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Mechanosensitivity of fibroblast cell shape and movement to anisotropic substratum topography gradients

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Abstract

In this report, we describe using ultraviolet (UV)-assisted capillary force lithography (CFL) to create a model substratum of anisotropic micro- and nanotopographic pattern arrays with variable local density for the analysis of cell-substratum interactions. A single cell adhesion substratum with the constant ridge width (1 μm), and depth (400 nm) and variable groove widths (1 μm to 9.1 μm) allowed us to characterize the dependence of cellular responses, including cell shape, orientation, and migration, on the anisotropy and local density of the variable micro- and nanotopographic pattern. We found that fibroblasts adhering to the denser pattern areas aligned and elongated more strongly along the direction of ridges, vs. those on the sparser areas, exhibiting a biphasic dependence of the migration speed on the pattern density. In addition, cells responded to local variations in topography by altering morphology and migrating along the direction of grooves biased by the direction of pattern orientation (short term) and pattern density (long term). Molecular dynamic live cell imaging and immunocytochemical analysis of focal adhesions and actin cytoskeleton suggest that variable substratum topography can result in distinct types of cytoskeleton reorganization. We also demonstrate that fibroblasts cultured as monolayers on the same substratum retain most of the properties displayed by single cells. This result, in addition to demonstrating a more sophisticated method to study aspects of wound healing processes, strongly suggests that even in the presence of considerable cell-cell interactions, the cues provided by the underlying substratum topography

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continue to exercise substantial influence on cell behavior. The described experimental platform might not only further our understanding of biomechanical regulation of cell-matrix interactions, but also contribute to bioengineering of devices with the optimally structured design of cell-material interface.

Keywords

Topography; Capillary force lithography; Grooves and ridges; Focal adhesions; Extracellular matrix; Cell migration; Fibroblast cells

1. Introduction

Living cells can sense the local geometry of complex and well-defined structures of extracellular matrix (ECM) to control their own shape, motility, and fate [1–3]. The organization of ECM is frequently hierarchical, with many proteins capable of forming large scale structures with feature sizes ranging from tens of nano-meters to several hundred microns [4]. In connective tissues, it is common to find bundles of collagen micro-fibrils running in parallel to each other, with cells of various origins attached to them [5]. Furthermore, cells present in the scar come in contact with clusters of severed fibrils that arrange themselves in an inhomogeneous pattern that can be perceived by cells as surfaces containing nano- and micro-scale grooves, ridges and pillars with variable local densities [6]. Cell migration in a wound site is mediated by matrix remodeling such as alterations in the composition, structural and mechanical properties of ECM [7]. While young skin has compact and normally arranged connective tissue fiber bundles, the higher expression of matrix metalloprotease (MMP) in aged skin causes degradation of the dermal connective tissue fibers and matrix proteins [8]. The resulting characteristic changes are seen in aged skin such as skin wrinkling, loss of elasticity, and impaired cellular movement [9], influencing various pathological phenotypes. It is therefore quite reasonable to expect that topographic features of the surrounding ECM might play a crucial role in regulation of the physiologically relevant migratory response *in vivo*. However, the extent and the importance of micro- and nanotopography of ECM in defining cell migration are currently poorly understood, in part due to an almost complete neglect of this factor in most *in vitro* experimentation.

It has been demonstrated that biomaterial surface topography that models features encountered in the native basement membrane can profoundly affect various cell behaviors such as morphology [10–12], cell-substratum adhesion [13,14], migration [6,15,16], proliferation [17,18], and differentiation [19–21]. In particular, arrays of parallel nano-grooves (alternatively nano-ridges) have been used as a popular nano-topography model in the previous studies focused on the effects of the substratum nano-topography on cell function. For example, the analysis of cultures of several primary corneal cells revealed differential elongation on smooth vs. nano-grooved substrata, accompanied by differences in actin microfilament and focal adhesion (FA) alignment, sizes and shapes [10,22]. The proliferation rates of these cells were reduced on grooved surfaces less than 800 nm in pitch compared to the smooth surface [13,17]. The nano-grooved patterns also affected cell morphology and formation of filopodia [13], although the percentage of aligned cells was constant on substrate topographies with lateral dimensions ranging from the nano- to the micro-scale, and increased with groove depth [10]. Adhesion strength was observed to be stronger on nanoscale than on microscale under the control shear stress in a laminar flow chamber [14]. Addition of serum to the culture medium resulted in a larger percentage of cells aligning along the topographic patterns vs. no-serum condition [10].

In spite of a considerable amount of ongoing research, however, current efforts in the area of bio-mimetic definition of the cell micro-environment still have important gaps [1]. First, virtually without exception [6,23,24], the state of the art analyses are performed with spatially homogenous patterns of ECM density or topography definition. For example, cell substrata are commonly patterned with micro- and nanoscale ridges of specified pitch, width and height, which, although possibly varied from experiment to experiment, are constant in any one experiment. However, supporting ECM structures in living tissues and the scaffolds that might be used for tissue engineering and engraftment have generally inhomogeneous, complex structures, variable on the scale of a single cell. Second, the measurements performed are commonly static, taking snapshots of cells on the topographically-defined structures with inferences about the mechanisms of cell polarity, locomotion and division made based on the analysis of cells fixed for staining or electron microscopy. Finally, the analyses are normally based on rather infrastructure-intensive and expensive nano-fabrication methodologies, barring this type of research from most labs interested in the basic and applied aspects of cell response to complex microenvironment.

Here we describe the design and fabrication of an anisotropic micro- and nanopattern array with variable local pattern density, and its use as a cell adhesion substratum, on which single cells can be exposed to variable topographic stimuli in the same experiment. This substratum was constructed using UV-assisted CFL, of transparent UV curable poly(urethane acrylate) (PUA) resin in a scalable and cost-effective fashion. Using the proposed cell culture substrata with variable anisotropically patterned topographic features, we measured FA dynamics at high resolution in space and time, using immunostaining and a live cell probe, as well as performing quantitative analysis of cell shape and migration. We show that the described lithographically constructed pattern presents cells with local variation in topographical cues and elicits a profound effect on cell shape and migration, with interesting implications for the wound healing response.

2. Materials and methods

2.1. Fabrication of the poly(urethane acrylate) (PUA) mold

Silicon wafers were spin-coated with a photoresist (Shipley, Marlborough, MA) and then patterned via electron-beam lithography (JBX-9300FS, JEOL). After photoresist development (MF320, Shipley), exposed silicon was deep reactive ion etched (STS ICP Etcher) to form arrays of sub-micron scale ridges with near-vertical sidewalls. The remaining photoresist on silicon wafers was removed using ashing process (BMR ICP PR Asher) and then diced into silicon masters for subsequent replica-molding.

To fabricate topographic nanopattern arrays, PUA was used as a mold material from the silicon master as previously described [25]. Briefly, the UV-curable PUA was drop-dispensed onto a silicon master and then a flexible and transparent polyethylene terephthalate (PET) film was brought into contact with the dropped PUA liquid. Subsequently, it was exposed to UV light ($\lambda = 200\text{--}400\text{ nm}$) for 30 s through the transparent backplane (dose = 100 mJ cm^{-2}). After UV curing, the mold was peeled off from the master and additionally cured overnight to terminate the remaining active acrylate groups on the surface prior to the use as a first replica. The resulting PUA mold used in the experiment was a thin sheet with a thickness of $\sim 50\text{ }\mu\text{m}$.

2.2. Fabrication of topographically variable micro- and nanopatterned arrays

The topographic nanopattern substratum with a density gradient was fabricated onto the glass coverslip using UV-assisted capillary molding techniques [6,26]. Prior to application of the PUA mold, the glass substrate was cleaned with isopropyl alcohol (IPA), thoroughly rinsed in distilled ionized water, and then dried in a stream of nitrogen. Subsequently, an adhesive agent

(phosphoric acrylate: propylene glycol monomethyl ether acetate = 1:10, volume ratio) was spin-coated to form a thin layer (~100 nm) for 30 s at 3000 rpm. A small amount of the same PUA precursor was drop-dispensed on the substrate and a PUA mold was directly placed onto the surface. The PUA precursor spontaneously filled the cavity of the mold by means of capillary action and was cured by exposure to UV light ($\lambda = 250\text{--}400\text{ nm}$) for ~30 s through the transparent backplane (dose = 100 mJ cm^{-2}). After curing, the mold was peeled off from the substrate using a sharp tweezer.

2.3. Culture of cells on topographic micro- and nanopattern arrays

NIH 3T3 fibroblasts were cultured at $37\text{ }^{\circ}\text{C}$ and 5% CO_2 in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 50 U ml^{-1} penicillin, and $50\text{ }\mu\text{g ml}^{-1}$ streptomycin with 10% fetal bovine serum (Invitrogen). The topographically patterned substratum was oxidized and sterilized using plasma treatment (Harrick) at 60 W for 1 min under a pressure of 50 mTorr. Fibronectin ($10\text{ }\mu\text{g ml}^{-1}$, BD Biosciences) was absorbed onto PUA pattern arrays within the culture dish for 1 hr. Cells are seeded at $5\times 10^4\text{ cells ml}^{-1}$ onto arrays of micro- and nanopattern and cultured up to 24 hr.

2.4. Transfection of GFP-vinculin fusion protein

For transient transfections, we used FuGENE6 transfection reagent (Roche) to express enhanced green fluorescent protein (EGFP)-vinculin (pEGFP/V1-1066) [27] in NIH 3T3 fibroblast cells. Briefly, $6\text{ }\mu\text{g}$ DNA was added to $600\text{ }\mu\text{l}$ OptiMEM 1 (Gibco 11058) followed by addition of $18\text{ }\mu\text{l}$ FuGENE6. The mixture was incubated at room temperature for 30 min before adding to 10 cm dish (Corning) containing NIH 3T3 fibroblasts at 60–70% confluency. Cell medium was changed to fresh non-antibiotic media to attain enhanced efficiency of transfection. After 24 hr transfection, NIH 3T3 cells were enzymatically detached using 0.25% trypsin-EDTA, washed with PBS and sorted using FACS Vantage SE system (BD Biosciences) with a 488nm Argon excitation laser. Sorted cells were cultured on fibronectin ($10\text{ }\mu\text{g ml}^{-1}$, BD Biosciences) coated PUA pattern arrays.

2.5. Immunofluorescence staining

NIH 3T3 fibroblasts were seeded onto the topographic micro- and nanopattern substratum and, allowed to spread overnight for 14 hr, then fixed with 3.7 % paraformaldehyde solution for 15 min, washed, made permeable with 0.1 % Triton X-100 in phosphate buffered saline (PBS) for 15 min, and then incubated for 1 hr with Texas Red conjugated phalloidin (Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI, Sigma) to stain for actin filaments and cell nuclei respectively. Cells were blocked using 10 % goat serum in PBS for 1 hr before incubated with a monoclonal mouse anti-vinculin antibody (Sigma) for 1 hr. Stained cells were imaged using a Zeiss LSM 510 Meta CLSM under 100x and 63x plan apochromat objective lens 1.4 N.A. Images were processed using the Zeiss Meta software version 3.5.

2.6. Time-lapse microscopy of live cells

For time-lapse analysis of individual cell movement, NIH 3T3 fibroblasts were cultured on the glass coverslip covered with the topographical pattern substratum, which was previously glued onto the bottom surface of the custom-made Mattek dish (P35G-20-C). For long-term observation, the environmental chamber containing the custom-made Mattek dish integrated with topographically patterned substratum was mounted onto the stage of a motorized inverted microscope (Zeiss Axiovert 200M) equipped with a Cascade 512B II CCD camera. Phase-contrast and epi-fluorescent images of the NIH 3T3 fibroblasts were automatically recorded using the Slidebook 4.1 (Intelligent Imaging Innovations, Denver, CO) for 12 hr at 15 min intervals.

2.7. Imaging cell morphology using scanning electron microscopy (SEM)

For scanning electron microscopy, cultured NIH 3T3 fibroblasts were washed with phosphate-buffered saline (PBS, pH 7.4, Gibco Invitrogen) and fixed in 3% glutaraldehyde (Sigma) in PBS for 1 hr. After fixation, samples were rinsed in 0.1 M sodium cacodylate for 30 min at 4 °C. They were then post-fixed in 2% osmium tetroxide for 1 hr at the same temperature. After a brief D-H₂O rinse, samples were en-bloc stained in 2% aqueous uranyl acetate (0.22 µm filtered) for 1 hr at room temperature in the dark. Following a graded ethanol dehydration cells were critical point dried with liquid CO₂, mounted onto SEM stubs with double stick carbon tape, and sputter coated with 10 nm gold palladium. Samples were viewed and photographed with a LEO FESEM 1530 operating at 1 Kv.

2.8. Atomic force microscopy (AFM)

AFM measurements were performed using a commercial AFM (Surface Imaging Systems, NANOSTATION). Measurement performed in non contact mode. The scan rate was 0.7 line/sec and oscillation frequency was 182 Hz. Data were processed using Surface Probe Image Processor software (Surface Imaging Systems).

2.9. Quantification of cell shape and orientation

For quantitative analysis of cell orientation and elongation, we cultured NIH 3T3 fibroblasts on the topographic substratum for 14 hr and then fixed and stained for F-actin. 15~20 fluorescent images with 1~5 cells per each image were subsequently acquired and analyzed using the SlideBook software. A morphometric parameter, the axial ratio was defined by L_y , the longest length of the cell along the substratum grooves and the width L_x of the cell perpendicular to the gradient of the grooves (ridges) on the substratum. The major axis was defined as the longest aspect of the cell.

The orientation angle of polarized cell was determined by measuring the acute angle between the major axis of the cell and the direction of grooves. A total of 100 cells were used to construct the polarization angle distributions with range -90° and 90° . Positive and negative angles were defined to be counter-clockwise and clockwise direction, respectively. An angle of 0° was defined as the angle when cells were perfectly aligned parallel to the ridge/groove pattern arrays.

2.10. Quantitative analysis of cell migration

Centroid of the image of the cell projection was used as the geometric center of a cell. Assuming that each point enclosed by the perimeter of a cell image is equally loaded, the centroid is calculated as the center of mass of the corresponding geometric shape. To determine the migration speed, the instantaneous speed values were computed from mean squared displacement of two consecutive images taken over a 15 min interval, with these values averaged for 9 hr period. The measured average speed values were grouped according to 5 equally spaced spatial ranges, indicating the position on the substratum, with each range having the width of 100 µm. Only single, ungrouped cells were used for all analyses.

To calculate the probability of a cell to encounter a given ridge density area, cell trajectories were plotted against the ridge array. For visualization, the initial points of all trajectories were centered at same point (0 µm) and trajectories traced, tracking the sign of extension: towards sparser (negative sign) and denser (positive sign) arrays. The dimension perpendicular to the orientation of the ridge array was binned at the resolution of 10 µm. For each trajectory passing through a given bin, a count was incremented by one for the bin to obtain histograms. Results were normalized to obtain the probability that a cell trajectory would pass through the zone associated with a give bin.

2.11. Quantification of focal adhesion (FA) orientation

The relative orientation of the vinculin-GFP bands assembled in FAs was manually determined by measuring the alignment angle with respect to the direction of the grooves. 40 to 70 vinculin-GFP bands at 0, 1, 2, 3, 5 and 8-hr time point images were analyzed to determine the orientation distribution with range -90° and 90° . Positive and negative angles were defined to be counterclockwise or clockwise direction, respectively.

2.12. Correlative analysis of the orientation of actin cytoskeleton relative to focal adhesions

To determine the correlation coefficient (R) of the co-localization of F-actin and vinculin at the protruding lamellipodial region, we analyzed these values using Zeiss Meta software version 3.5. We defined the lamellipodial region as extending over one-tenth of the total distance from the leading edge to the nucleus. Measurements were taken from 20–30 cells, grouped into those on ‘sparse’ (the range of groove width: $6.9\ \mu\text{m} \sim 9.1\ \mu\text{m}$) and ‘dense’ (the range of groove width: $1\ \mu\text{m} \sim 5.6\ \mu\text{m}$) ridge array areas depending on the position of lamellipodia extending in the direction of locomotion.

2.13. Analysis of collective cell movement

Stencils were molded using PDMS elastomer (Sylgard 184, Dow Corning) on a photoresist template, as previously described [28]. Briefly, 100- μm -thick rectangular prism structures were fabricated in negative photoresist (SU8–2025 or SU8–2075, Microchem) by conventional photolithography. The rectangular shaped ($6 \times 10\ \text{mm}$) PDMS stencils were made by cutting with razor blades. The PDMS stencil was then attached along the perpendicular direction of the ridged pattern arrays on the glass coverslip at the bottom of the custom-made Mattek dish (P35G-20-C), which was pre-coated with $10\ \mu\text{g ml}^{-1}$ fibronectin for 1 hr. After thermal curing at 37°C for 2 hr, cell suspensions were added according to standard tissue culture procedures. Once a confluent monolayer was formed, the PDMS stencil was slowly peeled off and collective migration of fibroblasts was allowed into the cell-free area for 24 hr.

2.14. Statistical analysis

The unpaired Student's *t* test was used for statistical analysis. Differences were considered significant at $P < 0.05$. Skewness (a measure of the lack of symmetry) was calculated using the corresponding function in Microsoft Excel.

3. Results

3.1. Fabrication of topographically variable micro- and nanopatterned arrays

Using UV-assisted CFL (see Fig. 1a), we fabricated topographically variable ridge pattern arrays on a large area ($>1 \times 1\ \text{mm}$), of which feature size is close to the size scale of individual focal adhesions ($\sim 1\ \mu\text{m}$). These patterns were expected to not only provide the local density variation of cell adhesion substratum topography, but also allow collection of sufficient data for statistically significant quantitative analysis from a single experiment. The fabricated topographic pattern was composed of an array of parallel ridges $1\ \mu\text{m}$ wide and $400\ \text{nm}$ high in ridge with variable inter-spacing (i.e. $100\ \text{nm}$ increments in groove width between neighboring ridges from the dense to the sparse pattern) as verified by SEM and AFM measurements (Fig. 1b,c). The groove width in the fabricated substratum ranged from $1\ \mu\text{m}$ to $9.1\ \mu\text{m}$. Compared to other methods, such as photolithography [29], colloidal lithography [30,31], polymer demixing [32,33], and nanoimprinting [11,34] that were used to create sub-micron topographic features in silicon substrata, the CFL method offers a fast and cost-effective way of producing topographically gradient ridge pattern arrays of transparent, biocompatible polymeric materials with high pattern fidelity and physical integrity. Furthermore, topographically patterned areas were surrounded by planar smooth polymer regions, allowing

for direct simultaneous assessment of cell behavior in controlled conditions on both smooth and textured substrata. The fabricated pattern was highly reproducible between experiments. To the best of our knowledge, this is the first demonstration of the use of lithographically-defined topographical pattern arrays of variable local densities in a single cell adhesion substratum for detailed analysis of cell migration through influenced by cell-substratum interactions.

3.2. Analysis of cell morphological response to density-variant substrates

Using topographically defined ridge/groove patterns with spatially graded features, fabricated as described above, we analyzed cell shape and locomotion simultaneously with collecting data from different locations in a single substrate, and thus from areas of different pattern densities. Several notable findings were derived from the experiments. First, we observed that NIH 3T3 fibroblasts polarized largely along the direction of micro- and nanostructured ridge/groove pattern arrays. However, the extent of cell alignment and elongation appeared very sensitive to the absolute local density of ridge pattern arrays. For instance, cells on denser patterns aligned more strongly along the direction of ridges/grooves, relative to those on sparser pattern areas (Fig. 2a). Correspondingly, cells became less oriented and more rounded on the ridged arrays of lower local densities which approached the lack of topographical cues to those on flat surfaces. Furthermore, fibroblasts appeared to be very sensitive not only to the local density of the topographic features, but also to its gradients. Indeed, SEM analysis revealed that cells, particularly in relatively dense local pattern areas, formed flattened lamellipodia at the leading edges preferentially up the pattern density gradients (Fig. 2b). In contrast, the lamellipodia in cells cultured on regularly spaced ridge/groove pattern aligned strongly along the direction of ridge patterns, similar to previous findings (Fig. 2c) [10,14]. These data suggest that the variation in the local densities of the ECM topography on the level of a single cell might constitute a more potent guidance cue than previously appreciated, and needs to be further explored in terms of its importance for regulation of *in vitro* and *in vivo* cell migration.

3.3. Effect of the density of the ridged pattern arrays on cell orientation, elongation, and migration

To quantify the observations obtained using low throughput electron microscopy imaging, we took advantage of the ability to assay multiple cells simultaneously present on substratum with graded local densities of topographic cues in a single substratum (Fig. 3a). In particular, we quantitatively characterized the dependence of polarization of cell morphology and movement on topographic variations of spacing between the substratum ridges under otherwise identical experimental conditions (Fig. 3a). The cell orientation angle relative to the grooves/ridges was measured to determine how cells are directionally sensitive to different local ridge densities grouped into 5 adjacent 100 μm pattern arrays. As indicated in Fig. 3b, the degree of the cell polarization response increased with increasing pattern density. For the cells adhering to the substratum zone with the intermediate ridge density (positioned between 100 μm and 400 μm), cells were not aligned as well as those adhering to the denser pattern area (positioned between 0 μm and 100 μm ; the range of groove widths: 1 μm ~ 3.8 μm). Cells adhering to the most sparsely patterned area (positioned between 400 μm and 500 μm ; the range of groove widths: 8.1 μm ~ 9.1 μm) were randomly oriented similar to those adhered on flat surfaces (positioned between 500 μm and 600 μm), consistent with the initial observations shown in Fig. 2a. Cell morphological change was also assessed by measuring the axial ratio, defined as the ratio of L_y , the length of cell parallel to the groove/ridge and L_x , the width perpendicular to L_y . We found a decreasing linear trend of the axial ratio with the substrate position, illustrating that cells elongate in the direction of ridge/groove arrays proportionally to the density of the ridged patterns (Fig. 3c). We consistently found highly elongated shapes of fibroblast cells with the range of the axial ratio from 2 to 6 along the density gradient as the inter-ridge distance decreased and ridges became closely packed. For comparison, the value of the axial ratio was

nearly unity on the flat surfaces devoid of topographic cues. These data suggest that cell elongation on the micro- and nanostructured substrata depends strongly on the density variations of the underlying substrate topography.

To investigate whether cell migration is affected by the density variation in ridged pattern arrays, we performed time-lapse live cell imaging using epifluorescence microscopy and then analyzed the migration speed in the five distinct areas on the substrate of different local topographic densities shown in Fig. 3a. We observed that cell polarization was often accompanied by directional cell migration along the ridges. Although both cell alignment and elongation was directly proportional to the density of the topography, we observed that the cell speed depended biphasically on the topographic density, with the fastest migration ($\approx 40 \mu\text{m/hr}$) occurring at an intermediate ridge pattern density (positioned between $200 \mu\text{m}$ and $300 \mu\text{m}$; the range of groove widths: $5.6 \mu\text{m} \sim 6.9 \mu\text{m}$) (Fig. 3d). Variation of fibronectin concentration also showed profound effect on cell migration speed on all the pattern density (data not shown). We note that although it is already well established that cells migrate optimally at intermediate coating densities of ECM protein [35,36], this is the first observation of a biphasic cell speed response to variation of the topographic density of the ridged cell adhesion substratum, with the substratum itself largely defining the direction of cell migration.

We then analyzed migration tracks of fibroblast cells from two substratum zones: the dense pattern array zone (positioned between $0 \mu\text{m}$ and $250 \mu\text{m}$; the range of groove widths: $1 \mu\text{m} \sim 6.3 \mu\text{m}$) and the sparse pattern array zone (positioned between $250 \mu\text{m}$ and $500 \mu\text{m}$; the range of groove widths: $6.3 \mu\text{m} \sim 9.1 \mu\text{m}$), over the 9 hr period (Fig. 4). Cells on the densely spaced array zone (the range of groove widths: $1 \mu\text{m} \sim 6.3 \mu\text{m}$, $n=27$) traveled mostly along the direction of the ridge pattern (Fig. 4a), whereas the directions of cell migration on the sparsely spaced array (the range of groove widths: $6.3 \mu\text{m} \sim 9.1 \mu\text{m}$, $n=55$) were considerably more dispersed (Fig. 4c). Further trajectory analysis indicated that cell trajectories were also biased toward the sparser pattern area for cells cultured on the dense ridged arrays (positively skewed distribution in Fig. 4b) and towards the denser pattern area for cells on the sparsely ridged arrays (negatively skewed distribution in Fig. 4d). Together, these data strongly suggest that the density of the topographic patterns present on cell substrata can serve as a strong graded cue defining both the directionality and speed of cell movement.

3.4. Quantitative and correlative analysis of the orientation of stress fibers and focal adhesions (FAs)

It has been well documented that micro- and nano-scale ridges elicit contact guidance in many cell types, but the underlying mechanism on the role of contact guidance in generating polarization of cell movement remains still unclear [37,38]. Furthermore, the crucial parameters in topographic characteristics determining cell migration are largely unknown. Previous studies suggested that substrate micro- and nanotopography might influence the organization and regulation of FAs, and hence the orientation of the cytoskeleton and the cell itself [6,10,22]. FAs are large, dynamic protein complexes through which the cytoskeleton of a cell connects to the ECM [39]. The dynamic assembly and disassembly of FAs also play a central role in regulation of cell migration [36]. Given the strong influence of the substratum pattern density on cell migration characteristics observed in our experiments, we next investigated whether the formation of stable FAs and the cytoskeleton remodeling was sensitive to topographic pattern density. We found that individual NIH3T3 fibroblasts aligned their actin cytoskeleton along the direction of the ridges, established focal contacts proximal to the ridges, and extended lamellipodia preferentially along more densely spaced ridges (the range of groove widths: $1 \mu\text{m} \sim 3.8 \mu\text{m}$) (Fig. 5a). Cells on the sparsely spaced one-dimensional (1D) ridge array (the range of groove widths: $8.1 \mu\text{m} \sim 9.1 \mu\text{m}$) also aligned their stress fibers, but their

focal contacts were more randomly distributed (Fig. 5b), similar to those on the flat continuous surfaces (data not shown).

To ascertain the role of density of ridge array in regulation of cell motility, and elucidate its underlying mechanism, we performed quantitative analysis of FA and stress fibers distribution, and determined their co-localization in the leading edge of the cell. We found that FAs were spatially co-localized with the actin cytoskeleton proximal to the ridges (Fig. 4c,d), clustering preferentially in the close vicinity of the edges of individual ridges. We further performed the correlative analysis of the direction of actin filaments and vinculin bands (vinculin is a constitutive part of FAs), at the protruding lamellipodia (Fig. 5e). We found a higher correlation between the orientations of actin filaments and vinculin bands (i.e., FAs) in the lamellipodia on the dense ridge pattern (the range of groove widths: $1\ \mu\text{m} \sim 5.6\ \mu\text{m}$), compared to the sparse ridge pattern zones (the range of groove widths: $6.9\ \mu\text{m} \sim 9.1\ \mu\text{m}$). Together, these findings suggested that the density of a topographically defined cell adhesion substratum can determine the average (or prevailing) orientation of FAs, which in turn can define the cell polarity and modulate cell migration speed.

3.5. Analysis of focal adhesion dynamics in living cells on variable density substrata

As indicated above, vinculin is one of the critical components in the early formation of FAs, and thus a convenient marker of FAs formation [27]. To further investigate the details of the influence of the topographic patterns on the dynamics of the FAs organization, we performed live cell fluorescence imaging of the vinculin-GFP fusion protein in fibroblasts cultured on the topographically defined substratum following their initial adhesion. We observed, consistently with previous observations [10,40,41] and the immunostaining analysis reported above (Fig. 5a,b), that vinculin-GFP localized in the form of linear bands indicative of the FA architecture (Fig. 6a). Furthermore, the vinculin-GFP bands in cells on the denser pattern area (the range of groove widths: $1\ \mu\text{m} \sim 5.6\ \mu\text{m}$) were in a close proximity to and aligned with the direction of the nanotopographic ridges throughout the cell, both at the front of the cells and at their rear. However, in cells on the sparser pattern area (the range of groove widths: $6.9\ \mu\text{m} \sim 9.1\ \mu\text{m}$), although the orientation of the vinculin bands generally increased over time, the bands in the front retained a more random orientation compared to those in cells on the denser pattern (Fig. 6b). Indeed, as the individual cells moved through the field of view, the distribution of FA was progressively more dominated by the well aligned FAs in the cell's rear in cells migrating in both sparser and denser substratum zones, leading to the tightening of the distributions (Fig. 6c,d). The results suggest that the increasingly isotropic shape and lower precision of movement directionality observed for cells localized to the sparser zone is in part due to the ability of these cells to retain the shorter lived FAs at the leading edge, FAs whose orientation is not completely defined by the underlying substratum topography. More generally, we found that FA dynamics can be strongly dependent on the local variation in topographical features.

3.6. Analysis of collective cell migration on a variable density cell substratum

Collective cell migration, e.g., in development or wound healing, is a complex process regulated by organization of motile interacting cells, and the organization and integrity of the surrounding extracellular matrix [42]. The topographically defined substratum examined here can provide a useful means to model and analyze potential effects of ECM density and organization on collective cell migration. To illustrate this, we extended our experimentation from the analysis of single cell locomotion using sparse cell seeding to investigation of migration of fibroblast monolayers developing as a result of dense cell seeding. Using stencils, we could seed only parts of the available substratum, studying the cell movement into the available substratum areas devoid of cells. This assay is analogous to the more common wound healing assays, in which areas devoid of cells are created by scratching of the existing cell monolayers [43]. However, scratching can introduce undue cell damage, easily avoidable by

using stencils. More specifically, we seeded NIH3T3 fibroblasts as monolayers on a part of the available the topographically defined substratum and allowed them to migrate to the cell-free area for 24 hr. The direction of the cell migration was designed to be along the substratum ridges. We observed that the aligned groove and ridge arrays significantly enhanced cell migration compared to flat continuous surface (Fig. 7a, b). The result was supported by a quantitative and statistical analysis (* $p < 0.05$) (Fig. 7c). Interestingly we also observed differential migration speed as a function of the topographical feature density with the fastest migration frequently occurring at an intermediate ridge pattern density (see examples in Fig. 7d), consistent with results from individual cell migration (Fig. 3d). These results suggested that the effects of differential density of the substratum topography on cell organization and migration might hold both for individual migrating cells and dense groups of cells characteristic of many types of organizing tissues.

5. Discussion

Cell migration *in vivo* occurs within complex topographically and chemically defined cell adhesion substrata whose properties can considerably vary between different tissues and between different regions of the same tissue. A migrating cell can therefore face a dynamically changing ECM organization, which in part can be experienced as altering topography and density of the cell adhesive matrix. Previous studies have strongly suggested that topographically defined substrata can profoundly influence cell polarization and movement [3,15,16]. However, to date, virtually no attempts have been made to study in detail the cells simultaneously moving on substrata of distinct feature densities. In this analysis, we addressed this gap in analysis by using a single substratum with a graded change in the density of the topographical features.

Simultaneous analysis of cells cultured in different zones of this substratum strongly suggested two general trends relating to dependence of cell organization and migration on the topographical feature density. First, the anisotropy of cell morphology and the instantaneous orientation of the cell elongation with respect to the orientation of the substratum progressively increased with increasing ridge density. Second, the cell speed displayed an optimal value at an intermediate ridge density. These surprising findings suggest that, within the ridge densities tested, mouse fibroblasts can display exquisite sensitivity to the exact nature of the micro-scale variation of ridge separation and adjust their behavior accordingly. Furthermore, the results implied that if a single cell can span areas of the substratum with different ridge densities, it might sense the density gradient and ultimately migrate towards the intermediate density areas, within which cells have the optimal speed.

What might be reason for the differential cell responses to the local ridge densities? The clue to the possible mechanism came from the analysis of the changes in the spatial and temporal distribution of FAs in the moving cells (Fig. 5 and Fig. 6). The FAs were enriched around individual ridges and appeared to be stabilized in the immediate ridge vicinity. The stabilization ultimately led to co-orientation of FAs and ridges for all ridge densities observed, although for cells in areas of lower ridge densities, the orientations of FAs at the cell front retained substantial randomness. This interdependence between FA stability and ridge location has strongly suggested that cells can measure local rigidity of the substratum, which can have strong anisotropy around the ridges. Due to the currently poorly understood feedback mechanisms, FAs and the actin stress fibers linking them appear to get increasingly stabilized with increasing resistance from the substratum to the forces applied by the stress fibers [44,45]. Furthermore, generally, the resistance to bending deformation of a ridge is lower than the resistance to compressive deformation, resulting in a pronounced local anisotropy in the substratum rigidity. Due to the resulting anisotropic resistance to deformation by adhering cells, one expects an increased stabilization of FAs along rather than across the direction of the ridges, consistent

with our findings. This effect can influence a lower fraction of FAs, if the number of ridges covered by a single cell is relatively small, leading to a lower average precision of FAs co-orientation and lower FAs stability observed by us at the fronts of cells at lower ridge densities (e.g., Fig. 5b). As a consequence, this further can lead to more rounded cell shapes and more random instantaneous cell orientations (Fig. 3, lower ridge density). On the other hand, at very high ridge densities, the FAs can be over-stabilized leading not only to more precise cell orientation control, but also to an increase in effective cell adhesion to the substratum (FAs are points of cell-substratum interactions). This, in turn, can lead to a decrease in the effectiveness of cell separation from the substratum, resulting in both an increase in cell elongation and a decrease in cell speed (Fig. 3, higher ridge density). Thus the internally consistent behavior of FAs and cellular phenotypes on the variable topography pattern strongly supports sensing of the effective substratum rigidity as the primary determinant of the cell responsiveness to the topographic cues in our experimental setting.

It might be of interest to explore potential physiological consequences of cell responsiveness to the differential substratum feature density. Fibroblasts can traverse connective tissues and are among the first cells to arrive at a wound site. Furthermore, they can modify ECM by secreting its components, especially in the context of a large scale damage inflicted in many wounds. As such, these cells not only migrate on chemically, topographically and mechanically diverse substrata, they also participate in a gradual restructuring of the tissue scaffold matrices. For instance, fibroblasts can contract, bringing polymeric fibrous structures together, and thus contributing to scar tissue formation. The differential sensitivity of cell movement to the substratum topography found in this study suggests that fibroblasts or other motile cells can gradually switch from random, amoeboid movement to a much more directed movement by more elongated and organized cells, as the structure of the matrix is progressively refined. Furthermore, cells can move from either well developed (dense) or poorly organized (sparse) ECM structures to the zones of an intermediate ECM density and organization. This might lead to a progressive expansion of the structured dense ECM zone as cells build it up within a wound site and collectively progressively move toward the less dense and developed ECM areas (Fig. 8). This re-arrangement of fibroblasts during the fibroplasia phase of wound healing might serve to mediate a gradual completion of deposition of prospective ECM, and, in addition to apoptotic cell clearance, ensure ultimate removal of fibroblasts prior re-epithelization.

6. Conclusions

Our study supports the notion that cells can be exquisitely discriminating in their responses to the mechanical and topographical cues present in natural and engineered complex cell adhesion substrata. These powerful cues can be used to define cell shape and orientation, and control cell velocity, which can be used to both mimic the complexity of *in vivo* conditions and design novel model tissue properties. We demonstrated that cells can also respond to gradients of the topographic cues, the response that might be related to existence of an optimal topographical feature density for cell speed. We also confirmed that the control of cell movement by the substrata can be preserved in collective cell movement. These findings, when combined, suggest that the proposed methodology for design and fabrication of complex cell substrata may be used to engineer the properties of engineered tissues and, possibly, further affect tissue self-organization through controlling the structure of the ECM [46]. We propose therefore that capillary force lithography-based approach to controlling the topography of the biomaterial-cell interface can provide a potential strategy for developing self-organized cell-derived matrix as a bridging material for tissue repair or other regenerative applications.

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Figure 1.

Fabrication of polymeric ridge pattern arrays with graded spacing. (a) Schematic of the microfabrication process used to generate PUA micro- and nanopattern arrays. Drawings are not to scale. (b) The three-dimensional AFM image of topographic ridge pattern arrays of $\sim 400\ \text{nm}$ height and $\sim 1\ \mu\text{m}$ width. (c) An SEM image of one-dimensional topographical ridge pattern with graded spacing ($\sim 100\ \text{nm}$ increments in groove width between neighboring ridges).

Figure 2.

The morphological response of fibroblasts to the local density of topographic pattern arrays with graded spacing. (a) A phase-contrast micrograph of NIH 3T3 fibroblasts shows the differential morphology associated with the density variation of the underlying topographic pattern arrays. (b–c) SEM images of critical point-dried NIH 3T3 fibroblasts plated for 14 hr (b) on variable ridge pattern arrays with graded spacing and (c) on regularly spaced topographic pattern arrays with 1 μm wide ridges and 1 μm wide grooves. The white arrow indicates membrane protrusion extending toward the more densely spaced ridges.

Figure 3.

Effects of the local density of ridged pattern arrays on cell shape and migration. (a) A schematic representation of the density gradient of the ridged pattern array, indicating the distance measured from the most densely patterned area (0~100 μm) to the smooth area (500~600 μm). The average groove width is 2.6 μm , 4.8 μm , 6.3 μm , 7.5 μm and 8.6 μm in these zones, ranging from the most densely patterned area (0~100 μm) to the smooth area (500~600 μm), respectively. (b) The distribution of orientation angles for cells adhered to the substratum with the graded ridge spacing (error bars represent mean $\pm$ SD). (c) Morphological changes of fibroblasts as a function of the density variations in the underlying substratum, described by

axial ratio of L_y/L_x as a function of position (error bars represent mean $\pm$ SEM). (d) A biphasic dependence of cell migration speed on the topographic density (error bars represent mean $\pm$ SEM).

Figure 4.

Analysis of cell migration trajectories. Migration trajectories of cells (a) on a densely spaced ridged arrays (positioned between 0 μm and 250 μm ; the range of groove width: 1 μm ~ 6.3 μm ; n=27) and (c) on a sparsely spaced ridged arrays (positioned between 250 μm and 500 μm ; the range of groove width: 6.3 μm ~ 9.1 μm ; n=55). Probability of a cell trajectory to encounter the zone of a given ridge density on the dense (b) or sparse (d) ridge arrays. Skewness of the probability distributions is shown in each panel (b, d). Distributions indicated in each panel suggest that cell trajectories are biased toward the sparser pattern area for cells on dense ridge arrays (positively skewed distribution in (b)) and towards the denser pattern area for cells on sparse ridge arrays (negatively skewed distribution in (d)).

Figure 5.

Representative immunofluorescent micrographs of fibroblasts cultured on the patterned ridged substrata of different local densities. (a) A cell on the most densely spaced ridged arrays (positioned between 0 μm and 100 μm ; the range of groove width: 1 μm ~ 3.8 μm). (b) A cell on the most sparsely spaced ridged arrays (positioned between 400 μm and 500 μm ; the range of groove width: 8.1 μm ~ 9.1 μm). (c–d) Spatial co-localization of the actin cytoskeleton and FAs in the protruding lamellipodium aligned proximal to the individual ridge. (e) Quantitative and correlative analysis of the actin cytoskeleton and FAs in the protruding lamellipodium (error bars represent mean $\pm$ SEM).

Figure 6.

Effects of substratum topography on adhesion dynamics. (a–b) Time-lapse photographs of FA orientations of NIH 3T3 fibroblast cells transfected with GFP-vinculin cultured on (a) the densely spaced ridged array and (b) the sparsely spaced ridged array. (c–d) Angular histograms showing the distribution of FA orientation dynamically changing over time after exposed to the underlying (c) dense (positioned between 0 μm and 200 μm ; the range of groove width: 1 μm ~ 5.6 μm) and (d) sparse (positioned between 300 μm and 500 μm ; the range of groove width: 6.9 μm ~ 9.1 μm) ridged pattern arrays. The average groove width of the underlying patterns is 3.1 μm in (a) and 8.1 μm in (b).

Figure 7.

Topography-induced change in collective fibroblast cell migration (a–b) Phase contrast micrographs of collective fibroblast migration on (a) anisotropic micro- and nanopatterns and (b) the flat substratum in an *in vitro* wound healing model. (c) Quantification of cell migration speed for 12 hr (error bars represent mean $\pm$ SEM). (d) Representative time-lapse photographs showing the progression of collective fibroblast migration from dense (the average groove width: 3 μm), intermediate (the average groove width: 6.8 μm), and sparse (the average groove width: 8.7 μm) pattern density areas, respectively. The range of groove width in (a) is 3.8 μm and 6.4 μm.

Figure 8.

Model of the role of fibroblasts in extracellular matrix remodeling during wound healing. During the wound healing process, fibroblasts are functionally essential cells that migrate toward and into the open wound over the fibrin clot, which results in deposition of collagen, restructuring of ECM and formation of granulation tissue. Migrating fibroblasts also exert tractional forces onto the collagen matrix, which results in its reorganization along the direction of the resulting stress. Our results suggest that fibroblasts can aggregate in zones of intermediate ECM density that might become the zones of active ECM repair, which can shift as cells progressively create denser matrix, following which cells clear these now denser zones and

shift to the adjacent zone. This, along with apoptosis, can be an efficient mechanism of cell clearance from the restructured wound zones prior to re-epithelialization.