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Laser microdissection-based axotomy model incorporating the use of biomimicking fiber scaffolds reveals microRNAs promote axon regeneration over long injury distance

Na Zhang¹, Junquan Lin¹, Jiah Shin Chin^{1,2}, Kunyu Zhang¹, Sing Yian Chew^{1,3*}

¹ *School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore 637459;*

² *NTU Institute of Health Technology, Interdisciplinary Graduate School, Nanyang Technological University, Singapore 639798;*

³ *Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore 308232;*

*Corresponding author:

Tel.: +65 6316 8812; Fax: +65 6794 7553; E-mail: sychew@ntu.edu.sg

Abstract

The regeneration of injured neurons over long injury distances remains suboptimal. In order to successfully stimulate nerve regrowth, potent biomolecules are necessary. Furthermore, reproducible and translatable methods to test the potency of candidate drugs in enhancing nerve regeneration over long axotomy distances are also needed. To address these issues, we report a novel laser microdissection-based axotomy model that involves the use of biomimicking aligned fiber substrates to precisely control neuronal axotomy distances. Correspondingly, we demonstrate that in the absence of therapeutics, dorsal root ganglion (DRG) explants (consisting of neurons) axotomized within short distances from the main cell somas regenerated significantly longer than axons that were injured more distally ($p < 0.05$). However, when treated with a cocktail of microRNAs (miR-132/miR-222/miR-431), robust neurite outgrowth was observed ($p < 0.05$). Specifically, microRNA treatment promoted neurite outgrowth by ~2.2-fold as compared to untreated cells and this enhancement was more significant under the less conducive regeneration condition of a long axotomy distance (i.e. 1000 μm from the cell soma). Besides that, we demonstrated that the treatment of miR-132/miR-222/miR-431 led to longer length of nerve regeneration as well as a bigger nerve extension area after sciatic nerve transection injury. These results indicate that distance effects on axonal regrowth may be overcome by the effects of microRNAs and that these microRNAs may serve as promising therapeutics for nerve injury treatment.

Key words: Nerve tissue engineering; sciatic nerve injury; electrospinning; RNA interference; nerve regeneration; axon local protein synthesis

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1. Introduction

The ability of an injured neuron to initiate its regeneration is under the

governance of different factors. While the post-injury environment exerts a highly inhibitory influence on axonal regrowth^{1–3}, the intrinsic response of the neuron itself (e.g. injury signals and transcription factors activated by the injury signaling⁴) is another key factor^{5,6} that determines nerve regeneration outcomes. Besides that, one factor that also influences the cellular reaction to axotomy is the distance of the lesion from the neuronal cell body⁷. Studies have demonstrated that a neuron mounts the strongest regenerative response if the site of axotomy is close to the cell body^{5,6,8}. On the contrary, axons injured farther away from the cell soma have diminished regenerative capacity. As such, finding a way to overcome the distance effects on axon regeneration may be crucial for treating traumatic nerve injuries. To do so, it is essential to develop a reproducible and translatable method to test the potency of candidate drugs in enhancing nerve regeneration over long axotomy distance *in vitro*.

Here, a laser microdissection-based axotomy model coupled with an aligned electrospun fiber platform was developed, with which the distance of injury site from the cell soma can be precisely controlled. The advantages of laser microdissection is its ease of operation and high reproducibility^{9,10}. On the other hand, electrospun fiber scaffolds can direct cellular response^{11,12}, ranging from proliferation to differentiation^{13,14} and maturation¹⁵. In the case of nerve regeneration, neurons extend their neurites along aligned electrospun fibers and these neurite outgrowths extend significantly longer when they were seeded on aligned fibers than on two-dimensional films or tissue culture plates^{16–18}. As such, this laser microdissection-based axotomy model which involves the use of biomimicking aligned fiber substrates

can combine the advantages from both laser microdissection and aligned electrospun fibers. More importantly, the design of aligned electrospun fibers can be easily extended to an implantable nerve guidance conduit for peripheral nerve regeneration applications.

Axon regeneration through enhancing the intrinsic growth ability of neurons provides a promising strategy for nerve repair after injuries¹⁹. MicroRNAs play significant roles during this process by regulating local protein synthesis at growth cones. Specifically, miR-21^{20,21}, miR-222²¹, miR-132^{22–25} and miR-431²⁶ are enriched in axons^{27,23,28}. When these miRs were overexpressed in injured adult neurons, enhanced axonal regeneration occurred from the growth cones^{20,21,26}.

In our previous work, extensive miR screenings consisting of miR-21, miR-132, miR-222 and miR-431 were carried out using primary intact neurons to evaluate their collective effects of these miRs on neurite outgrowth¹⁶. Building on that work¹⁶, we want to further evaluate how these miR cocktails perform on axotomized neurons. Additionally, we also sought to investigate if these miRs are able to overcome the reduced regenerative capacity of axons when they are injured farther away from the cell body. To do so, miR-222/miR-431 and miR-132/miR-222/miR-431 were selected to test their potentials on axotomized neurons as they demonstrated the longest neurite extensions when tested in primary intact neurons¹⁶. Besides that, we also picked up two miRs (i.e. miR-222, miR-431) which demonstrated the worst effects on

neurite outgrowth in intact neurons¹⁶ to verify whether they have similar sub-optimal effects on the regeneration of neurites from axotomized neurons.

Specifically, dorsal root ganglion (DRG) explants were seeded on the aligned fiber platform and cultured for one week. Thereafter, the DRGs were axotomized at two different distances away from the bulk of cell somas (i.e. 400 μm and 1000 μm). These distances chosen are in good agreement with the work done by S. W. You *et al.*, where axotomy was performed at 500 μm and 1000 μm away from the optic disc²⁹ respectively in order to compare the regeneration capabilities. Another aligned fiber scaffold, pre-coated with various combinations of miRs was then placed underneath to collect the axotomized DRGs (Figure 1). Thereafter, the axotomized DRGs were cultured for another 1 week before their regrowth of neurites were assessed. We hypothesize that the miR cocktails could promote significant axonal regrowth and overcome the effects of axotomy distance. The administration of miRs could demonstrate its potential in promoting nerve regeneration after traumatic nerve injuries.

2. Materials and Methods

Materials

Polycaprolactone (PCL, molecular weight 45 kDa and 80 kDa), 2,2,2-trifluoroethanol (TFE, $\geq 99.0\%$), (Polyethylene Glycol (PEG) Mn 8000), poly-D-Lysine (PDL) (P0899), Dopamine hydrochloride (Dopamine, H8502) were

purchased from Sigma-Aldrich, USA. Alexa-Fluor 488 goat anti-Rabbit, Alexa-Fluor 555 goat anti-Mouse, miR-132-3p (PM10166; mature miR sequence: UAACAGUCUACAGCCAUGGUCG), miR-222-3p (PM11376; mature miR sequence: AGCUACAUCUGGCUACUGGGU), and miR-431-5p (PM10091; mature miR sequence: UGUCUUGCAGGCCGUCAUGCA), DAPI (4',6-diamidino-2-phenylindole), paraformaldehyde (PFA, 7230681), 10x phosphate buffered saline (PBS; pH=7.4), goat serum, laminin (23017015), neurobasal medium, B-27 supplement, Glutamax supplement, Fluoromount-G™ (00-4958-02), Rabbit anti-GAP43 (AB5220) and Lipofectamine RNAiMAX (13778150) were obtained from Life Technologies, USA. TransIT-TKO (TKO) were purchased from MirusBio. Mouse anti- β III Tubulin (Tuj1) (801202) and Chicken anti-NF200 (822601) were purchased from Biolegend. Nerve growth factor (NGF) was purchased from PeproTech. Fetal Bovine Serum (FBS) was acquired from Research Instruments. Polyethylene naphthalate (PEN) membrane were obtained from Leica.

***In vitro* Scaffold fabrication**

Fabrication of suspended aligned fiber scaffold (collected on PCL frame) for laser microdissection

Figure 1A illustrates the fabrication process of the suspended aligned fiber platform, which was used for laser microdissection. Briefly, 70 mg of PCL pellets (molecular weight 45 kDa) were placed in between two pieces of metal sheets (15 cm x 25 cm) and pressed into a 50 μ m thin PCL film using a heat press machine (Carver,

Model 4128) at 80 °C and 0.2 GPa. The PCL film was then cut into thin frames with a customized cutter (Outer area: 11.5 cm x 1.5 cm, Inner area: 9.5 cm x 1 cm) before being fixed onto the rotating collector for collecting the electrospun fibers. Subsequently, another piece of thick PCL frame structure (20 mm x 15 mm x 0.5 mm thickness), formed by pressing melted PCL (molecular weight 45 kDa, ~25mg) manually on a hotplate, was glued onto it to provide structural integrity.

For electrospinning, PCL (molecular weight 80 kDa) was dissolved in TFE to obtain a 14 wt% solution. The homogenous PCL solution was then loaded into a syringe connected with a 21-gauge needle and dispensed at a fixed rate of 1.0 mL/h using a syringe pump (New Era pump Systems Inc., USA). Positive 4 kV (Gamma High Voltage, USA) and negative 4 kV were then applied to the tip of the syringe and the rotating collector, respectively. The syringe and the collector were separated at 11 cm apart. The electrospun fibers were then collected on the thin frame (i.e. 50 μ m in thickness) which was fixed on a rotating collector (speed: 1200 rpm) during the electrospinning process. Thereafter, the 2 cm PCL frame (i.e. 0.5 mm in thickness) was glued onto the collected fibers and dried at room temperature for overnight for further usage.

Fabrication the aligned fibers (collected on glass coverslip)

Figure 1B illustrates the fabrication process of an aligned fiber platform using a glass coverslip. Briefly, 50 mg of PCL (molecular weight 45 kDa) was melted at 60 °C

before being cooled down to room temperature. The resulting block of PCL polymer was then cut into pieces of 0.5 cm × 1.0 cm × 2.0 cm before they were sectioned into 20 μm thick films using a cryostat (Lecia CM1950). These 20 μm thick PCL sheets were then washed 3 times in distilled water at room temperature for immediate use.

The PCL sheets were dried at 37 °C for 2 h prior to sterilization with 70% ethanol. Thereafter, the sterilized PCL sheets were placed on glass coverslips (diameter: 18 mm). Once placed in position, the coverslips were heated at 50 °C to generate a 10 mm × 10 mm cell seeding area before six of these coverslips were placed on the rotating wheel for the collection of electrospun fibers.

For electrospinning, the homogenous solution (80 kDa PCL, 14wt%) was loaded into a syringe connected with a 21-gauge needle and dispensed at a fixed rate of 1.0 mL/h using a syringe pump. Positive 4 kV and negative 4 kV were then applied to the polymer solution and the rotating collector, respectively. The syringe and the collector (speed: 2400 rpm) were separated at 11 cm apart. Electrospun fibers were then collected on the coverslips. Finally, the scaffold was glued using silica glue so as to prevent the fibers from detaching.

The morphology of the aligned fiber substrates was evaluated by scanning electron microscopy (SEM) (JEOL, JSM-6390LA, Japan) under an accelerating voltage of 10 kV after sputter-coating with platinum for 100 s at 10 mA. The average fiber

diameters and alignment of the fibers were then quantified by measuring 100 fibers from high magnification images (2500x) using Image J software (NIH, USA).

Figure 1. Schematic of the experiments (A) Electrospinning procedure to obtain suspended fibers which were used for laser microdissection. A 50 μm thin PCL frame was used to generate a suspended fiber scaffold that is 2 cm in length and 1.5 cm in width. (B) Electrospinning procedure of fibers on glass coverslip. Coverslips fitting the wells of 12-well plates were attached onto the rotating collector to collect the electrospun fibers. (C) Leica laser dissection microscopy used for axotomy. (D) Specimen holder where the PEN membrane was placed. Suspended fiber scaffold with DRGs seeded on was mounted on the PEN membrane. A glass scaffold which was precoated with miR/miR cocktails was placed underneath the specimen holder. (E) Laser axotomy was performed. (F) Dropping of the axotomized DRG. (G) Axotomized DRGs attached onto the aligned fiber scaffold which was coated with miRs.

Preparation of *in vitro* miR-loaded PCL scaffolds

Scaffolds were placed in a 12-well plate and subjected to UV for 30 min before

being submerged into 70% ethanol for sterilization. Thereafter, the fibers were immersed into 0.5 mg/mL Dopamine solution, which was dissolved in Dopamine buffer (i.e. Tris, pH = 8) before being placed on an orbital shaker (120 rpm) for 2 h and 4 h, respectively. The amount of poly (dopamine) used for fiber coating was referred from our previous works where the coating procedure was optimized for neural cell cultures^{15,30}. Poly(dopamine)-coated fibers were then washed with deionized water for three times. Thereafter, the scaffolds were coated with 1 mL of PDL (100 µg/mL) for 2 h at 37 °C to facilitate the attachment of DRG neurons³¹. Any unbound PDL was then rinsed off with distilled water and the scaffolds were then coated with 1 mL of laminin (10 µg/mL) for another 2 h at 37 °C in order to promote the neuronal growth^{32,33}.

Following that, 1.5 µL of Lipofectamine RNAiMAX was diluted in DMEM before complexation with 1 µg of miRs. Lipofectamine RNAiMAX was selected to deliver miRs as it has been utilized in our previous studies for neural cell transfection when we used surface mediated delivery (i.e. poly(dopamine) or poly(dopa) coatings)^{30,34}. Importantly, Lipofectamine RNAiMAX demonstrated high transfection efficiency in delivering miRs into stem cells³⁵ and cell lines³⁶. Furthermore, Lipofectamine RNAiMAX was also used as the transfection reagent for delivering miR-21 into cortical neurons *in vitro*³⁷, resulting in a significant gene silencing of the downstream targets, PTEN and PDCD4³⁷. As such, Lipofectamine RNAiMAX was used here to deliver miRs to DRG neurons. Based on our previous work¹⁶, 1 µg of miRs was adopted here as this amount of miRs exhibited obvious gene silencing of their downstream targets when

delivered to neurons using our aligned fiber platform. The complexation process of Lipofectamine RNAiMAX and miRs was performed at room temperature for 15 min. After laminin coating, these complexes were added on the 10 mm × 10 mm cell seeding area directly to allow efficient adsorption onto the surface of the fiber scaffold at 37 °C for 2 h. Efficient adsorption can be obtained based on our previous work where siRNAs were absorbed on similar poly(dopamine)-coated PCL fibers^{14,30}. Specifically, the loading efficiency of siRNAs was 90-97%^{14,30}. All the aligned PCL fibers were then divided into 5 groups (i.e. Untreated, miR-222, miR-431, miR-222/miR-431, miR-132/miR-222/miR-431), as shown Table 1.

Table 1 MicroRNA cocktails and denotations

Groups		Denotation
Control	DRGs without any treatment	Untreated
Single miR	Lipofectamine with miR-222	MiR-222
	Lipofectamine with miR-431	MiR-431
2-miR cocktails	Lipofectamine with equal masses of miR-222 and miR-431	MiR-222/miR-431
3-miR cocktails	Lipofectamine with equal masses of miR-132, miR-222 and miR-431	MiR-132/miR-222/miR-431

Water contact angle characterization of *in vitro* fiber scaffolds

Contact angle measurement was performed to reveal the hydrophobicity of the aligned fiber scaffolds. Specifically, the contact angle of the uncoated and poly(dopamine)-coated aligned fibers (i.e. 2h and 4h of coating) were tested. Briefly, one drop of water was added onto the aligned fibers. The contact angle of the droplet with the aligned fiber was then measured using Contact Angle Analyzer (Kruss DSA 25). Thereafter, fibers were coated with PDL for 2h followed by laminin. The contact angle was measured after each coating step. Lastly, miRs were coated for 1 h and the

contact angle of the aligned fibers was subsequently measured again. Three scaffolds were tested per group.

Release profile of miRs from *in vitro* aligned fibers

To view the distribution of miRs on the aligned fibers, the scaffolds were coated with fluorophore tagged double stranded RNA (i.e. Cy5-RNA) which was used to mimic miRs due to their similarity in size. Thereafter, the Cy5-RNA coated scaffold were washed three times with 1 x PBS. The scaffolds were then mounted onto a glass slide and the distribution of Cy5-RNA was detected using a fluorescent microscope (Lecia, LMI81).

To calculate miR loading efficiency, scaffolds (n = 3) coated with miR complexes (1 µg each) were washed once with 1 x PBS. The amount of unbound miRs was then de-complexed by 4 µL of heparin (250 µg/mL) before being determined by RiboGreen Assay. This amount of heparin was optimized based on our previous works^{13,30,34}. The fluorescence intensity was then measured by a microplate reader (Tecan, Infinite 200). The loading efficiency of miRs was calculated based on the following equation:

$$\text{Loading efficiency (\%)} = \frac{(\text{Total mass of miRs loaded} - \text{Mass of unbounded miRs})}{\text{Total mass of miRs loaded}} \times 100\%$$

Thereafter, the miR-loaded aligned fiber scaffolds were completely submerged in 1 mL of 1 x PBS and incubated at 37 °C. At each time point, 1 mL of supernatant was

collected, and an equal volume of fresh PBS was added. The amount of miRs that were released at each time point were then determined using RiboGreen assay. Cumulative release profile was plotted as a percentage of the experimental mass of miRs loaded on the scaffolds.

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***In vitro* culture of DRG explants**

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Nanyang Technological University and approved by the Animal Ethics Committee, Institutional Animal Care and Use Committee (IACUC, Protocol number: A0309). Briefly, DRG explants were freshly isolated from P2-P3 rat pups and washed in ice cold DMEM/F12 medium. All the meninges from the DRGs were taken off before they were seeded on the precoated 2 cm suspended PCL aligned fiber platform. The attachment of the DRGs was ensured by slowly adding in the cell culture medium without disturbing the explants. The cultures were maintained at 37 °C and 5% CO₂ for 1 week in the Neurobasal medium containing 10% FBS, 2% B27, 1% Glutamax, 1% penicillin/streptomycin and 50 ng/mL of NGF. Medium was changed every 3 days.

***In vitro* DRG axotomy using laser microdissection**

After 7 days of culture, the construct along with the DRGs, were mounted onto the polyethylene naphtholate (PEN) membrane and fitted onto the laser dissection microscope (Leica, LMD7), as shown in Figure 1C and 1D. The power of the laser was

set as follows: Power 41; Aperture 12; Speed 3; Specimen Balance 3 and these parameters are consistent for all the experiments to ensure the reproducibility of axotomy. DRG soma was found under the microscope and a laser pen was used to cut the DRGs out at 400 μm and 1000 μm away from the center of each explant respectively (Figure 1E). The injured DRGs were dropped directly onto another aligned fiber scaffold, which was precoated with miRs, as illustrated in Figure 1F, 1G and S1. The axotomized DRGs were then cultured for another 1 week before the regrowth of neurites were assessed.

Fabrication and characterizations of *in vivo* nerve guidance conduit

PCL film

The fabrication process of nerve guidance conduits is highlighted in Figure 7A. Briefly, 70 mg of PCL pellets (molecular weight 45 kDa) were placed in between two pieces of metal sheets (15 cm x 25 cm) and pressed into a 50 μm thin PCL film using a heat press machine (Carver, Model 4128) at 80 °C and 0.2 GPa. The resulting PCL film was cut into strips (i.e. 34 cm in length and 1 cm in width) before they were fixed around a rotating collector for subsequent electrospinning.

Preparation of PCL-PEG polymer solution for the fabrication of nerve guidance conduits

The PCL-PEG solution was prepared and the volume ratio of PCL to PEG was

8:2. Briefly, PCL (molecular weight 80 kDa) was dissolved in TFE to obtain a 14 wt% solution. Thereafter, the PEG (i.e. 30 wt%) was dissolved in PBS containing 40 µg of miRs and 60 µl of transfection reagent, TKO. The choice of using TKO as the transfection reagent for *in vivo* experiments is based on our previous optimization studies^{16,38–41}. Specifically, we have demonstrated that TKO is more stable when being encapsulated inside the PCL scaffolds^{40,41}. In this work, the miRs were also encapsulated in the PCL aligned fibers before being implanted to bridge the injured sciatic nerve. Additionally, TKO has been used as an effective transfection reagent for *in vivo* nerve injury treatment^{16,38}. As such, we chose TKO as the *in vivo* transfection reagent. The homogenous solution (i.e. PCL and PEG) was then loaded into a syringe connected with a 21-gauge needle and dispensed at a fixed rate of 1.0 mL/h using a syringe pump. Positive 8 kV and negative 4 kV were then applied to the tip of the syringe and the rotating collector, respectively. The syringe and the collector were separated at 11 cm apart. The electrospun fibers were then collected on the PCL strip, which was fixed around a rotating collector (speed: 1200 rpm) during the electrospinning process. Thereafter, the PCL strip with aligned fibers was cut into small strips that were 17 mm in length before they were rolled into a conduit using a glass capillary with an outer diameter of 2 mm. This 34 cm PCL strip can be used to make twenty 17 mm nerve conduits and the theatrical loading of miRs was 2 µg each. Based on our own experience in spinal cord injury treatment, 2 µg of miR therapeutics is sufficient to achieve a biological effect such as enhanced myelination after spinal cord hemi-incision injury³⁸ or enhanced axon regeneration after spinal cord transection injury¹⁶. Besides that, 2 µg of miR loading is also based on extensive literature search

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where researchers used similar amount of nucleic acids^{42–45} for nerve injury treatment.

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Lastly, the edge of the conduit was sealed with 14 wt% of PCL polymer solution. The nerve guidance conduits were then stored at -20 °C for future usage.

The blank and miR-loaded nerve guidance conduits were cut in liquid nitrogen so as to preserve their cross-sectional structures. Part of the nerve conduit was cut open to expose the inner fiber surface. Both the cross-sectioned nerve guidance conduits along with their corresponding inner fiber surface were viewed under SEM. For inner fibers, 10 regions-of-interest (ROI) were imaged and the average fiber diameter and fiber alignment were obtained by quantifying 50 individual fibers using ImageJ software (NIH, USA).

MiR Release Kinetics from the nerve guidance conduit

To obtain miR release profile, nerve guidance conduits (n = 3) fabricated from the same strip of electrospinning containing miR-132/miR-222/miR-431 were completely submerged into 1 mL of 1 x PBS each and incubated at 37 °C. At each time point, 1 mL of supernatant was collected and an equal volume of fresh PBS was added. For the quantification of miRs, 4 µL of heparin (100 mg/mL) was added to 100 µL of supernatant and thoroughly mixed for 15 min to decomplex the miRs from TKO. The decomplexation of miRs was optimized based on the tests performed in our previous works^{14,41,46}. The concentration of miR present in the supernatant was then quantified using Quant-iT RiboGreen assay.

Sciatic nerve transection and nerve guidance conduit implantation

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Nanyang Technological University and approved by the Animal Ethics Committee, Institutional Animal Care and Use Committee (IACUC, Protocol number: A0334). Female Sprague-Dawley rats (12 weeks, 250-300g) were obtained from In Vivos Pte Ltd (Singapore) and divided into two groups, receiving either blank nerve conduits (n=4) or miR-132/miR-222/miR-431 (n=4) loaded nerve conduits. Rats were anesthetized with an intraperitoneal injection of ketamine (73 mg/kg) and xylazine (7.3 mg/kg). All animals were injected with buprenorphine subcutaneously (0.05 mg/kg) before surgery. The surgical field was shaved and cleaned with 70% ethanol and treated with betadine. Under the surgery microscope, the right sciatic nerve was exposed through a posterior thigh muscle-splitting incision and 5 mm of the nerve was resected. A 17 mm nerve conduit was then sutured to the proximal stump and distal stump for about 1 mm respectively, using a 11-0 nylon monofilament (Ethicon Inc) suture so as to bridge a 15 mm nerve lesion gap. The nerve guidance conduit was placed in parallel with the main trunk of the sciatic nerve. All nerve conduits were filled with 10 μ L of PBS prior to implantation. Lastly, the muscles and skin were closed.

Immunostaining

In vitro:

Successful regeneration of mammalian peripheral nervous system (PNS) neurons undergo a variety of changes in gene expression, such as the significant upregulation of growth-associated proteins⁴⁷ and tubulins⁶. High expression of regeneration-associated protein indicates a higher intrinsic propensity for axonal growth⁶. Therefore, GAP43 and β III-tubulin (i.e. Tuj1) were used to reveal the regeneration potentials. Briefly, after culturing for 7 days on miR coated aligned fiber scaffolds, DRGs were fixed with 4% PFA for 20 min. After washing with 1 x PBS for 3 times (5 min each), the cells were permeabilized in 0.1% Triton X-100 in 1 x PBS for 15 min. Thereafter, the DRGs were incubated in non-specific blocking solution (5% goat serum) for 1 h at room temperature, followed by incubation with primary antibodies, mouse-anti β III-Tubulin (Tuj1, 1:1000) and rabbit-anti GAP43 (1:500) for overnight at 4 °C. Following that, the explants were washed for three times with 1 x PBS and detected with Alexa Fluor 488 and Alexa Fluor 546 secondary antibodies (1:500) respectively for 2 h followed by DAPI staining for 10 min at room temperature. Stitched images of the DRGs were taken under Leica fluorescent microscope (Leica DMI8). The average longest neurite length, GAP43⁺ area as well as Tuj1⁺ area was then quantified to reveal the regeneration capability of the injured DRGs. All quantifications were done using ImageJ software.

For experiments that analyzed the distance effects on neurite regrowth, three DRG explants were tested for each group.

For experiments that evaluated the miR effects on distally axotomized DRGs

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(i.e. 1000 μm away from cell soma), three experimental repeats were performed by using a total of 3 untreated DRGs, 4 miR-222-treated DRGs, 8 miR-431-treated DRGs, 3 miR-222/miR-431-treated DRGs and 7 miR-132/miR-222/miR-431-treated DRGs.

For experiments that evaluated the miR effects on proximally injured DRGs (i.e. 400 μm away from cell soma), three experimental repeats were performed by using a total of 10 untreated DRGs, 10 miR-222-treated DRGs, 10 miR-431-treated DRGs, 10 miR-222/miR-431-treated DRGs and 8 miR-132/miR-222/miR-431-treated DRGs.

In vivo:

Three weeks post injury, the nerve guidance conduits were retrieved and post-fixed for overnight. Thereafter, the nerve conduits were transferred to 15% sucrose for 24 h followed by 30% sucrose at 4 °C until they were cryosectioned. Nerve conduits were sectioned into 20 μm thick horizontal sections and directly mounted on the glass slides. The frozen sections were washed three times with 1 x PBS and permeabilized with 0.1% Triton X-100 in 1 x PBS for 15 min. Following that, the sections were blocked with 10% goat serum for 1 h before they were stained with primary antibody, chicken anti-NF200 (1:1000). Samples were subsequently washed 3 times with 1 x PBS and incubated with the following secondary antibody: Alexa Fluor 488-conjugated Goat anti-Chicken secondary antibody (1:500) for 2 h followed by DAPI (1:1000) for 10 min at room temperature. The samples were washed for 3 time with 1 x PBS and mounted

with Fluoromount-G™.

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Stitched images were taken under 10x magnification using a fluorescent microscope (Leica DMI8). For nerve ingrowth measurements, the distance of nerve ingrowth from the proximal end, as well as the area occupied by NF200⁺ signals were quantified using ImageJ software, including four untreated samples and three miR-132/miR-222/miR-431-treated samples. Three sciatic nerve sections were quantified in each sample.

Statistical analysis

Outlier analysis was performed to exclude the outliers using GraphPad QuickCalcs software. One-way ANOVA and Tukey post-hoc tests were used when data were normally distributed and had equal variances. For data that was not normally distributed or had unequal variances, the Kruskal–Wallis and Mann–Whitney U tests were used for comparison when more than two groups were included. For comparison between two groups, Student's t-test was used. Statistical analysis was done using SPSS software. All values, unless mentioned otherwise, were represented as mean $\pm$ S.D.

3. Results

Characterization of *in vitro* aligned fibers

Figure 2 shows the SEM images of the aligned fiber scaffolds that were used for DRG culture before and after axotomy. The average diameter of the suspended fibers (i.e. fibers on PCL frame) was about $0.9\ \mu\text{m}$ and the fiber angle deviation of the suspended fibers was within 10 degree (Figure 2A-2C). In addition, the average diameter of the fibers that were collected on the glass coverslip was around $1.33\ \mu\text{m}$. Importantly, the deviation of fiber angle was within 5 degree (Figure 2D-2F).

Figure 2. Characterization of the suspended aligned fibers and aligned fibers on glass (A) Representative SEM image of the suspended aligned fibers which were used for laser microdissection. White box shows the enlarged image of the area of the small white box. (B) The measurement of fiber diameter and the frequency (%) of the fiber diameter. (C) The frequency of the deviation of fiber angle from the mean. (D) Representative SEM image of the aligned fibers collected on glass coverslip. White box shows the enlarged image of the area of the small white box. (E) The measurement of fiber diameter and the frequency (%) of the fiber diameter. (F) The frequency of the deviation of fiber angle from the mean. Data obtained here are from three technical repeats.

Characterization of *in vitro* fiber scaffolds and miR release

Figure 3A shows the water contact angle of the uncoated PCL fibers and fibers

after various steps of surface modification with PDL, laminin and miR coatings. For PDL-coated PCL aligned fibers, the water contact angle was about 100 degree. PCL fibers coated with PDL and laminin, as well as PCL fibers coated with PDL, laminin and miRs exhibited a significantly decreased water contact angle as compared to uncoated PCL fibers, indicating that PDL and laminin were successfully coated on the PCL fibers and resulted in a decreased hydrophobicity. Additionally, after 2h and 4h of coating with poly(dopamine), the water contact angle of PCL fibers was significantly decreased. PDL, laminin and miR coating did not alter the water contact angle of the poly(dopamine)-coated fibers (Figure 3A and 3B). Besides that, there is no significant difference between 2h and 4h of poly(dopamine) coating.

A uniform distribution of Cy5-RNA was observed after two hours of coating, regardless of the initial length of time taken for poly(dopamine) coating. The average loading efficiency of miRs on the aligned fibers was about 88% and there was no significant difference between 2 h and 4 h of poly(dopamine) coating (Figure 3C and 3D). As such, 2 h of poly(dopamine) coating was adopted for all subsequent experiments. Correspondingly, 15% of miRs was released from the aligned fibers at Day 7. As can be seen from the release curve, a sustained delivery of miRs was achieved using this aligned fiber platform for at least 21 days (Figure 3E). This suggests that the miR-loaded PCL scaffolds can be used for long time point studies, such as serving as artificial axons for studying oligodendrocyte myelination at day 14 or even day 21⁴⁸.

Figure 3. Poly(dopamine) coating changed the hydrophobicity of the fibers and the aligned fiber platform provided a sustained delivery of miRs (A) Representative images of contact angle tests of fibers undergoing various steps of coating. PCL @ PDL represents PDL was coated on the PCL fibers. PCL@PDL@LN means PDL was coated on PCL fibers, thereafter LN was subsequently coated on the PDL coated PCL fibers, and so on. (B) Water contact angle of fibers after various steps of coating. The poly(dopamine) coating significantly decreased the water contact angle of PCL fibers. Three scaffolds were measured per group. (C) The distribution of Cy5-RNA on the aligned fiber scaffolds after 2 h of coating. (D) The loading efficiency of miRs on the aligned fibers was about 90%. Three scaffolds were tested in each group. (E) Release profile of miRs obtained from three fiber scaffolds. The aligned fiber platform could provide a sustained delivery of miRs. Data represented as mean $\pm$ SD., *: $p < 0.05$, Shapiro-Wilk normality test followed by Kruskal-Wallis test and Mann-Whitney post hoc test.

More robust axonal regrowth was achieved when axotomy was done closer to DRG soma

One week after culture, the axotomized DRGs were fixed and stained with Tuj1

and GAP43. Specifically, the regenerated axons grew along the alignment of the fibers. By quantifying the average length of the longest neurite from the axotomy border, we demonstrated that axons grew longer when axotomy was done at 400 μm than that at 1000 μm (i.e. $2346 \pm 324 \mu\text{m}$ vs. $1539 \pm 219 \mu\text{m}$; $p < 0.05$) away from cell soma. Besides that, the quantification of GAP43⁺ area and Tuj1⁺ area also suggested that there was more GAP43⁺ (i.e. $1170000 \pm 213713 \mu\text{m}^2$ vs. $595767 \pm 233884 \mu\text{m}^2$; $p < 0.05$) and Tuj1⁺ (i.e. $872870 \pm 257603 \mu\text{m}^2$ vs. $332174 \pm 219313 \mu\text{m}^2$; $p < 0.05$) expression when axotomy was done closer to the cell soma. (Figure 4A-4D).

Figure 4. More robust axonal regrowth was achieved when axotomy was done closer to the DRG soma (A) Schematic diagram illustrating how the DRGs were axotomized and the distance of axotomy. Representative fluorescent images of the regenerated DRGs. White arrows indicate the terminals of the regenerated axons. (B-D) Quantification of the average longest neurite length, area of GAP43⁺ signals and area of Tuj-1⁺ signals suggested that the axotomized DRGs exhibited a more robust neurite outgrowth when injuries

occurred close to the cell soma. Three DRG explants were tested for each group. Data represented as mean $\pm$ SD., *: $p < 0.05$, Student's t-test.

Presence of miRs stimulated neurite outgrowth, among which miR-132/miR-222/miR-431 performed the best

MiRs/miR cocktails were coated onto the fibers before the distally axotomized DRGs (i.e. axotomized at 1000 μm away from the cell soma) were seeded on. Among the four selected miR/miR cocktails, miR-132/miR-222/miR-431 treatment resulted in the longest axonal regrowth (i.e. $3469 \pm 709 \mu\text{m}$) as compared to the other groups. More importantly, the axonal regrowth in miR-132/miR-222/miR-431-treated group was significantly longer than the untreated-(i.e. $1539 \pm 219 \mu\text{m}$; $p < 0.05$), miR-222-(i.e. $2262 \pm 638 \mu\text{m}$; $p < 0.05$) and miR-222/miR-431-treated (i.e. $1898 \pm 153 \mu\text{m}$; $p < 0.05$) samples. Additionally, the treatment of miR-132/miR-222/miR-431 also demonstrated the largest area of neurite extensions, as well as the largest area of growth associated protein expression, followed by the treatment of miR-431. Both treatments with miR-132/miR-222/miR-431 and miR-431 exhibited significant enhancement as compared to the untreated DRGs. On the contrary, the treatment with miR-222 and miR-222/miR-431 were relatively worse, in terms of neurite length and neurite extension area (Figure 5A-5D).

Figure 5. Presence of miRs stimulated neurite outgrowth, among which miR-132/miR-222/miR-431 performed the best (A) Representative fluorescent images of axotomized DRGs (1000 μm on both sides) after 7 days of culture. (B-D) Quantification of the average longest neurite length, area of GAP43⁺ signals and area of Tuj-1⁺ signals. Three experimental repeats were carried out by using a total of 3 untreated DRGs, 4 miR-222-treated DRGs, 8 miR-431-treated DRGs, 3 miR-222/miR-431-treated DRGs and 7 miR-132/miR-222/miR-431-treated DRGs. Data represented as mean $\pm$ SD., *: $p < 0.05$, Shapiro-Wilk normality test followed by Kruskal-Wallis test and Mann-Whitney post hoc test.

MiR-132/miR-222/miR-431 promoted significant axonal regrowth and overcame distance effects

MiR/miR cocktails were coated onto the fibers before the proximally axotomized DRGs (i.e. axotomized at 400 μm away from the cell soma) were seeded on. By quantifying the average longest neurite length, we demonstrated that the treatment of miR-222 (i.e. $3115 \pm 274 \mu\text{m}$; $p < 0.01$), miR-431 (i.e. $3055 \pm 327 \mu\text{m}$; $p < 0.05$) and miR-132/miR-222/miR-431 (i.e. $3205 \pm 426 \mu\text{m}$; $p < 0.01$) stimulated

significantly longer axonal regrowth as compared to the untreated DRGs (i.e. $2113 \pm 313 \mu\text{m}$, figure 6A-6B). In addition, the quantification of GAP43⁺ area and Tuj1⁺ area suggested that there was no significant difference among those four treatment groups (Figure 6C-6D).

To better visualize the effects of miR-132/miR-222/miR-431 on axon regeneration as the axotomy distance increased, a graph which showed the average longest neurite length in both untreated and miR-132/miR-222/miR-431-treated DRGs was plotted. Specifically, we demonstrate that neurite length of miR-132/miR-222/miR-431-treated DRGs with distal axotomy (i.e. $1000 \mu\text{m}$) was $3469 \pm 709 \mu\text{m}$, whereas neurite length of miR-132/miR-222/miR-431-treated DRGs with proximal axotomy (i.e. $400 \mu\text{m}$) was $3205 \pm 426 \mu\text{m}$ (Figure 6E). In contrast, the neurite length of untreated DRGs axotomized at $400 \mu\text{m}$ and $1000 \mu\text{m}$ away from the cell somas were $2113 \pm 313 \mu\text{m}$ and $1539 \pm 219 \mu\text{m}$, respectively. These results suggest that when axotomy was performed at $1000 \mu\text{m}$ away from the cell soma, neurite regrowth in the untreated DRGs was significantly decreased but the treatment with miR-132/miR-222/miR-431 still yielded similar, if not slightly longer, regeneration distance as compared to the proximal injury of $400 \mu\text{m}$. Additionally, we normalized the longest neurite length by that obtained in untreated DRGs. Correspondingly, when axotomy was performed more proximally (i.e. $400 \mu\text{m}$) away from the DRG soma, the average longest neurite extension in the miR-132/miR-222/miR-431-treated group was ~1.6-fold longer than the untreated samples (Figure 6F). In contrast, the average longest neurite extension after treatment with miR-132/miR-222/miR-431 was ~2.2-fold

longer than the untreated samples when axotomy was performed more distally (i.e. 1000 μm) from the DRG soma (Figure 6G).

Figure 6. MiR-132/miR-222/miR-431 promoted significant axonal regrowth and overcame distance effects (A) Representative fluorescent images of axotomized DRGs (400 μm at both sides) after 7 days of culture. (B-D) Quantification of the average longest neurite length, area of GAP43⁺ signals and area of Tuj-1⁺ signals. (E) Average longest neurite length in untreated and miR-132/miR-222/miR-431-treated DRGs, when the axotomy was performed at 400 μm and 1000 μm away from cell soma, respectively. (F-G) Fold change of average longest neurite length against untreated DRGs when axotomy was done at 400 μm and 1000 μm , respectively. Three experimental repeats were performed by using a total of 10 untreated DRGs, 10 miR-

222-treated DRGs, 10 miR-431-treated DRGs, 10 miR-222/miR-431-treated DRGs and 8 miR-132/miR-222/miR-431-treated DRGs. Data represented as mean $\pm$ SD., *: $p < 0.05$; **: $p < 0.01$, Shapiro-Wilk normality test followed by Kruskal-Wallis test and Mann-Whitney post hoc test.

MiR-132/miR-222/miR-431 slightly enhanced nerve regeneration in a sciatic nerve transection model

Figure 7B shows the SEM images of the blank and miR-132/miR-222/miR-431 loaded nerve guidance conduits, as well as their corresponding inner fiber surface morphologies. The diameter of blank fiber was around $1.325 \pm 0.6429 \mu\text{m}$ and the diameter of miR-132/miR-222/miR-431 loaded fiber was around $1.256 \pm 0.6266 \mu\text{m}$ (Figure 7C). Besides that, the deviation of fiber angle from the mean is about ± 10 degrees, suggesting that the inner fibers are quite aligned. Combining Figures 7B to 7D, we concluded that miR encapsulation did not alter the morphology of the electrospun fibers. Besides that, the nerve guidance conduits provided a sustained release of miRs for up to 35 days (Figure 7E).

Three weeks after implantation, the nerve conduits were sectioned longitudinally and stained with NF200. Figure 7F shows the representative fluorescent images of NF200 staining in untreated and miR-132/miR-222/miR-431-treated samples. By quantifying the distance of nerve ingrowth from the proximal end of the nerve conduits, we observed that the nerve ingrowth in the untreated DRGs was around $5463 \pm 1051 \mu\text{m}$ and the nerve ingrowth was about $6201 \pm 1197 \mu\text{m}$ in miR-132/miR-222/miR-431-treated samples (Figure 7F). In addition, the quantification of area occupied by NF200⁺ signals showed that in the untreated samples, the NF200⁺

area is around $0.935 \pm 0.417 \text{ mm}^2$ and the NF200⁺ area is about $1.488 \pm 0.876 \text{ mm}^2$ (Figure 7G).

Figure 7. miR-132/miR-222/miR-431 enhanced nerve regeneration in a sciatic nerve transection model (A) Schematic diagram illustrating the procedure of nerve guidance conduit fabrication. (B) Scaffold morphology for both blank nerve guidance conduit and nerve guidance conduit encapsulated with miR-132/miR-222/miR-431. (C-D) Quantification analysis of fiber diameter and deviation of fiber angle from the mean, suggesting that the encapsulation of miR-132/miR-222/miR-431 did not alter the morphology of the inner fibers. (E) The release profile of miR-132/miR-222/miR-431 from the nerve guidance conduit. (F) Representative fluorescent images of NF200 staining in untreated and miR-132/miR-222/miR-431-treated samples. (G) Quantification analysis of nerve regeneration which was calculated from the proximal end. (H) Quantification of area occupied by NF200⁺ signal.

4. Discussion

The failure of injured axons to regenerate over long distances and reestablish

connections destroyed by traumatic lesions have long been recognized as one of the bottlenecks for achieving optimal regeneration outcomes⁴⁹. In order to successfully promote nerve regrowth, potent biomolecules which stimulate the regrowth of injured axons are necessary. Furthermore, reproducible and translatable approaches to test the potency of drug candidates in enhancing regeneration over long axotomy distances are also needed.

Mechanical approach to carry out axotomy *ex vivo*, such as using microfluidic devices^{50,51}, has long been established. Although microfluidic devices have their own advantages, such as the ease of operation and high reproducibility, their low translational values (i.e. cannot be directly implanted into the body) has limited their applications to *ex vivo* stage. Besides creating nerve injury models by mechanical approaches, high throughput laser microdissection has been utilized for axotomy both *in vitro*⁵² and *in vivo*^{9,10,53,54}. The advantages of laser microdissection is its ease of operation, high reproducibility^{9,10} and most importantly, it can be used to axotomize axons *in vivo* with ultra-high accuracy using two-photon confocal microscopy^{10,53}.

Here, we specifically designed aligned electrospun scaffolds for laser microdissection so as to take advantage of the accuracy of the laser axotomy approach. In the context of axon regeneration, neurons extend their neurites along the aligned electrospun fibers and grow significantly longer than that on tissue culture plates^{16–18}. Additionally, the topographical cues provided by aligned fibers direct the regrowth of

neurites towards two directions, thereby facilitating subsequent imaging and quantification processes. Besides that, the ease of incorporation with different drug candidates onto the aligned fiber substrate^{15,30} makes it possible to serve as a drug screening platform¹⁶. Furthermore, the aligned fiber platform could provide a sustained delivery of therapeutics⁴⁶, enabling a prolonged availability of biochemical signalling. The bioactivities of the released therapeutics from the electrospun fibers have been verified in our previous works^{14,16,34,39–41,46,55,56}. Specifically, we showed that when siRNA targeting RE-1 silencing transcription factor (REST) was coated on similar poly(dopamine)-coated PCL fibers, these molecules were sustainably released from the PCL fiber scaffolds. Importantly, significant REST knockdown was achieved on both human mesenchymal stem cells¹⁴, as well as neural stem/progenitor cells (NPCs)³⁴. Besides that, scaffold-mediated delivery of siREST also enhanced the neuronal marker expression¹⁴. Additionally, in our previous work on neurons¹⁶, sustained delivery of miRs was achieved on the poly(dopa)-coated PCL scaffolds and the released miRs also exhibited significant target gene knockdown in the cultured cortical neurons. Besides surface-mediated drug/gene delivery, encapsulating the therapeutics in the electrospun fibers is also commonly utilized to deliver drugs and the fabrication procedure is based on our optimized published protocols^{39–41,46,55,56}. Specifically, we showed that the miRs and/or siRNAs can be sustainably released from the electrospun fiber regardless of the delivery vehicles used. More importantly, these molecules remain bioactive as they led to significant target gene silencing and notable biological outcomes^{39–41,46,56}. Given that the released miRs and/or siRNAs can elicit both biological effects and gene knockdown regardless of the delivery approaches

utilized, we concluded that the miR and/or siRNAs released from the PCL scaffolds remained functional. As such, the released miRs from similar PCL fiber scaffolds, as shown in this work, remain bioactive. Lastly, the design of aligned electrospun fibers can be easily extended to an implantable nerve guidance conduit for *in vivo* applications. Altogether, by incorporating laser axotomy with aligned electrospun fibers, we demonstrated a highly reproducible *in vitro* axotomy model with the above advantages.

Axon regeneration through enhancing the intrinsic growth ability of neurons provides a promising strategy for nerve repair after injuries¹⁹. MiRs play significant roles during this process by regulating local protein synthesis at growth cones. Specifically, miR-222²¹, miR-132^{22–25} and miR-431²⁶ were shown to enhance axonal regeneration when they were overexpressed in neurons. In particular, Ras GTPase, p120RasGAP (Rasa1), which is involved in cytoskeletal regulation, is targeted by miR-132²³. In addition, Wnt signaling, which is significant for neurogenesis and axon growth, is regulated by miR-431 as miR-431 decreases the expression of a Wnt antagonist, Kremen1²⁶. Lastly, miR-222 targets PTEN, an inhibitor of the PI3K pathway that is important to central axon growth²¹. Therefore, evaluating the potential effects of these miR/miR cocktails in promoting neurite outgrowth in neurons with axons that have been axotomized at long distances, as shown in this study, may be helpful in identifying new therapeutics for enhancing nerve regeneration after injuries.

In this work, DRG explants, instead of dissociated neurons were utilized to test the potential effects of miRs for various reasons. Firstly, the miRs tested (i.e. miR-222, miR-132 and miR-431) were found to be effective in promoting axon regeneration in dissociated DRG neurons by enhancing neurogenesis²⁶ or inhibiting molecular inhibitors^{20,21}. In this work, the same type of neurons was applied in order to have direct comparisons with the existing works. For instance, miR-431 was shown to be upregulated significantly in DRGs after sciatic nerve injury in the work by Wu *et al*²⁶. Specifically, when miR-431 was added to the cultured dissociated DRG neurons, the fold change enhancement of axon length was about 1.3-fold²⁶ whereas the fold change was around 2.2-fold (Figure 6G) when axotomized DRG explants were treated with similar amount of miR-132/miR-222/miR-431 cocktail. However, for high-throughput analyses, DRG explants were axotomized instead of doing the axotomy one by one in dissociated neurons. Another concern is the age of the DRG explants used. In this work, DRG explants were obtained from P2-P3 pups instead of the adult rats in order to facilitate laser microdissection and subsequent culture. However, we believe that the results shown here could also be relevant to adult DRG explants because we have previously demonstrated that the efficacy of the miR cocktails in promoting axonal regeneration was preserved regardless of the age and origin of neurons (i.e. embryonic day 14, postnatal day 1 cortical neurons as well as adult DRG neurons)¹⁶.

To elucidate the effects of miRs on injured neurons with different intrinsic regeneration capacity, the miRs were tested in both proximally and distally

axotomized neurons. We demonstrated that regardless of the injury distance of the neurites from the cell bodies, miR-132/miR-222/miR-431 always exhibited the best regeneration outcomes as the regenerated axon length was the longest and the extension area of neurite was also the largest (Figure 4 and 5). More importantly, when axotomy occurred at 1000 μm away from DRG soma, the neurite length was about 2.2-fold longer than the untreated cells (Figure 6G) whereas, the neurite length was only about 1.6 times longer when axotomy occurred at 400 μm away from DRG soma (Figure 6F). Additionally, we showed that even though the neurite regrowth in untreated DRGs (distally axotomized) was significantly decreased, miR-132/miR-222/miR-431 was still able to exhibit similar, if not longer, regeneration distance (i.e. $3469 \pm 708.9 \mu\text{m}$ vs. $3205 \pm 425.8 \mu\text{m}$; Figure 6E). These results suggest that miR-132/miR-222/miR-431 performs even better when the injury site of neurons is farther away from the cell soma. Hence, this miR cocktail may be able to overcome distance effects on neurite regrowth.

Looking back to our previous work when we used intact primary cortical neurons and dissociated DRG neurons, the fold change of neurite length after the treatment of miR-132/miR-222/miR-431 was only about 1.13~1.27-fold enhancement in contrast to the untreated cells¹⁶. In that work, the gene silencing effects of miR-132/miR-222/miR-431 was evaluated in cortical neurons using real-time quantitative PCR (qPCR). We found that the treatment of miR-132/miR-222/miR-431 significantly downregulated the downstream target, *Rasa1*. Besides that, the other two downstream targets, *PTEN* and *Kremen1* were also knocked down¹⁶. Future works

such as growth cone area evaluation and qPCR will be carried out to further prove the hypothesis that miR-132/miR-222/miR-431 downregulates the expression of its downstream targets in DRG neurons as well.

To further verify the efficacy of miR-132/miR-222/miR-431 in promoting nerve regeneration, we adopted a sciatic nerve transection model. Specifically, the miR cocktail was encapsulated in a nerve guidance conduit which was made by aligned electrospun fibers. The nerve guidance conduit was used to bridge a 15 mm critical nerve lesion gap as well as to direct the regrowth of the injured sciatic nerve. Due to the transient nature of nucleic acids, such as their fast degradation rate, repeated administrations are typically needed to achieve long-term therapeutic effects for the treatment of nervous system injuries. As such, a sustained delivery of nucleic acids is of great necessity. In this work, sustained delivery of miRs was achieved by encapsulating the miRs into the nerve conduits (Figure 7E) and hence the repeated administrations were avoided. MiRs effect was evaluated as early as three weeks post implantation, such that the effects of miRs was not masked by the spontaneous regeneration of sciatic nerves. We observed a trend of enhanced nerve regeneration after sciatic nerve transection (Figure 7F-7H) when miR-132/miR-222/miR-431 was administered. More in-depth studies, such as longer time point morphometrical studies and functional assessments (e.g. compound muscle action potential measurements) will be carried out in the future to further reveal the potential of the miRs. Additionally, microRNA array analysis and RNA sequencing will be included to further determine the expression level of each miR in L4-L6 DRGs after sciatic nerve

injury as well as the gene expression levels of the miRs' downstream targets, respectively.

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5. Conclusion

In this study, a biomimicking aligned fiber platform was established which can be used to carry out laser microdissection. The design of aligned fibers can be easily extended to an implantable nerve guidance conduit for peripheral nerve regeneration applications. By adopting this aligned fiber platform, laser microdissection-based axotomy can be carried out and more importantly, the distance of axotomy can be precisely controlled. Using this platform, we concluded that in the absence of miRs, DRGs axotomized proximally regenerated significantly longer than that injured distally. Besides that, miR-132/miR-222/miR-431 cocktail stimulated the most robust neurite outgrowth. Additionally, distance effects on neurite outgrowth was overcome by the effects of miRs. Lastly, treatment of miR-132/miR-222/miR-431 showed its potential in nerve regeneration after sciatic nerve transection, by exhibiting a longer axon length as well as a bigger nerve extension area.

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Conflict of interest

The authors declare that there is no conflict of interest.

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