Electric Conductivity on Aligned Nanofibers Facilitates the Transdifferentiation of Mesenchymal Stem Cells into Schwann Cells and Regeneration of Injured Peripheral Nerve

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1. Introduction

Peripheral nerve injury (PNI), as a worldwide clinical problem, which is often caused by trauma or surgery, can lead to the partial or total loss of motor function and sensory perception, and even lifelong disability.[1] Autologous nerve transplantation, which acts as an ideal immunogenically inert scaffold for axonal regeneration, is accepted as the “gold standard” for therapy of irreducible nerve defects.[2] However, the autologous nerve transplantation is limited by the shortage of sources, the considerable morbidity of the donor-site, the dimensional mismatch between graft and host nerves and other potential complications.[3] To overcome these limitations, various biomaterials and seed cells have been utilized to fabricate artificial nerve scaffold for bridging the nerve defects and have achieved varying degrees of success.[4] Among these approaches, artificial nerve composed of aligned nanofibers received wide attention.[5] It has been proved that alignment profile could influence cell adhesion and proliferation and it mediates stem cell differentiation.[6] For nerve tissue applications, the alignment is necessary to guide the cells migration in a particular direction and promote the axonal elongation.[7] And Chew et al. reported that, when cultured on the aligned electrospun polycaprolactone (PCL) fibers, Schwann cell (SC) cytoskeleton and nuclei were aligned and elongated along the fiber axes, emulating the band of Büngner,[8] which is

Schwann cells (SCs) are the most promising seed cells for peripheral nerve tissue engineering, but clinical applications are limited by the lack of cell sources. Existing data demonstrate that bone marrow mesenchymal stem cells (BMSCs) can be induced to differentiate into Schwann-like cells and aligned nanofibers can enhance the differentiation. Considering that SCs are living along with the electrical conductive axons, it is hypothesized that conductivity properties may play roles in SCs differentiation and then facilitate nerve regeneration. To verify this hypothesis, amine functionalized multi-walled carbon nanotubes (MWCNTs) are incorporated with polycaprolactone and gelatin to fabricate aligned or random conductive nanofibers by electrospinning. Current data demonstrate that MWCNTs can dramatically increase the electrical conductive properties but do not alter the biocompatibility of the nanofibers. It is found that endowing conductive properties into the aligned nanofibers can significantly enhance their capability to promote the SCs differentiation. Furthermore, the aligned and conductive nanofibers with induced BMSCs can dramatically promote peripheral axonal regeneration. Collectively, the present study demonstrates that the conductive properties in the aligned nanofiber plays significant roles in SCs differentiation and the aligned and conductive nanofibers can be used as a promising scaffold for SCs differentiation and peripheral nerve tissue engineering.

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an important supporting structure for axonal elongation during nerve development and regeneration.

As the most promising seed cells for peripheral nerve tissue engineering, SCs can synthesize and secrete various neurotrophic factors and extracellular matrix to guide and promote axonal growth.[9] Moreover, the regenerated peripheral axons have to be remyelinated by SCs for the functional nerve conduction.[10] Unfortunately, due to the damage of donor nerve for traditional SCs isolation, and SCs' low capability of proliferation, it is difficult to obtain and expand enough SCs to the therapeutic needs. To explore potential substitutes, kinds of stem cells were tried to be transdifferentiated into SCs.[11] For example, our previous study indicated that peripheral blood derived mesenchymal stem cells (MSCs) can be induced to express SCs' functional proteins and the induced cells could myelinate the axons after be transplanted.[11a] Recently, Xue et al. revealed that aligned nanofibers can significantly enhance the SCs transdifferentiation from bone marrow derived MSCs (BMSCs),[12] which indicates that the topology of scaffold play important roles in the SCs differentiation. Considering that SCs are living along with the axons, which are not only aligned but also electric conductive, we hypothesized that the conductivity property may also facilitate the SCs differentiation and then improve the efficacy of nerve tissue engineering. To verify this hypothesis, in the current study, the amine functionalized multi-walled carbon nanotubes (MWCNTs) were incorporated with PCL and gelatin to fabricate aligned conductive nanofibers by electrospun. For control, random conductive nanofibers, as well as aligned or random PCL/gelatin nanofibers were also fabricated. The nanofibers were evaluated by scanning electron microscopy, electrochemistry workstation, and the co-cultured cells live/dead staining assay, and then were used as scaffolds to culture the BMSCs, and the SCs induction was performed on its. The profiles of the induced SCs were assessed by immunocytochemistry, western blotting, co-culture with dorsal root ganglion (DRG) neurons as well as in vivo nerve tissue engineering. Overall data of the present study demonstrated that the conductive property in the aligned nanofiber plays significant roles in the SCs differentiation, the aligned and conductive nanofibers could be used as a promising scaffold for the SCs differentiation and peripheral nerve tissue engineering.

2. Results and Discussion

2.1. Characterization of the Electrospun Fibers

The key objective of this study is to explore the synergistic effect of electrical conductivity and aligned alignment profile of the electrospun nanofibers on the differentiation of BMSCs into SCs. To this end, electrospinning was used to fabricate aligned conductive PCL/gelatin/MWCNT nanofibers for control, random conductive PCL/gelatin/MWCNT nanofibers as well as aligned or random non-conductive PCL/gelatin nanofibers were also fabricated with electrospinning. After the mixtures of PCL/gelatin/MWCNTs or PCL/gelatin were uniformly dispersed in TFEA as described in the methodology, it can be seen that the PCL/gelatin solution was transparent, while PCL/gelatin/MWCNT solution was somewhat black (Figure 1a). Then, aided with the electrospinning system, the aligned PCL/gelatin nanofibers, random PCL/gelatin nanofibers, aligned PCL/gelatin/MWCNT nanofibers and conductivity, random PCL/gelatin/MWCNT nanofibers were developed. In the following description, these four kinds of nanofibers were abbreviated as A, R, AC, and RC respectively. A means aligned, R means random, and C means conductive. SEM images revealed that these fibers exhibited a smooth surface and have a range of diameter distribution from 200 to 900 nm (Figure 1b). Compared with the non-conductive fibers (R and A), the membranes composed of RC and AC fibers showed dramatically higher conductivity (Figure 1c). Collectively, MWCNTs can dramatically increase the electrical conductive property of the nanofibers. Therefore, the scaffolds of A, R, AC, and RC fibers can be used to assess the synergistic effect of electrical conductivity and aligned alignment profile on the SCs transdifferentiation in the following experiments.

2.2. Biocompatibility of the Electrospin Fibers

Carbon nanotube (CNT) is a promising and intriguing biomaterial for the regeneration and functional recovery of damaged nerve tissues.[13] Previous studies have shown that neurons can grow on the surface of CNTs, which also have the effect of improving neural signal transfer, promoting nerve regeneration and reducing scarring of nerve tissue.[14] Although there are still some challenges being faced in the clinical application because CNTs have certain cytotoxicity, many studies to overcome this limitation have been reported.[15] The dispersion and biocompatibility of CNTs can be improved by covalent or non-covalent modification. Amino group is a common modification group, and the carrier can carry the drug conveniently through the amide bond, so the surface of the carbon nanotube has the characteristics of amiation. The amine-functionalized MWCNT is characterized by good dispersion, low cytotoxicity and high reactivity.[16] Indeed, amine group has been shown to stimulate the growth of neurons.[17] In this study, we used amine-functionalized MWCNTs to fabricate electrospun nanofibers and the data revealed that the amine functionalized MWCNTs were well dispersed in 2,2,2-trifluoroethanol. In addition, we chose PCL as the core material due to its biocompatibility and extensively applications for tissue engineering.[18] Gelatin is a biocompatible protein derived from collagen, which has low antigenicity and promotes cell adhesion and migration.[19] So gelatin was combined with PCL to enhance the biocompatibility of the nanofibers. Nevertheless, since the scaffolds were designed for cell induction in vitro and then nerve repair in vivo, their biocompatibility and potential cytotoxicity must be considered in advance. To do that, the BMSCs derived from green fluorescent protein (GFP) transgenic rats (GFP-BMSCs) were cultured for 3 days on these scaffolds to illustrate the morphological changes of the cells. Notably, we selected GFP-BMSCs but not wild-type BMSCs for this assay since the cultured cells on the scaffolds are hardly to be observed clearly by phase-contrast microscopy (as shown in Figure 2). In case wild-type cells were used, they had to be immunofluorescent stained before observation but the true profile of the cultures might be changed during the
Figure 1. a) Digital photo of the electrospun solution. The PCL/gelatin solution was transparent, while PCL/gelatin/MWCNT solution was black. b) Representative SEM images of four different types of electrospun fibers. Quantitative analysis revealed that these fibers have a range of diameter distribution from 200 to 900 nm. c) The recorded curves and quantitative assessments of conductivity of the A, R, AC, and RC scaffolds. Data presented as mean ± SEM, n = 3, p-values are calculated using one-way ANOVA with Bonferroni correction. N.S., non-significance; *p < 0.05.
staining procedures. GFP-BMSCs can be observed directly alive in any time with a fluorescent microscope and therefore can overcome the weaknesses of wild-type cells. After the GFP-BMSCs were typsinized and the cells' suspension was planted into the culture dish with nanofiber scaffolds, we found the cells adhere onto the scaffolds as soon as the neighboring bare glass slides. All the cells extend their processes and increase their surface during the culture (Figure 2), which indicates that the scaffolds have good biocompatibility. Moreover, live/dead staining was also performed to assay the potential cytotoxicity of the scaffolds. Since the live/dead staining kit uses green fluorescence to label live cells and red fluorescence to label dead cells, we did this assay with wild type BMSCs. The live/dead staining illustrated that majority of cells were survived after they were cultured on the scaffolds for 3 days, as they were positive for Calicein-AM (green) and none of them were stained with PI (red). To verify the validation of this live/dead staining kit, we also treated the cells with 30% DMSO for positive control, which resulted in all cells being stained with red. Quantitative analysis showed that the ratios of survived cells (red) on all of the scaffolds were similar to the control BMSCs cultured on glass slides without any scaffold (Figure 3). These results indicate that our prepared electrospun nanofibers had excellent biocompatibility and negligible cytotoxicity.

Figure 2. Morphology comparison of GFP-BMSCs cultured on different nanofibers. BMSCs cultured on bare culture plate or random fibers (R, RC) had a disorganized arrangement, while BMSCs cultured on aligned fibers (A, AC) were arranged orderly on the fibers, and the direction of the long axis of the cells was consistent with the direction of the fibers.

2.3. The Aligned and Conductive Nanofibers Facilitate the BMSCs Transdifferentiating into SCs

After the electronic conductive, biocompatibility and cytotoxicity profiles were determined, the prepared nanofibers were further utilized to evaluate their efficiency on the transdifferentiation of BMSCs into SCs.

SCs are widely regarded as prospective seed cells for nerve tissue engineering, spinal cord injury, as well as traumatic brain injury. Because the autologous SCs are in short supply and encouraged by the widespread application of MSCs, many groups had tried to transdifferentiate MSCs into SCs with various outcomes.[11b,c,20] Even though most of the reports indicated that MSCs can be transdifferentiated into SCs or SC-like cells, scientists are obviously not satisfied by the existing achievements and are looking for more efficient and convenient approaches to obtain functional SCs. In 2017, our group reported a three-step induction protocol which could induce the peripheral blood derived MSCs to express SCs' specific neurotrophins, transcriptional factors, and functional structural proteins, and the induced cells could myelinated axons after being transplanted into the injured sciatic nerves.[11a] In the present study, the same protocol was used to induce BMSCs and then to compare the effects of different nanofibers on the
transdifferentiation. As shown in Figure 4a, the un-induced cells (BMSCs) were flat and negative for S100 and GFAP while the induced cells (iBMSCs) were transformed into spindle like and positive for S100 and GFAP which indicated this induction protocol was also working well in BMSCs’ transdifferentiation.

When the cells were cultured and induced on the nanofiber scaffolds, it was clear that the cells extended along the aligned fibers on A and AC scaffolds, while had a random distribution on the R and RC scaffolds as on the bare culture plate.

Furthermore, western blotting and quantification data indicated that all of the induced cells had significantly higher levels of S100 and GFAP than those of un-induced cells. Interestingly, we found that the aligned nanofibers (A and AC) significantly up-regulated the S100 and GFAP expression while the random nanofibers (R and RC) exhibited similar levels with iBMSCs. Moreover, the levels of AC group were statistically higher than those of A group (Figure 4b).

The above data indicated that, 1) the aligned nanofibers can promote the transdifferentiation of BMSCs into SCs, which is consistent with previous reports[12]; 2) the random nanofibers cannot promote this transdifferentiation, no matter whether they are conductive or non-conductive; 3) The conductivity can enhance the SCs differentiation efficiency on the aligned nanofibers.

2.4. The Transdifferentiated Cells Enhance the Neurite Outgrowth of the Co-Cultured Dorsal Root Ganglions

One of the most important functions of SCs is supporting the axonal outgrowth during the nerve development and regeneration.[25] In order to assess this function of the transdifferentiated cells, a DRGs co-culture was used to mimic the axonal regeneration in vitro and the length of neurites extended from DRGs was quantified. As shown in Figure 5a, when the DRGs were cultured on the R, RC scaffolds, or bare culture plate (control), the length and orientation of their neurites were similar. They outgrew in all direction, just like fireworks. However, on the A or AC scaffolds, the neurites projected from the DRGs were much longer and majority of them were in the direction of the aligned nanofibers. Statistically, both of average length and maximal length of the neurites were ranked first in AC group, second in A group, and there was no significant difference among R, RC, and control groups (Figure 5b).

2.5. Nerve Guidance Conduit Containing Aligned and Conductive Nanofibers Promotes the Nerve Regeneration

Though a variety of artificial nerve conduits have been evaluated for bridging the defected peripheral nerves, it is still a challenge to develop an ideal nerve guidance channel to replace autografts due to the difficulty in restoring proper signal transmission. To address this problem, it has become a new trend to design scaffolds to enhance electrical signals and modulate the cell functions.[21] Axonal regeneration through conduits can be enhanced and lengths increased in the presence of a biomaterial filler in the conduit lumen can be repaired.[22] Since the AC nanofibers play significant roles in promoting BMSCs differentiation into SCs and promote the axonal extension, we selected it as the filler materials to prepare conductive nerve guidance conduits (NGC) and investigated whether the NGC can promote nerve regeneration in the sciatic nerve defect model. We seeded GFP-BMSCs on the nanofiber membranes and induced in vitro for 2 weeks, then rolled up and inserted into PCL conduit. This conduit was named as PC-AC in the present study and the empty PCL conduit was named as PC as control. Both of them as well as a segment of sciatic nerve were utilized to bridge 10-mm rat sciatic nerve gap (Figure 6). At 12 weeks after transplantation, the nerves with the grafts were transversely or longitudinally sectioned for further assessments. In the NF200 or MBP immunostained transverse sections, the GFP positive cells were found wrapping with the NF200 positive axons just like the SCs’ profile. More importantly, the GFP signals around the axons were overlapped with the MBP immunoreactivity (Figure 7), which indicated that the grafted cells could differentiate into mature SCs and participate in myelin formation.
The main purpose of the nerve tissue engineering is bridging the defected nerve gap to support the axonal regeneration. To figure out this issue, NF200 immunohistochemistry was performed to assess the regenerated axons in the grafted conduits. First, the longitudinal sections with NF200 immunostaining showed robust NF200 positive axons penetrated into the grafts of PC-AC and AG across the entire length, while the regenerated axons sparsely emerged in the PC graft and most of them did not reach the distal end of the conduit (Figure 8). In order to quantify the regenerated axons, the transverse sections obtained at proximal, middle, and distal points of the grafts were also immune-stained with NF200 antibody (Figure 9a). Statistic analysis demonstrated that the number of axons at the proximal sections was similar among three groups while significant differences among three groups were found at middle and distal sites. Overall, PC-AC graft significantly improved the axon regeneration comparing to the PC graft (Figure 9b). Furthermore, MBP and NF200 double immunostaining was performed on the sections of the middle point of the grafts to assay the myelination of the regenerated axons (Figure 9c). In AG and PC-AC groups, almost half of NF200-positive axons were wrapped with MBP-positive myelin which indicated they were remyelinated. Statistics revealed the ratio of remyelination has no significant difference between AG and PC-AC groups but they were dramatically higher than that of the PC group (Figure 9d).

Except of morphological assessments of the axonal regeneration and remyelination as shown in Figures 8 and 9, the functional recovery of the injured sciatic nerves was also evaluated with the electrophysiological assay (Figure 10a). Electrophysiological examination can reflect the recovery of nerve conduction function of regenerated nerve fibers. The amplitude of CMAP is always used to signify the nerve...
conduction strength. Higher amplitude indicates that more axons regenerated and arrived at the measured muscles in the paws. The latency was analyzed for it reflects the nerve conduction speed and shorter latency means quicker nerve conduction which mainly attribute to the level of myelination. Quantification on the CMAP showed that the PC-AC graft resulted in significant higher amplitude and shorter latency than PC graft.

After PNI, the target muscles will atrophy and dysfunction due to the long-term denervation. When the damaged nerves regenerated quickly and reinnervated the muscle tissue effectively, the myoatrophy would be alleviated and even might be reversed. The present data illustrated that the wet weight and myofiber area ratio of the gastrocnemius muscle, both of which were widely used to reflex myoatrophy after sciatic nerve injury,
3. Conclusion

The key objective of this study is to explore the synergistic effect of conductivity and alignment profile on the transdifferentiation of BMSCs into SCs. The current data demonstrated that BMSC could be transdifferentiated into SCs, aligned nanofibers...
could improve the efficacy of the transdifferentiation while the random nanofibers had no effect on it. More importantly, we found that endowing conductive property into the aligned nanofibers could significantly enhance its capability to promote the SCs differentiation; however, the addition of electrical conductivity in the random nanofibers cannot change their negative results on the SC differentiation. Furthermore, both of in vitro and in vivo results demonstrated that the aligned and conductive nanofibers could promote peripheral axonal regeneration. Collectively, the present study indicated that the conductive property in the aligned nanofiber plays significant roles in the SCs differentiation, the aligned and conductive nanofibers could be used as a promising scaffold for the peripheral nerve tissue engineering.

4. Experimental Section

Fabrication of the Electrospun Fibers: The preparation of the electrospun solution was carried out by the following procedures. 0.2 g of PCL (Aldrich) and 0.8 g of gelatin (Sigma, USA) were dissolved in 10 mL of 2,2,2-trifluoroethanol (TFEA, Macklin) and stirred overnight. This solution was directly used to do electrospinning for the non-conductive fibers for control. To develop the conductive electrospun fibers, 0.01 g of amine functionalized MWCNTs (8–15 nm in diameter, 10–50 µm in length, Aladdin) were thoroughly dispersed in 10 mL of TFEA by ultrasonication for 2 h at 4 °C, then added into the PCL and gelatin solution and homogenized by stirring overnight at room temperature. The prepared solutions were electrospun by the electrospinning apparatus (Ucalery, Beijing) with the instructions of the system. Briefly, 5 mL solution was transferred into a syringe pump with a 21-gauge needle. A voltage potential of 17 kV and a distance of 15 cm were applied between the needle and the collector. The flow rate was controlled at 0.25 mm min⁻¹. Random fibers were collected on a fixed aluminum foil. Aligned nanofibers were deposited on an aluminum foil wrapped on a grounded rotating mandrel at a linear rate of around 15 m s⁻¹.

The surface morphology of the electrospun PCL/gelatin and PCL/gelatin/MWCNT nanofibers were evaluated by scanning electron microscope (SEM). The diameters of the electrospun fibers were measured from the image using ImageJ v1.8.0 software (NIH, USA). Cyclic voltammetry (CV) measurements were performed with an electrochemical workstation (Corrtest, CS150) as previously described. CV measurements were performed in the potential range of −0.2–0.6 V with a scan rate of 10 mV s⁻¹.

Isolation of Bone Marrow Derived Mesenchymal Stem Cells: The BMSCs were isolated from the bone marrow of the femurs and tibias of 2-week old mice. The BMSCs were cultured in complete Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine. The media was changed every 2 days and the cells were passaged when they reached 80–90% confluence. The cells were used for experiments when they were in the second to third passage.

Figure 9. a) Immunostaining with NF200 antibody on the transverse sections showed the regenerated axons in the proximal, middle, and distal segments of the grafts. b) Quantitative analysis demonstrated that the PC-AC group significantly improved the axon regeneration compared with the PC group. *p < 0.05. c) MBP (green) and NF200 (red) double immunostaining showing significant myelination of regenerated axons in the middle part of the AG and PC-AC grafts, but limited myelination in the PC graft. Yellow arrow, myelinated nerve fiber; white arrow, unmyelinated nerve fiber. d) Quantitative analysis revealed that the myelination rate of axons was significantly higher in the PC-AC group than in the PC group. Data presented as mean ± SEM, n = 6, p-values are calculated using one-way ANOVA with Bonferroni correction. N.S., non-significance; *p < 0.05.
transdifferentiation, the induced cells were immunostained with specific markers of SCs, including GFAP and S100 by routine procedures. Briefly, cells were fixed with 4% paraformaldehyde (PFA) for 30 min and permeabilized in 0.5% Triton X-100 in PBS for 30 min, and blocked in 5% deep sea fish gelatin (Sigma, USA) in PBS for 1 h. Then the cultures were incubated with diluted primary antibodies overnight at 4 °C and Alexa 568 fluorescent conjugated secondary antibodies (Molecular Probes) were incubated at room temperature for 2 h, followed by DAPI staining for cell nuclei. The following primary antibodies were used: mouse anti-GFAP (1:200, Abcam) or mouse anti-S100 (1:100, Millipore).

Western Blotting: After the cells were cultured in differentiation medium for 2 weeks, they were homogenized in RIPA buffer (Sigma, USA) containing 1% protease inhibitor cocktail (Cell signaling). Protein samples were separated on a 10% SDS-PAGE gel and then transferred onto PVDF membrane (Bio-Rad). The signals were detected by Super ECL Detection Reagent (Shanghai Yeasen Biological Technology, China).

Co-Culture of Induced BMSCs (iBMSCs) with DRGs: To assay the bioeffects of the induced BMSCs on the neurite outgrowth, DRGs were used as another model neuron for further study. DRGs were harvested from the spinal column in Sprague Dawley rat pups at the age of 1 day via sterile microdissection, and seeded onto the center of the nanofiber membranes, which have been pre-seeded with BMSCs and induced for 2 weeks in differentiation medium. The next day the medium was replaced by Neurobasal (Gibco) supplemented with 2% B27, 2 mM Glutamine and 50 ng/ml β-NGF and cultured for another 5 days. Thereafter, fixed DRGs and iBMSCs were immunostained with Tuj1 primary antibody and Alexa Fluor 568 secondary antibody. Fluorescence micrographs were captured using a fluorescence microscope. The average neurite lengths and the maximum neurite lengths of the projected neurites from the DRG bodies were derived from the fluorescence micrographs using ImageJ software.

Preparation of NGCs: Since the in vitro experiments illustrated that the electric conductivity efficiently promoted the transdifferentiation of BMSCs into SCs and enhanced the axonal regeneration, the further studies would like to test whether the aligned and conductive nanofibers scaffold can facilitate the nerve repair when it was used in
tissue engineering. To this end, a combined artificial nerve conduit was developed by a cylindrical conduit and AC nanofibers with induced BMSCs, and then used to bridge a long defected sciatic nerve in rat.

Ten thousands of BMSCs were seeded on the membrane (a rectangular of 10 mm × 5 mm) of aligned and conductive nanofibers, and then induced into SCs with the protocols as described in Section 2.4. 2 weeks later, the membrane was rolled up and inserted into a cylindrical conduit of 12 mm in length and 1.5 mm in diameter was prepared with PCL as described in the authors' previous report.[28] The empty PCL conduit without electrospun nanofiber membranes was set as a control group.

Animals and Surgical Procedure: Thirty-six adult Sprague Dawley female rats, weighing 200–250 g, were purchased from the Laboratory Animal Center of Southern Medical University, China. The animals were randomly divided into three groups with 12 rats each: 1) Autograft (AG, n = 12); 2) empty PCL conduits pre-seeded with iBMSCs (PC, n = 12); 3) PCL conduits containing aligned and conductivity nanofibers pre-seeded with iBMSCs (PC-AC, n = 12). The rats were anesthetized with 12 mg mL\(^{-1}\) tribromoethanol (90 mg per kg body weight). After routine skin preparation and disinfection, the right sciatic nerves were exposed and a 10-mm defect was created at the middle portion of the nerve trunk. The prepared conduits were used to bridge the gap; 1-mm segments of the proximal and distal nerve stumps were inserted into the conduit and sutured with 11-0 suture (Ethicon). For the positive control, the 10-mm nerve from the left sciatic nerve was reserved and re-bridged.

Electrophysiological Test: The electrophysiological test was performed in the same way as the authors' previous research. Briefly, 12 weeks after transplantation surgery, the animals were anesthetized with 12 mg mL\(^{-1}\) tribromoethanol (90 mg per kg body weight). Then, the involved sciatic nerve was exposed, stimulating electrodes were applied to the host nerve proximal to the injury site, and a pair of electrodes was placed in the intrinsic foot muscle to record the compound muscle action potential (CMAP) with a set of electrophysiological recorders (Axon Digidata 1500 Digitizer, Molecular Devices). The amplitude and latency of each animal were recorded and analyzed.

Tissue Collection and Histological Analysis: Immediately after the CMAP recording, the rats were euthanized with an overdose of anesthesia and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 mol L\(^{-1}\) phosphate buffer; then, the sciatic nerve and the gastrocnemius muscles were harvested for further histological analysis. After post-fixation with 4% PFA for 24 h and immersed in 30% sucrose (w/v) overnight, the samples were embedded in optimal cutting temperature compound for cryosectioning and immunofluorescence staining.

Every tenth section of each sample was immunostained with NF200 and/or myelin basic protein (MBP) antibodies to visualize regenerated axons and myelin. Selected sections were washed with PBS three times (10 min each) and permeabilized by 0.3% Triton X-100(Sigma) for 30 min, then blocked with 5% gelatin from cold water fish skin (Sigma) in PBS for 1 h at room temperature, followed by the incubation with polyclonal anti-NF200 (1:600; Sigma) and monoclonal mouse anti-MBP (1:400; Santa Cruz) diluted in 5% gelatin overnight at 4°C. Alexa 568-conjugated goat anti-rabbit and Alexa 488-conjugated secondary antibodies (1:400, Molecular Probes) were applied for 2 h at room temperature, and nuclei were stained by 4',6-diamidino-2-phenylindole (DAPI, Sigma) for 3 min. Digital images were captured with a fluorescence microscope (Leica, DM4000B).

Histomorphometry of the Gastrocnemius Muscle: After transcardially perfused, bilateral gastrocnemius muscles were explanted immediately and wet muscle weight was recorded on a laboratory scale. The weight ratio of the injury side/intact side was calculated as the recovery index of the gastrocnemius muscle. The mid-belly of the gastrocnemius muscle was trimmed for routine paraffin embedding, transversely section and hematoxylin-eosin staining. Six non-overlapping images of every eighth section of each animal were captured and the area of myofibers was quantified using Image-Pro Plus software.

Statistical Analysis: All the quantification data were analyzed with SPSS 22.0 (IBM, USA), OriginPro 8.0 (OriginLab, USA) and Graph Pad Prism 6.0 (CA, USA) software. Data were presented as mean ± standard deviation. Statistics for multiple comparisons were generated using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests. Differences between groups were considered significant at p < 0.05 and relevant significances were indicated as * for p < 0.05 in the figures.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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