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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, 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 microstructure and surface chemistry, 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 4 times that of pure PCL (7.1 MPa). Results from chondrogenic or osteogenic differentiation of progenitor cells on each scaffold indicate that the lower modulus PCL fibers provided more appropriate microenvironments for chondrogenesis, evident by marked upregulation 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 of a scaffold and the pliability of individual fibers may play a critical role in controlling stem cell differentiation. This may occur via regulation of distinct cytoskeletal organization and subsequent intracellular signaling events that control differentiation-specific gene expression.

Keywords

mechanical properties of scaffold; stiffness; stem cell differentiation; cartilage tissue engineering; bone tissue engineering

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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 its 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 forecasted, our knowledge of directed differentiation via cell-scaffold interaction is relatively limited as compared to 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.

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], 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 in the order of hundreds of nanometers to several micrometers, and exhibit distinctive genomic profiles in the absence of biochemical differentiation cues [15] and the degree of commitment to terminal-differentiation [16]. Although these studies have provided in-depth insights regarding the role of substrate microstructure on stem cell differentiation, the two-dimensional nature of these substrates limits their application in tissue engineering. Stem cells in 2-D environments have been shown to have significant differences in differentiation compared to 3-D culture [17,18].

Electrospun scaffolds are ideal matrices for three-dimensional culture of the cells providing non-woven nano- to micro-sized fibrous microstructure having 70 ~ 90% relative porosity. 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, the comparisons 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 modulus [20,28–30] but cannot account for potential effects by the changes of both nanofiber surface chemistry and microstructure on cellular behaviors.

In this study, electrospun pure poly(ϵ -caprolactone) (PCL) or core-shell poly(ether sulfone) (PES)-PCL scaffolds were synthesized. These scaffolds had identical microstructure 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 phenotypes of embryonic mesenchymal progenitor cells into 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, MO) dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma-Aldrich) was prepared and then electrospun using a high voltage DC 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/hr to produce approximately 100 μ m-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 the polymer solution of 8 wt. % PES dissolved in HFIP for the core, and connected to the 22 gauge needle and set to a 2 mL/hr flow rate 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/hr 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, PA) for scanning electron microscopy (SEM). The osmium-coated samples were observed under FEI XL-30 Sirion SEM 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, CA). The intra-fiber microstructure of the core-shell fibers was observed using an FEI Technai G2 Spirit at 80kV. In addition, the surface chemistry of electrospun nanofibers were analyzed by X-ray photoelectron spectroscopy (XPS) using a Kratos Axis Ultra XPS. Survey scans were acquired using an Mg K 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 #15, BD Medical Systems, NJ) was used to cut the straight edges while a 3 mm dermal biopsy punch (Miltex, 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, 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. Tensile properties were determined utilizing a 1 kg load cell (model 31, Honeywell Sensotec, OH) and a strain rate of 5 mm/min on a load frame (model 1322, Instron, 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, VA) were seeded (20,000 cells/cm²) in the pure PCL or core-shell PES-PCL electrospun fibers placed in 12-well tissue culture plates (Falcon, NJ). All scaffold samples were sterilized using an overnight exposure to 70% ethanol and washed with PBS (Gibco, CA) two times and HBSS (Gibco) containing 1% penicillin/streptomycin (Fisher scientific, NJ) prior to cell inoculation. The seeded cells were cultured in either chondrogenic media [35] (Ham's F-12 (Gibco) containing 10% FBS, 1% penicillin/streptomycin (Gibco) and 1% L-glutamine (Mediatech) supplemented with 100 ng/ml rhBMP-2 (GeneScript, NJ)) or osteogenic media [36] (DMEM containing 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine supplemented with 50 mg/ml L-ascorbic acid (Sigma-Aldrich), 10 mM β -Glycerophosphate (MP Biomedicals, 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 hours for subsequent characterization.

2.4. Analysis of Gene Expression by Real-Time Polymerase Chain Reaction (rt-PCR)

Three samples of cells cultured in the PCL or PES-PCL scaffolds under chondrogenic or osteogenic conditions were pooled after 48 hours of culture and total RNA extracted using the RNeasy Micro Kit (Qiagen, CA), followed by first strand cDNA synthesis as described earlier [37]. Real-time polymerase chain reaction (rt-PCR) was performed to assess gene expression using custom primers in Table 1. Collected data were analyzed by the comparative threshold cycle (C_T) method using *Rps18* as an endogenous control [38].

2.5. Measurement of Glycosaminoglycan (GAG) Synthesis and Alkaline Phosphatase (ALP) Activity

To evaluate GAG production, cells cultured under chondrogenic conditions in the PCL or PES-PCL scaffolds for 48 hour were fixed with 10% neutral formalin and stained with 1% Alcian blue 8GX (Sigma-Aldrich) in 3% acetic acid for 30 minutes. The stained samples were rinsed in distilled water, cleared with alcohol, and mounted for microscopic evaluation. For quantitative analysis of GAG synthesis, the Alcian blue stained samples were re-dissolved in 2% sodium dodecylsulfate solution with constant shaking [39]. The resultant solution was spectrophotometrically read at 620 nm with Wallac Victor³™ plate reader (Perkin Elmer, MA).

For the assessment of ALP activity, cells cultured in osteogenic conditions for 48 hours were stained with 0.01% Naphthol AS-MX Phosphate (Sigma-Aldrich, MO) and 0.06% Fast red LB salt (Sigma-Aldrich) dissolved in 0.04 M Tris, 0.008 M hydrochloric acid and 0.5% dimethylformamide solution for 30 minutes. The cells were then counterstained with Mayer's hematoxylin for 1 minute, followed by rinsing in distilled water. The ALP assay kit (Bioassay Systems, CA) was utilized for the quantitative analysis of ALP activity according to the manufacturer's instructions. Briefly, supernatants of the cells cultured in each scaffold for 48 hours were collected, and reacted with *p*-Nitrophenyl phosphate that is hydrolyzed by ALP into a yellow colored product. The absorbance of the resultant solutions was colorimetrically measured at 405 nm using Wallac Victor³™ plate reader.

To determine GAG production and ALP activity with per-cell basis, number of cells cultured in each condition was examined. The samples in triplicate for each condition were fixed with 10% formalin solution, followed by staining with 0.1% crystal violet (Sigma-Aldrich) for 20 minutes. The stained samples were thoroughly washed with DI water and taken to a separate 12-well plate in order to ensure that we counted only those cells adhering to the samples. 250 μ l of 1% SDS (National Diagnostics, GA) was added to each well and

the plate shaken for 30 minutes. Next, 200 μ l of the resultant crystal violet stain-dissolved SDS solutions was put into a 96-well plate (Falcon, NJ) and the absorbance of each well was read at 560 nm with a Wallac Victor³™ plate reader. Approximately 30,000, 60,000, 120,000 and 240,000 cells were stained with crystal violet using the same procedure and the absorbance reading used to produce a calibration curve.

2.6. Cellular Morphology

To examine the cellular distribution in the scaffolds, samples cultured in either chondrogenic or osteogenic conditions were fixed with 10% formalin followed by cryosection described elsewhere [40]. Briefly, the fixed samples were embedded in OCT compound (Sakura Finetek, CA) and cut to 20 μ m sections using a cryostat (CM3050S, Leica Microsystems, IL). The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and observed under an epifluorescence microscope (Axioplan2, Carl Zeiss, NY) with excitation wavelengths of 365 nm for DAPI and 490 nm for PCL autofluorescence.

To examine cellular morphology, the cells cultured in each scaffold under chondrogenic conditions for 48 hrs were 10% formalin-fixed followed by SEM sample preparation steps described elsewhere [40]. Briefly, the samples were sequentially dehydrated using DI water/Ethanol series followed by Ethanol/Hexamethyldisilazane (HMDS, Ted Pella, CA) series. The dried samples were subjected to osmium coating as previously described.

To observe intracellular organization of adhesion (integrin β 1) and cytoskeleton (F-actin) molecules, the cells cultured on each scaffold under chondrogenic conditions for 48 hours were fixed with 2% paraformaldehyde and permeabilized with 0.2% Triton-X (Sigma-Aldrich). The cells were then immunofluorescently stained using rabbit anti-integrin β 1 primary antibody (Santa Cruz Biotechnology, CA) and CY3 labeled goat anti-rabbit secondary antibody (The Jackson Laboratory, ME). Subsequently, the samples were counter-stained with fluorescein isothiocyanate (FITC) labeled phalloidin (Sigma-Aldrich) in PBS to reveal cytoskeletal organization of actin filaments. Finally, the samples were mounted in antifade reagent containing DAPI to stain nuclei, and observed under an epifluorescence microscope. Approximately 118 $\times$ 88 μ m sized images (1388 $\times$ 1040 pixels) were taken at fixed setting for brightness, contrast, gamma and exposure time.

To quantitatively assess stress fiber development in the cells on each of the scaffolds, densitometric analysis was performed by measuring the mean intensity of F-actin stained image using ImageJ (National Institutes of Health) similar to the image analysis method of Sbrana et al. [41]. First, intensity of each image was background collected by using 'subtract background' function with a "rolling ball" algorithm in the program [42]. After image standardization, total intensity was measured and divided by the number of nuclei counted to derive mean stress fiber intensity per cell. Six randomly chosen areas from three independent samples, a total of 18 areas, were analyzed in each condition.

2.7. Statistical Analysis

Unless otherwise noted in figure caption, all experiments were conducted in triplicate, and data represented as means $\pm$ standard deviation. Data were analyzed using SPSS (v.17.0) by either T-test or one-way ANOVA with Tukey's HSD post-hoc to test significances. $p < 0.05$ was regarded as statistically significant.

3. Results

Electrospinning PCL produced cylindrical random fibers (Fig. 1A), and core-shell PES (core)-PCL (shell) fibers synthesized by the standard needle-in-needle configuration exhibited microstructural characteristics that were very similar to PCL fibers (Fig. 1B). A

schematic illustration of core-shell structure is shown in Figure 1C. In the PES-PCL fiber, TEM revealed the expected coaxial configuration of a PES core surrounded by a PCL shell (Fig. 1D). The existence of a PCL shell continuous over the entire area of the fiber was further confirmed by XPS analysis (Fig. 2). Peaks specific to sulfur (S2s and S2p) are present in pure PES electrospun fibers but are absent in both pure PCL and, critically, the PES-PCL core-shell fibers. To our knowledge, this data provides the first demonstration of complete coverage of PES by PCL in these fibers. The average fiber diameters of pure PCL and core-shell structured nanofibers were 750 ± 490 and 867 ± 257 nm, respectively (Table 2). The average diameter of the PES core in the core-shell fibers was 185 ± 43 nm. Both structures generated a similar pore size based on the measured 'inter-fiber distance' at 8.44 ± 3.21 and 8.94 ± 3.90 μm for PCL and core-shell PES-PCL fibers, respectively (Table 2).

In spite of their similarities in fiber diameter and microstructure, pure PCL and core-shell PES-PCL nanofibers exhibited quite different mechanical moduli. At 30.6 MPa, the elastic modulus of the core-shell nanofibers was more than 4 times that of the pure PCL nanofibers (7.1 MPa). However, they both exhibited a similar ultimate tensile strength, 2.5 MPa (Table 2), indicative of largely microstructural control over failure [32].

To investigate how these different moduli affected stem cell differentiation, gene expression profiles of murine embryonic mesenchymal progenitor cells cultured in either chondrogenic or osteogenic conditions were examined. These cells were selected because 1) they constitute a homogeneous population of multipotential cells that can be differentiated into chondrocytes, osteoblasts, adipocytes and myoblasts, 2) their chondrogenic and osteogenic differentiations are compatible to those of primary mesenchymal stem cells derived from bone marrow, and 3) they do not spontaneously differentiate under normal culture conditions [35,36]. After 2 days culture in either condition, cellular infiltration throughout the depth (~ 100 μm) of the scaffolds was observed (Fig. 3).

To minimize the effects of cell-cell interactions and maximize cell-material interactions, the cells were cultured for a short period (48 hours) at which point the cell density reached approximately 70~80% confluence. Monolayer-cultured cells supplemented with differentiation media in tissue culture polystyrene (TCPS) plates were used as a control. The variation in scaffold stiffness led to differences in the degree of chondrogenesis when the cells were cultured under chondrogenic conditions (Fig. 4). The softer pure PCL nanofibers induced greater upregulation of chondrogenic gene expression including Sox9, aggrecan (*Acan*) and collagen type 2 (*Col2a1*) as compared to those of the cells cultured on the core-shell PES-PCL nanofibers. Sox9, a transcription factor regulating collagen type 2 gene expression [43] and chondrocyte maturation [44], was significantly upregulated only when cultured on the pure PCL nanofibers. Furthermore, cells cultured on the PCL nanofibers exhibited a significant upregulation of aggrecan expression that was more than 15 times greater than those grown on the stiffer core-shell PES-PCL nanofiber scaffolds. Collagen type 2 gene expression, a terminal-differentiation marker of chondrogenesis, was also upregulated only on the pure PCL scaffolds, but not on PES-PCL scaffolds.

The differential commitment of mesenchymal progenitor cells to chondrocytic phenotype was confirmed at the protein level by examining GAG production (Fig. 5). It was revealed that stronger Alcian blue staining on the pure PCL scaffold (Fig. 5A) indicates greater GAG deposition, when compared to the stiffer core-shell PES-PCL fibers (Fig. 5B). A quantitative spectrophotometric measurement showed statistically significant differences in GAG production (Fig. 5C).

Conversely, when the cells were cultured in osteogenic conditions, the stiffer PES-PCL fibers supported greater osteogenesis of mesenchymal progenitor cells compared to the softer PCL fibers. *Runx2*, an early osteogenic marker which competes with *Sox9* for eventual differentiation fate of mesenchymal cells to osteoblastic phenotype [44], was significantly upregulated only in the cells on the PES-PCL fibers (Fig. 6A). Similarly, other osteogenic markers, *Alp* and *Oc*, were upregulated greater by culture on the stiffer PES-PCL fibers (Fig. 6B and 6C). Interestingly, the gene expression of alkaline phosphatase on the pure PCL scaffolds was also significantly upregulated with respect to the monolayer on TCPS in the differentiation conditions.

The greater *ALP* gene expression in the cells grown on the core-shell fibers was further confirmed by higher alkaline phosphatase activity as compared to the cells on the PCL scaffolds by histological and quantitative analyses at the protein level (Fig. 7). It is shown that the cells on the core-shell fibers exhibit higher alkaline phosphatase activity (Fig. 7B) as compared to the cells on the PCL scaffolds (Fig. 7A). This observation agreed with the functional analysis of alkaline phosphatase activity by spectrophotometric measurements of exogenous *p*-Nitrophenyl phosphate conversion to *p*-Nitrophenol and phosphate (Fig. 7C).

To investigate whether the differences in mechanical moduli of substrates affect adhesion and/or cytoskeletal organization of the cells, the cells cultured under chondrogenic conditions, which showed more prominent differences in gene and protein expression between two scaffolds, were examined using immunofluorescent microscopy. The intracellular distribution of integrin $\beta 1$, an adhesion molecule, did not reveal significant differences between the two scaffolds (Fig. 8A and 8E). Integrin $\beta 1$ molecules in the cells on either scaffold showed a relatively uniform distribution with several agglomerates co-localized at the cell-nanofiber attachment sites. However, nanofiber modulus did appear to affect the cytoskeletal organization of F-actin (Fig. 8B and 8F). More stress fibers were observed in the cells cultured on the stiffer PES-PCL fibers. The differences in cytoskeletal organization could be observed in overall cellular morphology by SEM (Fig. 8D and 8H). The cells on the pure PCL showed relatively rounded morphology compared to the spread shape on the PES-PCL fibers. To quantitatively assess these differences, the F-actin stained samples were subjected to an image analysis (Fig. 8I and 8J). The analysis confirmed that the stiffer core-shell fibers induced significantly higher stress fiber density (stress fiber intensity/cell) (Fig. 8K).

Since mechanical microenvironments can affect density and alignment of stress fibers [45], a theoretical calculation of fiber deflection was performed to examine differential individual fiber pliability. A simple beam calculation [46] using 75 nN of applied tensile force [47] and data from Table 2, shows that these cells could cause substantial movement/deformation of either of the electrospun nanofiber compositions (Fig. 9). Simple bending of a nanofiber supported by two adjacent intersecting fibers was assumed and calculated using the beam deflection equation:

4. Discussion

In this study, we have demonstrated that scaffold stiffness is a dynamic regulator of stem cell-biomaterial interactions through the utilization of electrospinning to produce mechanically distinct scaffolds yet having both identical microstructure and surface chemistry. As the potential range of stem cell applications in tissue engineering continues to grow, scaffolding no longer provides mere structural support to developing engineered tissues. Instead, it creates tightly defined artificial microenvironments that instruct stem cell fate, via control over both differentiation and proliferation. The mechanical properties of a scaffold are thus another critical factor that influences stem cell fate, in addition to bulk

chemistry, surface chemistry, microstructure, topography, and three-dimensionality [15–18,28–30].

We show that the stiffness of electrospun fibrous scaffolds significantly alters gene expression in mesenchymal pluripotent cells during early chondrogenic and osteogenic differentiation. Unlike approaches using gel systems in which rigidity is changed by altered solid loading that also introduces modification in the density of adhesion sites, our use of core-shell nanofibers elegantly isolates the contribution of pure mechanical stiffness to the differentiation of stem cells. Furthermore, we deliberately avoided over-confluence of cells to examine changes in differentiation caused by cell-material interaction rather than by cell-cell interactions. Under these conditions, the modulus of scaffolds played a critical role in differentially regulating the expression of *Sox9* and *Runx2* transcription factors, the master switches of mesenchymal cell differentiation. *Sox9* and *Runx2* are known to compete with each other to control the terminal-differentiation of mesenchymal cells into chondrocyte and osteoblasts, respectively [44]. The mechanically ‘softer’ pure PCL fibers supported greater expression of *Sox9* leading to the upregulation of the chondrocyte-specific gene markers, *Acan* and *Col2a1*. The stiffer core-shell PES-PCL fibers supported better osteogenesis by upregulation of *Runx2* and subsequent *Alp* and *Oc* expression.

Cellular function can be modulated by adhesion to extracellular matrix (ECM). It has been recently shown that surface chemistry differentially mediates mesenchymal stem cell differentiation in a lineage-dependent manner by surface chemistry-induced changes in fibronectin conformation and integrin binding [49]. Integrins present on the cell surface in both chondrocytes and osteoblasts crucially affect cellular functions [50,51]. Therefore, the effect of substrate moduli on the intracellular/membranous distribution of integrin $\beta 1$ was investigated. We did not observe significant differences in integrin $\beta 1$ distribution or aggregation on the surfaces of the cells grown on PCL or PES-PCL. This was not surprising, as the surface chemistry of both PCL and PES-PCL fibers was identical, suggesting that cellular adhesion is driven more by surface chemistry and scaffold microstructure.

Plasma etching has previously been shown to provide a nanostructure that alters stress fiber development without changes in chemical composition [52]. In these electrospun substrates, the structure of the F-actin filaments in the cells on pure PCL versus those on core-shell fibers exhibited considerable differences in the chondrogenic conditions. The stiffer core-shell scaffolds induce substantial agglomeration and alignment of actin forming stress fibers. This agrees with the tensegrity model in which cells adapt their cytoskeletal organization to microenvironments around them [45]. Fiber pliability may provide a plausible explanation for the differences in cytoskeletal organization. For example, cellular traction forces on softer scaffolding material cause electrospun fiber motion during actin polymerization that could prevent the accumulation of F-actin filaments needed to create visible stress fibers. However, we did not observe statistically significant differences in stress fiber intensity between the cells cultured in the pure PCL and those in the PES-PCL (data not shown). This may be due to the fact that osteogenesis of mesenchymal cells involves the formation of thick actin bundles observed at the outermost periphery of the cells unlike chondrogenesis during which mesenchymal cells lose stress fibers [53]. It appears that soluble osteogenic factors compete with the mechanical environments of scaffolds to determine cytoskeletal organization.

Cells in the electrospun fibrous meshes span more than several fibers exerting isometric tensile forces on a ‘network’ of fibers rather than a single fiber. Given that cells engage with more than one fiber, small strain measurements of multiple fiber arrangements likely better capture nanofiber influences over the biological response than single fiber measurements. Currently, direct measurement of fiber deflection in a random fibrous network cannot be

accomplished due to the absence of a reliable testing method appropriate to that scale. To estimate fiber deflection, however, the model of a fiber supported by two intersecting fibers was employed in our theoretical calculation. Simple bending accounts only for deflections perpendicular to the supporting fibers. Therefore, the fibers can potentially be moved even longer distances by cells if we consider the random nature of electrospun matrices. The differences in the mutable nature of the structure of the two scaffolds is likely larger in reality. In addition, any given cell is surrounded by neighboring cells each of which exerts mechanical forces on the underlying scaffold. This suggests the importance of a truly “dynamic scaffold” in which an actively changing electrospun structure prevents sustained isometric tension and stress fiber development. Based on our theoretical calculations, we expect that the pure PCL nanofibers provide a more dynamic environment. However, the potential contributions of this dynamic behavior to genomic response have yet to be established.

In vivo, the absence of stress fibers in chondrocytes in mature articular cartilage is well documented [54]. The distance between focal adhesions at the ends of stress fibers is typically constant *in vitro* but is not constant *in vivo* as the tissues of the body undergo motion. In the absence of large stress fibers experiencing sustained levels of isometric tension, our data suggests that cellular functions other than adhesion could be more dominant. Here, chondrogenic gene (*Sox9*, *Col2a1*, *Acan*) expression and GAG production, activities associated with chondrocytic functionality, provide possible examples.

5. Conclusions

Utilizing a ‘needle-in-needle’ configuration in electrospinning, we have developed nano-fiber scaffolds with distinct mechanical moduli but identical surface chemistry and microstructure to reveal the contribution of nanofiber stiffness on the differentiation of mesenchymal progenitor cells. The modulus of the core-shell PES-PCL fibers (30.6 MPa) was more than 4 times that of the pure PCL (7.1 MPa). Results from the culture of mesenchymal progenitor cells on each scaffold indicate that the softer pure PCL fibers provide more appropriate microenvironments for chondrocytic differentiation marked by upregulation of chondrogenic genes including *Sox9*, collagen type 2 and aggrecan, and chondrocyte-specific ECM glycosaminoglycan production. In contrast, the stiffer core-shell PES-PCL substrate induced enhanced osteogenic differentiation by promoting *Runx2*, alkaline phosphatase and osteocalcin gene expression as well as alkaline phosphatase activity. Interestingly, the difference in mechanical moduli of scaffolds caused distinct cytoskeletal organization without changing the patterns of cellular adhesion to substrates. Differential fiber pliability of each scaffold may explain disparate stress fiber organization and differences in cellular differentiation to a specific phenotype.

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Figure 1.

The microstructure of (A) pure PCL and (B) coaxial PES-PCL 'core-shell' electrospun fibers. (C) a schematic illustrates the microstructure of a core-shell fiber. (D) TEM image of a single 'core-shell' PES-PCL fiber showing a continuous PES core surrounded by a PCL shell.

Figure 2.

XPS spectra of core-shell PES-PCL, pure PES and pure PCL fibers. O1s, C1s, S2s and S2p denote peaks detected from 1s atomic subshell of oxygen, 1s subshell of carbon, 2s subshell of sulfur and 2p subshell of sulfur, respectively.

Figure 3.

A cross-sectional image of cell/scaffold constructs showing cellular infiltration into the scaffold. Arrows indicate nuclei (blue) in the electrospun matrix with green autofluorescence.

Figure 4. Chondrogenic ((A) *Sox9*, (B) aggrecan (*Acan*) and (C) collagen type 2 (*Col2a1*)) gene expression of mesenchymal progenitor cells cultured on pure PCL and core-shell PES-PCL electrospun fibers under chondrogenic conditions as compared by real time-polymerase chain reaction (rt-PCR). Gene expression of the cells cultured on tissue culture polystyrene (TCPS) in chondrogenic conditions was used as a control (n=6; *: $p < 0.05$; **: $p < 0.01$). Expression level of each condition was normalized to the undifferentiated cells cultured on tissue culture plate.

Figure 5. Mesenchymal progenitor cells cultured on (A) pure PCL and (B) core-shell PES-PCL electrospun fibers under chondrogenic conditions stained with Alcian blue showing greater glycosaminoglycan (GAG) production on PCL fibers in comparison to core-shell fibers. Images were taken at 100X. (C) Spectrophotometric analysis of GAG synthesis by the cells grown on pure PCL fibers and core-shell PES-PCL electrospun fibers. (n=6; **: $p < 0.01$).

Figure 6.

Osteogenic ((A) *Runx2*, (B) alkaline phosphatase (*Alp*), and (C) osteocalcin (*Oc*)) gene expression of mesenchymal progenitor cells cultured on pure PCL and core-shell PES-PCL electrospun fibers under osteogenic conditions as compared by real time-polymerase chain reaction (rt-PCR). Gene expression of the cells cultured on tissue culture polystyrene (TCPS) in osteogenic conditions was used as a control (n=6, *: $p < 0.05$; **: $p < 0.01$). Expression level of each condition was normalized to the undifferentiated cells cultured on tissue culture plate.

Figure 7. Mesenchymal progenitor cells cultured on (A) pure PCL and (B) core-shell PES-PCL electrospun fibers under osteogenic conditions and stained to assess alkaline phosphatase activity (enzyme: pink to red, nuclei: blue). Images were taken at 100X. (C) Quantitative analysis of alkaline phosphatase activity by spectrophotometry in the cells grown on the pure PCL and core-shell fibers (n=6; *: $p < 0.05$, **: $p < 0.01$).

Figure 8.

The intracellular structure of mesenchymal progenitor cells on pure PCL (A–D) and core-shell PES-PCL (E–H) electrospun fibers under chondrogenic conditions by immunofluorescent analysis of the adhesion molecule integrin β 1 (red, A and E), F-actin (green, B and F), and SEM (D and H). The cells on both substrates show similarly uniform intracellular distribution of integrin β 1. However, the stiffness of the fibers significantly affected stress fiber organization. SEM examination shows more rounded cellular morphology on pure PCL (D) compared to the spread morphology on PES-PCL (H) fibers. (I, J) Inverted images of F-actin staining exhibiting greater development of stress fibers in the cells grown on the core-shell (J) versus pure PCL (I) scaffolds. (K) Quantitative analysis of the F-actin stained images to derive the mean intensity of stress fibers per cell basis (A.U.: arbitrary unit; $n = 18$; **: $p < 0.01$). Images were taken at 400X.

Figure 9. Theoretical deflection (under 75 nN of force) of an individual electrospun PCL (solid line) or PES-PCL (dashed line) fiber versus inter-fiber distance (Table 2). The pure PCL fiber can be moved significantly greater distances by adherent cells relative to the core-shell PES-PCL fiber.

Table 1

Custom primer sequence

Gene	Forward	Reverse
<i>Rps18</i>	5'-GGAAAATAGCCTTCGCCATCACT-3'	5'-GCCAGTGGTCTTGGTGTGCTGAC-3'
<i>Sox9</i>	5'-AACGGCTCCACTCAAGAACAAG-3'	5'-TCCGTTCTTCACCGACTTCCTC-3'
<i>Acan</i>	5'-ACTGTCAAAGCACCATGCCTT-3'	5'-CCATTCAGTCTGTTTCTTGCC-3'
<i>Col2a1</i>	5'-AAACTGGTGGAGCAGCAAGAG-3'	5'-ATCTGGACGTTAGCGGTGTTG-3'
<i>Runx2</i>	5'-ATGATGGTGTGACGCTGACG-3'	5'-GACTCATCCATTCTGCCGCTA-3'
<i>Alp</i>	5'-CGTTTTACATTTCGGTGGATAC-3'	5'-TTCTTGTCGGTGTGCTCA-3'
<i>Oc</i>	5'-CAAGCAGGAGGGCAATAAGGTAG-3'	5'-TGCTTGTGACGAGCTATCAGACC-3'

Table 2

Morphological and mechanical properties of pure PCL and core-shell PES-PCL fibers.

Electrospun fibers	Fiber diameter (nm)	Inter-fiber distance * (μm)	UTS (MPa)	Modulus (MPa)
PCL	750 $\pm$ 490	8.44 $\pm$ 3.21	2.43 $\pm$ 1.2	7.1$\pm$1.3
PES-PCL	867 $\pm$ 257	8.94 $\pm$ 3.90	2.54 $\pm$ 0.34	30.6$\pm$4.1

The data are represented as means $\pm$ standard deviation from five independent specimens.

* inter-fiber distance is the distance between two adjacent fibers intersecting a common fiber, UTS: ultimate tensile strength.