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Nanofiber topography and sustained biochemical signaling enhance human mesenchymal stem cell neural commitment

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ABSTRACT

Stem cells hold great promise in enhancing nerve regeneration. In particular, human mesenchymal stem cells (MSC) represent a clinically viable cell source due in part to their abundance and accessibility. Unfortunately, current methods to direct the fate of stem cells remains largely limited to biochemical-based approaches on two-dimensional substrates with restricted efficacies. Here we have evaluated a scaffold-based approach to directing stem cell differentiation. We demonstrate the combined effects of nanofiber topography and controlled drug release on enhancing MSC neural commitment. By encapsulating up to 0.3 wt.% retinoic acid (RA) within aligned poly(ε-caprolactone) (PCL) nanofibers (average diameter ~270 nm, AF750), sustained released of RA was obtained for at least 14 days (~60% released). Compared with tissue culture polystyrene (TCPS), the nanofiber topography arising from plain PCL nanofibers significantly up-regulated the expressions of neural markers, Tuj-1, MAP2, GalC and RIP at the mRNA and protein levels. Combined with sustained drug availability, more significant changes in cell morphology and enhancement of neural marker expression were observed. In particular, scaffold-based controlled delivery of RA enhanced MAP2 and RIP expression compared with bolus delivery despite lower amounts of drug (>8 times lower). The generally higher expression of the mature neuronal marker MAP2 compared with glial markers at the mRNA and protein levels suggested an enhanced potential of MSC neuronal differentiation. In addition, positive staining for synaptophysin was detected only in cells cultured on aligned scaffolds in the presence of RA. Taken together, the results highlight the advantage of the scaffold-based approach in enhancing the potential of MSC neuronal differentiation and demonstrated the importance of the drug delivery approach in directing cell fate. Such biomimicking drug-encapsulating scaffolds may permit subsequent direct cell transplantation and provide guidance cues to control the fate of endogenously recruited stem cells.

1. Introduction

Stem cells hold tremendous potential in the treatment of nerve injuries and neural diseases [1–3]. Amongst the various cell types explored, mesenchymal stem cells (MSC) probably hold the greatest promise for translational applications, due in part to their greater abundance and easier accessibility compared with neural stem cells and embryonic stem cells. However, neural differentiation in MSC remains ambiguous [4–6]. While multiple studies have demonstrated the potential of MSC to differentiate into progenitor cells expressing neural specific markers [7,8] and the ability of these cells to enhance nerve regeneration in vivo [2,9], there remains a search for a deeper understanding of the factors that govern MSC differentiation into non-mesenchymal lineages.

Furthermore, regardless of the choice of stem cell type, being able to specifically direct stem cell fate remains a limitation to the widespread clinical application of stem cell therapy. One of the major obstacles lies in the recapitulation of the stem cell niche. While studies thus far have demonstrated some degree of success in mimicking the biochemical signals required during stem cell differentiation [7,8], other works have suggested the critical and synergistic roles played by the extracellular matrix (ECM). In particular, substrate compliance and architecture signaling are important in directing cell fate [10,11]. Therefore, being able to combine several microenvironmental signals within a single scaffold construct would be attractive in achieving the ideal artificial stem cell niche design.

Nanofiber matrices mimic the architecture and size scale of the natural ECM. Compared with two-dimensional (2-D) substrates, nanofiber constructs provide more three-dimensional (3-D) biomimicking topographical signals to seeded cells and result in more

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physiologically relevant cellular phenotypes [12]. Applied to stem cell engineering, nanofibers alone support stem cell culture and neural differentiation, and effectively direct cell alignment and neurite extension along aligned fibers [13]. Endowed with sustained drug release capabilities, these nanofiber constructs enhance tissue regeneration [13–15] and may find useful applications in providing synergistic contact guidance and biochemical cues to direct stem cell fate [16]. Such biofunctional scaffolds may further permit subsequent direct cell transplantation and provide guidance signals to control the differentiation of endogenously recruited stem cells.

In this study we attempt to understand the effects of nanofiber topography and sustained release of a neuronal induction factor, retinoic acid (RA), on MSC neural commitment. We aim to evaluate the potential of RA-encapsulating poly(ε-caprolactone) (PCL) nanofiber constructs as an alternative platform for directing stem cell fate. Since MSC non-mesenchymal differentiation remains controversial, basic studies to understand the factors contributing to MSC phenotypic changes towards the neural lineage would be of scientific interest and relevance.

2. Materials and methods

2.1. Materials

PCL (M_n 80,000), bovine gelatin type B, bovine serum albumin (BSA), all-trans retinoic acid (>98%, gradient grade for HPLC), ammonium acetate (gradient grade for HPLC), 2,2,2-trifluoroethanol (TFE) of 99.0% purity, dimethyl sulfoxide (DMSO) (biotechnology performance certified), acetonitrile (gradient grade for HPLC), mouse anti-MAP2 monoclonal antibody, FITC-conjugated goat anti-rabbit antibody, ethanol, dichloromethane (DCM), hexamethyldisilazane (HMDS), Tris acetate–EDTA buffer (TAE), 10% formalin, Triton X-100, Tween 20, fluoromount and glycine were purchased from Sigma Aldrich. Phosphate-buffered saline (PBS) (pH 7.4), antibiotic and antimycotic 100×, phalloidin–Oregon green, DAPI, TRIzol reagent, Click-iT® EdU Alexa Fluor® 488 Imaging kits, Alexa Fluor488-conjugated goat anti-chicken antibody, and Alexa Fluor633-conjugated goat anti-mouse antibody were purchased from Invitrogen. RNeasy Mini kits, Sensiscript Reverse Transcription kits, and HotStarTaq Master Mix kits were purchased from Qiagen. Fast SYBR Green Master Mix was purchased from Taqman. Mouse anti-oligodendrocytes, clone NS-1 (RIP) monoclonal antibody and chicken anti-GFAP monoclonal antibody were purchased from Millipore. Rabbit anti-β-tubulin III (Tuj-1) polyclonal antibody was purchased from Covance. Mouse anti-synaptophysin monoclonal antibody was purchased from Dako. The Mesenchymal Stem Cell Growth Medium Bullet Kit (MSCGM) was purchased from Lonza. Fetal bovine serum (FBS) was purchased from Hyclone. Agarose was purchased from Bio-Rad and all primers were ordered from 1st Base, Singapore. All materials were used as received without further purification unless otherwise noted.

2.2. Fabrication of scaffolds

PCL (8 wt.%) and gelatin (1.5 wt.%) were dissolved in TFE. Thereafter PBS was added to the resulting solution at a 1:5 (PBS:TFE) volume ratio to form solution A. Solution A was subsequently used for all samples.

To fabricate plain PCL/gelatin electrospun scaffolds 2 ml of solution A was charged at 16 kV and fed at a flow rate of 0.8 ml h^{-1}. All fibers were then collected on a rotating drum (diameter 12 cm) that was charged at −5 kV. To obtain randomly oriented plain PCL/gelatin fibers (denoted RF, Table 1) the rotational speed of the drum was set at 500 rpm. To obtain aligned fibers (denoted AF, Table 1) a rotational speed of 2500 rpm was used. The syringe tip to collector distance was set at 12 cm.

Table 1
Optimized electrospinning parameters and average fiber diameters of scaffolds.

Sample	Applied voltage (kV)	RA loading level (%)	Rotation speed (rpm)	Fiber diameter (nm)
RF	16/−5		500	279 ± 30
AF	16/−5		2500	239 ± 37
AF250	15/−5	0.1	2500	270 ± 45
AF750	15/−5	0.3	2500	243 ± 93

All samples were fabricated using polymer solution comprising 8% PCL/1.5% gelatin, dispensed at 0.8 ml h^{-1}. The tip to collector distance was 12 cm.

To fabricate RA-encapsulating electrospun fibers, a stock solution of 3 mg ml^{-1} was prepared by dissolving RA in ethanol. To obtain aligned fibers comprising theoretical loading levels of 0.1 and 0.3 wt.% RA (with respect to PCL, denoted AF250 and AF750, respectively, Table 1), 85 and 250 μl of RA stock solution, respectively, were added into 2.2 ml of solution A for electrospinning. The resulting drug-loaded polymer solution was then charged at 15 kV and fed at a flow rate of 0.8 ml h^{-1}. All fibers were finally collected on the negatively charged rotating drum (−5 kV, 2500 rpm), which was set at 12 cm away from the polymer supply. The electrospinning process was protected from light to minimize the degradation of RA.

2.3. Characterization of nanofibers

The morphology of the electrospun fibers was investigated using a scanning electron microscope (JEOL JSM-6390LA) at an accelerating voltage of 15 kV after gold coating for 90 s. The fiber diameters were measured using ImageJ (NIH). Five images at 10,000× magnification were used and at least 100 fibers were measured for each sample.

The distribution of RA within the PCL/gelatin fibers was analyzed by electrospinning nanofibers directly onto a glass coverslip. Thereafter the autofluorescence of RA was detected using an Olympus IX71 microscope with a FITC filter.

2.4. Release kinetics of retinoic acid

AF750 scaffolds (average weight 228 mg, $n = 3$) were cut into pieces (4.71 × 2.5 cm each) and soaked in 5 ml of PBS. To account for possible dissolution of gelatin from the nanofibers, AF fibers (average weight 220 mg, $n = 1$) were used as the control. All samples were protected from light and incubated under static conditions at 37 °C. At various time points 2.5 ml of supernatant was retrieved and replenished with 2.5 ml of fresh PBS. To analyze the amounts of RA that were released within 1 day early time points of 3, 6, 12 and 24 h were chosen. The supernatants were 0.22 μm filtered before HPLC analysis. To obtain the standard curve for RA the supernatant from AF fibers was doped with 0.31–10 μg ml^{-1} RA. Thereafter an Agilent HPLC apparatus (Agilent 1100 series, consisting of a G1379A Degasser, G1311A Quat Pump, G1313A ALS, G1316A COLCOM, G1364C Analyt-FC, G1362A 1260 RID, and G1315B DAD) with a reverse phase column (Lichrospher® 100 RP-18 column, 4 × 25 cm, 5 μm) was used to measure the concentrations of the solutions. During the analyses the mobile phase consisted of a mixture of acetonitrile and aqueous ammonium acetate at a volume ratio of 9:1, delivered at a flow rate of 1 ml h^{-1}. HPLC DAD detection was carried out at 340 nm with a reference at 500 nm [17,18]. The data was quantified using the external standard method. A computer was connected to the detector for data acquisition and retention time and peak area calculations.