Mussel-Inspired Modification of Nanofibers for REST siRNA Delivery: Understanding the Effects of Gene-Silencing and Substrate Topography on Human Mesenchymal Stem Cell Neuronal Commitment

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Mussel-inspired modification of nanofibers for REST siRNA delivery:
Understanding the effects of gene-silencing and substrate topography on
human mesenchymal stem cell neuronal commitment

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Abstract

This study endeavors to direct neuronal differentiation of human mesenchymal stem cells (MSCs) through sustained release of siRNA targeting RE-1 silencing transcription factor (REST) from a nanofibrous microenvironment. Electrospun poly (ϵ -caprolactone) (PCL) nanofibers were surface modified with mussel-inspired DOPA-melanin (DM) coating for adsorption of REST siRNA. DM modification increased the siRNA loading efficiency and significantly reduced the initial burst release as compared to untreated PCL fibers. In addition, fiber alignment and DM modification induced greater REST knockdown efficiencies than randomly-oriented and untreated PCL nanofibers respectively. Under non-specific differentiation condition, synergistic REST silencing and nanofiber topography up-regulated neuronal markers of differentiated MSCs while reducing glial cell commitment. In neuronal specific medium, MSCs differentiated faster into mature neuronal cells, but showed modest improvement in neuronal differentiation with REST knockdown and fiber alignment. Such scaffolds may find useful applications in enhancing MSCs neuronal differentiation under non-specific conditions such as an in vivo microenvironment.

1. Introduction

Damage to the central nervous system (CNS) by chronic degenerative diseases and acute trauma often results in irreversible loss of neural functions in patients. Stem cell therapy and tissue engineering are possible treatment strategies to replace damaged neurons and facilitate axonal regeneration. Mesenchymal stem cells (MSCs) represent a clinically viable cell source due to their relative abundance, ease of accessibility, absence of teratoma formation and reduced ethical concerns as compared to neural stem cells (NSCs), embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Importantly, numerous studies have demonstrated the capacity of MSCs for cross-lineage neuronal differentiation on planar substrates ^[1-10] and three-dimensional (3D) scaffolds ^[11-15]. However, these studies were largely dependent on protein and biochemical cocktails to direct MSCs neuronal commitment. Small interfering ribonucleic acids (siRNAs) may serve as a better alternative to these traditional classes of drugs and protein therapeutics due to the ease of synthesis, lower costs of production, and higher target specificity ^[16]. By incorporating siRNAs into scaffolds, prolonged silencing of inhibitory factors against desired lineage commitment and combined scaffold topographical effects may potentially enhanced the efficiency of directed MSCs differentiation to neural cells.

One possible factor that could be targeted for MSCs neuronal differentiation is the RE-1 silencing transcription factor (REST). In general, REST is a transcriptional repressor for a myriad of neuronal specific genes ^[17, 18], and is known to down-regulate during endogenous neurogenesis ^[19, 20]. Several studies have also demonstrated the feasibility of enhancing stem cell neuronal differentiation through the deliberate silencing of REST ^[21, 22]. However, only two studies attempted REST silencing of MSCs; of which, neither was conducted with a scaffold environment and both harbored the potential risk of immunogenicity and tumor

transformation from the MSCs-derived neuronal cells due to the use of knockout mutant ^[4] and lentiviral transduction ^[5] respectively. These drawbacks may be overcome by adopting a safer and more dynamic approach of nanofiber-mediated REST silencing. Besides recapitulating the structural organization of the natural extracellular matrix (ECM) ^[23, 24], the use of nanofibers also present a characteristic high surface area to volume ratio that potentially allows greater incorporation and maximal exposure of REST siRNAs to seeded MSCs. In addition, by controlling fiber diameter and orientation, gene silencing efficiency may be optimized ^[25].

The functionalization of nanofibers with siRNAs may be achieved through mussel-inspired adhesive coatings due to its facile chemical reactivity towards biomolecules ^[26, 27]. Recent studies including our previous work have successfully utilized this strategy for the incorporation and transfection of nucleic acids into cells ^[28, 29]. Therefore, in this study, REST siRNA, complexed with transfection reagent, TransIT-TKO, was incorporated onto poly (ϵ -caprolactone) (PCL) nanofibers by mussel-inspired coating of 3,4-dihydroxy-L-phenylalanine (DOPA)-melanin (DM) to direct MSC neuronal commitment. By examining the differentiation outcomes of MSCs cultured under different differentiation conditions (non-specific vs. neuronal), we evaluated the potential of nanofiber-mediated REST silencing in directing MSCs neuronal commitment.

2. Materials and methods

2.1. Materials

REST siRNA (siREST) (ID #: S11933), non-targeting, negative siRNA (siNEG) (Catalog #: AM4635) and nuclease-free water were purchased from Ambion. The transfection reagent, TransIT-TKO, was obtained from Mirusbio. Diethylpyrocarbonate treated Tris-EDTA buffer

(DEPC-treated TE Buffer) was purchased from 1st Base, Singapore. M-MLV Reverse transcriptase (M3681) was purchased from Promega. iQ SYBR Green Supermix was purchased from Bio-Rad. 2, 2, 2-trifluoroethanol (TFE) was purchased from Acros Organics. Polycaprolactone (PCL, Mw:70000-90000), 3,4-Dihydroxy-L-phenylalanine (DOPA), chloroform, 10% formalin, tris-buffered saline (TBS), Triton X-100, fluoromount, bovine serum, albumin (BSA) were purchased from Sigma-Aldrich. Fetal bovine serum (FBS) was obtained from Hyclone. Goat anti-nestin was purchased from Santa Cruz. Rabbit anti-microtubule associated protein-2 (MAP2), chicken anti-glial fibrillary acidic protein (GFAP), mouse anti-O4, anti-Olig2 and anti-ALDH1L1 were purchased from Millipore. Rabbit anti- β III tubulin (Tuj1) was purchased from Covance. All other reagents were purchased from Life Technologies.

2.2. Human fetal mesenchymal stem cells (MSCs) isolation and cell culture

Human tissue collection for research purposes was approved by the Domain Specific Review Board of National University Hospital Systems (DSRB-D-06-154), in compliance with International Guidelines regarding the use of fetal tissue for research (Polkinghorne) as previously described^[30]. In all cases, patients gave separate written consent for the use of the collected tissue. Human fetal MSCs were isolated from bone marrow as previously described^[31]. Briefly, single-cell suspensions were prepared by flushing the bone marrow cells from femurs using a 22-gauge needle, passed through a 70 μ m cell strainer, and plated at 10^6 cells per milliliter. Adherent spindle-shaped cells were recovered from the primary culture after 4 to 7 days. At subconfluence, they were trypsinized and replated at 10^4 cells per square centimeter. MSCs were maintained in proliferative medium consisting of Dulbecco's Modified Eagle Medium (DMEM, Cat. #: 10569-010, Life Technologies), 10% FBS and 1%

1 penicillin-streptomycin. All cells were maintained in a humidified incubator at 37 °C with 5%
2 CO₂ under sterile conditions. The MSCs were kept within passage 6 to 9 to ensure similar
3 cellular activity for subsequent siRNA transfection and differentiation studies.
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10 **2.3. Electrospinning of randomly-oriented and aligned PCL nanofibers**

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12 To obtain randomly-oriented fibers (RF), PCL was dissolved in a mixed solvent
13 containing TFE and DEPC-treated TE buffer (5:1 v/v ratio) to obtain a 12 wt% polymer
14 solution. Thereafter, the solution was electrospun at a constant flow rate of 1.4 ml/h (New Era
15 Pump) onto a rotating collector (200 rpm). For aligned fibers (AF), PCL was dissolved in TFE
16 only, and electrospun at a flow rate of 0.8 ml/h onto the rotating collector (2000 rpm). For all
17 fibers, the collecting distance was maintained at 12 cm, with a voltage of +10kV and - 4kV
18 (Gamma High Voltage Research, USA) applied to the polymer solution and rotating collector
19 respectively. Prior to all experiments, nanofibrous meshes were cut with a circular punch into
20 an area of 1.9 cm² to fit into the wells of 24-well plates (Nunc).
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41 **2.4. DOPA-melanin coating and siRNA adsorption**

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43 PCL scaffolds were modified with DOPA-melanin coating as described ^[32]. DOPA was
44 dissolved in a buffer solution of 10 mM bicine in 250 mM NaCl (pH 8.5) to a concentration of
45 10 mg/ml. All scaffolds were rinsed with deionized water before immersing in 1 ml of DOPA
46 solution for 6 h on an orbital shaker (100 – 120 rpm, Sartorius Certomat® R, Germany).
47 Thereafter, DM-modified PCL (DM-PCL) scaffolds were rinsed thoroughly with deionized
48 water to remove any residual DOPA and lyophilized overnight. To obtain scaffolds that
49 incorporated siRNA or Cy5-labelled oligonucleotides (Cy5-ODN), 2 or 4 µg of siRNA
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(~3 or 6 μ l from a stock concentration of 50 μ M) was complexed with transfection reagent, TKO, at 1:1 v/v ratio in 50 or 100 μ l of DMEM respectively. The mixture was then incubated for 10 – 15 min at room temperature. Thereafter, the siRNA/TKO complexes were loaded onto the scaffolds and incubated for 30 min at 37 C for adsorption. Finally, the scaffolds were rinsed once with 500 μ l nuclease-free water to remove residual un-adsorbed siRNA.

2.5. Surface characterization of DM-PCL nanofibers

The surface elemental composition was analyzed by X-ray photoelectron spectroscopy (XPS) as reported previously ^[29]. All samples were vacuum-dried overnight prior to analysis. XPS spectra were obtained using Al K α X-ray source (1486.6 eV photon) at 200 ms dwell time with a step size of 0.5 eV for survey scan and 0.04 eV for high resolution element scan. The X-ray source was run at 300 W (15 kV and 20 mA) under 10⁻⁹ Torr of analysis chamber pressure during measurement. Morphological characterization was evaluated by scanning electron microscopy (SEM) (JOEL, JSM-6390LA, Japan) after sputter-coating with platinum. The average fiber diameters were determined using Image J (NIH, USA) by measuring 100 fibers per sample.

2.6. In vitro characterization of siRNA-loaded PCL and DM-PCL nanofibers: Zeta potential, loading efficiency, release kinetics and Cy5-ODN distribution

Zeta potential of siRNA/TKO complexes (prepared as described in section 2.4 in 1 ml deionized water) was characterized using Zetasizer Nano ZS (Malvern Instruments, UK). Randomly-oriented and aligned PCL and DM-PCL nanofibers (4 different scaffolds, n = 3 each) loaded with 2 μ g siRNA/TKO complexes were rinsed with 500 μ l of nuclease-free

water. The wash solution containing residual siRNA was retrieved before incubating the scaffolds in 3 ml of PBS at 37 °C for 40 days under gentle orbital shaking (70 - 90 rpm, Sartorius Certomat® R, Germany). At predetermined time points, 1 ml of supernatant was collected and replaced with an equal volume of fresh PBS. SiRNA concentration in the supernatant and wash solution were determined using RiboGreen® assay after treating with 1 mg/ml heparin solution to de-complex siRNA from the cationic TKO transfection reagent. Fluorescence intensity was measured using a microplate reader (Tecan®, Infinite 200, Austria). The experimental siRNA loading efficiency was computed using the following equations:

$$\text{Mass of siRNA loaded (ng)} = \text{Theoretical siRNA mass (ng)} - \text{Residual siRNA mass (ng)}$$

$$\text{Loading efficiency} = \frac{\text{Mass of siRNA loaded (ng)}}{\text{Theoretical mass of siRNA loaded (ng)}} \times 100\%$$

Meanwhile, the cumulative siRNA release was expressed as a percentage of the mass of siRNA loaded. For nucleic acid adsorption and distribution analysis, Cy5-ODN/TKO complexes were loaded onto the scaffolds as described above and imaged using confocal microscopy (Zeiss, LSM 710 Meta Laser Scanning Confocal Microscope, Germany) immediately after adsorption.

2.7. Transfection and differentiation of MSCs on siRNA loaded nanofibers

Scaffold-mediated transfection was evaluated with siRNA/TKO-loaded PCL nanofibers (denoted as ‘siRNA PCL-RF’ or ‘siRNA PCL-AF’ for randomly-oriented and aligned fibers respectively) and DM-PCL nanofibers (denoted as ‘siRNA DM-RF’ or ‘siRNA DM-AF’ for randomly-oriented and aligned fibers respectively). All scaffolds were sterilized under

1 ultraviolet radiation for 1 h before loading of siRNA/TKO complexes. MSCs were then
2 seeded at a density of 5.0×10^4 cells per scaffolds in 1 ml media containing DMEM with 1%
3 FBS. For non-specific differentiation, cells were maintained in this media throughout the
4 duration of the study. For neuronal differentiation condition, the media was replaced with
5 induction media containing DMEM, 1% FBS, 1% N2 supplement and 1% B27 supplement 72
6 h after initial cell seeding. Cells were differentiated for up to 14 or 21 days, with the media
7 refreshed every 3 days. Scaffolds adsorbed with siNEG/TKO complexes (denoted as 'siNEG
8 control') were used as negative control. The entire experiment was repeated at least three
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26 **2.8. Real-time PCR analysis**

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29 At specific time points, cells were lysed using TRIzol® reagent and pooled together to
30 obtain sufficient RNA for analyses (n=4 scaffolds). Reverse transcription was carried out
31 using M-MLV Reverse transcriptase according to the manufacturer's protocol. Real-time PCR
32 analysis was performed using iQ SYBR Green Supermix in a StepOnePlus™ Real-time
33 PCR system (Applied Biosystems) with β -actin as the housekeeping gene. The sequences of
34 the primers used are listed in Table 1. The real-time PCR cycling condition used was: 10 min
35 at 95 °C, 40 cycles at 95 °C for 15 s, followed by 60 °C for 1 min. Our preliminary studies
36 showed that these primers had similar amplification efficiency under the parameters used.
37 Therefore, the $\Delta\Delta$ CT method was used for fold change analysis. For the evaluation of REST
38 knockdown efficiency, results were normalized with respect to siNEG controls. Unless
39 otherwise stated, all other results were normalized with respect to undifferentiated
40 MSCs on TCPS.
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2.9. Immunofluorescence staining and quantification

For immunofluorescence staining of nestin, Tuj1, MAP2, synapsin, GFAP, olig2 and ALDH1L1, cells were fixed in 10% formalin, permeablized with 0.3% Triton X-100 in PBS for 15 min and blocked overnight with blocking buffer containing 3% BSA and 5% FBS at 4°C. For oligodendrocyte marker, O4, samples were fixed and blocked as indicated above without permeabilization in Triton X-100 to prevent the antigen from dissolving out of the cellular membranes. Samples were then incubated sequentially with primary antibodies for 2 h and secondary antibodies for 1 h at room temperature. The primary antibodies used were: goat anti-nestin (1:200), rabbit anti-Tuj1 (1:1000), mouse anti-Tuj1(1:500), rabbit anti-MAP2 (1:1000), rabbit anti-synapsin (1:2000), chicken anti-GFAP (1:500), mouse anti-ALDH1L1, mouse anti-olig2 and mouse anti-O4 (1:500). Due to basal expression of GFAP in MSCs, ALDH1L1 was included as an alternative astrocytic marker. Anti-Olig2 was used to stain samples at early (Day 7) and mid time points (Day 14) while O4 was used to stain samples at the later time points (Days 14, 21). The secondary antibodies used were: Alexa-Fluor 633 goat anti-mouse (1:500), Alexa-Fluor 488 goat anti-rabbit (1:500), Alexa-Fluor 488 donkey anti-goat (1:500) and Alexa-Fluor 568 goat anti-chicken. Nuclei were counterstained with DAPI. Samples were then mounted on glass slides and imaged by confocal microscopy under 40× and 63× magnification using the same laser settings. To quantify the neural marker expressions, the fluorescence intensity of each Tuj1⁺, MAP2⁺ or GFAP⁺ cell was measured using ImageJ after subtracting the background signals. All fluorescence intensity measurements were then normalized with the mean fluorescence intensity of respective markers expressed in undifferentiated MSCs. At least 50 cells were measured per sample group.

2.10. Western blot analysis

For western blot analysis, bolus transfection of MSCs with 2 µg siRNA/TKO complexes on 2D was included as a positive control for REST knockdown. Briefly, MSCs were seeded on TCPS at a density of 5.0×10^4 cells per well in 1 ml media containing DMEM with 1% FBS. After 24 h, the cells were transfected with siRNA/TKO complexes for another 72 h. Western blot was performed as previously described with slight modifications [22]. Briefly, total soluble protein from six scaffolds was extracted by RIPA buffer and protein concentrations were measured with BCA assay kit according to manufacturer's protocol. Thereafter, 5 µg of denatured protein was loaded into each well of an SDS polyacrylamide slab gel and resolved in a Mini-PROTEAN 3 electrophoresis unit (Bio-Rad) for 1 h at 120 V. The resolved proteins were then transferred onto a PVDF membrane, blocked overnight with 5% BSA in TBS with Tween-20 (TBST), and sequentially treated to primary antibodies (rabbit anti-REST or mouse anti-ACTB at 1:200× dilution) and secondary antibodies (HRP-conjugated anti-rabbit IgG at 1:10,000×, HRP-conjugated anti-mouse IgG at 1:6000×). Finally, the protein bands were detected with ECL Plus™ Western Blotting Detection Reagents (GE Healthcare).

2.11. Statistical analysis

All values were represented as the mean $\pm$ standard error of the mean (S.E.). Statistical analyses were carried out using Student's t-test for pairwise analysis. One-way ANOVA and Tukey's post hoc test were used for comparisons of more than two sample groups after verifying equal variances using F-test. $p < 0.05$, $p < 0.01$ and $p < 0.001$ were considered statistically significant.

3. Results and discussion

Despite initial skepticism regarding the capacity of MSCs for neural trans-differentiation, recent studies have produced encouraging electrophysiological evidences in support of this phenomenon^[1, 2, 4-6, 9]. By far, genetic manipulation via viral transduction appears to be the most promising in terms of generating mature neuronal cells capable of firing action potentials^[5, 9]. However, this method is associated with the inherent risk of mutagenesis and tumor formation that limits its clinical translation. These studies were also largely restricted to two-dimensional (2D) substrates and neglected the critical roles of microenvironment in regulating stem cell fate^[33-35]. Therefore, scaffold designs which recapitulate the structural organization of the natural extracellular matrix (ECM) while allowing sustained biochemical delivery may be advantageous for driving MSCs neuronal differentiation. In this regard, we explored the potential of REST siRNA incorporated nanofibers in enhancing MSC neuronal differentiation through synergistic topographical signaling and sustained REST knockdown.

3.1. Fabrication and characterization of PCL and DM-PCL nanofibers

PCL nanofibers were fabricated by electrospinning. Meanwhile, DM-PCL nanofibers were prepared by simple immersion of PCL scaffolds into an alkaline solution of DOPA. Macroscopically, uncoated PCL scaffolds appeared white while a surface colour change to light brown, indicative of DOPA-melanin deposition, was observed for DM-PCL scaffolds regardless of underlying nanofiber orientation (**Figure 1A**, inserts). Most importantly, SEM analysis showed no significant difference in nanofiber architecture and diameter for all scaffolds after DM coating. The average fiber diameters were 545 ± 9 nm, 553 ± 13 nm, 567 ± 12 nm and 574 ± 13 nm for PCL-RF, DM-RF, PCL-AF and DM-AF respectively. Meanwhile, XPS spectra also revealed an increase of the N 1s signal for DM modified PCL scaffolds (% nitrogen concentration = 2.6 and 2.3 in DM-RF and DM-AF vs. 0 in uncoated

PCL nanofibers respectively, Figure 1B, arrow) due to the presence of nitrogen in DOPA-melanin. Altogether, these results indicated successful deposition of DOPA-melanin onto PCL nanofibers to form DM-PCL scaffolds.

Figure 1

3.2. SiRNA adsorption, release kinetics and REST knockdown efficiency

Cy5-ODN/TKO complexes were successfully adsorbed onto all scaffolds (**Figure 2A**) and the loading efficiencies were > 95% regardless of fiber orientation. In general, DM modification enhanced the loading efficiencies of the complexes ($p < 0.05$, Table 2). Compared to randomly-oriented fibers, the deposition of complexes on aligned fibers showed a higher degree of orientation in response to the underlying aligned fiber topography (Figure 2A, white arrow). As compared to the punctate Cy5 signals that were observed for DM-coated samples, a higher intensity of diffused background signal was observed for uncoated PCL scaffolds. These observations indicated a more rapid release of Cy5-ODN/TKO complexes in the absence of DM coating.

Figure 2B shows the release profile of siRNA/TKO complexes (zeta potential = 32.5 ± 1.7 mV). Consistent with the observed release of Cy5-ODN by confocal imaging (Figure 2A), DM modification significantly reduced the initial burst release of siRNA ($6.6 \pm 0.2\%$ (DM-RF) vs. $9.7 \pm 0.5\%$ (PCL-RF) and $12.3 \pm 0.8\%$ (DM-AF) vs. $16.6 \pm 1.5\%$ (PCL-AF) respectively, $p < 0.05$, Figure 2B). In addition, the release of siRNA/TKO complexes was sustained over a longer period of time with DM coating (22 days vs. 7 days for uncoated PCL scaffolds, Figure 2B). Although the exact nature of interaction between siRNA and DM is unknown, these observations may be explained by the electrostatic attractions between the positively charged siRNA/TKO complexes and negatively charged DM layer. In addition, any amine and/or thiol functional group present in TransIT-TKO (proprietary composition) could potentially react with DM o-quinones via Schiff base or Michael addition reactions ^[26, 27, 36] to further enhance siRNA adsorption. Furthermore, the maximal cumulative release of siRNA was enhanced with DM coating ($59.4 \pm 3.6\%$ (DM-RF) vs. $30.1 \pm 3.1\%$ (PCL-RF) and $57.3 \pm 3.7\%$ (DM-AF) vs. $43.3 \pm 3.2\%$ (PCL-AF) respectively, $p < 0.05$, Figure 2B). This result was unexpected given the similar loading efficiencies of siRNA complexes onto nanofibers with or without DM modification. Since the mode of interaction between siRNAs and the TransIT-TKO molecules is non-covalent and non-specific, decomplexation of siRNA/TKO complexes may occur overtime. This, coupled with a more negatively charged surface of DM-modified fibers than plain fibers, likely repels a greater amount of free siRNAs into the external aqueous environment to cause an overall higher release of siRNAs. No significant difference in release profiles was observed between randomly-oriented and aligned nanofibers with DM coating. In contrast, randomly-oriented, uncoated PCL scaffolds showed significantly higher cumulative release than aligned PCL constructs ($p < 0.05$, Figure 2B). Collectively, DM-PCL nanofibers appeared to be a more promising siRNA delivery platform than untreated

PCL fibers in providing a more stable and sustained release of siRNA complexes regardless of underlying fiber orientation.

The siRNA/TKO complexes successfully transfected MSCs on both PCL and DM-PCL scaffolds. When cultured under non-specific differentiation condition with 2 μ g siRNA/TKO complexes, significant REST knockdown was observed on all scaffolds at day 3 as compared to siNEG controls ($p < 0.05$, Figure 2C). By day 7, significant REST knockdown was only observed for aligned DM-PCL scaffolds (~36% knockdown, $p < 0.05$, Figure 2C), and no significant knockdown was observed for all scaffolds by day 14. Fiber alignment and DM modification appeared to enhance REST knockdown efficiency. This trend was similarly observed at the protein level (Supplementary Figure S1). Specifically, significant knockdown of REST protein (vs. respective siNEG controls) was observed on DM-PCL scaffolds at day 3 regardless of fiber orientation. Meanwhile, fiber alignment induced a greater down-regulation of REST protein for most scaffolds by day 7, and the lowest expression was observed for aligned DM-PCL scaffolds with REST knockdown (Supplementary Figure S1). Compared to the trends observed at the mRNA level (Figure 2C), uncoated PCL scaffolds showed lower REST protein expressions than DM-PCL scaffolds. It is possible that DM modification facilitated MSCs proliferation^[37, 38], which delayed the onsets of neuronal differentiation and resulted a higher relative expression of REST protein on DM-PCL scaffolds than uncoated PCL scaffolds. Since REST is regulated post-translationally during stem cell differentiation to neurons^[39, 40], the effect of siREST while evident at the mRNA level, may be overwhelmed by the induced down-regulation of REST protein on untreated PCL scaffolds. Therefore, future studies on the influence of DM modification and substrate topography on MSCs proliferation, differentiation and endogenous REST protein regulation should be evaluated.

Figure 2

Nevertheless, the trends in silencing efficiency appear to correlate with the cumulative release of siRNA at early time points. For instance, siRNA release was approximately 1.2 times higher for all aligned fibers (regardless of DM modification) at day 3 and aligned PCL fibers at day 7 as compared to randomly-oriented fibers. Meanwhile, the cumulative siRNA release was also higher for DM-modified nanofibers versus untreated PCL nanofibers ($\sim 1.1\times$ and $\sim 1.8\times$ for DM-AF and DM-RF respectively) at day 7. Taken together, it is possible that REST silencing of MSCs was achieved predominantly through the uptake of siRNA/TKO complexes that were released into the media. This finding differs from our previous work with polydopamine-mediated siREST delivery^[29], in which direct internalization of surface-bound siRNA was proposed as the main uptake mechanism for driving REST knockdown in mouse neural progenitor cells. Cell type differences and unique chemical properties of DM

versus polydopamine could potentially explain this observation. A detailed comparison between DM- and polydopamine-mediated siRNA deliveries could be included in future studies to assess the superiority of either platform in effecting target gene knockdown of stem cells.

3.3. MSCs cultured under non-specific differentiation condition

3.3.1. With 2 µg siRNA/TKO complexes

Figure 3 shows the neuronal and glial differentiation of MSCs under non-specific differentiation condition on PCL and DM-PCL scaffolds that were loaded with 2 µg of siRNA. Similar to studies reported elsewhere, undifferentiated MSCs were nestin-positive^[41, 42]. Nestin expression was down-regulated on nanofibers relative to undifferentiated MSCs on coverslips (Figure 3A), and negligible difference was observed with DM coating, REST knockdown and fiber orientation effects (Figures 3A & 3B).

A

Undifferentiated MSCs

Day 7

B

Day 14

C

Day 21

Figure 3

Tuj1, an early neuronal marker, was up-regulated on nanofibers relative to undifferentiated MSCs on coverslips (Figure 3A). Similar to our previous study, this suggests that topographical feature alone is able to enhance neuronal commitment^[11]. No significant difference in Tuj1 expression was observed between siREST- and siNEG- treated MSCs on Day 7. By days 14 and 21, however, REST knockdown appeared to sustain Tuj1 expression on both randomly-oriented and aligned fibers while a decrease in Tuj1 expression was observed for siNEG-treated samples. This suggests a transient up-regulation of Tuj1 expression due to nanofibers topography and the benefit of a combined REST silencing approach in enhancing early neuronal commitment of MSCs. Significantly higher Tuj1 expression was also observed with DM-mediated REST knockdown than that mediated by plain PCL scaffolds (Figures 3B & 3C). Meanwhile, the expression of mature neuronal marker, MAP2, was only detected from day 14 onwards. Regardless of the scaffolds used, MAP2 expression was only observed on aligned fibers at day 14. By day 21, MAP2

1 expression was observed on randomly-oriented fibers, suggesting an accelerated neuronal
2 differentiation of MSCs with fiber alignment. Importantly, sustained siREST availability from
3 DM-AF (vs. PCL-AF, Figure 3B) appeared to enhance MAP2 expression as compared to all
4 other scaffolds, and this trend seemed to remain at day 21 (Figure 3C). No synapsin
5 expression was observed for all samples (data not shown). Altogether, fiber alignment appears
6 to be a critical factor for neuronal differentiation of MSCs since MAP2 was only expressed on
7 aligned fibers after 14 days and the strongest expression was detected with the combined
8 effects of fiber alignment and DM-mediated REST knockdown. However, the differentiated
9 cells remained immunonegative for synapsin after 21 days, suggesting that they were likely
10 still in the neural progenitor stage and prolonged culture may be needed in order for the cells
11 to reach full maturation. Independently, a similar study also reported the necessity for
12 cytokine induction to initiate and commit REST silenced MSCs to become mature neuronal
13 cells ^[4]. Therefore, without additional biochemical cues other than REST silencing, a non-
14 specific differentiation condition while able to direct MSCs towards the
15 neuronal lineage, may not be sufficiently potent in generating mature neuronal cells.

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41 Basal level of GFAP was detected on all scaffolds at day 7 which gradually reduced with
42 time. Importantly, REST knockdown mediated by DM coating (vs. siREST PCL-AF/RF,
43 Figures 3A & 3B) significantly reduced GFAP expression of differentiated MSCs at days 7
44 and 14. Finally, O4, a marker for oligodendrocyte, was detected on day 21 only. Since the
45 morphologies of these O4-expressing cells were unlike that of mature oligodendrocytes, it is
46 possible that these cells were primed towards the oligodendrocyte lineage, but may require
47 other factors such as transcription factors for direct reprogramming and final commitment to
48 functional mature cell types. REST knockdown mediated on aligned fibers of both PCL and
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DM-PCL scaffolds induced the lowest O4 expressions (Figure 3C). In general, glial differentiation was reduced with DM-mediated REST knockdown and/or fiber alignment.

To further increase the efficiency of MSCs neuronal differentiation, neuronal specific media containing N2 and B27 supplements were used in our subsequent experiments. Untreated PCL scaffolds were also excluded from later experiments since DM-mediated siRNA delivery to MSCs showed an overall improved REST knockdown efficiency and neuronal differentiation capability.

3.4. MSCs cultured under neuronal differentiation condition

3.4.1. With 2 μ g siRNA/TKO complexes

Figure 4 shows the differentiation of MSCs on DM-PCL scaffolds loaded with 2 μ g of siRNA. Comparing Figure 3B and Figure 4, the added biochemical cues in neuronal specific media appear to enhance MSCs differentiation to MAP2⁺ and synapsin⁺ cells. However, the effects of REST knockdown and fiber alignment became less prominent. Specifically, increased MAP2 expression with REST knockdown was largely observed on randomly-oriented fibers only. Likewise, enhanced MAP2 expression was only observed with fiber alignment on siNEG controls (siNEG DM-AF > siNEG DM-RF, Figure 4), while no synergistic effect was observed with REST knockdown (siREST DM-AF $\approx$ siREST DM-RF $\approx$ siNEG DM-AF, Figure 4). No significant difference in synapsin expression was observed with REST knockdown or fiber orientation.

Compared to non-specific differentiation condition, the neuronal biochemical cues enhanced GFAP expression on aligned fibers, while no significant difference was observed on randomly-oriented fibers (Figure 3B vs. Figure 4). Olig2, an oligodendrocyte marker, was undetected for all samples.

Figure 4

In summary, the added biochemical cues in neuronal specific media appear to induce faster MSCs differentiation towards mature neuronal cells. However, the lack of synergistic effects between REST knockdown and fiber alignment in enhancing neuronal marker expressions and the induction of GFAP expression in differentiated cells warrant an increase in REST siRNA loading as an attempt to further enhance MSCs differentiation and maturation to neuronal-like cells.

3.4.2. With 4 μ g siRNA/TKO complexes

SiRNA loading was doubled to 4 μ g. Correspondingly, REST knockdown was sustained for up to 14 days (~25% & 20% knockdown for siREST DM-RF and DM-AF respectively, Supplementary Figure S2A) as compared to only 7 days with 2 μ g siRNA

(Figure 2C). No significant difference in silencing efficiency was observed with fiber orientation.

However, similar to the trends observed with 2 μ g of siRNA/TKO complexes (Figure 4), up-regulation of neuronal markers expression by REST knockdown was more significant on randomly-oriented fibers than aligned fibers. For instance, Tuj1 expression was significantly up-regulated on randomly-oriented fibers with REST knockdown versus siNEG controls on both days 7 and 14 ($p < 0.001$, **Figures 5A & 5B**). On aligned fibers, REST knockdown only induced mild increase of Tuj1 expression on day 7 (vs. siNEG control, $p < 0.05$, Figure 5B). These trends were similarly observed at the transcript level (Supplementary Figure S2B), where significant up-regulation of Tuj1 expression was observed with REST knockdown on randomly-oriented fibers for all time points examined ($p < 0.05$), while that on aligned fibers was only observed at day 7 with respect to siNEG control ($p < 0.05$). Compared to undifferentiated cells, the expression of Tuj1 was also high for siNEG-treated samples (Supplementary Figure S2B), suggesting that fiber orientation alone could have enhanced neuronal differentiation, thereby masking the effects of REST knockdown. Meanwhile, MAP2 expression showed no significant difference with REST knockdown (vs. siNEG controls) at both the protein (**Figures 5C & 5D**) and mRNA levels (Supplementary Figure S2C). Instead, fiber alignment significantly enhanced MAP2 expression for both siNEG- and siREST-treated MSCs at day 14 ($p < 0.05$, Figures 5C and 5D). Low expression of synapsin was only observed at day 14, and no significant difference was observed with REST knockdown or fiber orientation effect (Figure 5C). Taken together, REST knockdown played a greater role in enhancing early neuronal commitment, while fiber alignment enhanced neuronal maturation to an extent which was limited to the MAP2 marker.

Figure 5

GFAP expression remained similar to that induced with 2 μ g siRNA treatment (Figure 4 vs. **Figure 6**). On randomly-oriented fibers, REST knockdown appeared to reduce GFAP expression at day 7 ($p < 0.05$, Figures 6B). On aligned fibers however, no significant difference in GFAP expression was observed with REST knockdown on both days 7 and 14. Lower expression of GFAP was also observed on aligned fibers versus randomly-oriented fibers (Figure 6). Meanwhile, ALDH1L1, another astrocytic marker, was undetected in all scaffolds throughout the study (Supplementary Figure S3A). This suggests that the differentiated cells expressing GFAP may not be true astrocytes. Likewise, oligodendrocyte markers, Olig2 and O4, were undetected in all scaffolds throughout the study (Supplementary Figure S3A). Overall, glial cell differentiation was negligible and likely attributed to the increased REST knockdown and selectivity of nanofibrous topography against astrocytes and oligodendrocytes as reported previously [43-45].

Figure 6

1 Taken together, significant synergistic effect of REST knockdown and nanofiber
2 topography was observed when MSCs were cultured under non-specific differentiation
3 condition. However, in the presence of favorable medium condition, there was little synergy
4 between the neuronal specific biochemical cues and REST knockdown in further enhancing
5 MSCs neuronal differentiation. A similar observation was also made where micro-RNAs
6 mediated silencing of oligodendrocyte precursor cells (OPCs) in specific induction medium
7 had little ability to further increase oligodendrocyte differentiation as compared to that in OPC
8 proliferation medium ^[46]. Although the exact reasons remain to be elucidated, it is likely that
9 the exposure of MSCs to neuronal specific biochemical cues may have triggered endogenous
10 signaling cascades that desensitized the cells towards REST knockdown effects. Overall, our
11 results demonstrated DM-mediated siRNA adsorption as a potential strategy for therapeutic
12 gene silencing applications. Such nanofibrous scaffolds may find useful applications in
13 enhancing neuronal differentiation of MSCs under non-specific conditions
14 such as an *in vivo* microenvironment.
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33 34 35 36 37 38 **4. Conclusion** 39 40

41 This study explores the feasibility of siREST delivery through DM-modified nanofibers
42 to induce REST knockdown of MSCs for enhanced neuronal differentiation. In general,
43 siRNA loading, release kinetics and REST knockdown efficiency were enhanced with DM
44 modification and fiber alignment. Under non-specific differentiation condition, the synergistic
45 effects of REST silencing and nanofiber topography improved MSC neuronal commitment
46 and decreased glial cell differentiation. Meanwhile, the added biochemical cues in neuronal
47 specific medium played a significant role in driving neuronal differentiation of MSCs, and
48 mitigated the effects of REST knockdown even with increased dosage of siRNA.
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1 Altogether, the synergistic effects of nanofiber topography and gene silencing under non-
2 specific differentiation condition suggest the potential of such nanofibers in enhancing
3 neuronal commitment in in vivo applications, where well-defined culture conditions are often
4 absent.
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13 007-271).
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22 Keywords: Sustained release, electrospinning, RE-1 silencing transcription factor, neuronal
23 differentiation, neural differentiation
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Figures captions:

Figure 1: *In vitro* characterization of randomly-oriented and aligned PCL nanofibers with and without DOPA coating. (A) SEM images and (B) XPS survey spectra of randomly-oriented and aligned plain PCL and DOPA-PCL scaffolds respectively. Inserts: digital photographs of plain PCL and DOPA-PCL scaffolds.

Figure 2: *In vitro* Cy5-ODN adsorption, siRNA release kinetics, and REST knockdown efficiency of siRNA-loaded PCL and DOPA-PCL nanofibers. (A) Confocal microscopy images showing adsorption and distribution of Cy5-ODN (red) on nanofibers. Scale bar: 100 μ m. White arrows: Fiber orientation (B) Percentage cumulative release of siRNA from nanofibers (C) Real-time PCR analysis showing the REST mRNA expressions. All results were normalised to siNEG-loaded PCL and DM-PCL nanofibers respectively. + indicates $p < 0.05$ (ANOVA). * indicates $p < 0.05$ (ANOVA) as compared to siNEG controls. Mean $\pm$ SE, $n=3$

Figure 3: Immunofluorescence analysis of MSCs cultured on siNEG or siREST loaded PCL and DOPA-PCL nanofibers for 7, 14 and 21 days under non-specific differentiation conditions. All scaffolds were loaded with 2 μ g siRNA. (A,B) Cells were co-stained for Tuj1 and Nestin, MAP2 and GFAP after (A) 7 and (B) 14 days. (C) Cells were stained for Tuj1, O4 and co-stained for MAP2 and GFAP after 21 days. Nuclei were counterstained with DAPI (blue). Arrows indicate the fiber orientation of aligned nanofibers. Scale bar: 50 μ m.

Figure 4: Immunofluorescence analysis of MSCs cultured on siNEG or siREST loaded DOPA-PCL nanofibers for 14 days under neuronal differentiation conditions. Scaffolds were

1 loaded with 2 μ g siRNA/TKO complexes. Cells were co-stained for MAP2 and GFAP,
2 Synapsin and Olig2 after 14 days. Nuclei were counterstained with DAPI (blue). Arrows
3 indicate the fiber orientation of aligned nanofibers. Scale bar: 50 μ m.
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10 Figure 5: Neuronal markers expression of MSCs treated to increased loading of siRNA on
11 DOPA-PCL nanofibers under neuronal differentiation conditions for 7 and 14 days. Scaffolds
12 were loaded with 4 μ g siRNA/TKO complexes. Cells were stained for (A) Tuj1, (C) MAP2
13 and Synapsin. Fluorescence intensities of (B) Tuj1+ and (D) MAP2+ cells normalized to the
14 mean fluorescence intensity of the respective markers expressed in undifferentiated MSCs.
15 Arrows indicate the fiber orientation of aligned nanofibers. Scale bar: 50 μ m. * and ***
16 indicate $p < 0.05$ and $p < 0.001$ (ANOVA) respectively. Mean $\pm$ SE, n=50.
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29 Figure 6: GFAP expression of MSCs treated to increased loading of siRNA on DOPA-PCL
30 nanofibers under neuronal differentiation conditions for 7 and 14 days. Scaffolds were loaded
31 with 4 μ g siRNA/TKO complexes. (A) Cells were stained for astrocyte marker, GFAP, after
32 both 7 and 14 days. Nuclei were counterstained with DAPI (blue). (B) Fluorescence
33 intensities of GFAP+ cells normalized to the mean fluorescence intensity measured in
34 undifferentiated MSCs. Arrows indicate the fiber orientation of aligned nanofibers. Scale bar:
35 50 μ m. * indicates $p < 0.05$ (ANOVA). Mean $\pm$ SE, n=50.
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Supplementary Figure S1: Representative western blots showing REST protein expressions of MSCs on randomly-oriented and aligned PCL and DM-PCL nanofibers loaded with 2 μ g siRNA/TKO complexes. Cells were cultured under non-specific differentiation conditions for up to 14 days.

Supplementary Figure S2: REST knockdown efficiency and neural marker expression of MSCs treated to increased loading of siRNA on DOPA-PCL nanofibers under neuronal differentiation conditions. Scaffolds were loaded with 4 μ g siRNA. (A) Real-time PCR results showing REST expression of MSCs on siREST loaded DOPA-PCL nanofibers for 3, 7 and 14 days. Results were normalized to siNEG loaded DOPA-PCL nanofibers. (B-D) Real-time PCR results showing (B) Tuj1 (C) MAP2 and (D) GFAP expressions of MSCs differentiated on siNEG or siREST loaded DOPA-PCL nanofibers for 3, 7 and 14 days. All results were normalized to undifferentiated MSCs. * indicates $p < 0.05$. # and ### indicate $p < 0.05$ and $p < 0.001$ when compared to randomly-oriented fibers. Mean $\pm$ SE, $n=3$.

Supplementary Figure S3: Glial markers expression of MSCs treated to increased loading of siRNA on DOPA-PCL nanofibers under neuronal differentiation conditions for 7 and 14 days.

Scaffolds were loaded with 4 μg siRNA/TKO complexes. (A) Cells were stained for astrocyte marker, ALDH1L1, after 7 and 14 days. ALDH1L1 and GFAP staining of primary rat astrocytes were included as positive controls. (B) Oligodendrocyte markers, Olig2 and O4, were stained after 7 and 14 days, respectively. Nuclei were counterstained with DAPI (blue). Scale bar: 50 μm .

Supplementary Figure S4: MSCs-derived, synapsin positive cells on DOPA-PCL nanofibers under neuronal differentiation conditions for 14 days. Cells treated to 2 μg and 4 μg siRNA/TKO complexes were selected and magnified by 4 $\times$ from the images in Figure 4 and Figure 5C respectively. Scale bar: 50 μm .

List of Tables:

Table 1: Real-time PCR primer sequences

Primers	Sequence (5' – 3')
β-actin	Forward: GGCACCCAGCACAATGAAGATCAA
	Reverse: ACTCGTCATACTCCTGCTTGCTGA
REST	Forward: CGCCATGCAAGACAGGTTTACAAT
	Reverse: AGCTGCATAGTCACATACAGGGCA
Tuj1	Forward: GCAACTACGTGGGCGACT
	Reverse: CGAGGCACGTACTTGTGAGA
Map2	Forward: ACAGGTGCTTTTTGGTGACCCAGT
	Reverse: AATGAGTGGTGTGGGTTTGCTCCT
GFAP	Forward: CCTCTCCCTGGCTCGAATG
	Reverse: GGAAGCGAACCTTCTCGATGTA

Table 2: SiRNA loading efficiency

SiRNA loading efficiency			
Scaffolds	Mass loaded (ng)	Mass per area (ng/cm ²)	Percentage relative to theoretical mass (%)
DM-RF	1952 ± 6	1027 ± 3	97.6 ± 0.3*
PCL-RF	1904 ± 10	1002 ± 5	95.2 ± 0.5
DM-AF	1938 ± 10	1020 ± 5	96.9 ± 0.5 ⁺
PCL-AF	1904 ± 2	1002 ± 1	95.2 ± 0.1

* and + indicates p < 0.05 (ANOVA) as compared to PCL-RF and PCL-AF respectively, n=3.

Table of contents (ToC):

DOPA-melanin (DM) coated nanofibers were functionalized with siRNA to induce REST silencing of mesenchymal stem cells (MSCs) for neuronal differentiation. DM modification enhanced the siRNA loading, release kinetics and REST knockdown efficiency. Under non-specific differentiation condition, the synergistic effects of REST silencing and nanofiber topography improved MSCs neuronal commitment. Such nanofiber-mediated gene silencing platform may find promising applications in vivo.