Aligned PCL Fiber Conduits Immobilized with Nerve Growth Factor Gradients Enhance and Direct Sciatic Nerve Regeneration

Lei Zhu, Shuaijun Jia, Tuanjiang Liu, Liang Yan, Dageng Huang, Zhiyuan Wang, Shi Chen, Zhengping Zhang, Wen Zeng, Ying Zhang, Hao Yang, and Dingjun Hao*

Topographical guidance and chemotaxis are crucial factors for peripheral nerve regeneration. This study describes the preparation of highly aligned poly(ε-caprolactone) (PCL) fiber conduits coated with a concentration gradient of nerve growth factor (NGF) (A/G-PCL) using a newly designed electrospinning receiving device. The A/G-PCL conduits are confirmed in vitro to enhance and attract the neurite longitudinal growth of dorsal root ganglion (DRG) neurons toward their high-concentration gradient side. In vivo, the A/G-PCL conduits are observed to direct a longitudinal stronger attraction of axons and migration of Schwann cells in 15 mm rat sciatic nerve defects. At 12 weeks, rats transplanted with A/G-PCL conduits show satisfactory morphological and functional improvements in g-ratio, total number, and area of myelinated nerve fibers as well as the sciatic function index, compound muscle action potentials, and muscle wet weight ratio as compared to aligned PCL fibers conduits with uniform NGF (A/U-PCL). The performance of A/G-PCL is similar to that of autografts. Moreover, mRNA-seq and RT-PCR results reveal that Rap1, MAPK, and cell adhesion molecules signaling pathways are closely associated with axon chemotactic response and attraction. Altogether, by combining structural guidance with axon chemotaxis, the NGF-gradient/aligned PCL fiber conduits represent a promising approach for peripheral nerve defect repair.

1. Introduction

Peripheral nerve injuries, which affect about 3% of trauma patients are common orthopedic disorders. There are over 90,000 new cases of peripheral nerve injuries each year in the world. Peripheral nerve injuries are catastrophic injuries of the nervous system, resulting in loss of sensory and motor function in the region of the damaged nerve and even in lifelong disability. Autologous nerve transplantation has long been considered the gold standard for the clinical repair of peripheral nerve defects more than 10 mm long. However, autologous nerve grafting involves the denervation of the donor nerve. Moreover, poor matching in size between the donor and the injured nerve and limited sources of donor nerves have limited its use in clinical practice. Many advances in tissue engineering technology and the development of new biomaterials stand for a promising alternative to autologous nerve grafting. Many topographical, chemical, and biological cues have been integrated into nerve conduit fabrication. The use of scaffolds with aligned fibers or their combination with various growth factors have demonstrated a positive effect in peripheral nerve regeneration. However, to date, the functional gap that exists between the regenerative nerve and the normal nerve remains a challenge. Nerve growth rate is an important factor in determining the quality of nerve regeneration. Therefore, during peripheral nerve regeneration, a biomimetic structure combined with biochemistry guidance cues may better promote neurite alignment, growth rate, and functional recovery than individual cues.

Many methods have been used to fabricate bioactive materials, such as electrospinning, lyophilization, gelation, and 3D printing. However, electrospinning offers several advantages, including simple operation, controllable scaffold structure, and fiber diameter, and applicability to a variety of materials. Thus, electrospinning is a promising method for producing nerve conduits with suitable mechanical properties and microstructure. In the past, a narrow rod was often used as a receiver to prepare nerve conduits with random fibers by electrospinning. Previous studies have described the fabrication of aligned fibers membranes with different materials, which were then rolled into tubes for peripheral nerve regeneration.
regeneration.\textsuperscript{[8]} However, this rolling-up approach requires the use of bio-glue to glue the membrane edges. Often, the mechanical properties, shape, and structure of the conduit are also not controllable. In order to fabricate a nerve conduit with an aligned fiber structure and controllable mechanical properties, in this study we modified the electrospinning receiver through a negative high voltage power source to force the positively charged fibers into an aligned arrangement on a rotating stainless steel rod. This new electrospinning method of one-piece molding offers an easier and more versatile approach to fabricate nerve conduits with longitudinally aligned fibers for a range of practical applications.

A variety of natural and synthetic materials have been used to fabricate an aligned structure for nerve regeneration both in vitro and in vivo. Highly aligned poly(L-lactic acid) (PLLA) fibers can guide the directional growth of neuritis and Schwann cells.\textsuperscript{[9]} Aligned poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV)/collagen fibers were also fabricated and used in vitro, resulting in the appearance of bipolar extensions in PC12 and Schwann cells along the alignment structure direction. In contrast, PC12 and Schwann cells grew multi-polar extensions when using randomly-organized PHBV/collagen nanofibers.\textsuperscript{[10]} Poly-ε-caprolactone) (PCL) has also been widely used in regenerative medicine owing to its suitable mechanical strength, biodegradability, toughness, and cytocompatibility. Aligned laminin-PCL blend nanofibers showed the potential capability to promote the outgrowth and elongating of neurites along the major fiber axis, and resulting in better tibial nerve functional recovery in rat.\textsuperscript{[11]} Over the past decade, the role of the alignment structure in directing nerve regeneration has been confirmed by many studies.\textsuperscript{[12]} Application of the alignment structure cue alone to guide nerve regeneration may achieve a certain degree of functional regeneration. However, combining alignment structures and other guiding cues may achieve more satisfying effects in nerve functional regeneration.

Chemotaxis, a phenomenon in which cell movement is directed by a gradient of chemoattractant, is an essential component of immune responses, wound healing, and formation of blood vessels in the nervous system.\textsuperscript{[13]} Functional peripheral nerve regeneration requires Schwann cell and axon directional extension. Gradients of biological molecules are known as an important guiding cue for directing regenerating nerve fibers.\textsuperscript{[14]} Thus, the growth cones of sensory neurons direct themselves toward nerve growth factor (NGF) and orient axon extension along the NGF gradient. The trkA receptor has also been shown to be involved in the neural chemotaxis phenomenon.\textsuperscript{[15]} There is accumulating empirical evidence that through their growth cones, axons sense gradients of signaling molecules as guidance cues.\textsuperscript{[16]} The underlying signaling mechanisms largely depend on the concentration gradients. Hydrogel and microfluidic chambers are often used to construct a concentration gradient of growth factors in 3D to promote axon directional extending. Hydrogels have been extensively used in a variety of tissue repair applications.\textsuperscript{[17]} However, it is difficult for axonal growth cones to maintain an aligned arrangement in hydrogels. Although microfluidic chambers could maintain the alignment of axonal growth cones, the existence of bubbles and pressure differences may injure cells, with the added concern of microbial contamination is also a great concern.\textsuperscript{[18]}

Therefore, in this study, we combined the fabrication of aligned PCL fibers with growth factors gradients to produce NGF-gradient aligned fibers nerve conduits for peripheral nerve regeneration. We propose a straightforward method to construct highly aligned fiber conduits by placing two parallel charged metal plates with a negative high voltage electric field in front of the rotating stainless steel rod and by manipulating three electrospinning parameters, namely the syringe pump flow rate, the negative voltage level, and the rotation speed of the steel rod. In order to coat the aligned fibers with and NGF gradient, we superimposed the two gradient aspects of reaction time and reaction solution concentration. To this end, we used a micro-injection pump to gradually add water to the ammonia reaction solution. As the liquid level rose, the concentration in ammonia gradually decreased. The ammonia concentration and time of exposure to ammonia were thus higher at the lower end of the PCL conduits, while the concentration and time of exposure gradually decreased toward their upper end. This superposed method could produce a steeper gradient than that using a single factor approach. The NGF-gradient aligned fiber nerve conduits were evaluated by in vitro and in vivo studies for their ability to support neurite directional extending and sciatric nerve regeneration. mRNA-seq was also performed to explore the molecular mechanisms underlying axon attraction and chemotactic axon growth induced by the NGF gradient and aligned fibers.

2. Results

2.1. Characterization of NGF-Gradient/Aligned PCL Fiber Conduits

The NGF-gradient/aligned PCL fiber conduits were fabricated through electrospinning by placing two parallel charged metal plates with a negative high voltage electric field in front of the rotating stainless steel rod (Figure 1A), and using ethylenediamine to react with PCL to produce an axially steeper gradient distribution of NH\textsubscript{2} groups (Figure 1B) and coating NGF by covalently bound heparin (Figure 1C). Following fabrication, SEM images confirmed that fibers were randomly distributed in random PCL fibers conduits (R-PCL) (Figure 2A–C) and aligned PCL fibers conduits (A-PCL) (Figure 2D–F). The hole diameter and the average wall thickness of R-PCL nerve conduits were 1.5 mm and 570 µm, respectively, versus 1.5 mm and 575 µm for A-PCL conduits. The thicknesses of aligned and random regions in A-PCL conduit was about 87 and 488 µm, respectively. In addition, the fibers in both R-PCL and A-PCL nerve conduits ranged from 3.1 to 4.6 µm. The average thickness of the inner layer of the conduit wall (aligned fibers layer) was 82 µm, while the outer layer (random fibers layer) thickness was 493 µm. The distribution frequencies of fiber directions in two types of conduits are shown in Figure 2G,H. Defining the longitudinal axis of the conduits as the reference direction, the fibers of R-PCL conduits were randomly distributed between...
-90° and 90°, while the 97% of fibers in A-PCL conduits were distributed between −15° and 15°. This indicated that the PCL conduits fabricated with our novel method showed a high degree of orientation. Mechanical testing results (Figure 2I) indicate no differences among R-PCL, random PCL fibers conduit with gradient NGF (R/G-PCL), A-PCL, and aligned PCL fibers conduit with gradient NGF (A/G-PCL) in Young’s modulus, maximum load, maximum stress, and strain at break properties (p > 0.05). These results indicated that the mechanical properties of the aligned fiber structure conduits were similar to those of the random structure conduits. The fabrication process did not require the aligned fiber conduits to be rolled and glued, as they were integrally formed and the mechanical properties were advantageous for regeneration. Moreover, the average water contact angle of A/G-PCL was 41.7 ± 2.9°, which was lower than that of the other types of conduits (p < 0.05) (Figure 2J). Finally, R/G-PCL and A/G-PCL had a lower water contact angle than that of the others conduits (p < 0.05). This suggested that protein coating greatly increased hydrophilicity.

2.2. Content, Distribution, and Release Pattern of NGF in PCL Conduit

SEM images of the five sequential conduit segments showed that there were different degrees of chemical reactions on the surface of the PCL fibers, with a gradient distribution from top to bottom (Figure 3A,B). This indirectly reflected the gradient distribution of NH₂ groups on the surface of PCL fibers. The total amounts of coated NGF in random PCL fibers conduits with uniform NGF (R/U-PCL), R/G-PCL, aligned PCL fibers conduits with uniform NGF (A/U-PCL), and A/G-PCL conduits were similar, approximating 3 ng (Figure 3C). The five segments of R/U-PCL and A/U-PCL conduits showed similar amounts of coated NGF of approximately 0.6 ng. However, the NGF amount in the five segments of R/G-PCL and A/G-PCL conduits gradually increased. The 1.2–1.5 cm segment contained 1.17 ng of NGF while the 0–0.3 cm segment contained 0.13 ng of NGF. The five sections of the A/G-PCL conduits showed an increasing trend of NGF cumulative releasing amount in vitro in different time periods (Figure 3D).

2.3. DRG Neurite Outgrowth and Orientation

The axons of DRGs grew randomly in multiple directions on disordered PCL fibers (Figure 4A–C), while axons grew longitudinally along the fiber direction on ordered PCL fibers (Figure 4D–F). In addition, DRG neurites preferentially grew toward the highest concentration in growth factor on NGF-gradient conduits, indicating a chemotactic phenomenon. The axons of DRGs grew significantly longer on the
Figure 2. Characterization of PCL conduits. Scanning electron microscopy images: A,D) cross sections of R-PCL and A-PCL conduits. B,E) Inner surface of R-PCL and A-PCL conduits. C,F) Fiber morphology at high magnification of R-PCL and A-PCL conduits. Distribution frequencies of fiber direction in G) R-PCL conduits and H) A-PCL conduits. I) Mechanical properties of different conduits (n = 4 per group; mean ± SD; p = 0.241, 0.588, 0.787, 0.899 for Young’s modulus, maximum load, maximum stress, and strain at break, respectively). J) Average water contact angle of conduits (n = 4 per group; mean ± SD; p < 0.001). * p < 0.01. Scale bar = 500 µm in panels (A,D), 200 µm in panels (B,E), and 50 µm in panels (C,F).
high-concentration side of the gradient-NGF conduits than on their low-concentration side (axon length 2824 µm versus −1120 µm) (Figure 4G,H). Axons on gradient-NGF conduits grew longer than axons in the R/U-PCL group (p < 0.05). DRG neurite length in the R/U-PCL group was similar in both sides of the conduits (2317 µm versus −2374 µm). Furthermore, DRG neurite length was significantly higher in the NGF-coated groups compared to that in the uncoated groups (p < 0.05).

These results indicated that the fibers’ topography played an essential role in axon regeneration guidance, and that the gradient distribution of the growth factor prompted a significant chemotactic growth of axons.

2.4. A/G-PCL Conduit-Directed Nerve Regeneration Involves a Series of Signaling Pathways Associated with Axon Attraction

As the volcano maps shown that there were 277 downregulated and 330 upregulated differentially expressed genes (DEGs) between the A/G-PCL and A/U-PCL groups (Figure 5A). The heat map of DEGs among A-PCL, A/U-PCL, and A/G-PCL is presented in Figure 5B. The gene ontology analysis showed that up-regulated and down-regulated DEGs in A/G-PCL group enriched in GO biological processes terms mainly related to neuron projection morphogenesis and neurotransmitter secretion, leukocyte migration, and regulation of cytokine production, respectively (Figure 5C). The up-regulated DEGs between A/G-PCL and A/U-PCL groups were enriched in 13 pathways and down-regulated DEGs in A/G-PCL group were enriched in 6 pathways by KEGG analysis (Figure 5D). These pathways are involved in neural regeneration, chemotactic response, and axon attraction. Using gene network analysis of KEGG-enriched pathways by Cytoscape’s ClueGo application (Figures S1 and S2, Supporting Information), the mRNA levels of key genes closely related to neural regeneration, chemotactic response, and axon attraction were verified by RT-qPCR (primers in Table S1, Supporting Information). The expression of these genes was significantly higher in the A/G-PCL group than that in the
A/U-PCL group in vivo (Figure 5E). As a possible underlying mechanism of chemotaxis guidance by NGF gradient (Figure S3, Supporting Information), distal axons may sense growth factor gradients and activate local receptors, promoting axon growth and axon attraction through Adcy1, Prckz, Rap1/P13K/AKT, and MAPK, etc. A variety of adhesion molecules could promote growth cone guidance, cytoskeleton regulation, myelination, and synaptogenesis. This comprehensive molecular network may be recruited during axon chemotactic growth through strong local excitation mechanism.

### 2.5. Morphological Evaluation of Regenerated Nerves

The detailed structure of myelinated nerve fibers is presented in Figure 6. The axons and myelin sheaths in the autograft and A/G-PCL groups were normally and evenly distributed, and the Schwann cells around the myelin sheath had a better morphology (Figure 6A–D). In the A-PCL group, there were significant differences in myelin sheath size, with more fibroblasts infiltration and presence of unmyelinated and hypomyelinated axons (Figure 6B). However, hypermyelinated axons were found...
Figure 5. Transcriptome profiles of axon attraction driven by A/G-PCL conduits. A) Volcano map of DEGs of DRG in A/G-PCL versus A/U-PCL. Red dots indicate significantly upregulated DEGs; blue dots indicate significantly downregulated DEGs; and gray dots indicate no significance. B) Heatmap of DEGs of DRG among A-PCL, A/U-PCL, and A/G-PCL. C) GO terms. D) KEGG pathway enrichment. E) mRNA expressions results of the DRG tissue in bridged side at 4 weeks in four groups using RT-PCR analysis (n = 3 per group; mean ± SD; all p < 0.001 among groups). * p < 0.05 for the comparison with autograft group. # p < 0.05 for the comparison with A-PCL group. ▲ p < 0.05 for the comparison with A/U-PCL group.
Figure 6. Morphological appearance of regenerated nerves in all groups after surgery. Transmission electron micrographs of regenerated axons and myelin sheath in the middle portion at 12 weeks in the A,E) autograft group. Normal-sized myelin sheaths (red asterisk), B,F) A-PCL group. Bundle of unsorted axons (red circles), Schwann cell (yellow), fibroblasts (blue), hypomyelination (purple arrowheads), C,G) A/U-PCL group. Hypermyelination (black arrowheads) and hypomyelination (purple arrowheads). Schwann cell (yellow), unmyelinated axon (green), D,H) A/G-PCL group. Normal-sized myelin sheaths (red asterisk), Schwann cell (yellow). I–K) Scatter plot showing g-ratio and axon diameter for all groups at three time points (n = 6 per group; p < 0.001 in G-ratio or axon diameter). L) Quantification of the cross-sectional area of regenerated nerve (n = 6 per group; mean ± SD; p < 0.001 in three time points among four groups). M) Number of myelinated axons in the middle portion of the conduits (n = 6 per group; mean ± SD; p < 0.001 in three time points among four groups). * p < 0.05 for the comparison with the autograft group. # p < 0.05 for the comparison with the A-PCL group. ▲ p < 0.05 for the comparison with the A/U-PCL group. Scale bar = 5 µm in panels (A–D). Scale bar = 1 µm in panels (E–H).
in the A/U-PCL group, where unmyelinated and hypomyelinated axons also appeared (Figure 6G). The ultrastructure of the myelin sheath in the autograft and A/G-PCL groups could be clearly seen at high magnification (Figure 6E–H). Furthermore, at 8 and 12 weeks, g-ratio and axon diameter in the nerve autograft (g-ratio: 8 w 0.7 ± 0.1, 12 w 0.6 ± 0.1; axon diameter: 8w 6.7 ± 1.4 µm, 12 w 9.9 ± 1.7 µm) and A/G-PCL groups (g-ratio: 8 w 0.7 ± 0.1, 12 w 0.6 ± 0.1; axon diameter: 8 w 6.3 ± 1.3 µm, 12 w 9.6 ± 1.7 µm) were significantly superior to those in the other groups (Figure 6I–K). As time progressed, the g-ratio analysis scatter plots distribution of the autograft and A/G-PCL groups revealed gradually widening differences with the increase of the axon diameter at 12 weeks. The fitting curve tended to be parallel. In addition, the total area of regenerated axons and number of myelinated axons in the A/G-PCL group were significantly superior to those in the A-PCL and A/U-PCL groups (p < 0.05), but they were similar to autograft group at 8 and 12 weeks (p > 0.05) (Figure 6L–M). These results indicate that A/G-PCL conduits can greatly promote axon regeneration, and that the axon morphology and related parameters mimic autologous nerve transplantation.

2.6. Immunofluorescence Stained Regenerated Nerves and Retrogradely-Traced Neurons

The regenerated Schwann cells (S100 marker, red color) and axons (NF200 marker, green color) arranged orderly in the autograft group and the A/G-PCL group in fluorescence images (Figure 7A–L). The fluorescence distribution of Schwann cells and axons in the A-PCL group was sparse and disorderly (Figure 7D–F). The distribution of green and red fluorescence in the A/U-PCL group was significantly greater than that in the A-PCL group, but its structure was disordered, with locally abnormally proliferative Schwann cells and axons (Figure 7G–I). The orderly structure of regenerated Schwann cells and axons was also found in high concentration side of A/G-PCL group (Figure S4, Supporting Information).

To determine the origin of regenerated sensory and motor axons, the fluoro-gold (FG) was used to retrograde lumbar DRGs (L3-L6), and the ventral horn of the spinal cord. FG-positive neurons in DRGs and anterior horn of the spinal cord are shown in Figure 7M–T. Our quantitative analysis results show that the number of sensory and motor neurons in the four conduit groups increased with time (Figure 7U,V). The number of sensory neurons and motor neurons in the autograft and A/G-PCL groups were statistically different at 4 weeks after surgery (Sensory: autograft 442.2 ± 30.6 vs A/G-PCL 367.6 ± 28.9; Motor: autograft 340.6 ± 32.7 vs A/G-PCL 310.5 ± 25.9; p < 0.05). There was no significant difference in sensory and motor neuron numbers between the autograft (Sensory: 8 w 615.6 ± 62.5, 12 w 704.5 ± 35.8; Motor: 8 w 519.6 ± 54.2, 12 w 634.9 ± 69.4) and A/G-PCL groups (Sensory: 8 w 588.1 ± 49.1, 12 w 692.7 ± 59.0; Motor: 8 w 490.3 ± 49.7, 12 w 603.9 ± 479) at 8, 12 weeks in sensory and motor neurons numbers (p > 0.05). At 12 weeks after surgery, the number of sensory neurons in the A/G-PCL group was 17.4% higher than that in the A/U-PCL group and 32.6% higher than that in the A-PCL group (p < 0.05). In addition, the number of motor neurons in the A/G-PCL group was 21.7% higher than that in the A/U-PCL group and 34.6% higher than that in the A-PCL group at 12 weeks (p < 0.05). These results suggest that the gradient distribution of NGF can significantly promote axon regeneration.

2.7. A/G-PCL Conduit Promotes Neurologic Functional Recovery

The sciatic nerve separates into the tibial nerve, which innervates the gastrocnemius muscle. The function and structure of the gastrocnemius muscle can reflect the degree of sciatic nerve regeneration. Masson’s trichrome staining of the gastrocnemius muscle showed that muscle atrophy in the A-PCL group was the most severe, followed by that in the A/U-PCL group. Muscle atrophy in the autograft and A/G-PCL groups was not significant (Figure 8A–D). Muscle wet weight ratio was proportional to the average muscle fiber diameter in all groups (Figure 8E). The muscle wet weight ratio in the A/G-PCL group was 74.9 ± 4.8%, which was not significantly different from that in the autograft group (78.3 ± 4.3%) (p > 0.05). But the muscle wet weight ratio in the A/PCL group was 42.9% and 208.2% higher than that in the A/U-PCL group and A/G-PCL group, respectively (p < 0.05). The average muscle fiber area in the A/G-PCL group (86.6 ± 4.4%) and autograft group (93.2 ± 1.7%) was significantly higher than those in the A/U-PCL and A/PCL groups (p < 0.05). The degree of muscle atrophy was related to the duration of denervation. In addition, mast cells hardly infiltrated and proliferated in the muscle adjacent to nerve autograft and conduits. The inflammatory factor levels of IL-6, TNF-α, and HMGB1 were not increased in conduit groups compared to autograft group (p > 0.05) (Figure S6, Supporting Information). These results indicate that A/G-PCL can increase the speed and quality of nerve regeneration and reduce denervation time of target muscles, and do not cause surrounding tissue inflammation.

Gait analysis can assess lower extremity motor function in rats. At three time points, the SFI in the autograft and A/G-PCL groups was significantly higher than that in the other groups (p < 0.05, Figure 8F). In contrast no significant differences existed between the autograft and A/G-PCL groups at 12 weeks (p > 0.05). In addition, the SFI in the A/U-PCL group was consistently higher than that in the A-PCL group (p > 0.05). These data suggest that NGF can promote the recovery of motor function, but the effect of gradient distribution is more visible. Electrophysiological results are objective indicators of muscle function. The electrophysiological indices in the A/G-PCL conduits were significantly superior to those in the A-PCL and A/U-PCL conduits at three time points (p < 0.05) (Figure 8G–I). In addition, the A/U-PCL conduits achieved significantly higher CMAP, faster motor nerve conduction velocity (MCV), and shorter latency of CMAP than did the A-PCL conduit (p < 0.05). Furthermore, CMAP, MCV, and latency of CMAP in the A/G-PCL group were similar to those in the autograft group at 8 and 12 weeks (p > 0.05).

3. Discussion

Surgical treatment of peripheral nerve defects still faces many obstacles. Although autologous nerve transplantation has
Figure 7. Immunohistochemical staining and fluorogold retrograde tracing. Representative images of regenerated nerves in the middle portion of the conduits in the A–C) autograft group, D–F) A-PCL group, G–I) A/U-PCL group, and J–L) A/G-PCL group at 12 weeks after surgery. Neurofilaments were labeled with NF-200, green color. Schwann cells were labeled with S-100, red color. Representative images of FG-positive sensory neurons and motoneurons in M,Q) autograft group, N,R) A-PCL group, O,S) A/U-PCL group, and P,T) A/G-PCL group at 12 weeks after surgery. The number of U) FG-positive sensory neurons and V) motoneurons in four groups (n = 6 per group; mean ± SD; all p < 0.01 among four groups in three time points). * p < 0.05 for the comparison with the autograft group. # p < 0.05 for the comparison with the A-PCL group. ▲ p < 0.05 for the comparison with the A/U-PCL group. Scale bar = 100 µm in panels (A–T).
always been considered the gold standard for the treatment of peripheral nerve defects, the limited source of graft, the permanent loss of donor nerve function, and the mismatch between the size of the donor and the defected nerve greatly limit its application in the clinical setting.[19] In the past few decades, a variety of nerve conduits have been developed and tested in experiments for nerve defect repair.[20] Despite intense research efforts, translatable methods for functional and clinical peripheral nerve regeneration remain scarce. The biomimetic structure and function of nerve conduits are key to achieving satisfactory bridging results. Structural guidance and chemotaxis are two crucial factors for peripheral nerve regeneration.[21]

Herein, we developed a novel technology to fabricate aligned PCL fiber conduits and immobilize NGF in a concentration gradient pattern. We then explored its effect on the guidance and enhancement of neuronal outgrowth, and analyzed the potential molecular mechanism network associated with axon growth and attraction.

First, we described a novel NGF-gradient/aligned PCL fiber conduit developed using an integrated molding pattern. The in vitro results showed that the A/G-PCL conduits enhanced and attracted DRG neurite longitudinal growth toward the high-concentration gradient side of the conduits. The biological characteristics were also evaluated in vivo using a 1.5 cm long segment sciatic nerve defect model in rats. The A/G-PCL implants were observed to longitudinally direct a stronger attraction of axons and migration of Schwann cells. At 12 weeks, rats transplanted with A/G-PCL conduits presented a significant recovery in SFI, electrophysiological parameters, muscle weight, and histological performance as compared to A/U-PCL conduits. In addition, the A/G-PCL conduits directed nerve regeneration through a series of signaling pathways associated with axon growth and attraction.

Figure 8. Masson trichrome staining of gastrocnemius muscle, sciatic functional index (SFI), and electrophysiological assessment. Representative images of gastrocnemius muscle for A) autograft group, B) A-PCL group, C) A/U-PCL group, and D) A/G-PCL group at 12 weeks postoperatively. E) Scatter plot showing muscle wet weight ratio and muscle fiber area for all groups at three time points. Asterisks represent the mean of each group ($p < 0.001$ for muscle wet weight ratio or fiber area, $n = 6$). F) Sciatic functional index ($p < 0.001$ among four groups at 4, 8, and 12 weeks after surgery). G) The peak amplitude of CMAP. H) Latency of CMAP onset. I) Conduction velocity. All data were expressed as the mean ± SD, $n = 6$ per group (F–I). $p < 0.01$ among four groups at 4, 8, and 12 weeks after surgery (G–I). * $p < 0.05$ for the comparison with the autograft group. # $p < 0.05$ for the comparison with the A-PCL group. ▲ $p < 0.05$ for the comparison with the A/U-PCL group. Scale bar = 50 µm in panels (A–D).
attraction, involving Rap1, MAPK, and cell adhesion molecules pathways. Thus, these results strongly indicate that the A/G-PCL conduits are functional and represent a potentially powerful strategy for clinical translational applications in peripheral nerve regeneration.

Cell arrangement is different in each tissue, and the tissue structure of the peripheral nerve is mainly longitudinally arranged.\[22\] Several studies suggested that the morphology of the material exerts a strong guiding effect on the cell growth direction and shape.\[23\] It is widely acknowledged that aligned fibers play an unidirectional guidance role in axon growth. To date, aligned filament structure and multiple parallel channels of nerve conduits have been constructed mainly using electrospinning and phase separation methods.\[24\] Aligned electrospun fibers have usually been prepared using a high rotation speed cylindrical collector, which straightened random fiber so they would form an aligned membrane. But this aligned fiber membrane needs to be cut and curled, and glued into a tube. Because this is not one-piece technology, the continuity and mechanical properties of the conduit are also poor. In view of this, we innovatively used a parallel high-voltage electric field to create the aligned electrospun fibers, which could then be fabricated into a conduit in one-piece. The mechanical properties of the conduit met the requirements of in vivo transplantation. Thus, our method can conveniently be used to produce aligned PCL electrospun fiber conduits and prepare nanofiber scaffold with higher-order structure suitable for the regeneration of various tissues. Moreover, it can be applied to all electrospun materials.

Chemotaxis is an important physiological phenomenon in organisms.\[25\] Chemotaxis is also an essential function for guiding axon regeneration. Growth cones, the specialized sensory-motor structures at the extending axonal tip, can probe axon guidance cues such as growth factor gradients and other stimulators to extend or retract constantly. As reported in a previous study, conduits harnessing gradient-controlled cell-secreted neurotrophic factors promote peripheral nerve regeneration.\[26\] In tissue engineering for nerve repair, the concentration gradients of YIGSR peptide direct Schwann cell migration.\[27\] There are several ways to achieve a gradient distribution of growth factors in conduits. The PCL/F127 cylindrical scaffolds with gradually increasing growth factor concentrations were fabricated by a centrifugation method.\[28\] In a previous study, scaffolds with gradients of the immobilized nerve growth factor PCLA were constructed through the growth factor adsorption time difference.\[29\] In this study, we innovatively developed an experimental set-up with a reaction liquid showing a gradually increasing concentration. The reaction time and concentration gradient produced a NH₂ group gradient on the conduit materials. Our results confirmed the gradient distribution of growth factors in the conduits. Our method is more convenient and efficient than current methods and can be used to bind growth factors and other guidance cues gradients covalently to conduit materials. Many physiologically entrapping growth factor strategies have often encountered problems due to burst release. On the other side, covalent binding may affect the biological activity of growth factors.\[30\] Therefore, we harnessed the high binding affinity of the thiol group to growth factors. The in vitro sustained release results showed that this strategy did not result in burst release. The release curve satisfied the requirements of in vivo regeneration. The multiple exogenous neurotrophic factors are required for in vitro culturing of adult rat DRG neurons owing to limited survival activity compared to DRG in neonatal or embryonic rats. These medium supplements may affect the chemotactic growth performance of axons in vitro experiment. Therefore, the DRG tissues from neonatal rats were used in this study. In the present study, NGF concentration gradients have notable axon chemotactic effect in both disordered and aligned PCL fibers. It is worth noting that axons extended longitudinally along the fiber direction, and the axon length in the high concentration site was significantly longer than that on the low-concentration side. These results demonstrate that our two innovative methods can be applied to construct biomimetic neural conduits for achieving axons longitudinal and chemotactic growth in vitro.

To assess the effect of NGF-gradient/aligned PCL fiber conduits to promote nerve regeneration in vivo, A/G-PCL conduits were transplanted to bridge a 15 mm long defect in rat sciatic nerve. Consequently, morphometric analysis showed that axonal regeneration and remyelination in the autograft and A/G-PCL groups were superior to those in the A-PCL and A/U-PCL groups. In addition, morphological parameters were similar between the autograft and A/G-PCL groups, demonstrating the advantages of structural guidance and chemotaxis in enhancing axonal regeneration. The longitudinal morphology of the regenerated nerve was showed by immunofluorescence staining. The results revealed that axons and Schwann cells arranged orderly in the autograft and A/G-PCL groups. Furthermore, the numbers of motor and sensory neurons in A/G-PCL groups were significantly higher than those in the A-PCL and A/U-PCL groups, and approximated the numbers found in the autograft group. These results indicate that the growth factor gradient can reduce neurons apoptosis and necrosis, and improve the regeneration rate and quality of axons. The degree of muscle atrophy is related to the denervation time.\[30\] The muscle wet weight and fiber area percentage in the A/G-PCL group were significantly higher than those in the A/U-PCL group. Therefore, it can be speculated that the gastrocnemius denervation time was shorter in the A/G-PCL group, and that the growth factor gradient increases the speed of axon regeneration. Furthermore, the A/G-PCL group achieved better motor functional recovery than the A-PCL and A/U-PCL groups, which was evidenced by higher SFI and good electrophysiological results. The conduction velocity can objectively reflect the electrical conduction velocity of regenerated nerve and the degree of myelination. The peak amplitude of CMAP can reflect the electrical activity state of the target muscle innervated by the regenerative nerve, and the degree of muscle atrophy to a certain extent. In fact, the degree of functional rehabilitation was very close between the autograft and A/G-PCL groups.

The mRNA-seq was performed to investigate the mechanisms underlying the nerve regeneration and axon attraction by the growth factor gradient. mRNA-seq and RT-PCR analysis confirmed that 19 KEGG pathways and 19 GO terms were involved in the growth factor gradient promoting neural regeneration, chemotactic response, and axon attraction. In this comprehensive molecular network, we analyzed and summarized three important signal pathways, involving Rap1, MAPK,
and cell adhesion molecules. Neuronal growth cone turning is a complex process in which actin-based motility is harnessed to produce persistent and directed microtubule advance.[31] A variety of adhesion molecules that were enriched in our study may be involved in growth cone guidance, synaptogenesis, myelination, and axon growth. More studies are needed to address the role of these screened and enriched differential genes in nerve regeneration and chemotactic axon growth.

Many efforts have focused on fabricating the best functional and biomimetic nerve conduits. Advances in materials science and conduits fabricating methods make the creation of physically biomimetic structures possible. The scientific integration of biomimetic structures with key chemical directional cues will achieve clinically translatable peripheral nerve regeneration.

4. Conclusion

In this study, we developed a NGF-gradient/aligned PCL fiber conduits to bridge a 15 mm gap in the rat sciatic nerve defect model. These A/G-PCL conduits directed the longitudinal and chemotactic growth of axons in vitro. Furthermore, they directed nerve regeneration through 19 enriched signaling pathways associated with axon attraction. The A/G-PCL conduits also significantly promoted axonal regeneration and functional recovery, which were similar to those of the autograft and superior to that of the conduits coated with uniform NGF. Altogether, these results suggest that NGF-gradient/aligned PCL fiber conduits have great potential as an alternative to autologous nerve graft in repairing peripheral nerve defects.

5. Experimental Section

Fabrication of NGF-Gradient/Aligned PCL Fiber Conduits: The procedure is outlined in Figure 1. In a first stage, A-PCL conduits were produced (Figure 1A). In brief, a PCL electrospinning solution at the concentration of 15% w/v was prepared by dissolving PCL pellets (Mn = 80,000, Sigma-Aldrich, USA) in a mixture of chloroform and methanol (5:1 v/v), and flowed at the rate of 4 mL h⁻¹ by a programmable syringe pump (Cole Parmer, USA). The positive high voltage power supply connected to the syringe needle was set to 16 kV. However, the electrospinning fibers were non-aligned, and the conventional receiver could not prepare nerve conduits with an ordered fiber structure. Therefore, we modified the receiver, which was placed 18 cm in front of the syringe needle, by placing two separate stainless steel saw blades 5 mm in front of the rotating stainless steel rod and applying negative high voltage power. This prompted the positively-charged disordered PCL fibers spouted from the needle to straighten between the two negatively-charged metal plates. The aligned fibers then continued to adhere to the uncharged rotating stainless steel rod under the influence of motion inertia (Figure 1A). The outer layer of the conduit was composed of random fibers to obtain appropriate mechanical properties. The R-PCL conduits were fabricated by turning the negative high voltage supply off.

In a second stage, the conduits were coated with a concentration gradient or uniform distribution of NGF by using ethylenediamine to react with PCL to produce NH₃ groups (Figure 1B–C). In brief, the axial surface of the conduits was placed in ethylenediamine solution (0.2 m). Distilled water was gradually added into the ethylenediamine solution with continuous gentle stirring. Through the continuous decrease of concentration of the ethylenediamine solution and the linear change of reaction time, the density of the NH₃ groups was made to show an axially steeper gradient distribution (Figure 1B). To obtain uniform distribution of the NH₃ groups, conduits were only saturated into 0.2 m ethylenediamine solution for 15 min. Then, the conduits were immersed in the mixed solution of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (0.2 m), N-hydroxysuccinimide (NHS) (0.1 m), and heparin sodium salt (0.5% w/v) in 2-(N-morpholino)ethanesulfonic acid (MES, Sigma) buffer (0.05 m, pH 5.6) for 4 h and vortexed briefly at room temperature to ensure that the covalent combination of the carboxyl groups of heparin with the gradient NH₃ groups of the PCL surface to form a gradient heparin distribution. Finally, gradient/uniform heparinized conduits were left to react with NGF (13.5 kDa, Peprotech, USA) at a concentration of 500 ng mL⁻¹ in phosphate buffered saline (PBS) at 4 °C for 6 h (Figure 1C). Thus, R/U-PCL, A/U-PCL, R/G-PCL, and A/G-PCL were obtained. A-PCL and R-PCL conduits were reacted with ethylenediamine and bound with heparin.

Characterization of Fibrous PCL Conduits: Static water contact angle was measured by the sessile drop method. The microstructure of PCL conduits and the surface of PCL fibers treated with ethylenediamine solution were observed by scanning electron microscope (SEM; S-3400N; HITACHI, Japan) at an accelerating voltage of 5 kV. The mechanical properties of the conduits were measured on a tensile-testing machine (MTS600, Schenck AG, Germany). The 1.5 cm length of the conduits was clamped with a 1.5 cm inter-clamp distance and pulled longitudinally at a rate of 1 mm s⁻¹ until rupture. The slope of the stress-strain curve in elastic region represented the Young’s modulus. The maximum stress and strain at rupture were measured.

NGF Gradient Detection in PCL Conduits: The 1.5 cm length of PCL conduits coated with NGF was sectioned into 3 mm long segments, with five of the segments being labeled from proximal to distal and weighed. The concentration of the NGF coating was determined by dissolving each segment in 1 mL 1:1 PBS/dichloromethane solution. Thus, while PCL can be dissolved in dichloromethane and transfer to the organic phase, NGF moves to the aqueous phase. The mixture was vortexed by centrifugation at 6000 rpm min⁻¹ for 3 min. Then, the supernatant containing NGF was collected for analyzing the coating amounts of NGF by ELISA kit.

Culture of DRG Explants on Various Fiber Conduits: DRG explants were cultured on the inner layer of aligned and random PCL fiber conduits coated with or without a concentration gradient of NGF following the procedures described previously.[32] Briefly, DRG explants from neonatal Sprague Dawley (SD) rats were cultured on R-PCL, R/U-PCL, R/G-PCL, A-PCL, A/U-PCL, and A/G-PCL fiber conduits in serum-free DMEM/F12 medium for 72 h before immunofluorescence staining. After immunostaining, the axonal length of DRG explants was measured by ImageJ software. Neurite outgrowth was evaluated based on the average length of the fifteen longest axons.

Transcriptome Sequencing (mRNA-seq) and Analysis: Total RNA from DRGs after 72 h of culture on various fiber conduits was extracted and then analyzed by PersonalBio Biotech (Shanghai, China). Libraries were constructed using a VAHTSTM mRNA-seq V3 Library Prep Kit (Vazyme Inc., USA) according to the manufacturer’s instructions. In brief, the mRNA separation/fragmentation reaction was performed and then 200–300 bp fragments were inserted using the hold fragmentation program at 4 °C. The first strand of cDNA was synthesized by using random primers and reverse transcriptase, and a specific library was established when the second strand was synthesized. The library was amplified and the total DNA was sequenced to isolate 320–420 bp strands. High-throughput sequencing was performed as single-end 75 sequencing using NextSeq 500 (Illumina, USA). Quantitative mapping was performed using FANSe2 algorithm and DESeq software was used to screen out the different genes according to the thresholds of ≥2-fold change in expression and a p-value < 0.05. Finally, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and gene ontology enrichment analyses were performed for the DEGs using the DAVID database.

Surgical Procedures and Groups: All animal procedures received ethical approval from the Animal Experimental Committee of Honghui Hospital affiliated to Xi’an Jiaotong University and were conducted in accordance with the animal care guidelines of Xi’an Jiaotong University. Healthy
adult male SD rats (n = 144, provided by the Laboratory Animal Center of Xi’an Jiaotong University) weighing 230–250 g, were randomly divided into four groups: i) the autograft group (n = 36); ii) the A-PCL group (n = 36); iii) the A/UPCL group (n = 36); and iv) the A/G-PCL group (n = 36). All rats were anesthetized by intraperitoneal injection of 1% sodium pentobarbital solution (40 mg kg⁻¹). The left sciatic nerve was carefully exposed and a segment of sciatic nerve was removed, leaving a 15 mm long defect after retraction of the nerve stumps. In the three conduit groups, the sciatic nerve gap was bridged with different conduits sutured by perineural 9/0 nylon sutures. In the autograft group, a 15 mm transected nerve segment was re-implanted under the microscope. Finally, wound were sutured in layers. The rats was evaluated at 4, 8, and 12 weeks post-operatively.

**Fluorogold Retrograde Tracing:** Retrograde labeling was performed at 7 days before three time points after surgery. 2 µL (2 wt%) FG (Fluorochrome, USA) was injected at a distance of 5 mm away from the distal nerve stumps/conduits. At each time point, the animals were anesthetized, perfused with saline and then 4 wt% paraformaldehyde, and the lumbar spinal cord and left DRGs (L3-6) were harvested. Tissues were fixed in 4% paraformaldehyde for 24 h and dehydrated in 30% sucrose for 24 h at 4 °C. Then, serial cross-sections of the L3-6 spinal cord and DRGs were obtained by a cryostat in transverse mode (thickness: 14 µm) or longitudinal mode (thickness: 14 µm), respectively. Since motor neurons were located in the ventral horn of the lumbar spinal cord and sensory neurons in DRGs, motor and sensory neurons could be completely separated before mounting after tissue collection. The numbers of FG-labeled sensory neurons and motoneurons were counted separately in every alternate section in the corresponding samples.

**Walking Track Analysis:** Functional muscle reinnervation was examined by walking track analysis at 4, 8, and 12 weeks after surgery. The parameters of the paw prints included the print length on both sides (EPL, NPL), toe spread (distance from toe 1 to toe 5) on both sides (EIT, NIT). The SFI was calculated as follows: SFI = (ETS – NIT) / NIT. SFI values approaching 100 represented better recovery, while SFI values close to 0 indicated total impairment.

**Electrophysiological Assessment:** Electrophysiological examinations were performed at three time points as previously described.[32] Electrical stimuli were applied to the proximal and distal ends of the nerve graft, and the CMAP was recorded on the belly of the gastrocnemius muscle. The peak amplitude of CMAP, latency, and the CMAP was recorded on the belly of the gastrocnemius muscle. The peak amplitude of CMAP, latency, and the CMAP was recorded on the belly of the gastrocnemius muscle. The peak amplitude of CMAP, latency, and the CMAP was recorded on the belly of the gastrocnemius muscle.

**Morphometric Evaluation of Axonal Regeneration:** All nerve conduits and grafts were retrieved at three time points after surgery and fixed in a solution of 4 wt% glutaraldehyde for 3 days. Transverse 50.0 nm thick ultrathin sections were obtained from the middle portion of the regenerated nerves. The ultrathin sections were stained with 5% uranyl acetate and 0.3% lead citrate. The sections were then examined by a transmission electron microscope (H-600; HITACHI) and the morphometric analysis of nerve regeneration was carried out by the image analysis software ImageJ (NIH, USA). Four parameters were quantified, namely the total axonal area, total number of myelinated axons, diameter of myelinated axons, and g-ratio of myelinated fibers. The g-ratio was calculated as the ratio of the inner axonal diameter to the total axonal diameter.

**Immunohistochemical and Histological Evaluations:** Regenerated sciatic nerves were harvested at 4, 8, and 12 weeks post-surgery. The biceps femoris muscles adjacent to conduits and nerve autograft were also collected at 12 weeks. The regenerated nerves and muscles were fixed in 4% paraformaldehyde for 72 h, and 14 µm thick serial longitudinal sections were obtained on a cryostat. For immunofluorescence staining, the sections were incubated with S-100 (1:100; Abcam, UK), NF-200 (1:100; Abcam, UK), and tryptase (1:100; Abcam, UK) primary antibodies at 4 °C for 12 h, and then with the secondary antibodies of fluorescin isothiocyanate (FITC)-labeled goat anti-rabbit IgG (1:200; Abcam, UK) and Cy3-labeled goat anti-mouse IgG (1:200; Abcam, UK), respectively. Cell nuclei were stained with DAPI (1:200; Sigma, USA). Then sections were rinsed and observed under a fluorescence microscope (DP70; Olympus, Japan). For histological analysis, both sides of gastrocnemius muscles were harvested at 12 weeks post-surgery. The wet weight of gastrocnemius muscles was weighed and recorded as a ratio (left/right). Samples were cut from the mid-belly of the gastrocnemius muscle and fixed in 4% parafomaldehyde before embedding in paraffin wax. The 10 µm transverse sections were cut and stained with hematoxylin and eosin. The five cross-sectional fields of muscle fibers were taken randomly and analyzed using the ImageJ. The percentage of muscle fiber area was defined based on the ratio of muscle fiber area to the whole area of the field.

**Real-Time Quantitative Polymerase Chain Reaction:** Total RNA was extracted from the L3-6 DRGs tissues in the injured site of rats at 4 weeks post-surgery with Total RNA Mini Kit (QIAGEN, Germany) according to the manufacturer’s instructions. cDNA was synthesized from the isolated RNA and qPCR was performed using SYBR Green PCR Master Mix (TAKARA BIO INC, China) with a Biorad CFX96 PCR system (Biorad, Australia). Primers were designed as described in Table S1, Supporting Information. The β-actin gene was used as control. The relative gene expression was analyzed with the 2^{-ΔΔCt} method.

**Statistical Analysis:** All values were expressed as means ± standard deviation. The data were analyzed using one-way analysis of variance with the SPSS 19.0 software package (SPSS Inc., Chicago, IL) followed by least significant difference for pairwise comparisons. A p-value less than 0.05 was considered to be statistically significant.

**Supporting Information**
Supporting Information is available from the Wiley Online Library or from the author.

**Acknowledgements**
L.Z. and S.J.J. contributed equally to this work. This research was supported by the grant from the Key Program of the National Natural Science Foundation of China (No. 81830077), and grant from the National Natural Science Foundation of China (No. 81772357).

**Conflict of Interest**
The authors declare no conflict of interest.

**Keywords**
aligned nanofibers, electrospinning, growth factor gradient, nerve guidance conduit, peripheral nerve regeneration

Received: March 22, 2020
Revised: June 4, 2020
Published online: August 13, 2020

[1] S. E. Stabenfeldt, A. J. Garcia, M. C. LaPlaca, J. Biomed. Mater. Res., Part A 2006, 77, 718.
[2] K. D. Bergmeister, M. Aman, S. Muceli, I. Vujaklija, K. Manzano-S zalai, E. Unger, R. A. Byrne, C. Scheinecker, O. Riedl, S. Salminger, F. Frommlet, G. H. Borschel, D. Farina, O. C. Aszmann, Sci. Adv. 2019, 5, eaau2956.
