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Integration of basal topographic cues and apical shear stress in vascular endothelial cells

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Abstract

In vivo, vascular endothelial cells (VECs) are anchored to the underlying stroma through a specialization of the extracellular matrix, the basement membrane (BM) which provides a variety of substratum associated biophysical cues that have been shown to regulate fundamental VEC behaviors. VEC function and homeostasis are also influenced by hemodynamic cues applied to their apical surface. How the combination of these biophysical cues impacts fundamental VEC behavior remains poorly studied. In the present study, we investigated the impact of providing biophysical cues simultaneously to the basal and apical surfaces of human aortic endothelial cells (HAECs). Anisotropically ordered patterned surfaces of alternating ridges and grooves and isotropic holed surfaces of varying pitch (pitch = ridge or hole width + intervening groove or planar regions) were fabricated and seeded with HAECs. The cells were then subjected to a steady shear stress of 20 dyne/cm² applied either parallel or perpendicular to the direction of the ridge/groove topography. HAECs subjected to flow parallel to the ridge/groove topography exhibited antagonistic effects of the two stimuli on cellular orientation and elongation. In contrast, flow perpendicular to the substrate topography resulted in largely antagonistic effects. Interestingly, the behavior depended on the shape and size of the topographic features. HAECs exhibited a response that was less influenced by the substratum and primarily driven by flow on isotropically ordered holed surfaces of identical pitch to the anisotropically ordered surfaces of alternating ridges and grooves. Simultaneous presentation of biophysical cues to the basal and apical aspects of cells also influenced nuclear orientation and elongation; however, the extent of nuclear realignment was more modest in comparison to cellular realignment regardless of the surface order of topographic features. Flow-induced HAEC migration was also influenced by the ridge/groove surface topographic features with significantly altered migration direction and increased migration tortuosity when flow was oriented perpendicular to the topography; this effect was also pitch-dependent. The present findings provide valuable insight into the interaction of biologically relevant apical and basal biophysical cues in regulating cellular behavior and promise to inform improved prosthetic design.

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Keywords

Endothelial cell; Shear; Nanostructure; Cell morphology

1. Introduction

Cardiovascular disease (CVD) remains the leading cause of mortality in the world [1,2]. A principal source of clinical CVD pathology is atherosclerosis, which involves the development of cholesterol-rich lesions in the arterial wall. Dysfunction of the vascular endothelium lining the inner surface of the arterial wall is known to be a key precursor to atherosclerotic lesion formation and progression [1]. In healthy vessels, vascular endothelial cells (VECs) exhibit an anti-inflammatory, anti-coagulative and hence atheroprotective phenotype. Atherogenic stimuli alter this phenotype, causing VEC inflammation and recruitment of circulating monocytes, which contribute to lesion development [3,4].

VEC function is influenced by a wide menu of biochemical and biophysical cues [5]. A large body of literature broadly documents the impact of biochemical cues on VEC homeostasis [6]. More recently, a growing body of literature has focused on the impact of biophysical cues on VEC homeostasis and pathogenesis. Local flow conditions have long been correlated with the progression of atherosclerosis [7,8], which itself can alter the substratum mechanics presented to the VECs [9,10]. There has additionally been recent interest in the potential role that these cues play in transplant and regenerative medicine [11–13]. In CVD transplant cases, surgical intervention often results in denuded endothelium that must be repopulated for proper transplant function [14]. Two critical biophysical cues that regulate VEC repopulation are substratum topography and the local flow regime in the blood vessel [12,15,16].

In vivo, VECs are anchored to a specialization of the extracellular matrix, the basement membrane (BM). The BM presents a variety of biophysical and biochemical cues that have been shown to regulate fundamental VEC behavior [11,13]. One of the biophysical cues provided to the basal aspect of the VECs by the BM is in the form of surface topography [17,18]. *In vitro*, topographic biophysical cues in the biomimetic size range (micro- and nanometer-scale features) have been shown to modulate fundamental VEC behavior as well as the expression of more than 3000 genes [11,12,19].

In addition to topographic cues presented to the basal aspect, VECs are presented with hemodynamic cues applied to their apical surface. The biophysical cues presented to the apical cell surface in the form of shear flow regulate fundamental behaviors and inflammation response of VECs [15,20–22]. Additionally, VECs are differentially responsive to flow with high, unidirectional shear resulting in an atheroprotective phenotype, in contrast to disturbed flow which is atherogenic [16,23]. The combination of basal topographic cues and apical flow cues experienced by VECs is schematically depicted in Fig. 1.

To date, *in vitro* investigations of biophysical cues in the form of substratum topography or shear flow have been largely conducted independent of one another. There are a few notable exceptions concerning other cell types [24,25] and VECs on larger microtopographies [26]. The simultaneous presentation of these two biophysical cues more accurately mimics the biophysical attributes of the microenvironment of VECs *in vivo*. With this aim in mind, we investigated the impact of combined basal and apical biophysical cues on fundamental VEC behaviors including cellular and nuclear shape and alignment as well as cellular migration in human aortic endothelial cells (HAECs).

2. Materials and methods

2.1. Cell culture

Human aortic endothelial cells (HAEC) (Lonza, Walkerville, MD) were cultured using standard conditions in endothelial basal media supplemented with the EGM-2 BulletKit containing: GA-1000, hEGF, fetal bovine serum, heparin, ascorbic acid, R3-IGF, VEGF, hFGF-B, and hydrocortisone (Lonza, Walkerville, MD). Cells between passages 2 and 7 were used for all experiments.

2.2. Topographic surface fabrication

Topographically patterned silicon masters were prepared at the Center for Nanotechnology (University of Wisconsin) and Nm2 LLC (Cambridge, MA) as previously described [27–29]. Briefly, ridge and groove as well as holed substrates were fabricated as arrays containing 6 different pitch sizes separated by planar control regions. As illustrated in Fig. 2A, pitch is defined as ridge + groove width, or hole diameter + lateral distance to the adjacent hole for holed substrates. The pitch sizes were 400 nm, 800 nm, 1200 nm, 1400 nm, 1600 nm, 2000 nm, and 4000 nm with the exception that there were no 1400 nm pitch surfaces for holed substrates. Feature depth was at least 300 nm in all cases. Polydimethylsiloxane (PDMS) sub-masters were fabricated from the silicon masters as previously described [28,30]. Following spin coating with Norland Optical Adhesive 81 (NOA81) poly-urethane (Norland Optical Adhesives, Cranbury, NJ) onto tissue culture polystyrene dishes or glass slides, surfaces were stamped using the submasters as previously described [11].

2.3. Microflow chamber fabrication and flow validation

Microflow chambers were designed and custom-fabricated for cell migration studies. We used #2 glass coverslips (Tedpella, Redding, CA) as mold inserts for fabricating polydimethylsiloxane (PDMS) flow chambers with dimensions of $220\ \mu\text{m} \times 2.2\ \mu\text{m} \times 2.6\ \text{cm}$, $H \times W \times L$. Briefly, the coverslips were coated with a monolayer of octadecyltrimethoxysilane (OTS) by placing them in 20 ml of 5 $\mu\text{l/ml}$ v/v OTS (90%, Sigma) solution in anhydrous toluene (<50 ppm water, Acros Organics) for 30 min. After 30 min, the OTS solution was removed and each coverslip was rinsed with toluene (99.8%, Fischer) and acetone (99.5%, Fisher) and dried under a stream of nitrogen. Sylgard 184 (Dow Corning, Midland, MI) solution was prepared by thoroughly mixing 40 g of the base and 4 g of the curing agent in a polypropylene weigh dish and degassed under vacuum. The Sylgard (PDMS) solution was poured over the coverslips and any air bubbles were removed. The PDMS was cured at 60 °C for 1 h. The cured PDMS was cut around the coverslips and the coverslips were removed. One short edge of the chamber was opened by removing the PDMS to allow liquid to flow in (Fig. 2B). An intake tube was inserted on the opposite side and attached flush with the roof of the flow chamber (Fig. 2B). Chambers were fixed inside TCP dishes with a thin layer of NOA 81 followed by 2 min of UV curing such that the chamber encompassed the topographic array.

The flow profile and shear stresses within the chamber were computed using the Navier-Stokes solver Elmer 6.2 (CSC, Espoo, Finland). We assumed symmetry across the centerline of the chamber and modeled half of the chamber and ~10 diameters of the outlet line. A parabolic velocity profile was assumed at the outlet, corresponding to a volume flow rate of 20 mL/min, and a constant pressure was assumed across the inlet. A total of 61076 nodes and 55920 hexahedral and wedge elements were used. Mesh density was varied in order to confirm mesh independence of the solution.

2.4. Shear flow effects on cellular and nuclear morphology

For all morphology experiments, approximately 20,000 HAECs/cm² were seeded onto glass slides containing two ridge/groove and one holed surface arrays and cultured to confluence. One of the two ridge/groove arrays was oriented perpendicular to the direction of the shear flow and the other parallel. The HAECs were then subjected to a steady laminar shear stress of 20 dyne/cm² for 24 h using parallel plate flow chambers as detailed in previous work [31]. After flow, the HAECs were immediately washed in warm PBS and then fixed and permeabilized with warm 3.7% formaldehyde and 0.2% Triton X-100 in PEM buffer for 5 min [32]. The cells were then labeled with Alexa fluor 555-phalloidin, FITC-tagged mouse anti-tubulin (Invitrogen, Carlsbad, CA) and the nuclear counter-stain DAPI (Invitrogen, Carlsbad, CA). The labeled cells were imaged with a Zeiss Axio Scope A1 upright microscope using a 10X/0.3NA objective and AxioCam HRc camera (Carl Zeiss Inc., North America). Triplicate samples of at least 174 cells per pitch were manually traced. Cellular and nuclear geometries were fit to an ellipse, allowing the extraction of alignment and aspect ratio. All processing was performed using Image J (NIH, Bethesda, MD) and Matlab 2007b (MathWorks, Natick, MA).

2.5. Cell migration

Approximately 30,000 HAECs were seeded onto each set of topographic pitches and incubated at least 3 h prior to flow treatment within the custom-fabricated microflow chamber. Cells were subjected to a nominal 20 dyne/cm² during the course of each migration movie. Images were taken every 10 min over the course of 12 h using a Zeiss Axiovert 200M inverted microscope with a 10X/0.4NA objective, and an AxioCam HRm camera (Carl Zeiss Inc., North America). The Zeiss migration tracking module was used to track individual cells that did not leave the field of view, contact another cell, or divide during the course of recording. At least 30 cells per pitch were tracked. Migration rate, tortuosity (total distance/straight distance) and migration path were then analyzed.

2.6. Statistics

Morphology was analyzed as population averages of 3 experiments and all groups, while migration analysis was based on individual cells. In all cases, ANOVA followed by Fisher's least significant difference post-hoc analysis was used to determine significance: $\alpha/\#/* = p < 0.05$, $\alpha\alpha/\##/** = p < 0.01$, $\alpha\alpha\alpha/\###/*** = p < 0.001$.

3. Results

3.1. Characterization of the flow field in microflow chambers

To determine the flow regime profile of the microflow chamber, the full velocity and pressure profile for the domain were computed; however, for the present studies only the shear stress magnitude and direction along the bottom surface were important. For the majority of the chamber, most notably the region containing the topographically patterned region (magenta border in Fig. 2C), there was little variation in the shear stress distribution, ranging from 19.2 to 20.8 dyne/cm² and deviating by no more than 4.3° from the nominal axial direction (Fig. 2B).

3.2. Combined effects of topography and flow on cell and nuclear alignment

Control HAECs grown on planar surfaces (i.e. surfaces with no topographic cues) under static conditions had a random net orientation (Fig. 3). Quantitatively, this translated to a net orientation angle of ~45° (Fig. 4). When cultured on ridge/groove patterned surfaces under static conditions, HAECs exhibited pitch dependent alignment with the long axis of the topographic features (Fig. S1, Fig. 4A). Addition of shear stress on planar surfaces resulted

in moderate alignment in the direction of flow with the cells oriented at an average angle of $\sim 30^\circ$ relative to the flow direction (Figs. 3 and 4).

When, HAEC monolayers cultured on topographically patterned ridges and grooves were subjected to a steady laminar shear stress of 20 dyne/cm² for 24 h, the combined effect of the delivery of apical (shear flow) and basal (anisotropically ordered topographic features) biophysical cues on cellular orientation was synergistic for ridges parallel to the direction of flow and antagonistic for ridges perpendicular to the direction of flow (Fig. 3 and Fig. 4A). HAECs on ridge/groove surfaces under perpendicular flow conditions generally resisted significant alignment in the direction of flow for pitch values greater than 800 nm. These results demonstrate a scale-dependent antagonistic interaction of the cues with HAEC orientation determined primarily by flow for small pitch values but by substrate topography for large pitch values. HAECs on ridge/groove surfaces under parallel flow conditions had a greater degree of alignment to both topography and flow for all pitch values, demonstrating synergism between the two stimuli. Comparisons between parallel and perpendicular flow conditions demonstrate that the flow effect was statistically significant at all except the largest topographies (Fig. 4A). Similar to planar and parallel ridge/groove topographies, isotropically ordered holed surfaces demonstrate alignment of cells in the direction of flow for all pitch values (Fig. 3 and Fig. 4B).

HAEC monolayers on topographic surfaces were also studied to determine the impact of perpendicular and parallel flow on nuclear orientation, 1200 HAEC monolayers on topographic surfaces were also studied to determine the impact of perpendicular and parallel flow on nuclear orientation. Under static conditions (i.e. no flow), nuclei on planar surfaces were randomly oriented (orientation angle of $\sim 45^\circ$). On ridge/groove patterned surfaces, there was a tendency for nuclear orientation in the direction of the long axis of the underlying topography (Fig. 5A). For planar surfaces, flow elicited a degree of nuclear reorientation in the direction of the applied flow (Fig. 5A). On ridge/groove surfaces, perpendicular and parallel flow had different effects on nuclear orientation at the smaller pitch values (< 1200 nm). More specifically, HAECs subjected to perpendicular flow exhibited nuclei that were significantly less aligned with the substrate topography than those subjected to parallel flow (Fig. 5A), reflective of the synergistic and antagonistic effects of these cues for parallel and perpendicular flow, respectively. For example, on 400 nm pitch ridge/groove surfaces, nuclei had a net orientation angle (relative to the topography) of $40.4^\circ \pm 1.8^\circ$ (mean $\pm$ SEM) under perpendicular flow conditions and $31.3^\circ \pm 1.7^\circ$ under parallel flow. In contrast, the net orientation angle of nuclei on 4000 nm pitch ridge/groove surfaces was $29.2^\circ \pm 3.2^\circ$ under perpendicular flow conditions and $25.9^\circ \pm 1.2^\circ$ under parallel flow conditions. At the higher pitch values (> 1200 nm), nuclear orientation appears to be primarily driven by substrate topography as cells under perpendicular and parallel flow exhibit similar nuclear orientations (Fig. 5A). The findings that nuclear orientation is sensitive to both flow and substrate topography at lower pitch values but is determined primarily by topography at the larger pitch values largely mirrors the trend seen for cellular orientation (cf: Fig. 3 and Fig. 4A), although the magnitude of nuclear reorientation with stimuli is lower overall. Overall, these data demonstrate a pitch dependent response to apical biophysical cues by HAECs on anisotropic surfaces. HAECs plated on isotropically ordered holed surfaces had a significantly greater degree of nuclei aligned in the direction of flow compared to static conditions for all pitch values (Fig. 5B).

3.3. Combined effects of topography and flow on cell and nuclear elongation

In addition to studying cellular and nuclear alignment, cellular and nuclear aspect ratios, measures of elongation, were analyzed. On planar surfaces, HAECs were significantly more elongated under flow conditions compared to static conditions (Fig. 6). On ridge/groove patterned surfaces, HAECs were significantly more elongated under parallel flow conditions

in comparison to static conditions on all pitch values larger than 400 nm (Fig. 6A). Under perpendicular flow conditions, on the other hand, HAECs tended to be more elongated than their static counterparts; however, the difference did not attain statistical significance for any pitch size ($p > 0.05$). HAECs on ridge/groove topographic features under parallel flow conditions were only significantly more elongated compared to perpendicular flow conditions on the 800 nm and 2000 nm pitch surfaces (Fig. 6A). Similar to parallel flow conditions, flow elicited significant HAEC elongation on holed surfaces for all pitch values (Fig. 6B).

To a lesser degree, similar trends were observed with nuclear elongation under static and flow conditions. On ridge/groove patterned surfaces, both parallel and perpendicular flow conditions tended to elongate HAEC nuclei relative to the static controls for all pitch values; however, the extent of the elongation was statistically significant only for parallel flow and only at the larger pitch values (> 1200 nm; Fig. 7A). On hole patterned surfaces, flow also tended to elongate HAEC nuclei for all pitch values; however, the effect did not attain statistical significance ($p > 0.05$; Fig. 7B).

3.4. Combined effects of topography and flow on HAEC migration

We also investigated the effect of substratum topography on various aspects of flow-induced migration in HAECs. Under perpendicular flow conditions on ridge/groove surfaces, HAECs migrated at similar speeds as cells on planar surfaces (data not shown). Surface topography did, however, alter migration direction with the average angle between the migration path and the flow direction increasing significantly for pitch values > 800 nm (Fig. 8A). Surface topography also influenced migration tortuosity with migration paths becoming significantly more tortuous on pitch sizes > 1600 nm (Fig. 8A). Representative dot plots of HAEC migration paths clearly demonstrate the competing effects of flow and surface topography on cell migration, particularly at the larger pitch values (Fig. 8B).

4. Discussion

Previous investigations into the impact of substratum topography on VEC morphology under static conditions have shown increasingly pronounced cellular alignment in the direction of topography as pitch size increases [11]. VECs on planar substrates have long been known to align in the direction of flow under steady laminar shear stress [16,23,33,34]. In examining the effects of topography or flow separately, our present results (Figs. 3 and 4) are similar to these previous reports. When the two stimuli were combined, however, we found a dynamic interaction between substratum topography and flow. When HAECs were exposed to flow parallel to the surface topography, they exhibited greater alignment than shown by HAECs exposed to either stimulus alone, demonstrating a pitch dependent synergistic interaction when the vectors of the biophysical cues are congruous. In contrast, flow perpendicular to the underlying anisotropically ordered topographic cues resulted in an antagonistic interaction that, particularly at smaller pitch values, significantly reduced alignment with the long axis of the ridges and grooves. In contrast, on isotropically ordered holed substrates realignment to flow did not change significantly with pitch size (Fig. 4B). This finding indicates that the presentation of an anisotropic topographic cue (ridges and grooves) was required to elicit the competitive interaction and that the presence of a topographic cue that does not provide a directional vector is insufficient for eliciting the observed synergistic/antagonistic alignment behavior.

The present cell alignment results with the simultaneous presentation of ridge/groove topographic cues on the cells' basal surface and flow on their apical surface indicate that VECs effectively integrate multiple biophysical cues. The dependence of the results on the pitch of the topographic features suggests that while the cell alignment response is driven

primarily by the topographic cue at high pitch values, the relative contribution of the apical flow cue increases as pitch size decreases. The mechanistic basis of this behavior remains unknown and merits future investigation.

Similar to the cell overall, the nucleus has long been known to align to directional cues, both for surface topography and flow [35,36]. Recent work has also combined the two stimuli in human mesenchymal stem cells [25]. Due to the importance of the nucleus, nuclear morphology has long been speculated to be an important component of cellular mechanotransduction [23,37–39]. Similar to published results, HAEC nuclei on planar surfaces had a net random orientation under static conditions but aligned in the direction of applied flow (Fig. 5A). Likewise, HAEC nuclei exposed to ridge/groove topography under static conditions align in a pitch dependent manner. By combining topography and flow, we found that when the direction of flow was parallel to the ridge/groove topography, nuclear alignment increased. However, when the direction of flow was perpendicular to the ridge/groove topography, cell nuclei resisted realignment in the direction of flow and remained largely aligned with the topography. Overall, the extent of nuclear realignment to flow was limited compared to cellular alignment and was broadly absent for pitch sizes greater than 1200 nm. This effect appears to depend on the directional nature of the biophysical cues as cell nuclei on holed surfaces did not resist realignment to flow (Fig. 5B).

The relationship between cellular alignment and nuclear alignment remains to be fully defined. The major components of the cellular cytoskeleton are coupled to the nuclear envelope via the nesprin family of proteins [40–43]; therefore, it is certainly conceivable that cytoskeletal alignment drives nuclear alignment directly through the mechanical connections between the cytoskeleton and the nucleus. Because nuclei are particularly rigid [38,44], it would not be surprising that the extent of nuclear alignment would be smaller than that of the cell overall as observed here. The alternative possibility that cytoskeletal and nuclear alignment occur independently of one another cannot be excluded at this point, however.

In addition to the alignment to directional biomechanical cues as discussed above, VECs are also known to elongate in response to biomechanical stimuli including unidirectional flow [16,23,36,45], topography [11], and substrate stretch [45]. *In vivo*, VEC morphology has been linked to atherosclerosis, where elongated VECs are associated with an anti-inflammatory phenotype and cuboidal VECs are found in pro-inflammatory regions of the vasculature [16,23]. Importantly, recent work has demonstrated that VEC shape alters the inflammatory state of the cells, implying the existence of a direct relationship between cell shape and inflammation [46]. VEC elongation by both shear flow [23] and by substratum ridge/groove topography [11] has been previously demonstrated, and our present findings are consistent with these reports. We have found that when substratum topography and apical shear flow are combined, cellular elongation is a function of the relative orientation of flow and topography as well as the pitch of the underlying topographic features (Fig. 6). When the two stimuli are applied parallel to one another, HAECs exhibit dramatic elongation beyond that induced by either stimulus on its own, demonstrating a clear additive effect. When flow is applied perpendicular to the long axis of the underlying topographic features, there is a trend towards higher cellular aspect ratios relative to cells under static conditions, and this trend is present at all pitch sizes. Although the differences in aspect ratios did not attain statistical significance, the observed trend is suggestive of a level of interaction between flow and topography when it comes to cellular elongation. These data, taken together with the cellular orientation results where flow perpendicular to the underlying anisotropically ordered topographic features had clearly antagonistic effects, suggest that orientation and elongation may be regulated independently in response to multimodal biomechanical stimuli. Again, there is a clear requirement for directional stimuli

provided by anisotropic surface order, as isotropically ordered holed surfaces had limited impact on aspect ratio under either static or shear conditions (Fig. 6B).

Similar to cell cytoplasmic results, HAEC nuclei were also observed to elongate, although the effect was much less pronounced (Fig. 7). The observed nuclear elongation was broadly in agreement with previous reports using flow [38], substratum topography [35], or both [25]. Combined, these data demonstrate that nuclear elongation is both pitch- and flow-dependent. Furthermore, these data demonstrate that apical shear flow is the more dominant factor in determining nuclear elongation whereas topography may be more dominant in determining cellular elongation.

In addition to regulating cell and nuclear shape and orientation, our results demonstrate that combined biophysical cues also regulate VEC migration. Under static conditions, VECs on ridge/groove substrates of pitch 1200 nm had previously been shown to migrate in the direction of the underlying topographic cues [11]. VEC migration in the direction of flow is also well documented [47,48]. In agreement with previous flow results, HAECs on planar surfaces exposed to a steady shear stress of 20 dyne/cm² migrated in the flow direction (Fig. 8) with an average angular deviation of less than 30° and limited tortuosity. When migrating in response to flow across perpendicular ridge/groove topographic features, however, the migration became much less directed along the axis of flow, demonstrating the impact of the biophysical cues associated with the substratum on cellular behavior. The migration paths were mixed with some cells moving smoothly in a vector that reflected the influence of both the topography and the shear flow whereas other cells switched between migration in the direction of the topography and that of flow. This switching, in contrast to the relatively smooth migration seen on planar surfaces, resulted in a more tortuous path on topographic surfaces. Interestingly, tortuosity is minimally impacted at pitches smaller than 1600 nm, while deviation from flow direction occurred at the smaller pitch values. These data provide insight into the impact of substrate topography on flow-induced migration and demonstrate that through complex dependence on topographic pitch size, various aspects of cellular migration can be independently modulated.

It is important to note that this study used a single shear condition, chosen as a physiologically representative condition. It is known that flow patterns *in vivo* can vary substantially and regions of disturbed flow correlate with the development of atherosclerotic lesions [7,8,49,50]. It remains poorly understood, however, what effect lesion development has on the substrate mechanics experienced by VECs. As both surface topography [19] and flow [5,16,23] are known to separately regulate gene expression in VECs, an important future direction will be determining the combined effects of topography and flow on gene expression and protein levels as well as establishing if gene and protein levels are sensitive to topographic pitch. Indeed, if the synergistic and antagonistic effects observed here for VEC orientation and elongation extend to gene expression, then the combination of substrate topography and flow promises to provide powerful strategies for controlling cell phenotype in a variety of applications including prosthetic design and rapidly evolving strategies in regenerative medicine.

5. Conclusions

We found that cellular behavior in response to the combined biophysical cues is highly dependent on pitch. We also found differences between the regulation of cell realignment to flow and nuclear realignment to flow. Importantly, we found that many of these relationships are differentially regulated. For example, topographic features orientated perpendicular to the direction of flow was found to cause HAECs to resist alignment with flow but not flow-induced elongation, while the synergistic effects of flow parallel to the

long axis of underlying topographic features were apparent in both elongation and alignment. Additionally, two measures of flow-induced migration, direction and tortuosity, exhibit sensitivity to the pitch of topographic features. These data reveal a complex role for substrate mechanics, especially when combined with other stimuli, and support the need for investigations into the signaling mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2012.02.047.

Fig. 1.

VECs experience biophysical cues on the basal and apical surfaces. VECs anchor to a specialization of the extracellular matrix known as the basement membrane. This basement membrane presents biophysical cues to the cell in the form of topography. Hemodynamic biophysical cues are presented to the apical aspect of the VECs in the form of shear flow.

Fig. 2. Design and Validation of microflow chamber. Synthetic planar, ridge and groove, and holed surfaces were fabricated from PDMS masters. Pitch is defined as ridge + groove width (or hole diameter for holed substrates) and lateral distance to the adjacent feature (A). Cell culture medium is drawn under the PDMS chamber and across the topography (B). Computed shear stress magnitude and direction within the chamber, demonstrating near-uniform shear magnitude and direction across the topography (magenta box) (C).

Fig. 3. HAECs respond in an interdependent manner to combined laminar shear stress and topographic cues. HAECs had a stochastic net orientation on planar surfaces under static conditions. A steady laminar shear stress of 20 dynes/cm² for 24 h resulted in cell elongation and alignment in the direction of flow. HAECs on topographic ridges/grooves oriented parallel to the direction of flow demonstrate a synergistic impact of the two cues on cellular orientation, whereas ridges/grooves oriented perpendicular to flow have an antagonistic effect. Cellular alignment changes are demonstrated by reorientation of actin stress fibers (red), microtubules (green), and nuclear morphology (blue). Scale Bar = 50 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Relative orientation of the basal and apical biophysical cues regulates HAEC orientation. (A) For ridge/groove surfaces under perpendicular flow, HAECs resisted alignment with flow for pitch values greater than 800 nm. Under parallel flow, HAECs had a greater degree of alignment to both topography and the direction of flow on all pitch sizes compared to static conditions. Significant differences: $\square$ = parallel flow vs. static, # = perpendicular flow vs. static, *, ** = perpendicular flow vs. parallel flow. (B) On holed surfaces, HAECs aligned in the direction of flow for all pitch values. Significant differences: * = flow vs. static. All data are mean $\pm$ SEM.

Fig. 5. Relative orientation of the basal and apical biophysical cues regulates the alignment of HAEC nuclei. (A) Under perpendicular flow, HAECs resisted alignment on all pitch sizes compared to static conditions. Under parallel flow, nuclei had a greater degree of alignment to both topography and the direction of flow on all pitch sizes compared to static conditions. Compared to parallel flow, perpendicular flow resisted the effects of substrate topography for pitch values ≥ 1200 nm. Significant differences: ## = perpendicular flow vs. static, * = perpendicular flow vs. parallel flow. (B) Holed surfaces increased the alignment of nuclei in the direction of flow for all pitch values. Significant differences: *, ** = flow vs. static. All data are mean $\pm$ SEM.

Fig. 6. Relative orientation of the basal and apical biophysical cues regulates HAEC elongation. (A) On ridge/groove surfaces, parallel flow-induced significant HAEC elongation compared to static controls for all pitch values larger than 400 nm. Perpendicular flow tended to elongate HAECs for all pitch values; however, the elongation did not attain statistical significance. Significant differences: □, □□ = parallel flow vs. static, # = perpendicular flow vs. static, *, **, *** = perpendicular flow vs. parallel flow. (B) On holed surfaces, flow increased cellular elongation for all pitch values. Significant differences: * = flow vs. static. All data are mean $\pm$ SEM.

Fig. 7.

Relative orientation of the basal and apical biophysical cues regulates HAEC nuclear elongation. (A) On ridge/groove surfaces, both parallel and perpendicular flow tended to elongate HAEC nuclei relative to static controls for all pitch values; however, the effect was statistically significant only for parallel flow and for pitch values ≥ 1200 nm. Significant differences: $\square$ = parallel flow vs. static, # = perpendicular flow vs. static, * = perpendicular flow vs. parallel flow. (B) On holed surfaces, flow tended to elongate HAEC nuclei for all pitch values; however, the effect did not attain statistical significance ($p > 0.05$). All data are mean $\pm$ SEM.

Fig. 8.

Relative orientation of the basal and apical biophysical cues regulates HAEC migration. (A) Under perpendicular flow on ridge/groove surfaces, HAECs exhibited significantly larger angles between migration direction and flow for pitch values ≥ 800 nm and more tortuous migration paths for pitch values ≥ 1600 nm. Significant differences: *, **, *** = pitch vs. planar. Data are mean $\pm$ SEM. (B) Dot plots of migration paths demonstrating cell migration in the direction of flow on planar surfaces. On ridge/groove surfaces oriented perpendicular to flow, substrate topography competes with flow in determining migration direction, and the effect is more pronounced at larger pitch values. Axes are in μm .