Nanofibrous scaffold-mediated REST knockdown to enhance neuronal differentiation of stem cells

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\textbf{Abstract}

At present, the recovery prospect for patients with chronic neurodegenerative diseases or acute trauma in the central nervous system is sub-optimal. The controlled differentiation of neural stem/progenitor cells (NPCs) to functional neurons is a possible treatment strategy. In contrast to the classical approach of biochemicals supplementation for guided stem cell commitment, this study explores the feasibility of directing neuronal differentiation through synergistic integration of three-dimensional nanofibrous topographical cues and scaffold-mediated knockdown of RE-1 silencing transcription factor (REST) in mouse NPCs. Taking advantage of the strong adhesive property and latent reactivity of mussel-inspired polydopamine (PD) coating, electrospun polycaprolactone (PCL) nanofibers were successfully functionalized with REST siRNAs (denoted as siREST PD-fiber). Sustained REST knockdown in NPCs was achieved for up to five days in vitro and the silencing efficiency was significantly higher than that mediated through siRNA adsorption onto non-PD coated sample controls. The silencing of REST, together with nanofiber topographical effect, significantly enhanced NPC neuronal commitment (57.5\% Map2\(^+\) cells in siREST PD-fiber vs. 43.5\% in siREST PD-film vs. 50\% in PD-fiber controls, \(p < 0.05\)) while reducing astrocytic and oligodendrocytic differentiation (10.7\% O4\(^+\) cells vs. ~30\% in siREST PD-film, \(p < 0.01\)). Taken together, the synergistic effects of scaffold-mediated REST knockdown and topographical cues from PD-modified nanofibers may be a useful strategy for generating functional neurons for therapeutic purposes.

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1. Introduction

The controlled differentiation of stem cells to functional neurons for cell replacement therapy is an alternative to treatment of damaged central nervous system (CNS). However, the conventional methods of generating neurons from stem cells, through bolus supplementation of small molecules or neurotrophic factors, is still lacking in the efficiency of neural conversion and lineage selection. One possible reason may be the absence of biophysical and topographical cues, which have recently been shown to modulate stem cell fate [1–3]. In addition, neuronal cells respond to contact guidance from underlying tracts of glial cells and orientated extracellular matrix (ECM) in vivo [4–7], as well as physical cues from imposed topography in vitro (micro-patterned surfaces, guidance conduits, nanofibers, etc.) [8]. Therefore, recent studies have begun to integrate biomolecules into tissue engineered scaffolds to recapitulate in vivo microenvironment of a developing or regenerating nervous system for more specific stem cell differentiation to neurons [9–11]. The incorporation of small interfering ribonucleic acids (siRNAs) as molecular mediators of differentiation to existing scaffold-based approach presents an interesting alternative to the encapsulation of lineage-specific inductive factors. By silencing repressive pathways against desired lineage commitment, and in combination with scaffold topographical effects, the efficiency of directed stem cell differentiation to neurons may be enhanced further.

Scaffold-mediated RNA interference (RNAi) has been carried out through surface immobilization or direct bulk encapsulation of siRNAs in hydrogels, nanofibers and sponge-based constructs [12,13]. However, these approaches faced restricted translatability...
to therapeutic implants due to compatibility issues that may exist between harsh scaffold treatment/fabrication process and siRNAs integrity. In this regard, a simple, substrate-independent strategy that can incorporate siRNAs into any biomimetic tissue engineered scaffold would be advantageous. Mussel-inspired polydopamine (PD) coating represents one such approach that can be easily adapted for siRNA functionalization due to its intrinsic ability to deposit on a wide array of material surface and facile chemical reactivity towards biomolecules [14,15]. This process only requires simple immersion of substrates in an alkaline dopamine solution which, by reactions between the amine and catechol functional groups of dopamine, polymerize to form an adherent PD layer. The latent reactivity of the deposited PD may then be exploited for further conjugation of biomolecules. Recent studies have already utilized PD as secondary reaction sites for incorporation and transfection of plasmid DNAs into cells [16]. Despite having molecular properties reminiscent of DNAs, siRNA delivery from PD-modified substrates has not been attempted, and the use of such substrate- or scaffold-mediated gene silencing platform to manipulate neural stem/progenitor cells (NPCs) differentiation remains unexplored. One possible factor that can be adapted into PD-mediated siRNA delivery for enhanced neuronal differentiation is that of NPCs is RE-1 silencing transcription factor (REST). In general, REST functions as a transcriptional repressor for a myriad of neuronal specific genes, which regulates the expression of ion channels, synaptic vesicles proteins and neurotransmitter receptors [17,18]. During neurogenesis, it has been established that REST is down regulated in embryonic stem cells (ESCs) and NPCs upon differentiation into neurons [19,20]. Additionally, several studies including our previous work [21–23], have already demonstrated the feasibility of enhancing neuronal commitment of stem cells through deliberate silencing of REST.

Therefore, by adsorbing REST siRNAs onto PD-modified electrospun polycaprolactone (PCL) nanofibers, we endeavor to further enhance the efficiency of neuronal differentiation through combined effects of REST silencing and scaffold topographical cues. Primary mouse neural progenitor cells (NPCs) were used for scaffold-mediated transfection and differentiation under chemically defined, serum-free condition. SiRNA adsorption on PD-modified PCL film was also included as a two-dimensional (2D) control and evaluated for its efficacy in directing NPCs neuronal commitment.

2. Materials and methods

2.1. Materials

Fibroblast growth factor-2 (FGF-2) was purchased from R&D systems. Epidermal growth factor (EGF) and heparin were purchased from Merck. HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG were purchased from Pierce. 2, 2, 2-trichloroethanol (TCE) and Green Supermix were purchased from Bio-Rad. BCA assay kit was purchased from Thermo Scientific. 

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2.2. Primary mouse neural progenitor cells (NPCs) isolation and cell culture

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and animals were treated in accordance with the IACUC guidelines in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved animal facility. NPCs were isolated from hippocampus of six days old C57BL/6 mice as described previously [22,24,25]. Primarily NPCs were cultured as a monolayer on 2 μg/cm² laminin-coated tissue culture plates or flasks. Poly-L-ornithine was pre-adsorbed at 2 μg/cm² concentration prior to laminin coating. Cells were maintained in serum-free Neurobasal medium incorporating Dulbecco’s Modified Eagle Medium: Nutrient Mixture, F-12 (DMEM/F12), 1% N-2 supplement, 1% L-glutamine, 1% penicillin-streptomycin and 20 ng/ml of EGF and FGF-2. All cells were maintained in a humidified incubator at 37 °C with 5% CO2 under sterile conditions. The NPCs were kept within passage 18–22 to ensure similar cellular activity for subsequent siRNA transfection and differentiation studies.

2.3. Fabrication of PD-modified films and scaffolds with surface adsorbed siRNAs

PCL was dissolved in a mixed solvent containing TFE and DEPC-treated TE buffer at 5:1 v/v ratio to obtain a 12% w/v polymer solution. Thereafter, the solution was loaded into a syringe and electrospun at a fixed flow rate of 1.5 ml/h (New Era Pump) with ± 10 kV and ± 4 kV potential (Gamma High Voltage Research, USA) applied to the polymer solution and rotating collector respectively. The collecting distance was maintained at 12–14 cm. Nanofibrous meshes were cut to an area of 2 cm² to fit into wells of 24-well plates (Nunc). For 2D controls, PCL films were spin-coated onto coverslips (15 mm diameter) with 8% w/v PCL polymer solution. For polydopamine (PD) deposition on PCL films and nanofibers, dopamine hydrochloride was dissolved in 10 ml Tris buffer (pH 8.5) to a concentration of 0.5 mg/ml. All PCL films and scaffolds were rinsed with deionized water before immersing in 500 μl of dopamine solution for 4 h, with constant agitation (90–100 rpm, Sartorius Certoimat® R, Germany) on an orbital shaker. PD-coated PCL films and scaffolds (denoted as ‘PD-film’ and ‘PD-fiber’ respectively) were then rinsed with deionized water to remove residual dopamine monomer and lyophilized overnight. For siRNAs or oligonucleotides (ODN) adsorption, PCL films and PD-fibers were loaded with 2 μg siRNA or Cy3-labeled ODN (Cy5-ODN) after complexing with transfection reagent. Lipofectamine RNAiMax at 1:2 v/v ratio. Specifically, 3 μl of Cy5-ODN or siRNA (50 μM stock concentration) and 6 μl of Lipofectamine RNAiMax were each diluted in sterile DMEM/F12 or PBS (pH 7.4) to a final volume of 50 μl. Thereafter, the two solutions were mixed by gentle pipetting and incubated for 15 min at room temperature. Finally, 100 μl of Cy5-ODN- or siRNA-Lipofectamine RNAiMax complexes (denoted as ‘Cy5-ODN/LP’ and ‘siRNA/LP’ respectively) was loaded onto each PD-film and PD-fiber and lyophilized overnight.

2.4. Surface characterization of PD-film and PD-fiber

The surface elemental composition was analyzed by Raman spectroscopy and X-ray photoelectron spectroscopy (XPS). All samples were vacuum-dried overnight prior to experiment. The Raman scattering analysis was performed at room temperature (Raman-1, Nanophoton). For data analysis, 532 nm laser was used for excitation. The spectra acquisition parameters were 10 s exposure time with line scan, 50,000 objective, and 0.07 mW of laser power. XPS spectra were obtained using Al Kα 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 a 300 W (15 kV and 20 mA) under 10⁻⁹ Torr or lower 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.

2.5. In vitro characterization of siRNA/LP and Cy5-ODN/LP loaded PD-fibers and PD-fibers: zeta potential, release kinetics and Cy5-ODN distribution

Zeta potential of siRNA/LP complexes (prepared as described in Section 2.3 in 1 ml deionized water) was characterized using Zetasizer Nano ZS (Malvern Instruments, UK). For release kinetics analysis, PD-fibers (average weight = 0.30 mg, n = 3) and PD-fibers (average weight = 6.18 mg, n = 3), loaded with siRNA/LP or Cy5-ODN/LP complexes were incubated in 3 ml PBS at 37 °C for 30 days under gentle orbital shaking (70–90 rpm, Sartorius Certoimat® R, Germany). At predetermined time points, 1 ml of supernatant was retrieved and replaced with an equal volume of fresh PBS. SiRNA concentration in the supernatant was determined using RiboGreen® assay after treating with 1 μg/ml heparin solution to de-complex siRNA from the cationic Lipofectamine RNAiMax. Fluorescence intensity was measured using a microplate reader (Tecan®, Infinite 200, Austria). For nucleic acid adsorption and distribution analysis, Cy5-ODN/LP complexes loaded on PD-film and PD-fiber were imaged using confocal microscopy (Zeiss, LSM 710 Meta Laser Scanning Confocal microscope, Germany) at specific time points. All experiments were performed in triplicates.

2.6. Transfection and differentiation of NPCs on siRNA/LP adsorbed PD-fibers and PD-fibers

Surface-mediated transfection was evaluated with siRNA/LP adsorbed PD-film and PD-fiber (denoted as ‘siRNA PD-film’ or ‘siRNA PD-fiber’) and siRNA/LP

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adsorbed uncoated film and fiber (denoted as ‘siRNA film’ or ‘siRNA fiber’). Bolus delivery of siRNA/LP complexes (0.67 μg siRNA with Lipofectamine RNAmax at 1:2 v/v ratio) onto PD-film and PD-fiber were also included. Prior to cell seeding, all films and scaffolds were coated with 10 μg/cm² laminin at 1 h at 37 °C. NPs were seeded at a density of 5.0 × 10⁶ cells/cm² in 1 ml proliferative medium. After 48 h incubation, differentiation was induced by EGF removal, reduced concentration of KGF-2 (5 ng/ml) and addition of 1% B27 supplement to the medium [26]. Cells were differentiated for up to 5 days (i.e. total of 7 days culture). PD-films and PD-fibers untreated with siRNA or adsorbed with siNEG/LP complexes were used as ‘film controls’ and ‘fiber controls’ respectively. The entire experiment was repeated at least three times.

2.7. Real-time PCR analysis

At specific time points, cells were lysed using TRIzolTM reagent and pooled together to obtain sufficient RNA for analyses (n = 4 for films; n = 6 for scaffolds). Reverse transcription was carried out using M-MLV Reverse transcriptase according to the manufacturer’s protocol. Real-time PCR analysis was performed using the LightCycler 480 (Roche) and the LightCycler 480 II (Roche) as previously described [27]. In all PD- films and PD-fibers, a lower REST silencing was observed on days 2 (67% vs. 0 on PD-film, Fig. 1A, arrow) and 4 (32% vs. 0 on untreated PCL film, Fig. 2A, arrow) due to the presence of nitrogen in PD. Macroporosity, a surface color change from white to light brown, indicative of PD deposition, was observed during the coating process (Fig. 1C, insets). The average fiber diameters were 542 ± 13 nm and 548 ± 20 nm for untreated and PD-coated PCL fibers respectively. On PD-films, similar trends in Raman survey spectra (Fig. 2A), XPS spectra (% nitrogen concentration = 5.0 in PD-film vs. 0 in untreated PCL film, Fig. 2B) and surface color change (Fig. 2C) were observed. SEM analysis (Figs. 1C and 2C) revealed no significant change in architecture for both scaffold and film after PD coating.

3.2. In vitro characterization of Cy5-ODN/LP and siRNA/LP loaded PD-films and PD-fibers

Cy5-ODN/LP complexes appeared well distributed throughout the nanofibers and planar surface of PD-fiber and PD-film respectively (Fig. 3A). Based on fluorescence intensity at day 0, PD-fiber showed greater extent of Cy5-ODN/LP adsorption than PD-film. Additionally, Cy5-ODN/LP complexes remained adsorbed on both PD-film and PD-fiber after 14 days of in vitro drug release. The zeta potential of siRNA/LP complexes was 13.6 ± 8.6 mV. From Fig. 3B, both siRNA PD-film and PD-fiber samples were characterized with rapid release of siRNAs within the first three days, followed by a slower rate of release for the rest of the study. The maximal cumulative release was higher for nanofiber substrates (206.9 ± 16.9 ng vs. 135.6 ± 8.2 ng for siRNA PD-film). However, the percentage cumulative release and loading efficiency could not be determined accurately due to difficulties in extracting residual siRNA/LP complexes from PD-modified scaffolds at the end of the release kinetics studies.

3.3. REST knockdown efficiency

Fig. 4A and B reflect REST knockdown of NPCs cultured on siRNA/LP adsorbed PD-films and PD-fibers. On PD-fibers, significant down-regulation of REST transcripts was observed on days 2 (67% silencing, p < 0.01, Figs. 4A and 5 (24% silencing, p < 0.05, Fig. 4A) as compared to the controls. On PD-films, a lower REST silencing efficiency was obtained, which remained fairly constant throughout the period of study (~20% silencing at days 2 and 7, p < 0.05). From Fig. 4B, western blot analysis showed significant down-regulation of REST protein at days 2 and 7 for PD-film and at days 2 and 5 for PD-fiber, when compared to film and fiber controls respectively (Fig. 4C). Additionally, REST protein expressions in all PD-fiber samples were slightly lower than its corresponding PD-film samples at all time points. Collectively, these results demonstrated successful loading of siREST/LP complexes onto PD-film and PD-fiber to effect significant REST knockdown at both transcript and protein levels.

**Table 1 Real-time PCR primer sequences.**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5′-3′)</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>Forward: TGTGATGGTGCAATGGGTGACAA</td>
<td>140</td>
</tr>
<tr>
<td>Reverse: TCTGTGCTTCAACCTGCTCATGTCATG</td>
<td>138</td>
<td></td>
</tr>
<tr>
<td>REST</td>
<td>Forward: AAGTCTGGACAGAAGCAGA</td>
<td>186</td>
</tr>
<tr>
<td>Reverse: TCTAGGCTGTTACCTAAGCAGC</td>
<td>143</td>
<td></td>
</tr>
<tr>
<td>TuJ1</td>
<td>Forward: TCTCTGGATGATAGGCAGA</td>
<td>186</td>
</tr>
<tr>
<td>Reverse: TCTCACTCATCTCCTCCAGCACAT</td>
<td>143</td>
<td></td>
</tr>
</tbody>
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3.4. Synergistic effects of REST knockdown and nanofiber topography on neuronal differentiation

3.4.1. Gene expression analyses

Fig. 5A and B show the transcript expressions of early neuronal marker, Tuj1, and late neuronal marker, Map2, of NPCs during differentiation on PD-modified scaffolds. On PD-film, REST knockdown up-regulated Tuj1 expressions as compared to film controls (Fig. 5A). The expression of Map2 was also significantly higher for siREST PD-film than film controls at day 7 (Fig. 5B). Similarly on nanofibers, REST knockdown induced higher expressions of Tuj1 and Map2 on siREST PD-fiber at day 7 and day 5 respectively, as compared to fiber controls.

Analyses on scaffold topographical effect revealed that nanofibers induced greater mature neuronal marker expression. In particular, Map2 expression was significantly higher for all nanofiber samples throughout the experiment (p < 0.001, Fig. 5B).

Comparing Fig. 5 and Supplementary Fig. S1, lower expressions of Tuj1 and Map2 were observed with non PD-mediated siRNA delivery, possibly a result of lower REST silencing efficiency as compared to PD-modified samples (Fig. 4 vs. Supplementary Fig. S2A). Increased neuronal markers expression due to nanofiber topographical effect was also observed (Supplementary Fig. S1).

3.4.2. Immunostaining of neuronal and glial cell markers

Nestin, a marker for neural stem/progenitor cells was undetected in all sample groups at day 7 (data not shown). From Fig. 6, REST knockdown significantly increased the proportion of Tuj1+ and Map2+ cells as compared to the film controls. On the other hand, the percentage of GFAP+ cells was significantly lower while no significant difference was observed for the percentage of O4+ cells. On nanofibers, the effect of REST knockdown on NPCs differentiation was similar. As shown in Fig. 7, PD-mediated siREST delivery generated increased percentage of Tuj1+ (19% increase, p < 0.05) and Map2+ cells (15% increase, p < 0.05) when compared to film controls. However, REST knockdown did not induce any significant difference in the percentage of GFAP+ and O4+ cells across all PD-fiber samples.
Comparing Figs. 6B and 7B, the proportion of neuronal to glial cells increased in response to nanofiber topography. Specifically, the percentage of Tuj1\(^+\) cells was significantly higher for all nanofiber samples than corresponding film substrates \((p < 0.01)\). In addition, twice as many Map2\(^+\) cells were generated on fiber controls than film controls \((p < 0.01)\), and a 32% increase was observed with REST knockdown. The percentages of GFAP\(^+\) and O4\(^+\) cells were also lower on nanofibers \((p < 0.01)\). In addition, the typical morphology for glial cells was absent on nanofibers (Fig. 6A vs. Fig. 7A).

### 4. Discussion

Chronic neural degenerative diseases and acute trauma to the CNS often result in irreversible loss of neurons sub-population and CNS functionality. Despite recent advances in cell therapies to replace damaged neurons or stimulate intrinsic regeneration capacity of CNS at the injured site, no single treatment has been therapeutically effective in promoting functional recovery \([27,28]\). One possible approach for treatment may be the use of siRNA-functionalized nanofibrous scaffolds for directed stem cell commitment towards neuronal lineage. By adsorbing siRNAs onto PD-modified PCL nanofibers, we demonstrated enhanced neuronal differentiation of primary mouse NPCs through synergistic effects of nanofiber topographical cues and scaffold-mediated REST knockdown.

PCL films and nanofibers were surface modified with PD before adsorption with siRNA/LP complexes. Although prolonged exposure of substrates to dopamine has been shown to increase nucleic acid loading efficiency \([16]\), the longer coating duration also leads to deposition of discrete PD particulates \([29,30]\) of heterogeneous sizes and distribution. This can potentially effect undesirable variation in topography, siRNAs adsorption and subsequent gene knockdown efficiency. Accordingly, the optimized coating protocol was applied to ensure deposition of PD without significant changes to the substrate topography.

Based on low cytotoxicity observed in our previous study \([22]\) and its potential for in vivo applications \([31,32]\), Lipoectamine RNAiMax was used as the transfection reagent for the present study. Successful deposition of Cy5-ODN/LP complexes on PD-film and
PD-fiber was achieved. PD-fiber showed greater adsorption efficiency than PD-film likely due to an overall higher surface area to volume ratio and enhanced physical entrapment of complexes by nanofibrous mesh. This coincides with the greater maximal cumulative release of siRNAs from siRNA PD-fiber (1.5x higher than PD-film). In both cases, rapid release of siRNAs within the first five days was followed by negligible release thereafter. However, the continued retention of Cy5-ODN/LP complexes after 14 days in vitro suggested that a substantial amount of siRNA/LP complexes still remained adsorbed on the PD-modified films and nanofibers.

The exact nature of interaction between siRNAs and polydopamine is unknown. Nevertheless, polydopamine has been reported to have a mild negative charge under neutral or slightly basic conditions [33,34]. This potentially facilitates interactions with positively charged siRNA/LP complexes via electrostatic attractions. Amine and/or thiol functional groups, if present in Lipofectamine RNAimax (proprietary composition), could also react with o-quinones in polydopamine via Schiff base or Michael addition reactions [14,15,35] to facilitate siRNA adsorption. Regardless of the mechanisms, residual siRNA/LP complexes on PD-modified film and fibers were difficult to extract, and the strong acid or base required to dissolve polydopamine may destroy the siRNAs in the process. Thus, the percentage cumulative release and loading efficiency could not be accurately determined in this study.

The stable adhesion of siRNA/LP complexes on PD-film and PD-fiber led to successful transfection and target gene knockdown in seeded NPCs. In general, PD-mediated siREST delivery showed greater REST knockdown than corresponding non-PD coated counterparts (Fig. 4A vs. Supplementary Fig. S1A). In particular, significantly higher REST knockdown efficiencies were observed with PD-modified nanofibers versus uncoated nanofibers on days 2 (67% vs. 20%, p < 0.01) and 5 (24% vs. 0%, p < 0.05). Although bolus delivery of siREST/LP complexes on PD-films induced the highest REST knockdown efficiency (Supplementary Fig. S2B vs. Fig. 4A), PD-mediated adsorption of siREST/LP complexes still possess greater applicability in vivo due to the feasibility of transfecting transplanted or endogenously recruited stem cells at scaffold implantation site. On PD-fibers in particular, adsorption of siREST/LP complexes resulted in significantly higher REST knockdown at day 2 and comparable silencing efficiency to bolus delivery of siREST/LP complexes at day 5 (Fig. 4A vs. Supplementary Fig. S2B) despite the low amount of soluble siRNAs released (maximum 206.9 ng vs. 670 ng for bolus transfection). Altogether, it is likely that REST silencing on siREST PD-fiber was achieved predominantly through direct internalization of surface-adsorbed siRNAs. Such delivery through localized and elevated concentration of surface-bound nucleic acids [36–40] has been demonstrated by earlier studies.

Fig. 3. In vitro characterization of siRNA/LP- and Cy5-ODN/LP-loaded PD-film and PD-fiber (A) Confocal fluorescent microscopy images indicating distribution of Cy5-ODN/LP complexes (red) on PD-film and PD-fiber at days 0 and 14 respectively. Scale bar: 100 μm. (B) Cumulative siRNA release from siRNA PD-film and PD-fiber. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Furthermore, the gene silencing efficiency of siREST PD-fiber at day 5 was comparable to and greater than that achieved by siRNA-encapsulated PCLEEP (29% vs. 31% in the latter [40]) and PCL (18% in plain PCL and 26% with PEG inclusion [39]) nanofibers respectively. Interestingly, the trend in gene silencing efficiency coincided with the cumulative release of siRNAs at day 5 (240 ng from siRNA-encapsulated PCLEEP > 200 ng from siREST(Ad) PD-fiber > 129 ng from siRNA-encapsulated PCL–PEG). This suggests

Fig. 4. REST knockdown efficiency with adsorption of siRNA/LP complexes on PD-coated films and fibers. (A) Real-time PCR results showing REST expression of siREST PD-film and siREST PD-fiber on days 2, 5 and 7. All results were normalized to siNEG PD-film or siNEG PD-fiber respectively. (B) Western blot analysis of REST knockdown in NPCs cultured on PD-film and PD-fiber with no siRNA (Lane 1), siNEG/LP adsorption (Lane 2) and siREST/LP adsorption (Lane 3) * and ** indicate $p < 0.05$ and $p < 0.01$ (Student’s $t$-test) respectively when compared to film or fiber controls. + indicates $p < 0.05$ (Student’s $t$-test) when PD-fiber samples were compared with PD-film samples. Mean ± SE, n = 3.

Fig. 5. Real-time PCR analysis of neural marker expression in NPCs differentiated on siRNA PD-film and siRNA PD-fiber. Expression of (A) Tuj1 and (B) Map2. All results were normalized to undifferentiated NPCs. * and ** indicate $p < 0.05$ and $p < 0.01$ (ANOVA) respectively. # and ### indicate $p < 0.05$ and $p < 0.001$ (ANOVA) when PD-fiber samples were compared to respective PD-film samples. Mean ± SE, n = 3.
that surface adsorption of siRNA/LP complexes onto PD-modified fibers may promote gene silencing at levels comparable to or better than bulk encapsulation in nanofibers, for the equivalent amounts of siRNAs present.

Studies thus far have suggested increased stem cell differentiation to neurons in the presence of REST knockdown on 2D cultures [21–23]. Likewise, our results on siREST PD-film showed significant increase in the percentage of neuronal cells and a lower percentage of astrocytic differentiation for mouse NPCs lacking REST [41]. However, a 2D microenvironment is...
simplistic and unable to recapitulate the complex three-dimensional in vivo microenvironment. Independently, studies have already shown favorable stem cell commitment to neuronal cells [42] and sub-optimal differentiation to astrocytes on nano-fibrinous scaffolds [43,44]. Our results appear to support these notions, but further demonstrate enhanced efficiency of neuronal differentiation through synergistic effects of scaffold-mediated REST knockdown and nanofibrous topography.

SiREST PD-fiber generated significantly higher percentage of neuronal cells than fiber controls. The enhanced neuronal commitment on siREST PD-fiber versus fiber controls was less obvious than that between siREST PD-film and fiber controls, suggesting an overall stronger signaling cue from nanofiber topology versus REST knockdown effect towards neuronal lineage commitment. Nevertheless, the efficiency of neuronal commitment with combined REST knockdown and nanofibers effect appears better than that achieved in comparative studies with poly(ethersulfone) (PES) nanofibers (57% Map2+ vs. 58% TuJ1+ cells in the latter [45]) and plain PCL nanofibers (47% and 38% TuJ1+ cells for aligned and random fibers respectively [42]) under serum-containing culture conditions. A lower extent of astrocytic commitment was achieved than that on randomly orientated PCL nanofibers (~2% with REST knockdown vs. ~30% in the latter [43]) and less oligodendrocytes were generated as compared to PCL and PES nanofibers respectively (>10% vs. <20% for both PCL [43] and PES [42] nanofibers). Overall, glial cell differentiation was adversely affected on PD-fibers and likely attributed to the selectivity of nano- and fibrous topography against astrocytes and oligodendrocytes as reported previously [42,43,46].

Recent reports on PD-mediated biofunctionalization of substrates and scaffold materials have shown encouraging results towards its application in neural stem cell engineering. Primary neurons demonstrated improved cell viability and maintenance of neural activities on substrates sequentially coated with polydopamine and poly-o-lysine [47]. Efficient immobilization of neurotrophic factors on PD-modified surfaces was also achieved, showing increased neuronal and astrocytic differentiation of human NPCs on selected substrates [48]. Independently, our results also demonstrated PD-mediated siRNA adsorption as a promising platform for therapeutic gene silencing applications. Therefore, future works can potentially utilize PD-modified nanofibrous scaffolds for delivery of topographical and multiple biochemical cues from neurotrophins and siRNAs functionalization, for highly efficient neuronal differentiation of NPCs. In vivo performance of such platforms in stimulating neuronal regeneration or axonal repair of traumatic nerve injuries may also be examined.

5. Conclusions

This study demonstrates the feasibility of delivering siRNA/transfection reagent complexes through PD-modified nanofibers, to induce scaffold-modified gene knockdown for enhanced neuronal differentiation of stem cells. Adsorption of siRNA/LP complexes on PD-fiber persisted for at least 14 days in vitro and sustained REST knockdown in NPCs was achieved for up to 5 days. The silencing of REST, together with nanofiber topographical effect, significantly enhanced NPC neuronal commitment and decreased glial cell differentiation. Accordingly, the synergistic effects of scaffold-modified REST knockdown and topographical cues from PD-modified nanofibers may be a useful strategy for generating functional neurons for therapeutic purposes.

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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.2013.01.093.

References


