSD95) demonstrated that grafted cells had a higher survival rate and were able to differentiate toward neuronal phenotype with ability to form synapse-like structure at the injury site; this was also observed under the electron microscope. Immunostaining of neurofilament-200 (NF-200) showed that the number of nerve fibers regrowing into the injury site in full treatment group was much higher than that seen in other groups. Furthermore, Nissl staining revealed that host neuron survival rate was significantly increased in rats with full treatments. However, there were no biotin dextran amine (BDA) anterograde tracing fibers crossing through the injury site, suggesting the limited capacity for corticospinal tract axonal regeneration. Taken together, although our artificial neural construct permits grafted cells to differentiate into neuronal phenotype, synaptogenesis, axonal regeneration and partial locomotor function recovery, the limited capacity for corticospinal tract axonal regeneration may affect its potential therapy in spinal cord injury.

© 2011 Elsevier B.V. All rights reserved.

Keywords:
Transected spinal cord
PLGA
Neurotrophin-3
TrkC
Neural stem cell
Artificial neural construct
Transplantation
neural stem cells seeded in PLGA, which generated an artificial
model of rat, implantation of PLGA scaffold with NSCs into the
cord model, but with lower survival rate of transplanted
hindlimb motor recovery in completely transected spinal
For instances, PLLA scaffold-Schwann cells construct improved
The transplantation reduced tissue loss and glial scarring
injured site resulted in a functional improvement for one year.
the lesion site (Novikova et al., 2002; Sayer et al., 2002).
One of the strategies to repair SCI was the transplantation
of neural stem cells (NSCs) (Nash et al., 2002), mainly as NSCs
have the capacity to differentiate into neurons. However,
grafted NSCs are rarely differentiated into mature neurons in
the lesion site (Novikova et al., 2002; Sayer et al., 2002).
Therefore, more and more attention has been focused on how
to pre-induce NSCs differentiation in vitro.
Molecular therapies, such as administration of neurotrophins (NTs) (Fernandez et al., 1993; Namiki et al., 2000; Schnell et al., 1994), are known to play important roles in neural survival, differentiation, and neurite outgrowth (Chao, 2003; Lu et al., 2005). NSCs transfected with NT-3 gene yield a higher percentage of differentiation toward neurons (Wang et al., 2007; Park et al., 2006). This percentage may be further increased when NSCs over-expressing NT-3 are mixed with NSCs expressing tyrosine receptor kinase C (TrkC), the NT-3 receptors (Wang et al., 2007). Our previous studies revealed that gene-modified NSCs promoted axon regeneration and functional improvement when transplanted into injured spinal cord of rats, but incapable of forming a neural network to bridge the gap at the injury site (Guo et al., 2007; Lu et al., 2003; Zhang et al., 2007).
Engineered biomaterials provide a platform to deliver therapeutic cells and/or neurotrophic molecules, which have been investigated for their ability to reconstruct spinal cord tissue architecture, to provide guidance for regenerating axons, and to prevent the infiltration of scar tissue and cyst formation in the SCI animal models (Friedman et al., 2002; Lee et al., 2003; Oudega et al., 2001; Teng et al., 2002; Xu et al., 1999). Natural biomaterials and synthetic biomaterials have been used as engineered scaffolds at the injury site of experimental animals, due to the advantages that they are biodegradable and have a good biocompatibility. In addition, the degradation products are easy to be absorbed and seldom to cause inflammation. Poly(lactic acid-co-glycolic acid) (PLGA) and poly(ε-lactic acid) (PLLA) polymers are representative in synthetic polymer biomaterials. For instances, PLLA scaffold-Schwann cells construct improved the hindlimb motor recovery in completely transected spinal cord model, but with lower survival rate of transplanted Schwann cells (Zhang et al., 2007). And in hemisectioned SCI model of rat, implantation of PLGA scaffold with NSCs into the injured site resulted in a functional improvement for one year. The transplantation reduced tissue loss and glial scarring (Novikova et al., 2002).
We recently reported that NT-3 and TrkC gene-modified neural stem cells seeded in PLGA, which generated an artificial
neural construct in vitro. The artificial neural construct
permits NSCs to differentiate into neurons establishing
connections with each other and exhibiting synaptic activities (Xiong et al., 2009). In the present study, we investigated whether the artificial neural construct transplanted into spinal cord transected completely could still retain NSCs-derived neurons in vivo, and promote axonal regeneration and functional improvement.

1. Introduction

It is still a major therapeutic challenge to promote the axonal regeneration and functional recovery from spinal cord injury (SCI). The pathophysiological processes in spinal cord injury are multifactorial, involving blood vessel rupture, ischemia, edema, metabolic derangement, and free radicals formation in acute phase, and followed by axonal degeneration/regeneration, loss of glial cells, demyelination/remyelination, and formation of cavities at the injury site (Faden, 1993). It has been proposed that these devitalized tissues be replaced by artificial neural tissues in order to restore the motor functions. There have been significant developments in experimental studies of this area in the past years.

One of the strategies to repair SCI was the transplantation of neural stem cells (NSCs) (Nash et al., 2002), mainly as NSCs have the capacity to differentiate into neurons. However, grafted NSCs are rarely differentiated into mature neurons in the lesion site (Novikova et al., 2002; Sayer et al., 2002). Therefore, more and more attention has been focused on how to pre-induce NSCs differentiation in vitro.

Molecular therapies, such as administration of neurotrophins (NTs) (Fernandez et al., 1993; Namiki et al., 2000; Schnell et al., 1994), are known to play important roles in neural survival, differentiation, and neurite outgrowth (Chao, 2003; Lu et al., 2005). NSCs transfected with NT-3 gene yield a higher percentage of differentiation toward neurons (Wang et al., 2007; Park et al., 2006). This percentage may be further increased when NSCs over-expressing NT-3 are mixed with NSCs expressing tyrosine receptor kinase C (TrkC), the NT-3 receptors (Wang et al., 2007). Our previous studies revealed that gene-modified NSCs promoted axon regeneration and functional improvement when transplanted into injured spinal cord of rats, but incapable of forming a neural network to bridge the gap at the injury site (Guo et al., 2007; Lu et al., 2003; Zhang et al., 2007).

Engineered biomaterials provide a platform to deliver therapeutic cells and/or neurotrophic molecules, which have been investigated for their ability to reconstruct spinal cord tissue architecture, to provide guidance for regenerating axons, and to prevent the infiltration of scar tissue and cyst formation in the SCI animal models (Friedman et al., 2002; Lee et al., 2003; Oudega et al., 2001; Teng et al., 2002; Xu et al., 1999). Natural biomaterials and synthetic biomaterials have been used as engineered scaffolds at the injury site of experimental animals, due to the advantages that they are biodegradable and have a good biocompatibility. In addition, the degradation products are easy to be absorbed and seldom to cause inflammation. Poly(lactic acid-co-glycolic acid) (PLGA) and poly(ε-lactic acid) (PLLA) polymers are representative in synthetic polymer biomaterials. For instances, PLLA scaffold-Schwann cells construct improved the hindlimb motor recovery in completely transected spinal cord model, but with lower survival rate of transplanted Schwann cells (Zhang et al., 2007). And in hemisectioned SCI model of rat, implantation of PLGA scaffold with NSCs into the injured site resulted in a functional improvement for one year. The transplantation reduced tissue loss and glial scarring (Novikova et al., 2002).

We recently reported that NT-3 and TrkC gene-modified neural stem cells seeded in PLGA, which generated an artificial
neural construct in vitro. The artificial neural construct
permits NSCs to differentiate into neurons establishing
connections with each other and exhibiting synaptic activities (Xiong et al., 2009). In the present study, we investigated whether the artificial neural construct transplanted into spinal cord transected completely could still retain NSCs-derived neurons in vivo, and promote axonal regeneration and functional improvement.

2. Results

2.1. Functional recovery

Before SCI, all the rats showed normal locomotor performance with BBB score of 21. When spinal cord was completely transected, the hindlimbs of the animals were immediately paralyzed with BBB score of 0. Two weeks after transplantation, locomotor performance improved gradually in all experimental groups. At the end of the 8th week, scores of the Co-culture group and NT-3 group were significantly higher than other groups, and the Co-culture group exhibited an average score of 8.78±1.78, which was the highest among the groups (P<0.05, Fig. 1). A BBB score of 8.78 indicates that the animals were able to sweep their hindlimbs with no weight support or/plantar paw placement with support in stance (when stationary). The 45° inclined grid test concerning limb coordination was also performed. Compared with the PLGA, NSCs and TrkC groups, the rest of groups could voluntarily place their paws on the rung and step their hindlimbs onto the grid occasionally. Especially, the Co-culture group occasionally exhibited the coordination movement of the fore-hind limbs and had more chance to step their hindlimbs onto grid (Supplementary Video 1 and 2, online),
but the PLGA, NSCs and TrkC groups showed no obvious hindlimb movement; they just struggled to climb onto the inclined grid with their forelimbs (Supplementary Video 3, online) (Table 1). In addition, the rats in the sham group reached the normal locomotor performance (i.e. BBB score = 21) within the 1st week and maintained the normal function until their sacrifice.

2.2. Improvement of tissue repair and local environment

The implants integrated with host tissue by bridging the rostral and caudal spinal cord stumps. In the lesion site, compared with the PLGA group, the cells-PLGA groups exhibited unobvious atrophy in the white, opaque bridging tissue, especially in the Co-culture group (Fig. 2A).

HE staining showed there was only a little of PLGA residue in the lesion site in the cells-PLGA groups. Whereas in the PLGA group, most of PLGA scaffold still remained and there were numerous cavities in the transected regions. Compared with other groups, there was a tissue-continuity across the transection site with minor cavities or gaps and integrating well with host cord stumps in the Co-culture group. But in all groups, there still was scar tissue in transected region although that of the Co-culture group lessened obviously (Fig. 2B–G). And we measured the area of cystic cavity at the injured spinal cord among all the groups except for the Sham group. The average area of the cystic cavity was significantly smaller in the cell-implanted groups (P<0.05) than that in the PLGA group, while that in the Co-culture group was the smallest (P<0.05) (Fig. 2H).

2.3. Neuronal survival after transection injury

As spinal cord contains not only populations of neuronal bodies but also quantity of ascending and descending nerve fibers, spinal cord transection causes all the nerve fibers to be cut down 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 represents the pyramidal system, RN represents extra-pyramidal system, and CN of L1 spinal segment represents afferent system. Cell counting showed more surviving neurons in the SMC, RN, and CN of the cells-PLGA groups than those of the PLGA group, and the maximum number of hindlimbs stepping inclined grid (x±s).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Frequency of hindlimbs stepping inclined grid</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA*</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>NSCs*</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Lac-Zf</td>
<td>5</td>
<td>0.8±0.84</td>
</tr>
<tr>
<td>TrkCfd</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>NT-3f</td>
<td>5</td>
<td>3.4±0.89</td>
</tr>
<tr>
<td>Co-culturef</td>
<td>5</td>
<td>6.4±1.14</td>
</tr>
</tbody>
</table>

a us. a; b us. c; a vs. d; b vs. c; b us. d; c us. d; P>0.05.
e us. a, b, c, d, f; f us. a, b, c, d; P<0.05.

To assess axon growth and regeneration, IFC and anterograde tracing were performed. NF-200 (neurofilament-200, mainly marking axon growth) positive nerve fibers were found in the epicenter and/or in both ends of the lesion site in the Co-culture group (Fig. 5A–D). The number of NF-200 positive nerve fibers was much higher in the Co-culture group than those in other groups (Table 3). Axonal regeneration in the CST was evaluated by anterograde transport of BDA from the SMC injection sites. In the Sham group, BDA-labeled integrity CST axons were tightly bundled along the dorsal white matter of spinal cord within the whole distance (Fig. 6A), but only a few axons irregularly distributed near the area rostral to the injury site (Fig. 6B). In other groups, we did not find any BDA positive fibers within T8–T12 spinal cord segments.

In SCI, demyelination is a common pathology contributing to dysfunction (Coyle et al., 1981). More importantly, this pathology is reversible if remyelination occurs on the denuded axons. We therefore assessed myelination by performing MBP (myelin basic protein, a protein on the compact myelin) in the transected areas. MBP immunoreactivity was conspicuously visible at the lesion region in the Co-culture group but always absent in other groups (Fig. 6C). Furthermore, electron microscopy was performed to examine remyelination at the injured site. Compared to other groups, the Co-culture group showed more myelinated axons with compacted myelin sheath (Fig. 6D).

2.4. Differentiation and synaptogenesis

IFC showed that transplanted NSCs differentiated into Map2 (microtubule associated protein 2, a marker for postmitotic neurons; Fig. 3A–D) and PSD95 (postsynaptic density 95, a postsynaptic density marker; Fig. 3E–H) positive cells in the injured cord. The percentage of Map2 and PSD95 positive cells in the Co-culture group was 32.06±4.17% and 15.20±1.29%, respectively, which was significantly higher than that in other cells-PLGA groups (P<0.05, Fig. 3I).

To further examine whether synaptic connections have been established at the injury site, TEM was performed at the transection area. Many neuron-like cells were visible with extending neurites in the Co-culture group. Their nuclei with a well-demarcated nucleolus, did not show dense chromatin. The cytoplasm contained abundant organelles, but in general had a low electron density. More importantly, a number of synapse-like structures were found frequently at the lesion site. Markedly thicken membranes could be seen where two cells contacted closely (Fig. 4A). In addition, some vesicle-like structures appeared at one side of contacting membranes (Fig. 4B). Moreover, more synapse-like structures were detected at the edge of the injury site. No synapse-like structure was found in the PLGA group.

2.5. Nerve fibers regeneration and re-myelination

2.6. Exogenous gene expression

Exogenous LacZ, NT-3 and TrkC gene expressed in vivo. By using X-gal staining, β-galactosidase positive cells were observed at the lesion site in the LacZ group (Fig. 7A). Additionally, numerous NT-3 and TrkC IFC positive cells (labeled with
Hoechst33342) were visible within or nearby the transplantation region in the Co-culture group (Fig. 7B, C). These results demonstrate the in vivo expression of exogenous gene, which persisted for the entire duration of this study (i.e. 8 weeks).

3. Discussion

In the present study, we demonstrated that gene-modified NSCs seeded in PLGA scaffolds over-express NT-3 and TrkC in vivo to promote functional recovery. Firstly, the most important finding of our results was our artificial neural construct could develop into neural network at the lesion site, which was consistent with our previous study in vitro (Xiong et al., 2009). For instance, 1) a high percentage of neural differentiation and synaptogenesis of NT-3/TrkC gene-modified NSCs was found in PLGA scaffolds. As we know, the microenvironments around injury site are not beneficial to planted cells for survival and differentiation (Coyle et al., 1981; Csernansky et al., 1994; Gwag et al., 1995). Therefore, the percentage of MAP2 positive cells was significantly higher than that in our previous study (Zhang et al., 2007), although it was sharply higher in vitro (Xiong et al., 2009). This was supported by our previous study that simultaneous expression of both NT-3 and TrkC may further facilitate the differentiation of NSCs into neurons and the synaptic formation (Xiong et al., 2009). TEM
Exogenous gene expression revealed that the expression of NT-3 and TrkC persisted for the entire duration in vivo in the present study. That is the reason why the above results are better in the Co-culture group than those in the NT-3 or TrkC groups. This is further supported by the results of neuronal survival after transection injury.

In this study, our artificial neural construct containing NT-3 and TrkC gene modified NSCs may be one of the reasons which were involved in the recovery of hindlimb locomotor function, because it may have become integrated with the local plasticity and locomotor circuitry in the lesion site. A significant, albeit modest, improvement in performance was observed in the Co-culture group (with BBB score of 8.78 ± 1.78), which was found despite the lack of CST axons growing into the injury site, and also has been caused by neurotrophin-mediated modifications at the neuronal level in the denervated T10 spinal cord segment. The gene-modified cells located at the injury site might have secreted enough neurotrophins to enhance neuronal excitability, stimulate neurotransmitter synthesis, and turnover in the neurons that are involved in the local locomotor circuitry (Altar et al., 1994; Siuciak et al., 1996). Recent study also showed that transplantation of cells engineered to produce NTFs (BDNF and NT-3) promoted locomotor recovery in untrained spinal cats. In the results, the potential for intraspinal delivery of NTFs supports the recovery of hindlimb locomotion in the cat of spinal cord transected completely, and this improvement was not associated with regeneration of host axons through the graft and into caudal spinal cord, but because of NTFs that was likely to involve plasticity of local neuronal circuitry (Boyce et al., 2007). And the consequences may be further facilitated by our functional neural construct.

Second, the relative high BBB score may be the result of limited loss of tissue evoked by the implantation of the bioengineered PLGA with gene-modified NSCs. Tissue engineering offers a potential solution to the massive loss of tissue structure after spinal cord injury. Pathological and imaging studies demonstrate tissue destruction with cysts and gliosis in the area of injury and atrophy in adjacent segments of cord (Bodley, 2002; Quencer and Bunge, 1996). The cystic and gliotic area is both a gap and a barrier to regeneration. Tissue engineering strategies would replace the cyst/scar with functional tissue. In this study, there was a tissue-continuity across the transected site with minor cavities or gaps and integrating well with host cord stumps in the Co-culture group. This may be another reason for improving the hindlimb function.

At last, axon regeneration is the main reason for functional impairment caused by spinal cord injury. 5-HT nerve fiber regeneration is partially related to locomotor functional recovery. 5-HT fibers are derived from a small group of neurons within the raphe nuclei of the brain stem, and play an important role in regulating voluntary movement as a neuromodulator. Recently, some studies showed that the recovery of 5-HT fibers and transporter or precursor increased motor function in injured rat spinal cord (Hayashi et al., 2010; Saruhashi et al., 2009). In our results, 5-HT positive nerve fibers were observed at the injury site and the area caudal to the injury site in the Co-culture group (Supplementary Fig. S1, online), but no 5-HT positive nerve fiber was observed in the caudal site near by injured spinal cord in other groups. This may be another reason that the recovery of hindlimb locomotor function in the Co-culture group was better than other groups. In addition, 5-HT

### Table 2 – Number of neuronal survival in SMC, RN and CN (×±s).

<table>
<thead>
<tr>
<th>Groups</th>
<th>SMC (neurons/0.16 mm²)</th>
<th>RN (neurons/slide)</th>
<th>CN (neurons/slide)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham¹</td>
<td>111.61 ± 19.11</td>
<td>97.58 ± 2.29</td>
<td>48.53 ± 4.63</td>
</tr>
<tr>
<td>PLGA²</td>
<td>51.15 ± 8.04</td>
<td>17.43 ± 2.03</td>
<td>6.38 ± 1.56</td>
</tr>
<tr>
<td>NSCs³</td>
<td>63.61 ± 7.78</td>
<td>38.60 ± 5.47</td>
<td>12.28 ± 1.58</td>
</tr>
<tr>
<td>Lac-Z⁴</td>
<td>70.54 ± 10.06</td>
<td>45.08 ± 3.65</td>
<td>19.33 ± 1.79</td>
</tr>
<tr>
<td>TrkC⁵</td>
<td>58.12 ± 7.88</td>
<td>36.08 ± 4.40</td>
<td>11.28 ± 1.47</td>
</tr>
<tr>
<td>NT-³⁶</td>
<td>80.49 ± 10.69</td>
<td>52.18 ± 5.67</td>
<td>27.38 ± 2.95</td>
</tr>
<tr>
<td>Co-culture⁷</td>
<td>100.48 ± 13.94</td>
<td>61.43 ± 8.09</td>
<td>37.95 ± 4.67</td>
</tr>
</tbody>
</table>

SMC: compared with any group, P < 0.01.
RN: c vs. e, P > 0.05.
a vs. b, a vs. c, a vs. d, a vs. e, a vs. f, a vs. g, b vs. c, b vs. d, b vs. e, b vs. f, b vs. g, c vs. d, c vs. f, c vs. g, d vs. e, d vs. f, d vs. g, e vs. f, e vs. g, f vs. g, P < 0.01.
CN: c vs. e, P > 0.05.
 a vs. b, a vs. c, a vs. d, a vs. e, a vs. f, a vs. g, b vs. c, b vs. d, b vs. e, b vs. f, b vs. g, c vs. d, c vs. f, c vs. g, d vs. e, d vs. f, d vs. g, e vs. f, e vs. g, f vs. g, P < 0.01.
nerve fibers that occurred in caudal site near by injured spinal cord can be attributed to the enhancement of exogenous NT-3 and TrkC under the combination of NSCs transplantation. Anterograde tracing for the CST axonal regeneration revealed that only a few axons irregularly distributed near the area rostral to the injured site in the Co-culture group, and no labeled fiber was found near the area rostral to the injury site in other groups. Previously, it was shown that a continuous infusion of NT-3 into the spinal cord promotes re-entry of descending spinal axons into the spinal tissue (Bamber et al., 2001). However, in our study the injury was confined to the lateral half of the cord and relatively high levels (1–10 μg/day) of neurotrophins were infused. The transection/implantation model used in our present study (i.e. 2 mm long spinal cord transection injury and 2 mm long PLGA scaffolds) undoubtedly caused more damage to the spinal tissue, which resulted in a graft-host cord interface highly inhibitory for axonal growth. This may be one reason why CST axons can not pass through the rostral cord to the grafts. Further more, in our previous study, the pH values of PLGA degradation solution is detected in narrow range of 4.1–4.5 from 2 to 20 weeks in vitro (He et al., 2009). The acid microenvironment may be another reason for preventing CST axons from regenerating. Therefore, our further investigation of inducing CST axons regeneration should provide more insight into (1) optimizing the ingredient of PLGA to lessen the acid degradation products, (2) improving its space structure to make neurotrophin easy exchange, and (3) supplementing some kind of adhesion proteins to promote grafted cells adhesion and axon growth, like laminin. These may lead to novel bioengineered scaffolds therapeutic strategies.

Fig. 3 – Differentiation and synaptogenesis of grafted NSCs in four groups. (A–D) Map2 positive cells (red) are visible in the grafted areas of the Co-culture (A), NT-3 (B), LacZ (C) and NSCs (D) groups. (E–G) PSD95 positive cells (red) are visible in the grafted area of the Co-culture (E), NT-3 (F), LacZ (G) and NSCs (H) groups. Many cells (arrows) with the Map2 or PSD95 positive cytoplasm were Hoechst33342-positive (blue). Insets: Hoechst33342-stained nuclei overlapped with Map2 (A) or PSD95 (B) immunoreactivity, suggesting they are grafted cells. Scale bar=20 μm (A–H). (I) Percentage of Map2 and PSD95-positive cells in Hoechst33342-labled cell. Data are presented as mean ± SD of the experiment. Asterisks (*) indicate significant differences between the NSCs (or TrkC) group and other groups (P < 0.05); pounds (#) indicate significant differences between the LacZ group and other groups (P < 0.05); ampersands (&) indicate significant differences between the NT-3 group and other groups. In the Co-culture group, the percentage of Map2 and PSD95 positive cells was significantly higher than other groups (P < 0.05).
aimed at stimulating or directing spinal cord plasticity to enhance the function of local locomotor neural circuits and to promote axon regeneration.

4. Experimental procedures

4.1. Preparation of PLGA scaffolds

Macroporous PLGA scaffold was synthesized as previously described (He et al., 2009; Xiong et al., 2009). In brief, the polymer scaffold was trimmed into a rod shape with 3 mm in diameter and with small channels parallel to the longitudinal axis. PLGA rods were formed various sizes of pores from a few \( \mu m \) to 200 \( \mu m \) that were suitable for seeded neurospheres with diameters of 100–300 \( \mu m \). The PLGA rod was cut into 2 mm long before seeding NSCs into it for culture.

4.2. Culture of neural stem cells

NSCs were prepared as described previously (Zeng et al., 2005). Briefly, one-to-three-day-old Sprague–Dawley (SD) rat pups were

Fig. 4 – Electron micrographs of synapse-like structures (arrows) in the Co-culture group. (A) At the rostral junction of host cord at the injury site, cells extended their processes to make contacts with other cells (arrow). (B) Cells in the grafted region also showed cell junction (arrow). Inset: synaptic vesicle-like structures near to presynaptic membrane (white arrows). Scale bar=0.5 \( \mu m \) (A and B).

Fig. 5 – NF-200 positive nerve fibers growing into the injury site in the Co-culture group. Boxes B, C, and D in A (longitudinal section through the injury site) represent rostral, central and caudal areas at the injury site, and are magnified in B, C, and D, respectively. Scale bar=160 \( \mu m \) (A). Arrows in insets B, C and D indicate cells with NF-200 positive cytoplasm and processes, which overlap with Hoechst-stained nuclei. Scale bar=20 \( \mu m \) (B–D) and 10 \( \mu m \) (insets of B and C).
anesthetized. The whole hippocampus was dissected and disassociated in D-Hanks’ balanced saline solution (HBSS). After centrifuging at 1000 rpm for 5 min, the supernatant was removed. Pellet was resuspended in 5 ml basal medium including DMEM/F12 (1:1) containing B27 supplement (20 μl/ml, Gibco, CA, USA) and bFGF (20 ng/ml, Invitrogen, CA, USA). Within 3–5 days at 37 °C with 5% CO2, cells proliferated and formed floating neurospheres. The neurospheres were neural precursor cells conformed by labeling nestin, an NSC marker (Xiong et al., 2009).

4.3. NSCs transfection and seeding in PLGA scaffold

Recombinant adenoviral (Ad) vectors (Ad-NT-3 and Ad-TrkC) were produced as described in our previous study (Wang et al., 2007). Ad-LacZ (gift from Dr. Huang WL) was used as the control (Wang et al., 2007; Park et al., 2006; Zhang et al., 2007). NSCs were transfected and seeded into the sterile PLGA scaffold as described previously (Xiong et al., 2009). In brief, neurospheres were infected with Ad-LacZ, Ad-NT-3 or Ad-TrkC at a multiplicity of infection (MOI) of 50. The cells were resuspended in 5 ml fresh basic medium and plated onto 25 ml-culture flasks. 48 h later, cells in 20 μl culture medium (including 1:1 DMEM/F12 and 10% fetal bovine serum) were seeded into per scaffold as follows: NSCs only, LacZ-NSCs, TrkC-NSCs, NT-3-NSCs and NT-3/TrkC-NSCs (Co-culture of NT-3-NSCs mixed with TrkC-NSCs). The scaffolds were incubated in 35 mm culture dish for 14 days. The culture medium was replaced every 2 days. NSCs were labeled by Hoechst33342 prior to being seeded into scaffolds.

4.4. Spinal cord transection and transplantation of cell-PLGA constructs surgery

Thirty nine adult female Sprague–Dawley rats (220–250 g, supplied by the Experimental Animal Center of Sun Yat-sen University) were anesthetized with an intraperitoneal injection (i.p.) of 1% pentobarbital sodium (40 mg/kg). Following laminectomy at the T9 vertebral level, the spinal cord was transected and a 2-mm cord segment including visible spinal roots was completely removed at the T10 spinal cord level. All visible spinal cord tissue in the transection gap was removed. After hemostasis was achieved, a 2-mm-long construct of cells-PLGA

---

**Table 3 - Number of NF-200 positive fibers in transplantation groups (x±s).**

<table>
<thead>
<tr>
<th>Groups</th>
<th>NF-200 positive fibers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rostral</td>
</tr>
<tr>
<td>PLGA (n=5)*</td>
<td>2.20±0.84</td>
</tr>
<tr>
<td>NSCs (n=5)</td>
<td>1.80±0.84</td>
</tr>
<tr>
<td>LacZ (n=5)</td>
<td>13.80±2.16</td>
</tr>
<tr>
<td>TrkC (n=5)</td>
<td>7.20±1.92</td>
</tr>
<tr>
<td>NT-3 (n=5)*</td>
<td>32.20±6.83</td>
</tr>
<tr>
<td>Co-culture (n=5)†</td>
<td>61.40±8.40</td>
</tr>
</tbody>
</table>

Rostral: a vs. b, a vs. d, b vs. d, P>0.05.
Epicenter: a vs. c, a vs. e, b vs. c, b vs. e, b vs. f, c vs. d, c vs. e, c vs. f, d vs. e, d vs. f, e vs. f, P<0.05.
Caudal: a vs. b, a vs. d, b vs. d, P>0.05.
a vs. c, a vs. e, a vs. f, b vs. c, b vs. e, b vs. f, c vs. d, c vs. e, c vs. f, d vs. e, d vs. f, e vs. f, P<0.05.

---

**Fig. 6 – CST axons at the injury site. (A) By using BDA anterograde tracing, numerous CST axons (red) were seen around the T9 spinal cord (T7–T11) in the sham group, but few labeled axons were found at the injured site in the Co-culture group (B). (C) Myelination was detected by performing MBP (arrows) in the transected areas in the Co-culture group. (D) TEM showed the newly formed thin myelin sheaths (arrows) located at the injury center in the Co-culture group. Scale bars=40 μm (A, B), 20 μm (C) and 1 μm (D).**
(or PLGA only) was implanted between the rostral and caudal stumps, and the muscles and skin were sutured separately. Bladders were emptied manually twice a day until bladder function returned. In case urinary tract infection occurred later, penicillin (160,000 U/ml/day) was administered daily post-surgery for a week. No immunosuppressant was used. All experimental protocols and animal handling procedures were approved by the Animal Care and Use Committee of Sun Yat-sen University, and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

4.5. Experimental groups

The 39 rats were randomly divided into 7 groups:

Group 1: Sham group (n=5), being performed with the T9 laminectomy.
Group 2: PLGA group (n=5), being transplanted with PLGA scaffold only.
Group 3: NSCs group (n=5), being transplanted with NSCs-PLGA.
Group 4: LacZ group (n=5), being transplanted with Ad-LacZ-NSCs-PLGA.
Group 5: TrkC group (n=5), being transplanted with Ad-TrkC-NSCs-PLGA.
Group 6: NT-3 group (n=5), being transplanted with Ad-NT-3-NSCs-PLGA.
Group 7: Co-culture group (n=9), being transplanted with Ad-NT-3/TrkC-NSCs-PLGA (Ad-NT-3-NSCs and Ad-TrkC-NSCs mixed with 1:1).

4.6. Assessment of locomotor performance

Hindlimb function of the rats was assessed weekly after surgery, using the Basso, Beattie and Bresnahan (BBB) open-field locomotor test (Basso et al., 1995) and inclined-grid climbing test as described by Ramon-Cueto et al. (2000). The former quantitatively evaluates voluntary movement and body weight support, and the latter assesses accuracy of foot placement and coordination qualitatively which differentiate local reflex activity from voluntary movement. Two independent investigators blind of the different experimental treatments determined the BBB scores.

4.7. Morphological analysis

4.7.1. Anterograde tracing

Eight weeks after SCI, rats were anesthetized and placed in a stereotaxic frame. Biotin dextran amine (BDA, 10,000 MW, Molecular Probes, Invitrogen) at a concentration of 10% in PBS was injected with a Hamilton syringe into 12 sites (0.5 μl per site) on both lesioned sides of the sensorimotor cortex (SMC) to mark the regeneration of the corticospinal tract (CST) axons (Brosamle and Schwab, 1997; Guest et al., 1997). Rats were allowed to survive for two more weeks.

4.7.2. Perfusion and cryosection

Once the survival period ended, all rats were perfused transcardially with normal saline containing 0.002% NaNO2 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. T8–T12 spinal cord successive segments, L1 segment and brain were cryosectioned by protocol as follows: T8–T12 spinal cord successive segments were cut longitudinally, and L1 segment was cut coronarily. According to anatomical atlas, part of the brain, which contains red nucleus (RN) or SMC, was cut coronarily. The thickness of all slices was 30 μm.

4.7.3. Tissue processes for immunofluorochemistry (IFC)
IFC has been described in our previous publications (Guo et al., 2007; Xiong et al., 2009; Zhang et al., 2007). In brief, the sections were incubated with primary antibodies mixed in 0.3% Triton X-100 overnight at 4 °C, followed by incubation with secondary antibodies of FITC- or Cy3-conjugated anti-mouse or anti-rabbit IgG (1:1000, Jackson Immunological Research). The slides were examined under the fluorescence microscopy (Leica, micro-system AG). All primary antibodies are listed in Table 4. Anterograde tracer BDA was labeled by Cy3.

To quantify NSCs differentiation, one of every ten sagittal sections containing cell implants was selected for microtubule associated protein 2 (Map2) or postsynaptic density 95 (PSD95) IHC staining. Cells with both Map2 or PSD95-positive and Hoechst33342 labeling were counted in four fields of each section (20×objective) at the interface of implant and host tissue. A total of five sections (at least twenty visual fields) in every rat were included for counting.

Quantitative analysis of NF-200 positive nerve fibers was performed. A calibrated reticle eyepiece was used to delineate regions 300 μm rostral to the grafted site, at the grafted site, and 300 μm caudal to the grafted site. The NF-200 positive nerve fibers were quantified in all regions at 200× magnification. The spinal cords were cut in longitudinal sections, and every 5th section was mounted on a gelatin-coated slide. Ten sections per rat were analyzed and the total number of labeled fibers in all regions of each experimental group was averaged. The NF-200 positive fibers longer than 50 μm were counted as positive fibers.

4.7.4. Nissl staining and neuron counting
The serial brain slices containing SMC and RN were selected one per six. Eight randomly selected slices from serial slices of L1 spinal cord segment of each rat were stained with neutral red (i.e. Nissl staining). The neuronal densities of the inner pyramidal layer of SMC, RN and Clarke’s nuclei (CN) of L1 spinal cord were counted under grid-equipped microscopy based on stereologic principle.

4.7.5. Hematoxylin and eosin (HE) and X-gal staining
The remaining T8–T12 spinal cord sections of each group were stained with HE. The T8–T12 spinal cord sections of LacZ group were stained with X-gal solution (Guo et al., 2007; Liu et al., 1997). 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), containing 35 mM K3Fe(CN)6, 35 mM K4Fe(CN)6, 2 mM MgCl2 in PBS, at 37 °C overnight.

4.7.6. Electron microscopy
The cord tissue centered on the injury epicenter from each experimental group was fixed in 2% glutaraldehyde overnight and then post-fixed in 1% osmium tetroxide for 1.5 h, dehydrated in ethanol solutions and embedded in Epon over night. Ultra-thin sections were cut to 70-90 nm with an ultramicrotome (Reichert E, Co, Vienna, Austria) and stained with uranyl acetate for 10 min, followed by staining with lead citrate for 6 min. The sections were examined under a transmission electron microscope (TME, Philips CM 10, Eindhoven, Holland).

4.8. Cystic cavity assessment
Every tenth hematoxylin-eosin stained sagittal section from the spinal cord was used to determine the cystic cavity volume in each group except for the Sham group (20 μm sagittal sections separated by 200 μm distance and spanning the entire width of the spinal cord; n=5 per group). All the sections spanning ±3 mm from epicenter were imaged at a 25× magnification and captured through a Leica DC300 camera mounted on a Leica microscope. The cystic volume (pixels) of those representative images was determined with Photoshop 8.0 software (Adobe, San Jose, CA). Measurements of cyst volume were conducted blindly, without knowledge of treatment groups. The average cystic cavity area was compared among six groups of rats.

4.9. Statistical analysis
The data were analyzed using one-way analysis of variance (ANOVA) or repeated-measure ANOVA. If equal variances were found, least significant difference test was applied, otherwise Kruskal–Wallis test and Dunnett’s T3 were used. The significant level was set at 0.05.

Supplementary materials related to this article can be found online at doi:10.1016/j.brainres.2011.05.019.

Acknowledgments
This study was supported by grants from Chinese National Natural Science Foundation (30270700 and 30771143 to Y.S.)

<table>
<thead>
<tr>
<th>Table 4 – Primary antibodies.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibodies</strong></td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Map2</td>
</tr>
<tr>
<td>PSD95</td>
</tr>
<tr>
<td>NF-200</td>
</tr>
<tr>
<td>MBP</td>
</tr>
<tr>
<td>TrkC</td>
</tr>
<tr>
<td>NT-3</td>
</tr>
</tbody>
</table>
REFERENCES


