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Focal adhesion kinase knockdown modulates the response of human corneal epithelial cells to topographic cues

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Abstract

A rapidly expanding literature broadly documents the impact of biophysical cues on cellular behaviors. In spite of increasing research efforts in this field, the underlying signaling processes are poorly understood. One of the candidate molecules for being involved in mechanotransduction is focal adhesion kinase (FAK). To examine the role of FAK in the response of immortalized human corneal epithelial (hTCEpi) cells to topographic cues, FAK was depleted by siRNA transfection. Contrary to expectations, FAK knockdown resulted in an enhanced response with a greater number of hTCEpi cells aligned to the long axis of anisotropically ordered surface ridges and grooves. Both underlying topographic features and FAK depletion modulated the migration of corneal epithelial cells. The impact of FAK knockdown on both migration and alignment varied depending on the topographic cues to which the cells were exposed, with the most significant change observed on the biologically relevant size scale (400 nm). Additionally, a change in expression of genes encoding perinuclear Nesprins 1 and 2 (SYNE1, 2) was observed in response to topographic cues. SYNE1/2 expression was also altered by FAK depletion, suggesting that these proteins might represent a link between cytosolic and nuclear signaling processes. The data presented here have relevance to our understanding of the fundamental processes involved in corneal cell behavior to topographic cues. These results highlight the importance of incorporating biophysical cues in the conduction of in vitro studies and into the design and fabrication of implantable prosthetics.

Keywords

Cell signaling; Cornea; Nanotopography; Biomimetic material; Focal adhesion kinase

1. Introduction

Corneal diseases affect over 10 million people worldwide and remain a major cause of blindness [1]. At this time, the only well-established treatment for corneal blindness is transplantation with corneal donor tissue or with a biosynthetic corneal prosthesis. However,

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Conflict of interest

The authors have no conflict of interest.

the number of suitable donor transplants does not meet the demand and there are few widely utilized keratoprosthesis available. Recently, bioengineering approaches for the development of improved corneal prostheses have begun to incorporate biophysical and chemical cues into their design to better mimic the native cellular microenvironment.

Corneal epithelial cells in vivo attach to the underlying stroma through the basement membrane, a specialized extracellular matrix (ECM) which possesses a rich three-dimensional felt-like topographic landscape comprising bumps, fibers, and pores with feature sizes in the nano- and submicron range [2–4]. The impact of topographic cues on fundamental cell behaviors including shape [5], adhesion [6], proliferation [5,7,8], migration [9,10], cell alignment [2,11–13], and survival [8] has been reported by our lab and others, documenting that in addition to chemical signals, cells respond to biophysical cues that are intrinsic attributes of their microenvironment. However, the mechanisms that allow the cells to identify and react to topographic features remain poorly understood. To study the impact of topographic cues on cell behavior, surfaces patterned with anisotropically ordered ridges and grooves spanning the nano- to micron size scale have been developed that have been shown to induce corneal epithelial cells to lose their random orientation and preferentially align parallel or perpendicular to the long axis of the underlying topographic features [9,14].

As cells attach to the extracellular matrix through transmembrane proteins, mechanical stimuli from outside of the cell can be transmitted to the nucleus through the cytoskeleton. Extracellular mechanical stimuli modulate adhesion, migration, and cytoskeletal dynamics, resulting in changes in nuclear shape and hence cellular gene expression patterns. However, the molecular signaling mechanisms involved in these processes are not fully understood. We therefore set out to investigate the effects of micro- to nanoscale topographic features on signaling events both near the extracellular matrix interface and near the nucleus.

The extracellular matrix is linked to the contractile cytoskeleton by focal adhesions which are formed upon binding of integrin family transmembrane receptors to extracellular ligands. Aside from this structural role, the formation of focal adhesions induces several intracellular signaling processes. Among the multiple signaling molecules that are recruited to focal adhesions upon integrin clustering is focal adhesion kinase (FAK, encoded by the PTK2 gene), an intracellular non-receptor protein-tyrosine kinase that localizes indirectly to integrins through interactions with integrin-associated proteins [15,16]. FAK is activated in focal adhesions through autophosphorylation at tyrosine 397 [17], which induces the recruitment of multiple signaling and structural proteins including vinculin [18], paxillin [19,20], talin [21], p130Cas [22], and Src family kinases [23] and initiates multiple additional signaling pathways [24]. Multiple reports have documented FAK to participate in diverse cell processes including cell cycle [25,26], survival [27], and proliferation [8]. In particular, several studies provide strong evidence for a role of FAK in migration events [24]. Most notably, cultured fibroblasts isolated from FAK null mice, which demonstrated an embryonic lethal phenotype, display decreased cell motility [28]. Additionally, FAK has been proposed to be implicated in mechanosensing based on the observation that compared to wild type cells, FAK null fibroblasts show a reduced response to differences in substrate stiffness [8] and to traction forces applied to flexible substrates [29].

The mechanical link between the cytoskeleton and nucleus is mediated by a number of nuclear membrane proteins, including SUN proteins [30–32], lamins [33], emerins [34] and Nesprins [35–37]. Nuclear envelope spectrin repeat proteins, a family of large proteins (Nesprin 1, 2 and 3, ~6000–8000 amino acids [38]) found in the outer nuclear membrane, are also known as “syne” and “myne” proteins due to their presence in synaptic nuclei [39] and muscle cells [40]. Like many other spectrin repeats, Nesprins provide a structural link between cell membranes and cytoplasmic proteins. Nesprins 1 and 2 link cytoskeletal F-

actin to the outer nuclear membrane (ONM) while Nesprin 3 links intermediate filaments to the ONM via plectin [38]. Among the various Nesprins, Nesprin 2 isoforms are spatially localized to multiple sites including the membrane of the nuclear envelope, intra-nuclear foci, mitochondria and the plasma membrane foci [41]. More recently, Warren et al. [42] demonstrated that extracellular signal regulated MAPK1 and 2 (ERK1/2) are capable of regulating Nesprin 2 variants. It is known that adhesion disassembly and FAK-Src signaling are mediated by ERK and MLCK [43]. However, the molecular signaling link between the proximal signaling pathways such as FAK with the peri-nuclear proteins such as Nesprins remains unexplored.

The aim of this study was to investigate the role of FAK in mechanosensing and migration in immortalized corneal epithelial cells on biologically relevant topographically patterned substrates and to determine the relationship, if any, between FAK and Nesprins.

2. Materials and methods

2.1. Fabrication of NOA81 topographically patterned substrates with ridge/groove features

Patterned silicon chips containing ridge and groove features were fabricated using X-ray lithography as previously described [6]. The silicon masters were fabricated either containing an array of six (2×2 mm) areas with pitches (pitch = ridge width + groove width) of 400, 800, 1200, 1600, 2000 and 4000 nm separated by planar control areas; termed “six-packs”, or larger surfaces (6.5 cm^2) possessing a single pitch of 400, 1400 or 4000 nm as well as chemically identical planar surfaces; termed “monotypic”. The dimensions of the various topographies were of equal ridge and groove width with a groove depth of 300 nm. A composite stamp of the silicon chip master was made of poly(dimethylsiloxane) (PDMS) to retain the topographic features. The pattern could then be replicated into a thin layer of NOA81 (Norland Products, Cranbury, NJ) optical adhesive [44] deposited onto 35 mm (for six-packs) or 60 mm (for monotypics) tissue culture plates or glass cover slips using a spin coater and cured in an XL-1500 UV cross-linker under 365 nm light for 100 min. NOA81, a proprietary mercapto-ester compound of Norland Products, is supplied as a single component liquid adhesive that readily cures as a rigid polymer with exposure to UV light. Research from our laboratory has previously demonstrated NOA81 as a suitable material for cell culture [7,45,46].

2.2. Preparation of substrates for cell culture

In preparation for cell culture, all NOA81 surfaces were sterilized by 280 nm UV light for 20 min in a laminar flow hood. Immediately prior to cell seeding the surfaces were briefly treated with FNC coating mix (Athena Enzyme Systems, Baltimore, MD).

2.3. Cell culture and isolation of primary human corneal epithelial cells

Immortalized human corneal epithelial cells (hTCEpi) [47], kindly provided by Dr. James V. Jester (UC Irvine), were maintained in EpiLife medium supplemented with epithelial defined growth supplement (EDGS, Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin (Gibco, Carlsbad, CA) and were used between passages 40–70.

The alternative medium for the culture of human corneal epithelial cells, termed EP medium, is composed of F12:DMEM (3:1), supplemented with fetal bovine serum (2.5%), hydrocortisone ($0.4 \mu\text{g ml}^{-1}$), cholera toxin (8.4 ng ml^{-1}), insulin ($5 \mu\text{g ml}^{-1}$), adenine ($24 \mu\text{g ml}^{-1}$), epidermal growth factor (10 ng ml^{-1}) and penicillin/streptomycin (1%).

Primary human corneal epithelial cells (HCECs) were isolated as previously described [7,11] with slight modifications. Briefly, scleral and limbal areas of the corneas were

trimmed and the remaining tissue was immersed in dispase solution (1.2 U ml^{-1} , Boehringer, Mannheim, Germany) at 37°C for 4 h. Corneal cells were removed by gentle rubbing of the anterior surface with a pipette tip. The resulting cell suspensions were pooled and centrifuged and the corneal epithelial cells were resuspended in EP medium. Cell suspensions from one to two corneas were plated onto feeder cells in T25 cell culture flasks. Primary HCECs were expanded and utilized between passages 2–5. Feeder cells for the initial culture were prepared by mitotically inactivating Swiss 3T3 fibroblasts with mitomycin C ($4 \mu\text{g ml}^{-1}$) and were removed by treatment with 0.02% EDTA before plating the HCECs for the experiments.

2.4. Transfection of hTCEpi cells with siRNA

At 60–80% confluence, siRNA transfections were done on hTCEpi cells (cultured in EpiLife) using the HiPerfect transfection reagent (Qiagen, Valencia, CA) following the manufacturer's instructions with final siRNA concentrations of 50 nM. We used Hs_PTK2_11 (FAK) and AllStars Negative Control siRNA (Qiagen, Valencia, CA). 48 h after transfection, cells were harvested for RNA isolation with the RNeasy kit (Qiagen, Valencia, CA). In all experiments, knockdown to expression levels below 20% was achieved as determined by real-time qPCR analyses using aptamers specific to FAK.

2.5. RNA isolation and real-time qPCR analyses

To determine the expression levels of FAK, Nesprin 1, Nesprin 2 and ROCK1 in cells cultured on topographically patterned surfaces, cells were harvested for RNA isolation 12 h after plating on FNC coated topographically patterned surfaces (400 nm, 1400 nm and 4000 nm pitch sizes) and planar control surfaces. The RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's instructions and equal amounts were used for the qPCR reactions.

Expression levels of FAK (PTK2), Nesprin 1 (SYNE1), Nesprin 2 (SYNE2), ROCK1 and GAPDH were determined by real-time qPCR analysis using the Taqman One-Step PCR kit (Applied Biosystems, Carlsbad, CA) and aptamers specific to FAK (Hs00178587_m1), Nesprin 1 (Hs00323942_m1), Nesprin 2 (Hs00794881_m1), ROCK1 (Hs00178463_m1) and GAPDH (Hs00178463_m1, all from Applied Biosystems, Carlsbad, CA). The reverse transcription reaction was performed in a StepOne qPCR machine (Applied Biosystems, Carlsbad, CA) with the following parameters: 30 min at 50°C followed by 10 min at 95°C ; 40 cycles of 60°C for 1 min followed by 95°C for 15 s. Relative expression levels of the genes of interest were normalized to the expression of GAPDH. The experiment was performed in triplicate and repeated three times.

2.6. Orientation/alignment analysis

Cells were plated at a density of $15,000 \text{ cells cm}^{-2}$ in 35 mm dishes containing two six-pack polyurethane substrates with topographic features ranging from 400 to 4000 nm pitch and intervening control planar regions. After 4 h, cells were fixed for 20 min with 2% paraformaldehyde in phosphate buffered saline (PBS, pH 7.2) and permeabilized with 0.1% Triton X-100 in PBS followed by staining with Alexa Fluor 568 phalloidin (Invitrogen, Carlsbad, CA) and DAPI (BioGenex, San Ramon, CA). Images were taken at $10\times$ magnification with an Axiovert 200 M microscope (Carl Zeiss, Germany). The orientation of the cytoskeleton of individual cells in relation to the underlying pitch was determined from fluorescent images using IGOR 6.2 (Wavemetrics, USA). Cells included for analysis had to be fully contained within the border of the image, not in physical contact with other cells, and not undergoing mitosis. The orientation of the cells was based on the angle between the major axis of the object and the underlying feature. For each cell, an ellipse was created automatically based on the defined boundary of the object; from this ellipse, the

major and minor axes were defined. Cells were considered aligned parallel with the ridges and grooves when this angle was between 0° and 10° and perpendicular when the angle was between 80° and 90°. Cell elongation is defined as the ratio between the length and breadth of each cell. Cells were considered elongated if this factor was >1.3. A total of 100–250 cells for a given pitch size in four replicates were analyzed.

2.7. Immunocytochemistry

Successful knockdown was confirmed on a protein level by immunocytochemistry. Cells cultured on glass cover slips were fixed with ice-cold methanol for 5 min and permeabilized in 0.05% Triton X-100. Nonspecific binding sites were blocked with 0.1% bovine serum albumin (BSA) overnight at 4 °C. The cells were incubated with primary antibody (rabbit anti FAK pY397, Invitrogen, 1:50 dilution) for 2 h at room temperature (RT), followed by three consecutive washes in PBS. Incubation with secondary antibody (goat anti rabbit 488, Thermo Fisher Scientific, 1:400 dilution) was done for 1 h at RT. To ensure that the signal was not due to nonspecific binding of the secondary antibody we included a control without the primary antibody which did not lead to any signal. Nuclei were stained with DAPI (BioGenex, San Ramon, CA). Cells were imaged at 40× magnification using the Axiovert 200 M microscope (Carl Zeiss, Germany).

2.8. Time lapse microscopy and migration analysis

For cell migration analysis, hTCEpi cells were seeded at a density of 8000 cells cm⁻² onto patterned NOA81 surfaces and allowed to adhere in an incubator for 3–4 h at 37 °C and 5% CO₂. Plates were transferred to a Zeiss incubated microscope stage to maintain cell culture conditions.

Using Axiovision 4.6 software (Carl Zeiss, Germany) and the automated stage of an Axiovert 200 M microscope (Carl Zeiss, Germany), areas containing each topographically patterned region (400 to 4000 nm pitch) as well as planar control areas were identified and marked. Sequential phase contrast images of each marked area were taken every 10 min over a 12 h period at 10× magnification. Only individual cells that remained within the field of view and did not come into contact with another cell or undergo cell division during the course of the experiment were analyzed. The centroid of each cell was identified and the *X* and *Y* coordinates were recorded using a cell tracker module in Axiovision 4.6 (Carl Zeiss, Germany). From the resulting data from four independent experiments, cell trajectories, total distance, migration rate, and the tortuosity as an expression of the directional motility were calculated for a total of 19–87 cells per topographic feature or planar control.

2.9. Scanning electron microscopy

Control and FAK siRNA transfected hTCEpi cells were plated 48 h post transfection on glass cover slips with six-pack patterned surfaces. Four hours after plating they were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The samples were then rinsed in 0.1 M phosphate buffer to remove fixative and then dehydrated in graduating elements of ethanol (EtOH), 15 min each step, with three changes of 95% and three changes of 100% EtOH. They were then finally dried and infiltrated with hexamethyldisilazane (HMDS, Electron Microscopy Sciences, Hatfield, PA) as follows: (i) 3:1 (EtOH:HMDS), 30 min, (ii) 1:1 (EtOH:HMDS), 30 min, (iii) 1:3 (EtOH:HMDS), 30 min, (iv) fresh 100% HMDS, 30 min twice, (v) over incubation of fresh 100% HMDS. HMDS was then allowed to volatilize in air. Samples were finally mounted on aluminum stubs using double sided carbon tabs, sputter-coated with gold using a PELCO SC-7 Auto Sputter Coater (Ted Pella Inc., Redding, CA). Imaging was performed using an FEI XL-30 TMP scanning electron microscope (FEI Company, Hillsboro, OR).

2.10. Statistical analysis

Statistical analyses were performed with SigmaPlot software (SYSTAT Software Inc., Chicago, IL) using one-way ANOVA followed by Dunnett's multiple comparison test compared with the respective controls (either cells cultured on planar surfaces or control siR-NA transfected cells) or Mann–Whitney test comparing the tortuosity of FAK knockdown cells and control siRNA cells for the same pitch. In all graphs statistically significant differences between control and FAK siRNA transfected cells for a given pitch or planar area are marked using the following symbols: ###, $p < 0.001$, ##, $p < 0.01$, #, $p < 0.05$. Statistically relevant differences between cells grown on planar surfaces and cells exposed to a given topographic pitch are marked as follows: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

3. Results

3.1. Expression of FAK was not altered by substratum topography in corneal epithelial cells

Substratum topography did not alter the inherent expression levels of FAK in hTCEpi cells cultured on topographically patterned surfaces in EpiLife medium. To determine if this effect was a result of soluble factors we repeated this analysis with hTCEpi cells in a different medium containing 10% FBS. Again, no alterations in the expression of FAK were observed. Similar results were obtained in primary human corneal epithelial cells, validating the relevance of the findings with immortalized cells (Fig. 1).

3.2. Knockdown of FAK

FAK has been reported to play a crucial role in cell migration. To investigate the significance of FAK as a signaling molecule in the response to anisotropically ordered topographic features, we depleted FAK in immortalized human corneal epithelial cells by siR-NA transfection. Successful knockdown of FAK mRNA to expression levels below 20% compared with control siRNA transfected cells was achieved as determined by qPCR analysis (Fig. 2A). This down-regulation of FAK was confirmed on a protein level by immunocytochemistry 48 h post transfection (Fig. 2B). The depletion of FAK mRNA was detectable as early as 90 min post transfection and remained stable until at least 72 h after the transfection (data not shown), ensuring that FAK remained stably downregulated throughout our experiments. In addition, we show that knockdown of FAK does not alter the ability of the cells to actively attach to the substratum (Fig. 3). By scanning electron microscopy, we demonstrate the ability to form extensions and processes for attachment onto the ridges of the topography in both control and FAK knockdown cells.

3.3. Alignment of FAK depleted cells on topographically patterned surfaces

It has been proposed that FAK functions as a mechanosensor; however, the role of FAK in the cellular transduction of biophysical cues such as topography is not understood. To determine the effects of FAK downregulation on the response of hTCEpi cells to substratum topography, their orientation with respect to the long axis of the underlying topographic features was determined. On planar surfaces, both control and FAK siRNA transfected cells were oriented in a random manner. Interestingly, on topographically patterned surfaces, FAK knockdown cells aligned to a greater extent than the corresponding control siRNA treated cells. Approximately 40–45% of FAK depleted cells aligned to the larger pitches (1200 nm or greater) compared with only 25–30% of control cells ($p < 0.05$). Even more striking, while control cells showed no response to the smaller pitches, a significant percentage ($p < 0.05$) of FAK downregulated cells were able to align to those smaller topographic features (Fig. 4). To ensure that the transfection method had no impact on the cellular ability to orient to topographic cues we additionally included untreated cells and

mock transfected cells, i.e. cells that were transfected with water instead of siR-NA, and found no significant differences in their response compared with control siRNA transfected cells (data not shown). These results indicate that FAK knockdown increased the ability of corneal epithelial cells to align with anisotropically ordered topographic cues.

3.4. Migration rate after FAK knockdown

The importance of FAK in cell migration is well established; however, the impact of FAK knockdown on the migration rate and directional motility of cells plated on topographically patterned surfaces has not been reported. Substratum topography inherently altered the migration rate of both control and FAK siR-NA transfected corneal epithelial cells. The interaction of topographic cues and FAK knockdown in modulating corneal epithelial migration was found to be complex and affected by the size scale of the topographic features:

1. Control siRNA treated cells demonstrated a statistically significant decrease in migration rate on the 400 nm pitch patterned surfaces compared with planar surfaces (Fig. 5A). No statistically significant differences were observed in control siRNA treated cells on other pitches.
2. In FAK depleted cells, we observed an increased migration rate on the 800 and 1200 nm pitch compared to migration on planar surfaces (Fig. 5B). On all other pitches the migration rate of FAK knockdown cells was similar to the migration rate on planar surfaces.
3. Compared with control siRNA treated cells on the corresponding surface, depletion of FAK significantly inhibited the migration rate of cells by at least 30–50%, when cultured on planar, 400, 1600, 2000 and 4000 nm surfaces (Fig. 5C). No alterations to migration rates were observed when FAK knockdown cells were cultured on 800 or 1200 nm pitch surfaces in comparison to control siRNA transfected cells.

To analyze the impact of FAK knockdown on directional motility in hTCEpi cells we compared the tortuosity, defined as the ratio of total distance traveled by the cells to the shortest distance between the initial and final location of the cells, for the migration on anisotropic topographic surfaces after transfection with control and FAK siRNA. A significantly reduced directional motility was observed comparing FAK knockdown cells with control siRNA transfected cells cultured on planar surfaces. This is likely due to the reduced migrational ability of cells following FAK knockdown. On the other hand, when cultured on surfaces with varying topographies (400–2000 nm pitch), there was no statistically significant difference observed in the directional motility of cells (FAK knockdown vs. control siRNA, Supplemental data). The directional motility was only reduced to a limited extent in FAK knockdown cells cultured on the 4000 nm surface. In addition, cell trajectories for all cells analyzed were plotted and compared. No substantial differences in the overall migration pattern on any substratum were noted after FAK knockdown (Supplemental data).

3.5. Exposure to topographic cues and knockdown of FAK leads to changes in SYNE1, 2 expression but does not alter ROCK1 mRNA levels

While the expression of FAK was not affected by substratum topography, mRNA levels of SYNE1 and SYNE2 were altered. Specifically, SYNE1 expression was significantly downregulated in cells on the 4000 nm pitch while upregulated on the 400 nm pitch (Fig. 6A). SYNE2 expression, on the other hand, was reduced by 40% on the 1400 nm pitch but remained unaltered on all other pitches compared to planar substrates (Fig. 6B). The expression of ROCK1, another signaling molecule in the focal adhesion signaling pathway, was not significantly changed by topographic cues (Fig. 6C). After FAK knockdown in

these cells, we observed an interesting molecular signaling event. Depletion of FAK in corneal epithelial cells led to a significant increase in the expression of SYNE2 accompanied with a decreased SYNE1 expression (Fig. 7). Expression of ROCK1 was not altered upon knockdown of FAK in hTCEpi cells (Fig. 7).

4. Discussion

4.1. Use of immortalized cells instead of primary cells

For most studies presented here immortalized human corneal epithelial (hTCEpi) cells were used, and these have been shown to be a viable model for molecular mechanisms in corneal epithelial cells [47]. Immortalized cells were used to obtain higher consistency and to avoid biased data, which may arise from the use of primary corneal cells, due to variation between donors. However, to validate our data, we also used primary human corneal epithelial cells to determine the impact of topographic features on the expression of FAK mRNA.

4.2. Modulation of gene expression

In this study no significant changes in expression of FAK were observed in immortalized corneal epithelial cells cultured on substrates possessing topographic features of differing size scales. We also repeated this analysis for hTCEpi cells cultured in a different medium containing serum since it has previously been reported that although serum is not a requirement for cellular alignment to topography, it was shown to increase the percentage of aligned cells [11,48]. In addition, we analyzed the expression of FAK in primary human corneal epithelial cells cultured on patterned surfaces to establish the generality of this result. In both experiments we failed to detect any significant changes in the expression of FAK. This concurs with a study on HUVECs cultured on nanopatterned surfaces [49] where no alteration in the expression of FAK was observed. However, changes in FAK expression have been observed in hMSCs and osteoblasts cultured on topographically patterned substrates [50,51]. We also did not detect any change of ROCK1 expression in hTCEpi cells cultured on topographically patterned surfaces. However, it is important to keep in mind that focal adhesions and the involved signaling pathways are very dynamic, so that a transient alteration in FAK or ROCK1 expression after exposure to topographic cues cannot be excluded. In our experiments, exposure to topographically patterned surfaces did, however, affect the expression levels of SYNE1 and 2 in hTCEpi cells. We found the expression of SYNE1 to be upregulated on the 400 nm pitch. This is congruous with previously published expression profiles of HUVECs, cultured on the 400 nm pitch, where the expression of SYNE1 was elevated compared with planar control surfaces [49]. Curiously, in hTCEpi cells on the 4000 nm pitch patterned surfaces SYNE1 expression was inhibited. The expression of SYNE2 was downregulated on the 1400 nm pitch but unaltered on all other pitch sizes. It has been observed that alterations in cell behavior can vary with the size scale of the topographic cues being presented [7,9,10,52,6]. Our observations on SYNE genes are consistent with the previously published reports. Also, the changes in SYNE genes correlate with a recent study from our lab in which biophysical cues in the form of topographic ridges and grooves alter the alignment and shape of cell nuclei and cytoskeleton accompanied by changes in nuclear volume and elastic modulus of immortalized corneal epithelial cells [46]. A decrease in SYNE1 expression may result in lowered tethering of the actin cytoskeleton to the nucleus and thus an increase in nuclear volume for cells on 400 nm pitch patterned surfaces.

4.3. Knockdown of FAK alters cell behaviors

FAK has been proposed to function as a mechanosensor based on observations that FAK null cells are unable to respond to mechanical forces, durotaxis [29] and the topographic cues of micron-sized pillars [53]. Furthermore, it has been reported that stretching of the cell

culture substratum leads to a transiently increased phosphorylation of FAK in periodontal ligament fibroblasts [54]. Also, low stiffness substrates have been shown to induce a decrease in FAK phosphorylation [55]. Based on these previous reports, we initially expected a diminished response to topographic cues of FAK depleted cells. However, we observed an increased percentage of aligned cells after FAK knockdown. We also demonstrated that even after FAK depletion this adhesion is in fact an active process. Interestingly, we observed that the expression levels of the nuclear envelope proteins Nesprin 1 and Nesprin 2 were altered by knockdown of FAK; while SYNE1 was significantly downregulated, SYNE2 was upregulated in expression. Chancellor et al. [37] showed that focal adhesions and alignment of cells along their long axis were increased in Nesprin 1 deficient cells. It is therefore possible that there is a balance between the FAK and SYNE genes that facilitates how cells perceive and transduce biophysical cues associated with the extracellular environment (Fig. 8).

The observation that the Nesprin 2 mRNA SYNE2 is upregulated upon FAK depletion suggests that SYNE2 may compensate for FAK loss to allow cellular responses to topographic cues. This result, contrary to previous reports, that FAK depletion does not inhibit the cellular ability to recognize and respond to topographic cues but is accompanied by altered expression of SYNE genes suggests that a complex signaling network is involved in the sensing of various biophysical cues which most likely differs in different cell types. Thus, the relationship between FAK and Nesprins may be important in regulating orientation and possibly migration. A feedback mechanism from the nucleus may play a role in cell signaling effects, at least in corneal epithelial cells, as indicated by the dys-regulation of Nesprins on topography and upon depletion of FAK. This might provide the link between the nucleus and extracellular and cytosolic signaling events that are involved in the cellular responses to biophysical cues. Based on our data we propose a model illustrating the interplay between FAK and nesprins that might provide a link between cytosolic and nuclear signaling processes (Fig. 8). While our observations are interesting, we realize that a full understanding of the underlying mechanisms will require further investigation. Also, since ROCK1 expression was not altered by knockdown of FAK, Rho kinases may not play a central role in FAK-Nesprin mediated signaling in hTCEpi cells.

4.4. Impact of topographic cues on the migration rate of corneal epithelial cells

Cell migration plays a critical role in a number of biological processes including embryogenesis, metastasis and wound healing. In healthy eyes, wounding of the cornea initiates the migration of epithelial cells surrounding the wound to promptly repopulate the injured area [56,57]. Motility and proliferation are essential aspects of wound healing. The data presented here showed a reduced migration rate in control cells on the 400 nm pitch compared to cells cultured on planar control surfaces. In addition, our lab previously observed that topographic cues in the nanoscale range lead to a decreased proliferation rate of corneal epithelial cells [7]. Topographic features in the nanoscale range most closely reflect the topographic attributes of the basement membrane (BM) that healthy corneal epithelial cells are exposed to in vivo. With this in mind, our data indicate that the rates of proliferation and migration were increased when the physical cues from the BM are missing which might occur when corneal wounding causes considerable damage to the BM. The lack of the BM nanoscale topography might signal a need for increased migration and proliferation to the corneal epithelial cells to enhance re-epithelialization upon wounding.

4.5. Migration rate after FAK knockdown

Several reports document that depletion or inhibition of FAK reduce the migration rate to about 50% while results from gain of function experiments indicate that FAK promotes increased cell migration speed and directional persistence of cell movement [24]. The first

observations of a role of FAK in cell migration were made analyzing fibroblasts isolated from FAK knockout mouse embryos which display a reduced motility [28]. Similarly, expression of a dominant negative FAK construct resulted in a reduced migration rate in fibroblasts, HUVECs and ovarian cell cancer lines [58–60]. Comparable observations were made with inactive FAK mutant fibroblasts [29]. Conversely, the stable overexpression of FAK in Chinese hamster ovary (CHO) cells led to an increased migration rate [61] and the reduced migration of FAK null fibroblasts could be reversed by FAK overexpression [59]. Interestingly, FAK null fibroblasts showed an increased number of focal adhesions (FAs) which were also larger in size. A continuous process of assembly and disassembly of FAs is required for cell motility and FAK might play a crucial role in cell migration by regulating FA turnover.

In our experiments, FAK knockdown led to a reduced migration rate on planar surfaces and on the majority of topographically patterned surfaces which concurs with the previously published data. Strikingly, on the 800 and 1200 nm pitch there was no change in the migration rate in FAK depleted cells compared to control. Also, FAK knockdown hTCEpi cells on the 800 and 1200 nm pitch patterned surfaces showed an increased migration rate compared to their migration on planar surfaces after knockdown. It is possible that these size scales act as an optimal threshold at which cells are able to perceive their extracellular environment with increased sensitivity and are able to overcome the effect of FAK knockdown on migration rate, possibly by concomitant up or down regulation of other signaling molecules. Earlier studies [6,11,12] from our group have attempted to define the limits of the physical dimensions of topographies that cells are able to sense and respond to.

4.6. Directional motility of immortalized corneal epithelial cells after FAK knockdown

FAK has been linked with cell polarity and directional motility [28,29,62,63]. FAK null MEFs exhibit defects in directional persistence which can be rescued by FAK re-expression [63]. Our data comparing the tortuosity values and the cell trajectories did not show any substantial differences between control and FAK siRNA transfected cells plated on anisotropically ordered topographically patterned surfaces. Though our data are contrary to previous reports, most studies documenting a role of FAK in directional persistence have been made on tissue culture plastic, lacking topographic cues. However, the topographic cues used in our study do not appear to affect the directional motility of FAK deficient cells, suggesting FAK alone may not be the determining element for directional migration.

5. Conclusions

Even though adherent cells in vivo exist in an environment with complex topographical features the majority of in vitro research is done in flat tissue culture plastic (TCP). The data presented here show that exposure to topographic cues can influence the impact of FAK knockdown on cellular behavior. We showed that on planar and some of our patterned surfaces FAK depletion leads to decreased rates of cell migration in corneal epithelial cells, in line with previous reports on other cell types. On the 800 and 1200 nm pitch patterned surfaces, however, FAK knockdown had no effect on the migration rate. This result emphasizes that caution is needed with making generalized statements based on data acquired using TCP that lacks biomimetic surface features.

Contrary to previous reports, we observed an increased ability of FAK depleted cells to respond to topographic cues. This was accompanied with alterations in the genes encoding the Nesprin proteins. However, a direct comparison between our results and previously published data is not possible due to variations in the substrates that were used. Another explanation for the conflicting results is the possibility of cell type specific effects, suggesting that more than one pathway is involved in the response to such stimuli. A cell

type specific response to biophysical cues most likely reflects the considerable variations in the relevance of such cues in the particular in vivo environment of each cell type.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 2 and 8, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at <http://dx.doi.org/10.1016/j.actbio.2012.07.004>.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.actbio.2012.07.004>.

Fig. 1.

Substratum topography does not alter FAK expression levels in corneal epithelial cells. Expression of PTK2 (FAK) in primary and immortalized (hTCEpi) human corneal epithelial cells cultured on topographic features and planar control areas as detected by qPCR. To analyze the effect of culture medium components, hTCEpi cells were cultured in two different media (EpiLife and EP medium) with similar results. Values were normalized to expression levels on planar surfaces, which were arbitrarily set as “1”. Shown are representative graphs of at least three experiments with similar trends. Error bars represent the standard deviation of $n = 3$ samples.

Fig. 2.

Successful siRNA-mediated FAK knockdown in hTCEpi cells. Cells were transfected with control or FAK siRNA and 48 h later either harvested for RNA isolation or fixed for detection of pY397FAK by immunocytochemistry. (A) Transfection with FAK siRNA led to a knockdown to levels below 20% of the FAK expression in control siRNA transfected cells as detected by qPCR using an FAK specific aptamer. The error bar represents the standard deviation. The expression level of FAK in control cells was arbitrarily set as “1”. Untreated and control siRNA transfected showed comparable expression levels of FAK (data not shown). *** $p < 0.001$. (B) In control cells, pY397FAK (green) is detectable by immunocytochemistry as punctate structures predominantly at the periphery of the cells. These structures cannot be detected in cells transfected with FAK siRNA, thus confirming the knockdown on a protein level. The nuclei are stained with DAPI (blue). Scale bar represents 20 μm ; 40 $\times$ objective.

Fig. 3.

Attachment of cells on topography remains an active process after FAK knockdown. Representative scanning electron microscopy (SEM) images of control and FAK siRNA transfected hTCEpi cells on patterned surfaces (control (4000 nm pitch), FAKsi (2000 nm pitch)). Cellular processes (arrows) on the ridges of the topography are suggestive of an active process for cell attachment despite FAK knockdown in corneal epithelial cells.

Fig. 4.

FAK knockdown affects cellular responses to topographic cues. 48 h post transfection with control or FAK siRNA, hTCEpi cells were plated on “six-pack” topographic surfaces. The graph shows the percentage of corneal epithelial cells aligned with the anisotropically ordered topographies after transfection with control and FAK siRNA. Statistically significant changes are indicated as follows: differences between topographic vs. planar surfaces for each condition (control and FAKsi) are marked with * (** $p < 0.001$; * $p < 0.05$) and differences between control and FAK siRNA transfected cells for any given pitch are marked with # (#, $p < 0.05$). On larger pitches (1200 nm and higher), control cells aligned preferentially along the anisotropies of the underlying surface. FAK knockdown led to a preferential alignment along smaller pitches and increased the percentage of aligned cells on all pitches.

Fig. 5.

Topographic cues and FAK depletion affect the migration rate of hTCEpi cells. The graph shows the migration rate of hTCEpi cells cultured on topographically patterned and planar surfaces after transfection with control and FAK siRNA. In control cells, the biophysical cues from the 400 nm pitch led to a reduced migration compared to migration on planar areas (A). In FAK depleted cells topographic cues induced an increased migration on the 800 and 1200 nm pitch compared to the migration on planar surfaces (B). Depletion of FAK resulted in a significantly reduced migration rate on planar surfaces and on topographically patterned surfaces of 400, 1600, 2000, and 4000 nm pitch but had no impact on the migration on the 800 and 1200 pitch (C). Shown are average values from all analyzed cells in a total of four experiments. Error bars represent the standard error of the mean (SEM). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ###, $p < 0.001$; ##, $p < 0.01$; #, $p < 0.05$.

Fig. 6. Substratum topography alters the expression of SYNE1 and SYNE2 but not ROCK1 in immortalized corneal epithelial cells. SYNE1 was significantly upregulated in cells cultured on 400 nm pitch and significantly downregulated on 4000 nm surfaces (A) while SYNE2 expression was downregulated on the 1400 nm pitch (B). On the contrary, ROCK1 expression was unaltered by topographic features of the substratum (C). Shown are representative graphs of at least three experiments.

Fig. 7.

Knockdown of FAK alters the expression of SYNE1 and 2 but not ROCK1 in immortalized corneal epithelial cells. Cells were harvested for RNA isolation 48 h post transfection with FAK and control siRNA. The expression of SYNE1 is reduced while the expression of SYNE2 is increased in FAK depleted cells compared to control cells. The expression of ROCK1 remained unaltered. Shown are representative graphs of at least three experiments with similar trends, displaying average values with error bars representing the standard deviation of three replicate samples. *, $p < 0.05$.

Fig. 8.

Proposed pathway illustrating the interplay between FAK and nesprins. In corneal epithelial cells, although expression of FAK is unaltered by topographic cues, expression of SYNE1/2 (Nesprin1/2) is differentially altered. Importantly, knockdown of FAK led to the suppressed expression of SYNE1 accompanied by an elevated expression of SYNE2. Our results suggest a role for SYNE2 in facilitating cell adhesion in the event of FAK knockdown.