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Sub-micron and nanoscale feature depth modulates alignment of stromal fibroblasts and corneal epithelial cells in serum-rich and serum-free media

Sarah A. Fraser¹, Yuk-Hong Ting², Kelly S. Mallon³, Amy E. Wendt², Christopher J. Murphy³, and Paul F. Nealey¹

¹Department of Chemical Engineering, School of Engineering, University of Wisconsin, Madison, Madison, Wisconsin 53706

²Department of Electrical and Computer Engineering, School of Engineering, University of Wisconsin, Madison, Madison, Wisconsin 53706

³Department of Surgical Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, Madison, Wisconsin 53706

Abstract

Topographic features are generally accepted as being capable of modulating cell alignment. Of particular interest is the potential that topographic feature geometry induces cell alignment indirectly through impacting adsorbed proteins from the cell culture medium on the surface of the substrate. However, it has also been reported that micron-scale feature depth significantly impacts the level of alignment of cellular populations on topography, despite being orders of magnitude larger than the average adsorbed protein layer (nm). In order to better determine the impact of biomimetic length scale topography and adsorbed protein interaction on cellular morphology we have systematically investigated the effect of combinations of sub-micron to nanoscale feature depth and lateral pitch on corneal epithelial cell alignment. In addition we have used the unique properties of a serum-free media alternative in direct comparison to serum-rich medium to investigate the role of culture medium protein composition on cellular alignment to topographically patterned surfaces. Our observation that increasing groove depth elicited larger populations of corneal epithelial cells to align regardless of culture medium composition and of cell orientation with respect to the topography, suggests that these cells can sense changes in topographic feature depths independent of adsorbed proteins localized along ridge edges and tops. However, our data also suggests a strong combinatory effect of topography with culture medium composition, and also a cell type dependency in determining the level of cell elongation and alignment to nanoscale topographic features.

Keywords

nanotopography; corneal epithelial cells; contact guidance

INTRODUCTION

A common approach to biomaterial design is to mimic the physical structures and chemistry of the native tissue, and to elicit the required cellular responses for tissue growth.¹ To this end, we have focused on the interaction of human corneal epithelial cells (HCECs) with nanoscale topography, motivated by detailed study of the architecture of the native corneal basement membrane through which these cells adhere to the underlying stroma.^{2–5} Corneal epithelial cells and stromal fibroblasts in their native environment reside on highly topographic surfaces, which are thought to provide a multitude of cues to the cells *in vivo*.

Basement membranes with which corneal epithelial cells interact are composed of a complex topography of intertwining fibers of varying nanoscale (1–100 nm) and submicron (100–900 nm) dimensions (Fig. 1). Dimensions of individual features range from ~30 to 400 nm and occur at varying frequencies.^{3,5–10} The vast majority of the corneal stroma, within which stromal fibroblasts are found, consists of 200–500 layers of flattened collagenous lamellae. In the anterior one third of the stroma, collagen lamellae are thin (about 0.2–1.2 μm thick and 0.5–30 μm wide). In the posterior stroma, collagen lamellae are arranged parallel to the surface and are thicker (1.0–2.5 μm thick and 100–250 μm wide). The rich nanoscale and submicron architecture ensures a single cell interfaces with thousands of topographic features of the underlying basement membrane. We have primarily focused on investigating the modulation of corneal cell behaviors by nano, submicron, and micro-patterned substratum topographic features in comparison to traditional flat culture surfaces.

To facilitate these studies we designed and fabricated silicon surfaces that contain a range of feature sizes from 400 to 4000 nm in pitch (pitch = groove width + ridge width) as well as incorporating planar control regions. The smallest feature sizes on these chips were 70-nm ridges on a 400-nm pitch. Thus we were able to replicate, on a single chip, features that mimicked the biologic length scale of native basement membranes. These chips also contain micron-scale structures that allowed us to investigate transitions between micro and nanoscale features and also provided a link to the bulk of the literature, as well as planar control regions^{11,12} (Fig. 2). It should be noted that human stromal cell morphology in the native tissue and on groove and ridge topography is elongated, and the cells are aligned with respect to each other and the underlying collagen fibers. However, corneal epithelial basement membrane has a random topography, which does not induce an ordered alignment, elongation, or a particular orientation of corneal epithelial cells. Therefore, the use of anisotropic features in this study is not typical of the native environment for both cell types but is used to provide a useful way to isolate the specific effect of topography by providing easily measured and comparable cell morphology responses to different feature dimensions and to planar controls.

Alignment of cells is intrinsically linked to cell elongation using our analytical methods and it should be assumed that an aligned cell is elongated in a specific direction, thus providing an elliptical shape rather than the rounded morphology seen on planar control surfaces. Orientation of cells with respect to the underlying anisotropic topography as described in this study is defined as the angle the longest axis of an aligned cell makes with the groove/ridge direction.

Specifically related to this study, we have previously published data documenting that the cell morphology, proliferation, adhesion, and migration of epithelial cells on nano and submicron spaced features with a constant depth (300 nm) can differ markedly from their behavior on substrates with feature spacing >1 μm and flat controls.^{7,11–16}

Early work by our group also demonstrates that the ratio of groove-to-ridge width has significantly less impact on the level of corneal cell alignment than the feature pitch (sum of

groove and ridge width). Previous work has shown that ridge widths of 70 nm with a pitch of 400 nm elicit the same level of alignment of corneal epithelial cells (~30%) to those in practice at a variety of groove to ridge width ratios within this pitch constraint.¹⁷ Work is currently underway to more closely investigate the impact on focal adhesion orientation under this working theory.

We have also previously determined that topography can work in concert with the culture environment to modulate fundamental cell behaviors. In particular reference to this study, it has been shown that the orientation angle of primary HCECs to underlying topography at sub-micron and nanoscale spaced feature sizes was modulated by the composition of the cell culture media. Cells cultured in Epilife™ (Cascade Biologics) on silicon oxide treated surfaces were found to align parallel to micron-sized grooves and ridges, but align perpendicular to these features at nanometer length scales.¹⁷ This differs from HCECs cultured in supplemented hormone epithelial medium (SHEM + 10% FBS) in which cells align parallel to all feature sizes. Cells are rarely, if ever, aligned and elongated at angles other than parallel or perpendicular on any topographically patterned substrates in either culture medium composition.¹⁷ We term “aligned” cells to be those that have a long axis aligned either parallel or perpendicular to the underlying anisotropic features, when compared to cell morphological characteristics of control populations cultured under identical conditions on planar surfaces of identical chemistry.

That the cell orientation at these chosen angles is strictly bimodal has been previously discussed in work by Teixeira et al. which illustrates the full range of cell distribution angles and the lack of orientation at any angle between 0 and 90° to the topography when cells were cultured in similar serum-rich and serum-free culture medium to that detailed in this study.¹⁸

Also pertinent to this study, the mechanism whereby lateral topographic feature spacing and protein adsorption influence cell behaviors remains poorly understood. Investigating micron scale features, Curtis and Clarke¹⁹ speculated that cells react to topography primarily at lines of discontinuity (i.e. at the surface) by actin nucleation. In addition, it has been postulated that the presence of feature edges provides mechanical “footholds” for adhesion and directed motility, thus driving cell body polarization and alignment.²⁰

Importantly, surface effects are not the only parameter that affects cell population alignment on topographically patterned substrates. We and others have demonstrated that feature depth impacts the level of cell alignment on topographically patterned surfaces.^{18,21}

However, the majority of these studies investigated depths that were both at the micron-scale or demonstrated extremes of cell alignment response and were undertaken in serum-rich culture medium, which only induced parallel alignment of cells. It was not yet known whether perpendicularly oriented cells would respond to changing feature depth in the same manner as those orientated parallel to the feature direction. Thus these studies did not adequately isolate and describe the role of topography from that of selective protein adsorption theories in these model systems, and there remains a fundamental question as to whether the topographic geometry is driving the cell alignment indirectly via selective adsorption of protein or directly via topographic effects.

Our aim in this study was to help to determine the role of topography in context with adsorbed proteins on the alignment of corneal cells. The ability to successfully culture corneal epithelial cells in significantly different (protein rich and essentially protein free) medium compositions, and advances in X-ray lithography and etching techniques have for the first time allowed us to practically investigate the role of well-controlled topographic feature spacing and depth at a variety of controlled depths and lateral spacing at the

nanoscale (at sizes commensurate with those of the native tissue) in context with changing the culture medium protein composition.²² In addition, our prior observation that serum-free medium induces a perpendicular and parallel orientation shift with respect to lateral feature spacing allowed us to investigate the impact of ridge edge directional cueing in context with feature depth on cell alignment.

In this study, we have systematically demonstrated that increasing nanoscale feature depth of 75–800 nm increased corneal epithelial cell alignment on micron, submicron, and nanoscale topography under serum-rich and serum-free conditions. In particular, deeper features increased cell alignment regardless of cell orientation angle to anisotropic topographic ridges or edges.

We further demonstrate that the level of cell population alignment was highly dependent on the culture medium composition, with significantly higher levels of alignment seen in serum-rich medium than in serum-free medium.

In addition, when comparing the alignment response of corneal epithelial cells and stromal fibroblasts the response was significantly cell type dependent. A significantly higher population of stromal fibroblasts aligned to shallower topographic features and larger populations aligned than corneal epithelial cells when cultured in serum-rich conditions.

MATERIALS AND METHODS

Fabrication of micron, submicron, and nanostructured substrates

Silicon wafers were cleaned using trichloroethylene, acetone, methanol, and finally rinsed in distilled water. They were then placed in a tube furnace (Wisconsin Center for Applied Microelectronics) at 1000°C, with an O₂ flow rate of 2 L/min for varying times, depending on the thickness of oxide layer required. The final thickness of the silicon dioxide layer following oxidation was measured using optical reflectance (Rudolph Research Analytical, NJ) and verified by scanning electron microscopy (SEM) postetching (Leo Electron Microscopy, Thornwood, NY). The wafers were primed with hexamethyldisilazane (HMDS) in a vacuum oven (Yield Engineering Systems, CA). They were then coated with UV3 photoresist (Shipley, MA) in a resist spinner (Solitec Wafer Processing) and baked on a vacuum hotplate at 130°C for 60 s. Photo-resist thickness was varied to obtain desired groove depths, and ranged from 300-nm-thick, spun at 4000 rpm for 45 s, to 675-nm-thick for the <550-nm-thick dioxide layers, spun at 800 rpm for 2 min, as determined by optical reflectance. The wafers coated with resist were exposed to X-rays through a patterned mask using a Suss 200/2M X-ray stepper at 700–750 mJ/cm² (Center for Nanotechnology, UW Madison). The wafers were then postbaked for 90 s at 130°C and the photoresist developed in MF320 developer (Shipley, MA) at 100% for 60 s. The photomask used was of a unique design that consisted of six patterned areas ranging from the micro- to the nano-scale of 4 mm² each within a total area of 10 mm².¹¹ Finally, the wafers were diced using a diamond saw into chips containing patterned areas separated by smooth areas. The patterned areas had pitches (distance from one feature to its repeat) of 4000, 2000, 1600, 1200, 800, and 400 nm, with a groove-to-ridge ratio of 1:1. The chips were then etched using the silicon etch stop technique described below.

Silicon etch stop etching

Etching was undertaken in a helicon plasma etcher equipped with a diode laser interferometer for *in situ* etch rate measurements.²³ The photo-resist acted as a mask allowing for etching only of the exposed silicon dioxide. Experiments were conducted in a 20 mTorr mixture of CHF₃ (20 sccm), Ar (50 sccm), H₂ (5 sccm), and helium (7 sccm). Etching was undertaken at 10 mT at 600 W using a sinusoidal waveform. Different etch

times were initially investigated and depth of silicon dioxide and over-etch into the underlying silicon wafer was determined using scanning electron microscopy (SEM) analysis (Leo Electron Microscopy, Thornwood, NY). Grooves and ridges thus created had a final depth determined by the initial thickness of deposited silicon dioxide. Sacrificial samples were fractured for cross sectional analysis and the actual depth verified by SEM (Fig. 2). Prior to cell culture, the remaining UV3 photo-resist was removed using acid cleaning in a 3:7 ratio hydrogen peroxide and sulphuric acid mixture for 30 min, the resultant surfaces were verified as clean by SEM. Finally, the samples were O₂ plasma treated for 20 s at RF power 100 W, gas flow 8 sccm, to ensure a uniform surface chemistry. All chips were sterilized by cleaning with ethanol and 15 min exposure to ultraviolet light in a sterile hood. No additional pretreatment of the surfaces with biological agents was undertaken prior to culture.

Cell source and culture

HCECs were harvested from corneas donated by the Lions Eye Bank of Wisconsin (Madison, WI) or the Missouri Lions Eye Bank (Columbia, MO). The corneal buttons were trimmed to exclude any scleral or limbal regions. The buttons were then immersed in a dispase II solution (1.2 U/mL, Boehringer, Mannheim, Germany) and placed in an incubator for 4 h. The epithelial cells became loosely adherent and were removed by gently rubbing the corneas with a pipette tip. The resulting cell suspension was centrifuged and the cells were resuspended in Epilife™ basal medium (Cascade Biologics, Portland, OR) with a growth supplement of defined composition. The growth supplement contained purified bovine serum albumin, purified bovine transferrin, hydrocortisone, recombinant human insulin-like growth factor type 1, prostaglandin, and recombinant human epidermal growth factor (EDGS, Cascade Biologics). The cell suspension was transferred to culture dishes that had been treated with fibronectin, collagen, and albumin in a buffer (FNC coating mix, Biological Research Faculty and Facility Inc., MD) at a density of 64×10^4 cells/mL in Epilife medium. The cells were incubated at 37°C and 5% CO₂ and were harvested with 0.025% trypsin–0.01% ethylene diamine tetra acetic acid (EDTA) (Cascade Biologics) when they had reached ~70% confluency. Trypsin neutralization was achieved with FBS. The HCECs were plated at a density of 10,000 cells per cm², with each well of a 24-well-plate containing one topographically patterned silicon chip. They were incubated at 37°C in 5% CO₂ for 24 h. Plating either occurred in Epilife (Cascade Biologics) or in Dulbecco's modified Eagle medium (DMEM/F12) media containing 10% FBS.

Human stromal fibroblasts were obtained from corneas donated as previously described.¹⁶ The corneal buttons were trimmed as before and the endothelial cells removed by gently scraping the posterior side of the cornea with a pipette. The epithelium was removed by incubating the corneas in a dispase solution for 4 h (1.2 U/mL Dispase II, Boehringer Mannheim, Germany) to render the cells loosely adherent, followed by careful scraping of the anterior corneal surface repeatedly with a pipette. The corneas, devoid of epithelial and endothelial components, were then placed with the concave side down, in 6-well-plates (one cornea per well) containing 4 mL DMEM/F12 supplemented with 10% FBS. The corneas were incubated at 37°C and 5% CO₂ until the fibroblasts were ~85% confluent. The corneas were then discarded and the fibroblasts harvested using trypsin (2.4 g/L)/EDTA (25 mM). Cells were centrifuged and resuspended in DMEM/F12 medium supplemented with 10% FBS, and plated at a density of 10,000 cells per cm², each containing one topographically patterned silicon chip. They were incubated at 37°C in 5% CO₂ for 24 h.

Immunocytochemistry

Cell culture medium was removed and the surface with adherent cells was rinsed with 1 × phosphate buffered saline (PBS) (Invitrogen) after 24 h of culture. The surfaces were then

fixed with 4% paraformaldehyde (Sigma) at room temperature for 20 min. Following a PBS wash, the cells were permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 5 min, washed, and then immersed in 1% bovine serum albumin (Sigma) for 20 min to block non-specific binding. Cells were rinsed with PBS, and then incubated with 5 $\mu\text{g/mL}$ of TRITC-phalloidin (Sigma) containing 0.1 μg 4',6-diamidino-2-phenylindole (Molecular Probes) in PBS for 30 min, to label filamentous actin, and to outline the nucleus, respectively. Following final washes, the cells were then imaged using a Zeiss Axiovert 200M fluorescent microscope.

Quantification of cell shape and orientation

Images of labeled cells on each of the chips were obtained using a 10 $\times$ objective lens, with four different images taken of each pattern pitch and smooth areas on the same samples. The smooth areas to be imaged were randomly determined prior to seeding and the same smooth regions imaged on each chip. On average 10–15 cells per image were present per pattern size/depth, thus allowing ~60 cells to be analyzed per pattern. Images were obtained using an epifluorescence microscope (Zeiss Axiovert 200M). Image analysis was performed using KS300 software (Zeiss). The percentage of cells aligned, cell area, and cell length measurements were taken from the images and collated using parameters previously described by our group.^{11,16} In brief, the angle between the long axis of the cell and the long axis of the underlying topographic patterns was obtained and the cells considered aligned if oriented less than 10° from the parallel or perpendicular axis (the parallel axis lining up with groove and ridge direction). Elongation was described by an extension factor, characterized as the ratio between the longest axis of the cell (the “length”) and the widest point of the cell body at 90° from the long axis deemed “width” (longest axis perpendicular to longest axis) determined for each cell type from our previous work.¹⁸ All cells in contact with others were manually removed from the data sets. The percentage of cells that were determined both elongated and aligned in each data set (which consisted of 10 silicon 6-pack chips) was determined. Results were presented as % cell, representing the mean average % of the cell population that was deemed both elongated and aligned at each feature pitch and depth. Each data set was repeated four times, with four different cell batches.

Statistical analysis

A two-way ANOVA test was used to reveal whether any of the experimental groups were affected by depth and lateral pitch. Statistical analysis using the Students *t*-test (on triplicate repeats) used for specific comparisons were undertaken following ANOVA testing, and a level of $p \leq 0.05$ was deemed statistically significant.

RESULTS

Substrate design

Feature pitches, defined as the sum of the groove and the ridge widths, ranged from 400 to 4000 nm in six different fields (400, 800, 1200, 1600, 2000, 4000 nm) on each silicon chip separated by smooth control areas. Dimensions of groove ridge widths and depths were documented using SEM and the ratio of groove to ridge width determined (Fig. 2). Samples in which the etch depth was deeper achieved with thicker photoresist layers had greater variability in etch depth at the smaller pattern pitches because of passivation of the etching when photoresist was knocked into the grooves. However, with careful control of chamber conditions this was minimized. We have demonstrated that the silicon etch stop is a reliable etching method that can provide excellent side-wall profiles combined with controlled nano-scale etch depth at a large range of micro- to nano-scale pitch sizes concurrently. The depths achieved were between 75 and 880 nm (Fig. 2).

Contact guidance of primary HCECs is more affected by groove depth than pattern pitch when cultured in medium containing 10% serum (Fig. 3)

HCECs cultured in DMEM/F12 media containing 10% FBS showed an increased elongation and alignment response to increasing groove depth at 265 nm depth and greater (Fig. 3). Contact guidance response (defined as cells both elongated and aligned) was equivalent to planar control regions (at the basal level of 10.1%) on substrates with 75–150 nm deep grooves, and peaked at 40–50% on 800 nm deep grooves at all lateral pitches. Corneal epithelial cell increase in alignment with increasing depth was not altered by pattern pitch. Using SEM, cell membranes were observed mainly in contact with ridge tops, and had not attached to groove bottoms at 400–1600 nm pitch size at any depth. The membrane of this cell type only was able to mold into the features at 75 nm depth at 2000–4000 nm lateral pitch, where the cells were not aligned or elongated to the topographic patterns, confirmed by SEM.

Filipodia and lamellopodia were observed localized to the leading and trailing edge of the elongated cell body with less elongated cell protrusions being noted around the sides of cells on all pattern pitches. A few instances of filipodia touching ridge bottoms of the larger pitches were observed, but all bridged the grooves on the 1600–400 nm pattern pitches [Fig. 3(B)].

Primary HCECs respond to nanoscale depth when cultured in Epilife culture media with hormone supplement (Fig. 4)

As previously reported by our group, HCECs cultured in Epilife media assumed a perpendicular orientation relative to the long axis of the features only on nanoscale features [Fig. 4(A)], and a parallel orientation only on microscale features [Fig. 4(B)]. A clear transition zone at 1200–1600 nm pitch was seen, with a 10% increase in cells designated perpendicularly oriented at 1200 nm pitch and smaller. Cells oriented perpendicular to small lateral features (400–1200 nm) exhibited increased elongation and alignment when groove depths were 550 nm or deeper. The number of cells oriented parallel to large feature sizes did not significantly increase until presented with grooves of 550–800 nm [Fig. 4(B)]. The largest numbers of aligned and elongated cells detected were perpendicular to 400 nm pitch patterns, which increased significantly on 550 nm and deeper grooves.

Contact guidance of primary stromal fibroblasts is affected by groove depth and pattern pitch when cultured in medium containing 10% serum (Fig. 5)

Primary stromal fibroblasts cultured under identical conditions also showed increased population of cells elongated and aligned with increasing groove depth (20–40% to 40–90%) but demonstrated differential response with respect to lateral feature spacing, not seen with HCECs. Similar levels of alignment were seen at 75–150 nm groove depth with all pattern pitches (20–40% population designated elongated and aligned parallel). Elevated levels of elongation and alignment were detected for 1200–1600 nm pitch at all depths above 265 nm, which became statistically significant at 550 nm and deeper grooves where it reached a maximum (90% elongated and aligned cells).

DISCUSSION

It is widely accepted that when immersing a substrate into a protein-rich environment such as culture medium, adsorption of a protein layer occurs across the surface. The selective adsorption of adhesive proteins is thought to mediate adhesion receptor–adhesion protein interactions. It is thought that the surface properties of a substrate determine the organization of the adsorbed protein layer, and that the nature of this layer in turn determines the cellular response to the adsorbed surfaces.^{24–27}

Previous cell signaling studies have demonstrated that cell adhesion alters integrin expression (up regulation by lack of adherence) with documented changes in gene expression being associated with changes in cell shape.²⁸ Indeed, substratum topography has been shown to directly affect the expression of integrin subunits in human gingival fibroblasts.²⁹ Cell geometry was able to switch cells from growth (in a more “stretched out” state) to apoptosis (occurs in a more rounded state)³⁰ and it was suggested that integrins are involved in the transduction process.³¹ We have previously reported substrate topography to affect the formation and architecture of focal adhesions and focal contacts.¹¹ All this data strongly supports the theory that topographic features in culture have a fundamental impact on cell behaviors, by influencing cell shape and attachment, which may be via selective adsorption of soluble proteins from the culture medium or maybe a direct interaction of the topography with the cell body.

Groups have previously rationalized the effect of topography on protein adsorption by three main theories, which are summarized and discussed below.

1. Where topography is present, but does not affect the surface composition or conformation of proteins, adsorbed proteins are presented as lines or stripes of contacts along ridge tops and it is the interaction of cells with this patterned protein surface that brings about cell alignment.

A model of protein stripes presented to cells on the ridge tops inducing alignment by virtue of being the only zone where cell attachment can occur is easy to imagine, since cells on these surfaces are localized solely on ridge tops of all dimensions.^{11,12,16} This should be differentiated from previous work, which has concentrated on shallow features combined with larger micron scale spacing of features, that elicited cell adhesion to ridge edges or “cliffs”³² where cells could easily touch the bottoms of the wide grooves.

2. Adsorbed attachment protein conformation is modified by ridge edges, which causes them to unfold and expose more attachment sites such as RGD present in fibronectin. The directed unfolding of attachment sites along ridges leads to attached cell elongation and alignment.

This model assumes that changes in feature depth will have no effect on surface chemistry, and as a result will not affect cell behavior; a result contrary to that which we and others have observed. In addition, the model does not account for changing alignment angles of cells when changing culture medium composition, or indeed the upregulation alignment perpendicularly aligned cells with increasing feature depth presented in this study.

3. The adsorbed protein layer on planar controls is different to that on topographic features, which is changed by topographic feature lateral spacing and potentially as a function of depth.

The second and third points that consider the surface effects of adsorbed proteins encountering topographic features and changing conformation and/or composition are clearly not mutually exclusive. A possible way that feature depth may affect ridge edge composition could be imagined if the adsorbed proteins were large enough to interact with both the ridge edges and the groove bottoms. Although typical attachment proteins such as fibronectin (Fn) have been measured ~120–140 nm long by 10 nm wide when fully extended in solution (~60 nm wide with double-loop conformation), ellipsometric measurements of dry adhesive proteins such as fibrinogen (similar in size to fibronectin) to self assembled monolayer substrates have demonstrated that a maximum film thickness of only 10 nm ($\pm 25\%$ error) occurs upon adsorption to the surface.³³ Optical waveguide lightmode spectroscopy has more recently been used to determine Fn adsorption at the solid

(waveguide)/liquid interface.³⁴ One such study has demonstrated that the thickness of adsorbed Fn is maximum at 17 nm when in contact with a negative surface polymer, and 20 nm when in contact with a positive surface polymer, which is estimated at 2.5–5 times larger than that on bare substrates.³⁴ It has also been demonstrated by AFM that when fibronectin is adsorbed in near physiological buffer conditions (0.1–0.2M), the Fn possesses a compact conformation, which tends to aggregate at interfaces forming clusters with approximate height of 20 nm.³⁴ In context, the protein layer thickness is significantly smaller than even the minimum depth at which corneal epithelial cell alignment and elongation was detected in this study (150–265 nm) and a mechanism by which large proteins span the gap between ridge tops and groove bottoms thus changing conformation with increasing depth and interacting with the cell membrane is unlikely from a geometric standpoint.

When considering theories 2 and 3, it cannot be conclusively determined without further work whether the feature depth alters surface protein composition or conformation. However, it is difficult to imagine that at a given nanoscale lateral spacing, changing depth would alter the surface composition of a thin layer proteins on ridge tops and edges enough to cause the increase in cell alignment observed here, and these models cannot adequately explain the increase in alignment of perpendicularly aligned cells.

The impact on alignment of cells by interaction of the cell membrane with shallow grooves was also considered, where at pitches of 2000–4000 nm some interaction of the filopodia of both cell types with the groove bottoms was observed using SEM. However, the lateral feature sizes over the range of 400–1600 nm prohibited the cell membrane from touching the groove bottoms even at the shallowest depths. No observations of cell membrane or extensions interacting with the groove bottoms at this lateral feature spacing range were made at any of the feature depths examined in this study. When the ratio of groove-to-ridge width was varied in previous studies undertaken by our group corneal epithelial cells were still seen only to interact with the ridge tops and edges when the lateral feature spacing was below 1600 nm.¹⁸ Interestingly, stromal fibroblast extensions, although generally significantly longer than those observed from corneal epithelial cells, were also not observed to interact with the groove bottoms at the same length scale implying that sensing of underlying topography by cell membrane fitting was not the stimulus for increasing cell alignment with increasing depth at this length scale.

It is possible that the filopodia and lamellopodia extending from the cell body sensed the increasing depth and transmitted signaling molecules indirectly through the cytoskeleton, resulting in elongation and alignment.³⁵ In fact, previous studies have linked alignment and elongation responses with the initiation of the Rho and Rac signaling pathways via the actin cytoskeleton and this warrants further study in light of our latest observations.^{36–41}

We propose that the issue is not whether protein adsorption or topography is in control of the situation, but whether or not the factors work individually and/or in combination. Although protein is essentially eliminated from the serum-free medium used in these experiments, many other biochemical cues are present in the medium compositions including hormones and growth factors and undoubtedly play an important role in the behaviors observed. Our data indicates an isolated individual effect of topography on cell alignment, independent of protein cues on ridge tops or edges. However, our data also supports the mechanism that topography acts in combination with soluble factors present in the culture environment to elicit elongation and alignment.^{22,42} In addition, the alignment response was extremely cell type dependent, with stromal fibroblasts demonstrating significantly more alignment under identical serum conditions than corneal epithelial cells. This data and that showing that no minimum limit of sensitivity with respect to depth was found for stromal fibroblasts suggests that not only is there a wide variability of cell

response, but also that fibroblasts, which are the traditional cell type used for such studies, may not be the ideal cell type of choice; no suitable serum-free medium is currently available to test alignment angle dependence on medium composition of stromal fibroblasts and they appear relatively insensitive to nanoscale changes in topographic features.

In summary, we have demonstrated that topography provides a fundamental and unique stimulus for corneal cell elongation and alignment and works co-operatively with cues from the culture medium and adsorbed proteins to mediate elongation and alignment of cells on topographically patterned silicon.

CONCLUSIONS

This study has demonstrated that the surface characteristics of topographically patterned surfaces are not the only driving forces for cellular alignment, and that nanoscale feature depth influences cellular alignment similar to that on the microscale. We have demonstrated a lower limit of depth for corneal epithelial alignment. This work strengthens the proposition that biomimetic nanoscale topography is a relevant and important stimulus for corneal epithelial and fibroblast cell behavior. Furthering our knowledge of the effect of topographic cues on cell behavior will allow for better design of biomaterials to be used within tissue engineering models.

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Figure 1.

The native basement membrane has a complex three-dimensional topography, containing nanometer length scale features. We can use these size scales to design synthetic basement membrane analogues. The SEM image depicts the layered outer structures of the cornea, the epithelial cell layer (which when intact, is stratified), the basement membrane (highlighted in the lower SEM image) and Bowman's layer and the underlying stroma, which contains stromal fibroblasts. Feature sizes previously determined by SEM analysis demonstrate that fibres of 50–100 nm diameter, and pores of 100 nm diameter are present in the native basement membrane of human corneas. The stroma comprises ~90% of the cornea, and consists primarily of water, layered protein fibers, and stromal fibroblasts. The unique shape, arrangement, and spacing of the protein fibers are responsible for the transparency of the cornea and influence fundamental cell behaviors. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 2.

Sub-micron and nanoscale features can be recreated in silicon substrates using photolithography and silicon etch stop etching. Grooves and ridges of 400–4000 nm lateral pitch were etched from 75–800 nm deep into silicon oxide substrates to generate cell culture arrays (6-packs) that fit into the well of a 24-well-plate. Planar regions separating the topographically patterned areas allow for direct assessment of cell behavior in controlled conditions on both smooth and textured substrates. Feature sizes were determined by SEM, and produced feature sizes of groove width 188.78 ± 56.78 nm, ridge width 217.39 ± 52.07 nm, and groove depths of 73.95 ± 1.2 nm, 145.15 ± 6.98 nm, 252.5 ± 16.26 nm, 550.88 ± 1.72 nm, 713.14 ± 5.32 nm, and 800.48 ± 1.96 nm, $n = 4$ of each etched wafer (from initial silicon oxide thicknesses of 75, 150, 265, 400, 550, 700, and 800 nm). Differences in groove depth between pitch sizes can be attributed to passivation of the etchant by photo-resist debris. These images show typical etched substrates following etching including photo-resist, and measured dimensions are indicated on each image. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 3.

Primary HCECs cultured in DMEM/F12 media containing 10% FBS showed an increased elongation and alignment parallel response to increasing groove depth independent of lateral feature dimensions. A: % cells designated both aligned and elongated at depths 265 nm and deeper at all lateral pitches were deemed significant (*) as determined by students t-test ($p = 0.05$). All depths represented as mean % alignment + s.d, and compared to smooth control areas where % elongated and aligned = 10.01, total cell number included in analysis 60–67 per lateral pitch at each depth, repeated three times with different cell batches (total $n = 4$). Significant alignment was only observed parallel to grooves of depth 265 nm and deeper. Dashed line indicates response to planar controls (10.01%). B,C: SEM micrographs show an

elongated and aligned cell on 265 nm deep features, lateral pitch 1600 nm. Cells are not designated elongated and aligned when compared to typical dimensions of HCECs cultures on sub 75 nm deep grooves, pitch 4000 nm (C). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 4.

Primary HCECs respond to pattern depth when cultured in Epilife™ culture media with hormone supplement. Cell alignment was related both to pitch and depth where a peak of response (A) perpendicular to features was seen on 400–1200 nm pitch and a peak of alignment parallel to features (B) of 4000 nm pitch, as previously reported. The perpendicular response to depth was stronger than the parallel, but both demonstrated larger aligned populations with increasing depth. Response was only significantly (* determined by Student's *t*-test $p = 0.05$) higher than smooth controls at 550 nm and deeper on 4000 nm pitch features (when oriented parallel) but from 400–1200 nm when oriented perpendicular (determined by ANOVA). Dashed line indicates response to planar controls (11.55%). C: Perpendicularly aligned primary HCECs on 400 nm pitch, higher magnification, cell in panel Figure 4(C). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 5.

Contact guidance and morphology of human stromal fibroblasts is affected by both groove depth and pattern pitch. A: Contact guidance response was significantly increased with increasing depth at all lateral pitches from smooth control areas (* determined by Students t-test ($p = 0.05$)). Dashed line indicates response to planar controls (11.55%). Stromal fibroblasts reacted to topography at the shallowest groove depths of 75 nm. A peak of response was seen at 1200–1600 nm lateral pitch at all depths >2150 nm but only became significant at 550 nm depth and deeper. B,C: Cell spreading was affected by groove depth, where no evidence of filopodia touching groove bottoms was detected at 400-nm-pitch.

[Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]