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The effects of nanofiber diameter and orientation on siRNA uptake and gene silencing

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ABSTRACT

While substrate topography influences cell behavior, RNA interference (RNAi) has also emerged as a potent method for understanding and directing cell fate. However, the effects of substrate topography on RNAi remain poorly understood. Here, we report the influence of nanofiber architecture on siRNA-mediated gene-silencing in human somatic and stem cells. The respective model cells, human dermal fibroblasts (HDFs) and mesenchymal stem cells (MSCs), were cultured onto aligned or randomly oriented electrospun poly(ε-caprolactone) fibers of different average diameters (300 nm, 700 nm and 1.3 μm). In HDFs, decreasing fiber diameter from 1.3 μm to 300 nm improved Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Collagen-I silencing efficiencies by ~ 3.8 and ~4.4 folds respectively ($p < 0.05$) while the effective siRNA uptake pathway was altered from clathrin-dependent endocytosis to macropinocytosis. In MSCs, aligned fibers generated significantly higher level of gene silencing of RE-1 silencing transcription factor (REST) and green fluorescent protein (GFP) (~1.6 and ~1.5 folds respectively, $p < 0.05$), than randomly-oriented fibers. Aligned fiber topography facilitated functional siRNA uptake through clathrin-mediated endocytosis and membrane fusion. Taken together, our results demonstrated a promising role of three-dimensional fibrous scaffolds in modulating siRNA-mediated gene-silencing and established the critical synergistic role of these substrates in modulating cellular behavior by RNAi.

1. Introduction

The manipulation of cell behavior and differentiation remains a major challenge in the field of regenerative medicine. Hence in attempt to steer differentiation in a more specific and delicate manner, alternatives to conventional biochemical cocktails have been explored. In particular, genetic modification of cells by small non-coding RNAs (sncRNAs, e.g. small interfering RNAs, siRNAs) to elicit transient RNA interference (RNAi) has emerged to be a safe and popular way to induce accelerated cellular differentiation and transdifferentiation [1–6]. Unfortunately, the efficacy of RNAi-induced differentiation has been limited by the delivery and transfection efficiencies of sncRNAs [7]. Although attempts have been made to overcome this limitation by sustained application of sncRNAs from delivery vehicles [8–10] or by modifying the composition of nanoparticle carriers to improve transfection efficiency [11,12], the outcomes remain suboptimal. Therefore, more in-depth understanding and alternative approaches to enhance RNAi are required.

One potential method of modifying RNAi outcomes may be to control the architecture of the underlying substrate on which cells are cultured. Besides biochemical cues, topographical features of the extracellular matrix (ECM) also play a significant role in dictating cell fate. In this context, nanofiber constructs have been

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widely explored due to its biomimicking architecture. Indeed, fiber diameter and orientation can affect the proliferation, apoptosis and differentiation of cells [13–17]. In particular, neural stem cells preferentially differentiated into glial lineage on thin fibers (283 nm) but into neuronal lineage on thicker fibers (749 and 1.452 μm) [13]. Fiber diameter effect on cell behavior has also been established *in vivo* where nanofibers better facilitated the generation of myelinated axons and myelin sheaths [18], while microfibers enhanced the growth of vascular constructs in electrospun conduits [19]. Additionally, fiber orientation also modulates cell behavior. Aligned fibers could enhance osteogenic differentiation of marrow stromal cells [20] and mesenchymal stem cells [21], as well as neuronal differentiation of neural precursor cells [22] as compared to random fibers.

With more extensive research on RNAi- and substrate topography-mediated differentiation, the synergistic effect of these two platforms has been explored [9,10,23]. When applying siRNA to cells cultured on scaffolds, the topography of the scaffold facilitated the extent of differentiation by RNAi [9]. However, the endocytic pathway utilized by cells on scaffolds may be different from that on a two-dimensional surface [24]. Therefore, there is a need to examine the combined effect of ECM topography and gene knockdown. Unfortunately, to date, little is known about the interrelationship of these two factors. The potential of nanotopography in improving endocytosis and enhancing drug and gene delivery has been highlighted in a few studies [24,25], but its effect on gene silencing has yet to be elucidated.

In this study, we examined the effect of fiber diameter and orientation on gene silencing efficiency. The nanotopography of the microenvironment was tailor-made by electrospun poly(ε-caprolactone) (PCL) fibers of different average diameters (300 nm, 700 nm and 1.3 μm) and orientation (random and aligned). Using human dermal fibroblasts (HDFs) and human fetal mesenchymal stem cells (MSCs) as model somatic and stem cells, we investigated the effect of substrate topography on siRNA uptake and gene knockdown efficiencies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was chosen as the knockdown target for both cells since it is a housekeeping gene and has a high endogenous expression level which is not sensitive to substrate topography. Additionally, collagen I and RE1-silencing transcription factor (REST) were chosen as the knockdown targets for HDFs and MSCs respectively in order to investigate the knockdown effect on targets of lower abundance. We hypothesize that gene silencing efficiency may be improved by modifying the topography of the ECM.

2. Materials and methods

2.1. Materials

PCL (M_w = 80,000) and 2,2,2-tetrafluoroethanol (TEF, ≥99%), chlorpromazine, filipin, amiloride, and DMSO (≥99.9%) were obtained from Sigma Aldrich. The LIVE/DEAD® Cell Viability Assay kit (calcein-AM and ethidium homodimer-1), Alexa Fluor® 488 phalloidin, Molecular Probes transferrin and dextran, Ambion In Vivo siRNA controls (siNEG), GAPDH siRNA, type I Collagen siRNA, REST siRNA, GFP siRNA and 96-well plate for real-time PCR were bought from Life Technologies. Dulbecco's Modified Eagle Medium (DMEM), DMEM F-12 medium, fetal bovine serum (FBS), and penicillin-streptomycin (p/s) were purchased from Hyclone. Cy5-conjugated oligonucleotide (Cy5-ODN) was obtained from Aitbiotech. PCR reagents, random (dT) primers, M-MLV Reverse Transcriptase were purchased from Promega. RNeasy Mini Kit was purchased from Qiagen. HDFs were purchased from Lonza. Coverslips were purchased from VWR. MSCs and GFP-MSCs were derived from human fetal bone marrow as previously described with ethical approval from institutional research ethics board [26]. SYBR green Supermix was purchased from Bio-rad.

2.2. Scaffold fabrication

PCL film was fabricated by spin-coating. PCL was dissolved in TFE to obtain an 8 wt% solution. Thereafter, 80 μl of polymer solution was used and the PCL film was spin-coated onto a round coverslip (diameter 18 mm). For electrospinning of PCL fibers, different parameters were used to generate fibers with different diameters, as indicated in Table 1.

2.3. Evaluation of fiber scaffolds

Fiber scaffolds were coated with platinum at 20 mA for 80 s and imaged by scanning electron microscopy (SEM) (JOEL, JSM-6390LA, Japan) with an acceleration voltage of 10 kV. Thereafter, Image J (NIH, USA) was used to measure the average fiber diameter. Over 100 fibers were counted for each sample.

2.4. Cell culture and transfection

HDFs were maintained in DMEM supplemented with 10% FBS and 1% p/s. MSCs were maintained in DMEM F-12 supplemented with 10% FBS and 1% p/s. Cells were maintained in a humidified incubator at 37 °C with 5% CO_2. HDFs and MSCs were seeded at 10,000 and 20,000 cells per cm^2 respectively onto PCL film or fibrous scaffolds. Cells were cultured for 3 days in 24-well plates and stained with the LIVE/DEAD® Cell Viability Assay kit according to manufacturer's protocol. The elongation factor and cell area of live cells were measured by Image J. Elongation factor was computed by the equation: (Cell length/cell width) − 1. The cell area was the surface area of live cells spreading on the fibers or film. Over 100 cells per sample were calculated for statistical analysis.

All siRNA transfection was carried out in 1% FBS with TKO as the transfection agent. Cells were seeded for 24 h in 12-well plates before transfection. HDFs were transfected with GAPDH or Collagen I siRNA for 48 h at a volume ratio of 1:3 (siRNA:TKO) following manufacturer's protocol. MSCs were transfected with GAPDH or REST siRNA for 48 h at a volume ratio of 1:1. Thereafter, cells were harvested for real-time PCR for evaluation of knockdown efficiency. Cells transfected with random strands of siRNA were taken as a negative control (siNEG). For GFP silencing, lentiviral transduced MSCs over-expressing GFP were transfected with GFP siRNA for 48 h [27,28]. To quantify the extent of GFP silencing in GFP-MSCs, GFP signal per cell was measured using Image J. The signal intensity relative to GFP-MSCs transfected with siNEG was calculated. A lower relative signal intensity denotes a higher knockdown efficiency and *vice versa*. GFP transfection efficiency was computed by the equation: (1-signal intensity after siGFP transfection/signal intensity after siNEG transfection) ×100%. The particle size of siRNA/TKO complexes freshly prepared or incubated in DMEM for 48 h were measured by Zetasizer (Malvern Instrument, UK). Three samples were measured for each group.

2.5. Cy5-ODN uptake assay

HDFs or MSCs were seeded on film or fibrous scaffolds for 24 h before they were transfected with Cy5-ODN/TKO complexes (using the same volume ratio as siRNA) for 48 h. After transfection, the cells were solubilized with RLT lysis buffer (Qiagen RNeasy Mini Kit) and fluorescence intensity of the lysate was measured by Infinite F200 microplate reader (TECAN). Two hundred micro liters of lysate was added to each well for intensity measurement. A standard curve was generated with known concentrations of Cy5-ODN. The amount of Cy5-ODN retained by the cells was then derived from the standard curve and further normalized by the total amount of RNA

Table 1
Parameters for electrospinning of PCL fibers.

	300 nm Random	300 nm Aligned	700 nm Random	700 nm Aligned	1.3 μm random	1.3 μm aligned
Concentration (wt%)	12	12	12	12	12	12
Solvent	TFE:PBS (2×) = 1:4	TFE:PBS (1×) = 1:4	TFE:PBS (1×) = 1:4	TFE	TFE	TFE
Needle	22 G	22 G	30 G	30 G	21 G	30 G
Flowing rate (mL/h)	0.7	0.7	0.3	0.5	0.5	1
Rotator speed (rpm)	300	2300	300	2000	300	2000
Voltage (kV)	15	15	10	7	7	10
Distance (cm)	15	15	12	12	12	12
Fiber diameter (nm)	317 ± 33.7	306 ± 55.6	712 ± 82.3*,#	709 ± 93.7*,#	1339 ± 99.3*	1297 ± 144*

*: $p < 0.05$ with respect to 300 nm fibers; #: $p < 0.05$ with respect to 1300 nm fibers.

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