Published in final edited form as:

Biomaterials. 2011 January ; 32(2): 538–546. doi:10.1016/j.biomaterials.2010.08.104.

Organ-derived coatings on electrospun nanofibers as *ex vivo* microenvironments

Sara N. Fischer^{a,b}, Jed K. Johnson^c, Christopher P. Baran^b, Christie A. Newland^b, Clay B. Marsh^b, and John J. Lannutti^{c,*}

^aThe Integrated Biomedical Science Graduate Program, The Ohio State University, Columbus, OH, USA

^bDivision of Pulmonary, Allergy, Critical Care, and Sleep Medicine, The Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University, Columbus, OH, USA

^cDepartment of Materials Science and Engineering, The Ohio State University, Columbus, OH, USA

Abstract

Idiopathic pulmonary fibrosis (IPF) is an interstitial lung disease characterized by irreversible scarring. Collagen deposition, myofibroblast expansion, and the development of fibroblastic foci are the hallmark pathological events. The origin and mechanism of recruitment of myofibroblasts, the key cell contributing to these events, is unknown. We hypothesize that the fibrotic lung microenvironment causes differentiation of arriving bone marrow-derived cells into myofibroblasts. Therefore, a method of isolating the effects of fibrotic microenvironment components on various cell types was developed. Electrospun nanofibers were coated with lung extracts from fibrotic or nonfibrotic mice and used to determine effects on bone marrow cells from naïve mice. Varying moduli nanofibers were also employed to determine matrix stiffness effects on these cells. At structured time points, bone marrow cell morphology was recorded and changes in fibrotic gene expression determined by real-time PCR. Cells plated on extracts isolated from fibrotic murine lungs secreted larger amounts of extracellular matrix, adopted a fibroblastic morphology, and exhibited increased myofibroblast gene expression after 8 and 14 days; cells plated on extracts from nonfibrotic lungs did not. Similar results were observed when the nanofiber modulus was increased. This *ex vivo* system appears to recapitulate the three-dimensional fibrotic lung microenvironment.

Keywords

Fibrosis; ECM; Electrospinning; Polycaprolactone; Fibroblast; Three-dimensional cell culture

1. Introduction

Tissue engineering is an emerging field that strives to synthesize biologically relevant tissue for a wide range of applications. A number of approaches have been taken to achieve this goal ranging from purely synthetic to purely biological techniques [1–4], each having their own advantages and disadvantages. To overcome limitations associated with a “blank slate” synthetic approach and controversies inherent to any attempt to chemically mimic the *in vivo* environment that begins with individual biomolecules, we have combined

homogenized *ex vivo* lung tissue with electrospun nanofiber mats. Electrospinning is a well-established procedure for creating three-dimensional (3-D) scaffolds that mimic the *in vivo* topography although the result often lacks realistic *in vivo* chemistries. Therefore, we have added homogenized *ex vivo* fibrotic and normal lung tissue to better recapitulate the chemistry inherent to specific microenvironments and disease states.

By ‘borrowing’ this tissue engineering technology to address a pathological disease state, we may be able to define new mechanisms of action for interstitial lung diseases, including Idiopathic Pulmonary Fibrosis (IPF). IPF is the most prevalent form of a group of interstitial lung diseases characterized by excessive matrix deposition and destruction of normal lung architecture. The incidence of IPF has more than doubled since 1990, and the disease is almost uniformly fatal, with typical survival of only 2–5 years after diagnosis [5]. While the etiology is unknown, several risk factors have been postulated to lead to the development of IPF, such as smoking, drug exposure, exposure to infectious agents, and genetic predisposition [6,7]. Although mouse models of pulmonary fibrosis have been utilized to better understand the mechanisms responsible for IPF pathogenesis, currently there are no effective treatments of this disease. Therefore, it is imperative that the molecular mechanisms of IPF pathogenesis are elucidated so that new targets for potential therapeutics may be discovered.

Although little is understood about IPF, it is clear that excessive collagen deposition, myofibroblast expansion, and the development of fibrotic foci are hallmark pathological events [8]. The myofibroblast is the key effector cell that produces collagen and other matrix materials [8] that lead to the loss of alveolar function [9–11]. The origin and mechanism of recruitment of the myofibroblast is unknown, however there are three leading hypotheses. The first hypothesis is that resident fibroblasts differentiate directly into myofibroblasts when exposed to a pro-fibrotic microenvironment [10]. The second hypothesis, termed epithelial–mesenchymal transition (EMT), contends that during injury to the lung epithelium, epithelial cells transition into mesenchymal fibroblasts/myofibroblasts. This process continues as the injury to the lung is not resolved, therefore leading to irreversible scarring and loss of normal lung function. This process has been repeatedly demonstrated *in vitro*, and recent *in vivo* evidence is accumulating [12–17]. A newer hypothesis proposes that myofibroblasts may be derived from circulating cells known as fibrocytes [18–20]. Discovered in 1994, these cells express type-I collagen and are derived from the bone marrow [21] as indicated by co-expression of CD45. Recent studies have demonstrated that fibrocytes traffic to the lung during injury [8,22,23]. However, the mechanisms by which fibrocytes differentiate into myofibroblasts and the origin of these cells are poorly understood. Tissue engineering technologies may allow us to interrogate bone marrow cells to identify the origin of these cells and the gene networks responsible for their expression of matrix-related genes, such as collagen I, which lead to organ fibrosis.

While it is important to study the individual cells responsible for mediating subepithelial lung fibrosis under ‘normal’ *in vitro* conditions, *in vivo* cells exist in a more complex microenvironment. The lung microenvironment is an intricate network of structural (epithelial, fibroblasts, and smooth muscle) and inflammatory (macrophages [24,25] and neutrophils) cells, cytokines, proteins, and growth factors. An *ex vivo* system capable of enhancing our understanding of the specific cell and molecular pathways governing the initiation, progression and resolution of pulmonary fibrosis is critical to the development of new diagnostics therapeutics.

2. Materials and methods

A 14 wt% solution of poly(ϵ -caprolactone) (PCL) ($M_n = 70,000$ – $90,000$, Sigma) in acetone (Sigma) was prepared by heating acetone to 50 °C followed by continuous stirring to dissolve the polymer. The solution was placed in a 60 cc syringe (BD Luer-Lok tip) with a 20 gauge blunt tip needle (EFD, Inc., RI) and electrospun using a high voltage DC power supply (FC50R2, Glassman High Voltage, NJ) set to +24 kV, a 20 cm tip-to-substrate distance and a 16 mL/h flow rate. A $3 \times 3'$ (7.6×7.6 cm) sheet approximately 200 microns (Nanofiber Solutions, Columbus, OH) in thickness was deposited onto non-stick aluminum foil. The resulting 40 g PCL sheets were then placed in a vacuum overnight to ensure removal of residual acetone [26]. High resolution ESI analysis (Esquire) was used to establish that the resulting acetone content is beneath our ability to detect it (less than 10 ppm).

A 8 wt% solution of polyethersulfone (PES) (Goodfellow, Cambridge, UK) in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) (Sigma) was prepared by continuous stirring within an Erlenmeyer flask at room temperature for approximately 12 h to dissolve the PES. The flask was kept tightly stoppered to prevent evaporative losses of HFIP and maintain the desired solids loading. The solution was then placed in a 60 cc syringe (BD Luer-Lok tip) with a 20 gauge blunt tip needle (EFD, Inc., RI) and electrospun using a high voltage DC power supply (FC50R2, Glassman High Voltage, NJ) set to +23 kV, a 20 cm tip-to-substrate distance and a 5 ml/h flow rate. A $3 \times 3'$ (7.6×7.6 cm) sheet approximately 0.2 mm in thickness was deposited onto non-stick aluminum foil. The resulting ~40 g PES sheets were then placed in a vacuum overnight to ensure removal of residual HFIP [26].

Core shell fibers [27–30] were prepared by 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. The core solution of PES + HFIP was prepared as described above. To optimize the electrospinning of core-shell fibers, the shell solution was prepared using 5 wt% poly(ϵ -caprolactone) dissolved in HFIP by continuous stirring at room temperature. One syringe (BD Luer-Lok tip) was filled with the polymer solution for the core, PES + HFIP, connected to the 22 gauge needle and set to a 2 mL/h flow rate using a syringe pump. Another identical syringe was connected via an extension to the T-junction, filled with the shell material, PCL + HFIP, and set to a flow rate of 2 mL/h using another syringe pump. A high voltage power source (FC50R2, Glassman High Voltage, NJ) was connected to the concentric needle structure and set to +30 kV for the PES + HFIP polymer solution core with a shell of PCL + HFIP and a tip-to-substrate distance of 20 cm. The resulting ~40 g sheets were then placed in a vacuum overnight to ensure removal of residual HFIP [26].

2.1. Microstructural characterization

Determination of microstructural change (or its absence) required that these samples be examined in a scanning electron microscope (XL-30 Sirion, FEI, OR). All samples were coated with a 15 nm thick layer of osmium using an osmium plasma coater (OPC-80T, SPI Supplies, West Chester, PA). The use of osmium plasma instead of Au or Au–Pd eliminated concerns regarding PCL melting during gold sputter coating [31] and allowed for higher resolution imaging [32] of the fiber surface.

2.2. Mice and bleomycin regimen

Male mice (FVB/N background), aged 6–12 weeks, underwent intra-peritoneal (IP) injection as previously described [33]. Briefly, mice were injected with 0.035U bleomycin/g or PBS

(vehicle control) on days 1, 4, 8, 11, 15, 18, 22, and 25. One week following the last injection, mice were sacrificed. The lungs were removed and solubilized with a glass homogenizer in RPMI 1640 medium supplemented with 5% FBS. The cell suspension was then passed through a 100 μ M filter and centrifuged at 1500 rpm for 5 min. The resulting homogenate was resuspended in 1 ml RPMI 1640 medium supplemented with 5% FBS.

2.3. Biological effects of lung extract coatings on PCL nanofibers

Nanofiber matrices were spun as previously described and then coated with this bleomycin-treated mouse lung extract [33] using a TC-100 Desktop spin coater (MTI Corporation, CA). Electrospun fiber sheets were cut into 22 mm diameter discs and glued to glass coverslips using 50 μ L of an FDA-approved silicone glue (Part# 40076 Applied Silicone Corporation, CA). Separated from it by the electrospun fiber sheet, the cells never made contact with the glue. The fiber discs were then placed on the spin coater at 1000 RPM and pre-wetted with 75 μ L of ethanol followed by the immediate addition of 75 μ L of the lung extract. After air drying, the extract was lightly cross-linked in place on the fibers as described elsewhere [34]. Briefly, the coated samples were submerged in a 7 mM solution of N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (Part# E1769, Sigma-Aldrich) in pure ethanol for 24 h at room temperature. Afterwards, they were rinsed three times using pure ethanol. For comparison purposes, other matrices were spin coated with PBS-treated lung extract using exactly the same procedure. All samples were then placed into wells within 12-well cell culture plates, the weight of the glass coverslip being more than sufficient to hold the samples down.

Bone marrow from wild-type FVB/N mice was extracted by passing RPMI Medium 1640 (Gibco-Invitrogen, Carlsbad, CA) through the femur bones into a 15 mL centrifuge tube. The bone marrow was centrifuged at 1500 rpm, and the resulting pellet was resuspended in sterile water to lyse red blood cells. The remaining white blood cells were resuspended in 2 mL RPMI medium. The cells were counted by trypan exclusion and were seeded at densities of 1×10^6 cells/well on top of the fibers in a 12 well plate. 500 μ L of RPMI medium supplemented with 5% fetal bovine serum (FBS) was added to each well. Fresh medium was replaced every 3 days. Cells were allowed to proliferate and were harvested at various time-points (2, 4, 8, and 14 days) for scanning electron microscopy and real-time PCR analysis.

2.4. Scanning electron microscopy

After various time points, cells and scaffolds were rinsed with PBS 3 $\times$ for 5 min each and fixed with a 4% paraformaldehyde aqueous solution for 30 min and then rinsed with an aqueous solution of 0.1 M phosphate buffer +0.1 M sucrose for 3 $\times$ 5 min. Samples were then dehydrated in a graded ethanol series: 50% EtOH 1 $\times$ 5 min, 70% EtOH 1 $\times$ 5 min, 80% EtOH 1 $\times$ 10 min, 95% EtOH 1 $\times$ 10 min, 100% EtOH 2 $\times$ 10 min. The samples were then chemically dried using a graded series of 1,1,1,3,3,3-hexamethyldisilazane (HMDS): 3:1 EtOH:HMDS 1 $\times$ 15 min, 1:1 EtOH:HMDS 1 $\times$ 15 min, 1:3 EtOH:HMDS 1 $\times$ 15 min, 100% HMDS 2 $\times$ 15 min, followed by 100% HMDS allowed to air dry overnight. Samples were then mounted on aluminum pin mounts (Part#16111, Ted Pella, Inc.) using double sided carbon tape (Part#16084-1, Ted Pella, Inc.) and coated with osmium as described earlier for viewing in the SEM.

2.5. Real-Time PCR

Total RNA was extracted from cells trypsinized off of the fiber surface using Trizol (Invitrogen, Carlsbad, CA) extractions at various time-points. Reverse Transcription was performed to amplify only mRNA by using oligo-dT primers (Invitrogen, Carlsbad, CA). The resulting cDNA was analyzed by Real-Time PCR with SYBR Green. All primers were designed using Primer Express software (Applied Biosystems, Foster City, CA). Real-Time

PCR analysis was done using an ABI 7700 machine (Applied Biosystems, Foster City, CA) with GAPDH as an internal control. Primer sequences: mouse GAPDH: 5'-GCACAGTCAAGGCCGAGAAT-3' (For); 5'-GCCTTCTCCATGGTGGTGAA-3' (Rev); mouse type-I pro-collagen: 5'-GGCTATGACTTTGGTTTTGAAGGA-3' (For); 5'-CGTTGTCGTAGCAGGGTTCTTT-3' (Rev); mouse-smooth muscle actin: 5'-CTGACAGAGGCACCACTGAA-3' (For); 5'-CATCTCCAGAGTCCAGCACA-3' (Rev); mouse CTGF: 5'-AAAGTGCATCCGGACACCTAA-3' (For); 5'-TGCAGCCAGAAAGCTCAAACCT-3' (Rev); mouse tenascin-C: 5'-TGTGTGCTTCAAGGCTATG-3' (For); 5'-GCAGACACACTCGTTCTCCA-3' (Rev).

2.6. Statistical methods

A student's *T*-test was used to compare cell response. ANOVA was used for multiple comparisons with Tukey's *post hoc* testing. These analyses were completed using Origin 7.0 (OriginLab, Northampton, MA). A *p*-value of less than 0.05 was considered significant for all comparisons.

3. Results

3.1. Biological effects of lung extract coatings on PCL nanofibers

Wild-type mouse bone marrow-derived cells plated on nanofibers coated with lung extracts from PBS or bleomycin-treated mice were fixed and prepared for scanning electron microscopy (SEM) at various time-points. Figs. 1 and 2 reveal minimal differences in the microstructural appearance of plain PCL nanofibers and PCL nanofibers coated with either PBS- or bleomycin-treated lung extract. Cells plated on plain PCL nanofibers and PCL nanofibers coated with PBS-treated lung extracts maintained a rounded appearance, did not form aggregates, and did not appear to secrete matrix materials. However, cells plated on nanofibers coated with bleomycin-treated lung extracts exhibited a spindle-like, fibroblastic morphology, tended to aggregate, and appeared to secrete substantially more matrix after 8 and 14 days (Fig. 3). These differences were not apparent at days 2 and 4 in culture (data not shown). Since differences were not observed until 8 days and continued through 14 days, for the remainder of the study cells were cultured for 8 and 14 day time-points. These observations indicated that our *ex vivo* system induced bone marrow cells to assume a fibroblast-like phenotype in culture.

RNA was isolated from these cells after they were plated on nanofiber matrices coated with lung extracts from either PBS or bleomycin-treated mice. As shown in Fig. 4, collagen I, alpha smooth muscle actin, and tenascin-C expression were significantly increased in cells plated on nanofibers coated with bleomycin-treated mouse lung extracts versus cells plated on plain PCL nanofibers or PCL nanofibers coated with lung extracts from PBS-treated mice on both days 8 and 14. No significant change in gene expression between the 8 and 14 day time-points. Fibrotic gene expression was not increased relative to PCL on days 2 and 4 (data not shown). This experiment provided significant indications that an appropriately-coated nanofiber matrix could give rise to myofibroblast differentiation signals in bone marrow cells. Current studies are underway in our laboratory to identify the origin of these bone marrow-derived cells.

3.2. Biological effects of nanofiber modulus

Cell differentiation and migration occurs in response to mechanical cues, such as matrix stiffness [35–40], from the surrounding microenvironment. In a recent study by Engler and co-workers, mesenchymal stem cells (MSCs) were cultured in matrices of different elasticity (modulus) [41]. Matrices with low modulus differentiated MSCs into neuron-like cells, whereas matrices with high modulus induced MSCs to become myogenic. To establish the

role of matrix stiffness independent of lung extract matrix interactions with plated cells, we next tested nanofiber matrices having different moduli spun from synthetic polymers. Bulk polyethersulfone (PES) has a modulus (385,000 psi) ~28.5 times greater than that of bulk PCL (13,500 psi). This modification provided a dramatically different modulus appropriate to determining the effects of modulus (if any) on cellular response. We also included a “core-shell” fiber [27–30] consisting of a thin ‘shell’ of PCL on a ‘core’ of PES. XPS (Fig. 5) was utilized to prove that the PES ‘core’ was obscured by the PCL as shown by the disappearance of the S2p peak at approximately 170 eV. Core-shell spinning provided an elegant means of retaining the PCL surface chemistry while increasing the overall fiber modulus from 7.1 MPa (pure PCL) to 30.6 MPa, an increase of more than a factor of 4 (Table 1). Figs. 6 and 7 show that PES core/PCL shell and plain PES possessed limited microstructural differences. However, bone marrow cells plated on PES core/PCL shell nanofibers exhibited a fibroblastic morphology similar to cells plated on PCL with bleomycin-treated extract. These Figs. 6 and 7 also show that culture on uncoated PCL (possessing a modulus used in the initial coating studies) and PES (higher modulus with change in coating) did not affect cell morphology. Mechanical property (modulus, tensile strength) changes (Table 1) associated with the presence of the lung extract coatings were not statistically significant. The only statistically significant change observed was a decrease in elongation associated with the bleomycin-treated lung extract coating. However, this difference was observed using strain values many times greater than the bone marrow cells would contact in a fibrotic lung matrix or in the coated native nanofiber matrix. Thus, this observation had little relevance aside from providing another example of how deposition of biologically-generated compounds can lead to restrictions on large-scale interfiber translation [42].

As shown in Fig. 8, type-I collagen, alpha smooth muscle actin and connective tissue growth factor were increased in cells plated on the core-shell nanofibers compared to both PCL and PES fibers after 8 days in culture. However, tenascin-C was not increased as observed with bleomycin extracted coated fibers. Given that this nanofiber composition has the *same* surface chemistry as PCL, these results suggested that the nanofiber modulus played a significant role in conditioning the fibrotic response of bone marrow-derived cells. However, after 14 days the core/shell fiber induction of collagen I and SMA expression were diminished while collagen I was increased in cells plated on PES fibers. At 14 days, expression of CTGF and TN-C were increased in cells plated on the core/shell fibers relative to that seen on day 8. Since these fibers did not have a biological coating, it is possible that the changes in gene expression relate to lack of cues in the matrix environment present in the lung extract-coated fiber. These data underscore the importance of both the chemical signals and modulus of the tissue that bone marrow-derived cells encounter in determining their cellular fate in health and disease.

4. Discussion

For reasons not well understood, mortality rates for idiopathic pulmonary fibrosis continue to climb and have surpassed those from acute myeloid leukemia, multiple myeloma and bladder cancer [43]. There are no available treatments for this disease. No *in vitro* models exist, making isolation of potential environmental effects difficult. Delineating specific cell and molecular pathways governing the initiation, progression and resolution of IPF [44] is critical to understanding these increases along with enabling development of new diagnostics and therapeutics. Useful *in vitro* models will allow the study of the impact of the lung microenvironment on the differentiation of circulating bone-marrow derived cells in a manner reminiscent of the *in vivo* disease state.

In our murine model of pulmonary fibrosis [33], bleomycin-treated lungs exhibit increased expression of extracellular matrix components such as collagen I, connective tissue growth factor, α -smooth muscle actin and fibronectin relative to PBS-treated mouse lungs. Fibrotic lungs are characterized by increased chemokine content, inflammatory cell infiltration, and increased numbers of myofibroblasts [33]. We hypothesize that this fibrotic milieu causes bone marrow cells to traffic to the lungs and differentiate into myofibroblasts, thus perpetuating the cycle of aberrant wound-healing and irreversible scarring.

The influence of nano- or micro-structures on cellular and intra-cellular characteristics has been repeatedly demonstrated [45–48]. Electrospinning allows efficient production of nanoscale tissue engineering scaffolds that resemble many of the topographical features of normal ECM. PCL has previously served as an optimal scaffold for cells to adhere to and move freely as they divide and differentiate [4,26,42,49,50]. We modified this system by coating the nanofibers with bleomycin- (or vehicle-) treated lung extracts. PCL nanofiber scaffolds coated with *ex vivo* lung extracts caused bone marrow-derived cells to differentiate into myofibroblast-like cells. We work with primary bone marrow cells to represent the non-RBC bone marrow environment; cell lines representing individual stem cell types from these tissues have not yet been developed. This system allows us to test our hypothesis that the fibrotic microenvironment may be responsible for the differentiation of bone marrow cells into (myo) fibroblasts *in vivo* using an *ex vivo* model.

This approach combines biological extracts reminiscent of a specific disease state with synthetic nanofibers that serve as a topographical “blank slate” upon which the specific biochemical characteristics of the disease state can be explored. When bone marrow cells adhered to bleomycin-treated lung extract coatings on electrospun PCL nanofibers, changes in morphology were accompanied by only increases in ECM deposition. In addition, we observed upregulation of genes associated with inflammatory cellular responses relative to PBS-treated controls. In the long-term, this system is particularly powerful in that specific conditions of the matrix can be altered to understand causal events in fibrosis. The addition or subtraction of specific cytokines, growth factors or proteins can be used to determine which of these factors are responsible for myofibroblast differentiation in the chemically complex microenvironment. This model system will allow us to determine which signals present in the fibrotic microenvironment drive differentiation and to develop compounds that either mitigate or reverse this influence. In addition, we can target the bone marrow population that gives rise to myofibroblasts in the lungs of patients with pulmonary fibrosis.

The incidence of IPF is known to increase with age [51]. In parallel, a variety of related fields [52–54] have observed an increase in the stiffness of various tissues with age. The increased condensation of intracellular and extracellular matrix components observed in aged tissues [55] cause an increase in tissue modulus. However, the contribution of these modulus increases, which could conceivably trigger excess scar formation in the lung [51], to IPF have not yet been elucidated. Bone marrow-derived cells are widely believed to play an important role in mediating inflammation and repair during aging [56–58]. The influence of the modulus of the microenvironment in which they arrive has not, to our knowledge, been previously examined.

To investigate the concept that matrix modulus might play a role in cell differentiation [41], nanofibers of increasing modulus yet constant surface chemistry were utilized to determine if the bone marrow cells in our study are capable of differentiating into myofibroblast-like cells following adherence to a ‘stiffer’ matrix. Interestingly, bone marrow-derived cells plated on PCL shell/PES core fiber displayed increased fibrotic gene expression and a morphology similar to cells plated on PCL fibers coated with fibrotic lung extracts. However, cells plated on PES fibers did not exhibit these changes. It is possible that the PES

fibers were either too stiff, thus presenting an environment not agreeable to the cells, or possess a specific surface chemistry unfavorable to the inflammatory response. PES is known to encourage stem cell proliferation [59] and this influence may prevent differentiation. Of note, after 14 days in culture the cells plated on core/shell fibers exhibited decreased expression of collagen I and smooth muscle actin. Expression of CTGF and TN-C were increased in cells plated on the core/shell fibers relative to day 8. This could be due to the deposition of biological factors on the “clean slate” of as-spun fiber that generally results in greater cell sensitivities at higher moduli. Previous work [38] has suggested that cells may use different integrin receptors as the stiffness of the matrix is varied and this could be responsible for the observed changes.

These data underscore the importance of both the chemical signals and modulus of the tissue that bone marrow-derived cells encounter in determining their cellular fate in health and disease. The long-term goal of this work is to elucidate the role of the fibrotic lung microenvironment in cellular activation and fibrotic gene and protein expression. By examining the phenotypic behavior of cells utilizing this *in vitro* model, their gene programs and the matrix factors that regulate and control their myofibroblast differentiation can be determined. We can then focus on determining new targets for targeted therapies and diagnostics in human pulmonary fibrosis. Furthermore, by creating a new means of pharmacological testing, this approach has considerable potential to utilize next generation materials and engineering technology to address other diseases beyond pulmonary fibrosis utilizing the same technology with different tissue extracts (e.g., cirrhotic or solid tumor microenvironments). This platform could allow interrogation of these microenvironments to develop new imaging and high throughput screening technologies to create biologically-inspired next generation materials. This method of testing biomolecular, cellular, and biochemical interactions *ex vivo* lends itself to agent based modeling approaches making robust cellular pathway development more efficient.

5. Conclusions

This *ex vivo* assay focuses on the impact of the lung microenvironment on cell-to-cell and cell-to-matrix interactions resulting in fibrotic lung disease and myofibroblast production. By combining PCL nanofibrous scaffolds with *ex vivo* lung extracts we demonstrate that 3D geometry and chemical signals provide environmental cues that are necessary for bone marrow cells to acquire a myofibroblast phenotype. Taken together, these observations suggest that bone marrow-derived cells exposed to a fibrotic lung microenvironment develop a more fibroblast/myofibroblast phenotype than cells exposed to a ‘normal’ lung microenvironment. In addition, the modulus of the underlying nanomatrix plays a significant role in this response. This system can be used to study any disease or organ, not only the lung, rendering it a powerful platform for studying various disease states. This technology also has the potential to be used in the clinic, with tissue extracts from patients used to create a matrix environment representing a disease of interest and then plating human cells onto the matrices as a diagnostic measure.

Acknowledgments

This work was partly supported by a research grant from the National Science Foundation under Grant Nos. EEC-0425626 and CMMI-0928315 (JJ and JJJ), and from research grants from the National Institutes of Health under Grant No. R03 HL095431-01 (CPB) and NIH RO1 HL067176 (SNF, CAN, SRW, and CBM). Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation or the National Institutes of Health.

References

1. Agarwal S, Wendorff JH, Greiner A. Progress in the field of electrospinning for tissue engineering applications. *Adv Mat.* 2009; 21(32–33):3343–51.
2. Piskin E, Bolgen N, Egri S, Isoglu I. Electrospun matrices made of poly(alpha-hydroxy acids) for medical use. *Nanomed.* 2007; 2(4):441–57.
3. Martins A, Araujo JV, Reis RL, Neves NM. Electrospun nanostructured scaffolds for tissue engineering applications. *Nanomed.* 2007; 2(6):929–42.
4. Lannutti J, Reneker D, Ma T, Tomasko D, Farson D. Electrospinning for tissue engineering scaffolds. *Mater Sci Eng C.* 2007; 27:504–9.
5. Meltzer E, Noble P. Idiopathic pulmonary fibrosis. *Orph J Rare Dis.* 2008; 3(8):1–15.
6. Strieter RM, Mehrad B. New mechanisms of pulmonary fibrosis. *Chest.* 2009; 136(5):1364–70. [PubMed: 19892675]
7. Thannickal VJ, Toews GB, White ES, Lynch JP, Martinez FJ. Mechanisms of pulmonary fibrosis. *Annu Rev Med.* 2004; 55:395–417. [PubMed: 14746528]
8. Camoretti-Mercado B. Targeting the airway smooth muscle for asthma treatment. *Transgenic Res.* 2009; 154(4):165–74.
9. Scotton CJ, Chambers RC. Molecular targets in pulmonary fibrosis – the myofibroblast in focus. *Chest.* 2007; 132(4):1311–21. [PubMed: 17934117]
10. Phan SH. The myofibroblast in pulmonary fibrosis. *Chest.* 2002; 122(6):286S–9S. [PubMed: 12475801]
11. Eyden B. The myofibroblast: phenotypic characterization as a prerequisite to understanding its functions in translational medicine. *J Cell Mol Med.* 2008; 12(1):22–37. [PubMed: 18182061]
12. Kim JH, Jang YS, Eom KS, Il Hwang Y, Kang HR, Jang SH, et al. Transforming growth factor beta 1 induces epithelial-to-mesenchymal transition of A549 cells. *J Korean Med Sci.* 2007; 22(5): 898–904. [PubMed: 17982242]
13. Wu Z, Yang LL, Cai L, Zhang M, Cheng X, Yang X, et al. Detection of epithelial to mesenchymal transition in airways of a bleomycin induced pulmonary fibrosis model derived from an alpha-smooth muscle actin-Cre transgenic mouse. *Respir Res.* 2007; 8:1. [PubMed: 17207287]
14. Kim KK, Kugler MC, Wolters PJ, Robillard L, Galvez MG, Brumwell AN, et al. Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. *Proc Natl Acad Sci U S A.* 2006; 103(35):13180–5. [PubMed: 16924102]
15. Barth K, Reh J, Sturrock A, Kasper M. Epithelial vs myofibroblast differentiation in immortal rat lung cell lines – modulating effects of bleomycin. *Histochem Cell Biol.* 2005; 124(6):453–64. [PubMed: 16187068]
16. Kasai H, Allen JT, Mason RM, Kamimura T, Zhang Z. TGF-beta 1 induces human alveolar epithelial to mesenchymal cell transition (EMT). *Respir Res.* 2005; 6:56. [PubMed: 15946381]
17. Willis BC, Liebler JM, Luby-Phelps K, Nicholson AG, Crandall ED, du Bois RM, et al. Induction of epithelial–mesenchymal transition in alveolar epithelial cells by transforming growth factor-ss 1-potential role in idiopathic pulmonary fibrosis. *Am J Pathol.* 2005; 166(5):1321–32. [PubMed: 15855634]
18. Barth PJ, Westhoff CC. CD34+ fibrocytes: morphology, histogenesis and function. *Curr Stem Cell Res Ther.* 2007; 2(3):221–7. [PubMed: 18220905]
19. Schmidt M, Sun G, Stacey MA, Mori L, Mattoli S. Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma. *J Immunother.* 2003 Jul; 171(1):380–9.
20. Bucala R, Spiegel LA, Chesney J, Hogan M, Cerami A. Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue-repair. *Mol Med.* 1994; 1(1):71–81. [PubMed: 8790603]
21. Mori L, Bellini A, Stacey MA, Schmidt M, Mattoli S. Fibrocytes contribute to the myofibroblast population in wounded skin and originate from the bone marrow. *Exp Cell Res.* 2005; 304(1):81–90. [PubMed: 15707576]

22. Saunders R, Siddiqui S, Kaur D, Doe C, Sutcliffe A, Hollins F, et al. Fibrocyte localization to the airway smooth muscle is a feature of asthma. *J Allergy Clin Immunol*. 2009; 123(2):376–84. [PubMed: 19081612]
23. Wang CH, Huang CD, Lin HC, Lee KY, Lin SM, Liu CY, et al. Increased circulating fibrocytes in asthma with chronic airflow obstruction. *Am J Respir Crit Care Med*. 2008; 178(6):583–91. [PubMed: 18583572]
24. Geiser M, Casaulta M, Kupferschmid B, Schulz H, Semirriter-Beinke M, Kreyling W. The role of macrophages in the clearance of inhaled ultrafine titanium dioxide particles. *Am J Respir Cell Mol Biol*. 2008; 38(3):371–6. [PubMed: 17947511]
25. Alexis NE, Lay JC, Zeman KL, Geiser M, Kapp N, Bennett WD. In vivo particle uptake by airway macrophages in healthy volunteers. *Am J Respir Cell Mol Biol*. 2006; 34(3):305–13. [PubMed: 16272458]
26. Nam J, Huang Y, Agarwal S, Lannutti J. Materials selection and residual solvent retention in biodegradable electrospun fibers. *J Appl Polym Sci*. 2008; 107(3):1547–54.
27. Sriker R, Yarin AL, Megaridis CM, Bazilevsky AV, Kelley E. Desorption-limited mechanism of release from polymer nanofibers. *Langmuir*. 2008; 24(3):965–74. [PubMed: 18076196]
28. Xie JW, Ng WJ, Lee LY, Wang CH. Encapsulation of protein drugs in biodegradable microparticles by co-axial electrospray. *J Colloid Interface Sci*. 2008; 317(2):469–76. [PubMed: 17945246]
29. Kim TG, Lee DS, Park TG. Controlled protein release from electrospun biodegradable fiber mesh composed of poly(epsilon-caprolactone) and poly(ethylene oxide). *Int J Pharm*. 2007; 338(1–2): 276–83. [PubMed: 17321084]
30. Diaz JE, Barrero A, Marquez M, Loscertales IG. Controlled encapsulation of hydrophobic liquids in hydrophilic polymer nanofibers by co-electrospinning. *Adv Func Mat*. 2006; 16(16):2110–6.
31. Powell, HM.; Lannutti, JJ.; Kniss, DA. Nanoscale modifications to tissue engineering polymers: physical control of cellular behavior. Berlin, Germany: VDM Publishing Ltd; 2010. p. 32
32. SPI. website: <http://www.2spi.com/catalog/osmi-coat.html>
33. Baran CP, Opalek JM, McMaken S, Newland CA, O'Brien JM, Hunter MG, et al. Important roles for macrophage colony-stimulating factor, CC chemokine ligand 2, and mononuclear phagocytes in the pathogenesis of pulmonary fibrosis. *Am J Respir Crit Care Med*. 2007; 176(1):78–89. [PubMed: 17431224]
34. Powell HM, Boyce ST. EDC cross-linking improves skin substitute strength and stability. *Biomaterials*. 2006; 27(34):5821–7. [PubMed: 16919327]
35. Bershadsky A, Kozlov M, Geiger B. Adhesion-mediated mechanosensitivity: a time to experiment, and a time to theorize. *Curr Opin Cell Biol*. 2006; 18(5):472–81. [PubMed: 16930976]
36. Discher DE, Janmey P, Wang YL. Tissue cells feel and respond to the stiffness of their substrate. *Science*. 2005; 310(5751):1139–43. [PubMed: 16293750]
37. Hinz B, Celetta G, Tomasek JJ, Gabbiani G, Chaponnier C. Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. *Mol Biol Cell*. 2001; 12(9):2730–41. [PubMed: 11553712]
38. Cukierman E, Pankov R, Stevens DR, Yamada KM. Taking cell-matrix adhesions to the third dimension. *Science*. 2001; 294(5547):1708–12. [PubMed: 11721053]
39. Beningo KA, Dembo M, Kaverina I, Small JV, Wang YL. Nascent focal adhesions are responsible for the generation of strong propulsive forces in migrating fibroblasts. *J Cell Biochem*. 2001; 153(4):881–7.
40. Pelham RJ Jr, Wang Y-L. Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proc Natl Acad Sci U S A*. 1997; 94:13661–5. [PubMed: 9391082]
41. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell*. 2006; 126(4):677–89. [PubMed: 16923388]
42. Johnson J, Niehaus A, Nichols S, Lee D, Koepsel J, Anderson D, et al. Electrospun PCL in vitro: a microstructural basis for mechanical property changes. *J Biomat Sci-Polym*. 2009; 20(4):467–81.
43. Olson AL, Swigris JJ, Lezotte DC, Norris JM, Wilson CG, Brown KK. Mortality from pulmonary fibrosis increased in the United States from 1992 to 2003. *Am J Respir Crit Care Med*. 2007; 176(3):277–84. [PubMed: 17478620]

44. Magro CM, Marsh CB, Allen JN, Ross P, Liff D, Knight DA, et al. The role of anti-endothelial cell antibody-mediated microvascular injury in the evolution of pulmonary fibrosis in the setting of collagen vascular disease. *Am J Clin Pathol.* 2007; 127(2):237–47. [PubMed: 17210529]
45. Flemming RG, Murphy CJ, Abrams GA, Goodman SL, Nealey PF. Effects of synthetic micro- and nano-structured surfaces on cell behavior. *Biomaterials.* 1999; 20(6):573–88. [PubMed: 10213360]
46. Powell HM, Kniss DA, Lannutti JJ. Nanotopographic control of cytoskeletal organization. *Langmuir.* 2006; 22(11):5087–94. [PubMed: 16700598]
47. Powell HM, Lannutti JJ. Nanofibrillar surfaces via reactive ion etching. *Lang-muir.* 2003; 19(21): 9071–8.
48. Von Recum AF, Van Kooten TG. The influence of micro-topography on cellular response and the implications for silicone implants. *J Biomat Sci-Polym.* 1995; 7(2):181–98.
49. Gaumer J, Prasad A, Lee D, Lannutti J. Source-to-ground distance and the mechanical properties of electrospun fiber. *Acta Biomater.* 2009; 5(5):1552–61.
50. Kang XH, Xie YB, Powell HM, Lee LJ, Belury MA, Lannutti JJ, et al. Adipogenesis of murine embryonic stem cells in a three-dimensional culture system using electrospun polymer scaffolds. *Biomaterials.* 2007; 28(3):450–8. [PubMed: 16997371]
51. Mora AL, Rojas M. Aging and lung injury repair: a role for bone marrow derived mesenchymal stem cells. *J Cell Biochem.* 2008; 105(3):641–7. [PubMed: 18759327]
52. MacNee W. Premature vascular ageing in cystic fibrosis. *Eur Respir J.* 2009; 34 (6):1217–8. [PubMed: 19948908]
53. Karrasch S, Holz O, Jorres RA. Aging and induced senescence as factors in the pathogenesis of lung emphysema. *Respir Med.* 2008; 102(9):1215–30. [PubMed: 18617381]
54. Berdyeva TK, Woodworth CD, Sokolov I. Human epithelial cells increase their rigidity with ageing in vitro: direct measurements. *Phys Med Biol.* 2005; 50(1):81–92. [PubMed: 15715424]
55. Dimri GP, Lee XH, Basile G, Acosta M, Scott C, Roskelley C, et al. A biomarker that identifies senescent human-cells in culture and in aging skin in vivo. *Proc Natl Acad U S A.* 1995; 92(20): 9363–7.
56. Rauscher FM, Goldschmidt-Clermont PJ, Davis BH, Tang W, Gregg D, Ramaswami R, et al. Aging, progenitor cell exhaustion, and atherosclerosis. *Circulation.* 2003; 108(4):457–63. [PubMed: 12860902]
57. Muschler GF, Nitto H, Boehm CA, Easley KA. Age- and gender-related changes in the cellularity of human bone marrow and the prevalence of osteoblastic progenitors. *J Orthop Res.* 2001; 19(1): 117–25. [PubMed: 11332607]
58. Zammit PS, Partridge TA, Yablonka-Reuveni Z. The skeletal muscle satellite cell: the stem cell that came in from the cold. *J Histochem Cytochem.* 2006; 54 (11):1177–91. [PubMed: 16899758]
59. Christopherson GT, Song H, Mao HQ. The influence of fiber diameter of electrospun substrates on neural stem cell differentiation and proliferation. *Biomaterials.* 2009; 30(4):556–64. [PubMed: 18977025]

Appendix

Figures with essential colour discrimination. Fig. 5 in this article may be difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi: 10.1016/j.biomaterials.2010.08.104.

Fig. 1. SEM images of wild-type mouse bone marrow cells grown on plain PCL matrix in RPMI supplemented with 5% FBS for 8 days (A–B) or 14 days (C–D). Scale bars: 100 μm (left), 20 μm (right).

Fig. 2. SEM images of wild-type mouse bone marrow cells grown on PCL matrix coated with PBS-treated mouse lung extracts in RPMI supplemented with 5% FBS for 8 days (A–B) or 14 days (C–D). Scale bars: 100 μm (left), 20 μm (right).

Fig. 3. SEM images of wild-type mouse bone marrow cells grown on PCL matrix coated with bleomycin-treated mouse lung extracts in RPMI supplemented with 5% FBS for 8 days (A–B) or 14 days (C–D). Note the evidence of greater extracellular matrix deposition compared to PCL alone and PCL coated with PBS-treated lung extract. Scale bars: 100 μm (left), 20 μm (right).

Fig. 4.

Relative real-time PCR analysis of fibrotic gene expression in mouse BM cells after culture on PCL, PBS lung extract-coated, or bleomycin lung extract-coated matrices after 8 and 14 days. A. Type-I collagen expression showing a 12-fold increase on bleomycin lung extract-coated PCL on day 8, and a 20-fold increase on day 14. B. Smooth muscle actin expression showing a 37-fold increase on bleomycin lung extract-coated PCL on day 8, and a 23-fold increase on day 14. C. Connective tissue growth factor expression. D. Tenascin-C expression. Fibrotic gene expression levels of cells plated matrices coated with either PBS or Bleomycin-treated lung extracts are shown relative to gene expression of cells plated on plain PCL matrices. * $p < 0.05$, with $n = 8$ for each condition.

Fig. 5.

X-ray photoelectron spectroscopy (XPS) surface analysis of electrospun PCL, PES and PES (core)/PCL (shell) fiber. The sulfur peaks clearly visible in the PES spectra are absent in that of the core-shell fiber. This shows that the PES 'core' is fully covered by the 'shell' and thus does not contribute a chemical component to the observed biological responses.

Fig. 6. SEM images of wild-type mouse bone marrow cells grown on PCL core/PES shell (A–B) or plain PES (C–D) matrix in RPMI supplemented with 5% FBS for 8 days. Scale bars: 100 μm (left), 20 μm (right).

Fig. 7. SEM images of wild-type mouse bone marrow cells grown on PCL core/PES shell (A–B) or plain PES (C–D) matrix in RPMI supplemented with 5% FBS for 14 days. Scale bars: 100 μm (left), 20 μm (right).

Fig. 8.

Relative real-time PCR analysis of fibrotic gene expression in mouse BM cells after culture on PCL, PCL core/PES shell, or PES matrices after 8 and 14 days. A. Type-I collagen expression showing a 4-fold increase on PES on day 14. B. Smooth muscle actin expression showing a 51-fold increase on PCL/PES on day 8, with a 3-fold increase on day 14. C. Connective tissue growth factor expression showing a 3-fold increase on PES core/PES shell fibers on day 14. D. Tenascin-C expression showing a 7-fold increase on PES core/PES shell fibers on day 14 relative to all other conditions. Fibrotic gene expression levels of cells plated on core/shell and PES matrices are shown relative to gene expression of cells plated on plain PCL matrices. $*p < 0.05$, with $n = 11$ for each condition.

Table 1

Mechanical properties of plain PCL (control), PCL with PBS or Bleomycin-treated lung extract coatings, and PES core/PCL shell fibers.

Fiber Type	Ultimate Tensile Strength (MPa)	Percent Elongation	Young's Modulus (MPa)
PCL	2.34 ± 0.11	174.5 ± 5.5	3.23 ± 0.14
PBS	1.96 ± 0.07	151.7 ± 8.6	3.73 ± 0.72
Bleomycin	2.17 ± 0.10	$122 \pm 10^*$	4.46 ± 0.38
PES Core/PCL Shell	2.55 ± 0.15	$64 \pm 10^*$	$30.7 \pm 2.3^*$

*
 $p < 0.05$.