Non-electric bioelectrical analog strategy by a biophysical-driven nano-micro spatial anisotropic scaffold for regulating stem cell niche and tissue regeneration in a neuronal therapy

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ABSTRACT

The slow regenerating rate and misdirected axonal growth are primary concerns that disturb the curative outcome of peripheral nerve repair. Biophysical intervention through nerve scaffolds can provide efficient, tunable and sustainable guidance for nerve regrowth. Herein, we fabricate the reduced graphene oxide (rGO)/polycaprolactone (PCL) scaffold characterized with anisotropic microfibers and oriented nanogrooves by electrospinning technique. Adipose-derived stem cells (ADSCs) are seeded on the scaffolds in vitro and the viability, neural differentiation efficiency and neurotrophic potential are investigated. RGO/PCL conduits reprogram the phenotype of seeded cells and efficiently repair 15 mm sciatic nerve defect in rats. In summary, biophysical cues on nerve scaffolds are key determinants to stem cell phenotype, and ADSC-seeded RGO/PCL oriented scaffolds are promising, controllable and sustainable approaches to enable peripheral nerve regeneration.

1. Introduction

Traumatic peripheral nerve injuries (PNIs) lead to poor clinical outcome and cause tremendous health care costs each year \cite{1,2}. Rapid reconstruction of structural integrity is essential to optimize repairing outcome, as the delay to surgery will result in significant function loss \cite{3}. However, the current gold standard treatment—autograft transplantation, generates unsatisfactory results, because only a few patients regain proper motor function and recuperate sense perception to a full extent. Also, the donor nerve is insufficient for patients with large-size (2-3 cm) or multiple nerve defects.

Tissue engineered scaffolds, constituted by cells, materials and bioactive molecules, are potential alternatives to autografts for nerve repair \cite{4,5}. Over the past decades, various bioactive molecules have been integrated to propagate regenerative signaling in the advancement of bionic nerve scaffolds for nerve repair \cite{6-8}. However, the integration of these biochemical cues also presents three major drawbacks: 1) rapid biodegradation and unsustainable bioactivity; 2) chance of severe complications, including tumor formation and mutation due to xenogenic contaminants; 3) uncertainty in in vivo experiments and lack of reproducibility \cite{9-11}. In contrast, biophysical cues have been examined to exert defined, sustainable and less-toxicant effect during nerve regeneration.

Due to the electroactive nature of nerve tissue, electroactive materials have been widely adapted to reconstruct the physiology of peripheral nerves. Electroconductive polymers could enable electrical
signal propagation, which not only promoted neurite outgrowth, but also accelerated functional maturation [12]. Sensory conduction function could be restored in paralyzed limbs via a bionic electrical interface [13]. A wireless neural stimulator elicited repeatable compound action potentials after implantation in a rat sciatic nerve defect and thus provided well-controlled and therapeutically relevant effect in sciatic nerves [14]. Graphene, as a kind of electroconductive material with carbon-carbon bonds and densely packed, hexagonal structure, proves to be an ideal platform for neural differentiation of stem cells [15,16]. Different from the stringently-arranged aromatic structure of graphene, reduced graphene oxide (rGO) is anchored with oxygenated groups on the surface of carbon atom sheet which enhance the biocompatibility [17,18]. Apart from electrical cues, topological cues also promise to alter cellular behavior and stimulate nerve regeneration by redefining the cell-material interface. Micro-scaled topologies are of a comparable size to cells and mediate whole-cell contact guidance while nano-scaled topologies orchestrate cell-material interaction at single molecule level and play a decisive role in cell spreading and intracellular mechanosensory signaling [19,20]. Integration of these longitudinally oriented topological cues on scaffolds yields inductive niches and guides in situ peripheral nerve regeneration across a critical defect [21]. Poly-caprolactone (PCL) is a kind of biodegradable and biocompatible semi-crystalline polymer with appropriate stiffness and elasticity. Other polymeric scaffold materials, including polyglycolide, polyl-lactide and poly(lactide-co-glycolide), are featured with superior cell affinity and hydrophilicity but less-intensified rigidity and stiffness [22]. Therefore, PCL-based scaffolds are ideal candidates for long-term in vivo implantation, as they can prevent nerve collapse or entrapment within the conduits due to their physical properties [11].

Living biological cells are one of the key components in nerve tissue engineering and Schwann cells (SCs) are considered to be one of the most favorable cells due to their axon-supporting and myelin-forming capacity [23,24]. However, the proliferation ability of SCs is poor and the isolation of autologous SCs requires for donor nerve. These drawbacks limit the further use of SCs in the clinical practice. In this context, adipose-derived mesenchymal stem cells (ADSCs) have emerged as highly attractive seeded cells with great clinical translational potential. ADSCs have emerged as one of the most attractive cell sources for tissue engineering and Schwann cells (SCs) are considered to be one of the most promising cell sources for tissue engineering. In this context, adipose-derived mesenchymal stem cells (ADSCs) have emerged as highly attractive seeded cells with great clinical translational potential. ADSCs have emerged as one of the most attractive cell sources for tissue engineering and Schwann cells (SCs) are considered to be one of the most promising cell sources for tissue engineering.

2. Materials and method

2.1. Fabrication of electrosprun aligned microfibers with longitudinal nanogrooves

We used the electrospinning technique to fabricate rGO/PCL conduits. RGO powder (0.25%, 0.5%, 0.75%, 1%, 2% and 4% W/W, XF247, XFNANO, China) was added into the mixed solution of dichloromethane/dimethylformamide (3:1, V/V) and subjected to sonication for 30 min at 15 °C. Then, PCL particles (10%, W/V) and poly(vinyl pyrrolidone) (PVP) powder (6%, W/V) were blended into the rGO suspension at 15°C and stirred evenly for 12 h to prepare the spinning solution. The electrostatic spinning procedure was maintained at relative humidity of 70%. The spinning voltage was set at 15 kV. An injectable syringe was placed at a distance of 15 cm from the receiving tubular mold. The rGO/PCL/PVP mixture solution was sprayed from syringe at a flow rate of 2.5 μl·h⁻¹ and the microfibers were deposited onto the surface of rotating tubular mold. Under the force of electric field, microfibers were aligned along the axis of the tubular mold. Aligned microfibers were collected for 5 min at a high speed of 10 rpm to form the inner layer of the conduit. The outer layer of neural conduit was produced by collecting random fibers for 35 min at a slow rotating speed of 70 rpm. Afterwards, the scaffold was peeled off from the PVP-coated tubular mold and washed thrice with alcohol and deionized water to remove PVP. The removal of PVP caused erosion on microfibers and thus nanogrooves were decorated on each fiber. The same procedure was applied in the PCL scaffold preparation.

2.2. Characterisation of the rGO samples and electrosprun fibers

The surface topography, rGO particle distribution, and fiber diameter of scaffold were observed and measured through scanning electron microscope (SEM) as well as energy-dispersive X-ray spectroscopy (EDS) mapping (SU8010 Field Emission-SEM, Hitachi, Japan). The samples of films and scaffolds were prepared by a 30-s gold coating which strengthened the electroconductivity of the samples. Both the films and the transverse ultrathin sections of scaffolds were photographed at 2000× magnification at 5 kV. Atomic force microscope (AFM, MFP-3D Bio, USA) was typically used for analysis of rGO/PCL and PCL topographical features. The scan size of each sample was 6 μm × 6 μm, and the scan speed was 12 μm s⁻¹. The surface morphology of rGO/PCL and PCL membrane was also measured in terms of surface area via Brunauer-Emmett-Teller (BET) analysis (ASAP 2460, USA). The nanostructure of rGO sheets was observed by transmission electron microscope (TEM) (JEM-2100, Japan).

The tensile mechanical properties were measured by two parameters, including elongation at break and elastic modulus constants by an electronic fabric tensile device (YG026MB, China) at a speed of 10 mm min⁻¹. The stress-strain curves were acquired at the same time. The measurement for Young’s modulus data was replicated six times for each sample. Water contact angles (WCA) of 2% rGO/PCL and PCL films were measured by a contact angle goniometer (OCA15EC, Germany).

The electroconductivity of the 2% rGO/PCL and PCL scaffolds was measured using a multimeter device (DL8460, China). Fourier transform infrared (FTIR) spectroscopy of rGO powder, PCL, PVP and 2% rGO/PCL composite scaffolds was investigated using a FTIR spectrometer (NEXUS-670, USA) in the 400-4000 cm⁻¹. Thermogravimetric Analysis (TGA) was performed by a thermal gravimetric analyzer (PerkinElmer TGA400, Holland) from 30°C to 800°C with a heating rate of 20°C min⁻¹, under a nitrogen atmosphere.

The Raman spectra of rGO particles, 2% rGO/PCL and PCL membranes were acquired with a Raman microscope (inVia-Reflex, UK) over the 0-4000 cm⁻¹ region via a laser source of 532 nm. The crystallographic structures of the 2% rGO/PCL and PCL fibrous membranes were
characterized by X-ray diffractometer (XRD) Bruker D8 Advance at angles 2θ from 5° to 80° with Cu radiation 1.542 Å. The reduction state of rGO in rGO/PCL membranes was characterized by X-ray photoelectron spectroscopy (XPS) analysis (Escalab 250Xi, USA).

2.3. Cell seeding and viability of ADSCs on rGO/PCL and PCL scaffolds

ADSCs of Sprague-Dawley (SD) rats were purchased from Cyagen Biosciences (RASMD-01001, Guangzhou, China). We seeded ADSCs on the rGO/PCL and PCL material films and fixed these films with sterilized metal rings of the appropriate size. The films were sterilized with 75% ethanol for 2 h and exposed to ultraviolet light overnight. Different scaffolds, including 0, 0.25%, 0.5%, 0.75%, 1%, 2%, and 4% rGO in PCL, were fabricated to determine the best rGO concentration by comparing their cell viability respectively. ADSCs were seeded at a density of 2 × 10⁵ cm⁻² and cultured with α-MEM (Invitrogen, Thermo Fisher Scientific), 1% (V/V) penicillin-streptomycin solution (Sigma-Aldrich) and 10% fetal bovine serum (FBS) (Gibco).

We used Cell counting kit 8 (CCK8) analysis to evaluate the cell viability of ADSCs. Following 24, 48, 72 and 96 h of culture, cells were subjected to 10% (V/V) CCK8 solution (Dojindo, Japan) (10 μL CCK-8 reagent diluted in 100 μL of α-MEM) based on the manufacturer’s instruction. Then the absorbance value of each concentration was measured through micro-plate reader at 450 nm. The experiment was repeated 6 times for each concentration.

The short-term effect of rGO/PCL on the viability of ADSCs was studied by cell apoptosis assay and quantified by flow cytometry. ADSCs (5 × 10⁴ in each sample, 3 samples for each group) were cultured for 12 h on rGO/PCL and PCL films before the test. Briefly, the supernatants were removed, and the ADSCs were digested with 0.25% Trypsin-EDTA for 5 min. Next, digested cells were washed with PBS for 3 times and re-suspended in PBS. Then ADSCs from both groups were incubated with the apoptosis detection kit (C1062L, Beyotime Biotechnology, China) in accordance to the manufacturer’s instructions. The ratio of apoptotic ADSCs was analyzed by flow cytometry. Early-stage apoptotic ADSCs were stained with annexin V-FITC only. In contrast, late-stage apoptotic ADSCs was analyzed by flow cytometry. Early-stage apoptotic ADSCs was analyzed by flow cytometry. Early-stage apoptotic ADSCs was analyzed by flow cytometry.

2.4. Alignment and attachment of ADSCs on microfibers with nanogrooves

Both rGO/PCL and PCL films were opaque materials and we identified the alignment of living ADSCs on different materials by using GFP-labeled ADSCs (RASMD-01101, Cyagen Biosciences). ADSCs were cultured on tissue culture plate (TCP) for 24 h to acquire the optimal state. Then, ADSCs at passage 4 were digested enzymatically and seeded on the scaffolds. After another 3 days of culture, ADSCs were observed under a fluorescence microscope (H600L, Nikon, Japan).

The attachment of ADSCs were observed by SEM. We fixed the ADSCs onto the material with 2.5% glutaraldehyde for 1 h and 1% osmium tetroxide for another 30 min. A graded ethanol immersion was performed for 20 min followed by vacuum drying for 2 days to dehydrate the samples. Finally, the samples were coated with a gold-layer and observed by SEM (VEGAS TESCAN).

2.5. Neural differentiation of ADSCs on rGO/PCL and PCL scaffolds

After the 24 h of culture, α-MEM was replaced with 2% B27 (Invitrogen)-supplemented DMEM/F12 (Gibco). Then, human brain-derived neurotrophic factor (BDNF) (10 ng mL⁻¹, Peprotech), human nerve growth factor (NGF) (10 ng mL⁻¹, Peprotech) and basic fibroblast growth factor (bFGF) (20 ng mL⁻¹, Peprotech) were added to produce the serum-free neural differentiation medium. After a week of neural induction culture, the differentiated ADSCs were characterized with immunofluorescence. Considering the fact that our materials had complex topographical surface, dyes could be absorbed and left in the materials. In order to prevent the interference of fluorescence from the materials, we digested the differentiated ADSCs and re-plated them on the coverslips. Their neural differentiation efficiency was evaluated after the overnight incubation. Cells attached on the coverslips were fixed with 4% paraformaldehyde for 30 min and then permeabilized by 0.2% tritonX-100 (Sigma-Aldrich). Next, ADSCs were blocked by 10% goat serum (SU038, Solarbio). Our samples were incubated with primary antibodies, including anti-γ-glial fibrillary acidic protein (GFAP, 1:200, ab7260, Abcam) and anti-Tuj1 (1:200, ab78078, Abcam), overnight. On the next day, the samples were incubated with secondary antibodies, including goat anti-rabbit IgG H&L (Alexa Fluor® 488, 1:200, ab150079, Abcam) and goat anti-mouse IgG H&L (Alexa Fluor® 488, 1:200, ab150113, Abcam) for 1.5 h. Finally, the samples were incubated with DAPI (No. H-1200; Vector Laboratories Inc) for 5 min. The coverslips were observed under a fluorescence microscope. The ratio of Tuj1 and GFAP-positive cells in the rGO/PCL and PCL groups were calculated from 6 randomly selected fields in each groups using Image J software.

We performed Western Blot to further evaluate the neurotrophic differentiation of ADSCs. After a week of cell neural induction culture on materials, ADSCs were lysed in cell lysis buffer with protease inhibitor (Cell Signaling Technology). Next, the collected protein samples were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis, followed by transferring onto PVDF membrane. The PVDF membranes were directly blocked with 5% nonfat milk in PBS for 30 min at room temperature. Next, PVDF membranes were incubated with the primary antibodies, including anti-NGF (1:500, ab52918, Abcam), anti-glial cell-derived neurotrophic factor (GDNF) (1:500, ab119473, Abcam) and anti-β-actin (1:500, ab8227, Abcam) followed by incubation with goat anti-rabbit IgG-HRP (1:1000, ab205718, Abcam) for 2 h. Finally, PVDF membranes were incubated in ECL Western Blotting Detection Reagent (Sigma-Aldrich, USA) to develop visualization signals. Image J software was used to measure the intensity of signals.

The neurotrophic function of ADSCs was directly verified by dorsal root ganglia (DRG) neuron co-culture in a Transwell® system (a six-well plate with six inserts which contain 0.4-μm pore size polyester membrane) (3450; Corning). DRGs were collected from rat intervertebral foramen using a stereo microscope and enzymatically digested. The DRG neurons were seeded in the lower chamber of the Transwell® culture, the length of longest axons in DRG neurons was analyzed to reflect the trophic effect of ADSCs. Briefly, DRG neurons were fixed, permeabilized and blocked as before-mentioned method. Then neurons were incubated at 4°C overnight with anti-Neurofilament 200 (1:100, 28365, Cell Signaling Technology) followed by goat anti-mouse IgG H&L (Alexa Fluor® 488, 1:200, ab150113; Abcam) for 2 h at room temperature. Finally, the DRG neurons were incubated with DAPI. The mean length of the longest axons was calculated based on 6 randomly selected fields of each sample using Image J.

2.6. Preparation of ADSCs for FACs and the RNA extraction

In order to specifically identify the neurotrophic identity of ADSCs in the in vivo condition, GFP-labeled ADSCs were suspended in Matrigel (Corning® Matrigel®) at 4°C and the cell-Matrigel mixture were injected into the lumen of rGO/PCL and PCL scaffolds. Briefly, 5 × 10⁶ GFP expressing ADSCs were suspended in 500 μL ice-cold Matrigel and slowly injected into the scaffolds by a Hamilton micro-syringe to reach the uniform distribution. In our experiment, 500 μL ADSC-gel mixture was injected into each conduit prior to surgery. The coagulation was achieved when the cell-suspended Matrigel solution reached the room
temperature.

All protocols involving the use of animals followed the ethical guidelines of the Animal Ethics Committee for Shanghai Jiao Tong University, and were approved by Institutional Ethical Committee of the Shanghai Sixth People’s Hospital for animal care and uses (DWLL2021-0867).

In brief, six SD rats, weighing 200-220 g, were selected for this experiment and uniformly divided into two groups. We injected 30 mg kg\(^{-1}\) pentobarbital sodium intraperitoneally for anesthesia. We exposed the right sciatic nerve of each rat through the gluteal muscle incision. A 15-mm-long sciatic nerve defect was made. Then, conduits filled with GFP-labeled ADSC-Matrigel mixture were sutured to both proximal and distal nerve stumps by 3 peri-neural 6-0 nylon. We then sutured the muscles and skins with 4-0 nylon sutures and all animals were returned to their cages. After a week post-operation, all the animals were sacrificed through over dosage anesthesia. The implanted nerve conduits were quickly harvested and the regenerated tissue inside the conduits were collected separately. The regenerated tissues were cut into small pieces and digested with 1 mg ml\(^{-1}\) collagenase type I, 0.1% papain and 0.05% trypsin at 37 \(\degree\)C for 15 min. Finally, the suspensions were triturated in α-MEM with 10% FBS to terminate digestion. All the suspensions were then centrifuged and the cells were re-suspended in 1 mL basal culture medium for each sample. GFP-expressing cells were then sorted using FACS technology (BD FACS ARIA III machine). Non-GFP-expressing ADSCs were set as control. All the cells were collected into ice-cold PBS and washed thrice. In the final step, suspensions were centrifuged and RNA was extracted using the Trizol (Invitrogen).

### 2.7. Transcriptome analysis

Total RNA isolated from ADSCs of rGO/PCL group was marked as experiment group and the RNA extracted from PCL group was marked as control. Subsequently, the samples were sent for standard RNA-seq at Oebiotech Co. Ltd (Shanghai, China). The global transcript profile of ADSCs was then compared between two groups to determine the differentially expressed genes (DEGs) by DESeq2 (1.24.0) analysis. The threshold was set as \(p < 0.05\) and fold change cutoff of 2, leading to the generation of 76 DEGs. We then conducted Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis to determine the enriched signaling pathway.

### 2.8. LY294002 treatment and subsequent Western blotting

To investigate the role of PI3K signaling on rGO/PCL induced neurotrophic phenotype changes and myelin protein secretion, we cultivated ADSCs on rGO/PCL scaffolds in neural induction medium for a week and incubated ADSCs with LY294002 (50 μmol L\(^{-1}\), 1,54447-36-6, MCE, USA) for 24 h. We selected 50 μmol L\(^{-1}\) as the concentration of the LY294002 solution on the basis of our experiments on ADSCs and manufacturer’s recommendations. Then ADSCs on rGO/PCL scaffolds treated with or without LY294002 were lysed in cell lysis buffer with protease inhibitor (Cell Signaling Technology). The electrophoresis and PVDF membrane transfer were performed in consistent with before-mentioned steps. PVDF membranes were then incubated with anti-pAkt (1:1000, 40605, CST), anti-Akt (1:1000, 46915, CST), anti-NGF (1:500, ab52918, Abcam), anti-myelin basic protein (MBP) (1:500, ab209328, Abcam) and anti-β actin (1:500, ab8227, Abcam) overnight. On the next day, the PVDF membranes were goat anti-rabbit IgG-HRP (1:1000, ab205718, Abcam) at room temperature for 2 h. Visualization signals were developed as was mentioned before. Image J software was used to measure the intensity of signals.

### 2.9. Animal surgeries and procedures

A total of 18 male SD rats weighing approximately 200-220 g were equally allocated into three groups, including rGO/PCL, PCL and autograft group, with 6 rats in each group. These animals were anesthetized and performed sciatic nerve defect as mentioned before. The 15-mm long-gap nerve defect was bridged with ADSC-laden nerve conduits. For autograft implantation, nerve defects were bridged by the previously-excised nerve segment with reversed polarity. At the end of animal experiment, autografts were retrieved and cut into sections for histological analysis. Other procedures were performed the same as conduit group. All the animals were injected with 10\(^5\) units of penicillin intraperitoneally and then sent back to their cages. Observations and procedures were performed at 18 weeks.

#### 2.10. Walking track analysis

At 18 weeks postoperatively, we performed walking track analysis and introduced the Functional Index (SFI) scoring system to measure the locomotor functional improvement. SFI was calculated as previously described [22]. SFI is a negative number ranging from 0 to -100 and a higher number indicates better function of sciatic nerves while a lower number stands for the worse function. The footprint of the hind limbs was collected and recorded. The experiment was repeated for three times on each rat.

#### 2.11. Electrophysiological assessment

After locomotor function evaluation, all the SD rats accepted electrophysiological testing under anesthesia. Electrical signals were given by the bipolar electrodes which were fixed at the two ends of regenerated nerves. Thereafter, another electrode was fixed at the belly of gastrocnemius muscle to collect the electromyography. Both distal compound muscle action potential (DCMAP) and nerve conduction velocity (NCV) were important indicators to evaluate the overall electrophysiological recovery in nerves. The experiment was repeated for six times in each group.

#### 2.12. FluoroGold (FG) retrograde tracing

After functional tests, all the rats were sent for retrograde tracer injection. Briefly, 10 μL of 4% FG tracing solution (Fluorochrome Inc., Denver, CO, USA) was injected into the sciatic nerve, 5 mm away from the distal end of the conduits, following the manufacturer’s instruction. The fifth day post-injection was chosen as the time-point for retrograde tracing analysis according to manufacturer’s instruction. The lumbar 4 (L4)-lumbar 6 (L6) lumbar spinal cord was exposed through ophthalmic scissors and excised together with DRGs of the injured side. The samples were fixed in 4% paraformaldehyde overnight and embedded. Afterwards, the spinal cord and DRGs were cut into 25 μm-thick transverse section and 14 μm-thick longitudinal section respectively, on a cryostat. FG-labeled motor neurons in the anterior horn of spinal cord and FG-labeled sensory neurons in the DRGs were counted under fluorescence microscope.

#### 2.13. Histological analysis

At day 5 post-injection, all the animals were sacrificed to harvest the L4-L6 spinal cord, gastrocnemius muscle and the implanted conduits with regenerated nerves inside. In brief, previously-implanted nerve scaffolds were exposed and harvested by cutting off the two ends of regenerated nerves inside the scaffolds. Gastrocnemius muscle of the injured side was carefully dissected through mosquito clamp and scalp. All the samples were rinsed with PBS thrice. The major functioning organs were also harvested carefully from each group and fixed in 4% paraformaldehyde overnight. The paraffin-embedded sections of heart, liver, spleen, lung and kidney were stained by hematoxylin-eosin (HE) reagent for systemic toxicity evaluation.

The regenerated nerve tissues within the grafted conduits were processed using 1% toluidine blue (TB) staining, immunofluorescence
analysis and TEM. The nerve samples were fixed in 4% paraformaldehyde overnight and subjected to dehydration process. These samples were then cut into 10 μm thick cross-sections on a cryostat. The sections were stained with 1% TB. NF200 and TuJ1 (red fluorescence) were employed to identify regenerated axons at the midpoint of nerves within the conduits. SCs and myelin sheath were identified by S100β and MBP respectively, which were labeled with green fluorescence. The expression and activation of PI3K-Akt signaling pathway were evaluated by Akt and phosphorylated Akt (p-Akt). The following primary antibodies were used to incubate sections overnight: rabbit anti-MBP (1:100, ab40390, Abcam), rabbit anti-S100 (1:100, 90393S, Cell Signaling Technology), mouse anti-βIII-tubulin (1:100, 4466S, Cell Signaling Technology), mouse anti-NF200 (1:100, 2836S, Cell Signaling Technology), rabbit anti-pAkt (1:1000, 4060S, CST) and rabbit anti-Akt (1:1000, 4691S, CST). Secondary antibodies were then applied to incubate the sections, including goat anti-mouse Alexa Fluor® 647 (1:200, ab150079, Abcam) and goat anti-rabbit IgG H&L (Alexa Fluor® 488, 1:200, ab150113, Abcam). Finally, the nuclei were counterstained with DAPI. The mean density of myelinated nerve fibers was calculated for each group. The nerve samples were prepared in 2.5% glutaraldehyde (Sigma Aldrich) and cut into 70 nm-thick ultrathin sections for TEM observation as previously described [23].

The L4 lumbar spinal cord was chosen to investigate the interaction between the peripheral nervous system and central nervous system (CNS) after sciatic nerve injury. The L4 spinal cord sections were blocked and incubated with primary antibodies, including mouse anti-nestin antibody (1:100, ab61422, Abcam) and rabbit anti-GFAP antibody (1:100, ab7260, Abcam). Afterwards, second antibodies including goat anti-mouse and goat anti-rabbit IgG H&L (Alexa Fluor® 647, 1:200; Alexa Fluor® 488, 1:200, Abcam), were dropped onto the sections. In the end, all the samples were counterstained with DAPI. The relative expression level of nestin and GFAP in the spine were calculated using Image J.

Gastrocnemius muscle samples were processed and sliced into 50 μm-thick paraffin-embedded transverse sections as previously described [23]. Thereafter, paraffin sections were dewaxed and subjected to Masson’s trichrome staining. The sections of muscle middle portion were observed under light microscope and analyzed in terms of muscular atrophy. The percentage of muscle fiber area (Pm) was assessed by Image J.

The angiogenesis in the regenerated nerves was determined by CD31 staining. In brief, the paraffin-embedded sections were rehydrated and subjected to antigen retrieval. Nerve sections were then incubated with anti-CD31 antibody (1:150, ab182981, Abcam) overnight. On the next day, the samples were incubated with biotin-conjugated donkey anti-rabbit antibody (1:500, Abcam). Finally, the samples were dehydrated and mounted and the angiogenesis of regenerated nerves was quantified by CD31 positive area.

Fig. 1. Schematic illustration of nerve conduit fabrication and characterization. (A) Electrospun microfibers were ejected from the syringe of electrospinning machine and deposited on the tubular mold along its longitudinal axis. The aligned microfibers were modified with nanogrooves and the so-produced scaffolds were employed for cell seeding and nerve repair. (B) Optical and SEM images of the scaffolds. The morphology of inner and outer surface of the scaffolds was enlarged. Nanogrooves and rGO nanoparticles (red) were exhibited on the PCL microfibers (green).
2.14. Statistical analysis

GraphPad Prism (version 9.0) and the SPSS software package (version 23.0) were used for the statistical analysis by employing unpaired t-test and one-way analysis of variance (ANOVA). P > 0.05 was considered as no significant difference (*P < 0.05, **P < 0.01 and ***P < 0.001).

3. Results

3.1. Fabrication and characterization of rGO/PCL and PCL scaffolds

The micro-nano structured rGO/PCL scaffold was produced using electrospinning technique (Fig. 1A). A tubular mold was employed to receive microfibers ejected from the syringe. PVP was mixed in the PCL solution to obtain a homogenous emulsion for electrospinning process. Microfibers were deposited layer by layer to generate rGO/PCL and PCL scaffolds. The removal of PVP results in the formation of nanoscaled grooves on the surface of PCL and rGO/PCL fibers due to the immiscibility between PCL and PVP. As shown in Fig. 1B, the inner wall of conduits was composed of longitudinally aligned microfibers and the outer wall was composed of random microfibers. The zoomed-in view of rGO/PCL and PCL microfibers revealed that longitudinally parallel nanogrooves were pitted on the surface of PCL microfiber and rGO particles were firmly adhered to the PCL interfaces, leading to excellent nanoparticle-microfiber interfaces (Fig. 1B).

In the SEM images of rGO/PCL scaffolds, we could see that the scaffolds with varying rGO contents had fiber-based grooves and micro-nano composite orientation topography (Fig. 2A). The average diameter of PCL fibers was approximately 5 μm. However, when the concentration of rGO particles exceeded 4%, the excessive rGO loading in the PCL substrates led to the clustering of rGO particles and the deformation of oriented fibers which might cripple the electroconductivity of the scaffold. Based on the results of cytocompatibility experiment (Fig. 4A), the

Fig. 2. Morphology, mechanical properties and electroconductivity of nerve conduits. (A) SEM images of 0.25%, 0.5%, 0.75%, 1%, 2% and 4% rGO/PCL microfibers and the diameter distribution of microfibers from all groups. Scale bar: 10 μm. (B–E) The scaffold thickness (B) and the mechanical properties, including elastic modulus (C), elongation at break (D) and wettability (E) were compared in 2% rGO/PCL and PCL electrospun membranes. (F) Stress-strain curve of 2% rGO/PCL and PCL scaffold. (G) The BET surface area of 2% rGO/PCL and PCL. P and P0 were the equilibrium and saturation pressures of N2 at 77 K respectively. STP was standard temperature and pressure. (H) The electrical conductivity of rGO/PCL scaffolds with different rGO contents. (I) The TGA curves of PCL and 2% rGO/PCL electrospun films. ns: no significant difference.
2% rGO/PCL microfibers were selected for the conduit fabrication and subsequent measurement. The thickness of 2% rGO/PCL and PCL was 0.724 mm and 0.718 mm respectively, as determined by examining the transverse section of both scaffolds (Fig. 2B, Fig. S1). The elastic modulus values for rGO/PCL and PCL films were 52.70 and 61.47 MPa respectively (Fig. 2C). The addition of rGO particles didn’t change the shear behavior of membrane as measured by the elongation at break (Fig. 2D). As observed in the strain-stress curve (Fig. 2F), the mechanical properties of rGO/PCL were similar to those of pure PCL scaffolds, indicating that rGO incorporation did not influence the membrane ductility. The contact angle values were 106° and 103° for the PCL and rGO/PCL membranes respectively (Fig. 2E). The nitrogen adsorption curves of rGO/PCL and PCL treated at standard temperature and pressure (STP) were displayed in Fig. 2G. Based on Fig. 2G, both samples exhibited type IV isotherms, and H3 hysteresis loops which might be attributed to the intrinsic porosity of electrospun fibers and the
nanogrooved surface architecture of the fibrous sheet. The BET surface areas of rGO/PCL and PCL were 6.36 m² g⁻¹ and 8.14 m² g⁻¹ respectively, with no significant differences in the surface area between two sheets. The electrical conductivity of 0.25%, 0.5%, 0.75%, 1% and 2% rGO/PCL composite scaffolds was 8.359, 9.181, 9.741, 11.502 and 14.748 × 10⁻³ S cm⁻¹, respectively (Fig. 2H). Clearly, the electrical performance of the rGO/PCL scaffolds was enhanced as the content of rGO increased. As shown in Fig. 2I. The TGA curves of both PCL and rGO/PCL membranes showed excellent thermal stability up to around 400°C. The mass loss of rGO/PCL and PCL was synchronous during the thermal decomposition process and the residual weights at around 500°C were also similar. Therefore, the addition of rGO particles did not alter the thermal stability of the resultant fibrous membrane.

Different from the stacked and agglomerated structure of GO, the separated accordion-like rGO sheets were exhibited in the TEM micrographs (Fig. 2A). EDS pattern detected the content of C and O elements in the composition of rGO sheets (Fig. 3B) and the distribution of C and O elements on rGO samples was subsequently detected by EDS mapping (Fig. 3C). It was identified that both C and O were distributed homogeneously with uniform morphology and size on rGO sheets. Therefore, oxygen-containing groups were grown evenly on the surface of graphene sheets in which carbon atoms formed the basic framework. From the FTIR spectra (Fig. 3D), the PCL characteristic peak appeared at 1241 cm⁻¹ and 1182 cm⁻¹ (stretching vibration of C–O–C) as well as 1727 cm⁻¹ (stretching vibration of C=O). The C–O (1667 cm⁻¹) peak was the characteristic peak of pure PVP. The absorption of C=O (1667 cm⁻¹) peak was not shown in the rGO/PCL conduit, indicating the complete removal of PVP from conduits. Identical to a previous report [28], the characteristic peak of rGO was undetectable in the infrared absorbance of rGO/PCL, due to the rather small content of rGO in the PCL fibers. However, the existence of rGO was clearly characterized by Raman spectroscopy, in which the characteristic vibration peaks of rGO/PCL were concomitantly seen in pure rGO samples (Fig. 3E). In the Raman spectra of rGO/PCL samples, two characteristic vibration peaks were observed at 1349.3 cm⁻¹ and 1601.4 cm⁻¹ corresponding to the D and G bands respectively. D bands represents the in-plane vibrations of sp²-bonded carbon atoms in rGO while D band reflects the structural defects which create out-of-plane vibrations [29]. The D/G intensity ratio of rGO was 1.0, similar to what was reported previously [30]. The surface roughness and submicron features of both rGO/PCL and PCL films were characterized by AFM profiling (Fig. 3F). XPS analysis of rGO/PCL composites revealed high values of carbon to oxygen atomic ratio (C/O) and strong graphitic intensity which confirmed the efficient ratio of oxygenated groups from rGO. In the XRD pattern of the PCL films, 4 main characteristic diffraction peaks (3359.6), (4092.5), (1718.4) and (1932.1) were obtained with corresponding angles (2θ) at 21.0°, 21.7°, 23.4° and 24.0° (Fig. 3H). The addition of rGO into PCL substrates didn’t significantly change the XRD pattern of PCL, indicating that rGO was completely exfoliated and homogeneously distributed in the PCL substrates. Although rGO is susceptible to aggregating and restack due to the high surface energy of rGO sheets following the rGO delamination process, according to our XRD pattern of rGO/PCL, no diffraction peaks were seen below angles (2θ) of 15.0°, which indicated the successful layer separation of rGO in PCL substrates. EDS identified that rGO/PCL microfibers were composed of 73.73% (wt%) of carbon and 26.27% (wt%) of oxygen while PCL fibers were composed of 73.54% (wt%) of carbon and 26.46% (wt%) of oxygen. Carbon (cyan) and oxygen (purple) atoms were homogeneously distributed in both rGO/PCL and PCL films (Fig. 3J).

3.2. Viability and neural phenotype plasticity of ADSCs on rGO/PCL and PCL films

In order to explore the optimal concentration of rGO in the composite scaffolds, we seeded ADSCs on different rGO/PCL scaffolds for 24, 48, 72 and 96 h. CCK8 kit can quantify the number of live cells by detecting the orange formazan dye produced by cellular dehydrogenases within live cells and optical density (OD) value is proportional to the number of viable cells. According to the results of CCK8 assay, 2% rGO/PCL exhibited lower cytotoxicity than 0.25%, 0.5%, 0.75% 1% and 2% rGO/PCL composites. In addition, cells on 2% rGO/PCL also displayed lower toxic effect than those on PCL control (Fig. 4A). Cell apoptosis assay by flow cytometric quantification further confirmed that the apoptosis of ADSCs was slight with no significant difference between 2% rGO/PCL and PCL group (Fig. 4B and C). We utilized GFP-expressing ADSCs derived from GFP transgenic mice to track cellular behavior on rGO/PCL and PCL sheets. The alignment of ADSCs on directional fibers was confirmed by immunofluorescence of the GFP-expressing ADSCs (Fig. 4D). As shown in Fig. 4D, when cultured on TCP, GFP-expressing ADSCs spread randomly without specific directionality. After 3 days of in vitro culture on the oriented conduit, ADSCs exhibited elongated spindle-like morphology and aligned along the longitudinal axis of microfibers, while ADSCs on PCL scaffolds were sparsely distributed with their morphology barely extended. In SEM images, ADSCs on rGO/PCL microfibers were manifestly bipolarized and displayed excellent orientation behavior in accordance with the fiber direction. In contrast, ADSCs from PCL group were less polarized and tended to assemble together.

When traumatic PNI happens, neurotrophins are released from nerve ending to activate repairing process which stimulates neurite outgrowth and neural differentiation [31]. In this context, we constructed the in vitro model of nerve microenvironment by adding a cocktail of growth factors according to previous reports [32,33]. After a week of pro-neurogenic culture on the rGO/PCL and PCL films, ADSCs were dissociated from the rGO/PCL and PCL films and replated on TCPs to observe their own morphology. The morphology of ADSCs changed greatly into round or polygonal shape, a sign of cell aging. However, under the stimulation of neural differentiation medium, ADSCs simultaneously expressed the neuronal marker TuJ1 (green) and glial marker GFAP (red) (Fig. 4G). Consequently, we analyzed the neuronal and glial expression of differentiated ADSCs. The TuJ1 and GFAP positive rate of ADSCs on rGO/PCL scaffolds were 35.18 ± 8.61% and 57.07 ± 18.60% respectively (Fig. 4I). In contrast, the TuJ1 and GFAP positive rate of ADSCs on PCL scaffolds were 21.33 ± 6.96% and 33.74 ± 10.04% respectively. The rate of GFAP positive and TuJ1 positive cells were both significantly higher in ADSCs of rGO/PCL group than in that of PCL group (Fig. 4I). These results indicated that such electroconductive material potentiated the neural differentiation of ADSCs.

SCs secrete a variety of neurotrophic factors following PNIs among which NGPs and GDNPs are vital factors for the health of sensory and motor neurons respectively [34]. SC-like differentiated ADSCs could also secrete detectable levels of neurotrophic factors in vitro [35]. Herein, we discovered higher expression of NGF and GDNF in the ADSCs cultured on rGO/PCL films than those on the PCL films (Fig. 4E and F). We then cultured the differentiated ADSCs in the upper chamber of Transwell® system and DRG neurons in the lower chamber, to investigate the trophic effect of ADSCs (Fig. 4H, Fig. 52). The mean axonal length of DRG neurons was 738.78 ± 140.10 μm when cocultured with ADSCs of rGO/PCL group, significantly longer than those cocultured with ADSCs of PCL group (394.21 ± 105.00 μm) (Fig. 4J).

3.3. Isolation of transplanted ADSCs and transcriptome analysis

Although different neurotrophic phenotypes of ADSCs from rGO/PCL and PCL group have been verified in vitro, the transcriptional changes of these ADSCs remain elusive in the in vivo nerve microenvironment. In our strategy, GFP-expressing ADSCs were suspended by Matrigel and were injected into the lumen of nerve conduits to realize localized delivery into sciatic nerve defect. Matrigel was biocompatible and biodegradable in an in vivo environment [36]. After a week of in vivo transplantation, we isolated these GFP-expressing ADSCs for RNA sequencing (Fig. 5A). We specifically chose this time-point to avoid the
(caption on next page)
Fig. 4. The survival, growth and neural differentiation of ADSCs and neurite development of DRG neurons on scaffolds. (A) Proliferation of ADSCs by CCK8 assays on day 1, 2, 3 and 4. *p < 0.05 for the comparison with PCL scaffold. ▴p < 0.05 for the comparison with 0.25% rGO/PCL scaffold. ●p < 0.05 for the comparison with 0.5% rGO/PCL scaffold. ■p < 0.05 for the comparison with 1% rGO/PCL scaffold. ▲p < 0.05 for the comparison with 4% rGO/PCL scaffold. (B–C) Cell apoptosis assay by flow cytometric quantification. PI reflected the late apoptosis or death of cells. Annexin-V revealed the early apoptosis of cells. (D) Cell growth morphology on rGO/PCL and PCL scaffolds. The cellular alignment and attachment were longitudinally oriented. Scale bar: 50 μm and 15 μm respectively. (E–F) The Western blot quantitative analysis of two neurotrophic factors in ADSCs. (G) The neuronal and glial differentiation of ADSCs identified by immunofluorescence of Tuj1 (green) and GFAP (red), after culturing in differentiation medium for 7 days. Scale bar: 100 μm. (H) NF200 fluorescence staining of DRG neurons co-cultured with ADSCs for 3 days. Scale bar: 100 μm. (I) Quantitative analysis of the average neurite length of DRG neurons cultured with ADSCs. *p < 0.05, **p < 0.01, ***p < 0.001, ns: no significant difference.

Fig. 5. RNA sequencing of acutely FACS-isolated ADSCs identified PI3K-Akt signaling as the key regulator in rGO-induced ADSC reprogramming. (A) Schematic illustration of isolating and purifying GFP-expressing ADSCs from regenerated nerve bridge via FACS. (B) Representative FACS plots of ADSC purification based on GFP expression level. Control ADSCs without GFP expression were used for gating. (C) Differentially expressed genes from PI3K and FA signaling were listed as heatmaps. (D) Visualization of KEGG pathway enrichment analysis for the up-regulated DEGs between the rGO/PCL and PCL groups. The size of dots indicates the relative number of up-regulated DEGs. (E) MBP and NGF expression of ADSCs on rGO/PCL scaffolds dramatically decreased in response to LY294002 treatment. (F) RNA-sequencing differential gene expression analysis of ADSCs from rGO/PCL and PCL conduits. Genes regulated over 2-fold changes (adj. p < 0.05) were highlighted in red and green. (G) Quantitative analysis of MBP, NGF and p-Akt showed the expression of these proteins decreased about 2.425, 3.885, and 2.436 folds respectively, after treatment of LY294002, as measured by Western blot. **p < 0.01, ***p < 0.001.
quenching of GFP fluorescence in vivo, and also, to be parallel to that of our in-vitro model. Then, the regenerated tissues within conduits were dissociated into single cell suspension. FACS isolated the previously-implanted ADSCs from a bunch of other derived cells based on their GFP expression (Fig. 5B). ADSCs without GFP expression were used as reference for FACS gate setting.

RNA-sequencing was then employed to analyze the molecular identity of these purified GFP-expressing ADSCs. The reproducibility and quality of the RNA-Sequencing data were confirmed prior to the differential expression analysis. The global transcript profile of ADSCs was then compared between rGO/PCL and PCL groups to discover the DEGs. The threshold was set as \( p < 0.05 \) and fold change > 2, leading to the generation of 76 DEGs. We then conducted KEGG enrichment analysis to investigate the underlying mechanism of the different cellular behaviors. The top 20 up-regulated KEGG pathways were listed based on the DEGs (Fig. 5D). Our findings revealed that PI3K-Akt signaling pathway was greatly up-regulated in ADSCs of rGO/PCL group, followed by FA pathways. PI3K-Akt is one of the most important pathways in the regulation of cell proliferation, adhesion, survival, apoptosis and differentiation [37,38]. FA signaling pathway is triggered by specialized transmembrane integrin adhesions which link intracellular cytoskeletons to the extracellular topological cues [27,39]. Based on the gene expression enrichment analysis, genes belonging to the PI3K-Akt pathway (e.g., Vegfd, Csf1r, Col9a3, Col6a5 and Lama1) and FA signaling pathway (e.g., Vegfd, Shc2, Col9a3, Col6a5 and Lama1) were derived from DEGs and listed as heatmaps for pairwise comparisons (Fig. 5C). Noteworthy, four DEG genes in the FA signaling pathway overlapped with those in PI3K-Akt pathways (e.g., Col9a3, Col6a5, Vegfd, and Lama1), emphasizing the considerable crosstalk between PI3K-Akt and FA signaling in the reprogramming of ADSCs (Fig. 5C). These results indicated that the addition of rGO reprogrammed the intracellular signaling of ADSCs which could be decisive to the

Fig. 6. Functional reconstruction of injured nerves and systemic toxicity analysis at 18th week after surgery. (A) Rat walking footprints from rGO/PCL, PCL and autograft group at 18th week after surgery. (B) SFI values calculated from walking footprint analysis. (C) DCMAP and NCV calculated from electrophysiological test of each group. (D) Schematic illustration of electrophysiological test in which electrodes were placed at the proximal and distal end of regenerated nerves. (E) Representative electrophysiological recordings of each group. (F) HE staining of major functioning organs. Scale bar: 100 \( \mu m \). *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \), ns: no significant difference.
acquisition of nerve reparative phenotype. We next validated the PI3K signaling expression of ADSCs on rGO/PCL interface in vitro. Following the treatment of LY294002, an inhibitor of PI3K signaling pathway, ADSCs cultured on rGO/PCL composites strongly reduced the phosphorylation of Akt (the downstream of PI3K), without changing the general expression level of Akt (Fig. 5E). The expression levels of NGF and MBP were reduced by 3.9 and 2.4 folds respectively, along with the blockage of Akt activation (Fig. 5G).

3.4. Gait analysis

To investigate the nerve repair capacity of different conduits, ADSC-laden rGO/PCL and ADSC-laden PCL conduits were implanted into a lengthy sciatic nerve defect model. Autograft group was the positive control in the assessment of nerve regeneration. Gait analysis was performed on the basis of SFI scoring system [40], to assess the motor function recovery of rats (Fig. 6A and Videos S1–S3). At 18 weeks postoperatively, the functional recovery of regenerated nerves was significantly faster in ADSC-loaded rGO/PCL nerve conduits than that in ADSC-loaded PCL conduits, but still not as good as that in the autograft group (Fig. 6B).

Supplementary video related to this article can be found at doi:10.1016/j.bioactmat.2022.05.034.

3.5. Electrophysiological examination of regenerated nerves

The restoration of bioelectrical signal conduction was reflected by electrophysiological activities in the proximal and distal end of nerve grafts (Fig. 6D and E). Although the DCMAP value was similar between rGO/PCL and PCL group, the NCV value of rGO/PCL group was evidently higher than that of the PCL group, and even slightly better than that of the autograft group (Fig. 6C). We consider it was due to the electroactive property of rGO conduit, that it could conduct the bioelectricity of the nerve tissue [7].

3.6. Systematic evaluation of scaffold biosafety in vivo

Right after the functional evaluation, all the animals were executed for further histological analysis. We harvested the major functioning organs to evaluate the biosafety of rGO/PCL and PCL scaffolds in vivo. According to the HE staining results, no evident toxicity effect was shown in any of these organs (Fig. 6F). Our systematic evaluation results confirmed the biosafety and biocompatibility of scaffolds for long-term in vivo application. A relatively low degradation rate guaranteed the structural integrity of nerve conduits during the long-term in vivo support of nerve repair. As for the biodegradation of conduits used in this study, we did not observe full degradation at 18 weeks post-implantation, although both rGO/PCL and PCL conduits were much softer than they were at implantation.

Fig. 7. Morphological analysis of regenerated sciatic nerves from different scaffolds and autograft groups in vivo. (A) TB staining of regenerated sciatic nerves. Scale bar: 50 μm. (B) Quantitative analysis of the mean density of myelinated nerve fibers. (C) TEM images of regenerated nerves after 18 weeks post-operation. Zoomed-in images exhibited the myelin sheath and axons from different groups. Scale bars of the first, second and third line were 10 μm, 2 μm, and 1 μm respectively. (D–E) Quantitative analysis of the myelin sheath thickness and axon cross-sectional area. *p < 0.05, **p < 0.01, ***p < 0.001, ns: no significant difference.
3.7. Histological evaluation of axonal regeneration and myelination

TB staining of peripheral nerve sections remains gold standard for the morphometric evaluation of cross sections of peripheral nerve [41]. According to our TB staining results, most of the regenerated nerves were well organized without excessive scar or fat tissues (Fig. 7A). The density of myelinated axons was significantly higher in the autograft group, followed by rGO/PCL groups (Fig. 7B). The results of both groups were significantly better than those of PCL groups. TEM analysis further calculated the thickness of myelin sheath and the area of myelinated axons (Fig. 7C). The thickness of myelin sheath in the rGO/PCL and autograft group was significantly higher than that in the PCL group (Fig. 7D). However, the averaged axon area in rGO/PCL and PCL group was similar and was comparable to that of autograft group (Fig. 7E). Both TEM and TB observation indicated the favorable performance of ADSC-laden rGO/PCL scaffolds in nerve regeneration.

To measure the axonal regeneration and remyelination in different groups, we assessed the expression of neural specific proteins, S100β, NF200, MBP and Tuj1 (Fig. 8A and B). Tuj1 is an early neuronal marker to judge axonal recovery while MBP has profound implications in the formation of myelin sheath [42,43]. S100β is SC-specific marker expressed in both immature and mature SCs [44]. Neurofilament-200 (NF200) is axon-specific marker and is positively correlated with the content of axons [2]. In our results, rGO/PCL scaffolds increased the expression of MBP, NF200 and S100β, compared to the PCL counterparts (Fig. 8C–F).

3.8. Retrograde labeling of spinal motoneurons and sensory neurons

Retrograde neuroanatomical tracing is frequently used to trace axons from the axon terminal back to neuronal cell body [45,46]. In the recovery of peripheral nerve injuries, axons successfully regenerating across the nerve conduits could be identified when the accumulation of FG tracer was found in the corresponding spinal cord anterior horn and DRG (Fig. 9A). Herein, FG fluorescence was observed in the L4 spinal cord anterior horn and L4 DRG of the injured side in each group (Fig. 9B). The numbers of FG-labeled sensory and motor neurons were similar between autograft and rGO/PCL groups, but was significantly less in PCL groups (Fig. 9C). Apparently, more nerve fibers have grown into the distal stump of injured sciatic nerves in autograft and rGO/PCL group.

3.9. The response of corresponding spinal cord to sciatic nerve repair

Nestin, a type VI intermediate filament protein highly expressed in neural progenitor cells (NPCs), is considered a biomarker of neural development, but its expression does not persist into adulthood and is mainly restricted to the ependymal cells in the adult spinal cord [47]. GFAP, a marker of reactive astroglia and astrocytes, reflects the astrocyte reactivity in the CNS insults and is widely used for the investigation of CNS neurogenesis [48]. After 3 days of PNIs, nestin-positive NPCs and glial cells in spinal cords were re-activated and the expression of nestin aggregates [49,50]. Herein, the relative expression level of nestin and GFAP was not significantly different in spinal cords of rGO/PCL and PCL group, but was much higher in the autograft group (Fig. 9D). The dual activation of central and peripheral nerve regeneration was seen in all groups.
Fig. 9. FG retrograde tracing and spinal cord responses in each group. (A) Schematic illustration of FG tracer injection and accumulation in corresponding DRGs and the anterior horn of spinal cords. (B) Immunofluorescent tracer was observed in the motoneurons and DRG sensory neurons of different groups. The first and second column were immunofluorescent images of FG-labeled spinal cords. The third column was the corresponding optical photographs of spinal cord. The last column was immunofluorescent images of FG-labeled DRGs. Scale bars of spinal cords were 300 μm. Scale bars of DRGs were 150 μm. (C) Quantitative analysis of FG-labeled motoneurons and DRG sensory neurons in three groups. (D) Quantitative analysis of nestin and GFAP relative expression levels in three groups. (E) Immunofluorescence staining of nestin (green) and GFAP (red) neural markers of spinal cords in different groups. Scale bars were 500 μm (left) and 50 μm (right) respectively. *p < 0.05, **p < 0.01, ***p < 0.001, ns: no significant difference.
three groups (Fig. 9E).

3.10. Assessment of angiogenesis and function restoration in the regenerated sciatic nerves

The expression of CD31, a specific endothelial cell marker, is commonly applied to reflect the neovascularization in tissues [51]. Based on CD31 immunohistochemistry, both rGO/PCL group and autograft group showed rich neo-vessels with higher CD31 positive area compared with that of PCL group (Fig. 10A and B). Improved CD31 positive area in rGO/PCL group indicated better angiogenic potential of rGO/PCL conduits. The enhanced neovascularization could provide sufficient nutrition supply to the structural and functional restoration of regenerated nerves.

Sciatic nerve controls the function and viability of gastrocnemius muscle and the denervation procedure can lead to muscular atrophy [52]. The morphological recovery of gastrocnemius muscle reflects the recovery of locomotor function as well as the reinnervation of axons. Therefore, we measured the muscle biopsy from the middle portion of gastrocnemius (Fig. 10C). According to our results, autograft group ranked the highest in the mean cross-sectional area of muscle fiber, followed by the rGO/PCL group and the PCL group (Fig. 10D), while the hyperplasia of collagen fibers was most frequently seen in the PCL group.

Fig. 10. The angiogenesis and muscle reinnervation performance of different groups and the PI3K-Akt signaling pathway investigation of rGO/PCL and PCL conduits in vivo. (A) CD31 immunochemistry staining revealed the neo-vessels in the regenerated nerves. Scale bar: 100 μm. (B) Quantitative analysis of CD31 expression area. (C) Masson trichrome staining of regenerated gastrocnemius muscle of rats. Scale bar: 200 μm. (D) Quantitative analysis of average Pm in each group. (E) Akt and p-Akt staining of regenerated nerves from rGO/PCL and PCL conduits. Scale bar: 200 μm (left) and 50 μm (right). (F–G) Quantitative analysis of p-Akt and Akt expression levels in rGO/PCL and PCL groups. *p < 0.05, **p < 0.01, ***p < 0.001.
3.11. The functional role of PI3K-Akt signaling pathway in the stimulation of nerve regeneration by rGO/PCL conduit

After confirming that rGO/PCL conduits improved the axonal regeneration and remyelination in vivo, we compared the expression level of Akt and p-Akt within the regenerated nerves of rGO/PCL and PCL conduits (Fig. 10E). The average intensity of immunofluorescence was used to measure the expression level of Akt and p-Akt. The p-Akt expression was enhanced in the rGO/PCL conduit, at 1.82 folds higher than those in the PCL groups which further verified the significance of PI3K-Akt signaling pathway enabled by rGO/PCL conduit.

4. Discussion

Recently, the combined use of topological, biophysical and biological cues has received more and more attention in the peripheral nerve tissue engineering [53]. Stem cells, as extensively self-renewing cells, are ideal candidates designed to seed a scaffold [54]. Combinatorial use of various extrinsic cues is an effective approach to optimize stem cell fate specification [55]. Biochemical cues of graded concentration can induce discrete changes in the stem cell fate and neural differentiation commitment via direct manipulation of intracellular signaling [56]. However, current breakthrough of chemical biomodification in tissue engineering also presented prominent limitations. First, the in vivo biosafety level of these chemical factors is largely uncontrollable, and their inappropriate exposure can lead to abnormal karyotype [57]. Secondly, the curative effects of biochemical stimuli are usually unsustainable due to their rapid degradation and unpredictable release pattern [58]. A relatively overlooked factor for orchestrating cell function is biophysical modification. Cells sense the inherent physical properties of materials, including stiffness, electroactive property and structure, and translate these extracellular signals into various cellular behaviors.

In this study, we utilized substrate anisotropy to provide an instructive microenvironment for seeded ADSCs. Controlling the lineage switching of stem cells by modulating substrate anisotropy has emerged as an effective approach in stem cell-based researches. In a previous study, Hu et al. designed a 3D-printed dual-anisotropic substrate with the scale hierarchy to maximize substrate anisotropy [59]. The 3D-printed stripes comprised the first scale while the magnetic nanoparticles assemblies, which matched the single ADSCs, comprised the second scale. Such dual anisotropy collaboratively enhanced the adhesion and osteogenic differentiation of ADSCs [59]. Kim et al. reported that anisotropic line patterns enhanced the osteogenic differentiation of ADSCs by modulating cell elongation, whereas, grid patterns stimulated the neuronal differentiation of ADSCs by mimicking the interconnected neuronal network [60]. Park et al. designed an anisotropic patterned substrate with topologically defined regions in which spatially aligned stem cells were spontaneously differentiated into smooth muscle cells [61]. Cell surface receptors, such as integrins, have been shown to sense topological signals from cell niche, leading to the formation of FAs [62]. Moreover, combinatorial hybrid-pattern arrays with various geometries and sizes, such as grooves, pillars, pores, dots and fibers in the micro/nanometer scale, have also been made to induce selective differentiation of ADSCs into specific cell lineages [63]. Huang et al. added nanogrooves, as the secondary structure, to the surface of aligned nanofibers [64]. The removal of PVP, which was immiscible with PCL, could result in the formation of nanogrooves on the PCL fiber surface [65]. These multi-scale topographies are intentionally controlled to have a substantial influence on stem cell phenotype.

To closely mimic the properties of native nerve tissues, bioelectrical signals are integrated in the fabrication of nerve scaffolds to accelerate nerve growth rate. Cumulative studies have affirmed the benefits of conductive substrates as compelling materials to restore the conduction of bioelectricity across nervous system [66,67]. Local voltage potential generated by piezoelectric materials could trigger the opening of ion channels on electroactive cells (e.g., neurons) and induce the intracellular calcium transients [68]. High-performance bioelectronic devices usually rely on flexible conductive materials with excellent micro-/nanostructures to further improve their sensitivity and responsivity [69, 70]. Flexible conductive microfibers fabricated by a co-axial micro-fluidic spinning and injection method can guarantee the encapsulation of conductive microfibers which shield the conductive components from oxidation or damage [71]. Guo et al. reported the conductive hydrogel microfibers with in situ encapsulation of MXene enabled the outstanding capabilities of sensitive responses to improve motion and photothermal stimulation for electronic skins [72]. Yu et al. fabricated ultra-elastic conductive microfiber with polyurethane shell and liquid metal core which could act as dynamic force sensor and motion indicator for wearable electronics due to the tunable morphologies and responsive conductivities [73].

As a typical conductive material, rGO nanoparticle can transmit free electrons in the nerve environment and create certain electrical currents through the rGO/PCL composites, which may accelerate the bioelectric evolvement of stem cell membranes [74]. Besides, graphene-based materials constitute a preconcentration platform which enables biochemical factors to stack on its unique non-covalent π-π bonds [75]. Moreover, the presence of oxygen functionalities disrupts the extensive sp² conjugation and the π-π binding ability and facilitate hydrogen bonding which leads to stronger cell attachment [76].

Following peripheral nerve transaction injuries, a nerve bridge composed of connective tissue is first constructed to rejoin the two stumps, followed by the formation of bands of Büngner in which dedifferentiated SCs are aligned into longitudinal cellular cords [77]. This SC-loaded fibrotic bridge connects the two nerve stumps and guides the regrowth of axons to their target tissue [78]. Inspired by the architecture of bands of Büngner, we seeded ADSCs onto the aligned microfibers. The experimental design and protocol were depicted and recapitulated in Fig. 11. Microfibers mimicked the structure of micro-sized components (e.g., basal laminae) while nanogrooves mimicked nano-sized extracellular matrix composition in the nerve tissue. Graphene-based materials could upregulate the endogenous expression of adhesion-related protein in cells (N-cadherin, vinculin, and integrin) which subsequently enhanced the section of growth factors [78,79]. According to our SEM images, ADSCs were well extended and polarized on rGO/PCL microfibers compared with those on PCL microfibers. Therefore, rGO supplements encouraged stem cells to sense and adhere to the material, which potentiated their neurotrophic phenotype. Consistent to this conclusion, the expression levels of GDNF and NGF were both up-regulated in ADSCs of rGO/PCL group, indicating an enhanced acquisition of neurotrophic phenotype.

The intracellular signaling in ADSCs was also different between rGO/PCL and PCL scaffolds. In our mRNA sequencing analysis, rGO supplement propagated an upregulation of PI3K-Akt signaling which accounted for the increased expression of neurotrophins and myelin protein. The enhancement of FA signaling was also observed in rGO/PCL group. Such concomitant upregulation indicated the possibility that the rGO supplements and anisotropic topology exerted synergistic effects on the cell biology. PI3K signaling is initiated by the assembly of cellular receptor-PI3K complexes, followed by the activation of Akt which regulates multiple cellular activities and other signaling pathways as well [80]. Previous research has demonstrated that PI3K-Akt signaling can be activated in response to PNIs and is required for axonal regeneration [81]. FA signaling pathway is responsive to the anisotropic topology of electrospun fibers and FA formation is known to convert topological signals into biochemical signals to determine the terminal of cell fate [82,83]. Such co-expression network of PI3K-Akt and FA pathways was also reported in a previous study where PI3K-Akt and FA signaling pathways were linked under the same co-expression cluster using pathway enrichment map [84]. We then evaluated the effect of PI3K-Akt signaling on the neurotrophic phenotype of ADSCs. ADSCs on rGO/PCL conduits were treated with LY294002 which blocked the
activation of Akt signaling and decreased the expression levels of NGF and MBP. We further performed in vivo assessment between rGO/PCL and PCL groups to verify the role of PI3K-Akt signaling pathway in sciatic nerve regeneration. The expression of both Akt and pAkt in the regenerated nerves exhibited higher level in rGO/PCL group than that in the PCL group which indicated higher PI3K signaling activities in the regenerated nerves as a response to rGO/PCL scaffolds.

During the regeneration process of injured nerves, SCs ensheathe axons and secrete myelin basic protein to construct myelin sheath around axons. Myelin sheath maintains the salutatory conduction of peripheral nerves and increases the conduction velocity by limiting the action potential propagation to the nodes of Ranvier [85]. Our electrophysiological assessment of NCV and DCMAP indicated better nerve conduction recovery by rGO/PCL conduits compared to PCL group. As obtained by TB staining, TEM and immunofluorescence of neural markers, rGO/PCL conduits showed more reparative capacity than PCL conduits.

Sciatic nerves contain efferent motoneuron axons and afferent sensory neurons axons which originate from L4-L6 spinal cord and adjacent DRGs respectively. DRG neurons have bifurcated axons with the central branches entering the CNS and the peripheral branches innervating DRGs respectively. DRG neurons have bifurcated axons with the central markers, rGO/PCL conduits showed more reparative capacity than PCL obtained by TB staining, TEM and immunofluorescence of neural pathological scar formation in CNS, the reorganization and modification of spinal cord levels [86]. Although the activation of glial cells can lead to pathological scar formation in CNS, the reorganization and modification of CNS in response to PNI may result into better adaptation, a positive sign of regeneration [87]. However, in the present study, the relative expression levels of these two markers were not significantly increased in rGO/PCL compared with PCL group. We consider it was due to the complexity and peculiarity of CNS remodeling which varied among the different injury models and experimental design.

In the existing literature, rGO-based nerve conduits have been widely explored as one of the most exciting platforms for neural tissue engineering. Vijayavenkataraman et al. revealed in vitro that 3D-printed rGO/PCL scaffolds promoted the proliferation and neural differentiation of PC12 cells [88]. Fang et al. also confirmed that rGO/GelMA/PCL facilitated the regenerative phenotype of rat Schwann cell line RSC96 and promoted the functional recovery of rats [89]. Sánchez-González et al. revealed that rGO/PCL improved the neural differentiation and maturation of neural stem cells [90]. Hoon et al. reported the enhanced neurogenesis of dental pulp stem cells by rGO/PCL nanofibers [91]. Despite all these studies have used rGO/PCL composites as an excellent pro-neurogenic cell-material interface, they failed to translate the in vitro results to those observed in vivo.

Several studies explored the stem cell fate after in vivo administration simply by seeding GFP labeled-stem cells into conduits and localizing the fluorescence signals of GFP and specific neural markers in the immunostaining of nerve tissue [92-94]. Due to the incomprehensive understanding of the interrelationships between stem cells and nerve conduits, the current design of cell-seeded scaffolds could hardly match the requirement of clinical translation. Our study successfully overcame difficulties of exploring the fate of cells after in vivo transplantation. Moreover, as far as we know, this is the first time that transplanted stem cells have been retrieved successfully to study their phenotypic reprogramming in biomaterial-based peripheral nerve repair.
5. Conclusions

In summary, our rGO/PCL anisotropic scaffolds induced neural differentiation of ADSCs and promoted efficient nerve restoration by mimicking the electroactive nature and oriented topography of peripheral nerves. Further exploration on the mechanism of topological cues and rGO supplements reprogramming ADSCs has revealed the involvement of PI3K-Akt and FA signaling, which unraveled the complex of this stem cell/scaffold interface.

Autologous fat tissue is easy to harvest by liposuction or open surgeries and ADSCs have been applied in clinical practices for years. Although the in vivo use of rGO/PCL scaffold in human body has not been clinically proven, its therapeutic effect has been confirmed in rat models of PNIs. As a preclinical assessment of ADSC-laden rGO/PCL scaffolds, this study centered on the advancement of their clinical translatability. Together, our ADSC-seeded rGO/PCL scaffold appears to have great biocompatibility, effectiveness and translational promise for clinical use as a novel therapeutic method.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2022.05.034.

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