Nanoscale Topography–Induced Modulation of Fundamental Cell Behaviors of Rabbit Corneal Keratocytes, Fibroblasts, and Myofibroblasts

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PURPOSE. Keratocyte-to-myofibroblast differentiation is a key factor in corneal wound healing. The purpose of this study was to determine the influence of environmental nanoscale topography on keratocyte, fibroblast, and myofibroblast cell behavior

METHODS. Primary rabbit corneal keratocytes, fibroblasts, and myofibroblasts were seeded onto planar polyurethane surfaces with six patterned areas, composed of anisotropically ordered grooves and ridges with a 400-, 800-, 1200-, 1600-, 2000-, and 4000-nm pitch (pitch = groove + ridge width). After 24 hours cells were fixed, stained, imaged, and analyzed for cell shape and orientation. For migration studies, cells on each patterned surface were imaged every 10 minutes for 12 hours, and individual cell trajectories and migration rates were calculated

RESULTS. Keratocytes, fibroblasts, and myofibroblasts aligned and elongated to pitch sizes larger than 1000 nm. A lower limit to the topographic feature sizes that the cells responded to was identified for all three phenotypes, with a transition zone around the 800- to 1200-nm pitch size. Fibroblasts and myofibroblasts migrated parallel to surface ridges larger than 1000 nm but lacked directional guidance on submicron and nanoscale topographic features and on planar surfaces. Keratocytes remained essentially immobile

CONCLUSIONS. Corneal stromal cells elongated, aligned, and migrated, differentially guided by substratum topographic features. All cell types failed to respond to topographic features approximating the dimensions of individual stromal fibers. These findings contribute to our understanding of corneal stromal cell biology in health and disease and their interaction with biomaterials and their native extracellular matrix. (*Invest Ophthalmol Vis Sci* 2010;51:1373–1381) DOI:10.1167/iovs.09-4074

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To maintain transparency, the cornea depends on a high degree of structural organization, which is likely the result of interplay between the various extracellular matrix (ECM) components maintained by resident corneal stromal cells or keratocytes.^{1–4} Keratocytes play a dominant role in the maintenance of corneal homeostasis and transparency through the production of high concentrations of water-soluble cytoplasmic proteins (corneal crystallins) and ECM components.^{2,3,5–8} Keratocytes contact each other through long cell extensions and communicate through gap junctions, forming a functional cell syncytium able to respond to changes in corneal homeostasis.^{9,10} Fibroblasts and myofibroblasts are stromal repair phenotypes that arise from the keratocyte and exhibit increased migratory and proliferative characteristics. They also have contractile properties and are involved in repopulation and remodeling of corneal tissues and wound contraction. The presence of α -smooth muscle actin (α -SMA) enables myofibroblasts to generate higher contractile forces than fibroblasts. α -SMA organized in stress fibers is a widely recognized marker for myofibroblasts populating healing wound sites.^{5,11}

Trauma-induced apoptosis of keratocytes and replacement by fibroblasts or myofibroblasts disrupts stromal organization and corneal transparency, resulting in corneal haze.¹² It is, therefore, important to understand the variables that influence the differentiation of keratocytes to myofibroblasts because this plays an important role in corneal pathology and wound healing.^{13–15}

Keratocyte-to-myofibroblast differentiation in vitro has been described in detail.^{5,16–18} This is a labile process with several factors recognized in promoting and inhibiting this process. Fibroblast growth factor 2 (FGF-2), platelet-derived growth factor (PDGF), and serum induce the differentiation of keratocytes to fibroblasts,⁵ whereas transforming growth factor- β 1 (TGF- β 1) induces keratocytes and fibroblasts to differentiate to myofibroblasts.^{17,18} In vitro reversal of the myofibroblast to fibroblast phenotype has been described in rabbit cells.¹⁹ However, the differentiation of fibroblasts and myofibroblasts to the keratocyte phenotype has not been reported to date.⁵ Various techniques reported to stabilize the keratocyte phenotype in vitro involve the use of serum-free medium^{20,21} and culturing keratocytes as nonattached spheroids^{22,23} or adhered to the stromal side of amniotic membranes.^{24,25}

Most publications have focused on the influence of soluble factors on the keratocyte differentiation process. Cells are influenced by their chemical and physical environment. Chemical cues include soluble factors¹² (autocrine, paracrine, or released by ECM²⁶), protein adhesion sequences in the ECM, pH, and osmolarity.^{27,28} Physical factors influencing cells include the topographic features and compliance of the ECM.^{29–34} In vivo, keratocytes are surrounded by a structurally rich three-dimensional (3D) environment consisting of collagen fibrils arranged in a lamellar orientation. We propose that the native nano and submicron topographic environment of

FIGURE 1. Collagen coating does not obscure topographic features. To verify the maintenance of the topographic features, tissue culture plates containing patterned surfaces were coated for 1 minute with a 50% collagen solution. The plates were analyzed using wet-field AFM. (A) One culture dish contains a 6-pack with the six different nanotopography pitch sizes (ranging from a 400- to a 4000-nm pitch) separated by a planar surface used as control. (B) AFM image of the anisotropic pattern on a polyurethane surface (400-nm pitch size). (C) AFM image of the anisotropic pattern on a polyurethane surface (400-nm pitch size) after coating the surface for 1 minute with a 50% collagen solution. After coating, the surface was rinsed three times with a serum-free medium. Collagen coating does not obscure the surface pattern. Scale bars, 400 nm.

the cell assists in stabilizing the keratocyte phenotype, whereas pathologic alterations of this environment may be permissive for the differentiation into myofibroblasts (Pot SA, et al. *IOVS* 2007;48:ARVO E-Abstract 1962).³⁵ The keratocyte phenotype-stabilizing properties of amniotic membrane stroma^{24,25} and certain 3D hydrogels³⁶ might well be caused by such topographic cues.

With that in mind, biologically relevant nano- and submicron-structured surfaces that mimic the fibrillar topography of the ECM were fabricated as previously described.^{29,33} The anisotropic pattern of these chemically identical cell culture surfaces enabled us to monitor the influence of a single stimulus (topography) on cell responses such as alignment and migration trajectories along grooves.

Cell shape and migration are important cell behaviors determining cell and tissue fate and morphology.^{37,38} Furthermore, cell orientation, the acquisition of polarity, and often-times cell elongation are the first steps in cell migration, which is an important factor in many biological phenomena such as embryogenesis, inflammation, wound healing, metastasis, and the colonization of biomaterial scaffolds.^{39–42} We were, therefore, interested in identifying topographic cues that influence cell morphology and migration. This study was undertaken to determine the influence of environmental topographic cues on the orientation and migration of corneal keratocytes, fibroblasts, and myofibroblasts.

MATERIALS AND METHODS

Tissue Collection

Rabbit eyes used for isolation of primary corneal keratocytes were obtained from PelFreez (Rogers, AR). The eyes were shipped overnight on ice in minimum essential medium and were processed within 36 hours after tissue harvesting. Anterior segments of all eyes were found to be normal after examination with a focal light source. Keratocytes were isolated using previously described methods.¹⁷

Cell Culture

Tissue culture plates were coated for 30 minutes with 97% collagen I and 3% collagen III (PureCol; Inamed, Fremont, CA) diluted in an equal volume of 0.012 M HCl (Acros Organics, Geel, Belgium) in filter-sterilized H₂O. The dishes were then rinsed twice with serum-free medium (Dulbecco's minimum essential medium [DMEM]) containing 1% nonessential amino acids (both from Invitrogen Corp., Carlsbad, CA), 1% RPMI 1640 vitamin solution, 0.055 mg/mL L-ascorbic acid (both from Sigma, St. Louis, MO), and 1% penicillin-streptomycin-amphotericin B (Cambrex Bio Science, Walkersville, MD) and were left hydrated until use.

Keratocytes were cultured at 37°C in 5% CO₂ on collagen-coated culture dishes in serum-free medium. As previously described, kerato-

cytes were exposed to specific growth factor-supplemented media for a minimum of 4 days to induce the fibroblast and myofibroblast phenotypes.¹⁷ 10% Fetal bovine serum (Sigma) and 10 ng/mL transforming growth factor- β 1 (TGF- β 1; Sigma) were added to the culture media for induction of the fibroblast and myofibroblast phenotype, respectively. Trypsin used for passing keratocytes was neutralized with soybean trypsin inhibitor (Invitrogen Corp.) at a concentration of 0.5 mg/mL in 1× phosphate-buffered saline (PBS).

Fabrication of Microscale and Nanoscale Surfaces

Patterned silicon surfaces were constructed at the Center for Nanotechnology (University of Wisconsin at Madison) using x-ray lithography, reactive ion etching, and low-pressure chemical vapor deposition silicon oxide coating, as previously described.²⁹

Each substrate contained six areas with anisotropic feature sizes, composed of grooves and ridges each with a 400-, 800-, 1200-, 1600-, 2000-, and 4000-nm pitch (pitch = groove + ridge width), separated by flat control surfaces ("6-packs"). The groove depth was 300 nm. These silicon surfaces were used as templates for the production of patterned polyurethane cell culture surfaces using soft lithography and composite stamping replication techniques, as previously described.³³

Directly before use in any of the experiments, the patterned polyurethane 6-packs were collagen coated, as described. The 6-packs were exposed to the collagen solution for 1 minute only, creating an adsorbed molecular collagen coating. Maintenance of the topographic features was verified using wet-field atomic force microscopy (AFM; Nanoscope IIIa Multimode scanning probe microscope; Veeco, Santa Barbara, CA) (Fig. 1). AFM is widely used for the imaging and physical characterization of synthetic and biological materials.⁴³ Advantages include the ability to image unfixed surfaces and tissues in liquid environments.

Immunocytochemistry

α -SMA was used as a marker to identify the myofibroblast phenotype. Cells were fixed in 1% paraformaldehyde (16% paraformaldehyde [formaldehyde] aqueous solution; Electron Microscopy Sciences, Hatfield, PA) in 1× PBS for 3 minutes and then permeabilized in ice-cold acetone (−20°C) for 5 minutes. Incubation with goat serum (Sigma; 1:10 dilution) in 1× PBS at 37°C for 30 minutes was used to block nonspecific protein binding. The sample was then incubated with monoclonal fluorescein isothiocyanate (FITC)-labeled anti- α -SMA (Sigma; 1:100 dilution) in blocking solution at 37°C for 60 minutes. Rhodamine-conjugated phalloidin (Sigma) was added for 30 minutes at room temperature to label intracellular actin. Cell nuclei were stained for 10 minutes at room temperature with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen Corp.). The cells were washed with 1× PBS between each step, except between the blocking and primary antibody incubation steps.

Finally, the cells were left in 1× PBS until immunofluorescence imaging with an epifluorescence microscope (Axiovert 200M; Carl Zeiss Imaging Solutions GmbH, Munich, Germany).

FIGURE 2. Keratocyte, fibroblast, and myofibroblast phenotype demonstrate cell identity. Epifluorescence microscopic images of (A) keratocytes, (B) fibroblasts, and (C) myofibroblasts on planar surfaces at 40 $\times$ magnification. Keratocytes and fibroblasts are stained with DAPI and rhodamine-conjugated phalloidin. Myofibroblasts are stained with DAPI, rhodamine-conjugated phalloidin, and FITC-labeled anti- α -SMA. Note the stellate morphology of the keratocytes, the loss of this stellate morphology in fibroblasts, and the expression and organization of α -SMA in stress fibers in the myofibroblasts. Scale bars, 20 μ m.

The following staining protocol was used for the analysis of cell shape and orientation. Cells were fixed in 4% paraformaldehyde for 15 to 20 minutes and then washed three times with 1 $\times$ PBS. Cells were incubated in 0.1% TritonX-100 (Triton X-100 Sigma Ultra; Sigma) in 1 $\times$ PBS for no longer than 5 minutes, blocked for 20 minutes with 1% bovine serum albumin (albumin from bovine serum; Sigma) in 1 $\times$ PBS, and incubated with rhodamine-conjugated phalloidin for 30 minutes. After a wash with 1 $\times$ PBS, the cells were incubated for 10 minutes with DAPI and left in 1 $\times$ PBS until imaging. All steps were performed at room temperature.

Analysis of Cell Shape and Orientation

Keratocytes were seeded on collagen-coated culture plates with 6-packs at a density of $1 \times 10^4/\text{cm}^2$. Fibroblasts and myofibroblasts were seeded at a density of $4 \times 10^3/\text{cm}^2$. To examine cell-substrate adhesion only and avoid biased results, the cells were seeded at the lowest density possible and cells in contact with other cells were manually removed from the data sets. Given that spontaneous fibroblast-to-myofibroblast transformation in cell cultures of extremely low density has been described,⁴⁴ we decided to use the density $4 \times 10^3/\text{cm}^2$. Keratocytes do not survive in serum-free cell culture at plating densities much lower than those that allow intercellular contact. The density used in this study ($1 \times 10^4/\text{cm}^2$) was the lowest density at which the keratocytes were viable and morphologically normal. The viability of keratocytes was significantly decreased at culture densities of $\leq 6 \times 10^3/\text{cm}^2$ (data not shown). After an adhesion period of 24 hours, the cell nuclei and cytoskeleton were stained according to the protocol described. Images were obtained with an epifluorescence microscope (Axiovert 200M; Carl Zeiss Imaging Solutions GmbH) with a 10 $\times$ objective lens. Cell shape and orientation were analyzed with image analysis software (KS300; Carl Zeiss Imaging Solutions GmbH) using a previously described protocol.²⁹ Cell elongation was defined as the ratio between the length and the breadth of each cell. In previous experiments conducted by this laboratory,^{17,29–31} corneal epithelial cells were defined as elongated when the ratio of cell length divided by cell width exceeded 1.3. The same cutoff factor was used for the purpose of continuity across cell types and phenotypes. The angle between the main cell axis and the surface topography pattern was also obtained. Elongated cells were considered aligned with the grooves when this angle was $<10^\circ$ and were considered to have a random orientation to the surface pattern when this angle was $>10^\circ$.

Migration

Cell migration analysis was performed using a previously described protocol.²⁹ Briefly, keratocytes ($1 \times 10^4/\text{cm}^2$), fibroblasts, and myofibroblasts ($4 \times 10^3/\text{cm}^2$) were sparsely seeded on collagen-coated culture plates with 6-packs and were allowed to adhere for 2 to 4 hours at 37°C in 5% CO₂. The cells were then placed on an incubated microscope stage, maintaining cell culture conditions at 37°C in 5% CO₂. Sequential phase-contrast microscopic images of cells on patterned and planar surfaces were obtained every 10 minutes for 12 hours using a phase-contrast microscope (Axiovert 200M; Carl Zeiss Imaging Solutions GmbH) with a 10 $\times$ objective lens. Individual cells

not contacting other cells along the observed migration trajectory (since contact inhibition of migration has been described⁴⁵) and not exiting the imaged field were manually tracked using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). Microsoft software (Excel; Microsoft, Redmond, CA) was used to plot the migration trajectories over time and to calculate the migration rates.

Statistical Analysis

Within one experiment, all data sets were compared with one-way analysis of variance (ANOVA). When variability between the data set means was determined to be significant, either a Dunnett multiple comparison test was used to compare all the experimental groups with one control group or a Bonferroni multiple comparison test was used to compare selected data sets with one another. The level for statistical significance was set at $P < 0.05$ for all comparisons. Commercial software (Prism 2003; GraphPad, La Jolla, CA) was used for all statistical analyses.

RESULTS

Verification of Cell Identity

Cell morphology and α -SMA expression were used to identify the different cell types. Keratocytes showed a stellate, dendritic morphology and did not express α -SMA. Fibroblasts were recognizable by their spindle-shaped morphology. Approximately 10% of fibroblasts showed cytoplasmic α -SMA staining. Myofibroblasts were large cells with abundant α -SMA-positive stress fibers in the cytoplasm (Fig. 2).

Impact of Topographic Cues on Cell Orientation

We observed a profound impact of surface topography on cell orientation and elongation in all three cellular phenotypes (Fig. 3). The percentages of keratocytes (Fig. 4A), fibroblasts (Fig. 4B), and myofibroblasts (Fig. 4C) aligned to 400-nm pitch size topography were not significantly different from the random orientation observed on planar surfaces. More than 40% of keratocytes, fibroblasts, and myofibroblasts aligned themselves within a 10° angle to the surface pattern on topography pitch sizes greater than 1 μ m. The degree of alignment to the surface topography pitch sizes larger than 1000 nm was significant for all three phenotypes compared with the cell orientation on planar surfaces ($P < 0.01$ for all comparisons except myofibroblasts on 1200-nm compared with planar, which was $P < 0.05$). Both keratocytes and fibroblasts showed significant alignment to the 800-nm pitch size topography (31.5% [$P < 0.05$] and 32.8% [$P < 0.01$], respectively). Myofibroblasts, however, did not show significantly greater alignment in response to the 800-nm pitch size (22.6%, $P > 0.05$) compared with the cell orientation on planar surfaces. Thus, a lower limit to the topography pitch size that cells respond to was identified for all three phenotypes, with a transition zone around the 800- to 1200-nm pitch size (Fig. 4).

FIGURE 3. Orientation of primary rabbit corneal keratocytes, fibroblasts and myofibroblasts cultured on collagen-coated planar and topographically patterned surfaces. All three phenotypes display a random orientation on (A–C) planar surfaces and (D–F) surfaces with a 400-nm pitch size pattern. All three phenotypes orient along the linear surface topography on surfaces with a (G–I) 1200-nm pitch and a (J–L) 4000-nm pitch size pattern. *Double-headed arrows:* direction of grooves and ridges on the patterned surfaces. Keratocytes were cultured in serum-free DMEM, and 10% FBS and 10 ng/mL TGF- β 1 were added to the culture medium for induction of the fibroblast and myofibroblast phenotype, respectively. Intracellular actin fibers were labeled with rhodamine-conjugated phalloidin. Cells were considered to be aligned to the underlying surface topography if the orientation of the main cell axis was within a 10° angle of the orientation of the surface grooves and ridges. The elongation factor of a cell was calculated by dividing the maximal cell length by the maximal cell width measured at a 90° angle to the main cell axis. Scale bars, 100 μ m.

The presence of serum (and thus the soluble factors it contains) in the culture medium has been shown to influence the response of primary human corneal epithelial cells to topographic cues.^{29,30} Therefore, we tested the orientation and elongation responses of myofibroblasts in TGF- β 1-enriched cell culture medium in the presence and absence of 10% fetal bovine serum. No significant differences were found (data not shown).

Guidance of Cell Migration by Surface Topographic Features

The centroids of the keratocytes remained largely stationary during the 12-hour imaging period, usually shifting less than one cell body diameter and migrating at an average rate of 2.9 μ m/h parallel to and 1.8 μ m/h perpendicular to the surface topography on a 1200-nm pitch surface (Figs. 5A, D, 6A). The keratocytes were essentially immobile, as has been previously reported.³¹ Though largely immobile, the keratocytes actively extended and retracted filopodia. Most of these filopodial extensions aligned with the surface topography (Figs. 3G, J).

Fibroblasts and myofibroblasts exhibited contact-guided cell migration parallel to the surface pattern of the topographic substrates, with limited movement perpendicular to the surface pattern, on all but the smallest surface pitch sizes (400 + 800 nm). This was in sharp contrast with the lack of directional guidance of fibroblasts and myofibroblasts on the planar surfaces (Figs. 5B, C, E, F). However, some persistence in the direction of migration was observed in a number of fibroblasts and myofibroblasts on planar, 400-, and 800-nm pitch size

surfaces (Figs. 5B, C). Interestingly, fibroblasts and myofibroblasts were aligned with the surface pattern on the same pitch sizes that also induced contact-guided cell migration. Average migration rates measured were 22.7 and 28.3 μ m/h parallel to and 9.0 and 8.9 μ m/h perpendicular to the surface topography on a 1200-nm pitch surface for fibroblasts and myofibroblasts, respectively (Figs. 6B, C).

Fibroblast and myofibroblast migration perpendicular to the surface pattern were significantly inhibited on topography pitch sizes greater than 1000 nm compared with planar surfaces ($P < 0.05$ for fibroblasts on a 1200-nm pitch and myofibroblasts on 1600- and 2000-nm pitches; $P < 0.01$ for fibroblasts on 1600-, 2000-, and 4000-nm pitches and myofibroblasts on 1200- and 4000-nm pitches; Figs. 6B, C). Otherwise, there were no significant differences in migration rates observed between patterned and planar surfaces for any of the three phenotypes (Fig. 6).

Fibroblasts and myofibroblasts migrate at a significantly increased rate compared with keratocytes on all surfaces. No significant differences were observed in migration rates between fibroblasts and myofibroblasts (Fig. 6).

DISCUSSION

We have demonstrated that surface topography has a significant impact on the shape and orientation of rabbit corneal keratocytes, fibroblasts, and myofibroblasts and that this effect is modulated by scale of the substratum features. We observed keratocyte, fibroblast, and myofibroblast elongation and align-

FIGURE 4. Surface topography pitch size has a profound impact on cell orientation in all three phenotypes. These graphs are representative of experiments with (A) keratocytes, (B) fibroblasts, and (C) myofibroblasts completed in triplicate. More than 40% of keratocytes, fibroblasts, and myofibroblasts orient themselves within 10° of perfect alignment with the surface pattern on topography pitch sizes greater than 1000 nm. A lower, but still significant, number of keratocytes and fibroblasts orient to the 800-nm pitch size topography. However, the myofibroblasts showed no significant response to surface features in the submicron range. A lower limit to the topography pitch size that the cells responded to was identified for all three phenotypes with a transition zone around the 800- to 1200-nm pitch size. Significant difference compared with planar surface (* $P < 0.05$; ** $P < 0.01$).

ment to anisotropic surface patterns on 2D substrates. Contact-guided cell migration of fibroblasts and myofibroblasts was also observed on all but the smallest surface pattern pitch sizes. Previous studies by other research groups have also shown significant changes in the morphologic appearance and migration patterns of cultured fibroblasts and various other cell types, depending on the culture substrates.^{46–53}

A lower limit to topography feature sizes to which cells can respond was observed. For keratocytes and fibroblasts, this limit lies between 400 and 800 nm, whereas myofibroblasts exclusively respond to features measuring 1200 nm or larger. Keratocytes and fibroblasts appear to have finer “tactile acuity” than do myofibroblasts. These findings are interesting because they suggest that the lower limit for tactile acuity is above the feature scale of individual stromal collagen fibers (± 30 nm).¹

Keratocytes were essentially immobile but were observed to actively probe their environment with lamellipodial and filopodial extensions aligning with the surface topography, presumably in an attempt to contact neighboring cells or to sample the extracellular space. These observations seem to be in agreement with the description of keratocytes as fairly sedentary cells in the corneal stroma that interconnect with one another and form a cellular syncytium.^{10,16} Keratocytes interact with both macrophages and neutrophils present in the cornea (Jester JV, et al. *IOVS* 2006;47:ARVO E-Abstract 1811).⁵⁴ This interaction may be part of an immunologic sentinel function carried out by keratocytes, which would explain the probing of the extracellular space. Microglial cells in the brain⁵⁵ and dendritic cells in the cornea⁵⁶ (Jester JV, et al. *IOVS* 2004;45:ARVO E-Abstract 1168) exhibit a similar “sampling” behavior. The alignment of keratocyte cell extensions with the surface pattern is consistent with previous experiments in our laboratory showing surface pattern guidance of epithelial cell filopodia²⁹ and neurites of PC12 cells cultured on nanotopographic surfaces.⁵⁷ Keratocytes are quiescent cells that on exposure to serum change phenotype to that of an activated fibroblast. Because the keratocytes are cultured in serum-free medium, many soluble factors that induce chemotaxis and chemokinesis present in serum are lacking. There is, therefore, no serum-induced stimulation of keratocyte migration, which is also absent within the normal, untraumatized cornea.

After corneal injury, keratocytes in the direct vicinity of the traumatized tissue undergo apoptosis. Adjacent keratocytes transform into fibroblasts, which then proliferate and migrate into the wound site.^{12,58} This *in vivo* behavior is consistent with the much higher migration rates exhibited by fibroblasts and myofibroblasts compared with keratocytes in this *in vitro* study.

The lack of difference in migration rates between fibroblasts and myofibroblasts observed in this study was surprising. Previous experiments by Dugina et al.⁵⁹ and Vaughan et al.⁶⁰ have demonstrated an increase in α -SMA expression and focal adhesion number and size in TGF- β 1-stimulated myofibroblasts in 2D and 3D culture systems, respectively. Given that increased adhesion strength is associated with a lower speed of migration,³⁹ we expected the myofibroblasts to exhibit a lower speed of migration than fibroblasts. This hypothesis was also supported by previous experiments conducted by Mar et al.⁶¹ in which two myofibroblast cell line clones were constructed, one with abundant stress fibers and focal adhesions and one with few stress fibers and downregulation of α -SMA. The clone with abundant stress fibers showed a significantly increased ability to contract a collagen matrix but exhibited significantly decreased cell motility compared with the second clone.⁶¹

Other studies have also shown that the presence of stress fibers was associated with increased contractility^{62–64} and decreased motility.⁶⁵ A substantial difference between our study and the two other studies evaluating cell migration^{61,65} is the nature of the observed cells. Our experimental model involves rabbit cor-

FIGURE 5. Migration pattern of primary rabbit corneal keratocytes, fibroblasts, and myofibroblasts cultured on collagen-coated planar and topographically patterned surfaces. Individual cell trajectories from five randomly chosen motile keratocytes (A, D), fibroblasts (B, E), and myofibroblasts (C, F) are shown. Each color represents the movement of a single cell. (A–C) Planar control surfaces. (D–F) Grid lines represent the direction of grooves and ridges on patterned surfaces with a 4000-nm pitch size. Most of the cell movement on the patterned surfaces is parallel to the surface pattern, with limited movement perpendicular to the surface pattern. Contact guidance was observed on all but the smallest surface sizes (400 and 800 nm). This is in sharp contrast to the orientation of movement of cells on the planar surfaces, which lacks directional guidance. These are representative images of experiments performed in triplicate on planar control and 400-, 800-, 1200-, 1600-, 2000-, and 4000-nm pitch size surfaces (two 6-packs were evaluated per experiment).

neal fibroblasts before and after transformation into α -SMA-expressing myofibroblasts. Lewis et al.⁶⁵ observed human foreskin fibroblasts with different quantities of stress fibers and unknown α -SMA content. Mar et al.⁶¹ observed increased migratory behavior in a gelsolin-overexpressing myofibroblast clone with decreased stress fiber and α -SMA expression and a significantly altered actin cytoskeleton. Because of the significant differences between the cell types used, it is difficult to attribute the changed migratory behavior to a single common trait: the presence of stress fibers. Many other mechanisms, including focal adhesion expression, likely play a role.

The greatest difference in cell behavior would be recorded while comparing a pure population of fibroblasts with a pure population of myofibroblasts. As previously described by Jester et al.,¹⁷ serum-induced myofibroblast transformation was observed in approximately 10% of our cultured fibroblasts. With myofibroblasts being less responsive to topographic cues, the “contamination” of our fibroblast culture with a small population of myofibroblasts likely diminished the difference we observed between these two populations. Future studies might focus on FGF- or PDGF-stimulated keratocytes, which show a purer fibroblast population.

Interestingly, our data show that the same environmental cues (topography pitch size) guiding cell orientation also direct migration of the cells along the surface topography. Thus, topography might influence migration-driven biological phenomena through the induction of cell orientation and the acquisition of polarity. Calculation and comparison of migration rates showed that the contact-guided migration on all but the smallest surface pitch sizes was caused by inhibition of migration perpendicular to the surface topography, not by increased migration parallel to the surface features (Figs. 6B, C). This might have been caused by inhibition of the formation

of cellular extensions perpendicular to the surface pattern. Preferential alignment of filopodial and lamellipodial cell extensions along surface topographic features has previously been described.^{29,30,57} The unguided directional persistence observed during fibroblast and myofibroblast migration on planar and on 400- and 800-nm pitch surfaces has previously been observed in various cell types.^{39,52,66} This phenomenon is thought to be a result of cell polarization during migration³⁹ and can be influenced by small changes in total Rac activity.⁵²

One potential caveat of this study was the plating density of the keratocytes. Because of cell death at densities lower than those allowing intercellular contact, we were unable to plate the keratocytes at very low densities. Intercellular contacts might have biased our results because these contacts are important regulators of cell behavior. However, given that keratocytes maintain intercellular contacts with neighboring cells and essentially operate as a cell syncytium *in vivo*,^{9,10} the results obtained with the plating densities used in this study may well be more reflective of the *in vivo* condition. Because the fibroblasts and myofibroblasts were seeded at densities that prevented intercellular contact, the results obtained were less biased but difficult to compare with the keratocyte results.

Two-dimensional culture systems lack biological relevance, especially regarding the topography of the cellular environment. The physical configuration of a 3D matrix seems to provide spatial signals to resident cells, changing cell morphology, and migration patterns.^{46–52} In concert with these complex topographic cues, 3D matrices also provide cells with a multitude of chemical and additional physical cues,^{50,51} making it difficult to separate out the effects of various stimuli on cell behavior and to monitor the influence of a single stimulus. The anisotropic pattern of our physically and chemically identical 2D substrates enables us to isolate cell responses to a

FIGURE 6. Fibroblasts and myofibroblasts exhibit contact guidance and migrate at a faster rate than keratocytes. (A) Keratocyte, (B) fibroblast, and (C) myofibroblast migration rates on all available surface pitch sizes. Discernible contact-guided migration was observed on the 800- and 1200-nm and larger pitch sizes for both fibroblasts and myofibroblasts. Both fibroblasts and myofibroblasts show a significantly decreased speed of migration perpendicular to the surface pattern on all surface pitch sizes larger than 1000 nm. A transition zone was present around the 800- to 1200-nm pitch size. Keratocyte migration was minimal on all surface pitch sizes and on planar surfaces, with no significant differences observed in migration rate between surfaces. Significant differences in migration rates compared with planar (* $P < 0.05$, ** $P < 0.01$).

single stimulus (topography). The morphologic appearance of fibroblasts cultured in various 3D matrices and on the 2D nanotopographic surfaces used in this study is essentially identical with the morphology of corneal stromal fibroblasts in vivo.⁴⁵ Therefore, patterned 2D substrates may embody the best of both worlds because they seem to simulate the in vivo situation better than planar substrates and yet enable researchers to look at cell behavior in response to environmental stimuli with a reductionist approach.

In summary, we have shown that fundamental cell behaviors of keratocytes, fibroblasts, and myofibroblasts such as shape, alignment, and migration are guided by environmental topography. Surprisingly, we found that all the stromal cells failed to respond to feature dimensions approximating individual fibers in the biomimetic range. It is possible that these results indicate that the corneal stromal cells respond to topographic cues that reside in the size range of lamellae ($\pm 2 \mu\text{m}$) rather than individual collagen fibers ($\pm 30 \text{ nm}$).¹ Given that shape and migration are powerful determinants of cell fate,³⁸ nanotopographic patterning may help in determining that fate. Thus, topography may be tailored to promote or inhibit specific cell behaviors to benefit the performance of prosthetic implants. Additionally, patterned 2D substrates improve the relevance of in vitro cell culture experiments, especially involving corneal stromal cells. The incorporation of nanotopography in the design and fabrication of prosthetic devices that require biointegration and of tissue culture plasticware should be considered.

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