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Topographical effects on fiber-mediated microRNA delivery to control oligodendroglial precursor cells development

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Abstract

Effective remyelination in the central nervous system (CNS) facilitates the reversal of disability in patients with demyelinating diseases such as multiple sclerosis. Unfortunately until now, effective strategies of controlling oligodendrocyte (OL) differentiation and maturation remain limited. It is well known that topographical and biochemical signals play crucial roles in modulating cell fate commitment. Therefore, in this study, we explored the combined effects of scaffold topography and sustained gene silencing on oligodendroglial precursor cell (OPC) development. Specifically, microRNAs (miRs) were incorporated onto electrospun polycaprolactone (PCL) fiber scaffolds with different fiber diameters and orientations. Regardless of fiber diameter and orientation, efficient knockdown of differentiation inhibitory factors were achieved by either topography alone (up to 70%) or fibers integrated with miR-219 and miR-338 (up to 80%, $p < 0.05$). Small fiber promoted OPC differentiation by inducing more RIP⁺ cells ($p < 0.05$) while large fiber promoted OL maturation by inducing more MBP⁺ cells ($p < 0.05$). Random fiber enhanced more RIP⁺ cells than aligned fibers ($p < 0.05$), regardless of fiber diameter. Upon miR-219/miR-338 incorporation, 2 μ m aligned fibers supported the most MBP⁺ cells (~17%). These findings indicated that the coupling of substrate topographic cues with efficient gene silencing by sustained microRNA delivery is a promising way for directing OPC maturation in neural tissue engineering and controlling remyelination in the CNS.

Keywords

Nanofibers; Electrospinning; Oligodendrocytes; Myelination; Sustained release; Gene silencing

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1. Introduction

Traumatic nerve injuries (TNI) in the central nervous system (CNS) result in devastating outcomes of functional impairment. Unfortunately, to date, the strategies for treating such neurological pathologies remain suboptimal. Oligodendrocyte (OL), which produces the multilamellar myelin sheaths that wrap around axons, is considered to be a pivotal cell type in supporting functional recovery. However, after TNI, the lack of surviving OLs often results in inefficient myelin regeneration and impaired signal transmission. To generate sufficient OLs, oligodendroglial precursor cells (OPCs), a widespread population of precursor cell, are recruited to the lesion site for differentiation and remyelination [1–3]. However, the spontaneous remyelination after TNI is very limited, particularly in humans [4,5]. As reported recently, OPC rapidly differentiates into OL in the absence of mitogens that maintain the undifferentiated states of OPC [6]. This suggests that myelination by OL might be a default pathway that is controlled at the level of inhibition. Therefore, regulating some of these inhibitory factors might enhance OL differentiation and myelination [7].

MicroRNAs (miRs), a class of small non-coding RNAs (sncRNA) containing 21–23 nucleotide base pairs, are increasingly recognized to play critical roles in various biological processes including stem/precursor cell differentiation, by silencing relative inhibitory factors for differentiation. Their roles in different stages of OL lineage progression have been elucidated [7–9]. Among these reported miRs, miR-219 and miR-338 have been identified to play critical roles in regulating OL development. Specifically, they work by silencing the expressions of negative regulators of OL differentiation, such as platelet-derived growth factor receptor alpha (PDGFR- α), SRY-box containing gene 6 (Sox6), hes family bHLH transcription factor 5 (Hes5), forkhead box J3 (FoxJ3) and zinc finger protein 238 (ZFP238) [6,10]. Correspondingly, the over-expression of miR-219 and miR-338 enhanced OPC differentiation while blockage of their expressions inhibited OL maturation [10]. Therefore, controlled delivery of miR-219 and miR-338 may act as a potential strategy for manipulating OPC differentiation and OL maturation.

Electrospun fiber scaffolds are promising substrates which can mimic the natural structure of the extracellular matrix (ECM). They provide topographical cues to manipulate cell fate [11–16]. In particular, aligned fiber enhanced human Schwann cell maturation and may promote myelination of the peripheral nervous system (PNS) [17]. On fibers with small diameters (283 nm), neural stem cells preferred oligodendrocyte differentiation, while on larger fibers (794 and 1492 nm), they underwent neuronal differentiation predominantly [13]. Expanded to gene silencing, upon combining with sncRNAs, electrospun fibers efficiently silenced target genes to manipulate cell fate commitment [18–21]. In particular, smaller fibers provided better collagen type I gene knockdown efficiency and altered RNA uptake pathway in human dermal fibroblasts, while aligned fibers favored the silencing of RE-1 silencing transcript factor (REST) in human mesenchymal stem cells [22]. The *in vivo* model was also established where nano-sized fiber was more beneficial for peripheral nerve regeneration than micron-sized fibers [23].

Here, we hypothesize that different fiber topographic cues can alter gene silencing outcomes, which in turn allows us to improve the efficiency of fiber-mediated miR delivery

to control OPC development. To incorporate miR-219 and miR-338 onto poly(ϵ -caprolactone) (PCL) electrospun fibers, mussel-inspired bio-adhesive 3,4-dihydroxy-L-phenylalanine (DOPA) coating was used. This bio-adhesive coating allowed easy incorporation of snRNAs onto nanofiber scaffolds, thereby facilitated neural stem/precursor cell differentiation by gene silencing [19]. Specifically in this study, we used poly-DOPA coated electrospun fibers with different diameter and orientation to investigate their effects on miR reverse transfection efficiency and enhancement of OPC differentiation and OL maturation.

2. Materials and methods

2.1. Materials

Polycaprolactone (PCL, Mw: 80,000), 3, 4-Dihydroxy-L-Phenylalanine (DOPA), Dnase I, 2, 2, 2-trifluoroethanol (TFE, 99.0%), L-glutamine (G8540), sodium pyruvate (P2258), bovine serum albumin (BSA, A9649), apo-transferrin (T2252), insulin (I6634), sodium selenite (S5261), D-biotin (B4501), hydrocortisone (H0888), 10% formalin, Triton X-100 and fluoromount were purchased from Sigma–Aldrich. LIVE/DEAD® Cell Viability Kit, Alexa-Fluro 488 goat anti-mouse, Alexa-Fluro 555 goat anti-rabbit secondary antibodies, scrambled miR, miR-219, miR-338-3p and miR-338-5p mimics were purchased from Lifetechnologies. DMEM (11960-044) and FBS (11995-065) were purchased from Gibco. PDGF-AA (100-13A) and bFGF (100-18B) were purchased from Peprotech. TransIT-TKO was purchased from MirusBio. 1 × Tris–EDTA (TE) was purchased from 1st Base, Singapore. RNeasy Mini Kit was purchased from Qiagen. M-MLV Reverse transcriptase was purchased from Promega. IQ SYBR Green Supermix was purchased from Bio-Rad. All primers were purchased from AITbiotech, Singapore. Rabbit anti-oligodendrocyte transcription factor 2 (Olig2) (AB9610), mouse anti-receptor interacting protein (RIP) (MAB1580) and mouse anti-myelin basic protein (MBP) (MAB387) antibodies were purchased from Merck. Small ribonucleic acid and 488-labeled double stranded oligonucleotides (ODN), both of similar size as miR (i.e. 21–23 bp), were purchased from Aitbiotech. All other reagents were purchased from Invitrogen.

2.2. Fabrication and characterization of fibers

PCL fibers with different diameters were electrospun using different parameters as listed in Table 1. The homogenized PCL solution was loaded into a syringe and injected at a fixed flow rate by a syringe pump (New Era pump systems Inc., USA). Suitable positive and negative voltages (Gamma High Voltage, USA) were applied to the solution and rotating collector respectively, with predetermined distances between the syringe and the collector. The morphology and thickness of dried as-spun fibers was evaluated by scanning electron microscopy (SEM) (JEOL, JSM-6390LA, Japan) under an accelerating voltage of 10 kV after sputter-coating with platinum. Over 100 fibers were measured using ImageJ (NIH, USA) to calculate the average fiber diameters and 3 scaffolds were measured for scaffold thickness. In the following text, 2 μ m random fiber, 2 μ m aligned fiber, 700 nm aligned fiber and 300 nm aligned fiber are denoted as 2 μ m RF, 2 μ m AF, 700 nm AF and 300 nm AF, respectively.

2.3. Poly-DOPA coating of PCL fibers and miR absorption

PCL fibers were cut into 2 cm² area and pre-wet with deionized water overnight. Then all fibers were immersed into 0.5 mg/ml DOPA that was dissolved in poly-DOPA coating buffer (10 mM Bicine and 250 mM NaCl, pH = 8.5). The samples were coated under constant agitation at 120 rpm on an orbital shaker for 4 h. The fibers were then rinsed with deionized water to remove residual monomer and lyophilized overnight. FTA200 Contact Angle Analyzer (First Ten Angstroms, USA) was used to measure the contact angle values of electrospun fiber scaffolds (1 cm²). Computer software provided by FTA precisely recorded and measured the contact angle values on the surface of each fiber.

2.4. Loading of miRs onto lyophilized Poly-DOPA coated scaffolds

4 mg of miR mimics were complexed with 6 ml of TransIT-TKO and incubated at room temperature for 15 min. The complexes were then dropped onto UV-sterilized fibers for complete absorption at 37 °C for 30 min. Next, all miR-absorbed fibers were coated with laminin at 10 µg/cm² for 2 h. All PCL fibers with same diameter were divided into 3 groups: TKO with scrambled negative miR (denoted as Scrambled miR), TKO with miR-219 (denoted as miR-219), TKO with equal mass of miR-219, miR-338-3p and miR-338-5p (denoted as miR-219/miR-338).

2.5. Primary OPC isolation, culture, viability and reverse transfection

The isolation of primary OPC from rats was approved by the Institutional Animal Care and Use Committee (IACUC) at Nanyang Technological University, Singapore. According to the reported protocol [24], for OPC isolation, DMEM20S medium was used, which comprised of DMEM, 20% FBS, 4 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin and 50 µg/ml streptomycin. Briefly, the cortices from P1–2 neonatal rats were digested with 0.01% trypsin and 0.01 mg/ml DNase I at 37 °C for 15 min. After mechanical homogenization, the suspension was passed through 70 µm cell strainer (BD Biosciences, USA) and seeded in T75 flasks at a density of 10⁷ cells. After 10-day culture, with fresh DMEM20S medium being replaced every 3 days, the OPC were purified by shaking on an orbital shaker for 18–20 h at 37 °C. Following that, the cell suspension was passed through a 20 µm sterile screening pouch (Sefar American, USA). The purity of OPC by using this approach was ~96% [25]. Thereafter, the OPC were seeded onto poly-DOPA-coated PCL fibers at a density of 50,000 cells per scaffold and cultured in OPC medium that comprised of DMEM, 4 mM L-glutamine, 1 mM sodium pyruvate, 0.1% bovine serum albumin, 50 µg/ml apo-transferrin, 5 µg/ml insulin, 30 nM sodium selenite, 10 nM D-biotin, 10 nM hydrocortisone, containing 10 mg/ml PDGF-AA and 10 ng/ml bFGF. At indicated time point post-transfection, three independent samples for each experimental group were collected for live–dead assay by using LIVE/DEAD[®] Cell Viability Kit and were quantified using ImageJ software. Either live cells or dead cells were quantified by using plug-ins of ImageJ. After changing the images to 8-bit, the “process → binary → watershed” function was used in order to distinguish the edge of each cell. Thereafter, cell numbers were quantified. All other samples were collected for real-time PCR or immunofluorescent staining.

2.6. In vitro characterization of small ribonucleic acid loaded fibers

Poly-DOPA coated fibers that were loaded with RNA/TKO complexes were incubated at 37 °C for 30 min followed by washing with 1 ml of PBS. After treating the supernatant with 1 µg/ml of heparin solution, the concentration of the de-complexed RNA in the supernatant was determined by RiboGreen[®] assay. Thereafter, the fluorescence intensity was measured using a microplate reader (Tecan[®], Infinite 200, Austria) and the mass of RNA was determined. The mass of RNA in the supernatant was termed m_{washed} while the mass of initially loaded RNA was denoted as m_{loaded} . The initial RNA loading efficiency was then calculated as $(m_{\text{loaded}} - m_{\text{washed}})/m_{\text{loaded}} \times 100\%$. Following that, the fibers were incubated in 3 ml of PBS at 37 °C for up to 30 days. At indicated time points, 1 ml of supernatant was collected and replaced with 1 ml of fresh PBS. The concentration of RNA in the supernatant was then measured using the same protocol as above. To determine RNA distribution, 488-labeled RNA was loaded onto the fibers in the same manner as indicated above, followed by visualization under a confocal microscope (Zeiss LSM710).

2.7. Real-time PCR

At day 2 post-transfection, OPC were lysed by Trizol[®] reagent and RNA was extracted using RNeasy Mini Kit. Thereafter 100 ng of RNA was used for reverse transcription using M-MLV transcriptase according to the manufacturer's protocol. Real-time PCR was carried out using iQ SYBR Green Supermix in a StepOnePlus[™] system (Applied Biosystems, USA) with the following program: 10 min at 95 °C, 15 s at 95 °C followed by 1 min at 60 °C for 40 cycles. The sequences of the primers are shown in Table 2 and β -actin was used as the housekeeping gene. All our primers showed similar amplification efficiency under the parameters used. Hence the Ct method was used for fold change analysis. All the results were normalized by two-dimensional control group, in which OPCs cultured on conventional poly-D, L-ornithine (p-DL-o) coated coverslips were transfected with bolus delivery of scrambled miR.

2.8. Immunofluorescent staining

At indicated time points, the samples were fixed with 10% formalin, permeabilized with 0.1% Triton-X 100 in PBS for 20 min and blocked with 1.5% goat serum at room temperature for 1 h. Thereafter, samples were incubated with primary antibody overnight at 4 °C followed by secondary antibody for 1 h at room temperature. The primary antibodies used were: mouse anti-MBP (1:200), mouse anti-RIP (1:500) and rabbit anti-Olig2 (1:1000). The secondary antibodies used were: Alexa-Fluoro 488 goat anti-mouse (1:700) and Alexa-Fluoro 555 goat anti-rabbit (1:1000). Nuclei were counterstained with DAPI. Samples were mounted and imaged under a confocal microscope (Zeiss LSM710). At least 700 cells were counted in each sample using ImageJ. We counted the number of Olig2-positive cells that were within RIP or MBP positive signals [10]. The proportion of RIP⁺ or MBP⁺ cells was then expressed as percentage of Olig2⁺ oligodendrocyte lineage cells, which indicates all stages of oligodendrocytes.

2.9. Statistical analysis

All values were represented as mean $\pm$ standard error of mean (S.E.M.). After F-test, one-way ANOVA and Turkey post-hoc tests were used when samples had equal variance. Otherwise the Kruskal–Wallis and Mann–Whitney *U* tests were used for comparisons between more than two groups. For paired comparisons, student's *t*-test was used.

3. Results

3.1. Characterization of PCL fibers

Electrospun scaffolds with different average fiber diameters ($\sim 2\ \mu\text{m}$ vs $\sim 700\ \text{nm}$ vs $\sim 300\ \text{nm}$, $p < 0.001$, Table 1) and orientation (random vs aligned) were prepared (Fig. 1A–D). There was no significant difference among the thickness of all fiber scaffolds (Supplementary Fig. 1). After DOPA coating, $700\ \text{nm}$ AF and $2\ \mu\text{m}$ RF scaffolds had larger water contact angle as compared with $300\ \text{nm}$ AF and $2\ \mu\text{m}$ AF scaffolds (Supplementary Fig. 2) As shown in Fig. 2, the distribution and loading efficiency of RNA was similar amongst all experimental groups regardless of fiber diameter and orientation. Specially, a loading efficiency of $\sim 98.7\%$ was achieved for all scaffolds (Fig. 2E). As shown in Fig. 2F, the initial burst release of RNA was $\sim 3\%$ for all experimental groups. After that, a sustained release of RNA was obtained for 3 weeks on all scaffolds. The ultimate accumulative release of RNA from the $700\ \text{nm}$ AF group was less than 20% , which is relatively lower than the other three groups. On $300\ \text{nm}$ AF and $2\ \mu\text{m}$ AF scaffolds, $\sim 30\%$ RNA was released during 3 weeks, which was slightly higher than $2\ \mu\text{m}$ RF group ($\sim 24\%$).

3.2. Viability of OPC on fiber scaffolds

The representative live–dead assay images of all groups are shown in Fig. 3A. Quantification of these images indicated no significant difference in live cell percentage between day 4 and day 7 within each experimental group (Fig. 3B). In $2\ \mu\text{m}$ RF group, significantly higher proportions of live cells were detected at both time points, as compared to $700\ \text{nm}$ AF and $300\ \text{nm}$ AF groups. However, no significant difference was observed with respect to $2\ \mu\text{m}$ AF group.

3.3. Target gene knockdown

In our preliminary studies, we compared target gene knockdown in cells that were cultured on fiber scaffolds that were treated with scrambled miR vs. plain scaffolds without any miR treatment. As shown in Supplementary Fig. 3, there was no significant difference in target gene expression between these two treatments. Coupled with the fact that scrambled miR transfection is often used as the standard control group in most studies involving miR delivery, instead of samples without any miR treatment [6,10,26–28], we used scrambled miR-treated scaffolds as controls in all subsequent experiments.

Fig. 4 shows the effects of substrate topography and miR treatment on target gene knockdown in OPCs after 2 days of fiber-mediated miR reverse transfection. The values of gene expression were normalized by the mRNA level of cells that were cultured on 2D coverslips with scrambled miR bolus transfection. Therefore, 100% knockdown efficiency represents a normalized relative mRNA expression level of 0% on the experimental sample

vs. 2D coverslip controls. The knockdown efficiencies of four downstream targets of miR-219 and miR-338, FoxJ3, Sox6, ZFP238 and PDGFR- α , were evaluated. Generally speaking, miR cocktail treatment resulted in more significant silencing of these targets than miR-219 treatment alone, regardless of fiber diameter or orientation.

To evaluate miR-treatment outcomes, gene knockdown efficiencies were compared with the respective Scrambled miR treated samples. As shown in Fig. 4, when treated with miR-219, no significant enhancement in gene silencing vs. scrambled miR treatment was observed regardless of fiber diameter. When treated with miR-219/miR-338 cocktail, 300 nm AF showed significantly higher silencing efficiency for all 4 repressors ($p < 0.05$). On 700 nm AF, three of these repressors, PDGFR- α , ZFP238 and Sox6, were efficiently knocked down once treated with cocktail miRs. On 2 μ m AF, 54% PDGFR- α knockdown ($p < 0.01$) and 64% FoxJ3 knockdown ($p < 0.01$) were achieved when treated with miR-219/miR-338. However, on 2 μ m RF, only one inhibitor, Sox6, was efficiently knocked down with a silencing efficiency of 46% under miR-219/miR-338 treatment.

To evaluate scaffold topographical effects in the absence of gene silencing, gene expression changes were evaluated after treatment with scrambled miRs. In the case of fiber diameter effect, similar extent of Sox6 knockdown efficiency was achieved in all the experimental groups (25–30%), regardless of fiber diameter. For PDGFR- α and ZFP238, 700 nm AF demonstrated higher knockdown efficiency than 2 μ m AF and 300 nm AF samples. However, the lowest gene knockdown of FoxJ3 was observed on 700 nm AF. Comparatively, 2 μ m AF achieved better gene silencing efficiencies for PDGFR- α ($p < 0.05$) and FoxJ3 ($p < 0.05$) than 300 nm AF. In the case of fiber orientation effects, the silencing efficiency of all targets genes, except Sox6, on 2 μ m RF appeared significantly higher ($p < 0.05$) than 2 μ m AF. Overall, as compared to conventional 2D bolus transfection of scrambled miR ($y = 0$ in Fig. 4), fiber topography reduced the expressions of target genes (except 700 nm AF on FoxJ3) regardless with fiber diameter or orientation ($p < 0.05$ for Scrambled miR). This implied that fiber topography alone enhanced OL differentiation by repressing one or more inhibitors.

Further gene silencing was achieved when different topographic cues acted in synergy with miR treatment. MiR-219 provided better Sox6 knockdown efficiencies ($p < 0.05$) on smaller fibers (700 nm AF and 300 nm AF) than on larger fibers (2 μ m AF). Cocktail treatment of miR-219/miR-338 exhibited higher ZFP238 ($p < 0.001$) but lower FoxJ3 ($p < 0.001$) silencing on 700 nm AF, as compared to 300 nm AF and 2 μ m AF. Synergistic effect of fiber orientation and cocktail treatment only affected the expression of ZFP238, where the silencing was significantly ($p < 0.001$) higher on 2 μ m RF (71%) than 2 μ m AF (60%).

3.4. Immunostaining of OPC differentiation and maturation markers

a) Effects of fiber-mediated miR transfection on OPC differentiation—To evaluate the extent of OPC differentiation, the expression levels of RIP, an immature OL marker, were analyzed as shown in Fig. 5. In general, miR-219 treatment significantly enhanced RIP expression only when coupled with 300 nm AF ($p < 0.01$). In contrast, miR-219/miR-338 cocktail treatment demonstrated positive effects on OPC differentiation in all experimental groups.

As shown in Fig. 5B, in the absence of gene silencing, fiber orientation played a more significant role in promoting OPC differentiation. Specifically, when treated with scrambled miR, randomly oriented fibers significantly enhanced the percentage of RIP⁺ cells, while similar extent of RIP expression was obtained within all aligned fiber groups.

When coupled with miR-219 treatment, aligned fibers with smaller diameter promoted OPC differentiation (300 nm AF vs. 700 nm AF and 2 μ m AF, $p < 0.05$). However, the effects of fiber orientation remained the dominant factor. Specifically, in 2 μ m RF group, RIP⁺ cell percentage remained the highest, although the difference with 300 nm AF decreased. Once synergized with cocktail treatment, no significant fiber diameter or orientation effect was observed, although comparatively, 2 μ m RF facilitated significantly higher extent of OPC differentiation than 700 nm AF ($p < 0.05$). Altogether, without gene silencing, 2 μ m randomly oriented fibers resulted in the highest percentage of RIP-positive cells among all experimental groups. However, with miR treatment, 300 nm fibers gave similar percentage of RIP-positive cell number as 2 μ m RF.

b) Effects of fiber-mediated miR transfection on OL maturation—MBP is specifically expressed by mature OL in the later stage of OL development. Here, we evaluated its expression levels as an indicator of the extent of OL maturation. In general, as shown in Fig. 6, in all experimental groups, miR-219 and miR-219/miR-338 cocktail treatments increased MBP⁺ cell percentage as compared to scrambled miR, which indicated enhanced OL maturation.

At both time points, with respect to scrambled miR incorporation, 2 μ m AF group significantly promoted MBP expression (~8.5%) as compared to 700 nm AF (~3.5%) or 300 nm AF (~2.3%) groups ($p < 0.01$, Fig. 6B). A slightly higher portion of MBP⁺ cell was detected on 2 μ m RF (~9.5%), although the results were not significantly different as compared to 2 μ m AF.

Once coupled with miR-219, the efficiencies of smaller fiber-mediated OL maturation increased (~5.5% on day 4 and ~7% on day 7). However, such differentiation extents remained lower than that obtained on larger fibers on day 4 (~11%, $p < 0.01$ for 2 μ m AF) as well as on 2 μ m RF on day 7 (~12%, $p < 0.05$). Cocktail treatment of miR-219/miR-338 further facilitated OL maturation on all scaffolds. In particular, 2 μ m AF group continued to enhance OL maturation more than 300 nm AF and 700 nm AF ($p < 0.05$) at both time points. Notably, in the case of OL maturation, aligned fibers appeared more beneficial by promoting more MBP⁺ cells in 2 μ m AF group than 2 μ m RF ($p < 0.05$).

Altogether, larger fiber diameter promoted OL maturation. Coupled with miR mediated gene silencing, the fiber diameter effects remained unaltered. In fact, the synergistic effect of miR-219/miR-338 appeared more obvious on large aligned fibers. The best condition to facilitate OL maturation was 2 μ m AF integrated with cocktail miR-219/miR-338.

4. Discussion

Within the CNS, OL is crucial in maintaining the normal structure and function of myelin sheaths that are formed around axons. Failure to myelinate axons appropriately can result in

defects in neuronal signal transduction and ultimately functional impairment as frequently seen in demyelinating lesions like TNI and multiple sclerosis. Hence, methods that provide control over OPC differentiation and OL maturation are attractive and useful for treating neurological conditions that require remyelination.

Due to topographical similarity between the natural structure of axons and nanofibers, electrospun fibers have been used to mimic OL-axon interactions, making them promising platforms for understanding neuronal–glial interaction and OL myelination [29,30]. In this study, we further expanded the use of electrospun fiber substrates and demonstrated that enhanced OL differentiation and maturation may be achieved by modulating scaffold fiber diameter and orientation, along with effective miR-based gene silencing.

The effective absorption (Fig. 2A–E) of RNA onto DOPA-coated nanofibers resulted in sustained release (Fig. 2F) in all the scaffolds. From day 4, different amount of RNA was released from different scaffolds, which was likely attributed to the differences in hydrophilicity of the samples. In particular, the more hydrophobic 700 nm AF and 2 μ m RF scaffolds with significantly larger water contact angles (Supplementary Fig. 2), demonstrated slower release kinetics than the other two experimental groups. However, the release profiles did not appear to affect gene silencing efficiency and subsequent differentiation outcomes in similar fashion. It is possible that the amounts of miRs that were released from all scaffolds had already reached a minimum threshold [31,32] that is required for repressing target gene expression and for OPC differentiation, although, there is also another possibility that OPCs may have taken up miR directly from the scaffolds, as seen with mouse derived neural progenitor cells [19]. Hence, further detailed work should be carried out using fluorescently labeled nanoparticles by live cell imaging to elucidate the exact cellular uptake mechanisms to provide further insights.

As shown in Fig. 4, fiber topography alone (with scrambled miR), regardless of diameter and orientation, down-regulated the expressions of inhibitory regulators (except FoxJ3 on 700 nm AF) as compared to 2D culture (indicated as $y = 0$ in Fig. 4). PDGFR- α is the only receptor that interacts with both PDGF and bFGF to enhance OPC proliferation and prevent OL differentiation [33,34] via at least two signaling pathways—MAPK and PKA [35]. In Fig. 4, with scrambled miR treatment, the smallest fiber, 300 nm AF, achieved the poorest PDGFR- α knockdown, as compared with other groups. This is quite consistent with the result of OL maturation marker, MBP, expression as shown in Fig. 6. As for PDGFR- α gene silencing, 700 nm AF was better than 2 μ m AF, but the effect was possibly eliminated by the 2.5-fold higher expression of FoxJ3, a newly discovered repressor of the OPC-OL transition [6]. Such may be a reason why 2 μ m AF achieved more percentage of MBP⁺ cells (Fig. 6). 2 μ m RF inhibited gene expression of most regulators (except sox6) more than 2 μ m AF, which may have resulted in higher portion of RIP⁺ cells in Fig. 5. These results confirmed our previous finding that fiber topography alone could enhance Schwann cell maturation [17] and OPC differentiation [25], which may be beneficial for remyelination applications. Furthermore, when coupled with miR-219, 2 μ m RF still achieved lowest gene expression of most inhibitors (except sox6), which resulted in the best outcome of RIP⁺ and MBP⁺ cells (Figs. 5 and 6). Cocktail treatment with miR-219/miR-338 further reduced the expressions

of those inhibitory factors by up to 50–60%, hence the expressions of RIP and MBP were enhanced accordingly.

RIP is an early-stage marker of OPC differentiation. Comparatively, the highest proportion of RIP⁺ cells obtained on 2 μ m RF suggested that fiber orientation played a critical role in OPC differentiation. However, the miR treatment, especially miR-219/miR-338 cocktail treatment, had more influence on aligned fibers. When transfected with miR-219, RIP expression by OPCs on 300 nm AF was increased by 1.4 fold, which was better than the other three groups. And with miR-219/miR-338 cocktail treatment, the enhancement in smaller aligned fibers were better (Fig. 5B, 300 nm AF vs 700 nm AF vs 2 μ m AF vs 2 μ m RF = 1.54 fold vs 1.34 fold vs 1.32 fold vs 1.22 fold). Altogether, in a more inducible environment such as random fibers, miRs appeared to have more limited ability in promoting OPC differentiation, as compared to aligned fibers.

MBP is a late myelin marker for indicating OL maturation. Regardless of miR treatment, large-diameter (2 μ m AF and 2 μ m RF) induced more MBP expression ($p < 0.05$), as compared to 300 nm AF and 700 nm AF. The effect of fiber orientation on OL maturation was only reflected with incorporation of cocktail miR treatment. As shown in Fig. 6, once coupled with miR-219/miR-338, the percentage of MBP⁺ cells on 2 μ m AF was ~1.5 times of 2 μ m RF on day 7. Regarding the extent of MBP⁺ cell increment, small fiber-mediated miR-219 or cocktail miR-219/miR-338 treatment appeared better than large fibers, increasing by 3–5 fold (on 300 nm AF and 700 nm AF) *versus* 1–2 fold (on 2 μ m AF and 2 μ m RF). The difference might be due to the different inductivities of different fibers. The large fibers were more inductive with a higher basal level of MBP positive cells (8–9%), while on small fibers, the basal levels were only 2–3% (Fig. 6B). Taken together with the similar phenomenon observed in RIP expression, miRs were more capable in enhancing OPC differentiation in a less inductive environment, which is consistent with previous report [6]. Hence, miR mediated OPC differentiation and OL maturation may find useful applications for disease treatment *in vivo*, where specific inductive factors are often missing.

Fiber diameter and orientation affected gene uptake and gene silencing in our previous study [22]. Similarly, in this study, we observed a dependence of OPC differentiation and maturation on fiber scaffold architecture. To date, many nanotopographical surfaces have been developed to mimic the architectural features of stem cell niches. While alterations in cell fate in response to different topographical features have been observed, many of the underlying biophysical mechanisms remain unclear [36]. Among many potential pathways analyzed, H3 acetylation as regulated by histone deacetylase (HDAC) [11] has been identified to play critical roles. HDAC activity is also required for OPC differentiation [37], and has been seen to affect the timing of OPC differentiation [38]. Hence, given the roles of HDAC, future works may focus on elucidating the involvement of HDAC in nanofiber-mediated OPC development.

5. Conclusion

In the present study, we explored the effects of different topographical cues, including diameter and orientation, on fiber-mediated miR delivery systems in directing OL

differentiation and maturation. Larger fibers and random fibers are more potent in promoting OPC differentiation and OL maturation. However, when coupled with miRs, aligned fibers are better. Specifically, smaller aligned fibers benefited OPC differentiation, while larger aligned fibers favored OL maturation. Altogether, the synergistic application of topographic cues and nanofiber-mediated miR reverse transfection may be a promising strategy for treating neural pathological diseases that require remyelination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2015.08.029>.

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Fig. 1. Scanning electron micrographs of (A) 300 nm AF, (B) 700 nm AF; (C) 2 μm AF and (D) 2 μm RF.

Fig. 2. RNA distribution on (A) 300 nm AF, (B) 700 nm AF, (C) 2 μm AF and (D) 2 μm RF scaffolds; (E) RNA loading efficiencies (Mean ± S.E.M); (F) RNA cumulative release profile (Mean ± S.E.M).

Fig. 3. Live-dead assay results. (A) representative fluorescent microphotographs and (B) quantification of live OPCs %. * $p < 0.05$ (ANOVA, mean $\pm$ S.E.M., $N = 3$).

Fig. 4.

Target gene knockdown efficiency on day 2 with respect to 2D bolus transfection of scrambled miR (mean $\pm$ S.E.M., N = 3). * $p < 0.05$ (ANOVA), ** $p < 0.01$ (ANOVA) and *** $p < 0.001$ (ANOVA) compared with respective Scrambled miR group. # $p < 0.05$ (ANOVA), ## $p < 0.01$ (ANOVA) and ### $p < 0.001$ (ANOVA) compared with respective miR-219 group. + $p < 0.05$ (ANOVA or Mann-Whitney *U*), ++ $p < 0.01$ (ANOVA) and +++ $p < 0.001$ (ANOVA) between two indicated groups.

Fig. 5. Immunofluorescent staining (day 4) of immature OL marker, RIP, indicating enhanced differentiation on PCL scaffolds with different diameters. (A) Representative fluorescent images and (B) quantification of RIP⁺ cells among Olig2⁺ oligodendrocyte lineage cells. **p < 0.01 and ***p < 0.001 *versus* respective Scrambled miR group. ##p < 0.01 *versus* respective miR-219 group. +p < 0.05 and ++p < 0.01 between two groups with same miR treatment. (ANOVA, mean $\pm$ S.E.M., N = 3).

Fig. 6.

Immunofluorescent staining of mature OL marker, MBP, indicating enhanced maturation on PCL nanofibers (day 4 and day 7). (A) Representative fluorescent images, and (B) quantification of MBP⁺ cells among Olig2⁺ oligodendrocyte lineage cells. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus respective Scrambled miR group; # $p < 0.05$ versus respective miR-219 group; + $p < 0.05$, ++ $p < 0.01$ and +++ $p < 0.001$ between two indicated groups. (ANOVA, mean $\pm$ S.E.M., $N = 3$) \$ $p < 0.05$ between day 4 and day 7 (Student's- t test).

Table 1

Parameters for electrospinning PCL fibers.

	2 μ m RF	2 μ m AF	700 nm AF	300 nm AF
PCL concentration (wt%)	14%	15%	12%	12%
Solvent	TFE	TFE	TFE	TFE:TE (1 $\times$) = 4:1
Needle	21 G	21 G	21 G	22 G
Flow rate (mL/h)	1.5	3.0	1.0	1.0
Distance (cm)	12	11	12	12
Rotating speed (rpm)	200	2100	2600	2400
Voltage (kV)	+12/-4	+12/-4	+10/-4	+10/-4
Diameter (nm)	2095.1 $\pm$ 31.3	2003.6 $\pm$ 38.7	690.0 $\pm$ 10.5 ***	291.8 $\pm$ 3.7 ***.###

p < 0.001 compared with 2 μ m groups.

p < 0.001 compared with 700 nm group.

Table 2

Sequences of primers for real-time PCR.

β-actin	Forward	5'-ACGGTCAGGTCATCACTATCG-3'
	Reverse	5'TGCCACAGGATTCCATAACCCAG-3'
PDGFR-α	Forward	5'-CGTCTGGTCTTATGGCGTTCTG-3'
	Reverse	5'-TCTCTTTTCGGGTTCAGTGTCC-3'
FoxJ3	Forward	5'-TCAGTTCTTCACACAGACGGGC-3'
	Reverse	5'-TATGAGGATAACCAGGGGGTGG-3'
ZFP238	Forward	5'-TGAAGACGAAGGCGAAGATGAC-3'
	Reverse	5'-AGGGGCTGGCTACTGTITTTCC-3'
Sox6	Forward	5'-TGGTATGAAGATGGACGGCG-3'
	Reverse	5'-TGTTGTTGTTGTTGGGAAA-3'