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Specificity of endothelial cell reorientation in response to cyclic mechanical stretching

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Abstract

Evidence suggests that cellular responses to mechanical stimuli depend specifically on the type of stimuli imposed. For example, when subjected to fluid shear stress, endothelial cells align along the flow direction. In contrast, in response to cyclic stretching, cells align away from the stretching direction. However, a few aspects of this cell alignment response remain to be clarified: (1) Is the cell alignment due to actual cell reorientation or selective cell detachment? (2) Does the resulting cell alignment represent a response of the cells to elongation or shortening, or both? (3) Does the cell alignment depend on the stretching magnitude or rate, or both? Finally, the role of the actin cytoskeleton and microtubules in the cell alignment response remains unclear. To address these questions, we grew human aortic endothelial cells on deformable silicone membranes and subjected them to three types of cyclic stretching: simple elongation, pure uniaxial stretching and equi-biaxial stretching. Examination of the same cells before and after stretching revealed that they reoriented. Cells subjected to either simple elongation or pure uniaxial stretching reoriented specifically toward the direction of minimal substrate deformation, even though the directions for the two types of stretching differed by only about 20°. At comparable stretching durations, the extent of cell reorientation was more closely related to the stretching magnitude than the stretching rate. The actin cytoskeleton of the endothelial cell subjected to either type of stretching was reorganized into parallel arrays of actin filaments (i.e., stress fibers) aligned in the direction of the minimal substrate deformation. Furthermore, in response to equi-biaxial stretching, the actin cytoskeleton was remodeled into a "tent-like" structure oriented out of the membrane plane—again towards the direction of the minimal substrate deformation. Finally, abolishing microtubules prevented neither the formation of stress fibers nor cell reorientation. Thus, endothelial cells respond very specifically to the type of deformation imposed upon them. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Endothelial cells; Mechanical stretching; Cell alignment; Stress fibers; Cellular mechanics

1. Introduction

Mechanical forces induce a variety of cellular responses including morphological changes, protein synthesis, and gene expression (Sadoshima and Izumo, 1993; Davies and Tripathi, 1993; Banes et al., 1995). For example, when subjected to cyclic stretching, almost every type of cells has been found to align nearly perpendicular to the primary stretching direction. The actin cytoskeletons of the stretched cells were also found to be remodeled into bundles of actin filaments (i.e., stress fibers) oriented near the perpendicular direction (Dartsch and Betz, 1989; Iba and Sumpio, 1991; Takemasa et al., 1997). The cell orientation appeared to depend on the stretching magnitude—the larger the stretch, the farther the cells oriented away from the stretching direction (Wang et al., 1995; Takemasa et al., 1997). However, it is not completely clear whether the change in cell alignment was due to actual cell reorientation or selective detachment of cells in certain directions. If only optimally aligned cells remained after stretching, this could give the false impression that the cells had reoriented. Cells subjected to 12% stretching oriented predominantly 60–70° about the stretching direction (Wang et al., 1995)—a direction in which the

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substrate deformation was minimal. Examination of the cells subjected to simultaneous elongation and compression suggested that the eventual stress fibers and cell alignment were in the direction of minimum deformation (Takemasa et al., 1998). A modeling study also predicted that stress fibers should form in the direction of minimal substrate deformation (Wang, 2000). Therefore, a reasonable conclusion is that deformation is an unfavorable condition for the formation of stress fibers. As a result, cells subjected to deformation will reorganize their actin cytoskeletons and eventually align their long axes in the direction of the minimal substrate deformation.

While this conclusion is intuitively reasonable, it has not been rigorously tested experimentally. This is mainly because most existing cell stretching devices produce complicated strain fields on the substrate, and hence cells are subjected simultaneously to different strains. For example, when a substrate is stretched in one direction, it will be compressed in the orthogonal direction—in fact, the larger the stretch, the larger the compression. Thus, it is not clear whether it is the elongation or compression, or both, that is responsible for the cell alignment and the reorganization of the actin cytoskeleton. Also, because mixed deformations (elongation and compression) are imposed on the cells, it is difficult to delineate whether the stretching magnitude or stretching rate was the predominant mechanical factor responsible for the cell alignment observed in previous studies. To address the above issues, we undertook studies to test the following hypotheses: (1) the alignment of endothelial cells subjected to cyclic stretching is due to reorientation rather than selective retention/detachment of cells; (2) this cell reorientation is specifically toward the direction with minimal substrate deformation; and (3) the rate and extent of reorientation are determined primarily by the stretching magnitude, as opposed to the stretching rate. In addition, since both the actin cytoskeleton and microtubules are involved in a variety of cellular responses to mechanical forces (Wang et al., 1993; Wang and Ingber, 1994; Malek and Izumo, 1996), we also examined their roles in the stretching-induced cell alignment.

2. Materials and methods

2.1. Materials

Human aortic endothelial cells, essential basal medium (EBM), and supplements were purchased from Clonetics (Walkersville, MD) and used according to the manufacturer's instructions. Deformable silicone membranes (0.5 mm thick) were obtained from Specialty Manufacturing (Saginaw, MI). ProNectin-F, a bioengineered polymer for promoting cell attachment, was purchased from Protein Polymer Technologies (San Diego, CA). Rhodamine phalloidin, fluorescein phalloidin, and FITC-labeled fluorescent microspheres were obtained from Molecular Probes (Eugene, OR). Monoclonal antibody against mouse tubulin, rhodamine-labeled goat anti-mouse immunoglobulin (IgG), and nocodazole were obtained from CalBiochem (San Diego, CA).

3. Methods

3.1. Cell cultures

The endothelial cells were cultured in EBM containing 2% fetal bovine serum and other supplements, and grown to subconfluence in uncoated plastic dishes at 37°C in a humidified 5% carbon dioxide atmosphere. Cells from 9th to 16th passages were used; they were transferred in 1 ml of growth medium to the central region (see below) of a silicone membrane pre-coated with 10 μg/ml ProNectin-F. The cells were incubated for about 1 h, after which they attached to the membrane. Then, the medium on the membrane was removed by extraction, and 20 ml of fresh growth medium was added. After incubation overnight (12–16 h), the cells were subjected to cyclic mechanical stretching.

3.2. Application of three types of mechanical stretching to the cells

The endothelial cells were cyclically stretched with a custom-built apparatus, as described previously (Wang et al., 2000a, b). A unique feature of this apparatus is its ability to apply three types of stretching: (a) simple elongation, in which the membrane is stretched in one direction and is allowed to compress in its orthogonal direction; (b) pure uniaxial stretching, in which the membrane is stretched in one direction, but the orthogonal edges are controlled so that there is no deformation in that direction; and (c) equi-biaxial stretching, in which the membrane is stretched equally in both orthogonal directions (see Figs. 1A–C). Note that with simple elongation or pure uniaxial stretching, the substrate deformation patterns differ depending on the angle with respect to the stretching direction (Figs. 1D, E).

To ascertain that uniform deformations were imposed on the cells, we first determined the region of the membrane that had uniform deformations for each type of stretching. This was done by imaging a grid of fine ink marks on the membrane with a video camera and analyzing their displacements off-line. It was found that the membrane strains were uniform in the central $20 \times 20\,mm^2$ region of the membrane. Specifically, under simple elongation, the strain along the stretching

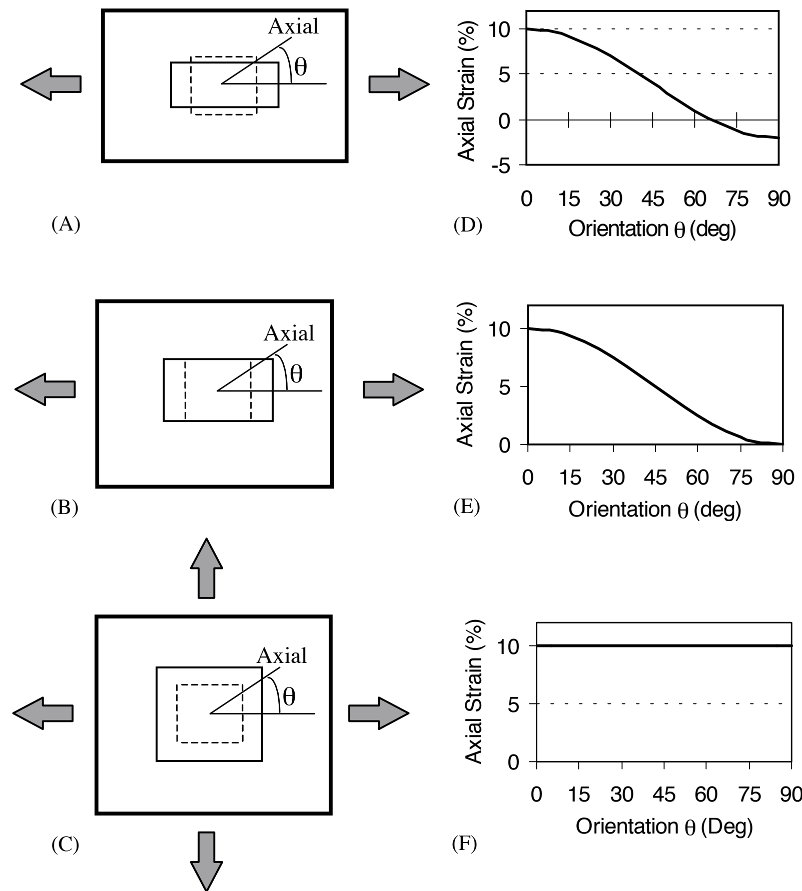

Fig. 1. Schematic of three types of stretching: simple elongation (A), pure uniaxial stretching (B), and equi-biaxial stretching (C). The dotted lines represent a central region on the membrane before stretching, and the solid lines represent the same region after stretching. For simple elongation, the membrane is stretched in the horizontal direction and is compressed in the vertical direction. Under this stretching condition, the membrane deformation varies from a maximum at 0° (i.e., the stretching direction) to minimum (negative, i.e., compression) at 90° (D). For pure uniaxial stretching, the membrane is also stretched in the horizontal direction, but there is no deformation in the vertical direction. The dependence of membrane strain on direction differs from that under simple elongation—it is maximum at 0° but is 0 at 90° and is never negative (E). For the equi-biaxial stretching, the membrane is stretched equally in both the horizontal and vertical directions, and therefore the axial strain is constant from 0° to 90° (F). Note that the axial strain in (D)–(F) represents the substrate strain at a specific angle (θ) with respect to the stretching direction, or horizontal direction.

direction (ε_x) was 0.10 ± 0.002 (mean ± SD), and the strain perpendicular to the stretching direction (ε_y) was -0.034 ± 0.002. Under pure uniaxial stretching, ε_x was 0.108 ± 0.008, and ε_y was 0.005 ± 0.003. Under biaxial stretching, ε_x and ε_y were 0.10 ± 0.003 and 0.098 ± 0.009, respectively. Finally, under the three types of stretching, the mean shear strain (γ_{xy}) was consistently small (<0.004). Therefore, the endothelial cells were plated only in this central region of each membrane to assure that they were subjected to similar membrane strains. In addition, to determine whether the membrane strains were transmitted to cells, we measured cell strains using the fluorescent microsphere technique, as described previously (Simon and Schmid-Schonbein, 1990; Barbee et al., 1994). We found that under biaxial stretching, the cell strain was 77.2 + 20.4% (mean ± SD) of the membrane strain, indicating that membrane strains were indeed transmitted to the cells on the membrane, but with a smaller magnitude.

3.3. Tracking the change of cell orientation in response to cyclic stretching

We used a simple method to determine whether or not individual cells reoriented after stretching. Four ink marks were placed within a $1 \times 1\,\mathrm{mm}^2$ area on the bottom surface of the silicone membrane. Cells, at a low density of 2000 cells/cm^2, were plated and grown on the membrane within the region demarcated by these ink marks. After overnight incubation, using the ink marks as a reference, the same group of cells was photographed on a phase contrast microscope before and after 3 h of 10% cyclic pure uniaxial stretching at a frequency of 0.5 Hz. The orientation and number of cells were

determined from photographs in this manner, from at least three separate experiments.

3.4. Determining the effect of stretching magnitude and rate on the cell alignment

To examine the influence of different stretching magnitudes and rates on the cell alignment, cells were subjected to 10% pure uniaxial stretching at 0.5 Hz (10%/s) and 0.25 Hz (5%/s), and 5% at 0.5 Hz (5%/s) and 1 Hz (10%/s). The cells were cyclically stretched from 30 min to 3 h. Upon the completion of stretching, cells in various fields were photographed and cell orientations were measured as described in Section 3.6.

3.5. Examination of actin filaments and microtubules

To determine the role of the actin cytoskeleton and microtubules in the cell alignment, they were examined in separate experiments after stretching for 3 h. The procedures for staining actin filaments and microtubules are as follows. Cells were briefly washed with PBS, fixed in 3.7% formaldehyde, permeabilized in 0.25% Triton X-100, and finally incubated with either 0.165 μM of rhodamine phalloidin or fluorescein phalloidin in PBS. For staining of microtubules, cells were fixed and permeabilized as above, then incubated with mouse anti-tubulin antibody (1 : 30 in PBS) followed by rhodamine-conjugated goat-anti-mouse antibody (1 : 20 in PBS). The stained cells were viewed on a Zeiss fluorescence microscope and photographed with Kodak 400 color slide film.

3.6. Data analysis

The orientations (defined by the longest aspects of the cells with respect to the stretching direction) of unstretched and stretched cells from separate experiments were measured from phase contrast microphotographs. The photographs were digitized and cell orientations were measured using NIH Image 1.6. A total of 77–144 cells were used to construct cell orientation distributions, defined in the orientation range between 0° (the stretching direction) and 90° (the perpendicular to the stretching direction). The symmetry of the silicone membrane and applied stretching about the 90° direction was the reason for defining this range.

For statistical analysis, the Kolmogorov–Smirnov test (Hoel, 1971) was used to compare the cell orientation distributions between two groups of cells. Also, since cell orientations were not normally distributed, the non-parametric Kruskal–Wallis ANOVA test (Rosner, 1990) was used to compare the median orientations of two groups of cells. A difference was considered to be significant if $p < 0.05$.

4. Results

Phase contrast microphotographs of the *same* group of endothelial cells before and after 3 h of pure uniaxial stretching were obtained (Fig. 2). Notice that whether in isolation (see the cell labeled with a and b) or in a cluster (labeled with c), cells changed their orientations to be nearly perpendicular to the stretching direction after the stretching. Furthermore, in three separate experiments,

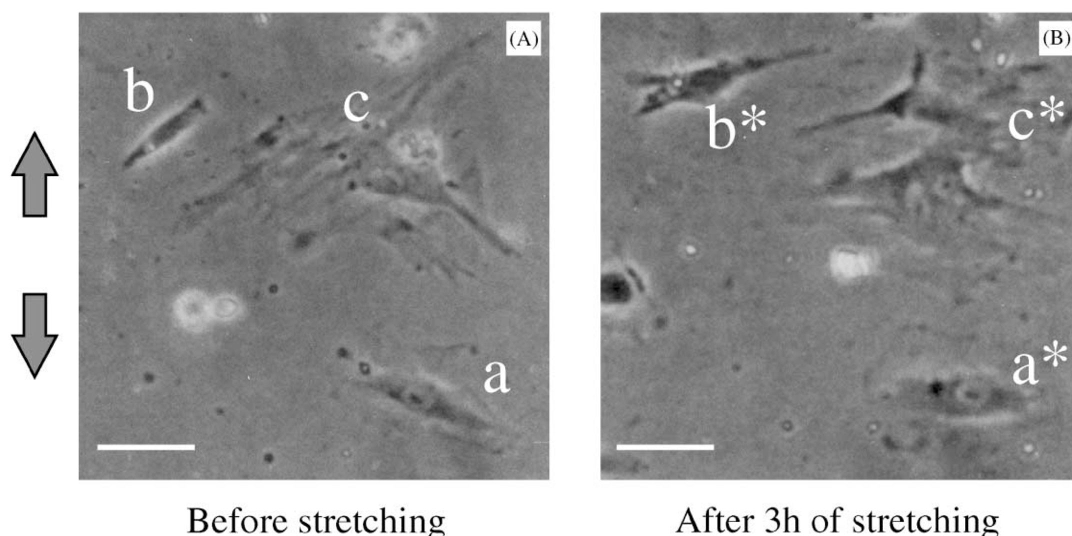

Fig. 2. A pair of representative phase contrast microphotographs of the same group of cells before (A) and after (B) 3 h of pure uniaxial stretching (arrows). Ink marks (not shown) were placed on the membrane for identification of the same cells before and after the stretching (a in A vs. a* in B; b in A vs. b* in B; and c in A vs. c* in B). Since there were only a few cells in the same view before and after stretching, and no cells were found to be detached, it is concluded that the *same* cells changed orientations, i.e., reoriented after stretching (Bar: 50 μm).

no discernible change in the number of cells in a given region was found before and after stretching (data not shown). Taken together, these results indicate that the alignment of cells observed after cyclic stretching is not likely due to selective detachment of cells with certain initial orientations, but rather due to the cells' reorientation in response to cyclic stretching.

In separate experiments, in response to the first round of stretching, almost all the cells reoriented perpendicular to the stretching direction (Fig. 3A). After the cells had been aligned by the initial stretching, they were then stretched in the direction of the cell alignment for an additional 3 h. In response to the second round of stretching, nearly all the cells reoriented once again, so that they ended up parallel to the initial stretching direction (Fig. 3B).

The specificity of the cell reorientation responses to stretching is shown in the three representative microphotographs (Fig. 4), which show cell orientations without stretching (Fig. 4A), with simple elongation (Fig. 4B), and pure uniaxial stretching (Fig. 4C). It is readily seen that the cells without stretching were randomly oriented. The cells with simple elongation were aligned obliquely to the stretching direction, whereas the cells with pure uniaxial stretching were aligned nearly perpendicular to the stretching direction. These visually apparent differences were confirmed by inspection of cell orientation distributions (Fig. 5). The unstretched cells were fairly evenly distributed from 0° (i.e., the stretching direction) to 90° (i.e., perpendicular to the stretching direction). However, the orientations of simply elongated and uniaxially stretched cells clustered around 70° and 90°, respectively. These two distributions are significantly different ($p < 0.01$), and they are both significantly different ($p < 0.01$) from that of the cells without stretching. However, after equi-biaxial stretching, the cells remained randomly oriented, and their orientation distribution was not significantly different from that of unstretched cells (data not shown). Note that for the membranes used in this study, the direction of minimum deformation was about 70° for simple elongation and 90° for pure uniaxial stretching (see Figs. 1D and E). Therefore, the modes of the orientation distributions of the cells under both simple elongation and pure uniaxial stretching coincided with the directions in which membrane deformation was a minimum.

To study the effect of stretching magnitude and stretching rate on cell reorientation, the median orientations of cell orientation distributions were obtained (Fig. 6). After 30 min at both stretching rates (10%/s and 5%/s), the cells subjected to 10% pure uniaxial stretching already had a significantly different orientation from that of unstretched cells ($p < 0.01$), whereas those subjected to 5% stretching at 10%/s did not. After each duration of stretching, the median orientation of cells subjected to 10% stretching was greater than that for 5% stretching, regardless of the stretching rate. Moreover, except at the 3 h duration, there was no significant difference between the orientations of cells subjected to 10% stretching at either stretching rate. Cells subjected to 5% stretching, however, showed statistically significant differences in orientations between the two stretching rates at the stretching durations (2 and 3 h). These results suggest that for the early cell reorientation response, stretching magnitude, not the stretching rate, is the predominant factor in determining both the rate and extent of cell reorientation. For the later cell reorientation response, however, stretching rate influences the cell reorientation. The exact mechanism for this is not clear and requires further studies.

The actin cytoskeletons of the cells also responded specifically to cyclic mechanical stretching. Compared to

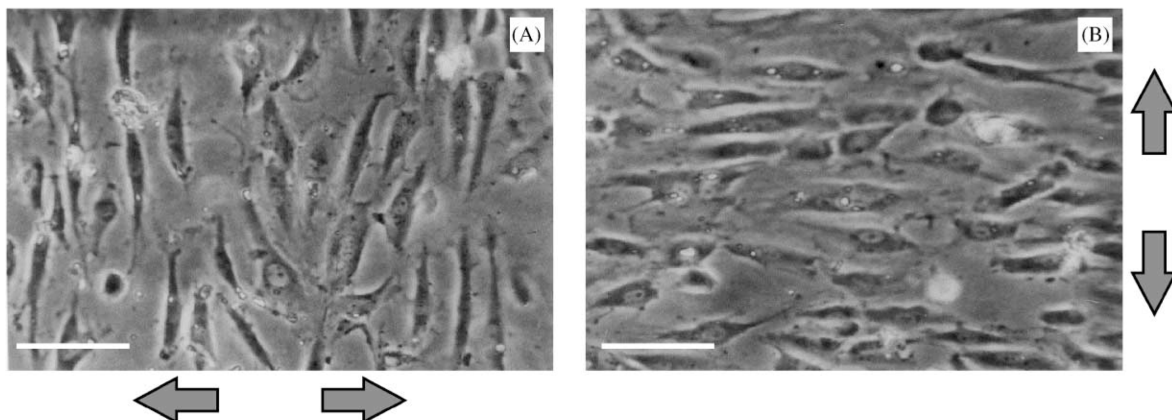

Fig. 3. Phase contrast microphotographs of cells in response to cyclic stretching. After 3 h of 10% pure uniaxial stretching, the cells reoriented perpendicular to the initial stretching direction (arrows, A). Then the cells were stretched in the direction of the cell alignment (i.e., the vertical direction in A). In response to the second round of stretching, the cells reoriented again and became oriented parallel to the initial stretching direction (B) (Bar: 100 μm)

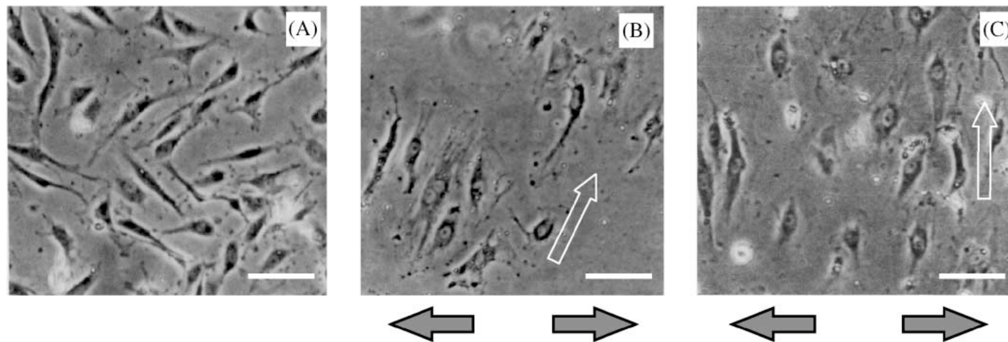

Fig. 4. Representative phase contrast microphotographs of endothelial cells: unstretched (A), after 3 h of simple elongation (B), and after 3 h of pure uniaxial stretching (C). Unstretched cells did not appear to orient in any specific direction, but with simple elongation and pure uniaxial stretching, the cells oriented about 70° and 90°, respectively (arrows). Note that these stretched cells were viable, since they reoriented again after a second round of stretching (see Fig. 3) and did not uptake trypan blue (Bar: 60 μm).

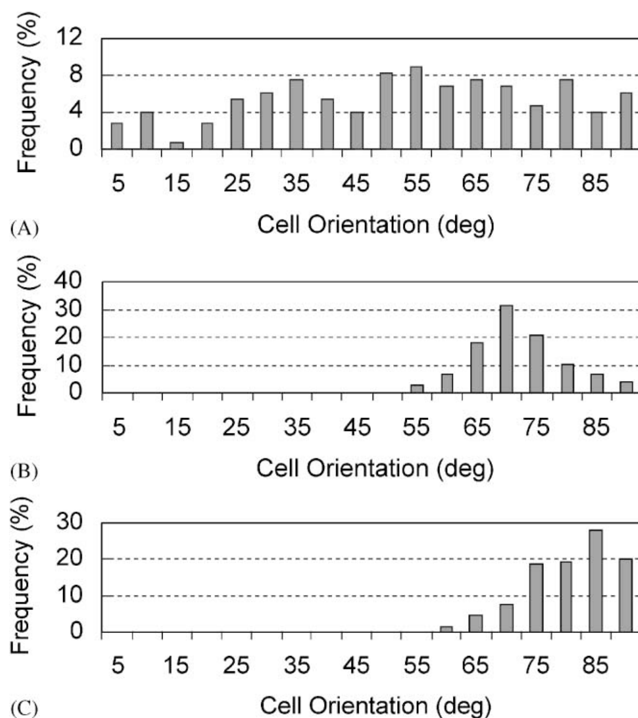

Fig. 5. Cell orientation distributions without stretching (A), with simple elongation (B), and with pure uniaxial stretching (C). Note that the cell orientations were categorized into groups encompassing a range of 5° between 0° and 90°. Thus, for each experiment we had 18 groups of cell orientations in 5° increments. Each of these distributions is significantly different from the other two ($p < 0.05$). Note that the cell orientation distributions were pooled from at least three separate experiments.

the more or less randomly oriented actin filaments in unstretched cells (Fig. 7A), after 3 h of simple elongation or pure uniaxial stretching, the actin cytoskeleton of the stretched cell was remodeled into stress fibers oriented obliquely (Fig. 7B) or nearly perpendicular (Fig. 7C) with respect to the stretching direction. Interestingly, under equi-biaxial stretching, a "tent-like" actin structure protruding from the substrate plane was formed (Fig. 7D), which was confirmed by confocal microscopy.

In addition, compared to unstretched cells (Fig. 8A), cells pre-treated with nocodazole for 30 min and then stretched in the presence of the drug were nearly devoid of microtubules (Fig. 8B). Despite the fact that the microtubules were largely eliminated, however, the cells still reoriented and stress fibers still formed—similar to the responses of the cells without the nocodazole treatment (Fig. 8C).

5. Discussion

There are four main findings in this study: (1) in response to cyclic mechanical stretching, either simple elongation or pure uniaxial stretching, human aortic endothelial cells reorient; (2) both the cell reorientation and stress fiber reorganization are specifically in the direction with minimum substrate deformation; (3) at least for the early cell reorientation response, the rate and extent of cell reorientation are primarily dependent on stretching magnitude, not stretching rate; and (4) microtubules are not essential for the cell reorientation and stress fiber formation.

Many studies have shown that cells align away from the stretching direction in response to cyclic stretching (Dartsch and Betz, 1989; Shirinsky et al., 1989; Iba and Sumpio, 1991; Wang et al., 1995). The altered alignment has been tacitly assumed to be due to the reorientation of cells. In those early studies, however, stretching was imposed for much longer time than in our study, possibly enabling cells aligned in unfavorable directions to detach from the underlying substrate and only cells aligned in the perpendicular direction to remain attached and proliferate, thus giving the impression that reorientation occurred. Our results indicate that the reorientation of the same individual cells instead of cell selective detachment occurred before and after stretching. Moreover, once cells had been aligned by one bout of pure uniaxial stretching, a second bout of the stretching in the cells' alignment direction caused the

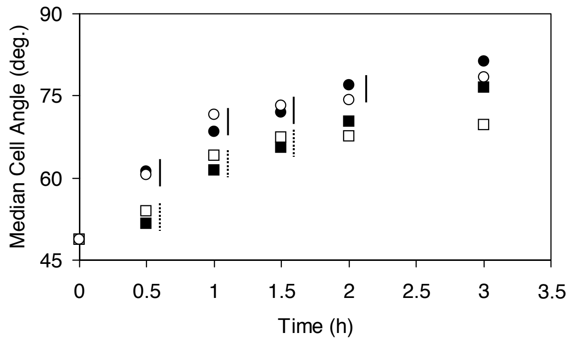

Fig. 6. The effect of stretching magnitude and rate on the median of cell orientation distribution. Cells were subjected to pure uniaxial stretching, and their orientations were measured after various stretching durations, as indicated. Note that each of the symbols (■, 5% at 10%/s; □, 5% at 5%/s; ●, 10% at 10%/s; ○, 10% at 5%/s) represents the results from three separate experiments. Also, the solid lines compare two median orientations of the two cell orientation distributions at two different stretching rates (10%/s vs. 5%/s) with the 10% stretching magnitude, whereas the dotted lines compare the two stretching rates at the 5% stretching magnitude. These pairs were not significantly different. Additionally, for either of two stretching rates (5%/s and 10%/s), all pairs between two stretching magnitudes (5% vs. 10%) at each stretch time were significantly different.

cells to reorient once again (Fig. 3). Also, note that for complete cell reorientation, the endothelial cells we used in this study needed to be stretched for only 3 h, compared to days in previous studies (e.g., 3 days in the study of Dartsch and Betz, 1989). As a result, the confounding effect of cell proliferation during cyclic stretching on the cell reorientation response in our study was largely eliminated. Thus, we have strong evidence of actual cell reorientation as opposed to selective detachment of cells oriented in particular directions.

It has been hypothesized that the direction of minimal substrate deformation is the preferred direction in which cells align after cyclic stretching (Wang et al., 1995; Takemasa et al., 1998). Due to the complicated strain fields produced by the devices previously used, however, it has not been clear which deformation—the elongation, the compression, or both—is responsible for the endothelial cell reorientation. For example, when deformable membranes are simply elongated, strain increases in the stretching direction, but compression also increases in the orthogonal direction. Thus, a cell

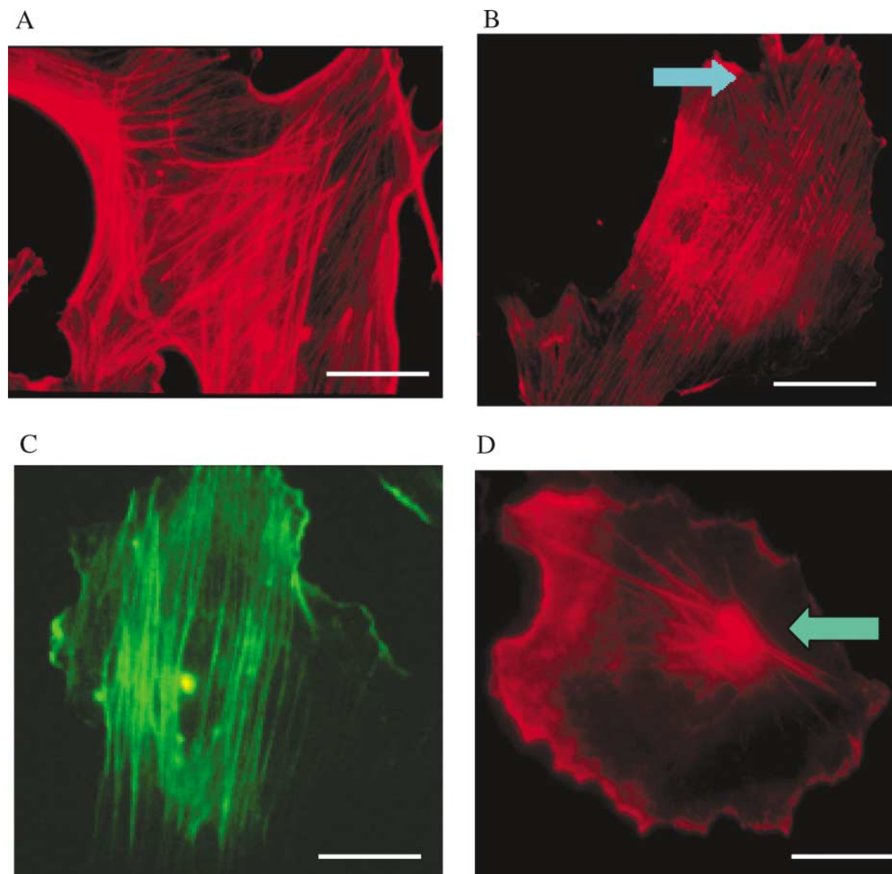

Fig. 7. The effect of cyclic stretching on the actin cytoskeletal remodeling of the endothelial cell. Unstretched cells had dense, randomly oriented stress fibers (A). After 3 h of simple elongation, the cells formed bundles of stress fibers, which in some cases exhibited "X" shapes (arrow) oriented around 70° with respect to the stretching direction, i.e., the horizontal direction (B). After 3 h of pure uniaxial stretching, the stress fibers were oriented nearly perpendicular to the stretching direction (C). After 10% equi-biaxial stretching for only 15 min, a "tent-like" actin structure (arrow) protruding in the direction normal to the membrane was observed (Bar: 30 μm).

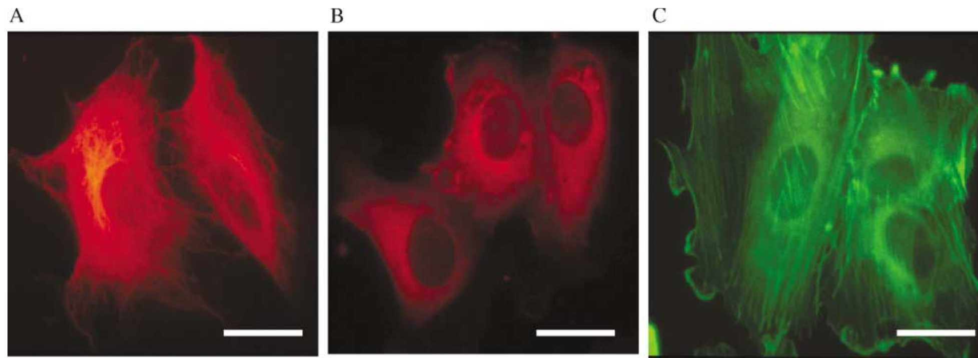

Fig. 8. The influence of microtubules on the cell reorientation and stress fiber formation. Unstretched endothelial cells had dense microtubules throughout the cells (A). When the cells were incubated with nocodazole (0.33 μM in DMSO) for 30 min and were then subjected to cyclic pure uniaxial stretching (10%, 0.5 Hz) in the presence of the drug, few microtubules were detected in the stretched cells (B). The cells still reoriented, however, and stress fibers still formed in the direction nearly perpendicular to the stretching direction (C). Note that higher dosages of nocodazole could not be used since the treated cells became rounded, presumably because of the toxicity of this drug at higher dosages. Also, it is apparent that the nocodazole-treated cells were less elongated than the untreated cells. The exact reason for this is not clear (Bar: 15 μm).

that is not aligned exactly in the direction of zero deformation will undergo some combination of elongation and compression, which is dependent upon the extent of elongation applied. Wang and Grood (2000) showed that dermal fibroblasts aligned away from both elongation and compression directions. Although Takemasa and co-workers (1998) showed that either predominantly stretching or compressing cells from their equilibrium lengths would result in the same pattern of realignment, the mixed strain fields obtained with their device precluded being able to differentiate the effects of pure elongation from those of pure compression. In contrast, our results from pure uniaxial stretching show that cells realign specifically in the direction perpendicular to stretching. Taken together, one can then reasonably predict that cells would also realign perpendicular to the direction of pure compression. That is, both cyclic elongation and compression are unfavorable conditions for cell alignment. The reason for this, however, is not clear from this study.

Furthermore, our results confirm that the stress fiber alignment occurs along the direction of minimal substrate deformation. Specifically, after simple elongation or pure uniaxial stretching, stress fibers were formed specifically in the direction of the minimal substrate deformation. Moreover, after equi-biaxial stretching, in which there was no direction with minimal substrate deformation in the plane of the membrane, a "tent-like" structure protruding from the substrate plane was formed. However, this unusual response is consistent with the notion that stress fibers can only be formed in a direction with minimal substrate deformation (Wang, 2000)—in this case, out of the plane of the membrane. It has been suggested that this remodeling of the actin cytoskeleton governs the subsequent cell alignment (Shirinsky et al., 1989).

It should be noted that there was only a small difference in the cell reorientation response between the two stretching conditions, i.e., simple elongation and pure uniaxial stretching. It was not an easy task to detect the small difference, considering there was a large variability in the orientation of cultured cells. We accomplished this by using a unique stretching device which can apply precisely both the simple elongation and pure uniaxial stretching. In addition, a non-parametric statistical technique, the Kolmogorov–Smirnov test (Hoel, 1971), was used to compare statistically two orientation distributions under the two stretching conditions. Finally, the small difference in the cell reorientation response to the two stretching conditions indicates that endothelial cells are exquisitely sensitive to mechanical stimuli, therefore, the cell orientations during mechanical stretching must be controlled if the specific role of these stimuli in the cellular biological responses can be properly interpreted.

In this study, we limited our observations to 3 h whereas other studies examined different durations of stretching (Iba and Sumpio, 1991; Takemasa et al., 1997, 1998). After 3 h of stretching, we found that cells subjected to 10% stretch had nearly completed their reorientation, but those subjected to 5% had not. The eventual reorientation of the cells subjected to 5% stretch requires further study. Thus, although our data show a clear difference in the reorientation angle between the two stretching magnitudes after comparable durations of stretching, it is not clear whether the extent of eventual reorientation is also dependent on stretching magnitude. Takemasa and co-workers (1997) found the angle of reorientation of human umbilical vein endothelial cells to be linearly dependent on stretching magnitude after 20 min of stretching. As our results indicate, however, the angle of reorientation varies with both magnitude and duration of stretching, so their interpretation may not be entirely correct. In a subsequent, more detailed study, Takemasa and co-workers (1998) showed that both the extent of reorientation and

the time to achieve complete reorientation apparently varied directly with the stretching magnitude. Since the direction of minimal substrate deformation also varied with the applied stretching magnitude, however, one may not definitively make those conclusions.

In this study, human aortic endothelial cells with relatively high passages (9–16th) were used. It is known that with increased passages, the cells tend to lose some phenotypic properties. For the cell reorientation we were concerned within this study, we found that all the cells with different passages almost complete their reorientations within 3 h. In other words, no passage-number dependent cell reorientation was noticed. As a result, to describe cell reorientation response, orientations measured from the cells with different passages were pooled together (Fig. 5).

Our finding of cell reorientation to around 70° about the stretching direction under simple elongation is inconsistent with that of a previous study (Dartsch and Betz, 1989), which reported that the cells oriented perpendicular to the stretching direction. While the exact reasons for this are unknown, there are several possibilities. First, there was a difference in species between the two studies. We used human aortic endothelial cells, whereas they used porcine aortic endothelial cells. It is possible that cells from different species may respond differently to mechanical stretching. Second, there was a difference in cell density. We used subconfluent cell culture, in which individual cells did not come closely into contact with each other. Hence, the cell reorientation response is expected to be independent. In contrast, the Dartsch and Betz's study used a highly confluent cell culture. Due to the cell-to-cell contact in the highly confluent culture, reorientation of individual cells would be expected to be influenced not only by mechanical stretching, but also by neighboring cells. Consequently, reorientations of individual cells would not be expected to be independent. Cell density may affect cell alignment response, e.g., confluent endothelial cells aligned in the flow direction (Malek and Izumo, 1996), whereas subconfluent endothelial cells did not respond in such a manner (Masuda and Fujiwara, 1993). Moreover, the difference in cell densities may mean that the cells in the two studies were in different growth cycles, which may also contribute to the difference in the cell reorientation response. Therefore, future studies are needed to examine cell reorientation response when cell cycles are controlled. Third, the cells can tolerate some degrees of strains (Wang et al., 1995) and the cells in a confluent culture may somehow tolerate larger strains than in a subconfluent culture. That the cells are tolerant to some degrees of strains may also explain why not all cells oriented exactly in the direction with the minimal strain, which was the 70° direction under simple elongation and the 90° direction under pure uniaxial stretching (Figs. 5B, C).

Our results show that at least for the early cell reorientation response, the rate of cell reorientation primarily depends on stretching magnitude. However, the rate of cell reorientation likely depends on many other factors, including the type and source of cells, culture conditions (such as the growth medium used), the coating protein on the substrate and the density of cells. All of these factors need to be considered when comparing different studies. We studied non-confluent cells, as this helped document reorientation by enabling us to examine the same cells before and after stretching, and also avoided potential complications which arise from cell to cell signals such as contact inhibition, which may alter the timing of stretch-induced responses. Finally, a previous study showed that, unlike most other cell types, macrophages did not align perpendicular to the stretching direction (Matsumoto et al., 1996). Macrophages do not have the typical actin cytoskeletal structure of other cells, such as endothelial cells, fibroblasts, and smooth muscle cells, which have all been shown to reorient in response to stretching. Thus, this finding lends a further support to the important role of the actin cytoskeleton in the cell response to cyclic mechanical stretching. Future studies should focus on quantitating cytoskeletal changes in the response of the endothelial cells to different types of mechanical stretching. Preliminary studies from this group have shown that the extent of actin cytoskeleton disruption depends on the type of stretching applied to the cells and the time after initiation of the stretching (Wang et al., 2000a, b).

Intact microtubules appear to be necessary for cell reorientation in response to shear flow (Malek and Izumo, 1996). In contrast, with cyclic mechanical stretching, disrupting the microtubules with nocodazole did not block the cell reorientation. The difference in the cell reorientation response to shear flow and cyclic mechanical stretching is further evidence of the specificity of cell responses to different mechanical stimuli. However, it is not clear from this study whether the reorientation rate of the nocodazole-treated cells in response to cyclic stretching is altered compared to untreated cells, and this requires further studies. In addition, note that although the microtubules in the nocodazole-treated cells were largely eliminated (compare Figs. 8A and B), there were some sparse microtubule fragments in the nuclear region. We found that, to completely eliminate the microtubule fragments, much higher dosages ($>1\,\mu$M instead of $0.33\,\mu$M) of nocodazole must be used. At the high dosages, however, many cells became rounded, presumably because of the toxicity of this drug. This made it impossible to examine cell reorientation response.

A striking feature of endothelial cells on healthy arterial walls in vivo is their elongated shape and alignment along the longitudinal direction of the aorta,

which is perpendicular to the predominantly radial vessel stretching direction. Since the arterial wall is undergoing uniaxial radial stretching, the endothelial cells are in the direction with the minimal stretching, and hence there is no stimulus for the cells to reorient. When a vessel wall is injured, however, e.g., denuded by balloon dilation, the cells that migrate to the injured area will be expected to reorient in order to be subjected to the minimal stretching like the cells in an intact vessel. In addition, it should be noted that many types of cells are generally embedded in extracellular matrix. The matrix itself can influence the cell reorientation response to cyclic mechanical stretching. For example, smooth muscle cells, when embedded in a collagen matrix, align in the stretching direction instead of orienting away from the stretching direction (Kanda and Matsuda, 1994). Moreover, the matrix influences phenotypic expression of the cells, including gene expression and protein synthesis (Ingber, 1991). Thus, to study biological responses of these cells with a surrounding matrix, the matrix should be included along with mechanical stretching.

In summary, this study shows that in response to cyclic stretching, human aortic endothelial cells reorient very specifically to the direction with minimal substrate deformation, regardless of the type of mechanical stretching applied. Furthermore, this study shows that the rate and extent of early cell reorientation depend predominantly on the stretching magnitude, not the stretching rate. The actin cytoskeleton of the stretched cell is remodeled into stress fibers which are also oriented in the direction of minimal substrate deformation, no matter what type of mechanical stretching is applied. Finally, microtubules are not necessary for the stress fiber formation and cell reorientation to occur in response to cyclic mechanical stretching.

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References

Banes, A.J., Tsuzaki, M., Yamamoto, J., Fischer, T., Brigman, B., Brown, T., Miller, L., 1995. Mechanoreception at the cellular level: the detection, interpretation, and diversity of responses to mechanical signals. Biochemistry and Cell Biology 73, 1–16.

Barbee, K.A., Macarak, E.J., Thibault, L.E., 1994. Strain measurements in cultured vascular smooth muscle cells subjected to mechanical deformation. Annals of Biomedical Engineering 22 (1), 14–22.

Dartsch, P.C., Betz, E., 1989. Response of cultured endothelial cells to mechanical stimulation. Basic Research in Cardiology 84, 268–281.

Davies, P.F., Tripathi, S.C., 1993. Mechanical stress mechanisms and the cell: an endothelial paradigm. Circulation Research 72, 239–245.

Hoel, P.G., 1971. Introduction to Mathematical Statistics. Wiley, San Diego, CA.

Iba, T., Sumpio, B.E., 1991. Morphological response of human endothelial cells subjected to cyclic strain in vitro. Microvascular Research 42, 245–254.

Ingber, D., 1991. Integrins as mechanochemical transducers. Current Opinion on Cell Biology 3, 841–848.

Kanda, K., Matsuda, T., 1994. Mechanical stress-induced orientation and ultrastructural change of smooth muscle cells cultured in three-dimensional collagen lattices. Cell Transplantation 3 (6), 481–492.

Malek, A.M., Izumo, S., 1996. Mechanism of endothelial cell shape change and cytoskeletal remodeling in response to fluid shear stress. Journal of Cell Science 109, 713–726.

Masuda, M., Fujiwara, K., 1993. Morphological responses of single endothelial cells exposed to physiological levels of fluid shear stress. Frontiers of Medical and Biological Engineering 5 (2), 79–87.

Matsumoto, T., Delafontaine, P., Schnetzer, K.J., Tong, B.C., Nerem, R.M., 1996. Effect of uniaxial, cyclic stretch on the morphology of monocytes/macrophages in culture. Journal of Biomechanical Engineering 118, 420–422.

Rosner, B., 1990. Fundamental of Biostatistics, 3rd Edition. Duxbury Press, Belmont, CA.

Sadoshima, J., Izumo, S., 1993. Mechanotransduction in stretch-induced hypertrophy of cardiac myocytes. Journal of Receptor Research 13, 777–794.

Shirinsky, V.P., Antonov, A.S., Birukov, K.G., Sobolevsky, A.V., Romanov, Y.A., Kabaeva, N.V., Antonova, G.N., Smirnov, V.N., 1989. Mechano-chemical control of human endothelium orientation and size. Journal of Cell Biology 109, 331–339.

Simon, S.I., Schmid-Schonbein, G.W., 1990. Cytoplasmic strains and strain rates in motile polymorphonuclear leukocytes. Biophysical Journal 58 (2), 319–332.

Takemasa, T., Sugimoto, K., Yamashita, K., 1997. Amplitude-dependent stress fiber reorientation in early response to cyclic strain. Experimental Cell Research 230, 407–410.

Takemasa, T., Yamaguchi, T., Yamamoto, Y., Sugimoto, K., Yamashita, K., 1998. Oblique alignment of stress fibers in cells reduces the mechanical stress in cyclically deforming fields. European Journal of Cell Biology 77 (2), 91–99.

Wang, H.C., Ip, W., Boissy, R., Grood, E.S., 1995. Cell orientation response to cyclically deformed substrates: experimental validation of a cell model. Journal of Biomechanics 7, 130–138.

Wang, J.H.-C., 2000. Substrate deformation determines actin cytoskeleton reorganization: a mathematical modeling and experimental study. Journal of Theoretical Biology 202, 33–41.

Wang, J.H.-C., Grood, E.S., 2000. The strain magnitude and contact guidance determine orientation response of fibroblasts to cyclic substrate strains. Connecticut Tissue Research 41 (1), 29–36.

Wang, J.H.-C., Goldschmidt-Clermont, P., Yin, F.C.-P., 2000a. Contractility and reactive oxygen species affect actin cytoskeleton remodeling of the endothelial cell to mechanical stretching. Annals of Biomedical Engineering 28, 1165–1171.

Wang, J.H.-C., Goldschmidt-Clermont, P., Moldovan, N., Yin, F.C.-P., 2000b. Leukotrienes and tyrosine phosphorylation mediate stretching-induced actin cytoskeletal remodeling in endothelial cells. Cell Motility and Cytoskeleton 46, 137–145.

Wang, N., Ingber, D.E., 1994. Control of cytoskeletal mechanics by extracellular matrix, cell shape, and mechanical tension. Biophysical Journal 66, 2181–2189.

Wang, N., Butler, J.P., Ingber, D.E., 1993. Mechanotransduction across the cell surface and through the cytoskeleton. Science 260, 1124–1127.