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Spatial patterning of endothelium modulates cell morphology, adhesiveness and transcriptional signature

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Abstract

Microscale and nanoscale structures can spatially pattern endothelial cells (ECs) into parallel-aligned organization, mimicking their cellular alignment in blood vessels exposed to laminar shear stress. However, the effects of spatial patterning on the function and global transcriptome of ECs are incompletely characterized. We used both parallel-aligned micropatterned and nanopatterned biomaterials to evaluate the effects of spatial patterning on the phenotype of ECs, based on gene expression profiling, functional characterization of monocyte adhesion, and quantification of cellular morphology. We demonstrate that both micropatterned and aligned nanofibrillar biomaterials could effectively guide EC organization along the direction of the micropatterned channels or nanofibrils, respectively. The ability of ECs to sense spatial patterning cues were abrogated in the presence of cytoskeletal disruption agents. Moreover, both micropatterned and aligned nanofibrillar substrates promoted an athero-resistant EC phenotype by reducing endothelial adhesiveness for monocytes and platelets, as well as by downregulating the expression of adhesion proteins and chemokines. We further found that micropatterned ECs have a transcriptional signature that is unique from non-patterned ECs, as well as from ECs aligned by shear stress. These findings highlight the importance of spatial patterning cues in guiding EC organization and function, which may have clinical relevance in the development of vascular grafts that promote patency.

Introduction

Cardiovascular disease is the major cause of morbidity and mortality in the US [1]. In particular, atherosclerotic diseases can lead to plaque rupture, thrombosis and/or embolism,

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causing myocardial infarction and stroke. Atherosclerosis is a chronic inflammatory disease that begins with endothelial activation [2, 3]. Upon exposure to hypercholesterolemia or other conditions that cause atherosclerosis, endothelial cells (ECs) begin to express adhesion molecules and chemokines. Vascular cell adhesion molecule-1 (VCAM1) and intercellular adhesion molecule-1 (ICAM1) are adhesion proteins that facilitate the adhesion of leukocytes to the arterial wall [4, 5] and eventually contribute to the accumulation of atherosclerotic plaque. Monocyte chemoattractant protein-1 (MCP1) is a chemokine that is associated with the formation of atherosclerotic lesions [6].

The spatial distribution of atherosclerotic lesions strongly correlates with endothelial morphology. ECs aligned along a straight segment of an artery appear to be resistant to vascular inflammation, whereas the non-aligned endothelial cells at a branch or bifurcation express biological properties that promote atherosclerosis [7]. The expression level of the pro-atherosclerotic molecules and the location of vascular lesions are often found in the sites of the vessel where the geometry is branched or curved [2]. At bends, branches and bifurcations, disturbed flow predominates, and the ECs are not aligned. We speculate that the biology of aligned ECs, possibly mediated by integrin-extracellular matrix (ECM) signaling, might be atheroprotective.

Interactions between the cell and the ECM are critical to cell phenotype and function, and should be considered in the design of medical devices [8]. Grooves, ridges, steps, pores, wells, and nodes patterned into substrates modify cellular response in fibroblasts, neurons, epithelial cells, macrophages, endothelial cells, and smooth muscle cells [9-19]. Micropatterning influences both the morphology and migration of ECs [20-26]. However, prior studies of the effect of patterned substrates on EC morphology have provided only limited information about function, and little is known about effects on EC adhesiveness, a key property regulating inflammation and thrombosis. [2]. In addition, these initial studies have been limited to patterned topography at the micron length scale, due to resolution limitations of the patterning techniques. Nanoscale features can be more biologically relevant to investigate than topography in the micron range since the cells interact with many features (pores, fibers, ridges) of the native ECM, which is in the nanometer scale [8]. A thorough understanding of cell-matrix interactions, specifically at the nanoscale, would significantly contribute to the design of biomaterials that provide inductive signaling cues to ECs.

We hypothesized that matrix-mediated parallel alignment of ECs may promote an atheroprotective phenotype by modulating cell morphology, adhesiveness, and transcriptome. To test this hypothesis, we used a micropatterned polydimethylsiloxane (PDMS) substrate to evaluate the effects of parallel-aligned microchannels on the phenotype and function of ECs, based on gene expression profiling of adhesion molecules, functional characterization of monocyte and platelet adhesion, and quantification of cellular alignment. We further extended this work to investigate the phenotype of ECs on oriented nanofibrillar collagen, which is a naturally-derived and biodegradable ECM.

Materials and Methods

Micropatterned substrates

Polydimethylsiloxane (PDMS) microgrooves were fabricated using soft lithography techniques [27] with some modifications. A 10- μ m thick layer of a negative photoresist (SU-8 3010, Microchem) was spin-coated onto silicon wafers (mechanical grade, University Wafer) for 30 seconds at 3500 rpm and UV-exposed for 8.5 seconds at 20 mW/cm² (OAI, light source centered at 365 nm) through a transparency mask (FineLine Imaging) and long pass UV filter (PL- 360-LP Omega Optical). The AutoCAD generated transparency mask

contained microgroove patterns of 30 μm width and spacing. Previous to UV exposure, SU-8 was baked on a hotplate (ramp from 65 to 95 $^{\circ}\text{C}$ and held for 6.5 minutes). Before development, SU-8 was submitted to post exposure baking (ramp from 65 to 95 $^{\circ}\text{C}$ and held for 2.5 minutes). After development of cured SU-8 features for 4.5 minutes in liquid with mild agitation (SU-8 Developer), N_2 gas dried wafers were silanized in a trimethylchlorosilane (Aldrich) atmosphere for 3 hours. PDMS (Sylgard 184, Dow Corning) prepolymer and curing agent were mixed 10:1 (Thinky), cast on the SU-8 microstructures, cured for 24 hours at 70 $^{\circ}\text{C}$, and then peeled from the SU-8 master.

To facilitate mold release, the cured PDMS-184 micropattern molds were O_2 plasma treated with 66.6 Pa and 80 W in an oxygen plasma asher (Branson/IPC-S3003) for 15 seconds, and then silanized as above described for 24 hours. PDMS-based micropatterned cell-culture substrates were fabricated by casting mixed 10:1 PDMS-182 (Sylgard 182, Dow Corning) [28] and degassed before curing at 70 $^{\circ}\text{C}$ for 24 h. The obtained micropatterned PDMS substrates had microchannel dimensions of 30 μm width, 30 μm spacing and 10 μm in depth (Fig. S1). The micropatterned substrates were plasma treated as above immediately before incubating with rat tail collagen type I (0.35 mg/ml, BD Biosciences) before seeding cells.

Fabrication of aligned collagen strips

Strips of anisotropic nanofibrillar collagen matrices were produced by a syringe needle extrusion-based fibrillogenesis method, modified from Lai *et al.* [29]. Briefly, high concentration rat tail collagen I was dialyzed to 30 mg/mL using polyethylene glycol (Fluka) at 4 $^{\circ}\text{C}$ using semi-permeable cellulose dialysis tubing (Fisher). To create anisotropic collagen, monomeric collagen was extruded in the form of a strip at a velocity 3.2 mL/min from a syringe into a 10X phosphate buffered saline (PBS), pH9 PBS buffer at 37 $^{\circ}\text{C}$ to induce fibrillogenesis along the direction of extrusion. The anisotropic collagen strips were then manually deposited onto a glass slide. Strips of non-aligned collagen fibrils, or the randomly oriented control, were produced by extruding at a slow rate of 0.02 mL/min to produce strips of random collagen, with similar thickness and width as the aligned collagen strips. The collagen then completely dried under a laminar flow hood for 12 hours and was then rinsed several times with MilliQ water.

Scanning Electron Microscopy (SEM)

Samples were processed for routine SEM, according to previous publications, using a Hitachi S-3400N VP scanning electron microscope [30].

***In vitro* culture experiments**

Primary human dermal microvascular endothelial cells (ECs) were grown in EGM-2 MV (Lonza) media, supplemented with EGM-2 MV SingleQuot kit (Lonza), at 37 $^{\circ}\text{C}$ and 5% CO_2 . The micropatterned PDMS and collagen substrates were sterilized in 70% ethanol for ten minutes and then washed with PBS before cell seeding. ECs were plated at a seeding density of 23,000-28,000 cells/ cm^2 and incubated for 24 hours on the substrates. The ECs were used below passage 14.

Where specified, cytoskeletal inhibitors were used to evaluate the role of actin or microtubules on cellular elongation and alignment. Immediately after plating the ECs on the collagen substrates, the ECs were incubated for 24 hours with cytoskeletal inhibitors cytochalasin D (100 nM, Sigma) or nocodazole (1 μM , Sigma), which disrupt the actin and microtubule networks, respectively (n=3).

Immunofluorescence staining

At indicated time points, samples were fixed in 4% paraformaldehyde (Alfa Aesar) and immunofluorescently stained for antibodies against Ki67 (Dako Cytomation), paxillin (BD Transduction Labs), focal adhesion kinase (FAK, BD Transduction Labs), and β -tubulin (Sigma), based on previous protocols [29, 31]. In addition, phalloidin (Invitrogen) and Hoechst33342 dye (Invitrogen) were used to visualize the F-actin and nuclei, respectively.

Analysis of cellular alignment

Cell alignment and shape were analyzed using ImageJ to conduct measurements, as previously described (n=3 micropatterned substrates; n=6 collagen nanofibrillar substrates) [29]. The angle of orientation was defined as the angle formed by the cell's principal axis, relative to the direction of the substrate (i.e. microchannels or nanofibrillar collagen). A minimum angle of 0° denoted parallel alignment from the substrate's axis and a maximum of 90° suggested perpendicular alignment. For quantification of cells on the random control, an arbitrary axis was selected.

We further validated the cell alignment analysis by quantifying the order parameter (S), where $S = \langle (3\cos^2\theta - 1)/2 \rangle$, and θ is the angle of alignment (n=3 microchannels; n=6 nanofibrillar collagen) [29]. A perfectly aligned distribution where θ is 0°, relative to the substrate axis, would indicate $S=1$. The cell shape index (CSI) provided an indicator of cell morphology by quantifying the extent of elongation in the cellular shape, where $CSI = (4\pi * \text{Area}) / (\text{Perimeter}^2)$ [19, 29]. A CSI value of 1 approximates the shape of a circle and a value of 0 depicts that of a straight line.

Monocyte adhesion and platelet adhesion assays

Confluent ECs on the PDMS or collagen substrates were stimulated with tumor necrosis factor- α (TNF- α , Sigma) at 2.5ng/mL for seven hours. Human monocytes (U937, ATCC) were fluorescently labeled with Cell Tracker Red and then allowed to adhere onto the substrates for 45 minutes with gentle rocking to promote even cell distribution. After 45 minutes, the unbound monocytes were washed away by rinsing twice with PBS before quantifying the number of bound monocytes. Cells cultured on aligned or random substrates were imaged in five representative fields, and the remaining monocytes in each image were quantified (n = 3). The level of monocyte adhesion was expressed as a relative fold change relative to the control, non-patterned substrate or randomly aligned collagen.

Similar experiments were performed using primary human apheresis platelets (Stanford Blood Center) to compare platelet adhesion onto ECs cultured on aligned or randomly oriented nanofibrillar collagen. The platelets were exposed to TNF-treated ECs on either aligned or randomly oriented collagen. After 30 minutes, the samples were fixed in paraformaldehyde and immunofluorescently stained for platelet marker CD41 (Abcam). Analysis was performed similar to the monocyte adhesion assay, taking images in five representative fields (n=6). The bound platelets were quantified and expressed as a relative fold change with respect to platelets on randomly aligned collagen.

Gene Expression Analysis

The ECs on the micropatterned or control PDMS substrates were lysed with Trizol (Invitrogen), followed by RNA isolation using the RNEasy kit (Qiagen). For DNA microarray analysis, RNA quality was verified by Agilent Bioanalyzer, and then the cDNA was synthesized and labeling according based on previous publications [32]. The samples were hybridized to Affymetrix Human Gene 1.0 ST GeneChips and then scanned on an Affymetrix 3000 scanner according to standard protocols (n=2). Fluorescence intensities

were normalized according to the manufacturer's instructions and are available in the Gene Expression Omnibus (GEO) database (accession no. GSE42596).

Gene expression on both the micropatterned substrates and nanofibrillar collagen was also assessed by quantitative reverse transcriptase-PCR (qRT-PCR) using Taqman primer sets (Applied Biosystems) on a 7300 Real-Time PCR system (Applied Biosystems), based on previous methods [33]. The $\Delta\Delta C_t$ method was used to evaluate the data, which was normalized to the 18S gene (n=4) [33].

DNA Microarray Bioinformatics

Expression data from control non-patterned ECs and micropatterned ECs was analyzed using the Significance Analysis of Microarrays (SAM) module in R [34] to calculate a D-score used to rank the most differentially expressed genes between groups, along with the corresponding q value for significance using false discovery rate (FDR) correction for multiple testing, using a cutoff value of $q < 0.05$. The top 100 up-regulated and top 100 down-regulated genes as ranked by D-score were then further curated, and hierarchical clustering of these genes using expression data was used to create a heatmap.

For gene ontology (GO) analysis, expression data was also analyzed using Gene Set Enrichment Analysis [35] to perform gene ontology enrichment analysis on the differentially upregulated and downregulated gene sets. GO categories meeting the cutoff of the more stringent familywise-error rate (FWER) p-value of at least 0.05 were considered significantly enriched.

The significantly up-regulated and down-regulated genes, as determined by the SAM algorithm as described above using a cutoff value of $q < 0.05$, were then compared to significantly upregulated and downregulated genes from a similar analysis of samples from GEO dataset GSE8852, using the subset of samples of lymphatic endothelial cells who underwent shear stress at 20 dyn/cm² vs. control (static), and GEO dataset GSE16706, using the subset of samples of human umbilical vein endothelial cells (HUVEC) exposed to 15 dyn/cm² of shear stress vs. control (static). The number of overlapping genes in the upregulated and downregulated gene sets from each dataset was then computed and the results used to create the Venn diagrams.

Statistical Analysis

All data are shown as mean $\pm$ standard deviation. For quantification of cell alignment, CSI, and order parameter analysis, phalloidin-stained samples were imaged in five representative fields (20X) and 25 cells in each field were evaluated (n = 3). Monocyte and platelet adhesion were quantified out of at least five representative fields (10X) and expressed as relative fold change. Statistical comparisons were carried out by a paired t-test. For quantitative PCR analysis, the level of gene expression was expressed as normalized relative fold change relative to the control substrate (n=4). Statistical significance was accepted at $P < 0.05$.

Results

EC morphology on micropatterned substrates

ECs were cultured on micropatterned channels to evaluate the effect of parallel aligned microgrooves on EC morphology by topographical confinement (Fig. 1A-C, Fig. S1). Non-patterned PDMS was used as the randomly oriented control. When the ECs were cultured on the control substrates, the cells demonstrated a random orientation and maintained a circular, cobblestone-like morphology, as demonstrated by both SEM and phalloidin stains (Fig. 1A,

Fig. S1). In contrast, the cells cultured on micropatterned substrates elongated their cytoskeleton along the direction of the grooved channels (Fig. 1A).

In addition to regulating the EC cytoskeleton, the micropatterned substrate also regulated the organization of the focal adhesion proteins, as shown by immunofluorescence staining of the punctate spots of FAK and paxillin (Fig. 1B-C). On the micropatterned substrates, FAK appeared to be localized at the front and back ends of the ECs, and punctate paxillin clusters often organized in clusters along the direction of the microchannels. In contrast, on the control PDMS substrates, the ECs adopted a random distribution of FAK and paxillin.

To quantify the differences in cellular morphology, we measured the average angle of orientation of the ECs, where cells parallel to the micropatterned substrate had a value of 0° and cells perpendicular to the micropatterned channels had a value of 90° . The cells on the micropatterned channels had a lower angle of orientation than the ECs cultured on the control ($7.9 \pm 0.4^\circ$ vs. $49.0 \pm 2.6^\circ$; $P < 0.0001$), indicating the micropatterned cells aligned along the direction of the grooved channels (Fig. 1D). In addition, the angle of orientation for micropatterned cells correlated to a higher value of order parameter (0.96 ± 0.01 vs. 0.17 ± 0.05 ; $P < 0.0001$), further confirming that the patterned geometries led to a higher degree of anisotropy in the cell orientation (Fig. 1E). Aligned ECs on the micropatterned substrates also had a lower CSI value (0.48 ± 0.03 vs. 0.60 ± 0.01 ; $P < 0.01$), suggesting the patterned ECs demonstrated a more elongated and polarized morphology than ECs on the control substrate. Therefore, the analysis of cellular alignment demonstrated that the anisotropy of the micropatterned channels directed cellular elongation, actin cytoskeleton, and focal adhesion assembly.

EC adhesiveness on micropatterned substrates

Since elongated ECs that are aligned along the direction of blood flow in straight vessel segments have a characteristic athero-resistant phenotype, we examined whether micropatterning also conferred an athero-resistant phenotype. A monocyte adhesion assay was also used to characterize the biological function of the micropatterned-ECs, since monocyte adhesion is an accepted surrogate for the inflammatory process linked to atherosclerosis [6, 36, 37]. We observed that the endothelium grown on micropatterned substrates had 49% fewer monocytes adhered, relative to the endothelium grown on control PDMS (Fig. 2 A-B, $P < 0.0001$).

To test the hypothesis that the reduction in monocyte adhesion was mediated by a reduction in the expression of adhesion molecules, we examined the expression level of genes known to have roles in the adhesiveness of ECs. As shown in Fig. 2C, ICAM1 gene expression was reduced by 50% ($P < 0.0075$) in micropatterning-aligned cells, where the expression levels in VCAM1, MCP1, and eNOS were not significantly different between the cells on micropatterned or control PDMS. A reduced expression of ICAM1 is consistent with an athero-resistant state of the ECs [5].

EC morphology on anisotropic nanofibrillar collagen

Since PDMS is not a suitable material for *in vivo* transplantation, we explored the utility of using collagen I, a biodegradable ECM, to align the ECs. We modified our previously reported extrusion technique [29], so as to reduce the fibril diameters to 31 ± 2 nm (from 45 ± 3 nm) which increased EC alignment along the nanofibril direction (Fig. 3A). Anisotropic nanofibrillar collagen induced EC alignment and elongation, similar to what was observed on the micropatterned PDMS, but in the absence of cellular confinement associated with micropatterned channels. On the anisotropic nanofibrillar collagen substrates, the ECs developed organized stress fiber assembly along the direction of the nanofibrils (Fig. 3B). In

addition, the matrix-aligned ECs demonstrated organized focal adhesions in which paxillin and FAK were polarized along the front and back ends of the matrix-aligned cells, in contrast to the uniformly distributed focal adhesions of ECs on randomly oriented collagen (Fig. 3C-D). We further quantified our observations of cellular alignment by demonstrating an angle of orientation of $12.7 \pm 1.8^\circ$ for aligned collagen (vs. $40.9 \pm 2.3^\circ$ for random collagen; $P < 0.0001$) and an order parameter of 0.90 ± 0.03 (vs. 0.34 ± 0.05 for random collagen; $P < 0.0001$) (Fig. 3E-F). As expected, the ECs incubated on the aligned collagen had a lower CSI, suggesting a more elongated cell shape (0.59 ± 0.05 vs. 0.67 ± 0.05 for random collagen; $P < 0.05$, Fig. 3G). Together, these results suggest that oriented nanofibrillar collagen regulated EC organization, as demonstrated by cellular alignment, cell shape, and focal adhesions.

Since cellular alignment was accompanied by organized stress fiber assembly, we used cytoskeletal inhibitors to target the actin cytoskeleton and microtubules and test whether these agents disrupted cell elongation and alignment on anisotropic collagen. The inhibited polymerization of either actin (cytochalasin D) or microtubules (nocodazole) significantly impaired the ability of the ECs to align along the collagen fibrils. The addition of either cytoskeletal inhibitor led to a higher angle of orientation, a lower order parameter, and a higher CSI for ECs on aligned collagen (Fig. 4A-C). Cytochalasin D interfered with the organization of the actin cytoskeleton, resulting in a higher angle of orientation ($28.1 \pm 2.9^\circ$ vs. $10.1 \pm 1.9^\circ$ control; $P < 0.01$) and lower order parameter (0.60 ± 0.05 vs. 0.93 ± 0.02 control; $P < 0.01$) when compared to the non-treated control on aligned collagen (Fig. 4B). The ECs on aligned collagen were also less elongated when treated with the actin inhibitor, as suggested by the higher CSI (0.66 ± 0.04 vs. 0.55 ± 0.03 control; $P < 0.05$) (Fig. 4C). Similarly, nocodazole disrupted the microtubule networks, leading to increased orientation angle ($29.3 \pm 6.3^\circ$ vs. $10.1 \pm 1.9^\circ$ control; $P < 0.05$), decreased order parameter (0.58 ± 0.13 vs. 0.93 ± 0.02 control; $P < 0.05$), and increased CSI (0.75 ± 0.01 vs. 0.55 ± 0.03 control; $P < 0.01$), compared to the non-treated control (Fig. 4B-C). Together, the disruption in alignment and cellular elongation in response to the cytoskeletal inhibitors suggested that both actin and microtubules play important roles in EC organization on aligned nanofibrillar collagen.

EC adhesiveness on anisotropic collagen nanofibrils

To determine if matrix anisotropy of the collagen fibrils could modulate EC function, we quantified monocyte adhesion to ECs grown on aligned or randomly oriented collagen nanofibrils. ECs on the aligned collagen matrices manifested a 30% reduction in monocyte adhesion ($P < 0.05$, Fig. 5A-B). We also and also observed a 50% reduction in platelet adhesion on the aligned collagen substrate ($P < 0.05$, Fig. 5A,C). To further verify the role of adhesion molecules and chemokines in mediating the reduction of platelet adhesion, we quantified the gene expression levels of ECs on aligned or randomly oriented collagen. As expected, ICAM1 was reduced by 24%, while MCP1 decreased by 18% when cells were aligned by a collagen matrix ($P < 0.05$, Fig. 5D). However, there were no statistically significant differences in expression for eNOS and VCAM1 between cells on randomly oriented and aligned collagen. Together these results suggested that matrix anisotropy may be promoting an athero-resistant phenotype in matrix-aligned ECs, correlating with less inflammatory signals and reduced EC adhesiveness.

Transcriptional profiling of ECs on micropatterned substrates

We further investigated whether ECs aligned by spatial patterning have a distinctive transcriptome signature by performing global gene expression analysis of ECs cultured on either micropatterned or non-patterned PDMS substrates. In a microarray representing over 28,000 well-annotated genes, 905 genes were significantly up-regulated in the

micropatterned cells, and 3,303 genes were significantly down-regulated in the micropatterned samples. Shown in Fig. 6A is a heat map of the top 100 most upregulated and 100 most downregulated genes, and the full list can be found in Tables S1 & S2. Transcriptional profiling confirmed our quantitative PCR results, revealing a 30% reduction in ICAM1 expression on micropatterned ECs, when compared to ECs on non-patterned substrates.

Having identified a set of genes that are differentially expressed between micropatterned and non-patterned ECs, we investigated the potential functional responses of these genes by classifying them according to GO enrichment analysis (Table S3). Among the upregulated categories include RNA processing, DNA repair, and DNA replication, whereas downregulated categories include G protein coupled receptor activity, ion channel activity, and transmembrane receptor activity. The GO enrichment analysis suggests that spatial patterning of ECs modulates a wide range of molecular processes.

Among the most differentially upregulated genes in ECs on the micropatterned substrate were matrix metalloproteinase (MMP)-1 and MMP16, which are involved in the degradation of ECMs, with a 60% enhancement in expression in the micropatterned EC samples (Table S1). Heat shock 27kDa protein family, member 7 (HSPB7), a molecular chaperone that modulates F-actin assembly [38], was downregulated by 40% in micropatterned samples (Table S2). MicroRNAs (miR)-222, miR186, miR21, and miR29a also showed significant upregulation on the micropatterned ECs. Whereas these miRNAs have been implicated in cancer, myocardial infarction, and thoracic aneurysms, the role of these miRNAs in endothelial alignment and function warrants further exploration [39-41]. In addition, we identified a large number of zinc finger proteins and small nuclear RNAs that are differentially expressed, but their role in matrix-mediated EC patterning is currently unknown.

Since laminar shear stress is also known to orient ECs along the direction of blood flow, we sought to determine whether the transcriptional signature of spatially patterned ECs bear resemblance to that of shear stress-aligned ECs. We therefore compared the differentially expressed genes from our micropatterning studies to the genes that were differentially expressed upon stimulation with shear stress. We used datasets from the GEO public functional genomics data repository, consisting of HUVEC (GSE16706) exposed to either shear stress or static (control) conditions. A similar analysis was performed to compare the differentially expressed genes in lymphatic ECs that were subjected to either shear stress or control (static) conditions (GSE8852). By comparing the differentially expressed genes due to spatial patterning or those modulated by laminar shear stress, we found surprisingly only a small overlap (Fig. 6B-C, Table S4 & S5). Between micropatterned ECs and shear-aligned HUVEC, there was an overlap of 33 upregulated genes and 69 downregulated genes. Between micropatterned ECs and shear-aligned lymphatic ECs, there were 6 common up-regulated genes and 20 downregulated genes. In particular, two up-regulated genes were common to micropatterned ECs, shear-induced lymphatic ECs, and shear-induced HUVEC. One of the genes was DNAJB9, an inducible member of the DnaJ protein family that protects cells from apoptosis in the presence of endoplasmic reticulum stress [42, 43]. The other gene was prostaglandin-endoperoxide synthase 2 (PTGS2/COX2), an inducible isoform of prostaglandin G/H synthase that is known to be upregulated in ECs as a result of shear stress [44, 45]. The common upregulation of DNAJB9 and PTGS2 suggests that matrix-mediated EC alignment recapitulates, in part, some functions of shear-mediated EC alignment in stimulating prostaglandin synthesis and in promoting cell viability.

Discussion

The salient findings of this work are that 1) matrix anisotropy is capable of directing the organization of the cytoskeleton and focal adhesions in ECs (Figs. 1, 3, 4); and 2) matrix anisotropy could regulate the biological function of ECs, as demonstrated by monocyte and platelet adhesion and gene expression (Figs. 2, 5, & 6). The effect of matrix anisotropy and ECs were investigated with two different platforms, parallel-aligned micropatterned PDMS and parallel-aligned nanofibrillar collagen. The micropatterned PDMS substrates provided a highly reproducible platform to regulate the morphology, transcriptional signature, and function of ECs. The analysis of cellular response on micropatterned substrates provides a foundation for the development of a more biologically-relevant scaffold to regulate EC behavior. The matrices of aligned collagen represent a tissue-engineered scaffold that can favorably influence endothelial response at the nanoscale, which is the length scale of native ECM structures.

More recently, researchers have been able to investigate how cells react to nanoscale structures due to the improvements with nanopatterning technologies. In general, however, nanopatterning research has been limited by the high expense and lengthy time requirements commonly associated with the large-scale production of nanofabrication techniques [46]. Ultimately, a simple, affordable, and straightforward technique that produces nanopatterned substrates is a highly desirable approach to investigate cell-matrix interactions. In this study, uniformly aligned nanofibrils of collagen were fabricated using a facile flow processing technique. Furthermore, the versatility of this fabrication technique has already been demonstrated in its ability to also produce three-dimensional conduits of aligned collagen with physiologically relevant strength for vascular tissue engineering applications[29].

The cytoskeleton is integrally involved in cell movement and organization. When treated with either cytoskeletal inhibitor, cellular alignment and elongation of ECs on the aligned nanofibrillar collagen was disrupted, as indicated by an increase in orientation angle, a decrease in order parameter, and an increase in CSI (Fig. 4). This trend has been similarly observed by Malek and Izumo [47]. Vartanian *et al.* further observed that nocodazole completely prevented the elongation of micropatterned ECs after one hour of treatment and reversed EC elongation after 24 hours [48]. The confluency of nocodazole-treated ECs decreased after the 24 hour incubation period in this study, however, suggesting that the microtubule inhibitor might have been slightly cytotoxic. The increase in the orientation angle and CSI for both cytochalasin D and nocodazole-treated ECs indicate that both actin filaments and microtubules play an important role in the organization of the EC cytoskeleton. The disruption of either actin or microtubule networks severely hinders the cell's ability to align or elongate in response to an anisotropic matrix.

The ability of matrix anisotropy to promote contact guidance is not completely understood, but the process may require cell surface integrin receptors. As heterodimeric integrins attach to matrix proteins, the integrin receptors aggregate together and attract cytoplasmic proteins to form focal contacts [46]. Integrin activation can lead to the activation of signaling pathways that modulate various aspects of cellular function [49]. In addition, matrix anisotropy can alter the conformation of cryptic molecular recognition sites such that they become accessible for cell binding [50]. Together, this evidence suggests a rational explanation for matrix-mediated changes in cellular morphology and function.

Vascular inflammation in ECs may be initiated by cytokine stimulation or metabolic perturbations, in association with an increase in the expression level of several cell adhesion molecules. VCAM1 and ICAM1 permit leukocytes to attach and adhere to the arterial wall, which is an initiating step in the development of vascular lesions [2]. The downregulation of

the adhesion molecules and MCP1 confers athero-resistance [51, 52]. The expression of these pro-atherosclerotic molecules and the location of fatty plaque are often found in the sites of the vessel of disturbed flow, i.e. bends, branches, and bifurcations [53]. At these sites, there is an early disturbance of normal endothelial functions, which is correlated with the development of atherosclerotic arterial disease [7]. Therefore, the ability to regulate these inflammatory genes, such as through substrate topography, might help in the prevention of vascular inflammation and/or thrombosis.

In addition to regulating EC morphology, both the micropatterned channels and aligned nanofibrillar collagen modulated EC adhesiveness and reduced the expression of ICAM1. Although the association of shear stress with EC function and biology is well established, there is less known as to how ECM patterning may effect EC adhesiveness. The anisotropic geometries, therefore, become useful tools to evaluate how morphology and function correlate in ECs, by regulating EC morphology in the absence of shear stress. We demonstrate that matrix-aligned ECs exhibit reduced monocyte and platelet adhesion, when compared to ECs cultured on randomly oriented collagen fibrils. The decreased level of monocytes or platelets adhered to matrix-aligned ECs strongly suggests that the matrix anisotropy induces not only an aligned cell morphology but also promotes an athero-resistant phenotype. It is possible that the reduction in monocyte adhesion correlates to changes in binding strength of the monocytes to surface ligands on the ECs. The regulation of gene expression in the adhesion molecules can lead to changes in either affinity, binding to an individual surface ligand, or avidity, aggregation of surface ligands to increase binding strength [2].

Gene expression profiling revealed a large number of differentially expressed genes, when comparing the ECs cultured on micropatterned substrates to non-patterned substrates. These genes included not only those related to inflammation such as ICAM1, but also genes that had not previously been associated with shear or cellular patterning (Tables S1-S3, Figure 6). The gene expression profiling reveals global transcriptional changes that accompany EC alignment on micropatterned substrates. Although micropatterned ECs share some similarities in differential gene expression with ECs aligned by shear stress, the distinctive population of non-overlapping genes suggests a unique signaling mechanism of matrix-mediated cellular alignment and function (Tables S4-S5, Figure 6). This finding sheds light on the differential roles of shear stress and matrix-mediated cell patterning in modulating EC behavior and function.

Conclusion

Micropatterned substrates and anisotropic nanofibrillar collagen provide useful platforms to examine the matrix-induced responses of ECs. The patterned topography was able to organize the EC cytoskeleton and focal adhesions and promote an athero-resistant phenotype. The alignment of ECs, induced by either the micropatterned channels or the aligned collagen fibrils, also correlated with the reduced adhesion of monocytes and platelets, as well as the regulation of inflammation-related genes. Transcriptional profiling revealed that micropatterned ECs possess a unique transcriptome signature, compared to non-patterned ECs, that shares some overlap with shear-aligned ECs. Therefore, we anticipate that spatially patterned substrates that guide EC morphology and function will have therapeutic applications in the development of vascular grafts that inhibit aggregation of platelets and monocytes, thus providing an approach to improve graft patency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Micropatterned ECs have spatially oriented alignment and morphology

A. ECs organize their F-actin assembly along the direction of the microgrooves. Comparative immunofluorescence staining of FAK (B) and paxillin (C) on micropatterned or non-patterned control PDMS substrates. Corresponding F-actin staining are shown for reference. D-E. Quantification of cellular alignment by the angle of orientation (D) and order parameter (E). F. Quantification of cellular elongation by the cell shape index. Arrows indicate direction of microgrooves. Scale bar: 100 μm (A); 50 μm (B-C). ** $P < 0.0001$ (n=3), * $P < 0.01$ (n=3)

Fig. 2. Effect of micropatterning on endothelial adhesiveness

A. Shown are immunofluorescently labeled monocytes adhered onto micropatterned ECs. B. Quantification of monocyte adhesion (** $P < 0.0001$, $n=6$). C. Gene expression of adhesion-related markers and chemokines (** $P < 0.0075$, $n=4$). Arrow denotes direction of microgrooves. Scale bar: 250 μm .

Fig. 3. Aligned nanofibrillar collagen modulates EC alignment and morphology

A. SEM of randomly oriented and aligned collagen. B. ECs organize their F-actin assembly along the direction of the aligned nanofibrils. Comparative immunofluorescence staining of FAK (C) and paxillin (D) on randomly oriented and aligned collagen. Quantification of EC alignment based on the angle of orientation (E) and order parameter (F). Quantification of cell elongation based on the cell shape index (G). Arrows indicate direction of nanofibrils. Scale bar: 600 nm (A); 100 μ m (B); 50 μ m (C-D). ** $P < 0.0001$ ($n=6$)

Fig. 4. Disruption of cytoskeletal proteins abrogate nanofibrillar collagen-mediated EC alignment and elongation

The addition of cytochalasin D (cyto D) or nocodazole (noco) inhibited F-actin assembly on aligned collagen substrates, based on immunofluorescence staining of F-actin or microtubules (A). Quantification of the angle of orientation (B) and cell shape index (C) in the presence of cytoskeleton disruption agents. Arrow denotes orientation of nanofibrils. Scale bar: 100 μ m (A). ** $P < 0.01$ ($n = 3$), * $P < 0.05$ ($n = 3$)

Fig 5. Effect of aligned nanofibrillar collagen on endothelial adhesiveness

A. Shown are immunofluorescently labeled monocytes or platelets adhered onto aligned nanofibrillar ECs. B. Quantification of monocyte adhesion (* $P < 0.05$, $n = 4$). C. Quantification of platelet adhesion (* $P < 0.05$, $n = 6$); D. Gene expression of adhesion related markers and chemokines (** $P < 0.01$, * $P < 0.05$, $n = 4$). Arrow denotes direction of nanofibrils. Scale bar: 250 μm .

Fig 6. Transcriptional profiling of micropatterning-induced EC alignment

A. Heat map and clustering analysis of the top 100 upregulated and downregulated genes. Red indicates $\log_2$ intensity >0 and Green indicates $\log_2$ intensity <0 . All Array data available from GEO (accession number GSE42596). B-C. Venn diagrams depict significantly upregulated (B) and downregulated (C) genes in common, when comparing ECs aligned by either micropatterning or shear stress.