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Original Article

Effect of Cyclic Stretching on Cell Shape and Division

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Abstract In multicellular organisms, cell fate and tissue organization are greatly affected by the orientation of the cell division axis. Cell division is aligned perpendicular to the long axis of a cell - the so-called 'Hertwig's rule'. Because mechanical strains on cells cause arrangement of cytoskeleton molecules which determines cell shape, it is expected that mechanical strains also play an important role on the orientation of the cell division axis. In this study, we used a microfabricated cyclic stretching device to understand how mechanical strains affect the orientation of the cell division axis, which determines the spatial arrangement of daughter cells. When RPE-1 (retinal pigmented epithelial) cells were cyclically stretched during their prophase and prometa phase at various stretching magnitudes (5% and 10%) and frequencies (0.1-10 Hz), the cells' aspect ratios were at their highest. Compared to unstretched cells with aspect ratios below 2.5, the stretched ones with aspect ratios higher than 4 better suited Hertwig's rule by showing a good alignment of spindle angle to the perpendicular direction to the long axis of their cell bodies. These results suggest that mechanical strains play important roles in determining the cell division axis by affecting cell shape and orientation.

Keywords: Mechanical strains, Stretching, Cell division axis, Cell elongation

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Introduction

The human body experiences diverse mechanical forces, from gravity to shear stress and tension[1,2]. Tissue matrices are distorted by strains in a range of 1-5% from daily activities such as walking or even breathing[3]. These mechanical strains cause changes in the structure and function of strain-sensitive cells in vivo. Especially, cells on soft surfaces such as fibroblasts receive mechanical strains through the cytoskeleton-adhesion interface.

In multicellular organisms including humans, cell fate and tissue organization are greatly affected by the orientation of the cell division axis[4]. Parental cells divide into daughter cells, and the spatial orientation of daughter cells is decided by the division axis of their parental cells. In the process of cell division, divided centrioles form asters and are positioned at opposite poles during prophase. The cell division axis is formed at the middle of the cell, where its chromosomes line up during metaphase. During anaphase, centromeres split and each half of the chromosomes move to each end of the holes. The cell membrane begins to pinch inward at the center, bringing about two daughter cells[5]. The division axis is usually aligned perpendicular to the long axis of the cell. This was discovered by Oscar Hertwig and is thus often called 'Hertwig's rule'[6].

Because mechanical strains on cells cause arrangement of cytoskeleton molecules and often lead to cell elongation, it is expected that mechanical strains also play an important role in the orientation of the cell division axis. However, little is known about how mechanical strains affect the orientation of the cell division axis.

Recently, we developed a microfabricated stretching device (Figure 1A) which enables a cell to be cyclically

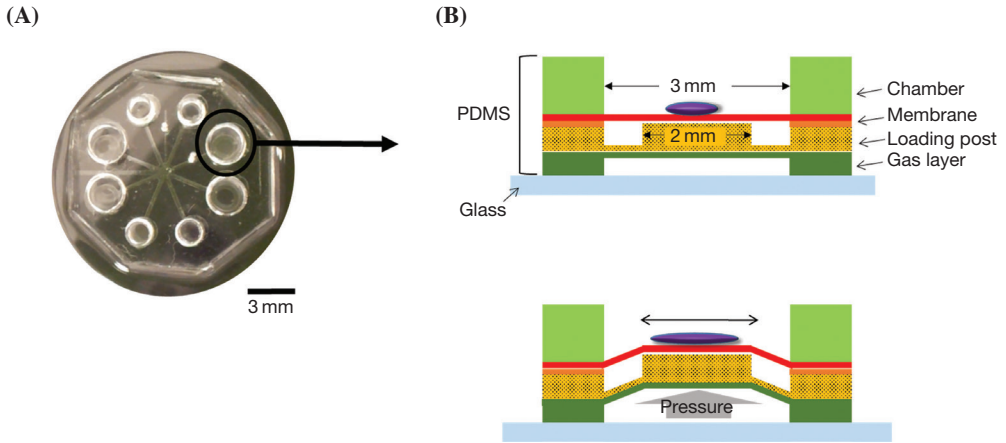

Figure 1. Images of the cyclic stretching device (A) and its cross-sectional design of (B). (A) Stretching device has eight chambers. Each chamber contains a cell culture membrane, a post, and a pneumatically-actuating channel. (B) The stretching device consists of four components of PDMS: a chamber (3 mm wide), a membrane (60 μm thick), and a loading post (2 mm wide and 100 μm tall) and a gas layer. When the gas layer is filled with CO_2 gas at various pressures, the flexible membrane is laterally stretched by lifting up the post to the top membrane. Cells on the top of the membrane are laterally stretched as well.

and equibiaxially stretched over a period of six hours without compromisingitsviability[3]. The device of cyclic equibiaxial stretching by gas pressure was used to stretch primary mouse embryonic fibroblasts (PMEFs) on a nanopillar array of the elastomeric polymer PDMS (polydimethylsiloxane). In this study, we found that when cells were 5% to 10% cyclically stretched, they became elongated and their viability increased by up to 50%[3]. Noticeably, we found that cyclic stretching caused cells to become aligned and elongated in the vertical direction to the strain direction, which was previously reported elsewhere[7,8].

However, it is still not known how the cell division axis is affected by mechanical strains. Herein, we studied the effect of cyclic stretching on cell elongation and orientation of the cell division axis, using the previously developed cyclic stretching device. Human retinal pigmented epithelial (RPE-1) cells, which are commonly used for mitosis research, were cultured for six hours under mechanical strains of 5% and 10% at various frequencies (0.1-10 Hz) (Figure 1B). Then, the aspect ratio of cell body and spindle angle was measured to investigate the effect of mechanical strains on cell elongation and orientation of the cell division axis.

Results and Discussion

Device characterization

The stretching magnitude was calculated by measuring changes in the center-to-center distances of 500 nm pillar arrays at various pressures as described elsewhere[3]. As shown in Figure 2A, 5% and 10% stretches were successfully accomplished. The homogeneity of stretch was estimated by plotting circumferential strain parameters with regard to changes in the circumferential pillar location. Figure 2B indicates that the device guarantees the homogeneity of the stretch. As previously reported, the consistency of the strains was maintained with an error of less than 1% over six hours (data not shown).

Effect of pre-incubation and stretching magnitude on cell elongation

Our previous study[3] showed that 5% or 10% cyclic stretching at 0.1 Hz increased the cell proliferation rate by up to 50%, while 20% or higher cyclic stretching decreased the cell proliferation rate. Based on that result, we tried to further identify an optimal condition for cell elongation by culturing RPE-1 cells under a cyclic strain of 5% or 10% at 0.1 Hz for four hours, both with and without pre-incubation for two hours. Since some cells need stabilization to inhibit cell detachment by stretching, the pre-incubation step was included before culturing cells under the cyclic strains.

As shown in Figure 3A, there is no difference in terms of cell area among the cells cultured under the four different conditions. However, the cell aspect ratio was increased only by the 10% cyclic strains at 0.1 Hz for 6 hrs, compared to those of the unstretched cells (Figure 3B) and normal culture. The results showed that cells needed sufficient stretching duration to be polarized

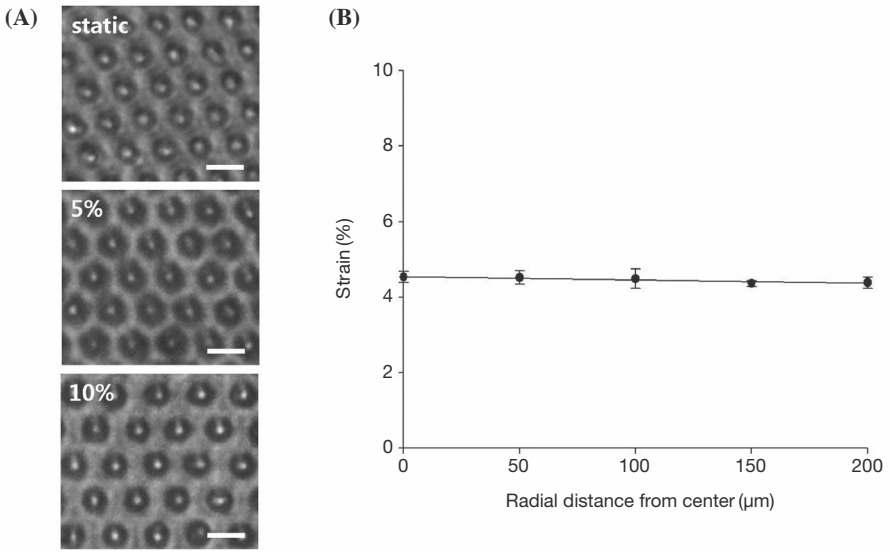

Figure 2. Device characterization. (A) Observation of strain magnitudes. To validate various pressures applied to the gas layer of the device, changes to the center-to-center distance of the array of PDMS pillars (500 nm diameter, 2 μm height, and 1 μm center-to-center distance) on the flat PMDS membrane on the device were observed. Scale bar: 1 μm. (B) Homogeneity of the strains over the membrane. It was examined by plotting circumferential strain parameters with regard to changes in circumferential pillar location. N = 5.

while they did not need the pre-incubation step for cell elongation. Our previous report[3] also showed that the aspect ratio of cells with 5% strain for 6 hrs was higher than that of cells with 5% strain for 4 hrs.

Effect of stretching frequency on cell elongation

Since 10% cyclic stretching at 0.1 Hz increased the cell aspect ratio (Figure 3B), we investigated which frequency of 10% cyclic stretching is the most suitable for cell elongation by culturing cells under 10% cyclic stretching at various frequencies (0.1-10 Hz) for six hours and measuring their cell areas and aspect ratios after the culture. Only the 10% cyclic stretching at 10 Hz significantly decreased the area of the stretched cells, compared to that of the unstretched cells (Figure 4A). The aspect ratios of stretched cells at 0.1 Hz and 10 Hz frequencies of 10% strain were significantly higher than those of the unstretched cells (Figure 4B). This discrepancy between the area and aspect ratio of stretched cells can be explained by the fact that when a cell is highly stretched, it would lose its circular shape and instead take on an elliptical shape. Since a circle reduces in area as it is stretched into an ellipse, the area of the highly stretched cell cultured under 10% cyclic stretching at 10 Hz was smaller than that of the circular unstretched cell. These results suggest that the aspect ratio of a cell is a good indicator for studying the effect of mechanical strains on cell shape, compared to the cell area.

Effect of cyclic stretching on orientation of the cell division axis

According to Hertwig's rule, the cell division axis is aligned perpendicular to the long axis of a cell body. Thus, we hypothesized that stretched cells have a higher tendency to follow the rule than do unstretched cells. To determine whether stretched cells are better suited to the rule than unstretched cells, the perpendicular alignment of the cell division axis to the long axis of a cell was examined by measuring the spindle angle (the red dotted line) difference from an imaginary line (yellow) drawn perpendicular to the long axis (the blue dotted line), all as shown in Figures 5A-C. For statistical accuracy, highly stretched and unstretched cells with their body's aspect ratios higher than 4 and lower than 2.5, respectively, were chosen for the measurement of the spindle angle difference from the imaginary line.

There was no statistical difference in the cell areas between stretched and unstreteched cells (Figure 5D). As shown in Figure 5E, the spindle angle difference of the stretched cells to the imaginary line was lower than 10 degrees, while that of the unstretched cells to the imaginary line was higher than 20 degrees. This indicates that cells elongated by stretching have a higher tendency to follow Hertwig's rule.

The nuclear and spindle positioning mechanism is

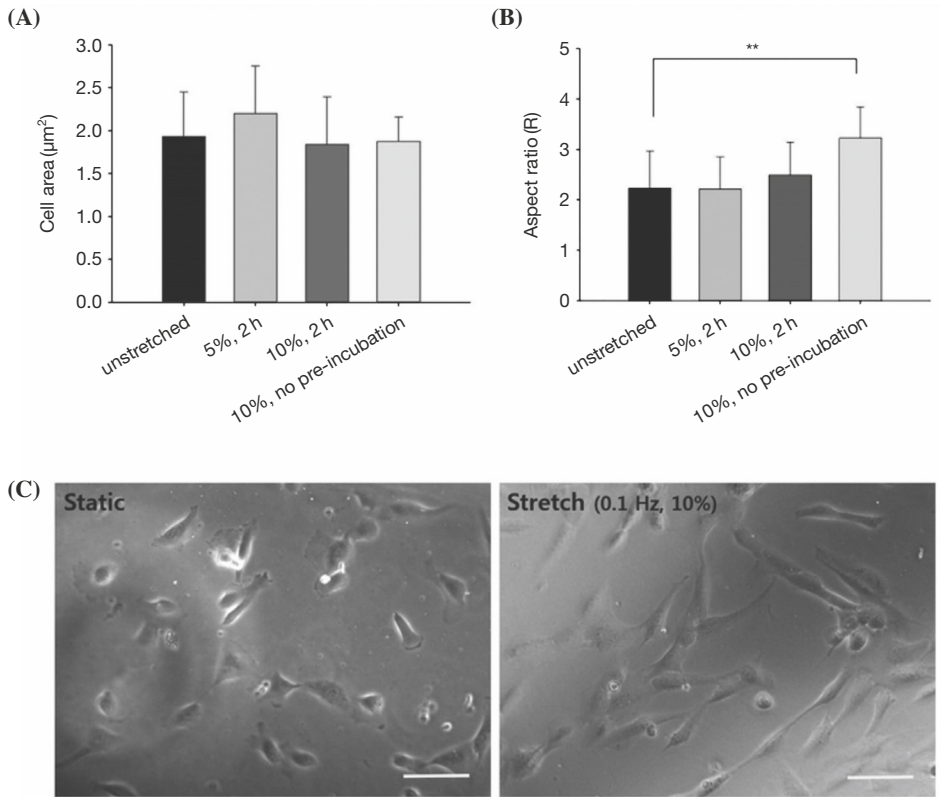

Figure 3. Effect of stretching magnitude (5% or 10%) and pre-incubation (two hours) on cell area and aspect ratio at 0.1 Hz for four hours. (A) Cell area and (B) aspect ratio of RPE-1 cells with various stretching conditions; no stretching, 5% or 10% stretch at 0.1 Hz for four hours after a two-hour pre-incubation,10% stretch at 0.1 Hz for six hours with no pre-incubation. Cell number (n) for each condition: 20. Aspect ratio was given by dividing the length of the long axis by that of the short axis of a cell. Images of cells without any stretching (C) and with 10% cyclic stretch at 0.1 Hz for six hours (D). Scale bar = 50 μm. Error bars, s.d. $^{**}P < 0.01$; data were analyzed using one-way ANOVA with a Turkey post hoc test.

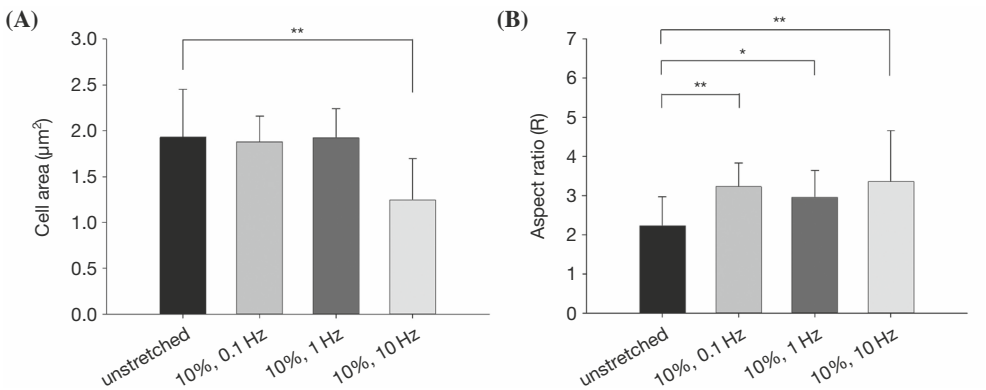

Figure 4. Effect of stretching frequency for 4 h (no pre-incubation). (A) Cell area and (B) Aspect ratio of RPE-1 with various stretching condition. 10% strain was applied without pre-incubation with frequencies of 0.1 Hz, 1 Hz or 10 Hz (n = 17). Error bars, s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$; Student's t-test.

involved with pulling and pushing forces of motor proteins from microtubule and actin cytoskeletons[9-12]. External mechanical stretch may affect the spindle orientation as spindle orientation is related with actin and myosin, and FA proteins talin and vinculin, which are also involved in actin organization. These spindle angle

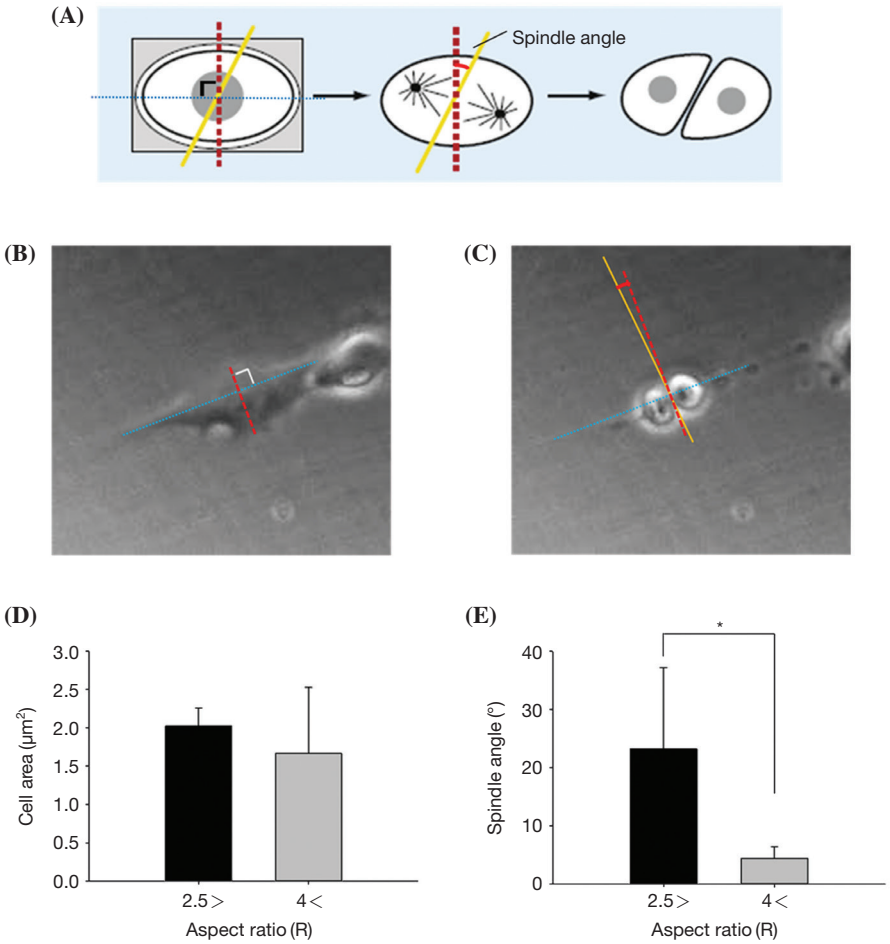

Figure 5. Measurement of the spindle angle difference. (A) Measurement ofthe spindle angle difference to the cell division axis. The perpendicular alignment of the cell division axis to the long axis of a cell was examined by measuring the spindle angle (red dotted line) difference to the imaginary line (yellow) drawn perpendicular to the long axis (blue dotted line). (B) An image of a cell with the longest shape right before cell division. (C) An image of a dividing cell. (D) The area of cells with different aspect ratios (lower than 2.5 or higher than 4). (E) Spindle alignment to a yellow line obtained from the cells with different aspect ratios (lower than 2.5 or higher than 4). The cells with the high aspect ratios were 10% stretched at 10 Hz for six hours without pre-incubation. Sample number per each condition: 4. Error bars, s.d. $*P<0.05$; Student's t-test.

data showed that the cell division axis can be aligned to the direction of mechanical strains. Since cell stretching on a substrate causes rearrangement of cytoskeleton molecules and also causes cell elongation, it is suggested that the orientation of the cell division axis on a substrate is greatly affected by mechanical force and force direction.

Conclusions

It is well known that cells in vivo live under mechanical strains from daily activities. However, questions remain regarding how the spatial and geometric arrangements of cells are affected by mechanical strains. Our results showed that mechanical strains cause cells to become elongated, and that the elongated cells reflect the direction and magnitude of the mechanical strains on the orientation of their cell division axis.

Materials and Methods

Design and fabrication of an equibiaxial cyclic stretching device

The design of the stretching device is shown in Figure 1. As shown in Figure 1A, the device has eight chambers of 3 mm diameter. Each chamber has a flat PDMS membrane. This is made with 1 : 10 w/w PDMS (Syl-

gard 184, Dow and Corning, USA) cured for three hours in an oven at 80°C and with elasticity of about 2.0 MPa[13,14]. At the bottom of the pillar membrane, a loading post of 2 mm diameter and 40 μm height (60 μm thickness) was placed (Figure 1B). The place between the loading post and the pillar membrane was filled with lubricant (90% glycerol) to prevent attachment of both layers and to facilitate equivalent stretching to all membrane surfaces (Figure 1B). A hole (3 mm diameter) was punched onto the top layer to make a cell chamber, while 300 μm holes were punched for lubricant insertion onto the loading post layer (Figure 1B).

The bottom layer was composed of a gas channel (height and thickness: 15 μm and 60 μm) and was connected to a commercial gas regulator (Fluidigm, USA) which enables CO_2 gas to be infused into the gas channel at various frequencies (0.1 Hz-10 Hz) and strains (5 % and 10%). The gas goes in and out through this channel and moves the loading post up and down, which causes the flat membrane to be stretched and relaxed. The membrane surface of the device was coated with 10 μg/mL of fibronectin (Sigma Chemical Co., USA) for two hours at 37°C, in a CO_2 incubator. Unbound fibronectin was washed with PBS (phosphate-buffered saline).

Device characterization

To examine the stretching magnitude of the stretching device, the PDMS membrane (60 μm thickness) with an array of pillars (500 nm diameter, 2 μm height, and 1 μm center-to-center distance) was attached to the top of the flat PDMS membrane on the device. The top of the pillars was imaged using fluorescence microscopy (Live EZ Widefield Fluorescence System, Olympus) and various gas pressures were applied to the device.

Retinal pigment epithelial (RPE-1) cell culture on a stretching device

RPE-1 cells were collected from T-75 cell culture flasks (Corning) by trypsin-EDTA treatment, and 5×10^5 cells of them were seeded on the fibronectin-coated membrane with 500 μL of cell culture media (Leibovits L15 media, Life Technologies, USA) containing 10% FBS (fetal bovine serum) and 1% penicillin/streptomycin. Five minutes after the cells were settled on the fibronectin-coated membrane of the device, and an extra 2.5 mL of media was added to the chamber on the device. The gas layer of the device was connected to a pressure pump by tubing. Various pressures were applied inside the gas layer at various frequencies (0.1-10 Hz). Cells were incubated and observed for live cell imaging under a Biostation IMQ with a motorized stage (Nikon, USA).

Data analysis for cell aspect ratio and spindle angle difference

To measure the aspect ratio of a cell, images of each cell under various conditions were obtained using the Biostation IMQ before mitosis. The images were analysed by the Image J program (NIH, USA) with a custom java algorithm, and the lengths of the long axis and the short axis of the cells in the images were measured. The aspect ratio of a cell was obtained by dividing the length of its major axis by the length of its minor axis.

For measuring the spindle angle difference, cells during mitosis were imaged using the Biostation IMQ. An imaginary line (in yellow) was drawn perpendicular to the long axis (blue dotted line) of each cell just before mitosis. The alignment of the cell division axis to the yellow line was examined by measuring the spindle angle (red dotted line) difference from the imaginary line during mitosis, as shown in Figures 5A-C.

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