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Patterning of Fibroblast and Matrix Anisotropy within Three-Dimensional Confinement is Driven by the Cytoskeleton

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Effects of three-dimensional confinement on cellular growth and matrix assembly are important in tissue engineering, developmental biology, and regenerative medicine. Polydimethylsiloxane wells with varying anisotropy are microfabricated using soft-lithography. Microcontact printing of bovine serum albumin is used to block cell adhesion to surfaces between wells. The orientations of fibroblast stress fibers, microtubules, and fibronectin fibrils are examined one day after cell seeding using laser scanning confocal microscopy, and anisotropy is quantified using a custom autocorrelation analysis. Actin, microtubules, and fibronectin exhibit higher anisotropy coefficients for cells grown in rectangular wells with aspect ratios of 1:4 and 1:8, as compared to those in wells with lower aspect ratios or in square wells. The effects of disabling individual cytoskeletal components on fibroblast responses to anisotropy are then tested by applying actin or microtubule polymerization inhibitors, Rho kinase inhibitor, or by siRNA-mediated knockdown of AXL

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Supporting Information

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or cofilin-1. Latrunculin A decreases cytoskeletal and matrix anisotropy, nocodazole ablates both, and Y27632 mutes cellular polarity while decreasing matrix anisotropy. AXL siRNA knockdown has little effect, as does siRNA knockdown of cofilin-1. These data identify several specific cytoskeletal strategies as targets for the manipulation of anisotropy in three-dimensional tissue constructs.

1. Introduction

Tissue engineering can provide powerful tools for regenerative medicine. Production of reproducible grafts or scaffolds for implantation is dependent upon insights into the development and regulation of the natural matrix microenvironment. These insights will pave the way for the creation of functional tissue constructs in precisely engineered, natural, tissue-specific scaffolds, which preserve complex matrix architecture without activating host immune responses.^[1, 2] Additional benefits to using natural, cell-derived scaffolds include the translation of distinct mechanochemical and signaling cues to guide cell proliferation, migration, and cytoskeletal reorganization.^[2, 3, 4-7] The current work advances efforts toward functional micro-tissue implants by creating three-dimensional (3D) matrices derived from human fetal lung fibroblasts (WI-38) with well-defined geometry and anisotropy. Anisotropy in 3D matrices is observed in a variety of important physiological contexts where the coordination of matrix and cell anisotropy is linked with proper cell function, such as in electrical and mechanical synchronization of cardiac tissue, mammary epithelial morphogenesis, collagen alignment in the human corneal stroma, cancer cell migration, wound healing, and the ordered function of neural and vascular tissues.^[5, 8, 9] Manipulation of anisotropy in 3D tissue constructs is an important frontier of tissue engineering.

Our approach combines state-of-the-art microfabrication, surface chemistry modification, and scaffold material characterization for the rational design of matrix scaffolds with controlled anisotropy. This platform is important from both fundamental and applied perspectives. On the fundamental side, it is important to understand biases that may be introduced by geometric and confinement cues during scaffold design. For this, our model system offers in situ imaging, the use of native matrix infrastructure, and spatial parameters that can be tuned with sub-micron precision. On the applied side, understanding how to build micro-tissues to pilot the development of modular tissue constructs with anisotropic organization is an important goal.

In this study, we patterned a system of simple 3D polydimethylsiloxane (PDMS) geometries and modified the surface with proteins to selectively promote and inhibit cell adhesion in spatially precise patterns. WI-38 growth was studied in these constrained, 3D PDMS wells of specific shapes and dimensions to isolate the effects of anisotropy (or polarity) on cell cytoskeleton and matrix deposition. Our hypothesis was that confined 3D geometries induced increases in early anisotropy in WI-38 populations through actin dynamics, thereby affecting actin organization and matrix deposition. The ability to influence early multi-cell behavior and matrix deposition through changes in external anisotropic cues would allow for the creation of oriented, natural matrix scaffolds in specific dimensions. This would improve the *in vitro* examination of difficult to study, *in vivo*, multi-cellular, physiological environments mentioned previously and give more precision to the scaffold design process.

We observed increasing actin and microtubule alignment within confined geometries as early as 24 hours due to increased aspect ratios in rectangular shapes. Interestingly, this increase in anisotropic spatial orientation was reflected in extracellular matrix (ECM) deposition as well. These findings contrasted with the less ordered mesh of matrix fibers that was observed in square isotropic confinements. For future studies, we aim to create patterned, vascularized, 3D tissue constructs as an *in vitro* model system for vasculogenesis and microvascular engineering.^[4, 5, 10]

2. Results

2.1. Design of Patterned PDMS Wells

Our microfabricated platform allowed for a reproducible and quantitative study of isolated multi-cell populations in 3D constrained environments of specific geometry and dimensions. The hypothesis that confined, 3D geometries would induce significant increases in early anisotropy in WI-38 fibroblasts and the matrix that they assembled was tested by systematically varying well anisotropy. Microfabrication and photolithography were used to create arrays of patterned PDMS wells (Figure 1). PDMS is gas permeable, optically clear, and elastic, which made it an optimal material to use. Length scales of several hundred microns were chosen to create simple geometries that directly isolated the effects of anisotropy, while still providing a large enough space for multi-cell organization (as oppose to single cell well studies) but not so large as to allow unconfined growth.^[11, 12] Moreover, the molding of PDMS set practical limits on the aspect ratios of structures that could be reproducibly fabricated. Six well shapes were chosen with the following dimensions: Rec 1 (rectangle 1) 1000 μm long by 500 μm wide; Rec 2 1000 μm $\times$ 250 μm ; Rec 3 1000 μm $\times$ 125 μm ; Sq 1 (square 1) 707 μm $\times$ 707 μm ; Sq 2 500 μm $\times$ 500 μm ; and Sq 3 354 μm $\times$ 354 μm (Figure 1b). Rectangular wells had aspect ratios of 1:2, 1:4, and 1:8, respectively. To isolate the effect of anisotropy and to use isotropic square shapes as controls, all other variables including depth, total surface area, and total volume were kept constant between the following rectangle-square pairings: Rec 1 and Sq 1, Rec 2 and Sq 2, and Rec 3 and Sq 3. All well shapes were approximately 100 μm deep. This depth was chosen because it is a relevant length scale in physiology, as this is the typical diffusion limit for oxygen, nutrient, and waste transport via the microcirculation.^[6, 13]

Wells were created using standard soft-lithographic approaches. Briefly, SU-8 was patterned with a negative photoresist using UV exposure through a CAD designed photomasks to create masters in a variety of shapes (Figure 1a; i-ii). Patterned PDMS molds were created from these SU-8 masters where the protrusions in the SU-8 master became the wells of the PDMS mold (Figure 1a; iii-iv). To limit cell growth to the interior of the wells, two surface chemistry modification approaches were used. First, microcontact printing (or stamping) of 10% bovine serum albumin (BSA) was used to make the top surface of the PDMS non-adherent to cells (Figure 1a; v-vi and Figure 1c). Secondly, cell adhesion inside the wells was promoted by soaking the patterned mold in fibronectin solution (Figure 1a; vii-viii). The fibronectin attached to exposed PDMS on the bottom and walls of the wells but not to the BSA-inked top PDMS surface. These dual surface chemistry manipulations allowed for isolated 3D growth on all surfaces within the wells but did not allow for cell growth and

communication between cells in neighboring wells. Thus, the resultant platform allowed for the study of isolated, multi-cell populations.

2.2 Effects of 3D Confined Geometries on Cell Cytoskeleton and Matrix Assembly

Previous work in our lab showed that WI-38 fibroblasts synthesized and assembled a heterogeneous, 5-10 μm thick, 3D matrix when grown on 12 mm, glass coverslips for 7-10 days.^[4, 14] The components and microelastic properties of this decellularized matrix were defined and measured in previous studies. Using the patterned PDMS well platform described above, matrices with approximately 20-45 μm depth were observed as early as Day 1 – an important step toward the production of *in vitro* micro-tissues.

The orientations of WI-38 stress fibers, fibronectin fibrils, and microtubules were documented at Day 1 (~24 hours) and demonstrated increased early development of anisotropic organization in the wells with higher aspect ratios (Figure 2 and Figure S1). These findings persisted on the second day after plating. For both days, anisotropy increased with rectangular aspect ratio and the 1:4 aspect ratio was a consistent threshold for the induction of significant increases in anisotropic cell and matrix organization (Figure 2b and Figure S2). As minimal differences were observed between Day 1 and 2, this study focused on Day 1 to study early and rapid macro effects of constrained environments on cell anisotropy.

WI-38 were grown in rectangles with aspect ratios of 1:2 (Figure 2a; i, vii), 1:4 (Figure 2a; ii, viii), and 1:8 (Figure 2a; iii, ix). Cells in square shapes served as controls for each corresponding rectangle (Figure 2a; iv, x; v, xi; vi, xii), as the squares imposed an isotropic confinement compared to the anisotropic confinement of rectangular shapes. Spatial organization of cytoskeletal elements and matrix deposition was qualitatively examined using laser scanning microscopy (Figure 2a and Figure S1) and quantitatively confirmed using a custom quantitative autocorrelation analysis (Figure 2b, and section 5). High anisotropy values for actin filament (F-actin) and microtubule organization were observed in rectangular shapes with aspect ratios of 1:4 and 1:8, and cells showed increasing alignment with the long axis as the aspect ratio of the rectangles increased (Figure 2a; i-vi, Figure 2b, and Figure S1). In squares shapes, this increasing cell polarity was not observed as squares progressed from Sq 1 to Sq 2 to Sq 3. Cells in square shapes aligned similarly to cellular alignment seen in unconstrained environments (Figure S3).

Interestingly, similar significant trends were seen for fibronectin anisotropy analysis (Figure 2a; vii-xii, and Figure 2b), indicating that the cells transmitted this orientation to ECM production and deposition. Overall, the 1:4 aspect ratio seemed to be a threshold for shape-specific effects of 3D confinement on the early orientation of both actin and fibronectin. Anisotropy coefficient variations between square shapes were small when compared to rectangle-square pairings and close to Rec 1 coefficients in value. Due to their spindle shape, WI-38 showed a natural tendency to align even in unrestrained monolayer culture on glass coverslips, thus anisotropy coefficients for squares were higher than 1 (Figure S3 and Figure 2b). However, the natural, baseline level of actin and fibronectin anisotropy for cells in unconstrained environments was found to be similar to that seen in minimally constrained

(Recs 1) and isotropically constrained (Sqs 1-3) environments, meaning cells in these shapes were orienting as they would in an unconstrained environment (Figure S3).

2.3 Biochemical Inhibitor Effects on WI-38 Anisotropy in Confined Geometries

Efforts to define the contributions of individual cytoskeletal components to the observed geometry-induced cellular anisotropy in Figure 2 began with biochemical inhibitors. We first tested the hypothesis that actin polymerization was the major cytoskeletal process determining the increased development of cellular and matrix anisotropy in response to 3D confinement. Latrunculin A (LatA) and nocodazole (NOC) were used to inhibit actin nucleation and polymerization or microtubule assembly, respectively.^[15, 16-18] Y27632 was then used to block actomyosin contraction and stress fiber formation catalyzed by the Rho-associated coiled-coil kinase (ROCK) to further test the functional role of actin.^[16, 19]

Titration experiments on glass coverslips were performed to identify optimal concentrations for each inhibitor that did not overly disrupt cytoskeletal organization or cell shape (Figure S4, S5, and S6). Concentrations ranging from 0.01 μ M to 0.5 μ M for LatA and from 0.01 μ M to 0.2 μ M for NOC were used,^[15, 16] and intermediate concentrations of 0.05 μ M LatA and 0.02 μ M NOC were chosen in order to inhibit, but not to ablate, actin or microtubule polymerization respectively. For Y27632, concentrations of 1 to 10 μ M were tested, and 4 μ M was chosen to create an inhibitory effect that did not also compromise cell adhesion or shape.^[16, 19]

2.3.1. Latrunculin A Decreases Anisotropy—Cells in patterned PDMS wells that were treated with 0.05 μ M LatA had significantly lower anisotropy in all three rectangle shapes when compared to untreated controls (Figure 3). Additionally, a higher threshold for the manifestation of the increase in cellular and matrix anisotropy was seen with LatA treatment - an aspect ratio of 1:8, as opposed to 1:4 in untreated controls (Figure 3b). With LatA treatment, there were no significant increases in anisotropy between Rec 1 and Rec 2 or between Rec 2 and Rec 3 in either the actin or the fibronectin analyses (although these differences were seen in untreated control cells). Thus, a higher external constrained anisotropy of aspect ratio 1:8 was needed before significant increases were observed in actin and fibronectin anisotropy above those seen in Rec 1 or square shapes. Matrix (fibronectin or Fn) organization had similar anisotropy values in squares in both control and LatA conditions. LatA treatment was also associated with a loss of the anisotropic increase in microtubular organization seen between Rec 1 and Rec 2 in the controls, and significantly lowered anisotropy values for all the rectangles and for Sq 1 as compared with controls (Figure S7).

2.3.2. Nocodazole Ablates Anisotropy—The actin and microtubule cytoskeletons of cells exhibited minimal anisotropy in rectangular shapes after treatment with 0.02 μ M NOC as compared with untreated cells (Figure 4a), and NOC treatment caused a significant loss of anisotropy in actin, fibronectin, and microtubules in almost all shapes by quantitative analyses (Figure 4b). Any anisotropy coefficient variations between NOC treated shapes were small compared to untreated variations. As in other data sets, most analyses of differences between rectangles and squares of identical surface area in control samples

showed significant differences, indicating that squares were functional isotropically constrained controls.

2.3.3. Y27632 Effects on Anisotropy—Actin, fibronectin, and microtubules in cells treated with 4 μ M Y27632 had both lower values and a smaller range of anisotropy coefficients when compared to untreated controls (**Figure 5b** and Figure S8). Cells in rectangles treated with Y27632 had fibronectin anisotropy coefficients that were closer to Rec 1 fibronectin values in untreated controls and thus, lacked the increase in anisotropy between Rec 1 and Rec 3 that was observed in fibronectin controls (Figure 5).

2.4 Effects of siRNA Knockdown on the Cellular Regulation of Fibroblast Cytoskeleton and Matrix Anisotropy

Specific molecular mechanisms that might drive increases in anisotropic fibroblast alignment in 3D geometries were tested next. Nontransfected cells and cells transfected with scrambled, nontargeted siRNA served as controls, and no statistical differences in anisotropy coefficients were observed between these two groups within the same patterned PDMS shapes (Figure S9). AXL and cofilin-1 were then examined using siRNA knockdown because they have both been implicated in geometry-dependent cell shape and fate determination.^[18, 20-22] AXL is a mechanosensor that plays a role in pre-polarizing cell adhesions and predicting overall cell anisotropy.^[22] Cofilin-1 destabilizes actin polymerization by depolymerization at the minus end of F-actin, and it is an indirect target of ROCK.^[18, 21]

2.4.1 AXL siRNA Knockdown Does Not Alter Anisotropic Fibroblast Responses—100% AXL knockdown was achieved using 100 nM AXL siRNA for 3, 6, 12, 24, 30, 36, 48 hours after AXL siRNA transfection. A waiting time of ~4 hour after transfection and before PDMS well seeding was chosen to ensure maximum knockdown during the 24 hour cell growth period inside PDMS wells. Western blot analysis showed ~100% AXL knockdown in AXL siRNA-treated cells and no knockdown in scramble siRNA control cells (**Figure 6c**). Epifluorescence and autocorrelation analyses for actin, fibronectin, and microtubules revealed that AXL siRNA-transfected cells had similar anisotropy to scramble siRNA controls, indicating that AXL did not play a major role (Figure 6). Square anisotropy coefficient values were more variable than in previous experiment, but both control and AXL values were in a similar range and lower than most Rec 2 and Rec 3 coefficients.

2.4.2 Effects of Cofilin-1 siRNA Knockdown on Anisotropy—Approximately 70% knockdown of cofilin-1 was achieved using 300 nM cofilin-1 siRNA at Days 2-4. Thus, ~52 hours after transfection was the chosen waiting time before seeding cells into PDMS wells. Western blot analysis showed ~70% cofilin-1 knockdown in cofilin-1 siRNA-treated cells and no knockdown in scramble siRNA control cells (**Figure 7b**). Cells transfected with cofilin-1 siRNA had higher anisotropy in rectangle shapes than compared to scramble siRNA controls, but differences in anisotropy were not statistically significant when compared to corresponding control cells in rectangles (Figure 7a, c). This lack of difference in anisotropy indicated that cofilin-1 did not contribute to the propagation of shape-specific

effects in a dominant fashion. Cellular anisotropies in squares were similar to each other within each condition and when comparing control to treated conditions (Figure 7c, Figure S10, and Figure S11). Epifluorescence labeling of scramble siRNA in cofilin-1 experiments looked similar to scramble siRNA images in AXL experiments (Figure 6a; i, iii, and Figure 6b; i, iii).

3. Discussion

Since the seminal work of Chen et al., micropatterning and microfabrication techniques have been utilized to investigate the effects of geometry on cell behavior.^[23] However, prior studies have focused predominantly on 2D patterns or the study of single cells in confined spaces,^[11, 24, 25] and only recently have 3D patterned wells and surfaces started to be explored to investigate the collective behavior of cells.^[26, 27] In our work, we utilized soft-lithographic techniques to address a fundamental question in cell biology: How does anisotropy alter the cell behavior of cell populations and their generated matrix in 3D anisotropic spaces? Anisotropic 3D spaces exist *in vivo*, and consequently *in vitro* model studies could shed light on early events in tissue morphogenesis, developmental biology, and more specific instances, such as cancer cell migration, neural function, wound healing, cardiac tissue synchronization, and vascular development.^[8] Soft-lithography and microcontact printing approaches afford high levels of reproducibility, tunability, and versatility. We were able to use microfabrication and cell non-adherent coatings, such as BSA, with adherent ones, such as fibronectin, to create a non-adherent top surface and isolated adherent wells of varying shapes.

Overall, we observed that confined anisotropic 3D geometries induced significant increases in early anisotropy in WI-38 fibroblasts and had profound effects on actin organization and matrix deposition. Actin, fibronectin, and microtubule alignment within confined geometries were observed as early as 24 hours, and increased with higher aspect ratios in rectangular shapes. Importantly, WI-38 grown in rectangular 3D shapes with 1:4 aspect ratios showed an increase in anisotropy from the less pronounced cellular alignment normally seen in cells grown in unconstrained conditions on coverslips, in minimally constrained Rectangles 1, or in isotropically constrained square shapes. Thus, we observed external confinement influencing the entire fibroblast cell population in a substantial way at widths of 250 μm (in the 1:4 aspect ratios of Recs 2), which was a confinement length scale that was several times that of a single cell. This distance was consistent with and exceeded by 25% the flat distances reported in Lückert et al. over which fibroblast alignment was affected by the orientation of neighboring cells.^[27] It would indeed be interesting to note how other cell types may respond in these confined 3D microenvironments, and our platform provides a convenient means to conduct such investigations. For example, single cell studies have shown that 3D confinement had a profound impact on HeLa cell division and tumor cell migration, and it would be interesting to investigate such behaviors in populations of other cells in confined microenvironments and their interactions with anisotropic 3D natural matrices.^[24, 28]

The wells used in our study had right-angled corners. Gomez et al. found that rounded convex corners induced high mechanical stress in cells.^[9] But in our study, right-angled

corners were not associated with heavy concentrations of cortical actin. It is therefore possible that if more rounded corners were used in our study (forming more ellipsoid, convex shapes) cytoskeletal tension might become concentrated and accentuate local anisotropy. Nikkah et al. found that fibroblasts stretched across curved sidewalls and did not adhere as often to areas of high curvature.^[29] Potentially, if corners were more rounded in our system and had less abrupt curvature than right-angled corners, fibroblasts would exhibit more robust adhesion and increased anisotropy.

Cytoskeletal functions span a diverse repertoire that encompasses the guidance of tissue morphogenesis and cellular mechanotransduction.^[30] The current work provided an in-depth examination of the role of individual cytoskeletal components in multi-cellular assembly of natural matrix during 3D confinement. The first step in this process was the synchronous orientation of the long axis of fetal lung fibroblasts. In the second step, these WI-38 fibroblasts served as cellular ‘looms’ that sculpted fibronectin fibrillogenesis through the application of cellular traction forces.^[31] We previously reported experimental data and a computational model indicating that cell traction force was proportional to the first moment of area at a given point on the cell surface, and that the cytoskeleton functioned as a cohesive unit in a dynamic interrelationship with cell shape.^[32] Data presented here in Figures 3, 4, and 5 indicated that assembly of both the microfilament (actomyosin) and microtubule (tubulin) cytoskeletons were essential in very early responses of cells to anisotropic 3D confinement, and that regulation of actomyosin contraction was also involved. These findings were consonant with the work of others. For example, detailed alterations in microtubule branching patterns have been described in response to both the geometry and the mechanical stiffness of confinement.^[33] Additionally, spatial confinement has been shown to elicit rapid localization of actin assembly, stress fiber contraction, and alignment of microtubule organizing centers during epithelial cell experiments with both monolayers and 3D morphogenesis.^[34] ROCK is a molecular mechanism that has been implicated in fibroblast responses to geometric cues from the 3D microenvironment and in actomyosin contraction and stress fiber growth.^[35] ROCK has also been found to mediate fibronectin matrix assembly in other cellular systems.^[36] Indeed by using Y27632 biochemical inhibition, our findings implicated the RhoA/ROCK pathway in fibroblast responses to shape-specific anisotropic effects - especially in the ability of WI-38 to produce aligned matrix. However, decreasing fibroblast expression of the actin depolymerizing protein cofilin-1, an indirect downstream target of ROCK, was not associated with robust differences in alignment of both F-actin and fibronectin (Figure 7). Potentially, more complete cofilin-1 knockdown would result in significant changes in anisotropy. Overall, the summation of observations from experiments with LatA, Y27632, and cofilin-1 siRNA pointed to an inverse relationship between filamentous actin turnover and cellular responses to confinement.

The observation that AXL knockdown had no significant effect on cellular and matrix anisotropy in this platform was surprising, as AXL had been shown to play a major role as a mechanosensor that pre-polarized cell adhesions to help define overall cell anisotropy.^[22] Recent research found that AXL was one of a number of proteins that modulated the force threshold for focal adhesion polarization on rigid surfaces. This defined an axis for cell anisotropy and led to cell polarization.^[22] In those studies, AXL knockdown resulted in

focal adhesion formation and cellular elongation that were independent of the rigidity of the substrate. Conversely in cancer cells, AXL knockdown caused a decrease in cell polarity, cell elongation, and filopodia.^[37] Clearly, AXL plays a complex role in the development of cellular anisotropy that may be altered by cell type and substrate rigidity. In our system, control and knockdown cells were both plated on a relatively flexible PDMS surface. Since force was not a variable component in this platform, and 3D confinement provided external stimulus that was not present in monolayer cultures, the contribution of AXL to anisotropy may have been dampened. These insights illustrate the importance of studying cell populations and matrix formation in physiologically relevant 3D platforms.

3D natural matrix scaffolds may be utilized to construct micro-tissues for use as implants in regenerative medicine. 3D scaffolds are superior to 2D cell systems for studies in *in vitro* platforms that merge materials science, developmental biology, and molecular signaling, and 3D scaffolds may also be advantageous for engineering *in vivo* implantation strategies. Additionally, while 3D polymeric synthetic biomaterials provide inexpensive, reproducible scaffolds with well-defined chemistries, these do not precisely mimic the physical and chemical complexity and heterogeneity of natural ECM.^[2, 38] Because of the important link between matrix orientation and cell function, there are many benefits to using natural ECM - a complex 3D mesh of proteins, proteoglycans, and bound growth factors - instead of synthetic materials as a scaffold. By providing a physical scaffold with distinct architecture, orientation, and biochemical and mechanical cues, the ECM creates a unique microenvironment for cells that influences cell proliferation, migration, and cytoskeletal reorganization.^[2, 3, 4] Therefore, understanding the natural strategies imbedded in the matrix for spatial guidance, anisotropy, and orientation and incorporating these natural cues into scaffold design is key to the successful development of 3D tissue grafts for regenerative treatments. Indeed, our lab has produced natural tissue constructs that support cell growth and tissue morphogenesis - specifically for fibroblast-derived, decellularized matrix that stimulated vasculogenesis.^[4, 5, 10] Soucy et al. observed that endothelial cells formed oriented blood vessels when cultured on decellularized, natural matrix.^[5] A near-term approach for creating 3D micro-tissue constructs could be to combine the 3D matrix scaffold described in this current study with the endothelial tubulogenesis shown by Soucy et al. and the promising method for retrieving intact microfabricated cell constructs using poly(N-isopropylacrylamide) (PNIPAm), which was described by Tekin and coworkers.^[39]

It is noteworthy that our study demonstrated an approach for growing an oriented 3D, natural matrix scaffold within a 3D microfabricated mold. In contrast to 2D patterns, we utilized a 3D mold that presented fibronectin-coated PDMS surfaces along both the bottoms and the sidewalls of the wells. This facilitated fibroblast adhesion and the production of natural ECM in the dimensions of the PDMS well shapes with z-dimensions as thick as ~20-45 μm in just 24 hours. This was a significant improvement over the 5-10 μm thick 3D matrix models previously grown on glass coverslips in our lab.^[4, 14] This observation suggested that strategies that promote cell adherence to both the bottom and sidewalls in 3D confined spaces could be utilized to produce thicker and also aligned natural ECM. Such thicker matrices would provide an ideal test bed to investigate the effects of geometry on cells within a realistic 3D natural matrix.

Our approach for micromold fabrication utilized AutoCAD tools to produce well-defined designs with considerable precision and versatility. Other biomimetic shapes, including those of integrated organoids, could readily be fabricated using the demonstrated soft-lithographic approaches to create more anatomically accurate 3D matrix models and confined environments for the study of cellular systems. This has important implications for creating a physiologically relevant microenvironment in a natural scaffold format. Our next steps include growing thicker matrices, seeding endothelial cells, and creating tissue-specific, oriented microvessels to define the effects of 3D confined geometries on blood vessel growth and orientation in natural matrix for tissue engineering applications.

4. Conclusion

Fundamental insights in matrix biology may guide successful engineering of cell-derived constructs in relevant 3D geometries for tissue regeneration. We presented a platform to control cell and matrix geometry and used it to test multi-cell behavior in 3D constrained environments. Human fetal lung fibroblasts cultured in constrained 3D microenvironments exhibited a significant increase in anisotropic alignment beginning at a 1:4 aspect ratio by the polarization of the actin and microtubule cytoskeletons. This anisotropy was propagated to the fibronectin matrix produced by these cells. Actin and microtubule nucleation both had substantial roles in these shape-specific early effects, as did ROCK-mediated actomyosin contraction. Our results suggest that modulating cytoskeletal dynamics and 3D confinement could be used to design natural, human, tissue-specific scaffolds with well-defined anisotropy for tissue engineering applications. This research provides an opportunity to bridge the gap between the biology of cell-matrix interactions and translational therapy in regenerative medicine.

5. Experimental Section

Microfabrication

Microfabrication and photolithography were used to create, polydimethylsiloxane (PDMS) wells of varying shapes and dimensions for isolated cell growth. SU-8 2100, a negative photoresist (MicroChem), was spun onto a silicon (Si) wafer (700 rpm for 10 seconds, and 2200-2300 rpm for 1 minute) using a spin coater to achieve a uniform ~100 um thick layer of SU-8. The resist layer was exposed to UV light through a CAD (AutoCAD, AutoDesk) photomask with a designed pattern of varying shapes using an automated bond alignment system (EVG620, EV Group). Non-UV exposed resist was chemically removed using SU-8 Developer (MicroChem), leaving only a series of solid, patterned SU-8 shapes. PDMS (Sylgard 184 Silicone Elastomer Kit, Dow Corning; prepolymer to curing agent ratio of 10:1) was poured onto the SU-8 master and allowed to harden overnight at 37 °C. PDMS was peeled away from the SU-8 master to create a PDMS mold (or PDMS sample) with 3D wells with specific shapes (rectangles, squares, etc.), straight walls, and a depth of ~100 um.

Microcontact Printing

The top, unpatterned surface area of the PDMS mold (the spaces in between well openings) was made resistant to cell adhesion by microcontact printing (stamping) of 10% bovine

serum albumin (BSA). BSA (Sigma-Aldrich; St. Louis, MO) was made in 1X PBS and sterile filtered through 0.22 μ m filter. An unpatterned piece of PDMS was soaked in 10% BSA, washed with PBS, and dried by pressurized air to create a BSA inks pad. A patterned PDMS well sample was inverted onto the BSA inks pad to treat the PDMS sample top surface for 30 minutes. The patterned PDMS sample was then peeled off and incubated at 70 °C for 10 minutes. The patterned PDMS sample was soaked in fibronectin (4 °C, overnight) to facilitate cell adhesion, coating wells where BSA did not make contact. Upon seeding, cell adhesion and growth was restricted to the interior of the PDMS wells. This was verified by phase contrast microscopy ~24 hours after cell seeding.

Cell Culture

WI-38 human fetal lung fibroblasts (primary cells from ATCC; Manassas, VA) were maintained in Eagle's Minimum Essential Medium with Earle's salts and L-glutamine (Sigma-Aldrich; St. Louis, MO), and supplemented with 1% non-essential amino acids (Sigma-Aldrich; St. Louis, MO), sodium pyruvate (1 mM, Sigma-Aldrich; St. Louis, MO), 1% antibiotic-antimycotic solution (Cellgro; Manassas, VA), and 10% FBS (Atlanta Biologicals; Lawrenceville, GA). Cells were grown at 37 °C and 5% CO₂. Both coverslips (12-mm no. 1, Thermo Fisher Scientific; Waltham, MA) and 3D patterned PDMS wells (fabrication described above) were coated with fibronectin (25 μ g/ml, Life Technologies; Grand Island, NY) overnight at 4 °C. The following controls were included for each 3D patterned PDMS well experiment to check for reagent and microcontact printing quality: 1. A patterned PDMS sample without BSA stamping was soaked only in PBS overnight at 4 °C, 2. A patterned PDMS sample without BSA stamping was soaked only in fibronectin overnight at 4 °C, and 3. A patterned PDMS sample without BSA stamping was soaked in 10% BSA for 30 minute at room temperature, washed in PBS, dried, incubated at 70 °C for 10 minutes, and soaked in fibronectin overnight at 4 °C. Using the microcontact printing method described above, WI-38 were seeded onto fibronectin-coated patterned PDMS wells at a range of $\sim 7 - 15 \times 10^4$ cell/ml in 1 ml of media per well (24 well-plate) and adhered to the interior portions of the PDMS wells. For glass coverslip experiments, WI-38 were seeded at $\sim 8 - 10 \times 10^4$ cell/ml onto fibronectin-coated glass coverslips.

Biochemical Inhibitors

WI-38 were seeded onto BSA-stamped, fibronectin-coated 3D PDMS wells. Cells were then grown for 2 hours to allow cell attachment to the PDMS well interior. After 2 hours, WI-38 media was changed to media containing latrunculin A (Santa Cruz Biotechnology; Dallas, TX), nocodazole (Sigma-Aldrich; St. Louis, MO), or Y27632 (Sigma-Aldrich; St. Louis, MO). Cells in patterned PDMS well samples were grown for ~24 hours with an inhibitor, and then fixed using permeabilization and fixation protocol described below. For titration experiments, WI-38 were seeded on glass coverslips and allowed to adhere for 2-4 hours. Then, WI-38 media was changed to media containing latrunculin A, nocodazole, or Y27632 at the appropriate concentrations. Cells were grown for 2-2.5 hours with an inhibitor treatment, and then prepared for analysis as described below.

siRNA Transfection and Knockdown

WI-38 from passage 4 to 6 were trypsinized (0.05% trypsin) and resuspended in Nucleofector transfection solution (100 μ l, Amaxa MEF 2 Nucleofector Kit VPD-1005, Lonza; Walkersville, MD) with AXL (100 nM, Hs_AXL_10 SI00605311, Qiagen; Valencia, CA), cofilin-1 (300 nM, Hs_CLF1_3 SI00299831, Qiagen; Valencia, CA), or the corresponding amount of All Stars negative siRNA control (SI03650318, Qiagen; Valencia, CA). The MEF Mouse T-020 program was used for the cuvette Amaxa Nucleofection System (Lonza; Walkersville, MD). Transfected cells were grown in warm media for ~4 hours (AXL siRNA) or ~52 hours (cofilin-1 siRNA), trypsinized, and seeded into patterned 3D PDMS wells. These times achieved maximal knockdown and were defined by time course experiments. After ~24 hours of growth in PDMS wells, cell-filled PDMS well samples were prepared for analysis as described below. Knockdown was verified by immunoblotting of lysates from parallel fibroblast samples.

Antibodies

For immunofluorescence, the following primary antibodies were used: rabbit polyclonal anti-beta tubulin (Abcam; Cambridge, MA) and mouse monoclonal anti-fibronectin (Abcam; Cambridge, MA). The secondary antibodies used were Alexa Fluor 647-conjugated donkey anti-rabbit IgG (Abcam; Cambridge, MA) and Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes; Grant Island, NY). Rhodamine phalloidin (Molecular Probes; Grant Island, NY) was used to stain actin. For western blotting, rabbit polyclonal anti-AXL (1:1500, Millipore; Temecula, CA), rabbit polyclonal anti-cofilin-1 (1:3000, Abcam; Cambridge, MA), and mouse monoclonal anti-GAPDH (1:1200-1:3000, Novus Biologicals; Littleton, CO) primary antibodies were used. Peroxidase-conjugated AffiniPure goat anti-mouse (1:8000, Jackson ImmunoResearch; West Grove, PA) and goat anti-rabbit (1:10000, Jackson ImmunoResearch; West Grove, PA) secondary antibodies were used.

Immunoblotting

Cells were harvested and lysed at appropriate time points. Protein quantification was done using the Bradford protein assay (Thermo Fisher Scientific; Waltham, MA). Cell lysate volumes of equal protein content were boiled for 5 minutes at 100 °C in 2X Laemmli sample buffer (Bio-Rad Laboratories; Hercules, CA) and subjected to SDS-PAGE on a 4-15% gradient gel (Bio-Rad Laboratories; Hercules, CA). Proteins were transferred to nitrocellulose membranes that were blocked with 1% BSA (Sigma-Aldrich; St. Louis, MO) in TBST (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.05% Tween-20) for 1 hour at room temperature. Membranes were immersed in primary antibody in 1% BSA in TBST, incubated overnight at 4 °C, and then washed three times for 10 minutes with TBST at room temperature prior to 1 hour incubations with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibodies at room temperature. Enhanced chemiluminescence (Thermo Fisher Scientific; Waltham, MA), X-ray film (Denville Scientific Inc.; South Plainfield, NJ), and an Epson Perfection scanner (Long Beach, CA) were used to detect proteins.

Epifluorescence and Confocal Microscopy

Cells were permeabilized in 0.5% Triton X-100 in 3% paraformaldehyde (Sigma-Aldrich; St. Louis, MO) for 2 minutes. Subsequently, cells were fixed for 20 minutes in 3% paraformaldehyde, washed with 1X PBS, and stored in 4 °C. Primary and secondary antibodies were diluted in 0.1% BSA in Tris-Cl buffer (50 mM Tris-HCL pH 7.6 and 150 mM NaCl). Samples were incubated with primary antibodies for 30 minutes at 37 °C, washed for 5 minutes in Tris-Cl buffer, incubated with secondary antibodies for 30 minutes at 37 °C, washed for 5 minutes in Tris-Cl buffer, and washed with 1X PBS. A Zeiss AxioExaminer 710NLO multiphoton confocal microscope was used for epifluorescence imaging of cells in 3D PDMS wells, and an Olympus BX61 motorized upright fluorescence microscope equipped with a Roper/Photometrics Coolsnap HQ CCD Camera was used for the merged image. Epifluorescence imaging of cells on glass coverslips was done using an inverted Nikon Eclipse TE-200 microscope (Melville, NY) equipped with epifluorescence, a Rolera EMCCD camera (QImaging; Surrey, British Columbia), and Volocity software (PerkinElmer; Waltham, MA).

Anisotropy Coefficient Analysis

Using MatLab (R2009b and R2014a, MathWorks), a custom quantitative autocorrelation analysis was developed for the analysis of raw confocal images (.ism file format). Fourier analysis speeded computation. From values greater than a 50% threshold of the two-dimensional autocorrelation, a statistical ellipse was calculated from the value-weighted second moment. This ellipse represented overall anisotropy (or polarity) of the image's fluorescence structure. For quantitative comparisons, we defined the anisotropy coefficient for each image as the ratio of the major to minor axes of this fitted ellipse. Anisotropic images had coefficients greater than 1, with higher anisotropy reflected as a correspondingly larger coefficient. Images with coefficients of 1 (or close to 1) were isotropic. Anisotropy coefficient data were output from MATLAB, sorted in Excel, and analyzed in Prism (Prism 6, GraphPad Software; La Jolla, CA).

Statistical Analysis

Anisotropy measurements were gathered from cells grown in N distinct and independent wells for each of the 6 shapes for every condition. For untreated cells in PDMS wells, control data points were gathered across all experiments and combined. The Mann-Whitney test for nonparametric data was used for statistical analysis (Prism 6), since the data were non-Gaussian. Standard error of the mean was included in relevant figures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Creating PDMS wells with varying aspect ratios. **(a)** SU-8 2100 negative photoresist was applied and exposed to UV light (i). Resist was removed, leaving a patterned SU-8 master (ii). PDMS was poured and hardened to create a patterned mold (iii). The PDMS mold was peeled away and applied to an unpatterned PDMS piece that was coated with bovine serum albumin (iv-v). The top PDMS mold area was made nonadhesive by BSA inking (vi). PDMS was coated with fibronectin solution (vii). Seeded cells attached only inside PDMS wells (viii). **(b)** 4X phase images of PDMS rectangular (R1-3) and square (S1-3) wells. All shapes were ~100 μm deep. Scale bar = 100 μm . **(c)** Merged top view epifluorescence image of actin (phalloidin, red), fibronectin (anti-fibronectin antibody, green), and nuclei (DAPI, blue) of WI-38 in PDMS wells on Day 1. BSA microcontact printing excluded cell adhesion from the top surface of the PDMS block. Scale bar = 100 μm .

Figure 2.

Actin, fibronectin, and microtubule anisotropy in wells with increasing aspect ratio. **(a)** Epifluorescence images of WI-38 actin (phalloidin; i-vi) and fibronectin (anti-fibronectin antibody; vii-xii) at the bottoms of individual PDMS wells on Day 1. Actin and fibronectin images were from different wells. Scale bar = 100 μ m. **(b)** Autocorrelation analyses for actin, fibronectin, and microtubule anisotropy were performed for each shape. N=87 data points for actin and fibronectin and N=66 data points for microtubules were collected at 24 hrs. Actin, matrix, and microtubule anisotropy increased with increasing aspect ratio in the following pairs of shapes: Recs 1-2, Recs 2-3, Recs 1-3, Rec 2-Sq 2, and Rec 3-Sq 3 ($p < 0.0001$). Squares were functional controls for isotropically constrained growth. Additionally, the rectangle-square comparisons indicated that the observed increases in rectangular anisotropy were due to aspect ratio and not due to volume or surface area variations. * indicates $p < 0.05$ between cells in the two compared shapes. Bars represent standard error.

Figure 3.

Latrunculin A decreased anisotropy. **(a)** Epifluorescence images show organization of actin (phalloidin) in untreated (i, iii) or LatA-treated (0.05 uM; ii, iv) WI-38 at the bottoms of PDMS wells one day after plating. **(b)** Autocorrelation analysis was performed on 9 independent PDMS wells for each of the 6 shapes used per experimental condition. For actin and fibronectin, latrunculin A increased the aspect ratio at which significant increases in anisotropy occurred, from 1:4 in the untreated samples, to 1:8 in LatA-treated samples. This is because LatA treatment muted differences in anisotropy between Rec 1 and Rec 2, and between Rec 2 and Rec 3 that were seen with untreated cells. When treated with LatA, only cells in Rec 3 demonstrated significant differences in anisotropy from cells in Rec 1 (actin $p < 0.01$; Fn $p < 0.05$). Additionally, analyses of Rec 3 and Sq 3 showed a difference in anisotropy ($p < 0.0001$ for actin and Fn in control cells; and $p < 0.01$ (actin) and $p < 0.05$ (Fn) for LatA-treated cells). * indicates $p < 0.05$ between cells in the two compared shapes. # indicates $p < 0.05$ for cells in that specific shape treated with LatA, as compared to cells with no treatment in the same shape. Bars represent standard error.

Figure 4. Nocodazole blocked anisotropic cell responses. **(a)** Epifluorescence images show organization of actin (phalloidin; i-iv) and microtubules (anti-beta tubulin antibody; v-viii) in the same fields in NOC-treated (0.02 uM; ii, iv, vi, viii) or untreated (i, iii, v, vii) WI-38 in PDMS well bottoms one day after plating. Actin and microtubule images of the same shapes were from the same wells. **(b)** Autocorrelation analyses for actin, fibronectin, and microtubule anisotropy were performed on N=9 for each shape in actin and fibronectin and N=6 for microtubules. Only cells in untreated control conditions had the following rectangular anisotropy: Recs 1-2 ($p < 0.01$ actin) and Recs 1-3 ($p < 0.001$ actin, $p < 0.05$ Fn, $p < 0.05$ microtubules). * indicates $p < 0.05$ between cells in the two compared shapes. # indicates $p < 0.05$ for cells in that specific shape treated with NOC, as compared to the untreated control for that shape. Bars represent standard error.

Figure 5.

Y27632 decreased anisotropy. **(a)** Epifluorescence images of actin (phalloidin; i-iv) and fibronectin (anti-fibronectin antibody; v-viii) in untreated (i, iii, v, vii) or Y27632-treated (4 uM Y27632; ii, iv, vi, viii) WI-38 in PDMS well bottoms one day after plating. Actin and fibronectin images of the same shapes were from the same wells. **(b)** Autocorrelation analyses for actin and fibronectin anisotropy were performed on 9 PDMS wells for each shape. Anisotropy coefficient values were lower and in a smaller range when cells were treated with Y27632. Fibronectin lost the difference in anisotropy between Recs 1-3 in Y27632-treated cells compared to control. * indicates $p < 0.05$ between cells in the two compared shapes. # indicates $p < 0.05$ for cells in that specific shape when treated with Y27632, in contrast to the untreated control for that shape. Bars represent standard error.

Figure 6.

siRNA knockdown of AXL did not affect anisotropic cell responses. WI-38 cells were transfected with 100 nM of AXL siRNA or 100 nM of scramble siRNA (control) and grown in warm media for ~4 hrs. Cells were then trypsinized and seeded into 3D PDMS wells, and a population of each condition was kept growing for future cell lysate collection. **(a-b)** Epifluorescence images one day after plating in PDMS well bottoms of actin (phalloidin) in WI-38 that were transfected with scrambled siRNA (a, i and iii; b, i and iii) or siRNA specific for AXL (a, ii and iv; b, ii and iv). **(c)** Protein lysates were collected from cell populations ~28 hrs after transfection for western blot analysis of AXL protein expression: Lane 1 was from untransfected controls, and 3 replicates of siRNA-transfected cells are in lanes 2-7 (AXL siRNA in lanes 2, 4, and 6, and scramble siRNA in lanes 3, 5, and 7). **(d)** Autocorrelation analyses for actin, fibronectin, and microtubule anisotropy were performed on 15 independent PDMS wells for each of the 6 shapes that were studied per condition. AXL siRNA-transfected cells had similar anisotropy coefficients and anisotropic trends as scramble siRNA controls. * indicates $p < 0.05$ between cells in the two compared shapes. # indicates $p < 0.05$ for cells in that specific shape that were transfected with AXL siRNA, as compared with cells transfected with control siRNA. Bars represent standard error.

Figure 7.

Effects of cofilin-1 siRNA knockdown on anisotropy. WI-38 were transfected with 300 nM of cofilin-1 siRNA or 300 nM of a scrambled siRNA control and seeded into 3D PDMS wells ~52 hrs later. **(a)** Epifluorescence images of actin and fibronectin from WI-38 with siRNA-mediated cofilin-1 knockdown one day after plating in PDMS well bottoms. The same cells in each shape were labeled with phalloidin (i, iii) and anti-fibronectin antibody (ii, iv). **(b)** Protein lysates from each cell population were collected ~76 hrs after transfection for western blot analysis of cofilin-1 protein expression: Lanes 1 and 8 were from untransfected controls, and 3 replicates of siRNA-transfected cells are in lanes 2-7 (cofilin-1 siRNA in lanes 2, 4, and 6, and scrambled siRNA in lanes 3, 5, and 7). **(c)** Autocorrelation analyses for actin and fibronectin anisotropy were performed on 15 total PDMS wells for each shape. Cofilin-1 knockdown did not significantly change cellular anisotropy for each comparison with cells transfected with scramble siRNA in the corresponding shape. * indicates $p < 0.05$ between cells in the two compared shapes. Bars represent standard error.