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Alterations in Gene Expression of Human Vascular Endothelial Cells Associated with Nanotopographic Cues

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Abstract

Human cells *in vivo* are exposed to a topographically rich, 3-dimensional environment which provides extracellular cues initiating a cascade of biochemical signals resulting in changes in cell behavior. One primary focus of our group is the development of biomimetic substrates with anisotropic nanoscale topography to elucidate the mechanisms by which physical surface cues are translated into biochemical signals. To investigate changes in gene expression as a result of nanotopographic cues, Human Umbilical Vein Endothelial Cells (HUVECs) were cultured on chemically identical flat and 400 nm pitch nanogrooved surfaces. After 12 hours, RNA was harvested for an Affymetrix HG U133 Plus 2.0 gene array. Of over 47,000 possible gene probes, 3171 had at least a two-fold difference in expression between the control flat and 400 nm pitch. The gene ontology groups with the most significant increase in expression are involved in protein modification and maintenance, similar to cells upregulating chaperone and protein synthesis genes in response to physical stresses. The most significant decreases in expression were observed with cell cycle proteins, including cyclins and checkpoint proteins. Extracellular matrix proteins, including integrins, collagens, and laminins, are almost uniformly down regulated on the 400 nm pitch surfaces compared to control. The down regulation of one of these genes, integrin beta 1, was confirmed via quantitative PCR. Together, these gene array data, in addition to our studies of cell behavior on nanoscale surfaces, contribute to our understanding of the signaling pathways modulated by topographical surface cues.

Keywords

gene array; nanotopography; endothelial cell; ECM; integrin

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Introduction

Cardiovascular disease is the most common cause of death in the United States [1]. While great strides have been made in medical and surgical interventions, current therapeutic approaches remain inadequate for many patients. For example, bypass grafts can fail shortly after the procedure or last for over ten years, but the reasons and mechanisms responsible for this wide variability in graft durability over time are poorly understood [2, 3]. We still lack the knowledge of many basic cell biology processes needed for the development of novel cardiovascular treatments and improvement of current therapies. Although the mechanisms are poorly understood, the extracellular matrix surrounding the cell is now believed to play an essential role.

Recent reports have shown that smooth muscle cell proliferation and contraction, behaviors which can lead to bypass graft failure and other diseases of the vasculature, are induced by upregulation of basement membrane-degrading matrix metalloproteases [4-6]. Other studies have demonstrated that the compliance (rigidity) of the extracellular matrix can induce changes in endothelial cell behavior resulting in cell spreading or differentiation into tubular structures that resemble blood vessels [7]. Based on these studies and others, it is clear that the extracellular matrix (ECM) plays a key role in the pathogenesis and progression of cardiovascular disease. However, while the biochemical signals and protein domains of the vascular ECM have been studied to a great extent, the biophysical cues that regulate vascular cell behavior have often been overlooked. Our laboratory and others have shown that the biophysical attributes of the basement membrane and extracellular matrix greatly influence a variety of cell behaviors [8-12].

Work from our laboratory and others have shown that the specialized ECM layer, the basement membranes (BM), possesses a wealth of 3-dimensional topographical information [13-18]. Quantification of high magnification electron micrographs reveals that pores, fibers, bumps, and other BM structures exist at the nanoscale level. This means that a single cell, which can have a diameter over 100 microns, interfaces with thousands of biophysical cues presented by the BM. Unfortunately, since the native BM is a complex meshwork of biophysical cues, it can be difficult to study the effects of so many variable topographies on vascular cell behavior. This problem has driven the need for artificial substrates that possess well defined, nano and submicron scale physical features for *in vitro* ECM models. Using electron-beam and soft lithography techniques, we have successfully fabricated biomimetic substrates that have repeating patterns of grooves and ridges. These substrates allow us to manipulate one biophysical variable at a time in order to understand how non-biochemical information affects the properties of a cell. Cellular behaviors such as proliferation, migration, orientation, and morphology have all been reported to be modulated by topographically patterned surfaces [8, 9, 19-25]. Although the cell behavior and resulting phenotypic changes from both epithelial and endothelial cell types on patterned versus flat surfaces have been documented, little is known about the mechanisms or genes that govern these behaviors. Isolating one gene or protein that initiates a cellular response to a biophysical cue can be a daunting task, and it is unlikely that any one gene is a “master molecule” for topography-induced behavior. An alternative approach is to observe changes in the expression of gene groupings, or ontologies, which occur when cells are exposed to biophysical information [26-28].

In this study, we have used Human Umbilical Vein Endothelial Cell (HUVEC) RNA isolated from cells grown on either flat or well-defined patterned substrates for Affymetrix gene array comparison. The results have provided insights into the genes and groups of genes that respond to a known biophysical cue. This information will be useful for

understanding the pathogenesis of cardiovascular disease and will aid in the intelligent design of vascular prosthetics and engineered tissues.

Materials and Methods

Fabrication of patterned substrates

Nanotopographic substrates were fabricated as previously described [9]. Briefly, 3 cm² substrates are created by patterning a photoresist on the surface of a silicon wafer via lithography. The photoresist is exposed to radiation through a mask and removed with a subsequent developer solvent. The unprotected silicon is etched to 300 nm depths by reactive ions to create 3-D relief structures. Finally, the remaining photoresist is stripped to reveal a nanostructured surface comprised of defined grooves and ridges and is coated with SiO₂ to provide uniform surface chemistry. Surfaces with pitches (ridge plus groove width) of 400, 1400 and 4000 nm, all with groove depths of 300 nm were used. The silicon surfaces were used as masters in a soft lithographic process to fabricate polyurethane (NOA-81, Norland Products, Inc, Cranbury, NJ) replicas of the original topography via a PDMS transfer stamp [29, 30].

Cell Culture

A pooled population of primary Human Umbilical Vein Endothelial Cells (HUVECs) (Lonza, Walkersville, MD, catalog #C2519A) from multiple donors were cultured in Endothelial Cell Basal Media-2 (EBM-2) with the single quote-2 pack, which contains fetal bovine serum, hydrocortisone, human FGF, VEGF, R³-IGF-1, ascorbic acid, hEGF, GA-1000, and heparin at 37° C and 5% CO₂. HUVECs were maintained on 100 mm polystyrene tissue culture dishes and passaged and expanded every 48 hours, which resulted in an average of 1.4 – 1.6 population doublings each time. HUVECs were used for experiments between the 4-7th passages, corresponding to about 7 to 11 population doublings. For the gene arrays, 60 – 80% confluent plates of 5th passage (7-8 population doublings) HUVECs were washed with HEPES buffered saline, trypsinized, pelleted, resuspended in a single tube, and 2.5×10^5 cells were plated onto each 3 cm² polyurethane NOA 81 substrate. All substrates were plated with the exact same pooled population of HUVECs and were lysed for RNA isolation after 12 hours. The cellular lysates from ten substrates were collectively combined for each sample. For quantitative PCR experiments, 2.5×10^5 4-7th passage HUVECs were seeded onto each 3 cm² NOA 81 substrate for varying times before lysis.

RNA isolation and purification

At the desired time point, HUVEC cells were quickly washed once with sterile 1× phosphate buffered saline (PBS, pH 7.4), and then harvested with a cell scraper in the presence of PBS. Cells were centrifuged, the PBS removed, and the pellet resuspended in Trizol (Invitrogen, Carlsbad, CA). RNA was isolated as per the manufacturer's instructions. After Trizol extraction, the RNA was passed through an RNA MinElute Cleanup Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol and the concentration and purity were quantified with a NanoDrop spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE).

Gene Array

Doubled-stranded cDNA was synthesized from 5 µg of purified total RNA using a kit (Superscript Double-Stranded cDNA Synthesis Kit, Invitrogen) with a T7-(dT)24 primer (Affymetrix, Santa Clara, CA). The double-stranded cDNA subsequently was purified by phenol-chloroform extraction and *in vitro* transcription reactions were performed (Bioassay

High Yield RNA Transcript Labeling Kit; Enzo Diagnostics, Farmingdale, NY) according to the manufacturer's protocol. Biotin-labeled cRNA was purified (Qiagen, Valencia, CA) and quantified before being fragmented into 35 to 200 base fragments in an alkaline buffer. Eight Affymetrix Human Genome HG U133 2.0 Plus chips containing 47,000 transcript targets were used. Washing, staining, and scanning were performed by using the Genechip Instrument System (Affymetrix) as recommended in the manufacturer's technical manual. The arrays were scanned and data were packaged with the Microarray Suite algorithm, ver. 5; (Affymetrix). Raw data were then uploaded into Genesifter (Geospiza, Seattle, WA) for further analysis. The data were normalized using robust multichip averaging with GC correction (GC-RMA). A pair-wise statistical analysis of all genes between the two groups, Flat (control) and 400 nm (experimental) were performed with a t-test and Benjamini and Hochberg correction. Genes that had a ± 2.0 -fold or greater difference and a t-test p value 0.001 were considered statistically significant. Groupings of genes with related functions, or ontologies, were considered significant if the groupings' standardized z-scores were more or less than 2.0. Higher or lower values indicate whether each ontology occurs more or less frequently compared to the control. Ontologies are based on functional gene groupings determined by the Gene Ontology project [31].

Quantitative PCR

Quantitative real-time PCR was performed with the TaqMan One-Step RT-PCR Master Mix (Applied Biosystems, Foster City, CA). Fifty ng of total RNA was mixed with either an integrin $\beta 1$ TaqMan probe or an 18S rRNA TaqMan probe per well. Samples were run in triplicate, and each experiment was performed three times with the Step-One real-time PCR machine (Applied Biosystems). The integrin $\beta 1$ samples were normalized against the 18S rRNA (a common QPCR normalization standard, and a gene shown to be unchanged in the gene array) and the fold change among the triplicate samples were calculated and averaged using the Step One software (Applied Biosystems, ver 2.0).

Statistics

Quantitative PCR data were analyzed using one way analysis of variance (ANOVA). When variability was determined to be significant ($p < 0.05$), the paired student's t-test was utilized to determine significance ($p < 0.05$) between groups. Within the figures significance is denoted by the following $* = < 0.05$, $** = p < 0.01$, $*** = p < 0.001$.

Results

HUVEC cells were plated onto chemically identical, flat control surfaces or surfaces that possessed anisotropically patterned grooves and ridges (Fig 1). The patterned substrates had a pitch of 400 nm (pitch = groove plus ridge width), with a 1:1 groove to ridge ratio, and a depth of 300 nm. After 12 hours, HUVECs (Group 1-“Flat” $n = 4$ samples, Group 2-“400 nm” $n = 4$ samples) were harvested in order to extract and purify the total RNA. Twelve hours was chosen as the end point because many visual phenotypic differences, including alignment and migration (Fig 1), have been observed between cells on flat and patterned surfaces at that time point [9, 25]. In Addition, over 90% of the HUVECs attached to both flat and 400 nm pitched surfaces within one hour (Fig 1C), and had similar cell densities at 12 hours (Fig 1D). This allowed us to quantitatively measure gene regulation in response to topography before the cells could deposit significant amounts of their own matrix. Total HUVEC RNA was reverse transcribed, and *in vitro* transcripts with fluorescent probes were created for hybridization with the Affymetrix HG-U133 Plus 2.0 gene array. The overall mean, upper and lower quartiles, and minimum and maximum raw signal intensities both within all the samples and between Flat and 400 nm groups was consistent, thus insuring the

quality of the array (Supplemental Figure 1). For this study, Flat is designated as the control group and the 400 nm group was the comparative group.

Analysis of the HUVEC microarray revealed 1584 and 1587 transcript targets were significantly upregulated and downregulated, respectively, on the 400 nm condition compared to Flat control (threshold 2.0-fold or greater, $p < 0.001$). Genes with altered expression were grouped together based on function using gene ontologies and scored for significance, which was assigned by a z-score. There were several upregulated ontologies with highly significant z-scores which ranged from 3.55 to 8.33. Most of these categories were related to amino acid metabolism, protein folding, lipid transport, cell size, apoptosis, and endothelial cell differentiation (Table 1). Upon examination, the downregulated ontologies had relatively higher z-scores compared to the upregulated categories ranging from 4.11 to 16.74. The most significant scores involve families of genes which regulate cell cycle (Table 2). Other downregulated categories involved cytoskeleton biogenesis, ribosome biogenesis, and organelle localization and organization. Further analysis of the cell cycle family of genes revealed almost universal downregulation of cyclins and cyclin dependent kinases (cdks) that govern the cell cycle checkpoints during mitosis (Table 3). In addition, inhibitors of the cdks were notably upregulated. These observations demonstrate the impact of nanotopographic cues, specifically 400 nm pitch, on cell cycle related genes.

We also investigated HUVEC genes potentially involved in the direct response to biomimetic topographic cues which include cell adhesion and the induction of genes involved in extracellular matrix production. Individual adhesion and ECM genes including alpha and beta integrin subunits, collagen and laminin family members demonstrated statistically significant changes ($p < 0.001$) of 2-16 fold in HUVEC cells on flat vs. 400 nm pitch topography (Table 4). The majority of genes are downregulated with the exception of collagen VI $\alpha 2$ and laminin $\beta 3$, which are upregulated.

In our investigation into the genes involved in the initial response to topographic cues, one known gene involved in substrate adhesion, $\beta 1$ integrin, was identified to exhibit significant downregulation in HUVEC cells on 400 nm pitch. This candidate was selected for further analysis based on reports in the literature demonstrating alterations in the amount of $\beta 1$ messenger RNA on fibroblasts seeded onto micron-sized grooved topographical substrates when compared to smooth surfaces at both 1 hour and 24 hour timepoints [32]. To thoroughly examine potential changes in integrin $\beta 1$ expression levels and confirm results obtained with the gene array data, we utilized a quantitative real-time polymerase chain reaction (qPCR). HUVEC cells were plated onto a range of nanotopographic cues including 400 nm, 1400 nm, and 4000 nm pitch. Two separate time points of 2 and 12 hours were examined to include potential changes in expression levels of $\beta 1$ integrin that are involved in initial adhesion to the topographic substrate. Two hours post-plating, qPCR revealed that HUVEC integrin $\beta 1$ messenger RNA (mRNA) is upregulated on all three topographically patterned substrates, with significant differences in the 400 nm ($p < 0.01$) and 4000 nm ($p < 0.01$) compared to flat control (Fig 2A). In contrast, at the 12 hour time-point, $\beta 1$ integrin message is downregulated 3-fold on the 400 nm ($p < 0.05$) and 4000 nm ($p < 0.01$) pitches, and 2-fold on the intermediate 1400 nm pitch substrates (Fig 2B). Our resulting qPCR data on $\beta 1$ integrin at the 12 hour time point also verifies the efficacy of our HUVEC gene array with a similar 2 fold reduction in expression of message.

Discussion

In this report as well as previous studies, the impact of the biophysical environment on HUVEC cell behavior has been established. However, the molecular basis that modulates essential cell behaviors remains poorly understood. In this study, we exposed endothelial

cells to a repeating pattern of ridges and grooves with dimensions in the biomimetic range and screened for significant differences in expression among 47,000 gene probes. With over 3,100 gene targets identified as being significantly up- or downregulated, it is clear that physical cues, independent of biochemical signals, can greatly influence molecular changes in cell behavior. While the immense amount of data obtained from a gene array study cannot confirm the role of one specific signaling pathway in the tested response, it can still serve as a useful starting point to make intelligent assumptions as to potential molecular candidates that are most likely involved in the specific response to biophysical cues.

The upregulated and downregulated ontologies when comparing HUVEC cells on nanoscale topography vs. flat control show a consistent theme across most of their categories. Namely, the 400 nm pitch topography seems to in effect, “stabilize” the endothelial cell phenotype. For example, in vascular tissue homeostasis, endothelial cells are normally non-proliferative with exceptions during development and wound healing. Our results demonstrate that cell cycle genes are significantly downregulated on topography, while apoptosis genes are upregulated. Recent experiments from our laboratory demonstrate that while HUVECs do indeed grow and proliferate on all of our polyurethane surfaces, their proliferation rate is significantly decreased on topographic features less than 800 nm in size. The impact on HUVEC proliferation is observed regardless of the geometry (the pitch of ridges and grooves or diameter of pores)[25]. It is also important to note that the scale of the topographic features contained within our substrates were fabricated based on the characterization of native vascular basement membranes [17]. Therefore, we hypothesize that topographical cues in the biomimetic range most likely provide a more *in vivo* like environment for HUVEC cells by influencing and decreasing their proliferation rates and preventing unwarranted expansion via maintenance levels of programmed cell death.

To maintain vascular tissue homeostasis there is a balance between proliferation, differentiation and cell death. With the observed decrease in proliferation we anticipated potential upregulation in endothelial differentiation genes. As expected, we observed a significant upregulated z-score in the endothelial differentiation ontology. Our results are comparable to past reports demonstrating the impact of biophysical cues on endothelial cell differentiation. The compliance, or measured stiffness of a substrate, has been demonstrated to influence the ability of endothelial cells to organize and differentiate into tube-like structures resembling capillaries when they are seeded onto substrates with various compliances [33-35]. Revascularization of engineered tissue is a critical component for a successful therapeutic outcome and using topographic cues to promote this process is a novel approach.

Another interesting aspect of the gene array data is the downregulation of several ECM and adhesion molecules at the twelve hour time point. However, we observed an early upregulation of $\beta 1$ integrin at the 2 hour time point. One hypothesis is that the patterned substrates provide the necessary cues to quickly (1-2 hours after plating) initiate the expression of key ECM molecules necessary for attachment to the surface leading to the upregulation observed over the planar control. The subsequent significant decrease in $\beta 1$ expression at 12 hours may be due to the lag in $\beta 1$ expression in the planar control because the cells, while attached within the same time frame, were not exposed to topographic stimuli. At the 12 hour time point, the cells exposed to topographic cues have fully formed attachments and are contact guided by the underlying surface. As a result, the HUVECs have reached an equilibrium, and stabilization of their endothelial cell phenotype, leading to decreased production of ECM products. In support of this, several groups have observed a decrease in $\beta 1$ expression after exposure to topographic cues or other surface modifications [32, 36]. Studies from Dalby et al. using anisotropic patterns of nanoscale hexagonal pits reported a similar decrease in ECM expression from fibroblasts [37]. It is possible that

surfaces with topographical cues act as a negative feedback loop to decrease the production of proteins that would otherwise act as adherence support. Understanding the influence of different biomimetic scales of anisotropic patterns influence cell behavior and gene expression could greatly aid the intelligent design of vascular, and other clinical prosthetics.

The results from our study strengthen the theory that biophysical cues are an essential parameter to incorporate into vascular prosthetics. Despite recent progress, tissue engineering has yet to fully deliver on its promise of clinical efficacy. Currently, creating *de novo* tissues in culture is often limited by the inability to grow complex, 3-dimensional cultures that share physiological functions to the organs or tissues they are designed to mimic and replace. This study and others demonstrate that not all information presented to cells is biochemical in nature, but rather biophysical cues also play a direct role in the regulation of many genes in various cell types [24, 27, 37].

Consideration should be given to the topographical substratum for the design of cardiac and vascular artificial tissues. For example, a recent report successfully created a new beating heart after 14 days in culture using an acellularized cardiac ECM scaffold seeded with cardiac cell-types [38]. Unfortunately, acellularized starting material is not easily obtained, but fabricated scaffolding imprinted with defined, biomimetic topography could be developed as a potential replacement. The topographic cues provided could modulate specific genes and pathways in different areas of the engineered heart (in this example), or in another tissue. This act could then “stabilize” the unique behaviors of the different cell types present in the tissue, independent or in conjunction with biochemical supplementation.

Conclusions

In conclusion, we found that the groups with the most significant increase in gene expression are involved in protein modification and maintenance and the most significant decreases in expression were observed with cell cycle proteins including cyclins and checkpoint proteins. We also found that extracellular matrix proteins, including integrins, collagens, and laminins, are almost uniformly down regulated on the smallest topographic features compared to control. In summary, the physical aspects of the cellular substratum are an important variable to consider when designing *in vitro* experiments, clinical prosthetics and engineered tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Contact guidance of HUVEC cells on nanotopography

(A) A schematic representation of the control flat and anisotropic ridge and groove nanotopographic surfaces. The pitch is defined as the width from the start of one ridge to the start of the next ridge. (B) Fluorescence images of HUVECs that were fixed and stained with rhodamine-phalloidin 12 hours after plating on flat control and 400 nm topography surfaces. Individual cells on flat control exhibit no preferred orientation or alignment to the underlying substrate while HUVECs on 400 nm pitch exhibit contact guided orientation to the topographic cues. Phase contrast images demonstrating attachment and density of HUVECs plated onto flat control and 400 nm topographic surfaces (C) 1 hour and (D) 12 hours after plating. Scale bars = 100 μm .

Figure 2. Nanoscale topographic cues modulate $\beta 1$ Integrin levels in HUVEC cells

QPCR data of Integrin $\beta 1$ message from HUVECs seeded onto control flat, 400 nm, 1400 nm and 4000 nm pitch topographies at both 2 and 12 hour time points. Integrin $\beta 1$ message from HUVEC cells normalized to the Flat condition at (A) 2 hours post-plating and (B) 12 hours post-plating. One way analysis of variance was determined to be significant for both the 2 hour ($p < 0.0001$) and 12 hour ($p < 0.01$) Timepoints. A students t-test was run to determine further significance. *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$ (Data shown as $\pm$ SEM)

Table 1
Nanoscale topography induces significant upregulation of a range of ontologies

The “Array” number denotes the total number of genes available on the Affymetrix chip that belong to each ontology category while the “List” category value represents the number of genes that were significantly upregulated on the 400 nm pitch substrates within that specific ontology category. The z-score is the statistical likelihood that the ontology is significantly upregulated in the 400 nm group. A z-score over 2.0 is considered a significantly positive upregulation.

Ontology	List	Array	Z-score
serine family amino acid biosynthetic process	6	9	8.33
Golgi organization and biogenesis	7	12	8.32
response to unfolded protein	13	56	6.11
amino acid metabolic process	30	217	5.81
apoptosis	70	728	5.58
carboxylic acid metabolic process	49	461	5.39
negative regulation of DNA replication initiation	3	6	4.96
cell redox homeostasis	10	51	4.67
lipid transport	14	100	4
regulation of cell size	20	180	3.63
endothelial cell differentiation	3	10	3.55

Table 2
Topographic cues initiate significant downregulation of genes involved in cell cycle control

The “Array” number denotes the total number of genes available on the Affymetrix chip that belong to that ontology category while the “List” category value represents the number of genes that were significantly downregulated on the 400 nm pitched substrates within that specific ontology category. The z-score is the statistical likelihood (better- use this in previous.) that the ontology is significantly downregulated in the 400 nm group. A z-score over 2.0 is considered significant and a true positive downregulation.

Ontology	List	Array	Z-Score
M phase	82	269	16.74
Cell cycle	141	685	16.11
DNA replication	58	195	13.78
Spindle organization and biogenesis	15	18	13.61
Response to DNA damage stimulus	60	290	10.39
Organelle organization and biogenesis	137	1049	9.68
Cytoskeleton organization and biogenesis	61	447	6.68
Microtubule-based movement	21	92	6.66
Ribosome biogenesis and assembly	16	85	4.86
Establishment of organelle localization	6	22	4.11

Table 3
Cyclin and cdk proteins expressed at significantly different levels from HUVEC cells grown on the 400 nm group compared to Flat

UniGene Title	Gene ID	Up or Down	Fold Induction
Cyclin A2	CCNA2	Down	4.6
Cyclin B1	CCNB1	Down	3.48
Cyclin B2	CCNB2	Down	2.56
Cyclin D1	CCND1	Down	2.41
Cyclin D2	CCND2	Up	2.56
Cyclin E1	CCNE1	Down	4.6
Cyclin E2	CCNE2	Down	9.15
Cyclin F	CCNF	Down	2.57
Cyclin G2	CCNG2	Up	5.88
Cyclin dependent kinase 2	CDK2	Down	4.28
Cyclin dependent kinase inhibitor 1A (p21)	CDKN1A	Up	2.02
Cyclin dependent kinase inhibitor 2A (p16)	CDKN2A	Up	2.87
Cyclin dependent kinase inhibitor 2B (p15)	CDKN2B	Up	7.15

Table 4
ECM and integrin proteins expressed at significantly different levels from HUVEC cells grown on the 400 nm group compared to Flat

UniGene Title	Gene ID	Up or Down	Fold Induction
Integrin alpha 5	INTGA5	Down	2.16
Integrin alpha V	INTGAV	Down	2.27
Integrin beta 1	INTGB1	Down	2.37
Integrin beta 5	INTGB5	Down	2.67
Integrin beta 8	INTGB8	Down	16.42
Collagen I alpha 2	COL1A2	Down	3
Collagen IV alpha 1	COL4A1	Down	4.08
Collagen VI alpha 2	COL6A2	Up	2.52
Collagen XII alpha 1	COL12A1	Down	3.22
Laminin alpha 4	LAMA4	Down	2.36
Laminin beta 1	LAMB1	Down	2.24
Laminin beta 3	LAMB3	Up	12.05