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# Controlling Cell Responses to Cyclic Mechanical Stretching

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**Abstract—**In most cell culture studies, cells are grown on smooth culture surfaces. Using microfabrication technology, we have developed microgrooved silicone surfaces to grow cells and subject them to repetitive mechanical stretching. When human patellar tendon fibroblasts were plated on these microgrooved surfaces, the cells had an elongated shape and underwent cyclic uniaxial stretching parallel to their long axes, all of which closely mimic conditions of tendon fibroblasts *in vivo*. Also, when fibroblasts were grown on microgrooves oriented at 45 and 90 degrees with respect to stretching direction, they did not change alignment or shape under cyclic mechanical stretching. Furthermore, compared to nonstretched cells, 8% cyclic stretching of tendon fibroblasts oriented at 0 (i.e., parallel to stretching direction), 45, and 90 degrees was found to increase $\alpha$-SMA protein expression level by 46, 31, and 14%, respectively. In addition, 8% cyclic stretching tendon fibroblasts for 4 and 8 h oriented parallel to stretching direction increased $\alpha$-SMA protein expression level by 25 and 57%, respectively. Thus, the results of this study showed that $\alpha$-SMA protein expression levels of tendon fibroblasts depend on cell orientation with respect to stretching direction and stretching duration. We suggest that microgrooved silicone substrates can be used to study biological responses of tendon or ligament fibroblasts to repetitive mechanical stretching conditions in a more controlled manner.

**Keywords—**Silicone microgrooves, Fibroblasts, Alignment, $\alpha$-Smooth muscle actin, Mechanobiology.

## INTRODUCTION

*In vitro* model systems are widely used to investigate the effects of mechanical forces on cells.[3,15,18,22] Typically, deformable materials with smooth culture surfaces are used in these systems to apply cyclic mechanical stretching to cells. Studies that utilize these model systems have shown that cyclic stretching of cells induces various biological responses, including cell reorientation,[3,22] actin cytoskeletal remodeling,[9,24] altered cell proliferation,[4,17] gene expression, and protein synthesis.[5,15] It is now well recognized that mechanical forces alter the phenotypic expression of various types of cells.

However, there are some considerable limitations in many of these model systems used in these studies. First, the cells on smooth culture surfaces randomly orient initially and reorient in a direction with minimal deformations after stretching.[22] The consequence of cell disorganization and subsequent reorientation is that individual cells are subjected to different combinations of surface strains, depending on their orientation to the stretching direction. To eliminate this problem, equibiaxial stretching systems have been developed.[14,20] These systems produce isotropic strains on smooth culture surfaces; therefore, all cells are subjected to the same surface strains regardless of their orientations. Equibiaxial stretching, however, may not be physiologic for many types of cells. For example, tendon or ligament fibroblasts are subjected to uniaxial instead of biaxial stretching *in vivo*.

Second, cells in previous model systems are disorganized and have multiple shapes. In contrast, cells *in vivo* are well organized, and have a defined shape. For example, tendon fibroblasts *in vivo* are organized in a parallel fashion and assume an elongated shape.[19] As a result, cellular responses may not closely mimic those *in vivo*, because cell organization and cell shape influence cell function.[6,13] Therefore, it is necessary to develop a culture system, with which cell organization, shape, and mechanical loading conditions can be closely controlled.

Using microfabrication technology, we have developed a novel culture system, in which cells are grown on microgrooved silicone surfaces, instead of commonly used smooth surfaces. We show that with or without mechanical stretching, human patellar tendon fibroblasts (HPTFs) on microgrooved silicone surfaces maintained their elongated shape and alignment and also formed parallel actin filaments. We also show that cyclic stretching of HPTFs increased $\alpha$-smooth muscle actin ($\alpha$-SMA) protein expression, where its expression levels appeared to depend on cell orientation to stretching direction and stretching duration.

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## MATERIALS AND METHODS

### *Fabrication of Microgrooves on PDMS Membranes*

The MEMS Exchange (Reston, VA) provided service for fabrication of microgrooved silicon wafers using photolithographic technologies. The fabrication procedures involved (a) spin-coating Shipley 1827 positive photoresist on the silicon wafer [Fig. 1(A)]; and (b) exposing UV light through the microgrooved photomask on mask aligner [Fig. 1(B)]. After development, the features (i.e., microgrooves) on the silicon wafer were obtained. Using the microgrooved silicone wafers as molds, microgrooved silicone membranes were obtained by a molding procedure. Briefly, poly(dimethylsiloxane) (PDMS) was prepared by thoroughly mixing two silicone fluid components, 601A and 601B (Wacker Silicones Corporation, Adrian, MI) in a volume ratio of 10:1, then slowly poured onto the microgrooved silicon wafers. After curing for 24 h at room temperature and then de-molding, the microgrooved silicone membranes were obtained [Fig. 1(C)].

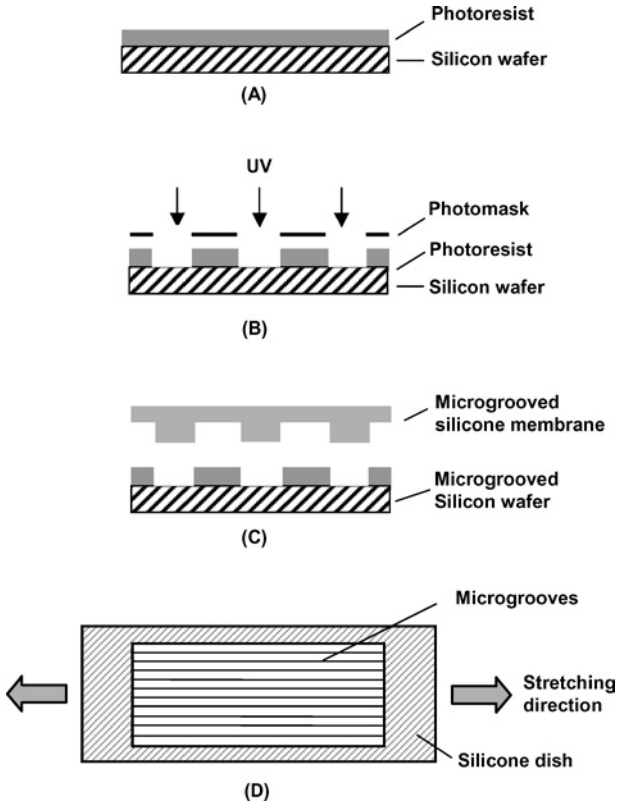


**FIGURE 1. An illustration of procedures for fabricating microgrooved silicon wafers and microgrooved silicone membranes. The procedures involved (A) spin-coating photoresist on the silicone wafer, (B) fabricating the microgrooved silicone wafer through exposure of UV light; (C) molding microgrooved silicone membrane using the microgrooved silicone wafer; and (D) fabricating the silicone dish with microgrooved culture surface. The direction of the parallel microgrooves in the dish was 0, or 45, or 90 degrees with respect to the long axis of the silicone dish, which is also the stretching direction (arrows). The microgrooves were rectangular in profile, with 10 $\mu$m ridge and groove width, and 3 $\mu$m groove depth.**

### *Fabrication of Microgrooved Silicone Dishes*

Silicone dishes were fabricated according to the following molding procedures. Silicone components (601A and 601B) were mixed and poured slowly into a multiple-dish mold made of acrylic (Plexiglass). The mold, corresponding to the culture surface of each dish, was covered with a piece of glass to form a smooth surface. After de-molding, silicone dishes with smooth culture surfaces were obtained. The "smoothness" is defined here as no specific features (e.g., "ridges" or "channels") on the silicone surface under examination of a regular light microscope. The microgrooved membranes, 1.5 mm thick, were cut to a size of about 3.5 cm $\times$ 7 cm and were bonded, using silicone adhesive (Dow Corning, MI), to the bottoms of silicone dishes whose smooth culture surfaces had been removed. Note that the dimensions of the culture surface were 3 cm $\times$ 6 cm, and the direction of the parallel microgrooves in the dish was either 0, 45, or 90 degrees with respect to the long axis of the silicone dish, which is also the stretching direction. The microgrooves were rectangular in profile, with 10 $\mu$m ridge and groove width, and 3 $\mu$m groove depth [Fig. 1(D)].

### *Cell Stretching Experiments*

HPTFs were derived from tendon pieces trimmed from patellar tendon grafts of healthy donors. The protocol for obtaining the tendon samples was approved by the Institutional Review Board of the University of Pittsburgh (IRB # 0108109). The fibroblasts were transferred to silicone dishes, whose culture surfaces had been coated with 10 $\mu$g/ml ProNectin (BioSource International, Inc., Camarillo, CA) to promote cell attachment. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen, CA). After 24 h, microphotographs of these fibroblasts in culture were taken using a digital camera (RT Color Digital Camera, Diagnostic Instruments, Inc., MI). Then, using the custom-made stretching apparatus, which is similar to the one described previously,[17] tendon fibroblasts in silicone dishes containing regular growth medium (DMEM + 10% FBS) were cyclically stretched at 8% (a "grip-to-grip" engineering strain), at 0.5 Hz, for up to 48 h. Nonstretched cells were used for control. After the end of stretching, digital microphotographs were again taken to document cell alignment.

In separate experiments, tendon fibroblasts in microgrooved silicone dishes were cyclically stretched at 8% for 4 h, followed by 4 h rest. Still in separate experiments,

cells were cyclically stretched at 8% for 4 and 8 h and then rested in stretching-conditioned medium for an additional 4 h. The cells, including those stretched and nonstretched for control, were then lysed to collect total cellular proteins for measuring total actin and $\alpha$-SMA protein expression levels.

### *Staining Actin Filaments and Nuclei*

Actin filaments of stretched and nonstretched fibroblasts were stained with rhodamine phalloidin (Molecular Probes, Eugene, OR) according to the following protocol. Immediately after tendon fibroblasts were stretched for 4 h, the medium in the silicone dish was quickly extracted, and the cells were washed once with ice-cold PBS and fixed in 3.7% para-formaldehyde for 15 min. After washing with PBS, the cells were permeabilized in 0.25% Triton X-100 for 10 min, followed by incubation with a solution of rhodamine phalloidin (0.165 $\mu$M) for 1 h. In separate experiments, fibroblasts were incubated with propidium iodide (1 $\mu$l propidium iodide dissolved in 3.3 ml of PBS) at room temperature for 15 min. After extensive washing with PBS, the cells that were stained with rhodamine phalloidin or propidium iodide were viewed on a fluorescent microscope (Eclipse TE200, Nikon) and photographed with a digital camera to record the pattern of fibroblast's actin filaments and nuclei on the microgrooved surfaces as well as on smooth surfaces.

### *Assaying Total Actin and $\alpha$-SMA Protein Expression*

Western blot was used to measure the actin expression levels of tendon fibroblasts. Briefly, fibroblasts were lysed to collect cellular proteins. A 20 $\mu$g protein sample was loaded into a 10% SDS-polyacrylamide gel for electrophoresis, and separated proteins were then transferred to a nitrocellulose membrane. After blocking with 5% nonfat milk in 0.1% PBS-Tween 20, the protein-containing membrane was incubated with a polyclonal antibody to actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or monoclonal antibody to $\alpha$-SMA (Sigma, St. Louis) with a ratio of 1:1000 in PBS-0.1% Tween 20 containing 1% nonfat milk at room temperature for 2 h. Then, the membrane was washed extensively and incubated for 2 h with the secondary antibody conjugated with horseradish peroxidase (1:5000 dilution ratio; Jackson Immunoresearch Lab., Inc., West Grove, PA). Finally, actin bands on the membrane were detected using the ECL system (Amersham, Piscataway, NJ). Note that in some experiments, GAPDH, a house-keeping protein, was also assayed using Western blot to serve as an internal control of protein loading. The protein bands were scanned and quantified by image analysis (NIH Image J 1.30v). The density of each band was normalized to that of GAPDH.

### *Statistical Analysis*

Wherever appropriate, one-way ANOVA was used for statistical analysis of the stretching effects on total actin and $\alpha$-SMA protein expression, followed by Duncan's test for multiple comparisons at a significance level of 0.05.

## RESULTS

Tendon fibroblasts aligned with microgrooves without stretching and remained so after stretching, regardless of the orientation of the microgrooves with respect to stretching direction (Fig. 2). A striking feature of these aligned fibroblasts was that they were uniformly elongated in shape. Staining of these fibroblasts on the microgrooved surface revealed that actin filaments aligned with the microgrooves [Figs. 3(A) and 3(B)], and that the nuclei also oriented along the microgroove direction [Fig. 3(C)].

Moreover, total actin expression levels of these fibroblasts were not altered by mechanical stretching, which was independent of cell orientation (0, 45, or 90 degrees) with respect to stretching direction (Fig. 4). However, 8% cyclic stretching of tendon fibroblasts at 0 and 45 but not 90 degrees significantly increased $\alpha$-SMA protein expression compared to nonstretched fibroblasts ($p < 0.05$). On average, $\alpha$-SMA protein expression levels of stretched tendon fibroblasts oriented at 0, 45, and 90 degrees were 46, 31, and 14% higher than that of nonstretched cells, respectively (Fig. 5).

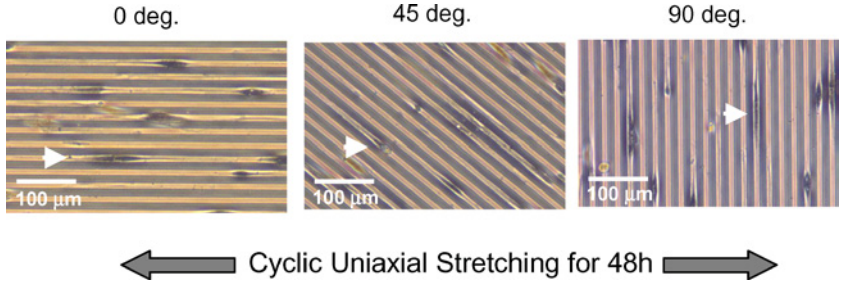


**FIGURE 2. The alignment of tendon fibroblasts in microgrooved silicone surfaces. Regardless of cell orientation with respect to stretching direction, the stretched tendon fibroblasts aligned with the microgroove direction. The cells were stretched at 8% for 48 h. Arrows point to fibroblasts residing in microgrooves.**

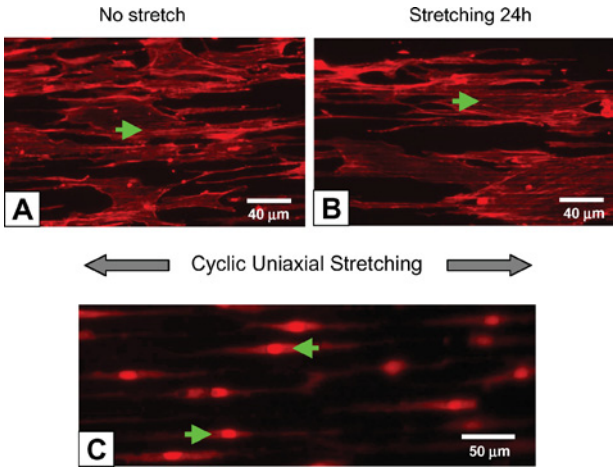


**FIGURE 3. The actin cytoskeletal organization of tendon fibroblasts on microgrooved silicone surfaces. Actin filaments (arrows) aligned with the direction of microgrooves without stretching (A) and with 8% stretching (B). With staining of propidium iodide, the nuclei (arrows) of individual cells on the microgrooves were also visualized to show the relative position of the fibroblasts on the microgrooved surface (C).**

In addition, compared to nonstretched cells, 8% stretched tendon fibroblasts on horizontal microgrooves (i.e., 0 degree, parallel to stretching direction) increased $\alpha$-SMA protein expression level by an average of 25 and 57% at 4 and 8 h, respectively (Fig. 6).

## DISCUSSION

This study showed that without cyclic stretching, the fibroblasts on microgrooved surfaces aligned with the microgrooves and had an elongated shape. These results are consistent with previous studies that used various types of cell grown on microgrooved substrates made of various materials.[2,7,11,26] Cell contact guidance may be responsible for the cell alignment on microgrooved surfaces, which impose mechanical restrictions on the formation of linear bundles of filaments involved in cell locomotion.[12]

Furthermore, regardless of cell orientation (0, 45, or 90 degrees) with respect to stretching direction (Fig. 2),

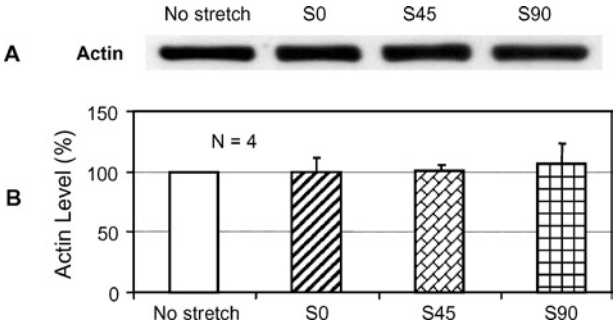


**FIGURE 4. The effect of cyclic stretching on total actin expression. Stretched tendon fibroblasts did not change total actin expression levels compared to nonstretched cells. Tendon fibroblasts were grown in microgrooves oriented at three angles (0, 45, and 90 degrees) with respect to stretching direction, and 8% cyclic stretching was applied for 4 h, followed by 4 h rest. A representative Western blot result is given here (A). Four separate experiments were performed, and consistent results were obtained (B). (S0: Stretching fibroblasts on the 0 degree microgrooves; S45: stretching fibroblasts on the 45 degree microgrooves; and S90: stretching fibroblasts on the 90 degree microgrooves.)**

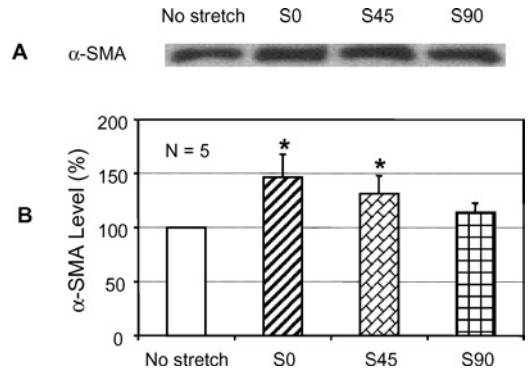


**FIGURE 5. The effect of cyclic stretching on $\alpha$-SMA expression. A representative Western blot result is shown here (A). Stretched tendon fibroblasts increased $\alpha$-SMA protein expression levels compared with nonstretched cells. Also, the stretching-induced $\alpha$-SMA protein expression levels depended on cell orientation. A total of five separate experiments were performed, and consistent results were obtained (B). (S0: Stretching fibroblasts oriented at 0 degrees; S45: stretching fibroblasts oriented at 45 degrees; and S90: stretching fibroblasts oriented at 90 degrees).**

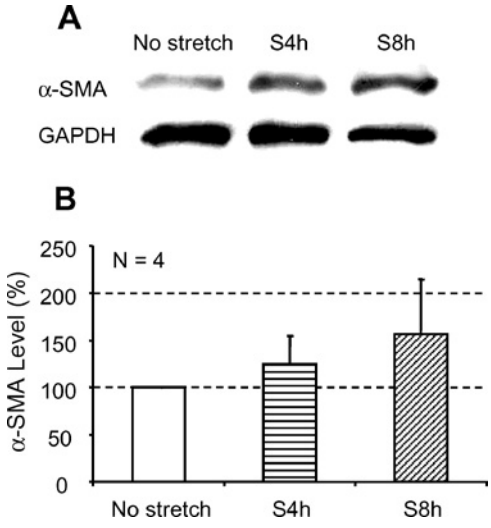


**FIGURE 6. A representative Western blot result of the stretching-duration effect on $\alpha$-SMA protein expression (A). From four separate experiments, it was found that on average, 8% cyclic stretching of human tendon fibroblasts for 4 and 8 h increased $\alpha$-SMA protein expression levels by 25 and 57%, respectively (B). (S4h: stretching fibroblasts for 4 h; and S8h: stretching fibroblasts for 8 h).**

cyclic stretching of the fibroblasts on the microgrooved surfaces did not change cell alignment. It is known that cells on smooth culture surfaces reorient in response to cyclic uniaxial stretching.[9,25] Therefore, the results suggest that cell contact guidance provided by microgrooves on the silicone surfaces is a much stronger cue[16] that prevents cells from reorienting under cyclic uniaxial stretching conditions.

The unaltered cell alignment on microgrooved surfaces under cyclic stretching conditions indicates that substrate surface strains on these cells do not change. However, cells grown on smooth surfaces change orientation when subjected to cyclic uniaxial stretching.[3,22] Consequently, the three surface strains (axial, transverse and shear strains) that act on a cell also change with cell orientation. Since microgrooves maintain cell orientation/alignment, the microgrooved silicone substrate may be used to deliver controlled uniaxial stretches to those cells aligned with the stretching direction *in vivo*, such as tendon or ligament fibroblasts. The silicone substrate with oriented microgrooves can also be used to study how different combinations of substrate strains influence the gene and protein expression of tendon or ligament fibroblasts.

It should be noted that the presence of microgrooves on silicone culture surface influences surface strains. Therefore, the strains experienced by cells on the microgrooved surface is likely different from those of the smooth surface. But, because the depth of the microgrooves is small (3 $\mu$m) compared to the thickness of the silicone substrate (1.5 mm), the influence of microgrooves on surface strains is expected to be small. However, this "microgroove factor" on the surface strains must be taken into account when one interprets biological responses (e.g., $\alpha$-SMA protein expression) of tendon fibroblasts on the microgrooves.

On the microgrooved surface, the tendon fibroblasts contacted each other longitudinally as well as laterally via cell processes between rows, as revealed by staining of actin filaments [arrows in Figs. 3(A) and 3(B)]. Such organization appears to mimic that of fibroblasts within a tendon *in vivo*.[19] Also, tendon fibroblasts on the microgrooves exhibited actin filaments, which were not clearly separated but aligned with the microgrooves, that is, the stretching direction. This is consistent with a previous study using cardiac fibroblasts,[11] and it also appears similar to that of fibroblasts in tendon. For example, tendon fibroblasts were shown to contain actin filaments running longitudinally.[19] Further, it is known that the actin cytoskeletal pattern determines cell shape, which in turn influences cell phenotypic expression.[6,13] Therefore, the similar organization of actin structures of tendon fibroblasts on the microgrooved surface suggests that the phenotypic expression of these cells may be more homogenous than they would be on a smooth surface. This makes it possible to more closely mimic mechanobiological responses of tendon fibroblasts *in vivo*.

This study showed that total actin expression levels of stretched tendon fibroblasts do not change under cyclic stretching conditions. This is consistent with a previous study, which showed that the actin expression levels of tendon fibroblasts on smooth culture surfaces were not changed by cyclic mechanical stretching.[19] This fact indicates that total actin can be used as a house-keeping protein that serves as an internal control protein with or without mechanical stretching. Moreover, this study showed that cyclic stretching increased $\alpha$-SMA expression. Interestingly, although the same level of uniaxial stretching (8%) was applied, stretched tendon fibroblasts with a different orientation expressed different levels of $\alpha$-SMA (Fig. 5). The mechanisms for this are not clear. But because different combinations of axial, transverse, and shear strains act on cells with different orientations (0, 45, and 90 degrees), it is believed that different "mechanotransduction signals" could be involved, which result in different $\alpha$-SMA expression levels. Furthermore, since $\alpha$-SMA is a specific marker for myofibroblasts,[8] the result of this study suggests that, like TGF-$\beta$1,[10] cyclic mechanical stretching can promote fibroblast differentiation into myofibroblasts. Previous studies have also shown that mechanical tension *in vitro* and *in vivo* regulates cellular $\alpha$-SMA expression.[1,21] However, it is not known from this study whether mechanical loading is an independent factor for regulation of $\alpha$-SMA expression or its effect is mediated by other factors such as TGF-$\beta$1, which was most likely present in 10% FBS used in this study. Also, to confirm stretching-induction of myofibroblast differentiation, future studies should investigate other markers for myofibroblasts, such as ED-A fibronectin, vimentin, and absence of desmin,[21,23] under different mechanical stretching conditions (e.g., various stretching magnitudes and frequencies).

In summary, this study shows that human tendon fibroblasts grown on microgrooved silicone substrates maintain an elongated shape and alignment under cyclic stretching conditions, and that cyclic mechanical stretching of the tendon fibroblasts increases $\alpha$-SMA protein expression in a stretching-duration dependent manner. We suggest that microgrooved silicone substrates may be used to study biological responses (e.g., gene induction and protein synthesis) of tendon or ligament fibroblasts and possibly other types of cells (e.g., endothelial cells, smooth muscle cells, and myocytes) to repetitive mechanical stretching conditions in a more controlled fashion.

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