Directional control of lamellipodia extension by constraining cell shape and orienting cell tractional forces

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ABSTRACT **Directed cell migration is critical for tissue morphogenesis and wound healing, but the mechanism of directional control is poorly understood. Here we show that the direction in which cells extend their leading edge can be controlled by constraining cell shape using micrometer-sized extracellular matrix (ECM) islands. When cultured on square ECM islands in the presence of motility factors, cells preferentially extended lamellipodia, filopodia, and microspikes from their corners. Square cells reoriented their stress fibers and focal adhesions so that tractional forces were concentrated in these corner regions. When cell tension was dissipated, lamellipodia extension ceased. Mechanical interactions between cells and ECM that modulate cytoskeletal tension may therefore play a key role in the control of directional cell motility.—Parker, K. K., Brock, A. L., Brangwynne, C., Mannix, R. J., Wang, N., Ostuni, E., Geisse, N. A., Adams, J. C., Whitesides, G. M., Ingber, D. E. Directional control of lamellipodia extension by constraining cell shape and orienting cell tractional forces. *FASEB J.* 16, 1195–1204 (2002)**

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Directional cell migration is critical for many important biological processes, including angiogenesis, tumor metastasis, wound healing, and the immune response. Most work on directional control of cell motility has focused on the role of gradients of motility factors such as PDGF, FGF, and EGF, with the general concept that cells physically move up the gradient of a soluble attractant (1–3). These factors promote cell migration by activating members of the Rho family of GTPases—*Rac* and Cdc42—which induce formation of actin-based lamellipodia, filopodia, and fascin-containing microspikes that drive cell extension (4–6). Other experiments have focused on the role of cell adhesion to extracellular matrix (ECM) and associated integrin receptors in the motility signaling response (6–8). However, recent findings suggest that mechanical interactions between cells and their ECM adhesions may influence cell migration. For example, cell motility rates vary depending on the mechanical rigidity of the culture substrate (9, 10), and migrating cells exert the greatest force in the region of focal adhesions positioned just behind the cell's main forward-extending lamellipodia (11). This is important because alterations in the level of mechanical stress transmitted across transmembrane integrin receptors that link the cytoskeleton to the ECM in focal adhesions can activate signal transduction pathways directly (12–14) and thus potentially influence the motile response (15). Directional cell movement can be promoted by direct mechanical distortion of cells, for example, by pushing or applying fluid pressure with a pipette (16). Yet, although the pattern of forces exerted by a motile cell is influenced by the shape of the cell and the direction of its movement, it is not clear that the local stress field distribution directly controls where lamellipodia will extend (9, 11).

Thus, in the present study, we set out to explore whether it is possible to redirect the position of the cell's leading edge and hence control the direction of cell movement, by experimentally manipulating the spatial distribution of tractional stresses that cells exert on their ECM substrate. To do this, we cultured cells on adhesive ECM islands of defined shape and size created on the micrometer scale with a microcontact printing technique (17, 18). By constraining the shape of the cell to that of the adhesive island, we were able to consistently control where cells formed focal adhesions and exerted the greatest tractional stresses. By redirecting mechanical stress distributions within cells, we were able to control the position of lamellipodia, filopodia,

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and microspikes that drive forward motion. These results, combined with the finding that lamellipodia extension abruptly ceased when cytoskeletal tension generation was inhibited, suggest that mechanical interactions between the cytoskeleton and ECM within the focal adhesion may help to determine the direction in which cells move.

MATERIALS AND METHODS

Experimental system

Micropatterned substrates containing square or circular adhesive islands were created on glass slides by microcontact printing (18) and coated with high-density (50 μg/mL) fibronectin (17) or 50 nM thrombospondin-1 (19), as described. Bovine capillary endothelial cells and mouse NIH 3T3 fibroblasts were allowed to grow to confluence in serum-containing medium, as described (17, 20), then were serum-deprived for 1 to 2 days before use in experiments. Subconfluent mouse skeletal C2C12 myoblasts were maintained as described (19). The quiescent cells were then trypsinized and plated sparsely (3×10^3 cells/cm^2) on the micropatterned substrates to ensure that individual islands were seeded with single cells. Studies of the microscope were carried out in experimental bicarbonate-free minimum essential medium (MEM) containing Hank's balanced salts lacking phenol red and bicarbonate (Sigma Chemical Co., St. Louis, MO), MEM amino acids (Sigma), MEM vitamins (Sigma), 2 mM L-glutamine, 1 mM sodium pyruvate, 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, D-glucose (1 g/L), hydrocortisone (1 μg/mL), and 1% bovine serum albumin (BSA; Intergen Co., Purchase, NY; Cohn fraction V, pH 7.0). This medium was supplemented with 20 μg/mL high-density lipoprotein (Bionetics Research, Rockville, MD) and 5 μg/mL transferrin (Collaborative Research, Lexington, MA) for studies with endothelial cells. Studies with fibroblasts used the same medium with high glucose (5 g/L). Lamellipodia extension was synchronously activated in NIH 3T3s by addition of human PDGF-BB (5 ng/mL) and in capillary endothelial cells by addition of basic FGF (5 ng/mL) or 10% calf serum. Microspike formation in myoblasts was activated by plating on thrombospondin-1 for 1 h. F-actin, vinculin, fibronectin, and DNA (nuclei) were visualized in paraformaldehyde-fixed cells using fluoresceinated-conjugated phalloidin (300 ng/mL), rabbit anti-fibronectin antibody, mouse anti-vinculin antibody, and DAPI staining (all from Sigma), respectively. Fascin was visualized in methanol-fixed cells as described (19). In some experiments, fibroblasts were transfected with *Rac1* linked to green fluorescent protein (GFP; 21; kindly provided by N. Hotchin and R. Horwitz) to better visualize lamellipodia or the control plasmid pGFP using Effectene transfection reagent (Qiagen, Chatsworth, CA); lamellipodia staining was observed only in cells containing *Rac*-GFP. Twenty-four hours later, the transfected cells were plated on fibronectin-coated adhesive islands and cultured in serum-free medium overnight. The medium was replaced with experimental bicarbonate-free medium for analysis by time-lapse digital microscopy. BDM (2,3-butanedione 2-monoxime; Sigma) was added at a concentration (5 mM), which has been shown not to significantly alter intracellular calcium concentration (22).

Morphological studies

Living cells were visualized with a Hamamatsu CCD camera on a Nikon Diaphot 300 inverted microscope equipped with phase contrast optics and epifluorescence illumination. Temperature was controlled by a stage mount (Micro Video Instruments, Avon, MA) equipped with a temperature controller (Omega technologies Co., Stamford, CT). The total projected area of lamellipodia per cell was quantitated using the computerized image acquisition and analysis tools of IP Lab Spectrum and RatioPlus software (Scanalytics, Fairfax, VA). Images of fibroblasts on 30 × 30 μm islands and stained with fluoresceinated phalloidin were projected over images in which the square limits of the fibronectin-coated islands were visualized using anti-fibronectin antibodies and a rhodaminated secondary. All cells that covered the entire 900 μm^2 surface of each island were included in this analysis whether or not they appeared to respond to PDGF stimulation. Any cell projection that extended over the nonadhesive region surrounding the square and stained positively for fluoresceinated phalloidin was considered a lamellipodium, provided it was greater than 1 μm^2 in area (to account for registration error) and had a pixel intensity greater than background. To determine relative changes in lamellipodia length in different regions of the cell, corners were defined as parts of the square perimeter within 6 μm from the intersection of the two sides; sides were defined as the 18 μm interval between these corner regions. To control for bias in morphometric calculations due to the geometry of the orthogonal corner regions relative to the linear sides and the large range of lamellipodial morphology, the normalized lamellipodia length was determined by transforming the total lamellipodia area measured in each region into a similar shaped region (corner or side) composed of a lamellipodium that extended equally from all points along its perimeter. Total cumulative data were presented by overlaying 20 to 40 images of fibroblasts cultured on 30 × 30 μm square fibronectin islands and stained with fluoresceinated phalloidin. The pixel occupancy at each position relative to the fibronectin island was determined using IP Lab software and the pixel distribution was color coded for frequency. Immunofluorescence microscopy was carried out using the epifluorescence optics of the Nikon Diaphot microscope. Atomic Force Microscopy was carried out with a Dimension 3000 atomic force microscope (Digital Instruments, Santa Barbara, CA) attached to a Nanoscope IIIa Controller using a modification of a published technique (23). For these studies, serum-stimulated NIH 3T3 fibroblasts were trypsinized and cultured on micropatterned substrates for 6 h in defined medium without growth factors before experimental analysis. Deflection and height data were collected at 512 × 512 pixels over scan sizes of 67 μm; images were flattened to a zero or first order polynomial fit.

Traction force microscopy

Traction force microscopy (9, 24) was adapted for use with cells cultured on micropatterned adhesive islands, as described (25). Flexible polyacrylamide gels (0.25% bis and 2% acrylamide; ~70 μm thick; Young's modulus=1300 Pa) were fabricated containing 0.2 μm diameter fluorescent beads (Molecular Probes, Eugene, OR). Polydimethylsiloxane (PDMS) membranes containing square holes the same size and shape as the desired adhesive islands were created using microfabrication techniques (26). ECM-coated adhesive islands of desired size and shape were created on the surface of the gel by chemically conjugating type I collagen (0.2 mg/mL) to its surface through the holes in the PDMS membrane. After removal of the PDMS membrane, serum-free medium containing 1% BSA was added for 30 min to block protein binding on the newly exposed, uncoated surface of the polyacrylamide gel. Human airway smooth muscle cells isolated and cultured as described (27) were trypsined and plated on the micropatterned gels at high-density (100,000

cells per membrane); nonadherent cells were removed after 2 h by changing the medium. The displacement field of the gel beneath individual adherent cells was determined from images of the same region of the gel taken at different times before or after experimental intervention and the traction field was calculated from the displacement field.

RESULTS

Directional extension of lamellipodia

We have used culture substrates containing micrometer-sized adhesive islands fabricated with microcontact printing and coated with ECM to analyze the effects of ECM-dependent changes in cell shape on capillary endothelial cell growth, differentiation, and apoptosis (17, 28). In those studies, we observed that when single cells were cultured on individual round or square adhesive islands coated with fibronectin and surrounded by nonadhesive regions, cells spread to take on the shape of the islands. In the course of studies in which endothelial cells were cultured on these islands for 1–2 days in the presence of serum, we noticed that whereas cells on round islands (50 μm diameter) extended cell processes at random points along their circumference (**Fig. 1*A***), cells on square (40×40 μm) islands seemed to preferentially extend these processes from their corners (Fig. 1*B*). These cell processes were comprised of lamellipodia and linear filopodia containing F-actin, as confirmed by staining with fluoresceinated phalloidin (Fig. 1*A*, *B*) or antibodies to β-actin (not shown). Time-lapse video microscopic analysis revealed that these processes exhibited dynamic ruffling movements characteristic of lamellipodia: they extended from the cell periphery over the nonadhesive regions, swept side to side, and raised and lowered relative to the plane of the substrate (Fig. 1*C*). In cells that exhibited ruffle formation in multiple corners, each lamellipodium appeared to extend and retract independently of the others (Fig. 1*C*), as observed in cells cultured on conventional substrates (29).

We therefore used these substrates to artificially constrain cell shape in order to explore the possibility that ECM geometry and mechanical interactions between cells and ECM could influence the direction of lamellipodia extension. We carried out these studies using a well-characterized model of migratory behavior: stimulation of lamellipodia formation by PDGF in NIH 3T3 fibroblasts (20, 30). Fibroblasts cultured on square adhesive islands (30 μm edges) for 6 to 24 h in the absence of serum remained limited to the square shape of the islands and did not extend cell processes onto the surrounding nonadhesive substrate. In contrast, within minutes after addition of the motility factor PDGF (5 ng/mL), lamellipodial ruffles could be observed by time-lapse digital recording to flow out synchronously from one or more corners of each square cell and to cantilever over the surrounding nonadhesive substrate (**Fig. 2*A***). The membrane ruffling occurred in the vertical direction, as evidenced by occasional darkening in the corner regions. Fluoresceinated phalloidin staining confirmed that the membrane ex-

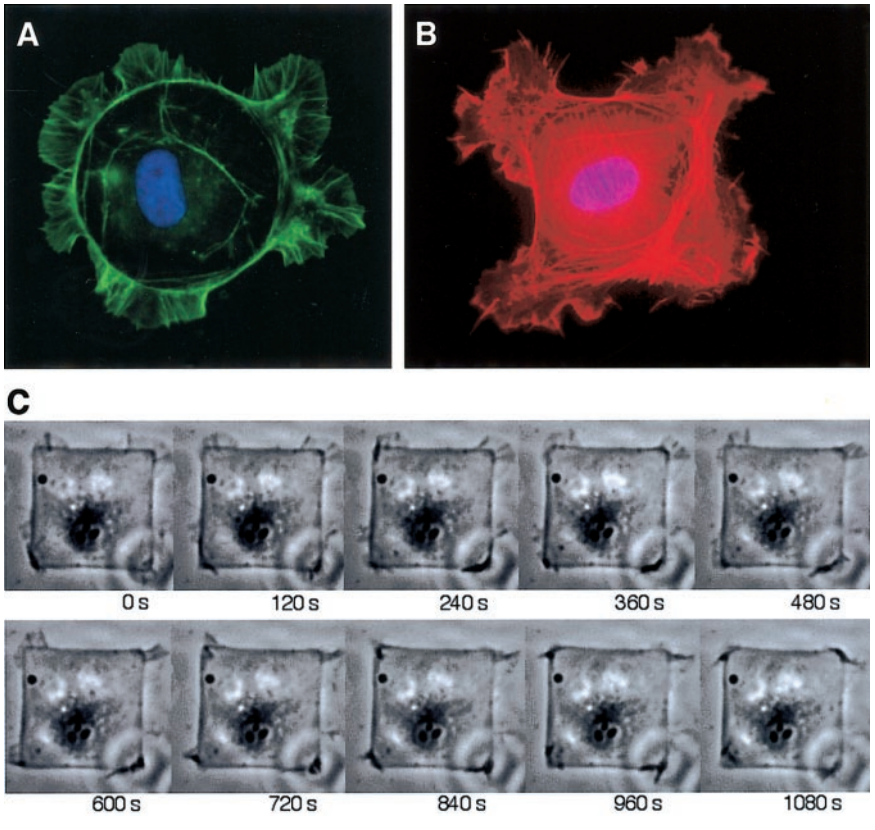

Figure 1. Endothelial cells on square fibronectin islands preferentially extend new cell processes from their corners. Fluorescence microscopic images of cells cultured on 50 μm diameter circular islands (*A*) or square (40×40 μm) islands (*B*) and stained with fluorescent phalloidin to visualize F-actin within lamellipodia and filopodia and with DAPI to visualize nuclei. *C)* Time sequence of phase contrast microscopic images recorded over 18 min of a living endothelial cell cultured in serum-containing medium on a square (50×50 μm) island. Note the unsynchronized, undulating movements of ruffling lamellipodia that are restricted to the corners of this cell.

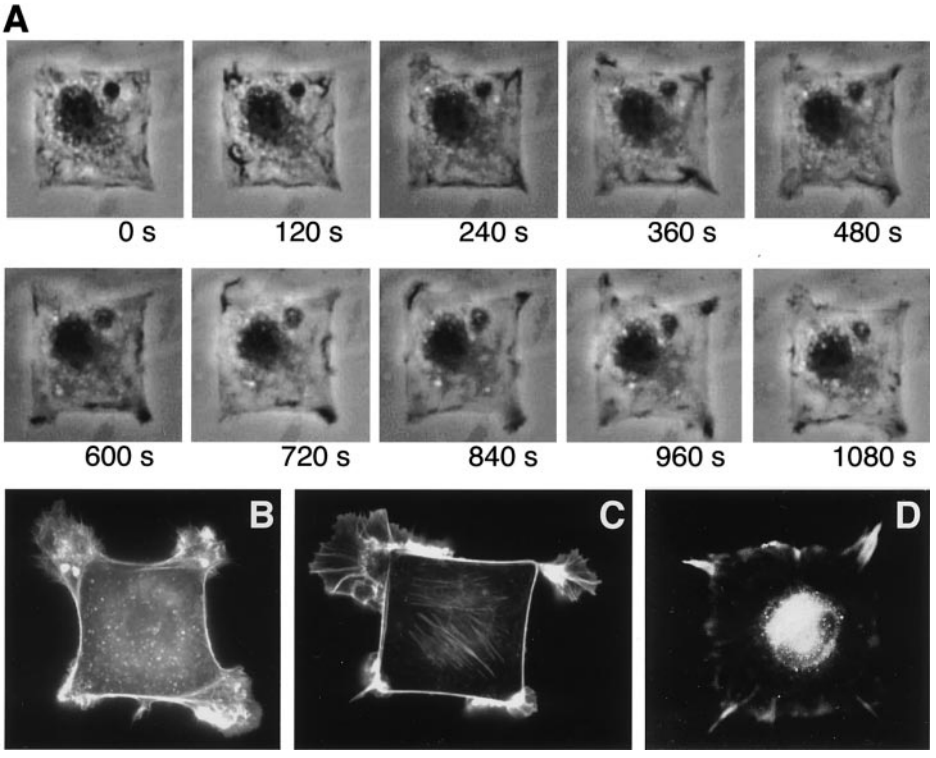

Figure 2. Synchronized extension of lamellipodia, filopodia, and microspikes from cells on square ECM islands in response to stimulation with motility factors. *A)* Time sequence of phase contrast microscopic images recorded over 18 min of a living fibroblast after stimulation with PDGF in serum-free medium. Note that new lamellipodia simultaneously extend from multiple corners of this single square cell. Fluorescence images of a fibroblast (*B*), endothelial cell (*C*), and skeletal C2C12 myoblast (*C*) cultured on square adhesive islands coated with fibronectin (*B, C*) or thrombospondin-1 (*D*) and stained with fluoresceinated phalloidin (*B, C*) or anti-fascin antibodies (*D*). *A, B)* 30 × 30 μm island; *C, D)* 40 × 40 μm islands. Note that newly formed lamellipodia, filopodia, and fascin microspikes all extend preferentially from the corners of these cells.

tensions that formed primarily in the corners of square fibroblasts when stimulated with PDGF were rich in F-actin and that cell processes often extended simultaneously from multiple corner regions of the same cell (Fig. 2*B*). A similar propensity for directional lamellipodia extension in corners was observed when quiescent, serum-deprived capillary endothelial cells cultured on square fibronectin-coated islands were stimulated with a different motile agonist, FGF (Fig. 2*C*). Mouse skeletal C2C12 myoblasts cells that have been shown to move by extending fascin-containing microspikes when plated on thrombospondin-1 (6, 19) preferentially extended microspikes from their corners when cultured on square adhesive islands coated with this ECM protein (Fig. 2*D*). Thus, cell processes formed in the corners of square cells regardless of the cell type, molecular nature of the ECM coating, or the morphology of the cell extension.

Quantitation of changes in cell shape using computerized image analysis demonstrated that PDGF doubled the total projected area of lamellipodia formed by each cell within 5 min of addition and increased it by almost 10-fold at 30 min (**Fig. 3*A***). When the projection lengths of all lamellipodia were quantified within multiple cells using image analysis, the average lamellipodia length doubled in both the corner and side regions of the square cells within 5 min after PDGF addition (Fig. 3*B*). However, after 30 min the mean lamellipodia length was ~60% higher in the corners compared to the sides (1.61±0.15 μm vs. 1.05±0.14 μm; $P<0.007$). This preference for cell processes to form in the corner regions was observed when the images of multiple cells recorded at 0, 5, and 30 min after PDGF stimulation were registered at the corners and overlaid. When the distribution of their pixel occupancy was color coded for frequency, the propensity for lamellipodia extension from the corner regions again was not detectable at 5 min, but was clear by 30 min (Fig. 3*C*), even though F-actin staining had already appeared to concentrate within some of the corners of these square cells at early times (Fig. 3*D*).

Moreover, when lamellipodia movement was analyzed at higher resolution by transfecting fibroblasts with *Rac* linked to enhanced GFP to visualize new process formation, waves of lamellipodia formation could be seen to initiate precisely from the corners of the square cells and to propagate back over the cell surface and along its sides (**Fig. 4*A***). Given these results, the relative increase in lamellipodial length in corners relative to sides measured in fixed cells (Fig. 3*B*) may be an underestimate because waves that propagated from the corners but were present in the side regions or on the apical surface of the cell would be scored as side-based lamellipodia or not scored at all in that quantitative analysis. In any case, these data clearly indicate that the position in which cells initiate formation of lamellipodia, and hence the direction in which they move, can be significantly influenced by the geometry of the ECM substrate or the shape of the cell.

Link between cell tension and directional extension

The finding that various cells preferentially extended new cell processes from their corners when constrained to square adhesive islands raised the question of control. How could altering ECM geometry or cell shape influence the direction in which cells move? One

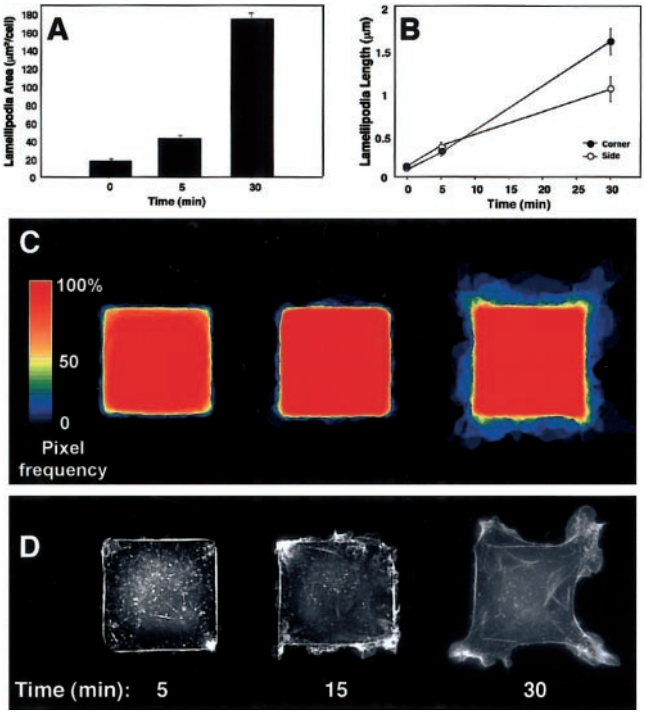

Figure 3. Quantitative analysis of spatial control of lamellipodia extension. Time-dependent effects of PDGF stimulation on mean lamellipodia area (*A*) and normalized lamellipodia length (*B*) in fibroblasts cultured on square (30×30 μm) fibronectin islands. The average length of lamellipodia that extended from corners (filled circles) vs. sides (open circles) was determined by computerized image analysis as described in Materials and Methods; error bars indicated SE. *C)* Visualization of cumulative data obtained by overlaying images from 20 to 40 cells recorded at 0, 5, and 30 min after PDGF addition (left to right) and pseudo coloring the data for frequency of pixel occupancy at each position, as indicated in the scale bar at the left. Yellow indicates the median position of the cell edge for the entire population studied. *D)* Fluorescence images of F-actin staining within three different representative fibroblasts recorded at 0, 5, and 30 min after PDGF addition (left to right). Note the presence of increased F-actin staining in some of the corner regions at 5 min even though preferential extension of lamellipodia from corners could not be detected at this time.

possibility is that altering cell shape somehow caused motility receptors and associated signaling complexes to preferentially distribute to the corner regions of the square cells before addition of the motile stimulus (PDGF). However, immunolocalization studies with anti-PDGF receptor antibodies revealed a homogeneous distribution of these motility receptors over the cell surface (not shown). Thus, the localized lamellipodia extension we observed in square cells did not appear to be biased due to the distribution of PDGF receptors.

One clue to the mechanism of direction control came from analysis of cytoskeletal structure within cells cultured on square, fibronectin-coated adhesive islands. When square endothelial cells were analyzed using fluoresceinated phalloidin, actin-containing stress fibers were observed to preferentially align diagonally within these square cells (**Fig. 5*A***) and similar results were obtained with cells cultured on square islands with edges 30, 40, or 50 μm in length. Similar diagonal alignment of stress fibers could be detected in fibroblasts using atomic force microscopy (Fig. 5*B*) This highly oriented distribution of actin bundles was consistent with the finding that the focal adhesion protein, vinculin, became concentrated within adhesion plaques that localized within the corners regions of the square cells where the actin stress fibers terminated at the cell base and exhibited similar diagonal orientation (Fig. 5*C*). Moreover, the fibroblasts accumulated elongated fibronectin fibrils in a diagonal pattern directly beneath the vinculin-containing focal adhesions (Fig. 5*D*).

Past studies have shown that cells preferentially transfer mechanical stresses across their surface through transmembrane integrin receptors that mechanically couple ECM proteins, such as fibronectin, to the actin cytoskeleton (31) and that vinculin contributes significantly to this response (32). Fibronectin fibril assembly is promoted by mechanical tension exerted by contractile cells (33, 34). Thus, these results suggested that physical constraint of cells within square islands may cause them to focus cytoskeleton-based tractional forces in their corners. To explore this possibility, the spatial distribution of cell tractional forces exerted by cells cultured on square adhesive islands was analyzed and quantitated using a modified form of traction force microscopy (24, 25). Microcontact printing was used to create square, collagen-coated adhesive islands on the surface of flexible polyacrylamide gels in which small (0.2 μm) fluorescent beads were embedded. By using the beads as fiducial markers, displacements and stresses could be visualized and quantitated within the ECM beneath individual square cells.

When human airway smooth muscle cells were cultured on these square islands, cell processes were again observed to preferentially extend from the corners of the cells (**Fig. 6**, left). Traction force analysis confirmed that the greatest bead displacements (Fig. 6, middle) and associated tractional stresses (Fig. 6, right) were similarly observed in the corners of the cells near where focal adhesions formed (Fig. 5*C*), just micrometers away from where new cell processes initiated.

These results suggested that tensional forces generated within the cytoskeleton could actively contribute to control of the direction of cell migration. In support of this hypothesis, we found that the waves of lamellipodia formation that initiated in the corners of square fibroblasts transfected with *Rac*-GFP and cultured on fibronectin islands (Fig. 4*A*) ceased abruptly within minutes after we inhibited actomyosin-based tension generation using the myosin inhibitor BDM (Fig. 4*B*). Lamellipodia formation and movement ceased after tension generation was suppressed using the more specific ROCK inhibitor Y27632 or by chelating intracellular calcium using BAPTA-AM (not shown). Thus, cytoskeleton-based tractional forces appear to play a key role in control of lamellipodia extension.

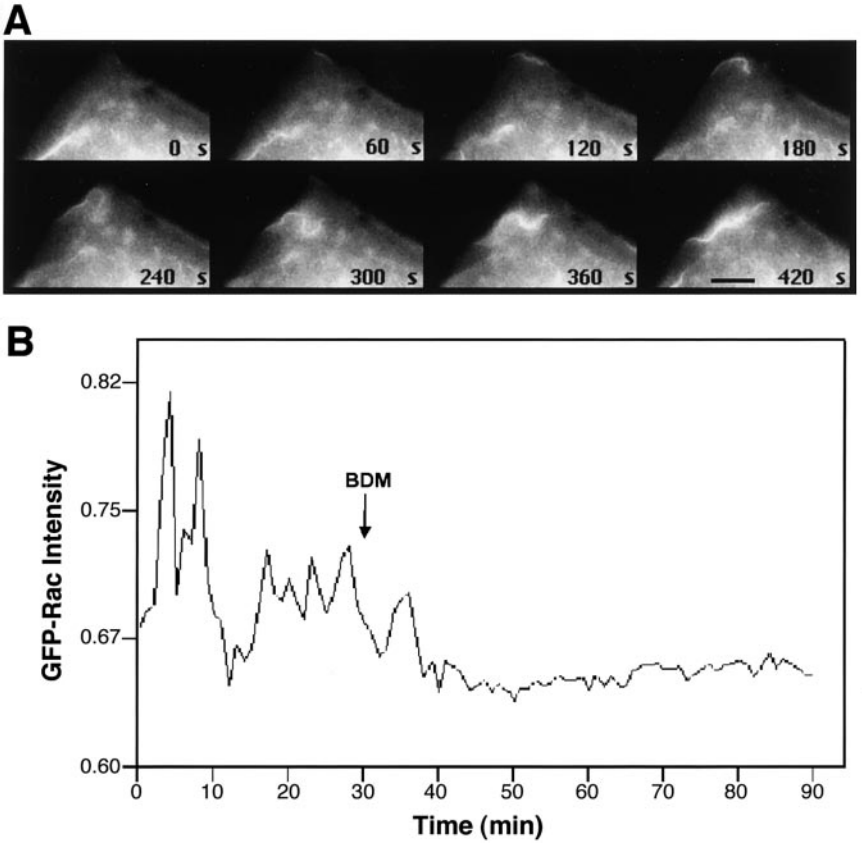

Figure 4. Analysis of ruffling dynamics in fibroblasts expressing *Rac*-GFP. *A)* Time sequence of fluorescence microscopic images recorded in a fibroblast expressed *Rac*-GFP cultured on a square (40×40 μm) fibronectin adhesive island. In these images, which were recorded continuously over 7 min in the presence of PDGF, *Rac*-GFP-labeled lamellipodia can clearly be seen to initiate at the top corner and to actively flow back and downward over the cell surface. Bar = 3 μm. *B)* A representative tracing recording depicting changes in *Rac*-GFP intensity measured along a fixed line (1×0.13 μm) located ~5 μm from one corner of a square cell in which real-time fluorescence imaging was carried out, as depicted in panel *A*. Each peak in the recording indicates the passage of an individual GFP-labeled lamellipodium. Note the rhythmic movement of lamellipodia ceases within 10 min after cytoskeletal tension generation was inhibited by the addition of the myosin ATPase inhibitor BDM (5 mM). Similar results were obtained with multiple cells in two different experiments.

DISCUSSION

Cell migration plays a critical role in normal developmental processes such as embryogenesis and wound healing, as well as in pathological conditions including tumor angiogenesis and cancer metastasis. Although a great deal is known about the soluble factors that stimulate cell movement and the molecular regulators of cytoskeletal filament polymerization that mediate the response, the mechanism by which the direction of cell motility is established remains poorly understood. This is an important control mechanism because random movement has little physiological significance; for example, formation of nerves in the embryo, vessels in tumors, and reconstitution of injured epithelium in wounds all involved concerted movement of cells in precise directions.

The present study was carried out to explore the possibility that mechanical interactions between cells and the ECM play a central role in the control of directional cell migration. Past studies have demonstrated a spatial correlation between where cells apply their greatest tractional forces on the ECM and where they extend new cell processes, such as lamellipodia, that lead cell movement (9). The direction of cell motility has been shown to change in response to external mechanical perturbation by either applying fluid pressure using a micropipette (16) or physically distorting flexible ECM substrates (35, 36). However, due to technical limitations, it has not been possible to

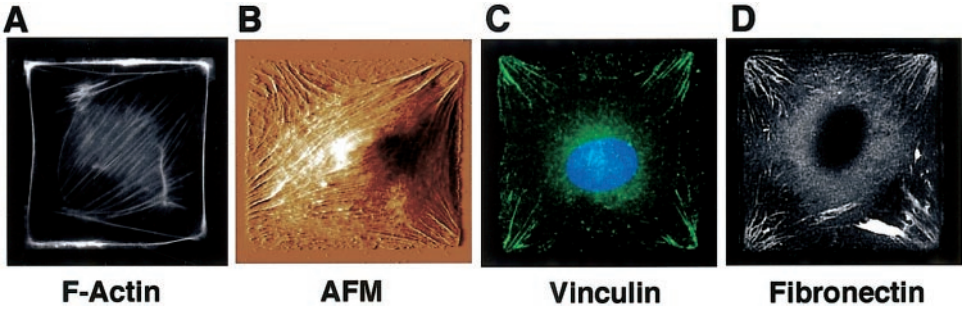

Figure 5. Coordinated reorganization of the cytoskeleton, focal adhesions, and ECM within cells on square islands. Oriented distribution of stress fibers (*A, B*), vinculin-containing focal adhesions (*C*), and underlying fibronectin fibrils (*D*) in endothelial cells (*A*) and fibroblasts (*B–D*) cultured on square fibronectin islands, as detected by fluorescence imaging (*A, C, D*) or atomic force microscopy (*B*). *A, B)* 50 × 50 μm islands; *C, D)* 30 × 30 μm islands.

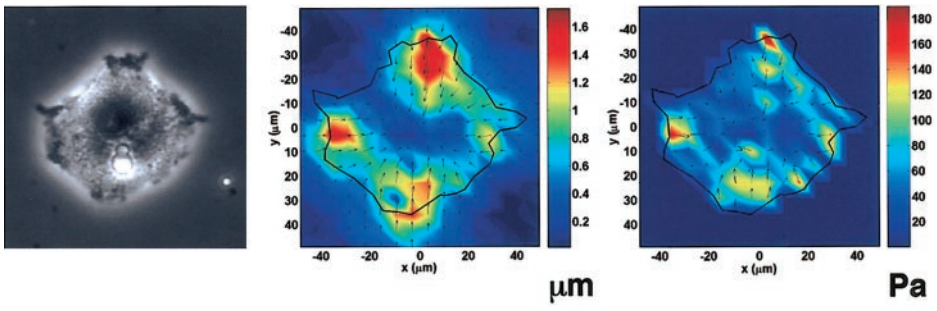

Figure 6. Quantitation of cellular tractional stresses using traction force microscopy. A phase contrast image of a cell cultured on a square collagen-coated adhesive island (40×40 μm) created on a flexible polyacrylamide gel and maps of its displacement field (middle) and traction field (right). Arrows indicate directions of the bead displacements (middle) and associated traction stresses (right). The color scale indicates the magnitudes of the displacements (in μm) and tractions (in Pa). Note that the greatest displacement and tractions preferentially concentrate in the corner regions of the square cell where new cell processes appear to preferentially extend.

directly test whether the mechanical forces naturally generated within the cytoskeleton of a cell adherent to ECM play an active role in determining the direction of its own movement.

In this study, we adapted a described microcontact printing method (17, 18) to create adhesive ECM islands of defined size and shape on the scale of individual cells that were separated by nonadhesive barrier regions. As demonstrated, various cell types adhere and spread on these islands, but extend only up to the limiting boundary along the periphery; in this manner, individual cells take on the shape of the island on which they adhere, although they can locally extend lamellipodia over the nonadhesive boundary regions (37). Cells on symmetrical circular islands extended new cell processes at random along their periphery. In contrast, whatever the nature of the ECM, cells on square islands extended lamellipodia, filopodia, and microspikes at their corners. By physically constraining cells to square islands, we were able to consistently reorient where cells formed their focal adhesions and exerted cytoskeletal tension. This structural reorientation resulted in consistent control of the direction of lamellipodia extension: cell processes were redirected to the corners of these cells where greatest tractional stresses were observed.

When the time course of the cells' response to stimulation with motile agonists was analyzed using computerized morphometry, lamellipodia were found to demonstrate a clear preference for the corner regions after 30 min of stimulation. Although no significant difference could be detected within 5 min using this morphometric approach with fixed cells, staining with fluoresceinated phalloidin revealed that F-actin could be seen to locally concentrate in regions near the corners (i.e., within the square cell boundary) even at these early times (Fig. 3*D*). Moreover, studies with living cells that expressed *Rac*-GFP in order to visualize new membrane process formation with higher fidelity clearly demonstrated that pulses of new lamellipodia generated from a highly localized position oriented precisely within the corners of the square cells. These processes appeared to be primarily lamellipodia with small filopodia in fibroblasts stimulated with PDGF and in endothelial cells activated with FGF when cultured on fibronectin-coated islands. Skeletal myoblasts shown to extend fascin microspikes when plated on thrombospondin-1 (19) and airway smooth muscle cells cultured on collagen-coated polyacrylamide gels (for traction force microscopy) also redirected new leading edge formation to their corners when cultured on square islands. Taken together, these data indicate that the ability of island geometry to influence the direction in which cells extend membrane processes appears to be a general phenomenon and not one limited to a single cell type, ECM component, or motile stimulus.

Analysis of the mechanism by which cells redirected the orientation of their lamellipodia on the square islands revealed that changes in cytoskeletal organization and mechanical force distributions were central to this process. In contrast to cells on circular islands, square cells both repositioned their focal adhesions within their corner regions and redirected their actin stress fibers along their longest axis—the diagonal. Cells generate mechanical tension within stress fibers and focus this force via integrins on their insertion points on the ECM, which promotes focal adhesion formation (38). This was confirmed by the demonstration of fibronectin fibril assembly beneath vinculin-containing focal adhesions, a process that is promoted by mechanical distortion (33, 34). Furthermore, direct mapping and quantitation of cell tractional forces using the modified traction force microscopy approach confirmed directly that cell tractional stresses are indeed concentrated in the corner regions of square cells. This ability to focus mechanical tension locally appeared to be critical for control of the formation and movement of lamellipodia because inhibition of cell tension generation using BDM, BAPTA, or a ROCK inhibitor suppressed this response. Inhibitors of Rho and ROCK have similarly been shown to inhibit cell movement in past studies (39–41), even though they appear to increase Rac activity (42–44).

How can changing the shape of an adhesive island produce these effects? The mechanism by which cells redirect the position of the leading edge appears to involve a change in mechanical stress distributions within the cell. The diagonal of a square adhesive island offers a longer axis of advance along which the cell may spread relative to a circular island that is radially symmetrical. This relative increase in cell distortion (stretching) along the diagonal may preferentially pro-

mote actin filament alignment due to tension molding within the actin lattice (45) in a manner analogous to the way in which actin bundles align with the applied stress field when cells are exposed to fluid shear stress or mechanically stretched (46, 47). Stress fibers have been shown to align with the direction of movement in migrating cells (48). This alignment is thought to be required for isometric contraction along the axis of movement, which in turn is necessary to break adhesions in the trailing edge of the cell as it moves forward (2). However, actin bundle formation also concentrates and focuses actomyosin-based tension forces on the insertion sites of the stress fibers within the focal adhesions. In addition to promoting focal adhesion assembly, the resulting increase in cytoskeletal tension may create a positive feedback loop that further increases stress fiber formation inside the cell (45) as well as fibril assembly outside the cell in the ECM (34), as we observed with fibronectin. Increased cytoskeletal tension in these regions could provide local shape stability of the cell necessary for extension of cantilevered lamellipodia, filopodia, and microspikes (45, 49). This local response, however, must be differentiated from global changes in cell stiffness (e.g., produced by altering extracellular matrix rigidity), which can influence the rate of motility (9, 50) rather than the direction of movement.

The novel finding here is that this targeting of isometric tension to the corners of square cells somehow creates a localized microcompartment in which formation of lamellipodia, filopodia, and microspikes is promoted in the absence of a gradient of soluble chemoattractants. One possibility is that localized tension transfer across integrins in the focal adhesion could locally activate small *Rho* GTPases such as *Rac* and CDC42, which drive formation of lamellipodia and filopodia as well as fascin microspikes (4–6). In fact, integrin signaling has been shown to be linked to *Rac* activation (8) as well as other signaling molecules involved in control of the cytoskeleton and the motile response (15, 51). Furthermore, previous studies have revealed precise spatial control of *Rac* activation in membrane ruffles at the leading edge of motile cells in response to stimulation with soluble motility factors (52). However, recent experiments show that constitutively active *Rac* cannot induce lamellipodia formation in cells that lack vinculin and, hence, fail to efficiently transfer mechanical forces across the focal adhesion (53). Moreover, whereas integrin binding can activate *Rac* in unattached cells, cells must be anchored to a substrate for activated *Rac* to stimulate its downstream effector, PAK (8). More recent studies show that although Rac may be activated globally, the local distribution of integrins determines where activated Rac will bind to effectors and facilitate lamellipodia extension (54). Thus, although we did not directly quantitate Rac activity in the present study, our results support a mechanism in which local mechanical force transfer across focal adhesions may similarly influence *Rac* action by spatially modulating its downstream signaling activities rather than by promoting its activation directly.

Taken together, these findings suggest that it is the ability of square cells to localize focal adhesions to their corners and concentrate tensional stresses at these sites that influence where activated Rho GTPases manifest their ability to promote formation of lamellipodia, filopodia, and microspikes that comprise the leading edge of a migrating cell. This mechanically controlled motility response appears to be compartmentalized within small regions of the cytoplasm. This result is reminiscent of the finding that local mechanical stress application to integrins promotes local formation of a microcompartment specialized for protein synthesis near the site of integrin binding (55). However, since multiple lamellipodia often formed in a consistent manner within the corners of square cells, alterations in the overall shape of the cell or cytoskeleton may provide a way to spatially orchestrate the activities of these different microcompartments at the whole cell level.

The navigational methods of crawling cells have been studied extensively in vitro in response to a variety of environmental cues (2–4, 9–11, 20, 30, 36). However, these studies lack physical constraints that cells may experience within living tissues (21). Perhaps for this reason, past work on the control of directional migration has focused on the role of soluble chemoattractant gradients and contact guidance by the ECM. Mechanical forces that act over long (millimeter to centimeter) distances have been recognized as pattern regulators in tissue morphogenesis, however, they are often assumed to act by reorienting ECM bundles and thereby altering contact guidance (35, 36). Our results suggest that cell-generated mechanical forces that act on the micrometer scale may play an equally important role in development by dictating the direction of cell motion. This mechanism is distinct from the role that cell contractile forces play during retraction of the cell's trailing edge, which is thought to contribute to cell locomotion by increasing forward cytosolic flow (2, 15). It may be distinct from the mechanism of lamellipodia formation itself, which involves activation of small *Rho* GTPases and associated increases in actin polymerization. Instead, the role of mechanical forces we have uncovered appears to function in two ways: *1)* cells apparently must be able to generate tension to support mechanical stiffening in the cytoskeleton and the action of chemical signals that are required for formation of the cell's leading edge, and *2)* the spatial distribution of forces within the cell and related positioning of basal focal adhesions appear to act like a steering mechanism in that they govern where the cell will form its leading edge and hence the direction in which it will move. Thus, cell-generated mechanical forces may play a much more central role in directional cell migration and developmental control than has been appreciated. FJ

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