Microtopography and flow modulate the direction of endothelial cell migration

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Uttayarat P, Chen M, Li M, Allen FD, Composto RJ, Lelkes PI. Microtopography and flow modulate the direction of endothelial cell migration. Am J Physiol Heart Circ Physiol 294: H1027–H1035, 2008—The migration of vascular endothelial cells under flow can be modulated by the addition of chemical or mechanical stimuli. The aim of this study was to investigate how topographic cues derived from a substrate containing three-dimensional microtopography interact with fluid shear stress in directing endothelial cell migration. Subconfluent bovine aortic endothelial cells were seeded on fibronectin-coated poly(dimethylsiloxane) substrates patterned with a combinatorial array of parallel and orthogonal microgrooves ranging from 2 to 5 μm in width at a constant depth of 1 μm. During a 4-h time-lapse observation in the absence of flow, the majority of the prealigned cells migrated parallel to the grooves with the distribution of their focal adhesions (FAs) depending on the groove width. No change in this migratory pattern was observed after the cells were exposed to moderate shear stress (13.5 dyn/cm²), irrespective of groove direction with respect to flow. After 4-h exposure to high shear stress (58 dyn/cm²) parallel to the grooves, the cells continued to migrate in the direction of both grooves and flow. By contrast, when microgrooves were oriented perpendicular to flow, most cells migrated orthogonal to the grooves and downstream with flow. Despite the change in the migration direction of the cells under high shear stress, most FAs and actin microfilaments maintained their original alignment parallel to the grooves, suggesting that topographic cues were more effective than those derived from shear stress in guiding the orientation of cytoskeletal and adhesion proteins during the initial exposure to flow. In physical terms, cell migration proceeds in three coordinated steps: 1) membrane extension and formation of FAs at the leading edge, 2) forward movement of the cell body through contraction of the actin cytoskeleton by myosin-based motors, and 3) detachment of the trailing edge of the cell, which completes the cycle of the migration process (25, 30, 35). When cultured cells are exposed to a steady, laminar flow, lamellipodial protrusions develop within minutes from the cell periphery in the direction of flow (27, 29), followed by the recruitment of focal adhesion kinase at FA sites underneath the newly formed lamellipodia (27). Shear stress also polarizes FAs to localize at the leading edge and the trailing edge of the cell before its persistent migration downstream with the flow (27). In addition to the alignment of cell shape, both actin microfilaments and microtubules remodel and align in the direction of flow (16, 18, 28). Thus, the integrated system of FAs and cytoskeleton is essential in mediating the physical process of cell migration.

Directional endothelial cell migration in vitro can be induced by chemical stimuli, either soluble in the medium (chemotaxis; Refs. 1, 10, 11, 14) or immobilized on the substrate surface (haptotaxis; Refs. 22, 24), as well as by mechanical stimuli (mechanotaxis), such as fluid shear stress (24, 27, 35). The increase in shear stress magnitude from 5 to 45 dyn/cm² enhances the migration distance of human umbilical vein endothelial cells about twofold into the denuded area after a 24-h exposure to flow (38). With the combination of both haptotactic and mechanotactic cues arranged orthogonally to each other, these two stimuli competitively direct endothelial cell migration (24). The increase in shear stress from 2 to 10 dyn/cm² is shown to effectively switch the direction of ~50% of endothelial cells to migrate with the flow across the haptotactically derived collagen tracks over a 16-h period (24). This result suggests that above a certain threshold, fluid shear stress can significantly modulate cell migration on adhesive substrata.

In this study, we examined the migration of subconfluent bovine aortic endothelial cells (BAECs) on poly(dimethylsiloxane) (PDMS) substrates patterned with a combinatorial array of three-dimensional (3D) microgrooves under static (no shear) conditions and when exposed to two different levels of fluid shear stress. Based on our previous study (40) of BAEC alignment on the 3D microgrooves, we hypothesized that the spatial localization of FAs along the groove depth would guide the directional cell migration. Results indicate that while the majority of cells migrated parallel to the grooves under static...
condition, the direction of cell migration under flow depended on the magnitude, as well as the direction, of fluid shear stress with respect to the orientation of microgrooves. This interplay between substrate microtopography and shear stress suggests that the direction of cell migration can be modulated by the spatial localization and the forced reorganization of FAs. For the development of vascular prostheses, these microgrooved surfaces can promote the endothelialization process in small caliber prosthetic grafts and facilitate the healing of the grafts after bypass surgery.

MATERIALS AND METHODS

Microfabrication of the combinatorial array of microgrooves. The combinatorial 4 × 8 array of microgrooves, containing four sets of groove patterns organized in the horizontal and vertical directions, was first fabricated on a silicon (Si) wafer (Fig. 1A) by electron-beam (e-beam) lithography and reactive ion etching at the Microfabrication Laboratory (University of Pennsylvania). For the symmetric patterns 5 × 5, 3 × 3, and 2 × 2, microgrooves contained ridges and channels of equal widths, whereas for the asymmetric 5 × 2 pattern, the widths of the ridges and channels were 5 and 2 μm, respectively. Each groove area, 500 × 500 μm², was separated by a distance of 500 μm.

For e-beam lithography, the precleaned Si wafer (Silicon Quest International, Santa Clara, CA; Ref. 40) was spin-coated with poly(methyl methacrylate) (PMMA; MicroChem, Newton, MA) at 3,000 rpm for 45 s to obtain a 400-nm resist layer. The PMMA-coated wafer was then baked at 110°C for 10 min before irradiation with e-beam. The microgroove patterns were designed and generated using commercially available software (Raith, Ronkonkoma, NY). E-beam lithography was performed on a JEOL (JSM-6400, Peabody, MA) scanning electron microscope (SEM) at a 15-mm working distance, ×50 magnification, 10-nA e-beam current, and 400-μC/cm² area dose. After e-beam writing, the resist was developed for 45 s in a solution of 1:3 (vol/vol) methyl isobutyl ketone/isopropanol, followed by rinsing in isopropanol for 45 s. After the evaporation of Nichrome IV to serve as a photomask (40), the wafer was then dry etched by reactive ion etching to create groove depth of 1 μm. This depth dimension was chosen based on our previous study of BAEC alignment (40). The finished Si wafer served as a mold to replicate the combinatorial array of microgrooves on the PDMS substrates.

Preparation and characterization of PDMS substrates. All PDMS substrates were prepared from Sylgard 184 Silicone Elastomer Kit (Robert McKeown, Branchburg, NJ) and sterilized as previously described (40). The microgroove profiles on the PDMS replicas were inspected by SEM, FEI Strata DB235 Focused Ion Beam (FEI, Hillsboro, OR), at an acceleration voltage of 10 keV. To observe cell migration in real time, the PDMS substrates were cut into circles (2-cm diameter) for static experiments and rectangles (2.5-cm wide × 6.0-cm long × 0.1-cm thick) for experiments conducted under flow. After sterilization, all substrates were incubated in a 10 μg/ml fibronectin (Fn) solution for 15 h. The nonspecific adhesion was blocked with 1% BSA (Fraction V; Sigma; Ref. 40). As we have previously demonstrated (39, 40), this extended incubation period in the Fn solution produced a uniform coverage of Fn coating on both grooved and smooth surfaces.

Assembly of the flow system. The parallel plate flow chamber device (21), with sealed glass windows on both the upper and the lower plates, was custom built from polysulfone (McMaster-Carr, Dayton, NJ) at the Research Instrumentation Shop (University of Pennsylvania). A Teflon gasket (McMaster-Carr) was inserted between the two plates, creating a rectangular flow channel (1.35-cm wide × 4.30-cm long × 0.025-cm high) for the circulation of medium. After each substrate was assembled on the lower plate, the two plates were screwed tight and connected to the flow loop, which consisted of a peristaltic pump (Cole Palmer, Vernon Hills, IL); two pulse dampeners to generate a steady laminar flow (17); a parallel plate flow chamber; and a medium reservoir (16, 17, 24, 36). The medium was maintained at 37°C throughout the entire experiment outside the incubator by keeping the reservoir and the pulse dampeners in temperature-controlled water baths.

Cell culture. BAECs were isolated and cultured in gelatin-coated T75 flasks using complete DMEM (Mediatech) supplemented with 10% FBS (Hyclone), 1 mg/ml glucose, 0.3 mg/ml L-glutamine, 10 μg/ml streptomycin, 10 U/ml penicillin, and 25 mg/ml amphotericin at 37°C in 95% air/5% CO₂ as previously described (40). Cells were subcultured every 2 days and used at passages 8–12.

Cell labeling with fluorescent tracker. For real-time fluorescence visualization, BAECs were prelabeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) in suspension in serum-free DMEM according to the manufacturer’s instructions. In brief, the cell suspension was first diluted to ~500,000 cells per 1 ml of serum-free DMEM containing a 1:100 (vol/vol) solution of DiI and incubated for 15 min at 37°C. The cells were then pelleted by centrifugation at 400 rpm for 4 min at 25°C, washed twice, and finally resuspended in serum-free DMEM. The uniform DiI labeling was confirmed by flow cytometry and fluorescence imaging (data not shown).

Time-lapse observation of individual BAEC migration. The DiI-labeled cells were seeded on the PDMS substrates at a density of 5,000 cells/cm² (~100,000–150,000 cells in total) in serum-free DMEM to reduce the interference of serum proteins with the pre-coated Fn on the PDMS substrates. Cells were allowed to attach and spread for 2 h inside the incubator. Before the start of all time-lapse recording on the microscope outside the incubator, cells were refreshed in DMEM without bicarbonate, supplemented with 25 mM HEPES (Cellgro) to maintain a physiological pH in the absence of CO₂, 2 μg/ml ascorbic acid (Sigma) as an antioxidant to minimize bleaching of DiI, and 0.5% FBS.

For the static experiment, circular PDMS substrates, preseeded with BAECs as described above, were placed inside a 12-well tissue culture plate. A heat pad underneath the well plate provided a constant
temperature of 37°C. To record cell migration under flow, the pre-seeded PDMS substrates were placed inside the parallel plate flow chamber and assembled onto the microscope stage (Nikon TE2000U equipped with a Prior ProScan II motorized stage). A steady, laminar shear stress of 13.5 or 58 dyn/cm² was applied for the entire 4-h duration of the experiments. These two magnitudes of shear stress were chosen based on previous studies (24, 31, 38). The corresponding Reynolds numbers for the two shear stress levels are 25 and 106, respectively.

Phase contrast images of selected cells, ~10–20, on each microgroove pattern within a given row (four parallel and four perpendicular to the flow, see Fig. 1A) were first captured using the Northern Eclipse software (P3I, Glen Mills, PA) to specify the initial locations of these cells. The motorized stage then moved accordingly to the specified locations on all eight patterns, while the time-lapse fluorescence images were acquired with a ×10 objective lens every 15 min for 4 h. Cell viability was assessed with the Live-Dead assay (Molecular Probes) at the termination of each experiment. Viability remained at ≥95% for all experiments. Scratch wound assay. Similar to the effect of cholesterol that slows down cell migration (17), we consider that the Dil accumulation in the lipid bilayer of the cell membrane might cause a decrease in BAEC migration velocity. The effect of Dil on endothelial cell migration was evaluated by a scratch wound assay. Nonlabeled and Dil-labeled BAECs were seeded at a density of 130,000–150,000 cells/cm² onto six-well tissue culture plates, which had been precoated with 10 μg/ml BSA solution for 10 min. This cell density was chosen such that a confluent monolayer was reached within 16 h to minimize Dil dilution after cell division. Multiple strips within the same well were scratched using a 200-μl pipette tip (17). Detached cells were washed away with PBS, followed by the addition of fresh DMEM without bicarbonate supplemented with 25 mM HEPES and 0.5% FBS. The temperature was maintained at 37°C by the heat pad underneath the well plate. Time-lapse phase contrast images of cells migrating away from the wound edge were acquired with a ×4 objective lens every 15 min for 4 h. Approximately eight distances between the two wound edges on each image were measured at 1, 2, 3, and 4 h, respectively. The migration velocity was calculated by dividing the total distance each wound edge had migrated by the set time points. The migration velocity of Dil-labeled BAECs was slower by 29 ± 3.4% (P < 0.05; n = 4) than that of unstained BAECs. This decrease in cell migration velocity remained constant throughout the entire duration of 4 h after wounding.

Fluorescent visualization of FAs and actin microfilaments. Actin microfilaments and FAs were visualized by fluorescent staining and indirect immunostaining, respectively, as previously described (40) at the termination of the migration experiments. In brief, BAECs without Dil labeling were fixed in 4% formaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 20 min at room temperature. Cells were then permeabilized in 0.5% Triton X-100 for 5 min, washed twice with PBS, and blocked with 1% BSA solution for 30 min. To stain for FAs, we used a mouse monoclonal anti-vinculin IgG (1:200; Chemicon) as a primary antibody and Alexa Fluor 488-conjugated chicken anti-mouse IgG (1:1,000; Molecular Probes) as a secondary antibody. The primary and secondary staining steps took 1 h and 45 min, respectively, at room temperature, with three washes in PBS after each staining step. To visualize actin microfilaments, cells were further incubated with tetramethylrhodamine isothiocyanate-conjugated phalloidin (1:1,000, Sigma) for 45 min at room temperature. After the last wash in PBS, the samples were mounted on microscope glass slides and a 20-μl drop of ProLong Gold (Molecular Probes) was added before covering with glass coverslips. The samples were then examined by conventional (Leica DMRX) and confocal (Nikon Eclipse TE-300 equipped with BioRad Radiance 2000-MP) fluorescence microscopy. For confocal microscopy, the confocal image was prepared by overlaying a stack of images taken at a step size of 0.2 μm over a distance of ~8 μm from the floor of the channels up to the apical surface of the cells.

Data analysis. Cell orientation on microgrooves was characterized as previously described (40). Briefly, cells were defined as “aligned” if they exhibited an elongated shape and their major axes were within 20° with respect to the grooves. Elongated cells with their major axes oriented between 20° to 90° to the grooves were defined as “non-aligned.” Cells were described as “isotropic” if they spread uniformly or randomly extended cell processes equidistant from the center of the cell body.

Cell migration was traced by the movement of cell nuclei using the tracking function in Image-Pro Plus software (version 5.0, P3I). The directions of cell migration were categorized as parallel, transverse, randomly oscillating, or mixed, with respect to the grooves. BAECs that migrated parallel to or across several ridges and channels were grouped into the parallel and transverse directions, respectively, whereas cells that randomly migrated in several directions but ended up near their original locations were defined as randomly oscillating. The term “mixed” describes cells that exhibited a mixture of parallel, transverse, or randomly oscillating migration. The velocity of cell migration was calculated from the traced total distance over the experimental time of 4 h.

Changes in cell shape due to flow were quantified by calculating the shape factor of individual cells. The shape factor is defined as the ratio 4πA/P², where A and P are the cell area and cell perimeter, respectively. The shape factor approaches 0 as the cells assume a highly elongated shape and 1 as the cells fully spread and assume a more rounded geometry. After the termination of cell migration experiments, the shape factor was determined using with tetramethylrhodamine isothiocyanate-conjugated phalloidin to delineate cell boundaries and Northern Eclipse software to calculate cell area, cell perimeter, and shape factor.

Statistics. All values are expressed as means ± SE. For cell orientation data, were collected from three independent experiments, each with two replicates. Approximately 200 cells were analyzed for their orientation on each of the microgroove patterns. For cell migration data, were collected from five to seven independent experiments for each of the static and flow conditions. To calculate shape factor, data were collected for static and high shear conditions, each from independent experiments with a total of ~80–110 cells. Statistical analysis was performed by one-way ANOVA, followed by the post hoc Tukey’s test with P ≤ 0.05 being statistically significant.

RESULTS

Characterization of microgroove patterns. The dimensions of all four microgroove patterns were characterized by SEM (Fig. 1, B and E) and are tabulated in Table 1. The average depth dimension of microgrooves was 1.2 ± 0.02 μm. Due to the surface tension of the base elastomer, the mixture did not totally wet the channels before it was cross-linked, resulting in mixture that tapered slightly by 27.0 ± 1.9° from a perfect vertical step. To account for this, the symmetry and the dimensions of microgrooves were determined by measuring the widths of the ridges and channels at the position half way down the groove depth.

Table 1. Dimensions of microgrooves on PDMS

<table>
<thead>
<tr>
<th>Patterns</th>
<th>Ridge, μm</th>
<th>Channel, μm</th>
</tr>
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<tbody>
<tr>
<td>5×5</td>
<td>5.04±0.07</td>
<td>4.84±0.07</td>
</tr>
<tr>
<td>5×3</td>
<td>2.86±0.03</td>
<td>2.97±0.04</td>
</tr>
<tr>
<td>2×2</td>
<td>2.12±0.03</td>
<td>1.73±0.03</td>
</tr>
<tr>
<td>5×2</td>
<td>5.00±0.05</td>
<td>1.69±0.04</td>
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Values are means ± SE. PDMS, poly(dimethylsiloxane). The ridge and channel widths are measured at a height that is at the half maximum between the ridge and channel.
Endothelial cells elongate and align in the direction of grooves. In agreement with our previous data (40), the vast majority of BAECs, 92.1 ± 1.0%, aligned within 20° parallel to the grooves, independent of the groove width within 1 h of attachment (Fig. 2). In this aligned population, 56.7 ± 2.8% of the cells had their major axes formed within 5° with respect to the groove direction. About 8.4 ± 0.9% of the total cells remained nonaligned or isotropic. These data demonstrate that the microgrooved substrate induces the rapid initial BAEC elongation and alignment before the onset of time-lapse measurements of cell migration.

Microgrooves guide the direction of cell migration in the absence of flow. The direction of BAEC migration on the microgrooved PDMS substrates was classified as belonging to one of the four distinctive groups: parallel, transverse, randomly oscillating, and mixed. The direction and the velocity of BAEC migration on microgrooves are presented as a function of groove width in Fig. 3, A and B, respectively. A majority of BAECs, 54.5 ± 3.9%, migrated parallel to the grooves (Fig. 3A), independent of the groove width on the symmetric patterns, whereas there was a slight decrease ($P > 0.05$) from 43.6 ± 4.4% on the asymmetric $5 \times 2$ pattern. As the groove width decreased from 5 to 2 μm, more cells were able to migrate across the channels, with a maximum found on the $5 \times 2$ pattern where 16.0 ± 3.6% of the cells ($P < 0.05$) migrated transverse to the grooves. Thus, the directional cell migration on microgrooves can be modulated by fine tuning the groove dimension at the micron scale. This increase in BAEC migration transverse to the grooves on the $5 \times 2$ pattern with the decrease in cell migration in the parallel direction suggests that the directional control also depends to some extent on the inherent symmetry of the pattern.

The migration velocity of BAECs (Fig. 3B) parallel to the grooves was similar on all microgroove patterns ($P > 0.05$) and statistically not different from that on smooth surfaces, where the migration velocity of the cells was 19.9 ± 1.5 μm/h. This latter value is in good agreement with the reported migration velocity (35) of endothelial cells on a glass substrate coated with a similar Fn surface density. Averaged across all the patterns, the cell migration velocities in the parallel and the transverse directions were 19.0 ± 1.0 and 15.4 ± 1.6 μm/h ($P > 0.05$), respectively. In our system, the migration velocity in the parallel direction was significantly higher ($P < 0.05$) than the transverse direction only on the $3 \times 3$ and the $2 \times 2$ patterns. In addition, the rate of cell migration (migration distance/30-min interval) remained constant (data not shown) in both parallel and transverse directions over the duration of 4 h in agreement with the constant cell migration rate on Fn-coated glass slides (27). This finding assured us that the cell migration velocity was steady throughout the experimental time, allowing the comparison of our data to previous studies, both longer term, i.e., 24 h (3, 4, 7, 16–18, 24, 28, 38, 41), and shorter term, e.g., 2 h (27, 35).

Fluid shear stress can add to or compete with microgrooves in guiding the direction of endothelial cell migration. Depending on the groove orientation with respect to the flow, fluid shear stress can add to or compete with microgroove-derived cues in directing BAEC migration. When flow was applied parallel to microgrooves (Fig. 4A), shear stress enhanced BAEC migration parallel to the grooves. At the high shear stress level (58 dyn/cm²), 89.0 ± 2.5% of BAECs migrated in the direction of both grooves and flow on all four microgroove patterns, significantly higher ($P < 0.05$) than the moderate shear stress (13.5 dyn/cm²) and static conditions. By contrast, during our observation time of 4 h, only 4.3 ± 0.5% of BAECs migrated in a straight line with the flow at high shear stress on a smooth surface. As seen in Fig. 4A, top, upon entering the $3 \times 3$ pattern under flow, a cell (solid arrows) immediately migrated parallel to the grooves, while another cell (arrowheads) on the same area preferentially extended filopodium from the leading edge of the cell along the channels. This result demonstrates that microgrooves can potentiate accentuate directional cell migration parallel to the flow. There was also a trend of a slight increase in cell migration velocity with increasing magnitude of shear stress from 19.6 ± 1.4 μm/h at moderate shear to 24.3 ± 1.0 μm/h at high shear.

Fig. 2. Bar graph of endothelial cell alignment after 1 h of cell attachment. Data are means ± SE from 3 independent experiments with a total of ~200 cells. Above the angle of orientation 0–5 and 10–15 (see measurement in MATERIALS AND METHODS), dashed lines represent the major axes of the cells and solid lines represent the groove direction.

Fig. 3. Endothelial cell migration in the absence of flow. Data are means ± SE from 7 independent experiments. A: cells are grouped according to their migration directions during 4 h time-lapse observation. *$P \leq 0.05$ vs. transverse direction on $5 \times 5, 3 \times 3,$ and $2 \times 2$ patterns. B: migration velocities of the cells in A are calculated from the traced total distance over 4 h. *$P \leq 0.05$ vs. transverse direction on $3 \times 3$ and $2 \times 2$ patterns.
At moderate shear stress, \( \sim 5.9 \pm 1.1\% \) of the individual, nondividing BAECs migrated upstream against the flow direction. For cells dividing under moderate shear stress, two daughter cells separated and then migrated in their respective downstream and upstream directions, whereas at high shear stress, the daughter cell that first migrated upstream against the flow eventually changed its direction to migrate downstream with the flow. We conclude that cell migration parallel to the grooves is much more sensitive to the magnitude of the applied fluid shear stress than the variation in groove widths (2–5 μm) or the groove symmetry.

When flow was applied perpendicular to microgrooves, cues derived from shear stress competed with those from the topography of the microgrooves in guiding BAEC migration. At high shear (Fig. 4B), 68.6 ± 1.50% \( (P < 0.05) \) of BAECs migrated transverse to the grooves on all four patterns, independent of the groove width. Within our observation time of 4 h, moderate shear stress only minimally affected cell migration transverse to the microgrooves. Interestingly, at high shear, the cells migrated transverse to the grooves in two different manners (×5 pattern, Fig. 4B, top). In approximately half of the observed events, cells extended protrusions from both ends of the cell body (arrowheads) to the next downstream ridge and then moved forward in the downstream direction, which was orthogonal to the grooves. In the other half, the cells extended single protrusions from one end of the cell body, causing them to reorient away from the groove direction. The cells then continued their movement in this downstream direction with a cell body alignment at a 30.0 ± 1.1° angle (solid arrows) to the grooves. In addition, the cell migration velocity at high shear was 17.8 ± 1.0 μm/h, slightly greater \( (P > 0.05) \) than the velocity at moderate shear (14 ± 1.4 μm/h). Similar to the switch in the migration direction of endothelial cells on glass surfaces patterned with collagen gradients (24), a threshold level of shear stress is required to compete with the adhesive interaction between cells and their underlying microgrooved substrata. In our study, the shear stress threshold appears to be between 13.5 and 58 dyn/cm².

Actin microfilaments align in the direction of grooves. Under static condition (Fig. 5), most actin microfilaments formed a
perimembrane web around the cell periphery, with a few bundles randomly stretched across the cells on the smooth surface (Fig. 5A), or aligned parallel to the grooves on the patterned surfaces (Fig. 5, B and C). On the $5 \times 5$ and $5 \times 2$ patterns, the cell protrusions were also observed in the $5 \mu m$ (Fig. 5B) and the $2 \mu m$ (Fig. 5C) wide channels, in agreement with the real-time observation (Fig. 4A, top) that the cells can extend filopodia inside the channels.

In response to high shear stress, the actin cytoskeleton remodeled to form extensive actin stress fibers on both smooth (Fig. 5D) and microgrooved (Fig. 5, E and F) surfaces. Similar actin reorganization was also observed at moderate shear stress (data not shown). When the cells were attached to the microgrooved surfaces, the actin stress fibers maintained their alignment parallel to the grooves regardless of flow direction (Figs. 5, E and F). On the microgrooves oriented parallel to the flow (Fig. 5E), the channels provided tracks to guide the protrusions at the front of the cells (solid arrows) along and inside the channels, while those at the back (arrowheads) of the cells remained attached to the edge of the ridge. Orientation of the microgrooves perpendicular to the flow (Fig. 5F) resulted in the preferential termination of protrusions at the downstream side of the cell (solid arrows). Our data suggest that cues derived from fluid shear stress are important for actin remodeling, while the microtopographic cues provide a more effective guidance in orienting the alignment of these stress fibers within the 4-h period of exposure to flow.

**Localization of FAs is groove-width dependent.** The distribution of FAs, as inferred from the localization of vinculin, was visualized by confocal microscopy. On smooth surfaces under static condition, FAs were concentrated at the cell periphery (Fig. 6A). Most FAs adopted an elongated shape of $2.3 \pm 0.2 \mu m$ in size, with the exception of a few highly elongated FAs ($>5 \mu m$, Fig. 6A, solid arrow). On microgrooved surfaces, the localization of FAs depended on the dimension of groove width. For the $5 \times 5$ pattern with the widest groove width, FA distribution was almost equal inside the channels ($42.2 \pm 3.0\%$, Fig. 6B, solid arrows) and on the ridges ($46.7 \pm 2.4\%$, Fig. 6B, arrowheads). A few FAs were observed to span along the grooves (Fig. 6B, open arrows). For narrower groove widths, fewer FAs ($5.7 \pm 1.3\%$) remained localized inside the channels as the majority of FAs formed along the grooves (Fig. 6B, open arrows). For the $5 \times 2$ pattern, most FAs ($78.2 \pm 4.4\%$, Fig. 6C, arrowheads) formed on the ridge edges and aligned parallel to the grooves, similar to the $2 \times 2$ pattern (data not shown). These results indicate that when endothelial cells are seeded on microgrooved surfaces in the absence of flow, the width of the groove is an important parameter that dictates the spatial distribution of FAs on the patterned substrate.

**Most FAs align parallel to grooves.** Upon exposure to high shear, FAs became elongated and polarized towards the downstream and the upstream sides of the cells (Fig. 7, A–E). On smooth surfaces where BAECs had already aligned (Fig. 7A) or were in the process of realigning the major axes with the flow (Fig. 7B), most FAs could be found at the edges of the cell. The alignment of FAs on microgrooved surfaces (Fig. 7, C and D) was reminiscent of our observations of actin stress fibers on patterned surfaces with the majority of FAs maintaining their alignment parallel to the grooves regardless of groove orientation with respect to flow. For example, on the $3 \times 3$ pattern oriented parallel to the flow (Fig. 7C), most FAs formed along the ridge edge, with a long stretch of FA (solid arrow) spanning the groove sidewall before terminating on the ridge at the rear of the cell. Similarly, when the flow was applied perpendicular to the orientation of the grooves ($5 \times 2$ pattern, Fig. 7D), most FAs formed along the ridge edge and aligned parallel to the grooves (dashed arrow), whereas a few FAs aligned in the direction of the flow (arrowheads). Interestingly, for this perpendicular orientation of grooves and flow ($5 \times 2$ pattern, Fig. 7E), we also observed the formation of FAs in the flow direction underneath the cell protrusion on the downstream side of the cell (solid arrow) as the cell migrated across microgrooves. The remaining FAs around the cell periphery either realigned their orientation (Fig. 7E, arrowhead) with the flow or maintained their alignment (Fig. 7E, dashed arrow) with the grooves. This preferential alignment of both FAs and actin cytoskeleton with microgrooves may help strengthen cell retention during exposure to a high level of fluid shear stress.

**Subconfluent endothelial cells become flattened under flow.** In addition to visualizing the FA distribution, confocal microscopy allowed us to estimate the height of the cells by measuring the distance between the ridge plane and the apical surface of the cell. The average height of subconfluent endothelial cells after exposure to high shear stress for 4 h was $1.4 \pm 0.1 \mu m$, compared with an average cell height of $2.5 \pm 0.11 \mu m$, in the absence of flow. This reduction in the cell height is in agreement with the atomic force microscopy data (4) that the cell height decreased by $50\%$ in endothelial monolayers exposed to a shear stress of $12 \text{ dyn/cm}^2$ for 24 h.

**Flow induced endothelial cell spreading in the flow direction.** As inferred from the shape factor, the 4-h exposure to flow induced cell spreading in the direction of flow on all smooth and microgrooved surfaces (Table 2). On microgrooves oriented parallel to flow, there was a trend of increasing shape factor at high shear stress compared with static
conditions irrespective of groove widths. On microgrooves oriented perpendicular to flow, the same high shear stress significantly ($P < 0.05$) induced the extension of cell membrane downstream with the flow, resulting in ~38% increase in the shape factor of the cells compared with static condition. This increase in shape factor of the cells that were initially aligned perpendicular to flow suggests that the flow-induced downstream extension of cell membrane can gradually guide a cell through the process of realigning its major axis with flow.

**DISCUSSION**

In this study, we demonstrated that 3D microgroove topography provides a significant stimulus to mediate the directional migration of endothelial cells without confining individual cells to a single ridge or inside a channel. In the absence of fluid shear stress, our microgrooved substrate induces the spatial localization of FAs along the groove depth and the alignment of actin microfilaments parallel to the grooves. On the substrate with the widest channel (5-μm ridge × 5-μm channel), the equal distribution of FAs inside the channels as well as on top of the ridges directs the majority of cells to migrate parallel rather than perpendicular to the grooves. On the narrower channels (3 × 3, 2 × 2, and 5 × 2 patterns), most FAs localize and align on the ridge edge, thus allowing the cells to migrate both parallel and transverse to the grooves. The spatial localization of FAs over the 3D microgrooves is a unique feature of our substrate that guides the directional endothelial cell migration and differs significantly from endothelial haptotaxis along two-dimensional tracks coated with gradients of adhesive proteins (24).

The ability of the cells to extend their protrusions across the channels may be attributed to the dynamics of actin polymerization and depolymerization. Using fluorescence speckle microscopy, Gupton et al. (20) showed that the lamellipodial protrusion was driven by the polymerization and depolymerization of actin located within 2–4 μm of the edge of the cell membrane, commensurate with the length scale of our 2-μm channel width on the 2 × 2 and 5 × 2 patterns. This dynamic region of the lamellipodium can propel a cell to successfully protrude across the channel and form a new adhesion site on the nearby ridge, thus enhancing cell migration transverse to the grooves.

Upon exposure to fluid shear stress, FAs and actin microfilaments in migrating endothelial cells undergo significant reorganization and remodeling (16, 27, 28). Within the time frame of our experiments, high, but not moderate, levels of shear stress can effectively compete with microgrooves in guiding cell migration, possibly due to the dynamic reorganization of FAs (27). In our system, the cells respond to the high shear stress level of 58 dyn/cm² by extending their lamellipodia by 10.2 ± 0.3 μm. The ability of the cells to extend their protrusions across the channels may be attributed to the dynamics of actin polymerization and depolymerization. Using fluorescence speckle microscopy, Gupton et al. (20) showed that the lamellipodial protrusion was driven by the polymerization and depolymerization of actin located within 2–4 μm of the edge of the cell membrane, commensurate with the length scale of our 2-μm channel width on the 2 × 2 and 5 × 2 patterns. This dynamic region of the lamellipodium can propel a cell to successfully protrude across the channel and form a new adhesion site on the nearby ridge, thus enhancing cell migration transverse to the grooves.

**Table 2. Shape factor of endothelial cells on smooth and microgrooved surfaces**

<table>
<thead>
<tr>
<th>Surfaces</th>
<th>Static</th>
<th>Parallel to Grooves</th>
<th>Perpendicular to Grooves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smooth</td>
<td>0.40 ± 0.04</td>
<td>0.48 ± 0.03</td>
<td>0.48 ± 0.03</td>
</tr>
<tr>
<td>5 × 5</td>
<td>0.35 ± 0.03</td>
<td>0.46 ± 0.03</td>
<td>0.47 ± 0.03*</td>
</tr>
<tr>
<td>3 × 3</td>
<td>0.36 ± 0.03</td>
<td>0.37 ± 0.02</td>
<td>0.51 ± 0.03*,†</td>
</tr>
<tr>
<td>2 × 2</td>
<td>0.35 ± 0.03</td>
<td>0.44 ± 0.05</td>
<td>0.51 ± 0.03*</td>
</tr>
<tr>
<td>4 × 2</td>
<td>0.39 ± 0.02</td>
<td>0.45 ± 0.04</td>
<td>0.53 ± 0.03*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Data were collected for static and high shear conditions from 3 independent experiments with a total of ~80–110 cells.

* $P < 0.05$ vs. static. † $P < 0.05$ vs. parallel.
have shown it generally takes around 24 h for endothelial cells on a smooth surface to adapt to flow under moderate shear stress (4, 41) and significantly longer at very low shear stress ($\leq 1$ dyn/cm$^2$; Ref. 33).

The microgroove topography can create local shear stress gradients (9), similar to the distribution of shear stress over an endothelial monolayer (2, 4). The magnitude of shear stress reaches the maximum on the apical surface of the cells above their nuclei and is minimal near the basal surface (4). Analogous to the distribution of shear stress over the cell surface, numerical simulations of flow over a groove geometry (9) as well as a spherical protuberance (32) predicted a significant reduction of shear stress inside the grooves and the near the base of the protuberance, respectively. For the groove geometry described by Daxini et al. (9), the fluid velocity and the wall shear stress acting parallel to the grooves decreased as a function of the groove depth. The average shear stress inside the 95-μm-wide channels was found to be $\sim 28$% less than that of a smooth surface, resulting in the retention of more cells inside the channels (9). Similarly, the numerical simulation of parabolic flow over a spherical cap also yielded a significant reduction in shear stress at the base level, compared with the top of the cap despite the formation of eddies at corners between the base level and the cap sidewall (32). In our study, the ridge-like protuberance (see Fig. 1) on the microgrooves oriented perpendicular to flow resembles this spherical cap geometry, with the channels formed at the base level. Based on these studies (4, 9, 32), we infer that the magnitude of shear stress inside the channels is lower than that at the ridges. This conclusion is supported by our observation of the cellular protrusions preferentially extending down the channels (Fig. 4A, top).

The increase in directional cell migration and the cell retention under high shear stress can improve the endothelialization process in prosthetic vascular grafts. The groove-induced directional cell migration can promote the transanastomotic ingrowth of endothelial cells towards the midgraft, a process that remains absent in current graft models (42). In contrast to the nonunidirectional patterned surfaces, microgrooves alone mediate the unidirectional migration of endothelial cells and such directional cell migration is enhanced under the application of fluid shear stress acting parallel to the grooves. This guidance from microgrooves can facilitate the directional endothelial cell migration beyond the anastomotic region and further into the midgraft area. Unlike a smooth surface, the topography of microgrooves can also provide additional anchorage sites for cellular attachment as demonstrated in the preferential alignment of FAs and actin microfilaments and the termination of cellular processes at the ridge edge (see Fig. 5F). In the presence of fluid shear stress, this groove-induced organization of FAs and actin microfilaments might strengthen cell anchoring on the substrate surface and retard the potential cell detachment with shear force. Even at the steady, shear stress of 58 dyn/cm$^2$, a level equivalent to the peak systolic shear stress in the abdominal aorta (5, 31), the endothelial cells in our study remain attached on the microgrooved substrate. This cell retention under high shear stress suggests that an endothelial monolayer grown inside a microgrooved vascular graft should be able to sustain the peak shear stress of the in vivo pulsatile arterial flow.

In summary, the width dimension of a 3D microgroove topography plays an important role in guiding the direction of endothelial cell migration in the absence of flow as the groove width determines the spatial localization of FAs. The critical groove width at which the cells can significantly migrate across the microgrooves was found to be $\sim 2$ μm. Cues derived from shear stress on cell migration can be additive to or competitive with those derived from microgrooves, depending on the orientation of the grooves and flow, as well as the magnitude of shear stress. In terms of actin cytoskeleton, microgrooves, but not shear stress, guide the orientation of actin stress fibers parallel to the grooves after exposure to flow for at least 4 h. Local variation in shear stress over the microgroove topography may contribute to the preferential establishment of cellular protrusions inside the channels as the cells adapt to the area of reduced shear stress within the grooves. With all these considerations, the microgroove topography can facilitate endothelialization of prosthetic grafts by increasing directional cell migration and strengthening cell retention under shear stress.

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