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Extracellular matrix production and regulation in micropatterned endothelial cells

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ABSTRACT

Production and maintenance of extracellular matrix (ECM) is an essential aspect of endothelial cell (EC) function. ECM surfaces composed of collagen type IV and laminin support an atheroprotective endothelium, while fibronectin may encourage an atheroprone endothelium through inflammation or wound repair signaling. ECs maintain this underlying structure through regulation of protein production and degradation, yet the role of cytoskeletal alignment on this regulation is unknown. To examine the regulation and production of ECM by ECs with an atheroprotective phenotype, ECs were micropatterned onto lanes, which created an elongated EC morphology similar to that seen with unidirectional fluid shear stress application. Collagen IV and fibronectin protein production were measured as were gene expression of collagen IV, fibronectin, laminin, MMP2, MMP9, TIMP1, TIMP2, and TGF-β1. ECs were also treated with TNF to simulate an injury model. Micropattern-induced elongation led to significant increases in collagen IV and fibronectin protein production, and collagen IV, laminin, and TGF-β1 gene expression, but no significant changes in the MMP or TIMP genes. TNF treatment significantly increased collagen IV gene and protein production. These results suggest that the increase in ECM synthesis in micropattern-elongated ECs is likely regulated with TGF-β1, and this increase in ECM could be relevant to the atheroprotection needed for maintenance of a healthy endothelium *in vivo*.

1. Introduction

Endothelial cells (ECs) are a critical component of blood vessels, serving as the interface between the blood and the bulk of the vessel—the predominately smooth muscle cell tissue layer. Healthy endothelium provides a smooth, anti-thrombotic, atheroprotective surface over which the blood flows. Under steady, unidirectional fluid flow, ECs will typically exhibit an elongated morphology and a healthy phenotype, while disturbed flow or static culture will lead to an atheroprone phenotype and a rounded, cobblestone morphology [1]. These morphology changes have been widely documented in both *in vivo* and *in vitro* systems. Morphological alterations to elongate ECs *in vitro* result from not just the external application of fluid shear stress, but also through confinement of their spreading, forcing them into an elongated phenotype through physical or biochemical barriers. There are a large variety of micropatterning techniques that have been used for EC manipulation that are reviewed elsewhere [2].

Results have varied somewhat regarding the specific phenotypic changes that physical EC elongation induces. The majority of previous work has focused on cytoskeletal or proliferative changes of the cells. Functional EC observations have been largely restricted to various immunogenic markers to determine the inflammatory capability of the ECs. Elongated, micropatterned ECs have been shown to exhibit a decrease in nitric oxide production [3], increase in monocyte detachment [4], decrease in VCAM-1 gene expression [4], and decrease in e-selectin gene expression [4]. Similar results were seen with flow-elongated cells [4]. The changes in these inflammation markers indicate an atheroprotective phenotype for the micropatterned cells in the absence of cytokines.

Tumor necrosis factor-α (TNF) is a pro-inflammatory cytokine that has a strong effect on ECs—increasing their ability to promote leukocyte adhesion through e-selectin, ICAM-1, and VCAM-1 [5]. TNF also decreases anti-inflammatory EC markers such as thrombomodulin [6] and eNOS [7]. Recently, high levels of TNF and other cytokines were found in patients with diabetes-induced peripheral artery disease, suggesting its powerful role on endothelial functions *in vivo* [8]. These effects suggest the usefulness of applying TNF to ECs *in vitro* as a model for simulating EC injury.

Extracellular matrix (ECM) production and maintenance is an important aspect of EC functionality. The ECM proteins collagen IV and laminin are components of the basement membrane, which is essential for EC adhesion and the maintenance of a smooth, anti-thrombotic endothelium. Fibronectin, however, is generally considered a matrix protein produced during wound healing and contributes to intimal hyperplasia *in vivo* [9]. This suggests that a matrix consisting of collagen IV and laminin would support

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atheroprotective EC functions, while fibronectin would promote an atheroprone EC phenotype. As mentioned before, previous work with micropatterned ECs showed a decrease in VCAM expression and monocyte attachment with aligned cytoskeleton [4]. When these micropatterned ECs were examined for important ECM protein deposition, the elongated ECs produced significantly more collagen type IV and less fibronectin compared to rounded ECs [10]. Laminin production was not different between the patterned and nonpatterned ECs [10]. Therefore, the micropatterned ECs, which are atheroprotective in nature, also produce ECM indicative of a healthy basement membrane; however, previous work did not examine if the ECM changes were a result of protein production or an alteration of the matrix remodeling. Since ECM breakdown and deposition is a dynamic process controlled by external forces, both biochemical and mechanical, it is important to examine the ECM regulating molecules to understand how cytoskeletal elongation of ECs can alter the underlying ECM composition. This study examined the effects of EC micropatterning on ECM and the regulators of ECM remodeling. Production of collagen IV and fibronectin proteins was quantified as was the gene expression of collagen IV, fibronectin, laminin, matrix metalloproteinase-2 (MMP2), MMP9, tissue inhibitor of MMP-1 (TIMP1), TIMP2, and transforming growth factor-β1 (TGF-β1). Micropatterned and nonpatterned ECs were also stimulated with TNF to serve as an injury model. Since TNF is such a powerful inflammatory cytokine that induces an atheroprone phenotype in ECs, its presence examines the atheroprotective ability of the elongated cells compared to controls. The overall objective of this study was to determine changes in ECM and ECM regulator gene expression of cobblestone and micropattern-elongated ECs under normal and injury conditions. We hypothesized that (1) elongated EC morphology, independent of flow, induces an increase in atheroprotective ECM through a decrease in the breakdown and remodeling of ECM with MMPs and (2) micropatterned ECs will retain more atheroprotective ECM in the presence of TNF than the nonpatterned ECs.

2. Materials and methods

2.1. Endothelial cell isolation and maintenance

Baboon carotid endothelial cells were isolated similarly to methods described previously [11]. 600 U/mL collagenase type II (Worthington) was put in the artery lumen, incubated for 5 min, massaged, and rinsed into a collagen type I-coated (BD Biosciences) tissue culture treated plastic containing endothelial growth media-2 (EGM, Lonza) with an additional 18% fetal bovine serum (FBS, Hyclone). Colonies of ECs were grown and/or passaged until a sufficient quantity was obtained for bead sorting. Cells were sorted with Dynabeads® (Invitrogen) for positive expression of CD31 according to the manufacture's protocol. Cells were passaged with TrypLE (Invitrogen), and ECs after P0 were maintained in EGM with 8% additional FBS. ECs were used between passages 3–6 for experimentation. When TNF was used to treat the ECs, samples were given 100 U/mL TNF (R&D Systems) in EGM for 4 h prior to testing.

2.2. Micropatterning

Patterns of elongated ECs were created from a modified method to that described previously [11]. Briefly, 50 μg/mL collagen type I solution was pulled with a vacuum through a polydimethylsiloxane stamp with 100 μm lanes on non-tissue culture treated polystyrene. Samples were then incubated with 0.1% Pluronic® F 108NF Prill Poloxamer 338 (BASF) in water to block the areas surrounding the collagen. Nonpatterned control samples were created similarly but using a drop of collagen rather than the channel pattern. Cells were plated for 1 h before a thorough rinsing and fresh EGM applied. ECs were cultured as described above for 48 h before protein and gene assessment.

Table 1
Forward and reverse primer pairs designed for quantitative RT-PCR.

	Forward primer	Reverse primer
COL IV	CCTCAACGACCACTTTGTCA	TTACTCCTTGGAGGCCATGT
FN	TATCCCTCGTGGCCATAAAG	AGTCACCTCGGTGTTGTAAG
LMN	CAGCAGGACTGTCGAAAGTG	CAGTCAGAGCCGTTACAGTG
MMP2	TGAGAAGGATGGCAAGTACG	TGCAGCTGTCATAGGATGTG
MMP9	CTACCACCTCGAACTTTGAC	CTCAGTGAAGCGGTACATAG
TIMP1	GTTGTTGCTGTGGCTGATAG	AAGGTGGTCTGGTTGACTTC
TIMP2	AGGGCCTGAGAAGGATATAG	TGAAGTCACAGAGGGTGATG
TGF-β1	ATTGAGGGCTTTCGCCTTAG	AACCCGTTGATGTCCACTTG

2.3. Protein quantification

Fluorescent microscopy was used to quantify the production of collagen IV and fibronectin as described previously [10]. Briefly, cells were fixed in paraformaldehyde and incubated with a primary antibody for collagen type IV, fibronectin, or a negative IgG control. An Alexa Fluor 488 secondary antibody and DAPI nuclear counterstain were used. Intensity of the ECM staining was quantified using MATLAB and cell counts of the same area done with NIH ImageJ. This provided a semi-quantitative metric for the amount of ECM produced per cell.

2.4. Quantitative RT-PCR

Gene expression was quantified for ECM and ECM regulators, specifically, collagen IV (COL IV), fibronectin (FN), laminin (LMN), MMP2, MMP9, TIMP1, TIMP2, and TGF-β1. Cells were removed from the patterned surface, and RNA was isolated with a Mini RNA kit (Zymo Research), according to the manufacturer's instructions. Then the DNA clean up kit (Zymo Research) was used to ensure elimination of any genomic DNA. Samples were reverse transcribed to cDNA using random primers and SuperScript® III RT (Invitrogen). The PCR reaction was performed on an ABI 7500 Fast System using primers designed and tested in house (see table 1) at a 250 nM concentration and was quantified using Platinum® SYBR® Green with a ROX reference dye (Invitrogen). GAPDH served as a housekeeping gene and samples were normalized to nonpatterned, nontreated controls with the 2^(−ddct) method. Fold changes values were then normalized to a continuous set of data (ranging from $-\infty$ to ∞) by subtracting one from positive values and taking the negative of the inverse plus one from the negative values. A Pearson's correlation coefficient, which is commonly used for analysis of gene co-expression [12], was calculated to determine the relationship between TGF-β1 gene expression and the gene expression of the ECM genes—LMN, FN, and COL IV.

2.5. Statistical analysis

All data were analyzed using a 2-way analysis of variance (ANOVA) with factors of patterning and TNF treatment—each factor having 2 levels. Significance was indicated using an *F*-test when $p < 0.05$.

3. Results

3.1. Cell morphology

Cells were successfully patterned into an elongated morphology without the application of fluid shear stress. Fig. 1 shows

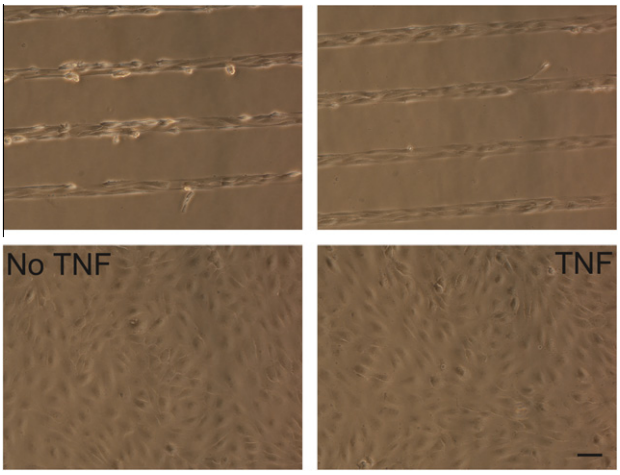

Fig. 1. Morphology of patterned and non-patterned ECs. Scale bar = 50 μm.

representative images of the ECs from each of the treatment groups. The TNF treatment did not appear to alter the morphology nor cell density of either the patterned or nonpatterned ECs.

3.2. Protein quantification

Representative images from the collagen IV and fibronectin staining are illustrated in Fig. 2. Cell nuclei are shown in blue with the corresponding ECM protein in green. Fluorescent intensity per cell for each of the treatment groups are also illustrated in the graphs in Fig. 2. Micropatterning significantly increased both collagen IV and fibronectin production by the ECs compared with non-patterned ECs using a 2-way ANOVA. The TNF treatment also significantly increased the collagen IV production compared to non-treated controls.

3.3. Quantitative RT-PCR

Fig. 3 and Table 2 show the quantitative RT-PCR results for collagen IV, fibronectin, laminin, and TGF-β1 and MMP2, MMP9, TIMP1, and TIMP2 genes, respectively. Collagen IV gene expression directly mirrored the results seen with protein production. Specifically, micropatterned ECs and cells treated with TNF showed a significant increase in collagen IV gene expression. Fibronectin gene expression, however, did not significantly increase with micropatterning. Laminin and TGF-β1 expression significantly increased with micropatterning, but MMP2, MMP9, TIMP1, and TIMP2 did not significantly change with either treatment.

The direct correlation of TGF-β1 gene expression to the ECM genes resulted in significant correlations with fibronectin (Fig. 3E) and laminin (Fig. 3F) when using a Pearson's coefficient. The Pearson's value for fibronectin to TGF was 0.619 and the laminin to TGF was 0.980, which were both statistically significant. The collagen IV to TGF Pearson's coefficient was not significant with a value of 0.502 ($p = 0.10$).

4. Discussion

Understanding the formation and maintenance of healthy endothelium is of critical importance in our current society, which has

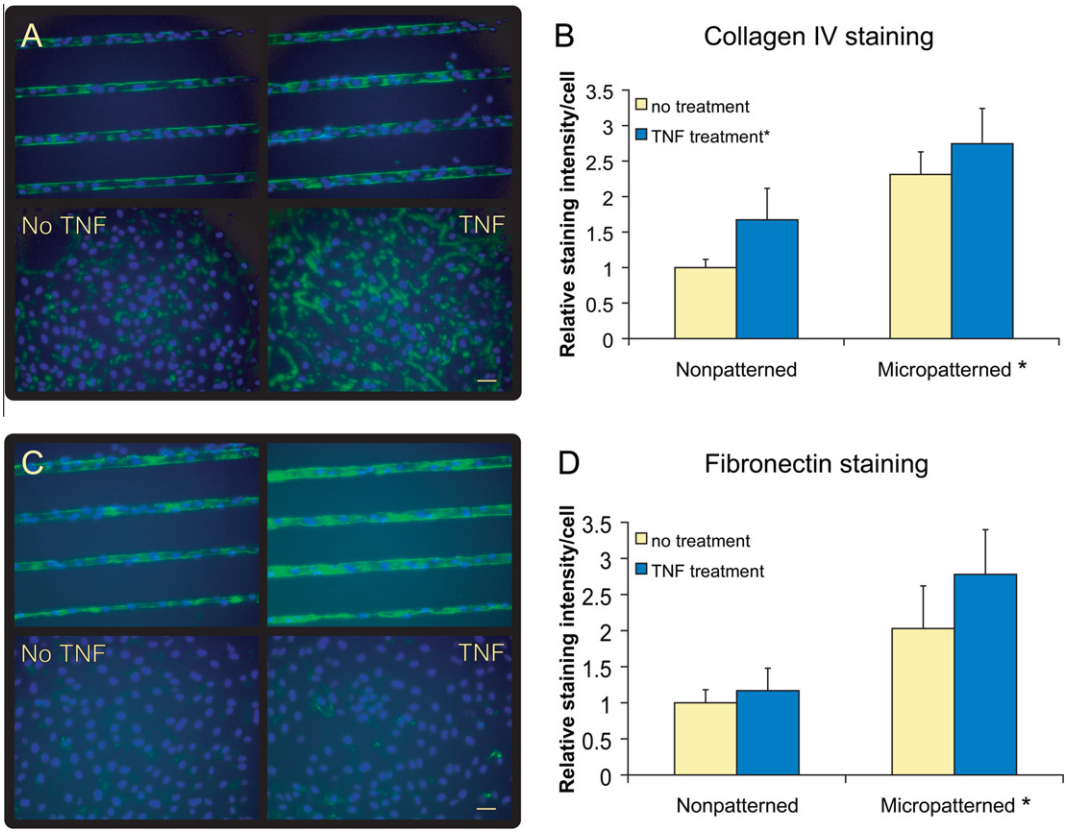

Fig. 2. Collagen IV (A) and fibronectin (C) staining in green with cell nuclei in blue for each treatment group. Scale bars = 50 μm. Fluorescent intensity per cell for collagen IV (B) and fibronectin (D). ∗ indicates a statistically significant increase in the factor using a 2-way ANOVA where $p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

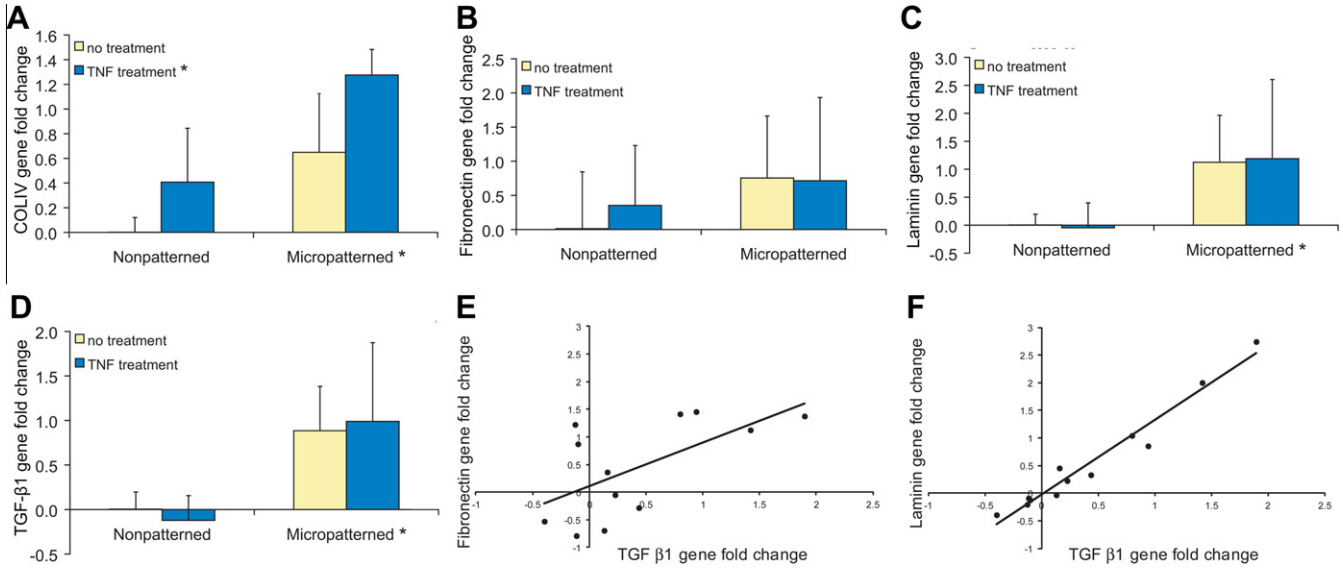

Fig. 3. Gene expression of collagen type IV (A), fibronectin (B), laminin (C), and TGF-β1 (D) for micropatterned compared to nonpatterned ECs with and without TNF treatment. Fold change values are compared to nonpatterned, nontreated controls. ∗ indicates a statistically significant increase in the factor using a 2-way ANOVA where $p < 0.05$. Correlations of fibronectin (E) and laminin (F) gene fold change to TGF-β1 fold change. The Pearson's coefficient for the former is 0.619 and the latter is 0.980, which are both statistically significant where $p < 0.05$.

Table 2
Gene expression of the ECM regulators for micropatterned and nonpatterned ECs with and without TNF treatment. Fold change values are compared to nonpatterned, nontreated controls. No statistically significance differences were seen with these data.

	Nonpatterned ECs		Micropatterned ECs	
	No TNF	TNF	No TNF	TNF
MMP2	−0.04 ± 0.64	0.56 ± 0.47	1.05 ± 2.10	2.83 ± 2.37
MMP9	0.00 ± 0.94	2.22 ± 0.44	1.57 ± 0.56	2.28 ± 0.10
TIMP1	0.04 ± 0.90	0.22 ± 0.49	1.20 ± 1.51	1.15 ± 1.19
TIMP2	0.00 ± 0.35	0.18 ± 0.37	0.68 ± 0.93	0.97 ± 1.65

such a high prevalence of cardiovascular disease. Heart disease continues to be the leading cause of death in the United States [13]. Appreciating the ECM production and regulation by ECs is just one aspect of cardiovascular health that has implications in stroke, heart disease, and vascular dysfunction. Previously, ECs on a fibronectin matrix exhibited an increase in a transcription factor associated with inflammation, NF-κB, while ECs on laminin, collagen type I, or basement membrane ECM showed a decrease in inflammatory signaling, supporting the idea that fibronectin is an atheroprone ECM protein while collagen and laminin are atheroprotective [14,15].

This work found that elongating ECs without flow led to an increase in collagen IV gene expression and protein production. Fibronectin protein production and laminin gene expression also increased. These results partially supported the first hypothesis of this study. That is, the elongated morphology did increase atheroprotective ECM without flow; however, fibronectin also increased. The fibronectin protein quantities increased significantly with micropattern-induced elongation while the fibronectin gene expression only showed a trend to increase, due primarily to the larger variability in the gene data. This difference may also be attributed to the gene expression being a single time point measurement while the protein quantities measured the amount of protein that was deposited over the entire 48 h period. The gene expression also does not take into account translation or post-translational modifications of the fibronectin protein, which may also contribute to differences between protein and gene expression. The results seen here for ECM genes and proteins also confirmed some of the previous work, which used very similar methods and found an increase in collagen IV production [10]. Unlike the previous work, fibronectin significantly increased in this study while previous results showed a decrease in fibronectin production; however, the latter results were not statistically significant [10]. This previous work also used a somewhat different micropatterning protocol—blocking with BSA rather than the polymer used here. The BSA block is less robust making the results less repeatable, whereas the polymer strongly prevents cell migration making a more reproducible cell pattern. Additionally, these studies used primary ECs from different animal sources, which can cause variation.

The results of this work corresponded well with results seen with the application of fluid shear stress. Previous work found that after stimulation with unidirectional fluid flow, ECs increased their production of fibronectin [16], laminin [16], and collagen IV [17]. Another study showed that fibronectin protein increased at the immediate onset of atheroprotective flow (4 h), then decreased over the next 20 h, but fibronectin increased steadily over 24 h with atheroprone shear stress [18]. These results suggest that the production of fibronectin does not clearly indicate an atheroprotective or atheroprone phenotype. Rather, the overall synthesis of the ECM may be an important component of the atheroprotective phenotype necessary to maintain a healthy endothelial layer.

Surprisingly few changes in the ECM regulating genes were observed with this work. The only regulating factor that changed significantly was TGF-β1 gene expression, which increased with micropatterned ECs compared to nonpatterned ECs. Micropatterned cells did not exhibit significant changes in MMP2, MMP9, TIMP1, or TIMP2 gene expression. The first hypothesis of this study stated that EC morphology, independent of flow, would induce an increase in atheroprotective ECM through a decrease in the breakdown and remodeling of ECM with MMPs, but this did not appear to be true. While the MMPs and TIMPs trended toward increasing

in the micropatterned samples compared to controls, the lack of statistically significant changes in any of the MMPs or TIMPs suggest that the elongated cells, while producing more ECM were not increasing degradation or remodeling of the matrix. Several *in vivo* experiments have shown that changing blood flow from normal physiologic flow conditions leads to an increase in MMPs between 4 and 21 days in mouse and rabbit models [19–21]. Perhaps changes in the production of MMPs or TIMPs would occur in this micropatterned EC system over longer time points than the ones examined here. The increase in TGF-β1 gene expression supports the observed increase in the production of ECM. The TGF-β superfamily is well understood to promote ECM synthesis and integrin expression while decreasing cell proliferation [22]. TGF-β1 likely regulates the increase in ECM seen with the elongated EC morphology. The significance seen with the direct correlation of TGF-β1 to the LMN and FN gene expression further supports the importance of TGF-β1 in ECM regulation. Previous work showed that blocking a TGF-β1 receptor after balloon injury decreased vessel size, suggesting the potential benefit of TGF-β1 in arterial remodeling [23]. ECs elongated by fluid shear stress in previous studies also did not increase MMP2 expression [17] and did increase TGF-β1 [24] further supporting the similarities between the micropatterned ECs and ECs stimulated by flow.

The increase in collagen IV with TNF treatment suggests that an elongated morphology may provide protection in this injury model, supporting this study's second hypothesis; however, since increases were seen in both the patterned and nonpatterned ECs, this is likely not the case. Rather, it appears that while TNF has been shown to elicit a strong inflammatory response in ECs, it results in few changes to the ECM production or regulation. Examining the TNF changes on various inflammatory signals with micropatterned ECs could be an important area of future research to determine if their elongated morphology provides atheroprotection.

The similar results seen for ECs elongated with micropatterning compared to fluid flow elongation suggest the usefulness of this technique in studying this atheroprotective phenotype *in vitro*. Other researchers have also found that micropatterned ECs may be useful in a tissue engineering approach to establish an elongated cell morphology while eliminating the need for preconditioning of the cells using fluid flow [25–27]. The micropatterning of biomaterials to create topographical features to influence EC behavior is an important area of research [28–31]. Using nanometer sized features to simulate ECs demonstrated that even subcellular length scale cues alter cell cycle and ECM gene expression [28]. However, more work is necessary to understand these micropatterned ECs and their matrix production. It would be interesting in future work to confirm the correlation of micropatterned ECM increase to TGF-β1 by disrupting the pattern either during or after cell alignment.

While more work is needed to understand the phenotypic changes that occur with EC elongation, the results shown here clearly show the usefulness of these micropatterned cells for studying the atheroprotective nature of ECs in the absence of fluid flow. This work also suggests the importance of TGF-β1 and the relative unimportance of MMPs and TIMPs in the observed ECM changes with elongated ECs. Future work with TNF will help to elucidate the specific ways in which elongated ECs aligned in the absence of flow can provide atheroprotection in an injured state.

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