NEURAL REGENERATION RESEARCH
November 2014, Volume 9, Issue 22
www.nrronline.org

Dorsal root ganglion-derived Schwann cells combined with poly(lactic-co-glycolic acid)/chitosan conduits for the repair of sciatic nerve defects in rats

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doi:*10.4103/1673-5374.145374*
http:*//www.nrronline.org/*

Accepted: *2014-06-03*

Abstract

Schwann cells, nerve regeneration promoters in peripheral nerve tissue engineering, can be used to repair both the peripheral and central nervous systems. However, isolation and purification of Schwann cells are complicated by contamination with fibroblasts. Current reported measures are mainly limited by either high cost or complicated procedures with low cell yields or purity. In this study, we collected dorsal root ganglia from neonatal rats from which we obtained highly purified Schwann cells using serum-free melanocyte culture medium. The purity of Schwann cells (> 95%) using our method was higher than that using standard medium containing fetal bovine serum. The obtained Schwann cells were implanted into poly(lactic-co-glycolic acid)/chitosan conduits to repair 10-mm sciatic nerve defects in rats. Results showed that axonal diameter and area were significantly increased and motor functions were obviously improved in the rat sciatic nerve tissue. Experimental findings suggest that serum-free melanocyte culture medium is conducive to purify Schwann cells and poly(lactic-co-glycolic acid)/chitosan nerve conduits combined with Schwann cells contribute to restore sciatic nerve defects.

Key Words: *nerve regeneration; Schwann cells; dorsal root ganglia; melanocyte medium; fibroblasts; poly(lactic-co-glycolic acid); chitosan; sciatic nerve defect; NSFC grants; neural regeneration*

Funding: *This study was supported by the National Natural Science Foundation of China, No. 30973060.*

Zhao L, Qu W, Wu YX, Ma H, Jiang HJ. Dorsal root ganglion-derived Schwann cells combined with poly(lactic-co-glycolic acid)/chitosan conduits for the repair of sciatic nerve defects in rats. Neural Regen Res. 2014;9(22):1961-1967.

Introduction

Schwann cells contribute to the mechanisms that underlie neuronal survival, growth and regeneration in the peripheral nervous system (Xin et al., 2013; Xu et al., 2013). However, isolation and purification of Schwann cells are usually a complex process. Because Schwann cells are easily contaminated by fibroblasts and proliferate poorly, the enrichment of a large population of Schwann cells within a short time span is a difficult task (Niapour et al., 2010). Although several methods have been developed to enrich Schwann cells over the past 30 years, these reported methods enrich Schwann cells with different purities and yields, and possess certain drawbacks (Wei et al., 2009).

The development of tissue-engineered artificial nerves provides new direction for identifying ideal substitutes for nerve transplantation (Fu et al., 2013; Georgiou et al., 2013; Zhang et al., 2013; Zong et al., 2013). To date, tissue-engineered artificial nerves have good histocompatibility, which provides a micro-environment for axonal growth of nerve tissue and induces correct orientation for axonal growth. Furthermore, artificial scaffold is eventually degraded. Schwann cells are the most commonly used cells for construction of tissue-engineered conduits. The combination of tissue-engineered conduits with Schwann cells may promote nerve regeneration and fibrous maturation in the process of nerve restoration (Mosahebi et al., 2001).

The present study aimed to develop an efficient and easily applicable method for obtaining highly purified Schwann cells from dorsal root ganglia, and to explore the efficacy of poly(lactic-co-glycolic acid) (PLGA)/chitosan nerve conduits combined with Schwann cells for the treatment of sciatic nerve defects.

Materials and Methods

Isolation and primary culture of Schwann cells from dorsal root ganglia

Twenty specific pathogen-free neonatal Sprague-Dawley rats, both male and female, 1–3 days post-birth, were supplied by the Animal Experiment Center, Dalian Medical University, China (animal delivery license No. SCXK (Liao) 2004-0017, animal use license No. SYXK (Liao) 2004-0029). Animals were housed at 20–29°C with a relative humidity of 50–60%. The study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996), and was approved by the Animal Ethics Committee, Dalian Med-

ical University, China.

Neonatal Sprague-Dawley rats were sacrificed by freezing on ice, immersed in iodophors for 5 minutes and decapitated in a curved plate under aseptic conditions. Fibrous connective tissues were cut open and the muscles beside the spinal column were bluntly isolated. The spinal process was removed and the spinal column was decompressed with microscissors (both sides were not expanded to prevent ganglion loss), prior to dissociation from the cervical to caudal segments. Redundant muscles were removed and the spinal column was placed in an additional aseptic dish containing D-Hanks solution.

The vertebral canal and vertebral foramen were exposed. While in the prone position and after pulling out the spinal cord, round yellowish translucent dorsal root ganglia with two nerve roots were visible at the intervertebral foramen. The dorsal root ganglia were isolated using microforceps and placed in D-Hank's solution at 4°C to maintain cell viability. A total of over 40 dorsal root ganglia were obtained from each rat.

The nerve root was cut off using microforceps. The dorsal root ganglia were washed twice with D-Hanks solution to remove redundant fibroblasts. Surface membranes were stripped from the dorsal root ganglia and cut into small pieces for digestion with 0.25% trypsin and 0.03% collagenase I. This step was carried out at 37°C in a water bath with shaking, prior to incubation in a 5% CO_2 incubator at 37°C for 5 minutes. When tissue pieces became flocculent, they were treated with 3 mL of fetal bovine serum, centrifuged at 1,000 r/min for 5 minutes. After the supernatant was discarded, the cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 ng/mL basic fibroblast growth factor (Sigma, St. Louis, MO, USA). Samples were stirred to achieve a single cell suspension. Samples (20 μL) were obtained with a micro-sample injector (Sigma) for cell counts and cell density was adjusted to 10^5/mL (24-well plate).

A total of 24 μL of rat-tail collagen (Sigma) was added in 10 mL of 0.01 mol/L HCl to prepare 20 pmol/mL of coating buffer. Coating buffer (200 μL) was then added into each well of the 24-well plate, which was placed in a 37°C 5% CO_2 incubator overnight. After this, the liquid was removed and samples were washed three times with aseptic PBS, and air-dried for further use.

Cells at a density of 1×10^5/mL were incubated in uncoated 24-well plates (1 mL in each well), and were purified twice by the differential adhesion method (Kaiser and Spatz, 2010), for 30 minutes. Once most of the fibroblasts were attached, the cell suspension was moved into an additional rat-tail collagen-coated 24-well plate. Once the Schwann cells had attached, the upper medium was removed and a pure Schwann cell population was obtained and incubated in a 37°C/5% CO_2 incubator.

Schwann cells experimental groups

Schwann cells were divided into melanocyte, normal control and arabinoside (Ara-C) groups. Schwann cells in the melanocyte group were treated as follows: after 4 hours, culture medium was replaced with melanocyte growth medium (PromoCell, Heidelberg, Germany; including 500 mL of basal medium, 5 mL of melanocyte growth supplement, 2.5 mL of fetal bovine serum and 5 mL of penicillin/streptomycin solution). Bovine pituitary extract (5 μg/mL, Sciencell Research Laboratories, Carlsbad, CA, USA) was then added to amplify the Schwann cells. The medium was replaced every 4 days thereafter. Schwann cells in the normal control group were treated as follows: after 4 hours, culture medium was replaced with DMEM medium (1% penicillin-streptomycin + 10% bovine serum albumin). The medium was replaced every 4 days thereafter. Schwann cells in the Ara-C group were treated as follows: Schwann cells were incubated in DMEM medium (1% penicillin-streptomycin + 10% bovine serum albumin) containing Ara-C at a final concentration of 10^{-9} mol/L (Pfizer Inc., New York, NY, USA) for 24 hours. Schwann cells were washed to remove Ara-C, and then placed in DMEM for 12 hours, incubated in the medium containing Ara-C for 24 hours, and then in DMEM (1% penicillin-streptomycin + 10% bovine serum albumin). Medium was replaced once every 2–3 days. Cell growth was observed and cells were quantified under an inverted phase contrast microscope (Nikon, Tokyo, Japan) daily.

Identifying Schwann cells using immunofluorescence staining

Cells in each group were incubated on coverslips in 24-well plates. After the medium was removed, cells were washed once with 0.01 mol/L PBS for 5 minutes, and fixed in 4% paraformaldehyde for 5 minutes, followed by three washes with 0.01 mol/L PBS, each for 5 minutes. Cells were perforated with perforating solution containing 3% bovine serum albumin and incubated in 0.01 mol/L PBS containing 0.5% Triton X-100 at 37°C for 30 minutes (200 μL in each well). Schwann cells were characterized immunocytochemically by antibodies directed against S-100 protein, based on the protocol previously described by Komiyama et al. (2003). Briefly, cells cultured on coverslips were fixed with 4% paraformaldehyde in PBS for 10 minutes and then permeabilized with 0.05% Triton X-100 for 10 minutes. Non-specific sites were blocked with 5% goat serum for 1 hour. Then, the cells were incubated with rabbit anti-rat S-100 monoclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 hour, washed three times with PBS, and incubated with FITC-conjugated goat anti-rabbit IgG (1:200; Santa Cruz Biotechnology) for 2 hours at room temperature. Positive cells were observed and quantified using a fluorescence microscope (Nikon).

Subculture of Schwann cells

Cells were subcultured on reaching confluence. In the melanocyte group, liquid in the wells was removed using the Cold Jet method (Jirsová et al., 1997). D-Hank's solution (1 mL, 4°C) was slowly added into the plate and immediately removed; then melanocyte medium (1 mL, 4°C) was added. Specimens were stirred at the bottom of the plate and cell morphology was observed under an inverted phase contrast microscope. When the majority of cells were floating, the cell suspension was moved to a new rat-tail collagen-coated well plate. In the normal control and Ara-C groups, after two replacements of medium, cells at 90% confluence were subcultured and digested with 0.125% trypsin. When the Schwann cell processes had retracted, digestion was terminated by addition of DMEM containing

10% fetal bovine serum. Non-detached cells were discarded and detached cells were collected and centrifuged.

Cell proliferation assay

Schwann cells were resuspended in fresh pre-warmed (37°C) DMEM containing Ara-C or melanocyte growth medium, and were counted and plated at a density of 2×10^5 cells/mL on 96-well plates. 5-Ethynyl-2-deoxyuridine (EdU; 50 μmol/L) was added to allow culture for an additional 2 hours. Schwann cells were then fixed with 4% formaldehyde in PBS for 30 minutes, and detected using the Cell-Light™ EdU DNA Cell Proliferation Kit (Ribobio, Guangzhou, China) according to the manufacturer's protocol. The number of EdU-positive and total cells was counted using fluorescence microscopy (Nikon). The percentage of EdU-positive cells was calculated (the ratio of EdU-positive cells to total cell number) and assays were carried out three times in triplicate wells.

Preparation of PLGA/chitosan nanofiber mesh tubes

For preparing PLGA/chitosan nanofibrous scaffolds, PLGA (PURAC; Gorinchem, the Netherlands) was first dissolved in hexafluoroisopropanol and further blended with chitosan solution (Dalian Chemical Physics, Chinese Academy of Sciences, Dalian, Liaoning Province, China) in trifluoroacetic acid/dichloromethane, to obtain a weight ratio of PLGA/chitosan (75:25). Briefly, chitosan was dissolved in trifluoroacetic acid/dichloromethane, and the solution was stirred for 3 days for complete dissolution. PLGA was dissolved in hexafluoroisopropanol separately and stirred for a day. Chitosan solution was then added to the PLGA solution to obtain a 75:25 weight ratio of PLGA:chitosan, and this blend mixture was stirred for another day to obtain a homogenous solution before use for electrospinning. To fabricate the PLGA/chitosan nanofiber mesh, a positively-charged jet ejected from the PLGA/chitosan solution at a rate of 2–8 mL/hour was sprayed at the negatively-charged collector. PLGA/chitosan nanofibrous structures were immersed in 99.5% ethanol, air-dried at room temperature for 2 hours, and then used for cell culture study after overnight sterilization. To fabricate PLGA/chitosan nanofiber meshes, the linear rate of the rotating drum was set to 5.2 meters/second (1,000 r/min). A high voltage of 25 kV was applied by a voltage-regulated DC power supply to generate the positively-charged jet. The resulting oriented nanofiber mesh sheets, with a thickness of 0.02 mm, were unrolled from the drum. Likewise, the obtained mesh was reeled onto the stainless steel bar with the nanofiber orientation parallel to the axis of the bar to form the PLGA/chitosan mesh tube. These tubes had an inner diameter of 1.2 mm, an outer diameter of 1.6 mm, and a length of 15 mm (Wang et al., 2009).

Culture of Schwann cells on the PLGA/chitosan conduits

Schwann cells (passage 2) derived from the melanocyte group were seeded on coverslips at a density of $5 \times 10^4/cm^2$ in DMEM supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 g/mL streptomycin and incubated in 5% CO_2 at 37°C for 4 hours to allow the cells to attach. Thereafter, the coverslips were submerged in the plastic dishes by adding 3 mL of feeding medium and incubated in 5% CO_2. Schwann cells were cultured for 4 days and seeded onto these sheets.

Cell growth on the sheets was observed and cells were quantified using an inverted phase contrast microscope (Nikon, Tokyo, Japan) daily. S-100-positive Schwann cells on the sheets were identified using immunofluorescence staining.

Implantation of PLGA/chitosan tubes in a rat model of sciatic nerve injury

A total of 10 specific pathogen-free Sprague-Dawley rats, both male and female, aged 2–3 months and weighing 200–250 g (Dalian Medical University, Dalian, China), were divided equally into two groups: (1) implantation and (2) control. Sprague-Dawley rats in each group were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). The right sciatic nerve was exposed, and a section of 10 mm in length was excised at the center of the thigh. A graft of 10 mm in length was performed by end-to-end suturing with 8-0 monofilament nylon to connect these PLGA/chitosan nanofiber mesh tubes (15 mm) and both nerve ends (2 mm). The PLGA/chitosan nanofiber mesh tubes cultured with Schwann cells were the implantation group, while the PLGA/chitosan nanofiber mesh tubes alone were the control group.

Evaluations of the motor and sensory function recovery of the sciatic nerve

The recovery of motor and sensory functions associated with sciatic nerve regeneration was evaluated with the von Frey hair test and static toe spread factor assessment (Wang et al., 2009), respectively.

The von Frey hair test was carried out as follows. The test rat was placed on a wire mesh plate and permitted to adapt to the new environment. Nylon monofilaments were then used to stimulate the soles of the hind feet from below. The monofilament size was recorded when a reaction of paw withdrawal or lick was observed repeatedly. Sensitivity to these filaments was used to evaluate heightened (neuropathic) pain responses, as well as recovery of normal nociception or tactile sensation in this study. Both sides were tested, and the results were calculated according to the following formula: (size in the experimental side – size in the normal side)/size in the normal side.

The static toe spread factor assessment was carried out as follows. The test animal was placed on a transparent plastic plate, under which a digital camera was set at a distance of 10 cm beneath. After an interval to permit the animal to adapt to the new environment, three frames of both hind feet were taken for each rat. Images were then transmitted into a personal computer system, and the distance between the spread first and fifth toes (toe spread factor; TSF) was quantified. Measurements were taken for three consecutive images and averaged for each side. The results were assessed by the following formula which is simplified from the static sciatic index: (TSF in the normal side – TSF in the experimental side)/TSF in the normal side. This was carried out every 4 weeks until 4 months post-implantation.

Evaluations of the pathological recovery of the sciatic nerve

At 3 months post-surgery, specimens were stained with hematoxylin-eosin for observation by light microscopy. The

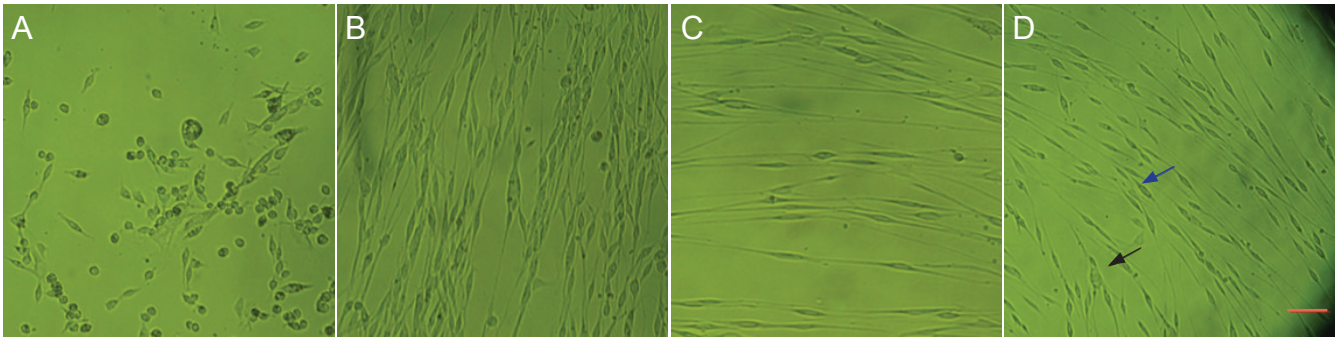

Figure 1 Morphology of primary cultured Schwann cells (inverted microscope).
In the normal control group after 24 hours of culture, Schwann cells became elliptic or short spindle-shaped (A) and at 1 week, cell number was high, fibroblasts proliferated and spread on the bottom of the well, and Schwann cells attached to the surface of the fibroblasts (B). (C) In the Ara-C group at 1 week after Ara-C treatment, fibroblasts were scavenged, however Schwann cell proliferation was slow. (D) In the melanocyte group after 1 week of culture, Schwann cells were pure and their proliferation rapid. Schwann cells proliferated and spread on the bottom of the well. Black arrow shows fibroblasts and blue arrow shows Schwann cells. Scale bar: 50 μm.

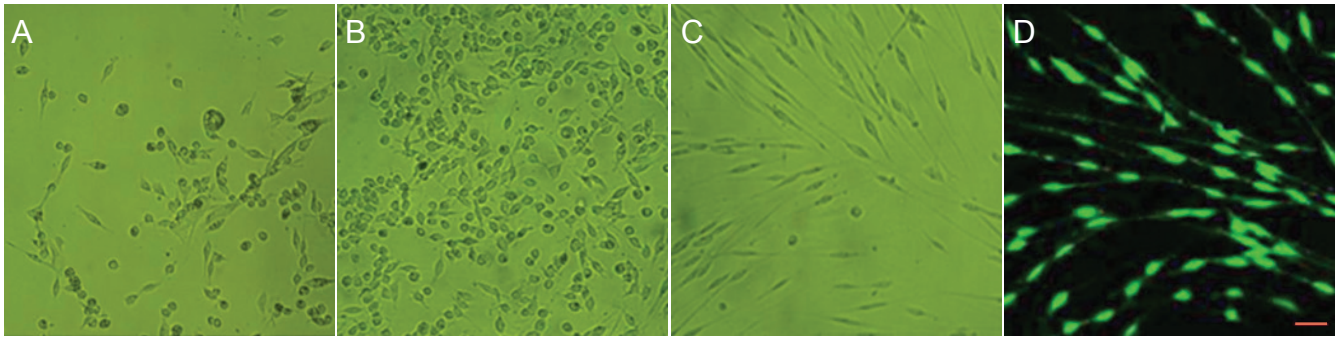

Figure 2 Purity and identification of Schwann cells in the melanocyte medium group.
(A–C) Schwann cells were cultured for different times and observed using inverted microscopy. (A) Primary cells were cultured for 24 hours. (B) Primary cells were cultured for 10 days, and reached high confluence (> 90%). (C) Schwann cell purification after subculture. (D) S-100 expression in cultured cells (immunofluorescence staining), and fluorescent indicator is fluorescein isothiocyanate (FITC) (green). Scale bar: 50 μm.

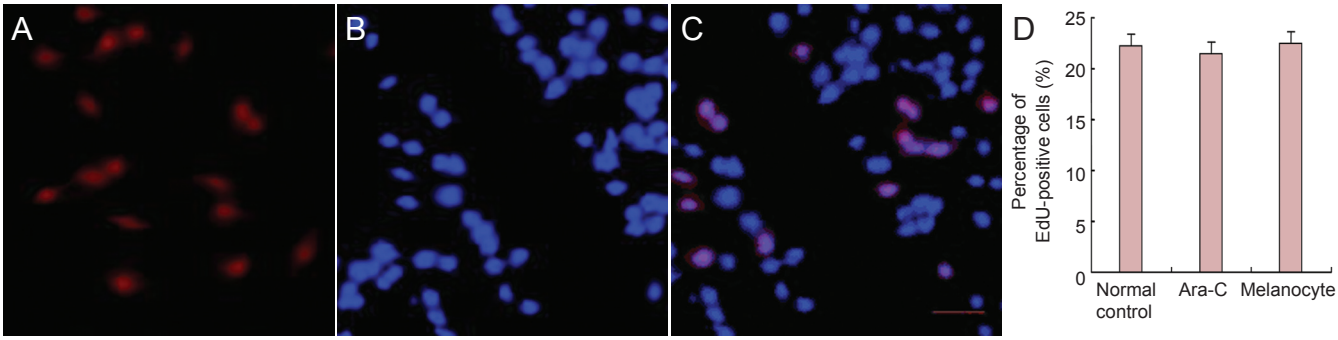

Figure 3 Effect of melanocyte medium on Schwann cell proliferation (double-label immunofluorescence staining).
(A) Representative images of EdU-positive cells (EdU staining, red). (B) DAPI-positive cells (blue). (C) Merged image. Scale bar: 50 μm. (D) Quantification of the percentage of EdU-positive cells. Data are expressed as the mean ± SD, and one-way analysis of variance followed by the least significant difference test was performed. Ara-C: Arabinoside; EdU: 5-ethynyl-2-deoxyuridine; DAPI: 4′,6-diamidino-2-phenylindde.

rats were sacrificed by an overdose of pentobarbital. The regenerative facial nerves with conduits and the control nerves were removed and examined. For the light microscopy examination, nerve sections were fixed in 10% buffered formalin for 24 hours, dehydrated, and embedded in paraffin blocks. Sections (5 μm) in each subfield were cut across the transverse axis. The sections were de-waxed and stained with hematoxylin-eosin. The stained sections were examined using a light microscope (Nikon).

Statistical analysis

Statistical analysis was performed with SPSS 10.0 software (SPSS, Chicago, IL, USA). Data were expressed as the mean ± SD, and one-way analysis of variance followed by the least significant difference test was performed. A level of $P < 0.05$ was considered statistically significant.

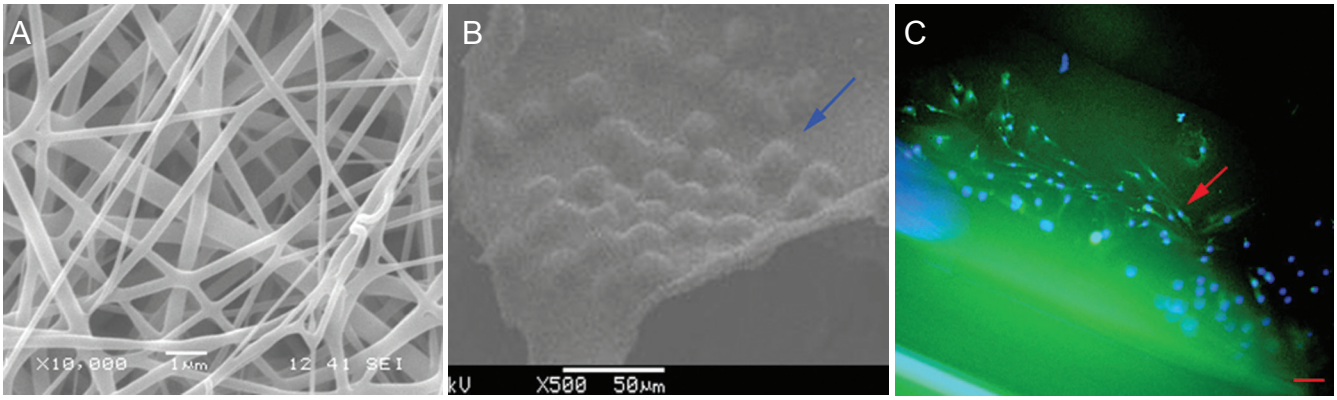

Figure 4 Morphology of Schwann cell alignment along oriented electrospun poly(lactic-co-glycolic acid) (PLGA)/chitosan nanofibers.
(A) Morphology of oriented electrospun PLGA/chitosan nanofibers (scanning electron microscopy, scale bar: 1 μm). (B) Morphology of Schwann cells (arrow) cultured on the oriented electrospun PLGA/chitosan nanofibers (scanning electron microscopy, scale bar: 50 μm). (C) S-100 expression (arrow) in Schwann cells cultured on the oriented electrospun PLGA/chitosan nanofibers (scale bar: 50 μm).

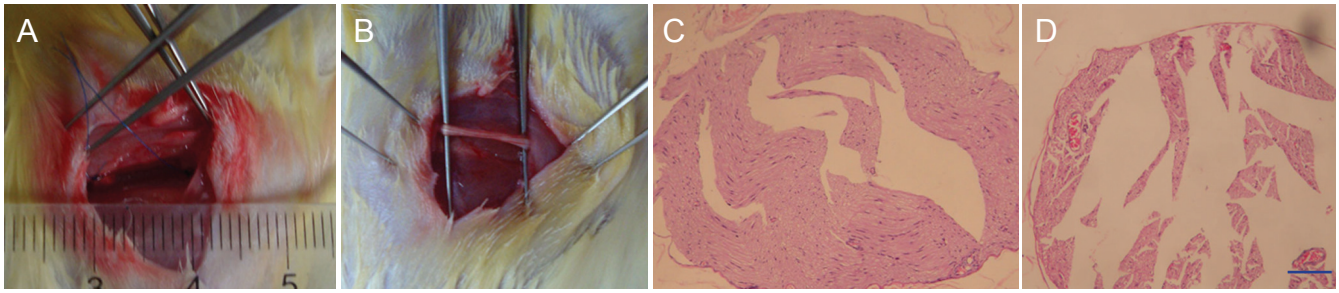

Figure 5 Schwann cells combined the oriented electrospun poly(lactic-co-glycolic acid) (PLGA)/chitosan nanofibers for regeneration of sciatic nerve defects in rats.
(A) The PLGA/chitosan nanofiber mesh tubes were bridge-grafted into rat sciatic nerve defects (10 mm long). (B) At 3 months post-operatively, the degradable biomaterials used for the nerve defects were completely degraded and absorbed. The appearance of the nerve showed that neurogenesis had occurred, and defects were healed. (C) In the implantation group, hematoxylin-eosin staining revealed bundled regenerative nerve fibers that were highly vascularized. (D) In the control group, hematoxylin-eosin staining showed the regenerated axons were scattered in the loose connective tissue. Scale bar: 100 μm.

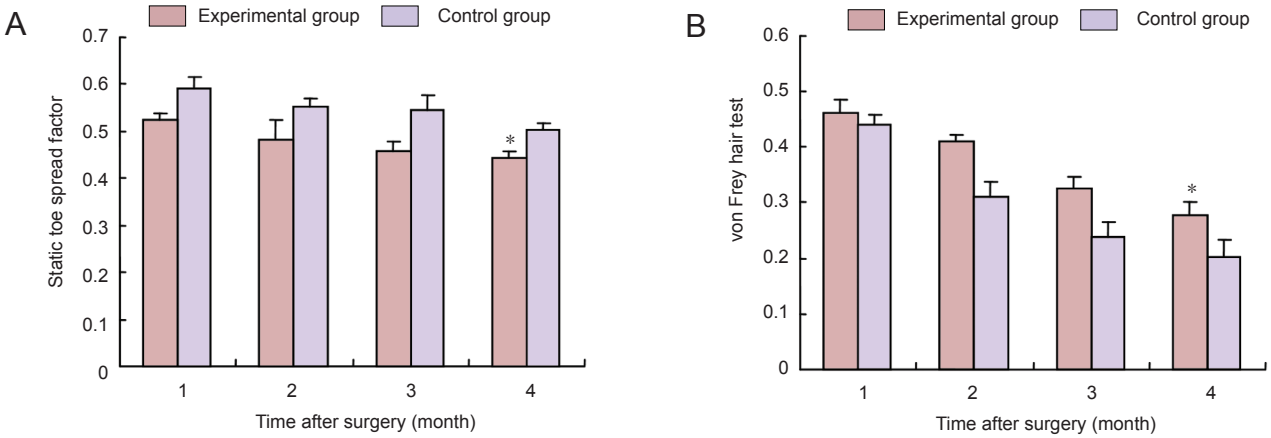

Figure 6 Schwann cells combined with oriented electrospun poly(lactic-co-glycolic acid) (PLGA)/chitosan nanofibers improved neurological function in rats with sciatic nerve injury.
(A) STSF values were calculated by the following formula: STSF = (TSF in the normal side – TSF in the implantation side)/TSF in the normal side. (B) The vFHT values were calculated by the following formula: vFHT= (size in the implantation side – size in the normal side)/size in the normal side. Data are expressed as the mean ± SD of 16 rats in each group, and one-way analysis of variance followed by the least significant difference test was performed. ∗$P < 0.05$, *vs.* control group. STSF: Static toe spread factor; vFHT: von Frey hair test; TSF: toe spread factor.

Results

Purity and identification of Schwann cells in melanocyte medium

As observed using the inverted microscope, Schwann cells began to adhere at 4 hours and showed small bodies. Twenty-four hours later, Schwann cells became elliptic or short spindle-shaped, mostly dipolar, seldom monopolar or multi-polar, with clear nuclei (**Figure 1A**).

After primary Schwann cells were cultured in the melanocyte medium for 10 days, they reached high confluence (> 90%). The subcultured Schwann cells were further purified. Immunocytochemical staining showed that adult rat Schwann cells expressed S-100, a marker of Schwann cells, which indicated that the cultured cells were Schwann cells (**Figure 2**).

Melanocyte medium promotes proliferation of Schwann cells

In the normal control group, the number of fibroblasts increased by 72 hours, and the cells were spread over the bottom of the plate at 1 week, which inhibited proliferation of Schwann cells. Schwann cells attached to the surface of the fibroblasts (**Figure 1B**). In the Ara-C group, after primary cells were treated with Ara-C for 1 week, only a few fibroblasts were observed, however proliferation of Schwann cells was slow (**Figure 1C**). In the melanocyte group, the speed of SC proliferation was faster than in the normal control and Ara-C groups, but this was not statistically significant. Schwann cells gradually reached confluence by day 5. One week later, Schwann cells were spread over the bottom of the culture plate. Fibroblast growth was inhibited and fibroblast proliferation was not obvious (**Figure 1D**). The double-label immunofluorescence results indicated that compared with the normal control and Ara-C groups, melanocyte growth medium resulted in no significant change in the Schwann cells proliferation rate ($P > 0.05$; **Figure 3**).

Morphology of Schwann cell alignment along the oriented electrospun PLGA/chitosan nanofibers

We constructed a PLGA/chitosan nanofiber mesh tube consisting of oriented fibers by the electrospinning method. Schwann cells were cultured on these sheets. Using scanning electron microscopy and immunofluorescence staining, we showed that on coverslips coated with oriented PLGA/chitosan nanofibers, Schwann cells aligned in the same direction as a result of secure adhesion to the oriented fibers (**Figure 4**).

Effect of Schwann cell alignment along oriented electrospun PLGA/chitosan nanofibers in the sciatic nerve injury rat model

Chitosan nanofiber mesh tubes were bridge-grafted into rat sciatic nerve defects (**Figure 5A**). Histological evaluation of the regenerated nerve tissue in the implanted tube, showed that the inflammatory response around the tube wall was scarcely observed in each group 3 months post-operatively (**Figure 5B**). In the implantation group, hematoxylin-eosin staining revealed bundled regenerative nerve fibers that were highly vascularized (**Figure 5C**). In the control group, regenerated axons were scattered in the loose connective tissue, and some blood vessels with large diameter were observed in the lumen (**Figure 5D**). These histological findings suggest that the regenerated nerve remained immature in the control group. The total axon and nerve areas in the implantation group were significantly larger than those in the control group ($P < 0.05$). Both motor and sensory nerve functions were recovered with time in each group, and the static toe spread factor and von Frey hair test values in the implantation group were significantly higher than in the control group 3 months post-implantation ($P < 0.05$; **Figure 6**). The results indicated rich vessels in the epineurium, bundled regenerative nerve fibers, and many neo vessels in the nerve fiber bundles in the NGF-microspheres group.

Functional recovery was recorded until 4 months after operation. The results of sciatic sensory nerve function, the static toe spread factor value, and motor nerve recovery, the von Frey hair test, revealed that both motor and sensory nerve function recovered with time in each group, but the overall effect of the experimental group is better than that of the control group.

Discussion

In this study, we used a serum-free culture approach combined with conventional methods, including melanocyte growth medium and the secondary enrichment Cold Jet procedure, to remove fibroblasts from Schwann cells cultures. Mauritz et al. (2004) verified that in medium containing 10% serum, Schwann cells grow in large numbers for a short time, even in the presence of small numbers of fibroblasts. Komiyama et al. (2003) confirmed that serum concentration (exceeding 2.5%) enhances fibroblast growth. It is therefore believed that a fetal bovine serum concentration of 2.5–3.0% in the Schwann cells medium is a critical factor for cell purification, and a serum concentration below this value could effectively suppress fibroblast proliferation (Haastert-Talini, 2012). In our study therefore, a serum-free culture approach was used to isolate, culture and passage Schwann cells from dorsal root ganglia *in vitro*. By direct comparison of the serum and serum-free culture conditions, optimal results to selectively support viable Schwann cells and effectively suppress fibroblast overgrowth were achieved using melanocyte growth medium as a defined minimal medium, with added bovine pituitary extract. Furthermore, applying the Cold Jet protocol as a secondary enrichment procedure, significantly elevated the percentage of Schwann cells. Cold Jet is a fast and easy method for cell purification, however frequent operation of this technique on Schwann cells over such a short time interval might result in low yield of Schwann cells, especially when performed by unskilled workers (Mauritz et al., 2004). In the present study, we proved that these two methods can be used to obtain highly purified and therapeutically applicable Schwann cells. Cell proliferation studies enable better understanding of the orchestration of Schwann cells behavior during peripheral nerve injury and regeneration (Yao et al., 2014). As EdU incorporation appears to be variable depending on the cell type and concentration, with respect to specific effects on cell proliferation, despite its routine use for labeling DNA, it is essential to evaluate the extent of any cytotoxic effects (Kohlmeier et al., 2013). We therefore performed EdU incorporation using a transwell-based assay, to monitor the rate of proliferation of primary cultured Schwann cells within the three groups. The cell proliferation assay demonstrated that there was no difference in the cell proliferation rate of primary Schwann cells. Therefore, the culture medium did not affect DNA synthesis of Schwann cells, and the Schwann cells cultured by melanocyte growth medium had good pro-

liferative potential, able to perform normal functions during the progression of neuro-tissue engineering.

It is well known in the field of neural tissue engineering, that artificial nerve grafts must be biocompatible, hydrophilic, non-immunogenic and inert in mediating scarring and fibrosis (Tian et al., 2005; Brännvall et al., 2007). Based on this requisite for further investigation into the neuro-trophicaction of Schwann cells, we transplanted Schwann cells within PLGA/chitosan conduits for peripheral nerve regeneration. Chitosan, a predominant absorbable material, has received much attention because of its physicochemical and mechanical properties. Chitosan is one of the most commonly used naturally derived polymers in tissue engineering. Chitosan fibers have been shown to support the adhesion, migration and proliferation of Schwann cells (Yuan et al., 2004). However, a major drawback of using chitosan alone for tissue engineering applications is its enhanced dissolution due to its hydrophilicity and swelling characteristics (Sencadas et al., 2012). Consequently, chitosan conduits do not function as well as nerve autografts in most cases (Guo and Dong, 2009; Liu et al., 2013). Comparatively, synthetic polymers, such as PLGA, have been used widely in a variety of biomedical applications because of their physicochemical and mechanical characteristics (Piao et al., 2013). However, these materials are bioinert and do not possess biological functions. In this study, we blended PLGA/chitosan nanofibers to improve biomaterial strength and act as a potential growth substrate for Schwann cells. The results of our study support the use of PLGA/chitosan nanofibers in providing sufficient mechanical strength and permitting enhanced nerve regeneration. We also found that this simple blend of PLGA with chitosan had a positive effect on the biocompatibility of the composite nanofibrous scaffold. Schwann cell growth and morphology after seeding on PLGA/chitosan scaffolds was good. Schwann cells rapidly attached and spread on the PLGA/chitosan scaffolds, which may permit enhanced nerve regeneration clinically.

In conclusion, this study proves the feasibility of PLGA/chitosan conduits loaded with Schwann cells for promoting nerve regeneration. This conduit created a barrier between the regenerating axons and surrounding environment and provided directional guidance to regenerating axons. When seeded with Schwann cells, this conduit can potentiate normal growth or reconstruction of nerves, as well as biomechanical alignment, and represents a new possible alternative to autografting for nerve repair.

Author contributions: *Zhao L evaluated the experiments, wrote the manuscript and performed statistical analysis. Wu YX and Ma H collected experimental data and conducted statistical analyses. Jiang HJ designed the study and revised the manuscript. Qu W supervised the study. All authors approved the final version of the manuscript.*
Conflicts of interest: *None declared.*

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Copyedited by Finnie E, Norman C, Yu J, Yang Y, Li CH, Song LP, Zhao M