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Effect of Static Pre-stretch Induced Surface Anisotropy on Orientation of Mesenchymal Stem Cells

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Abstract

Mechanical cues in the cellular environment play important roles in guiding various cell behaviors, such as cell alignment, migration, and differentiation. Previous studies investigated mechanical stretch guided cell alignment pre-dominantly with cyclic stretching whereby an external force is applied to stretch the substrate dynamically (i.e., cyclically) while the cells are attached onto the substrate. In contrast, we created a static pre-stretched anisotropic surface in which the cells were seeded subsequent to stretching the substrate. We hypothesized that the cell senses the physical environment through a more active mechanism, namely, even without external forces the cell can actively apply traction and sense an increased stiffness in the stretched direction and align in that direction. To test our hypothesis, we quantified the extent of pre-stretch induced anisotropy by employing the theory of small deformation superimposed on large and predicted the effective stiffness in the stretch direction as well as its perpendicular direction. We showed mesenchymal stem cells (MSC) aligned in the pre-stretched direction, and the cell alignment and morphology were dependent on the pre-stretch magnitude. In addition, the pre-stretched surface demonstrated an ability to promote early myoblast differentiation of the MSC. This study is the first report on MSC alignment on a statically pre-stretched surface. The cell orientation induced by the pre-stretch induced anisotropy could provide insight into tissue engineering applications involving cells that aligned *in vivo* in the absence of dynamic mechanical stimuli.

Keywords

Mesenchymal stem cells; Mechano-sensing; Static pre-stretch; Anisotropy, Orientation

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ELECTRONIC SUPPLEMENTARY MATERIAL

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CONFLICT OF INTEREST

The authors do not have any conflict of interest to disclose.

INTRODUCTION

While chemical stimuli have long been understood to regulate cell behavior, in recent decades mechanical stimuli have gained increasing attention in tissue engineering. Engineers are able to induce various changes in both morphology and intracellular signaling by manipulating the mechanical environment of the cell. For example, mechanical stretching can regulate cell behavior through the induction of cell orientation, remodeling of the cytoskeletal fibers, and changes in cell signaling.^{19,28,29} When subjected to an external stretch, stem cells have been found to align in a specific direction with certain protein synthesis up-regulated, showing promising differentiation potential.^{11,14} Uniaxial cyclic stretching has been shown to direct cell orientation perpendicular to the stretching direction.^{10,17,27} However, compared to the large body of research focused on cyclic stretching, few studies have examined the effects of static stretch on cell orientation and morphology. Collinsworth *et al.*⁶ found that after applying static stretch, the originally randomly oriented mammalian skeletal muscle cells showed clear alignment in the direction of static stretch. In contrast, Goli-Malekabadi *et al.*¹⁰ recently claimed that with MSCs static stretch is not as influential as cyclic stretching in directing cell alignment or changing cell morphology.

While the alignment of cells on a statically stretched substrate is intuitive, the mechanism by which the cells sense the stretch is not easily understood. That is due in part to the confusion that arises from the preponderance of research where static stretching meant the cells are seeded first to ensure attachment prior to applying an external stretch. As a result, any change in the cell orientation after the stretch was attributed to adaption to the sudden change followed by a slow recovery. In other words, the so-called static stretch in those prior studies is actually a one-time dynamic stretch without repetition. To address this issue, we undertook this study to create a purely statically stretched environment by seeding cells on a pre-stretched membrane. The major difference between our static pre-stretch and previous static stretch studies is that we applied the stretch prior to seeding the cells, and attributed the cell alignment to the pre-stretch induced anisotropy. A prior study by Haston *et al.*¹² used a similar procedure to ours; however they attributed their results to the topography induced by the fibrous collagen on the surface. In contrast, in our study the surface was coated with poly-L-lysine (PLL), which is not a fibrous matrix, and cell alignment was produced primarily as a result of the mechanical pre-stretch. We then proposed that cells behave actively by pulling the substrate to sense the mechanical environments and respond to the environmental condition by modulating their morphology and orientation after seeding, which can be predicted by using the theory of finite elasticity.

We have previously applied an “active mechano-sensing” model to show that the size of a cell depends not only on the mechanical properties of the substrate, but also on the energy consumption during active cellular probing.²⁰ Following an investigation of cell alignment on anisotropic surface generated by fixed and free boundaries (unpublished data), the experimental results agreed with our “cell active mechano-sensing” model which suggests that the cells can feel and respond to the surface anisotropy by orienting in the direction of maximal effective stiffness (unpublished data). In this study, we applied this concept on a static pre-stretched (previously stretched) polydimethyl-siloxane (PDMS) membrane. By culturing the cells on the pre-stretched PDMS membrane, the effective stiffness the cells experienced in the stretched direction was significantly larger than that in the unstretched direction. This increase of the effective stiffness results in the cells orienting in the stretched direction as well as modulating the cytoskeleton and focal adhesion.

This stretch-induced directional difference can be explained using Fig. 1. Let the rectangular body on the far left of the figure be an isotropic material in the stress-free configuration. Let

us first stretch this material in the vertical direction and deform it with a load F^0 on a square shape, which we denote as the “pre-stretched configuration.” From the pre-stretched state, if we deform it equi-biaxially (same deformation in both directions) then, in addition to the original loading F^0 , we have to apply F^1 and F^2 in the vertical and horizontal directions, respectively. Now we will find that F^1 should be larger than F^2 in order to achieve the same deformation in both directions from the pre-stretched state, because the material will be stiffer in the vertical direction due to the prior deformation. Similarly, if F^1 and F^2 are the same, then the deformation in the vertical direction would be smaller than in the horizontal direction. Thus, when a cell is cultured on a pre-stretched substrate, F^0 can be considered as the force holding the substrate in this intermediate or pre-stretched state, F^1 and F^2 are traction forces applied by the cell, and the deformation from this pre-stretched substrate are the forces that the cell senses. We will denote this anisotropic response of the cells to the pre-stretched substrate under active cellular probing as “stretch-induced anisotropy.” We propose, hence, that stretch is a natural mechanism to induce an anisotropic environment in the substrate for guiding cell morphology and functions. We should note that this stretch-induced stiffening arises from the change of the reference configuration for the strain measurement, which is different from a nonlinear material response called strain-hardening. We cannot capture this stiffening by linear elasticity because it uses only one unchanged reference configuration to measure the strain. Instead, the theory of small deformation superimposed on large (often called “theory of small on large”) based on finite elasticity enables us to accurately estimate the degree of anisotropy by measuring the pre-stretch of the substrate.²

In the present study, we predict the anisotropy induced by the theory and found that the distribution of cell alignment is in the stretch direction; therefore we interpret the cell alignment as the resistance to active cellular pulling. We employ the concept of active cellular mechano-sensing, and present a method that uses mechanical quantities (i.e., linearized stiffness) which can be obtained from mechanical analysis. Specifically, we theorize that cells actively pull on the substrate and sense the mechanical resistance of the substrate, i.e., the deformation upon active probing, and the cells thereby align in the direction of highest resistance upon active pulling. Based on our previous study,²⁰ we define an effective stiffness of the substrate that correlates with the stiffness that the cells sense. For the experiments, we created surface anisotropy with a uniaxially sustained stretching of the PDMS substrate and cultured MSCs on the pre-stretched substrate. The distributions of orientation of the cells during culture were quantified and the surface properties were examined on surfaces with and without pre-stretch. The impact of the magnitude of the pre-stretch on cell orientation and morphology were evaluated. We demonstrate that the effective stiffness can be estimated using conventional finite elasticity analysis, and predict the cellular orientations that were experimentally confirmed. In addition, the pre-stretch not only affects MSC morphology, but also promotes early myoblast differentiation of the MSC.

MATERIALS AND METHODS

Materials

PDMS substrate was prepared using the 184 silicone elastomer kit purchased from Dow Corning (Midland, MI). FlexiPERM ConA Silicone chamber was purchased from Greiner bio-one (Monroe, NC). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, 0.25% trypsin-EDTA, 1× phosphate buffered saline (PBS), and immunostaining components (mouse anti-vinculin antibody, Alexa Fluor 488 goat anti-mouse IgG secondary antibody, Texas Red-X phalloidin, and DAPI) were purchased from Invitrogen (Carlsbad, CA). Bovine serum albumin (BSA) was purchased from US Biological (Marblehead, MA). Cultrex® PLL was purchased from Trevigen (Gaithersburg,

MD). Fibronectin was purchased from Sigma-Aldrich (St. Louis). MyoD primary antibody (Santa Cruz) was kindly provided by Dr. Bruce D. Uhal at Michigan State University.

PDMS Substrate Preparation

The PDMS substrate was cured in polystyrene tissue culture dish by mixing a 35:1 solution of base and curing agent and pouring the mixture into the dish with a thickness of 1 mm. The mixture was kept under vacuum for 20 min to remove air bubbles, prior to curing overnight at 60 °C. The PDMS surface was further cleaned with a PX-250 plasma cleaning/etching system (March Instruments) for 3 min at 165 mTorr and 65 sccm flow of O₂. A piece of rectangle membrane (5 × 3.5 cm²) was then stretched evenly with 10, 20, and 30% elongation in the longer axis and fixed on top of a plastic slide with a 1 inch diameter hole in the center. This was followed by placing a silicone chamber on top of the membrane. The silicone chamber without a bottom provides a circular well to hold the cell medium and at the same time enable a separate, flat PDMS membrane at the bottom (on which the cells are to be attached) to be pre-stretched. The entire device design is shown in Fig. 2. Before seeding the cells, 1.5 mL PLL was added to the chamber and incubated for 20 min to enhance cell attachment. Alternatively, fibronectin (1 µg/mL) was added to the chamber and incubated for 2 h to enhance cell attachment.

PDMS Surface Characterization

The surface topography was analyzed by scanning electron microscope (JSM-7500F cold field emission SEM, JEOL Corporation) in secondary electron imaging mode. An acceleration voltage of 12 kV was used for all experiments. Images were taken at a magnification of 3000×.

Cell Culture

All procedures for cell isolation were approved by the Institutional Animal Care and Use Committee at Michigan State University.

Bone marrow mesenchymal stem cells (MSCs) were isolated from 6 to 8 week old Sprague–Dawley female rats. In brief, femurs and tibias from a 6 to 8 week old rat were dissected and both ends were cut off. The marrow was flushed using a needle and syringe. The cell suspension was then filtered through a 65 µm nylon mesh to remove bone debris and blood aggregates. Cells were cultured in DMEM with 10% FBS, 100 µg/mL streptomycin and 100 U/mL penicillin added, and placed in the incubator with a humidified atmosphere containing 5% CO₂ at 37 °C. The medium was replaced every 3–4 days until the cells reached 80–90% confluence. Confluent cells were detached using 0.25% trypsin–EDTA and plated at a density of 20,000 cells per mL with 2 mL added to the chamber.

Quantification of Cell Orientation Distribution and Cell Length

Phase contrast images were collected with Leica DM IL inverted microscope (Bannockburn, IL) equipped with SPOT RT color camera (Diagnostics Instruments, MI) using 10× objective. Image-Pro Plus Version 4.5 was used to measure the cell orientation angles. Briefly, a straight line was drawn perpendicular to the stretch direction which was used as the reference to indicate 0° orientation. For each cell, the cell orientation was measured by drawing a straight line through the longest axis of the cell body, and the angle between the cell axis and the reference line was automatically given by the software and recorded for further analysis. All experiments were repeated at least three times for statistical verification. For the stretched vs. unstretched comparison, the cells seeded on the stretched and unstretched membranes were from passages 12 to 16. For each experiment, we took four images in each stretch chamber everyday and quantified the cell orientation angles. Since

the cells proliferated quickly, from 100 cells in four images on the first day to about 800 cells total on the fourth day, we normalized the cell number at each 10 degrees of orientation to the total number of cells on that day. Two-sample Kolmogorov–Smirnov test were applied to evaluate the statistical significance. For the stretch magnitude analysis, we quantified the number of cells oriented at different angles (90° represents parallel to the stretch direction) for each 10° on 10, 20, and 30% pre-stretched surfaces as compared with the unstretched (control) surface. We counted the number of cells in the parallel orientation ($90 \pm 10^\circ$ angle) which was normalized to the total number of cells on each surface. The parallel orientation ratios were reported by the mean value and standard deviation. The cell length was measured using Image-Pro Plus by drawing a straight line through the longest axis of cell body starting from one end to the other end.

Immunocytochemistry

Immunocytochemistry was performed at room temperature on cells seeded on the stretched surface for 6 days. Cells were rinsed with PBS, followed by fixation with 4.0% paraformaldehyde in PBS for 15 min, rinsed 3 times in PBS, then permeabilized with 0.1% Triton X-100 in PBS for 15 min and washed 3 times with PBS. After washing, cells were blocked in 1% BSA for 30 min. After BSA blocking, the cells were incubated with Texas Red-X phalloidin (5 μ L stock per 200 μ L of 1% BSA solution) for 20 min to visualize actin filaments, and then incubated with mouse anti-vinculin primary antibody (1:400 dilution in 1% BSA solution) or rabbit anti-MyoD primary antibody (1:200 dilution in 1% BSA solution) for 1 h followed by three washes in 1 $\times$ PBS, and then incubated with Alexa Fluor 488 goat anti-mouse or anti-rabbit IgG secondary antibody (1:500 dilution in 1% BSA solution) for 1 h. Cells were washed again, three times in 1 $\times$ PBS and then incubated for 5 min in 300 nM DAPI to visualize the nucleus. The PDMS substrates were air dried and the silicone chamber was removed from the membrane, the stained PDMS membrane was finally kept in the dark to cure for 24 h at room temperature. Confocal laser scanning microscopy (CLSM) images were obtained with Olympus FluoView1000 laser scanning confocal microscope using 60 $\times$ oil, 40 $\times$ oil, and 10 $\times$ objectives. Fluorescence intensity analysis was performed using software FluoView1000. Briefly, fluorescence images were taken using confocal microscopy. Each cell on the images was traced and the average cell intensity was measured automatically. The average intensity of each image was then calculated automatically by the software and the average intensity of four images was then calculated using Excel. For each condition, three samples were measured and the standard deviation and *p* value were analyzed.

Statistical Analyses

Data were expressed as mean $\pm$ standard deviation of the mean. ANOVA-Tukey's test was applied to assess for significant difference in Figs. 9 and 10h, Two-sample Student *t*-test was used to determine statistical significance for Fig. 11e. Two-sample Kolmogorov–Smirnov test was applied to evaluate the statistical significance for Figs. 8 and S-1. In all cases, a *p* value of <0.05 was considered statistically significant.

ESTIMATION OF STRETCH-INDUCED ANISOTROPY

Estimation of Pre-stretch-induced Anisotropy of the Substrate in Active Cellular Sensing: Small on Large Theory

The deformation of the substrate can be divided into two parts: large deformation during pre-stretch and small deformation due to cell traction (Fig. 3). A pre-stretch was applied before the cells were cultured on the substrate to induce anisotropy of the substrate prior to active cellular sensing/probing. The finite elastic behavior of the substrate was characterized by performing uniaxial tests of the substrate, and the effective stiffness and degree of

anisotropy during cellular traction were calculated using the theory of small on large,² which allowed us to accurately estimate the structural stiffening due to the pre-stretch (i.e., change in reference length for strain measurement) as well as the material stiffening (i.e., change of the slope in stress–strain curve of Fig. 4). The effective stiffness and degree of anisotropy represent the substrate responses sensed by the cells during active probing (i.e., small deformation).

Characterization of Finite Elastic Behavior of the Substrate

The mechanical properties of the substrate (i.e., PDMS membrane) were measured by the uniaxial tensile test, and the mechanical behavior was described by the incompressible, isotropic neo-Hookean model. Figure 4 shows the engineering stress of the PDMS membrane with respect to the change in the engineering strain obtained from the experimental data (dots) and a theoretical fit (solid line) using the neo-Hookean model.

The deformation gradient of the substrate during uniaxial deformation can be obtained from

$\mathbf{F} = \text{diag} \left[\lambda_1, \frac{1}{\sqrt{\lambda_1}}, \frac{1}{\sqrt{\lambda_1}} \right]$ From classic theory of invariants, the strain energy density of an isotropic material is given by $W = (I_C, II_C, III_C)$, where

$I_C = \text{tr} \mathbf{C}$, $II_C = \frac{1}{2} \left\{ (\text{tr} \mathbf{C})^2 - \text{tr} (\mathbf{C}^2) \right\}$, $III_C = \det \mathbf{C}$ for $\mathbf{C} = \mathbf{F}^T \mathbf{F}$. The constitutive relation

between the stress and strain is then given by $\boldsymbol{\sigma} = -p\mathbf{I} + 2\mathbf{F} \left(\frac{\partial W}{\partial \mathbf{C}} \right) \mathbf{F}^T$, where $\boldsymbol{\sigma}$ is the Cauchy stress tensor and p is a Lagrange multiplier. Our preliminary study showed that the finite elastic behavior of a PDMS substrate could be described well with a neo-Hookean

function, $W = \frac{c}{2} (I_C - 3)$ with a material parameter c (Fig. 4). The substrate was tested with the uniaxial tensile tester, and the material parameter determined by a nonlinear parameter estimation technique. See previous study³⁰ for details on the parameter estimation using a finite elastic constitutive model.

Estimation of Stretch Induced Anisotropic Response of Substrate in Active Sensing

In the theory of linear elasticity, the relationship between stress and strain is linear, and the stress induced by two-step loading is expressed by the sum of each stress (i.e., $\boldsymbol{\sigma} = \mathbf{E}(\boldsymbol{\varepsilon}^0 + \boldsymbol{\varepsilon}) = \boldsymbol{\sigma}^0 + \mathbf{E}\boldsymbol{\varepsilon}$, where $\boldsymbol{\sigma}$, $\boldsymbol{\varepsilon}$, and $\mathbf{E}$ are stress, strain, and fourth-order elasticity tensor, respectively, and the superscript “0” represents the pre-stress values). However, this superposition principle is not valid when the pre-stress $\boldsymbol{\sigma}^0$ is induced by finite deformation, because the reference configuration of the second strain part $\boldsymbol{\varepsilon}$ is significantly different from the stress-free state which is the reference for the first strain part $\boldsymbol{\varepsilon}^0$. In this case, the stress–strain relationship could be derived from the finite elasticity theory (theory of small on large) and the incremental stress during small deformation is written as $\boldsymbol{\sigma} - \boldsymbol{\sigma}^0 = \hat{\mathbf{E}}\boldsymbol{\varepsilon}'$, where $\boldsymbol{\varepsilon}'$ is the strain measured from the pre-stretched state, and the stiffness obtained with respect to the pre-stretched state is, in index notation,^{2,9} $\hat{E}_{ijkl} = \delta_{ik}\delta_{jl}\hat{\sigma}_{ij}^0 + \delta_{jk}\delta_{il}\hat{\sigma}_{ij}^0 + E_{ijkl}$, where E_{ijkl} is the fourth-order tensor evaluated from the strain energy function at $\boldsymbol{\varepsilon}^0$, δ_{ik} is the Kronecker delta, and $\hat{\sigma}^0 = \boldsymbol{\sigma}^0 + p\mathbf{I}$. Since the cells were cultured on the stretched substrate and did not experience the pre-stretch, the strain induced during active cellular probing ($\boldsymbol{\sigma} - \boldsymbol{\sigma}^0$) is estimated by $\boldsymbol{\varepsilon}'$, and the stiffness that the cells sense is represented by the stiffness of pre-stretched substrate, $\hat{\mathbf{E}}$. As explained above and in previous studies,^{2,9} the $\hat{\mathbf{E}}$ depends on not only the material stiffness (i.e., tangent of stress–strain curve) but also on the pre-stress $\boldsymbol{\sigma}^0$ at $\boldsymbol{\varepsilon}^0$. These two components are calculated from the finite deformation of the PDMS membrane and the mechanical properties using the uniaxial test. The stretch-induced anisotropy of the PDMS membrane is then represented by the ratio of effective stiffness in

the stretched direction to its perpendicular direction ($\hat{E}_{1111}/\hat{E}_{2222}$). Figure 5 shows the change in effective stiffness in both directions with respect to the change in strain. When there is no pre-stretch, $\hat{E}_{1111} = \hat{E}_{2222} = 268$ kPa, which equals the elastic modulus of the material (35:1 crosslinked PDMS membrane). While the stiffness in the stretched direction increases, the stiffness in the perpendicular direction decreases as the PDMS membrane experience more strain during uniaxial stretching. At 10, 20, and 30% of strain of the PDMS membrane, the predicted anisotropy ratio ($\hat{E}_{1111}/\hat{E}_{2222}$) is approximately 1.33, 1.73, and 2.20, respectively. MSCs aligned along the stretched direction on a PDMS membrane (see Fig. 7) and were supported by the prediction based on the theory of small on large.

EXPERIMENTAL RESULTS

Surface Topography Characterization

Images of the surface topography were collected by applying the secondary electron imaging mode in SEM on PLL coated substrates, both unstretched (Fig. 6a) and stretched (Fig. 6b). As shown in Fig. 6, due to the dehydrated imaging condition, the PLL peptides aggregated to be large particles sitting on the surface. However both surfaces show clear and smooth background without any orienting direction; thereby confirming the cell alignment (under the medium condition) was not due to contact guidance. Comparing Fig. 6b with Fig. 6a, the stretch did not change the surface topography.

Orientation Quantification

Figure 7 shows a comparison of MSC orientation on stretched and unstretched PDMS substrates. Cells orient parallel to the stretched direction (Fig. 7a), whereas they show random orientation on the unstretched surface (Fig. 7b). The orientation of the cells changes slightly over time as the cells become confluent but the general trend remained. The cell orientation was quantified by determining the percentage of cells that aligned at each 10° angle during a 4 day culture (Fig. 8). As shown in Fig. 8, the pyramid shape of the bar graph is maintained for the stretched substrate. This indicates the cells oriented in the stretch direction and did not change significantly over time, while on the unstretched surface the cells oriented more randomly. Two-sample Kolmogorov–Smirnov test applied to evaluate the statistical significance found the distribution of cell orientations on the unstretched samples for days 1, 2, 3, and 4 were statistically different from their respective stretched samples ($p < 0.05$). The p values are $2.85\text{E-}04$ for day 1, $2.85\text{E-}04$ for day 2, $1.16\text{E-}05$ for day 3, and $6.12\text{E-}05$ for day 4.

To further explore the impact of the magnitude of pre-stretch on cell alignment, we evaluated the cell alignment vs. the magnitude of pre-stretch. We quantified the number of cells oriented at different angles (90° represents parallel to the stretch direction) for each 10° on 10, 20, and 30% pre-stretch surfaces as compared with the unstretched (control) surface. We counted the number of cells in the parallel orientation ($90 \pm 10^\circ$ angle) which was normalized to the total number of cells on each surface. The ratio of cells in parallel orientation was determined as the number of cells that orient in the pre-stretched direction ($90 \pm 10^\circ$ angle) divided by the total number of cells. We found the ratio of cells that oriented in the parallel direction increased significantly when the stretch magnitude increased from 10, 20, and 30% pre-stretched surfaces as compared with the unstretched (control) surface (Fig. 9).

Actin Cytoskeleton Alignment and Cell Morphology Change

To further confirm the cell orientation on the pre-stretched surface, we stained actin filaments and focal adhesion, which illuminated the cytoskeleton organization. As shown in Figs. 10a and 10c, the actin filaments aligned in the pre-stretch direction on the stretched

surface, but aligned randomly on the unstretched surface (Figs. 10b and 10d). Similar results were obtained with the focal adhesion staining (Figs. 10e–10g), where vinculin staining showed clear alignment on the stretched surface. Thus the cytoskeleton also aligned on the pre-stretched surface, which likely resulted in the cell orientation and morphology observed. In addition to alignment, we found the cell morphology changed with the magnitude of pre-stretch. As the magnitude of pre-stretch increased from 0 to 30%, the cell morphology became more elongated and the average cell length increased (Figs. 10e–10g). The cell length quantification was shown in Fig. 10h.

MSC Differentiation Potential on Pre-stretched Surface

We evaluated whether the pre-stretch impacted myoblast differentiation potential of the MSCs. The MSCs cultured on PLL coated 10% pre-stretched PDMS membrane for 4 days stained for MyoD1 (Fig. 11). The MSCs on the pre-stretched surface stained positive for MyoD1 (green) through the entire cell body (Fig. 11a) and an overlay with F-actin staining (red) showed orange (Fig. 11b). In contrast, on the unstretched surface the MSCs did not stain for MyoD1 (Fig. 11c) and an overlay with F-actin showed more red than orange (Fig. 11d). The increased expression of MyoD1 was quantified (Fig. 11e) and showed that the expression level of MyoD1 on the stretched surface is significantly higher than on the unstretched surface, thereby indicating that pre-stretch is able to induce early myoblast differentiation of MSCs.

DISCUSSION

Bio-Mechanical Environment

The mechanical environment is involved in the regulation of tissue structure and function. It is well known that the mechanical microenvironment has a significant impact on cell morphology and behavior. Cells are anchored on the substrate through focal adhesion and the cell morphology is dependent on the cytoskeleton organization. By changing the substrate stiffness,⁵ applying external force⁴ or surface topography,¹³ the cytoskeleton organization can be manipulated, resulting in changes in cell morphology as well as orientation. *In vivo*, there are several types of cells that align directionally, such as cardiomyocytes¹⁶ and blood vessel endothelial cells.⁷ These cells align and are located in an environment that involves mechanical stress and strain. To properly study these cells in an *in vitro* environment that more closely mimics the *in vivo* conditions, i.e., to achieve aligned cells, external mechanical forces are required, such as cyclic stretch on cardiomyocytes or shear stress on blood vessel endothelial cells.^{25,31} However, even in the absence of cyclic external mechanical stimuli or shear stress certain types of cells remain aligned, e.g., alignment of cells (neuron, glial, etc.) in the cerebellar cortex.^{1,24} Therefore, the objective of this study is to elucidate a possible mechanism of cell alignment independent of external mechanical forces, namely, through pre-stretch induced surface anisotropy. We believe that insights gained through this study could inform on tissue engineering applications.

Static vs. Cyclic Stretching

It is well established that cyclic stretching is able to induce cell orientation as well as impact cell proliferation and differentiation.^{11,26,29} Cyclic stretch is a much stronger interference process as compared to our static pre-stretch and the cells' response to cyclic stretch has been shown to depend on the frequency of the cyclic stretch. The cell orientation appeared to be dependent on the magnitude and the frequency of the stretch. In other words, the higher the frequency the faster the cells respond,³ while the lower the frequency the slower the cells respond. When the cyclic stretching frequency is low, e.g., 0.5 Hz, the MSCs do not orient, even after 8 h.¹⁸ Since our pre-stretch is static, it could be extrapolated to a zero frequency (cyclic) stretch, suggesting that the cells would need more time to respond to the

anisotropy induced by the stretch and could explain why we have to wait until 24 h to see significant alignment as oppose to <6 h with cyclic stretch.

Wang *et al.*^{17,27} found that orientation was due to actual reorientation of the cells rather than a selective detachment, and propose the cells subjected to cyclic stretching orient in the direction of minimal substrate deformation. In contrast, our results indicate that cells seeded on a pre-stretched substrate preferentially aligned along the pre-stretched axis, which is in agreement with our “active mechano-sensing” model.²⁰ Nevertheless both studies, Wang *et al.*²⁷ and ours, can be explained by cytoskeleton remodeling even though different mechanical inputs were imposed. When subjected to a dynamic mechanical environment (i.e., cyclic stretching), the cells re-organize their cytoskeletons and attempt to maintain a favorable environment by avoiding the external stimuli of cyclic elongation as well as compression by the substrate. In contrast, on a pre-stretched substrate, the cytoskeleton reorganizes due to the surface anisotropy experienced by the cells. Previously, we showed that cells attempt to maintain a favorable environment by maintaining similar levels of energy consumption through active cellular probing, which manifests as changes in their cell morphology.²⁰ Here, the cells orient in the stiffer direction, changing their morphology, as a result of active cellular probing because the cells can sense the pre-stretch induced surface anisotropy. These results illustrate that the cells can sense the pre-stretch-induced surface anisotropy without having to experience an external stretching.

Pre- vs. Post-stretch

In this study, a static stretch was achieved by pre-stretching the PDMS membrane prior to seeding the cells. In contrast, in almost all the previous studies on the effect of static stretching, the cells were seeded prior to the stretching process. In a recent study static stretching was found not to be as influential as cyclic stretching in inducing MSC orientation.¹⁰ However, our results suggest that static stretch was sufficient to direct significant MSC orientation. We attribute this difference to the different experimental design, as the cells in this experiment did not experience dynamic changes from the external mechanical environment as compared with the previous studies.¹⁰ The major difference between our study and the Goli-Malekabadi's¹⁰ study is that their static stretch started 24 h after seeding of the cells to allow the cells to attach. In other words, they seeded the cells and allowed the cells to attach for 24 h then stretched the cell-attached membrane by 10% and analyzed the results 24 h after the stretch was performed. In contrast, our substrate was pre-stretched prior to seeding the cells. Another more minor difference is that in their study they followed the cell orientation for another 24 h after the stretch, whereas we followed the cell alignment on a pre-stretched surface for up to 4 days. Nevertheless we performed the same experiment as in Goli-Malekabadi *et al.*¹⁰ and found the percentage of cells that align in the stretched direction (using their procedure) was 36%, which was not as high as that on the pre-stretched surface (69%). In their study, the alignment of the cells on the statically stretched surface was not sustained after 24 h. The cells oriented randomly when cultured on an unstretched PDMS surface for 24 h (Fig. S-1a, cell orientations are quantified in Fig. S-1d). The cells appeared to align on the PDMS substrate 24 h after a 10% static stretch of the cell-attached PDMS substrate (Fig. S-1b, cell orientations are quantified in Fig. S-1e). The alignment disappeared after 48 h on the 10% static stretch PDMS substrate (Fig. S-1c, cell orientations are quantified in Fig. S-1f). However applying a two-sample Kolmogorov–Smirnov test on the orientation distribution from days 1 to 3 suggest the three orientation distributions are not statistically different from each other. This is in accordance with the results of the static stretch reported in their paper.¹⁰ The *p* values from our statistical analysis are 0.0982 for day 1 vs. day 2, 0.4255 for day 1 vs. day 3, and 0.2182 for day 2 vs. day 3.

The exact mechanism for the difference is currently unknown. We hypothesize that stretching after cell attachment creates a disturbance to the cell environment, but the previous “memory” or “condition” of the cells in response to the substrate impacts their subsequent response upon stretching of the substrate, similar to pre-conditioning,²¹ to diminish the cells alignment. In other words, in the post-stretch case the cells are pre-conditioned to an isotropic surface for 24 h and then a disturbance occurs such that the randomly aligned cells experience an “anisotropic” surface but the pre-conditioning or “memory” causes the cells to show a reduced response to the anisotropy. However in the pre-stretched condition, the cells are not experiencing a disturbance or change in the environment. Instead one could liken it to cells being preconditioned to an “anisotropic” surface for the first 24 h, and then this environment continues, such that the cells experience a “memory” of the anisotropy and when the anisotropy persists, the cells continue to align.

Mechanical Force vs. Contact Guidance

Researchers have long observed significant effect of surface anisotropy on cell behavior, where the anisotropy is generated by changing the surface topography, which results in changes in both the mechanical properties and topography. In those previous studies, the anisotropic cellular behavior was due to the “contact guidance.”^{22,28} However, it might falsely indicate that anisotropy equals contact guidance. In this study, we used a pre-stretched surface and successfully induced surface anisotropy without modifying the surface topography.

Even without topography, not all anisotropic surfaces are due to effective stiffness. In an earlier experiment, Haston *et al.*¹² stretched collagen matrix to induce fibroblast orientation. Although their experiment applied a static pre-stretched membrane they found the cells aligned as a result of the stretch-induced oriented collagen fibers. They suggested that contact with the aligned collagen fibers induced cytoskeleton reorganization and the cell alignment. However, in this study a thin layer of PLL coating was added to promote cell adhesion on the pre-stretched membrane, the surface characterization shows that this protein layer was evenly distributed so it is not likely affecting the cell alignment. Taken together, in this study we successfully created a purely mechanical force induced anisotropic surface without the influence of contact guidance.

PLL vs. Fibronectin Coating

PLL is a commonly applied coating that can enhance cell adhesion. There are a number of studies that show cells align on PLL coated patterned surfaces, such as James *et al.*¹⁵ and Dowell *et al.*⁸ It is possible that the nonspecific binding of PLL could block potential chemical crosstalk and signaling between the substrate and the cell. Therefore to ensure our results are not due to nonspecific effects of PLL, we repeated the experiment with fibronectin and found the cells also aligned, as was observed with PLL, as shown in the Fig. S-2. Figure S-2a shows MSCs seeded on fibronectin coated pre-stretched surface and Fig. S-2b shows MSCs seeded on fibronectin coated unstretched surface.

Application of Mechanical Stretch in Tissue Engineering

Mechanical stretch has been applied in tissue engineering to guide cell behavior. It is especially important for inducing cell alignment in connective tissues such as tendons and ligaments, cardiac muscle tissues, and blood vessel endothelium.^{16,23,25,31} Along these lines, we created, alternatively, a static pre-stretched anisotropic surface, in the absence of dynamic external mechanical stimuli that also induced cell alignment. The results of this study could have potential implications to neural cell alignment during development where dynamic mechanical stimuli are not likely playing a role.^{1,24}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIGURE 1.

Schematic illustrating stretch induced anisotropy. The cells experience a stiffer surface in the direction of stretch and this stretch-induced anisotropic cellular response on an isotropic substrate can be explained with a simple two-step deformation model (see text for details).

FIGURE 2.
Design of static pre-stretching device.

FIGURE 3.

Schematic drawing of the two-step deformation of the substrate in the experiment. For the test, the substrate is stretched in one direction to adjust the effective stiffness and anisotropy prior to culturing the cells. The relationship between the force applied by the cells and deformation of the substrate is then calculated by using the theory of “small on large”.

FIGURE 4. Stress-strain plot. Plot of the uniaxial test data (dots) of a 35:1 PDMS substrate and the theoretical fit (solid line) using an incompressible neo-Hookean model.

FIGURE 5.

Effective stiffness. An effective stiffness plot in the stretched direction and its perpendicular direction with respect to the change of strain ($\hat{E}_{1111}/\hat{E}_{2222} = 1:33$ at 10% strain).

FIGURE 6.

Surface topological characterization. SEM images of the 3D topography of PLL coated (a) unstretched PLL coated 35:1 PDMS membrane, (b) 10% pre-stretched PLL coated 35:1 PDMS membrane, (c) unstretched 35:1 PDMS membrane without PLL coating, and (d) 10% pre-stretched 35:1 PDMS membrane without PLL coating. The images were taken at a magnification of 3000 $\times$.

FIGURE 7. MSCs on stretched vs. unstretched PDMS substrate. Phase contrast (10×) images of MSCs on (a) 10% pre-stretched and (b) unstretched PLL coated 35:1 PDMS membranes after 4 days of culture.

FIGURE 8.

Quantification of the orientation angles on stretched vs. unstretched membrane. The percentage of cells that orient at every 10°. The 90° angle represents the direction of pre-stretch. Cell orientation angles were quantified by ImagePro Plus for up to 4 days of culture on stretched (left) and unstretched (right) 35:1 PDMS membranes. Two-sample Kolmogorov–Smirnov test were applied to evaluate the statistical significance. The results showed that the unstretched samples for days 1, 2, 3, and 4 have statistically different distributions ($p < 0.05$) as compared to their respective stretched samples. The p values are 2.85E–04, 2.85E–04, 1.16E–05, and 6.12E–05 for days 1–4, respectively.

FIGURE 9.

Ratios of parallel orientation vs. magnitude of pre-stretch. Cell orientation angles on the pre-stretched substrate was quantified after 4 days of culture on PLL coated 35:1 PDMS membrane at 0, 10, 20, or 30% pre-stretch. The ratio of cells in parallel orientation was determined as the number of cells that orient in the pre-stretched direction ($90 \pm 10^\circ$ angle) divided by the total number of cells. ANOVA-Tukey's test indicates the unstretched surface is significantly different from the stretched surfaces and as the % pre-stretch increases, the alignment increases at $p < 0.0000001$.

FIGURE 10.

Actin filaments and focal adhesion staining of MSCs on stretched vs. unstretched surfaces. Fluorescence confocal microscopy images were taken of MSCs after 5 days of culture on 10% pre-stretched (a, c) and unstretched (b, d) 35:1 PDMS membranes stained with Texas Red-X phalloidin for actin. Images were taken at 10× (a, b) and 40× (oil) (c, d). F-actin filaments (red) and vinculin (green) staining of MSCs after 5 days of culture on (e) 0%, (f) 10%, and (g) 30% pre-stretched 35:1 PDMS membrane with confocal microscopy (60× oil). The average lengths of the cells on 0, 10, and 30% pre-stretched surfaces were quantified in (h). ANOVA-Tukey's test indicates the unstretched surface is significantly different from the stretched surfaces at $p < 0.0001$.

FIGURE 11.

MyoD1 staining of MSCs on stretched vs. unstretched surfaces. Fluorescence images of MSCs after 4-day culture on 10% pre-stretched (a, b) and unstretched (c, d) 35:1 PDMS surface. (a) MyoD1 staining of MSCs on 10% pre-stretched PDMS substrate and (c) unstretched substrate. An overlay of MyoD1 (green), F-actin (red), and nuclei (blue) staining on (b) PLL coated 10% stretched 35:1 PDMS substrate and (d) PLL coated unstretched 35:1 PDMS substrate. Images were generated using confocal microscopy at magnification of 60 $\times$ (oil). (e) Quantification of fluorescence intensity of MyoD1 staining on the (a) stretched and (c) unstretched surfaces, * p value = 0.0012.