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Modulation of embryonic mesenchymal progenitor cell differentiation via control over pure mechanical modulus in electrospun nanofibers

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ABSTRACT

As the potential range of stem cell applications in tissue engineering continues to grow, the appropriate scaffolding choice is necessary to create tightly defined artificial microenvironments for each target organ. These microenvironments determine stem cell fate via control over differentiation. In this study we examined the specific effects of scaffold stiffness on embryonic mesenchymal progenitor cell behavior. Mechanically distinct scaffolds having identical microstructures and surface chemistries were produced utilizing core–shell electrospinning. The modulus of core–shell poly(ether sulfone)–poly(ε-caprolactone) (PES–PCL) fibers (30.6 MPa) was more than four times that of pure PCL (7.1 MPa). The results for chondrogenic and osteogenic differentiation of progenitor cells on each scaffold indicate that the lower modulus PCL fibers provided more appropriate microenvironments for chondrogenesis, evident by a marked up-regulation of chondrocytic *Sox9*, collagen type 2, and aggrecan gene expression and chondrocyte-specific extracellular matrix glycosaminoglycan production. In contrast, the stiffer core–shell PES–PCL fibers supported enhanced osteogenesis by promoting osteogenic *Runx2*, alkaline phosphatase, and osteocalcin gene expression, as well as alkaline phosphatase activity. The findings demonstrate that the microstructural stiffness/modules of a scaffold and the pliability of individual fibers may play a critical role in controlling stem cell differentiation. Regulation of cytoskeletal organization may occur via a "dynamic scaffold" leading to the subsequent intracellular signaling events that control differentiation-specific gene expression.

1. Introduction

Tissue engineering is a clinically driven field that seeks to replace or support damaged organs. A common strategy is culturing target-specific cells *in vitro* in a scaffold followed by implantation. As a logical cellular source for tissue engineering stem cells have attracted a great deal of attention due to their relatively fast proliferation rate and diverse differentiation potential to various phenotypes. These include cells derived from several origins: induced pluripotent stem cells (iPS) from fibroblasts [1,2], mesenchymal stem cells from bone marrow [3,4], and adult stem cells from adipose tissue [5,6]. Stem cells distinctively self-renew and their terminal differentiation depends on the influence of soluble molecules (growth factors, cytokines) as well as physical and biochemical interactions with scaffolds. Cellular behavior and subsequent tissue development at the cell–scaffold interface therefore involve adhesion, motility, proliferation, differentiation and functional maturity. Even though many promising clinical applications of stem cells are forecast, our knowledge of directed differentiation via cell–scaffold interaction is relatively limited compared with the well-documented effects of soluble growth factors and cytokines. Hence, understanding how stem cell fate is influenced by the mechanical properties of scaffolds, a determinant of cell–material interaction, is of fundamental importance to functional tissue engineering.

The physicochemical properties of a scaffold, such as bulk chemistry, surface chemistry, topography, three-dimensionality and mechanical properties, all influence cellular response. Bulk chemistry can control cytotoxicity, as most scaffolds are made of biodegradable materials and must eventually release the by-products of their degradation. The effect of surface chemistry is often mediated by instantly adsorbed proteins such as fibronectin, collagen, fibrinogen, vitronectin, and immunoglobulin [7] that affect phenotype [8], viability [9], and morphology [10], as well as proliferation and differentiation [11]. A number of studies regarding the effect of surface topography/texture on cellular response have been conducted [12–14]. Stem cells recognize topographical features of the order of hundreds of nanometers to several micrometers, and exhibit distinctive genomic profiles in the absence of biochemical differentiation cues [15] and a commitment to terminal differentiation [16]. Although these studies have provided in-depth insights

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regarding the role of substrate microstructure on stem cell differentiation, the two-dimensional (2D) nature of these substrates limits their application in tissue engineering. Stem cells in 2D environments have been shown to have significant differences in differentiation compared with three-dimensional (3D) cultures [17,18].

Electrospun scaffolds are ideal matrices for 3D culture of the cells providing non-woven nano- to micro-sized fibrous microstructures having relative porosities of 70–90%. Natural biodegradable materials such as collagen [19], gelatin [20], elastin [21], chitosan [22], and hyaluronic acid [23], as well as synthetic biodegradable polymers such as poly(ε-caprolactone) (PCL) [24–26], poly(glycolic) acid (PGA) [27] and poly(lactic) acid (PLA) [27], have been electrospun for chondral and osseous applications. In general, the utility of electrospun scaffolds for tissue engineering is clear, however, comparison of cellular responses to chemically identical but mechanically diverse scaffolds is yet to be explored. Previous studies have investigated different polymer compositions as a means of modifying the modulus [20,28–30], but cannot account for potential effects of changes of both nanofiber surface chemistry and microstructure on cellular behavior.

In this study electrospun pure poly(ε-caprolactone) (PCL) or core–shell poly(ether sulfone) (PES)–PCL scaffolds were synthesized. These scaffolds had identical microstructures and surface chemistries but distinct moduli allowing dissection of the role of electrospun fiber modulus on stem cell differentiation. Morphological and mechanical properties of the scaffolds were first characterized, followed by evaluation of their potential to differentially regulate the phenotype of embryonic mesenchymal progenitor cells entering the chondrogenic and osteogenic lineages using phenotype-specific gene expression and protein synthesis as markers of differentiation.

2. Materials and methods

2.1. Electrospun pure and core–shell nanofibers

Pure PCL nanofibers were electrospun as described elsewhere [31,32]. Briefly, a 6.7 wt.% solution of PCL (M_w 65,000, Sigma–Aldrich, St. Louis, MO) dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma–Aldrich) was prepared and then electrospun using a high voltage d.c. power supply (model FC50R2, Glassman High Voltage, NJ) at +26 kV, a 20 cm tip to substrate distance [33] and a flow rate of 15 ml h^{-1} to produce approximately 100 μm thick scaffolds. The core–shell form of PES (Goodfellow, Cambridge, UK)–PCL nanofibers was prepared using a 22 gauge hypodermic needle (Integrated Dispensing Solutions, Agoura Hills, CA) inserted through a 16 gauge hypodermic T-junction (Small Parts Inc., Miramar, FL) to create two concentric blunt needle openings. A Swagelok stainless steel union was used to hold the needles in place and ensure the ends of the needles were flush with each other. One syringe was filled with a polymer solution of 8 wt.% PES dissolved in HFIP for the core and connected to the 22 gauge needle and set to a flow rate of 2 ml h^{-1} using a syringe pump. An identical syringe was connected via an extension to the T-junction, filled with the shell material of 6.7 wt.% PCL dissolved in HFIP and set to a flow rate of 2 ml h^{-1} using yet another syringe pump. A high voltage power source (Glassman High Voltage) was connected to the concentric needle structure and set to +30 kV, again using a tip to substrate distance of 20 cm.

2.2. Morphological and mechanical characterization of electrospun fibers

The electrospun fiber meshes were coated with an 8 nm thick layer of osmium using an osmium plasma coater (OPC-80T, SPI Supplies, West Chester, PA) for scanning electron microscopy (SEM). The osmium-coated samples were observed in an FEI XL-30 Sirion microscope with a field emission gun (FEG) source. For transmission electron microscopy (TEM) examination of the core–shell PES–PCL fibers, the polymer solutions were directly spun onto TEM 200 mesh copper grids with a carbon film backing (Ted Pella Inc., Redding, CA). The intra-fiber microstructure of the core–shell fibers was observed using an FEI Technai G2 Spirit at 80 keV. In addition, the surface chemistry of electrospun nanofibers was analyzed by X-ray photoelectron spectroscopy (XPS) using a Kratos Axis Ultra XPS. Survey scans were acquired using an MgK_α (1253.6 eV) X-ray source operating at 10 mA and 13 kV with a chamber pressure of approximately 2×10^{-9} Torr.

For mechanical property evaluation tensile dogbones with a gauge length of 20 mm and a gauge width of 2.4 mm were cut from the electrospun sheets as before [34] using 2 mm thick aluminum templates. A surgical blade (Bard–Parker No. 15, BD Medical Systems, Franklin Lakes, NJ) was used to cut the straight edges while a 3 mm dermal biopsy punch (Miltex, York, PA) was used to form the radii. Great care was taken in cutting the gauge length to avoid tearing or smearing which might conceivably influence the results. Tensile sample thickness was measured using a digital micrometer (Starrett, Athol, MA) by placing the gauge length of each specimen between two glass microscope slides and subtracting the thickness of the two slides from the total. The tensile properties were determined utilizing a 1 kg load cell (model 31, Honeywell Sensotec, Columbus, OH) and a strain rate of 5 mm min^{-1} on a load frame (model 1322, Instron, Norwood, MA) using lightweight carbon fiber grips (A2-166 Fibre Clamp Assembly, Instron). The tests were performed in a dry environment without pre-straining of the samples. Tensile testing utilized five samples for each condition.

2.3. Cell seeding and culture in scaffolds

C3H10T1/2 murine embryonic mesenchymal progenitor cells (ATCC, Manassas, VA) were seeded (20,000 cells cm^{-2}) in the pure PCL or core–shell PES–PCL electrospun fibers placed in 12-well Falcon™ tissue culture plates (BD Biosciences, Franklin Lakes, NJ). All scaffold samples were sterilized using an overnight exposure to 70% ethanol and washed with phosphate-buffered saline (PBS) (Gibco™, Invitrogen, Carlsbad, CA) twice and Hank's balnced salt solution (Invitrogen) containing 1% penicillin/streptomycin (Fisher Scientific, Fairlawn, NJ) prior to cell inoculation. The seeded cells were cultured in either chondrogenic medium [35] (Ham's F-12 (Invitrogen) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Invitrogen) and 1% L-glutamine (Mediatech, Manassas, VA) supplemented with 100 ng ml^{-1} rhBMP-2 (GeneScript, Piscataway, NJ)) or osteogenic medium [36] (Dulbecco's modified Eagle's medium containing 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine supplemented with 50 mg ml^{-1} L-ascorbic acid (Sigma–Aldrich, 10 mM β-glycerophosphate (MP Biomedicals, Solon, OH), 10 nM dexamethasone (Sigma–Aldrich)). The cells cultured on tissue culture polystyrene (TCPS) at the same cellular density in each differentiation medium were used as controls. Samples were harvested after 48 h for subsequent characterization.

2.4. Analysis of gene expression by real-time polymerase chain reaction (rt PCR)

Three samples of the cells cultured in the PCL or PES–PCL scaffolds under chondrogenic or osteogenic conditions were pooled after 48 h culture and total RNA extracted using an RNeasy Micro Kit (Qiagen, Valencia, CA), followed by first strand cDNA synthesis as described earlier [37]. rt PCR was performed to assess gene expression using the custom primers listed in Table 1. Collected