Vascular tissue engineering: microtextured scaffold templates to control organization of vascular smooth muscle cells and extracellular matrix

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Abstract

The in vitro construction of tissue-engineered small diameter (<6 mm) blood vessels with sufficient strength and mechanical compliance has evaded researchers. We hypothesize that the high spatial organization of the medial layer of vascular smooth muscle cells (VSMCs) and their surrounding matrix provides high burst strength, compliance, and stability. We investigated the effect of micro-fabricated polydimethylsiloxane (PDMS) scaffolds with various groove widths on VSMC organization. We found that the presence of these grooved topographical cues significantly enhanced VSMC aspect ratio, alignment, and oriented remodeling of the underlying extracellular matrix. This study suggests that topographical patterning of tissue scaffolds can influence cellular and matrix spatial organization and could provide a framework for achieving the required organization and physical properties for blood vessels.

Keywords: Microtopographical cues; Vascular smooth muscle cell alignment; Vascular tissue engineering; Fibronectin

1. Introduction

The development of tissue-engineered vascular grafts is critical not only for vessel replacement therapies but could also serve as model in vitro platforms for drug screening. A major goal in vascular tissue engineering, in particular small diameter (<6 mm) vessels, is to recapitulate the in vivo function of native blood vessels. While there has been much progress towards creating functional small diameter blood vessels in vitro, the structural integrity and vasoactivity of current engineered vessels are not yet comparable to that of native vessels [1–4]. It is also important to note that tissue-engineered vessels often lack the unique structural and hierarchical organization of cells and extracellular matrix (ECM) found in native blood vessels [1].

The medial layer provides the main structural support of the vessel (i.e. strength, elasticity, and contractility) and consists of multiple layers of vascular smooth muscle cells (VSMCs) and ECM that are arranged in distinct spiral configurations [5] (Fig. 1A). While the significance of this spiral organization is unclear, it is interesting to note that the helical pitch varies between species as well as in vessels with different functions [5]. In addition, the organization and structure of the ECM proteins such as fibronectin (FN), elastin and collagen play an important role in controlling the structural integrity of tissue engineered blood vessels [3]. We therefore hypothesize that the spatial arrangement of VSMCs and the ECM plays a critical role in the function of the medial layer.

Several attempts to tissue-engineer vascular constructs that mimic various aspects of the in vivo cellular
and extracellular matrix arrangement have demonstrated a correlation between structural organization and function. For example, collagen fibers oriented circumferentially using a magnetic field exhibit enhanced mechanical properties compared to unoriented fibers [6]. Three-dimensional microstructures [7–10] have been used to provide topographical cues to control cellular organization for a number of different cell types [11,12], but have not yet been used to organize and investigate VSMCs.

In this study, we fabricate polydimethylsiloxane (PDMS) tissue scaffolds with grooved relief structures of varying dimension to assess the effectiveness of these topographical cues to elicit VSMC orientations similar to that seen in the herringbone helical arrangement. We directly compare cellular orientation, morphology, and organization of FN on textured and nontextured substrata.

2. Materials and methods

2.1. Microfabrication of microtextured PDMS scaffolds

To create topographical features in scaffolds, photolithography and soft lithography are used to create multiple parallel grooves in polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning). Groove width is varied (~20, 50, and 80 μm), and groove depth and spacing between grooves are kept constant at 5 and 12 μm, respectively. These groove widths were chosen because most cell types are known to orient along fibers with diameters in a fairly narrow range of 5–50 μm [10].

Polymer scaffolds are fabricated as described previously by Deutsch et al. [13] (Fig. 1B). Briefly, a positive resist layer (Shipley’s Microposit 1818) is spun onto a silicon wafer (University Wafers) and soft baked at 100°C. The resist is patterned by UV exposure through a patterned chrome mask using a contact mask aligner (Karl Suss MJB-3). After exposure, the patterned silicon wafer is developed (Shipley’s Microposit Developer diluted 1:1 with de-ionized water) and shaken continuously for approximately 35 s to remove the exposed areas of the 1818 layer from the wafer. This process creates an 1818 master that could be used several times to mold polymer tissue scaffolds. The pattern dimensions on the master are verified with a profilometer (Alpha-Step 500 KLA Tencor). Microtextured scaffolds are formed by pouring PDMS (10:1 prepolymer: curing agent) onto the master and curing for 2 h at 80°C. Control nontextured PDMS scaffolds are created by curing PDMS onto the surface of an unmodified silicon wafer. Pattern transference from the 1818 master to the PDMS scaffold is verified by optical microscopy (Olympus BX60, 20X).

2.2. Cell culture on PDMS scaffolds for cell alignment and morphological studies

To test the effects of microtopography on cell response, human umbilical smooth muscle cells (HUVE-112D, American Type Culture Collection (ATCC), Manassas, VA) are cultured on microtextured and nontextured PDMS scaffolds. The cell media used for all experiments is 90% Ham’s F-12K medium supplemented...
with 2 mM L-glutamine, and 1.5 g/L sodium bicarbonate (ATCC), 10% fetal bovine serum (FBS, Sigma, St. Louis, MO), 0.1 mg/mL heparin (Sigma), and 15 mg Endothelial Cell Growth Supplement (Sigma). Circular disks (3.8 cm²) of the PDMS scaffolds are autoclaved and coated with fibronectin (Sigma) at a plating concentration of 1.3 µg/cm² in 1X phosphate-buffered solution (PBS) for 1.5 h at 37 °C [14]. Cells (10⁵ cells/cm²) are seeded onto the fibronectin-coated PDMS scaffolds and are allowed to attach for approximately 24 h before unattached cells are removed by rinsing. After 5 days, the orientation and morphology, of the VSMCs are quantified.

### 2.3. Determination of VSMC orientation and morphology on PDMS scaffolds

To analyze VSMC orientation and morphology on the PDMS scaffolds, cells are fixed and stained. Cells on PDMS scaffolds are fixed with 3.7% formaldehyde in PBS for 15 min at room temperature with gentle shaking, followed by sequential rinses in PBS (3 × 4 min), cold acetone (−20 °C, 5 min), and PBS (3 × 2 min). F-actin is stained using rhodamine phalloidin (1:200 in PBS, 30 min; Molecular Probes, Eugene OR) and nuclei are stained with fluorescent nuclear stain Hoechst (1:1000 in PBS, 30 min; Molecular Probes).

The aspect ratios and orientation of the VSMCs on the various PDMS scaffolds are calculated to determine the effect of scaffold microtexture on VSMC response. Micrographs of the cultured VSMCs are taken for randomly chosen fields of view using an Olympus BX60 fluorescence microscope (20X) equipped with an Olympus digital camera. Images are then captured using Image Pro data acquisition software and approximately 15-30 cells are analyzed for each condition. VSMC aspect ratios are calculated by outlining each cell and using Image Pro software to calculate the length to width ratio of the cell. High aspect ratios indicate elongated cellular morphology, typical of VSMCs in vivo, while low aspect ratios indicate more rounded and abnormally shaped cells [15].

The degree of orientation of a single cell is assessed by calculating the percent alignment [16]. Briefly, a grid of parallel lines (with spacing between grid lines equivalent to the groove widths for microtextured scaffolds and ~35 µm for nontextured scaffolds) is placed over the image such that the lines of the grid are aligned with the long axis of the grooves of the scaffold (denoted as 0°). A second grid of parallel lines is then positioned perpendicular to the first grid (denoted as 90°). The percent alignment of the cell with respect to the microtextured surface of the tissue construct is then calculated as follows:

\[
\text{Alignment} = \frac{B - A}{B + A} \times 100
\]

where \(A\) is the number of intersections between the 0° grid lines and the cell edge, and \(B\) is the number of intersections between the 90° grid lines and cell edge.

### 2.4. Statistical analysis

Student’s \(t\)-tests are used to determine the statistical significance of the differences between results. A significance level of \(p < 0.05\) is considered significant.

### 2.5. Fibronectin and F-actin studies

VSMCs are grown under similar cell culture conditions as used in the morphology and alignment studies. Circular disks (1.75 cm²) of the PDMS scaffolds are sterilized under UV for 12 h and then coated with fibronectin (FN, Sigma) at a plating concentration of 1.3 µg/cm² in 1X PBS for 1.5 h at 37 °C [14]. Cells (5000 cells/cm²) are seeded onto the FN-coated PDMS scaffolds and are allowed to attach for approximately 24 h before unattached cells are removed. After 5 days, cells are stained for F-actin (as described above) and FN.

To stain for FN, VSMC-seeded scaffolds are first fixed with 4% glutaraldehyde for 10 min at room temperature and then incubated with potassium borohydride at (0.5 mg/ml) for 5 min. Cells are then permeabilized with 0.5% TritonX-100 for 15 min at room temperature, followed by three washes with 1% bovine serum albumin (BSA) solution in PBS and then a 30 min incubation in 1% BSA. Next, the samples are incubated with the primary antibody (1:200 anti-fibronectin mouse IgG, BD Biosciences) at room temperature. The substrates are washed with 1% BSA and then incubated with the secondary antibody (1:500 anti-mouse IgG-FITC, BD Bioscience) for 1.5 h and Hoechst stain (1:1000) for 10 min at room temperature. The samples are then washed in 1% BSA (3 × 5 min). The samples are imaged with a Zeiss Axiovert S100 Microscope equipped with fluorescence and a digital camera and images are processed using Metamorph Data Acquisition software (Universal Imaging).

### 3. Results

#### 3.1. Evaluation of tissue scaffold microtexture

The silicon master substrates characterized with a profilometer show that the microtextured feature sizes are within 0.3 µm of the mask designs. Optical microscopy further verifies the successful transfer of these textures to the PDMS scaffolds (Fig. 1B) with dimensions within 0.3 µm of the mask design. Measured dimensions (groove width X spacing between grooves X groove depth, in µm) of the 1818 masters are as follows: 18.33 ± 0.577 × 11 ± 1 × 4.7, 47.66 ± 0.577 × 12 × 4.7,
and $78.4 \pm 0.547 \times 11.2 \pm 0.477 \times 4.7$. The respective approximate measured dimensions of textured PDMS substrata are as follows: $19 \times 12 \times 5$, $48 \times 12 \times 5$, and $79 \times 12 \times 5$.

### 3.2. Fibronectin adsorption to microtextured scaffolds

Characterization of the FN coating using immunofluorescence shows that the substrata are evenly coated with FN. A representative fluorescence intensity line scan over a region spanning several ridges and grooves (Fig. 2A) shows that the FN coverage is uniformly distributed over the entire surface. We observe fluorescence only from the grooves when the focal plane is located at the groove surface (Fig. 2B), but uniform fluorescence over the entire surface is observed when the focal plane is on the ridge (Fig. 2B, boxed region). Similar uniform fluorescence is seen on nontextured PDMS and textured substrata regardless of groove width (data not shown).

### 3.3. Cell orientation on microtextured scaffolds

We observe a significant effect of scaffold microtopography on VSMC morphology (Fig. 3). After 5 days of culture, cells are randomly oriented on nontextured substrata (Fig. 3A). In contrast, on grooved substrata, we note that regardless of groove width, the cells are predominantly located in the grooves (Fig. 3B–D). The basis for preferential adhesion to grooves cannot be related to differences in protein adsorption, as we observe uniform coverage of FN (Fig. 2).

For all groove widths investigated, VSMCs are aligned with their long axis parallel to the micropattern. Quantification of VSMC alignment shows 95% alignment on microtextured substrata, compared to 15% on nontextured substrata (Fig. 3E). However, there are no statistically significant differences between the VSMC % alignment on the three types of microtextured substrata. There are, however, differences in the arrangement of multiple cells in a single groove: for the smallest groove width, only one cell spans the width of the groove (Fig. 3B), whereas in the larger groove widths (Fig. 3C–D) multiple cells can span the width of the groove.

### 3.4. Cell morphology on microtextured scaffolds

Similarly, we observe differences in cell morphology on nontextured and textured scaffolds. VSMCs on microtextured scaffolds are more elongated than on nontextured scaffolds (Fig. 3F). However, there are differences in the sensitivity of VSMC alignment and cell shape to groove widths in the range of $20–80\,\mu m$: Alignment does not change significantly with increased groove width, but cell shape does. For example, the aspect ratio for VSMCs on relatively small groove widths ($20$ and $50\,\mu m$) is $11.5$. However, as the groove width is increased to $80\,\mu m$, the aspect ratio drops to $6.9$ and further decreases to $4.3$ for nontextured scaffolds (Fig. 3F).

It has been previously suggested [17] that cells do not react to topographical features at high cell densities. On our microtextured scaffolds, VSMCs retain their elongated morphology and alignment even after reaching confluency (Fig. 4). Moreover, note that the cells are no longer confined to only the groove regions but are also attached to the ridges, thereby covering the entire scaffold. Cells on $50\,\mu m$ grooves (Fig. 4B) appear to be slightly more spread than on $20\,\mu m$ (Fig. 4A) grooves, but it is difficult to quantify the aspect ratio because of

![Fig. 2. Fluorescence intensity line scan of microtextured FN-coated PDMS scaffolds after 5-day exposure to serum-containing media. (A) A line scan along a 170\,µm path of the boxed-in image in (B) shows the average fluorescent intensity versus position. (B) Fluorescent image of a 48\,µm-grooved scaffold stained for FN. Image was taken with focus on the grooves (2). Ridges (1) appear dark because of the focal plane. Boxed-in image was taken of same scaffold area shown in (B) but with the focal plane on the ridge. Distinction between groove and ridges cannot be seen indicating even coverage of FN over scaffold as confirmed by the constant intensity value seen in the line scan (A). Line scan and fluorescent images are representative of all other pattern dimensions (including nontextured) studied. Scale bar represents 12\,µm.](image-url)
the high density of cells. In contrast to the textured substrata, cells seeded at a high density on nontextured substrata do not exhibit any ordering (Fig. 4C).

3.5. ECM reorganization on VSMC-scaffold constructs

Another difference between VSMCs on textured and nontextured substrates is the ability to reorganize FN. Regions of intense fluorescence staining of FN are observed on textured substrata but are absent on nontextured substrata (Fig. 5). These fibrillar structures of FN are most likely the result of FN (pre-adsorbed and cell-derived) reorganization. Moreover, the orientation of the FN fibrils on textured substrata is predominantly parallel to the grooves and the amount of fibril-like structures appears to decrease as the groove width...
increases. No FN fibrils are observed on nontextured substrata.

The reorganization of FN into fibrils is a cell-mediated process [18] because no fibrils are observed on FN-coated scaffolds incubated in serum-containing media without cells (Fig. 2B). Furthermore, cells appear to deposit ECM onto the scaffold and migrate away: There are regions where fluorescence microscopy (Fig. 5B and F; C and G black arrows) shows the presence of FN fibrils while brightfield microscopy shows no cells present. The FN fibrils are characterized by a consider-

ably higher fluorescence intensity compared to nonfibrillar FN (Fig. 6D). We also observe the formation of fibril-like networks bridging between cells in the same groove and between cells in adjacent grooves (Fig. 6). We observe less network formation and bridging as the groove width increases, and no FN fibrils or network are detected when VSMCs are on nontextured substrata.

3.6. F-actin organization of VSMCs on microtextured scaffolds

We observe distinct differences in the organization of F-actin in VSMCs cultured on textured and nontextured substrata. The F-actin filaments are highly aligned and parallel to the pattern on the smallest groove widths (Fig. 7A). As the groove width is increased to ~50 and ~80μm, some alignment with the grooves is still observed, but there is a clear decrease in the number of highly aligned fibers, whereas F-actin shows no preferred direction on the nontextured substrata (Fig. 7D).

4. Discussion

4.1. VSMC morphology and orientation can be controlled on microtextured scaffolds

From our studies, we show that scaffold microtopography is a very effective means of aligning VSMCs and
can potentially maintain the alignment even when the cells are confluent. There is a striking difference in VSMC alignment on textured compared to nontextured substrata, but variations in groove width from $\sim$20–80 $\mu$m do not significantly affect cell alignment. In contrast, the aspect ratio has a stronger dependence on groove width, decreasing from $\sim$12 to 7 as the width is increased from $\sim$50 to 80 $\mu$m. The aspect ratio of VSMCs on the smallest groove widths is comparable to that of primary contractile VSMCs freshly isolated in vivo.

Fig. 7. Alignment of F-actin in VSMCs on FN-coated, textured (A–C) and nontextured (D) PDMS scaffolds after 5 days in culture. Cells are stained for F-actin (stained red) and cell nuclei (stained blue). Ridges and grooves are denoted by (1) and (2), respectively. Groove widths are 19 (A), 48 (B), and 79 $\mu$m (C). F-actin filaments in (A) align with the long axis of the cell (arrows). Cells in (B) and (C) also have F-actin fibers aligned along the long axis of the cell (arrows) but unaligned F-actin fibers are present as well. F-actin in (D) is randomly distributed within the cell and filaments are in random orientations with respect to the long axis of the cell (arrows). Scale bar is 12 $\mu$m.

4.2. Tissue scaffold microtopography promotes FN assembly and organization

Groove structures have a notable effect on FN remodeling. In particular, VSMCs remodel FN into fibrils and deposit fibrillar FN on textured scaffolds regardless of groove width ($\sim$20, 50, 80 $\mu$m). Furthermore, networks of FN-rich structures are seen bridging between cells and over ridges creating physical connections between cells and the scaffold. The presence of fibrillar FN has important implications in the resulting elasticity of the ECM because it has been shown that fibrillar FN is required for the successful assembly of fibrillar collagen [19], a major component of blood vessels. Therefore, microtextured substrata provide a more effective platform than nontextured substrata for promoting a cohesive and dense tissue construct with potentially increased mechanical strength.

Another important cellular process thought to be regulated by FN fibril assembly is cell motility. The FN fibrils that we observe on textured surfaces appear to be aligned in the direction of the groove and can potentially provide directional cues for cell movement. These findings have also been demonstrated by Brock et al. [20], where cell motility was shown to be sensitive to the concentration, composition, and geometry of the ECM. Our results show that topographical cues can be used to organize cells and ECM which in turn could potentially also direct cell migration. These processes could work in concert as a feedback loop to generate a physiologically relevant structural organization of the cells and ECM that ultimately could be assembled into a tissue-engineered blood vessel.

4.3. Tissue scaffold microtopography promotes F-actin alignment

Actin, the main cytoskeletal protein, is important in maintaining structural integrity and cell shape. Studies have suggested that FN fibril assembly requires cell-generated contractile forces and an organized actin cytoskeleton [21]. Our findings are consistent with this hypothesis, i.e. FN fibrils and organized actin filaments are observed on textured substrates, and no FN fibrils and randomly-oriented actin filaments are found on nontextured substrates.

Others have reported co-localization of extracellular FN fibrils and actin filaments, indicating a strong correlation between cytoskeletal organization and FN fibril assembly [22]. These regions of FN fibrils and actin
may serve as sites for cell-generated contractile forces to propagate throughout the ECM [21,22]. On microtextured substrates, both actin filaments and fibrillar FN are aligned with the direction of the grooves. Our findings therefore suggest that the cells in textured substrates may be generating contractile forces along the direction of aligned actin filaments and FN fibrils. Moreover, specific microtopographical dimensions could directly impact tissue organization and pattern formation by controlling the direction of cytoskeletal-generated contractile forces.

5. Conclusions

Creating practical tissue-engineered small diameter blood vessels remains a major challenge. To date, no single approach has been able to recapitulate the full functional properties of these vessels. The strong correlations between structure, properties, and function in tissues suggest that full functional activity requires the development of scaffolds that mimic the distinct in vitro microstructural organization of cells and ECM in blood vessels.

In this study, we demonstrate that VSMC response and ECM remodeling can be controlled by tuning tissue scaffold microtopography, i.e. microgroove width. Though PDMS is an excellent model system to investigate the effect of microtexture dimensions on biological response, a functional tissue-engineered vessel will most likely be fabricated on a resorbable scaffold. However, the technique used in our studies for generating microtextured substrata is general and can be applied to form microtextured biodegradable polymer scaffolds such as poly-lactic acid. Our findings that fibrillar FN and actin filaments are oriented with the grooves are promising, but it will be important to determine whether cells and ECM maintain this oriented structure after the scaffold degrades. By manipulating the spatial arrangement of VSMCs and the ECM in an engineered blood vessel, we can test whether such structural organization can lead to the enhancement of the functional properties of the vessel.

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