Altered phenotypic gene expression of 10T1/2 mesenchymal cells in nonuniformly stretched PEGDA hydrogels

W. J. Richardson,¹ E. Wilson,² and J. E. Moore, Jr.¹
¹Department of Biomedical Engineering, Texas A&M University, College Station, Texas; and ²Department of Systems Biology and Translational Medicine, Texas A&M Health Science Center, College Station, Texas

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Richardson WJ, Wilson E, Moore JE Jr. Altered phenotypic gene expression of 10T1/2 mesenchymal cells in nonuniformly stretched PEGDA hydrogels. Am J Physiol Cell Physiol 305: C100–C110, 2013. First published May 8, 2013; doi:10.1152/ajpcell.00340.2012.—Disease-related phenotype modulation of many cell types has been shown to be closely related to mechanical loading conditions; for example, vascular smooth muscle cell (SMC) phenotype shift from a mature, contractile state to a proliferative, synthetic state contributes to the formation of neointimal tissue during atherosclerosis and restenosis development and is related to SMC mechanical loading in vivo. The majority of past in vitro cell-stretching experiments have employed simplistic (uniform, uniaxial or biaxial) stretching environments to elucidate mechanobiological pathways involved in phenotypic shifts. However, the in vivo mechanics of the vascular wall consists of highly nonuniform stretch. Here we subjected 10T1/2 murine mesenchymal cells (an SMC precursor) to two- and three-dimensional nonuniform stretch environments. After 24 h of stretch, cells on an elastomeric membrane demonstrated varied proliferation [assessed by 5-bromo-2'-deoxyuridine (BrdU) incorporation] depending on location upon the membrane, with maximal proliferation occurring in a region of high, uniaxial stretch. Cells subjected to a nonuniform stretching regimen within three-dimensional polyethylene glycol diacrylate (PEGDA) hydrogel constructs demonstrated marked changes in mRNA expression of several phenotype-related proteins, indicating a sort of “hybrid” phenotype with contractile and synthetic markers being both upregulated and downregulated. Furthermore, expression levels of mRNAs were significantly different between various locations within the stretched gel. With the proliferation results, these data exhibit the capability of nonuniform stretching devices to induce heterogeneous cell responses, potentially indicative of spatial distributions of disease-related behaviors in vivo.

stiffness; smooth muscle phenotype; mechanobiology; atherosclerosis

It is widely known that mechanical loads can alter many cellular behaviors such as migration, orientation, proliferation, protein synthesis, phenotype modulation, and others. Knowing how mechanical signals are transduced into cell responses is therefore vital to our overall understanding of tissue and organ function in both health and disease. For instance, numerous studies have demonstrated the role of biomechanics in atherosclerosis initiation, progression, and treatment success. Spatial distributions of both blood flow patterns and artery wall stress have been linked to early intimal thickening (12, 23), and biomechanical changes following stent implantation are thought to be involved in restenosis (29, 30).

Linking these loading conditions to particular in vivo cellular responses is made difficult by the complexity of physiological mechanical environments, which can be nonuniform, anisotropic, and constantly remodeling. For example, in bypass grafts, concentrated zones of high stress are created at suture lines (3). In stented arteries, intimal circumferential stresses are elevated well beyond physiological norms, exacerbating transmural stress gradients (4, 24). Also, in the carotid sinus locations prone to atherogenesis exhibit a high ratio of inner wall to outer wall circumferential stress (12). In all of these pathological developments, smooth muscle cell (SMC) proliferation and phenotype transformation are key events contributing to intimal hyperplasia (39, 40). Additionally, in all of these pathologies the mechanical environment is highly nonuniform, with greater levels of stress at the inner edge of the wall compared with the outer edge. Thus there exists a need to investigate SMC phenotype modulation in the presence of nonuniform stretch environments.

SMCs in the normal adult vasculature undergo proliferation at a very slow rate and exhibit very low synthetic activity; however, they maintain a plasticity that allows them to respond to local environmental stimuli and undergo profound changes in phenotype, characterized by increased proliferation and synthesis and decreased expression of contractile proteins (32, 33, 52). While there is a short-term advantage to this response to injury, prolonged phenotypic changes in SMC phenotype lead to the pathogenic developments mentioned above. The specific molecular mechanisms regulating these responses remain unclear.

The literature contains reports of numerous devices that have been used to subject SMCs to cyclic stretch in order to reveal effects of stretch on SMC phenotype. These devices vary greatly in the types of mechanical stimuli employed, most of which do not invoke mechanical conditions representative of the in vivo environment. Accordingly, there is huge variety in the findings of these studies. For example, investigations subjecting SMCs to uniaxial stretch have reported phenotype shifts toward both the contractile and synthetic states as evidenced by higher levels of contractile apparatus (22) or increased synthesis of collagen (26), respectively.

Mixed phenotypes are also found in cells under biaxial stretch. A variety of studies have employed commercially available (e.g., FlexCell) or custom cell stretching devices to biaxially stimulate SMCs and have found increased expression of caldesmon (5), PKCδ translocation to the cytoskeleton (27), and matrix compaction (45), exhibiting contractile behavior, while similar studies have also found lower levels of actin and calponin (8, 14), increased production of early growth response factor 1 (31), and increased proliferation (5, 45), indicating a synthetic phenotype. This disparity in the literature is likely...
due to the drastic differences in mechanical loading parameters—stretching amplitude varied from 5% to 25%, duration from 10 min to 4 wk, and frequency from 0.5 to 2 Hz—and little clarity is assimilated from the findings (1, 2, 5, 10, 22, 27, 31, 55). While each of these studies has partly contributed to our knowledge of SMC phenotype modulation, the simplistic loading regimens do not adequately represent the in vivo mechanical environment. Therefore, our ability to predict cell phenotype transitions within a healthy or diseased artery remains limited.

To investigate SMC behavior in nonuniform stretch environments representative of in vivo conditions, we have previously designed an experimental stretching device capable of subjecting cells in two (2D)- and three (3D)-dimensional cultures to gradients in biaxial stretch (38). A proper investigation of SMC phenotype change in response to mechanical stimuli should come as close as possible to the fully 3D in vivo case. Here, we initiate a progression toward this idealized situation by subjecting cell-seeded polyethylene glycol (PEG) diacrylate (PEGDA) hydrogels to gradients in stretch. To investigate the effects on cell phenotype modulation, quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was employed to measure changes in mRNA expression related to contractile and synthetic phenotypes. Proliferation was also assessed with 5-bromo-2'-deoxyuridine (BrdU) immunostaining.

Methods

Cell culture and PEGDA hydrogel construction. 10T1/2 cells (ATCC CCL-226), a murine mesenchymal cell line, were purchased and maintained in a smooth muscle growth medium (SmGM) with 5% fetal bovine serum and antibiotics (SmGM kit, Lonza, Basel, Switzerland), incubated at 37°C and 5% CO₂, and passaged just prior to dishes reaching confluence. A smooth muscle precursor cell type, the 10T1/2 line is commonly used for in vitro SMC phenotype studies because of its ability to maintain a stable phenotype in culture and be pushed toward an SMC state with SmGM, as done here (20). For experiments, 10T1/2 cells at passages 8–12 were used.

PEGDA was synthesized by methodology adapted by Hahn and colleagues (16). Briefly, 10-kDa PEG (Sigma-Aldrich, St. Louis, MO) was acrylated by the addition of acryloyl chloride to a solution of PEG and triethylamine in anhydrous dichloromethane. The reaction was stirred for 24 h, subsequently washed with potassium bicarbonate, and dried with anhydrous sodium sulfate, at which point the product was precipitated in cold diethyl ether, dried under vacuum, and stored at −20°C.

For hydrogel manufacturing, PEGDA was allowed to warm to room temperature and then dissolved in sterile cell culture medium (SmGM, Lonza) to yield a 20% PEGDA solution. To allow cell adhesion to the polymer network, acrylated RGD peptide groups (Peptron, Daejeon, South Korea) were dissolved in sterile ultrapure water and added to the solution at a concentration of 1 mM. 10T1/2 cells were collected from culture dishes via trypsinization (Invitrogen, Grand Island, NY), centrifuged, and then resuspended into the medium-PEGDA-RGD solution at a density of 2 × 10⁶ cells/ml. Irgacure 2959 (Sigma-Aldrich), a photoinitiator curing agent, was dissolved in 70% ethanol and added to the bulk at 0.1% wt of total solution. The final solution was pipetted into a disk-shaped Lexan mold with a central post in order to produce gels with the following dimensions: Rᵣ = 35 mm, Rₗ = 6 mm, and thickness = 2 mm. Another disk mold was used to produce gels for unstretched control samples (R = 9 mm, thickness = 1 mm; smaller in order to conserve construct material). Molds were covered by thin glass sheets, and the gels were cured by long-wave UV exposure for 6 min (Ultraviolet Products High Performance UV Transilluminator, 365 nm; Upland, CA). After removal from the molds, gels were moved to petri dishes, submerged in culture medium, and incubated for 48 h prior to stretching experiments. This period allowed ample time for free swelling of gels and adequate cell adhesion to the RGD groups incorporated in the polymer network.

For elastomeric membrane experiments, circular membranes with central defects (Rᵣ = 7.5 mm, Rₗ = 50 mm) were cut from 0.5-mm-thick silicon sheeting (Specialty Manufacturing, Saginaw, MI) and sterilized by autoclave. Membranes were then coated for 1.5 h with bovine fibronectin (Sigma-Aldrich) diluted in phosphate-buffered saline (PBS) in order to yield a surface area protein concentration of 5 μg/cm². After coating, membranes were washed with successive changes of PBS and culture medium before finally being submerged with medium containing 10T1/2 cells. This process yielded adequate cell adhesion with a seeding density of 10⁴ cells/cm². Like the hydrogels, cell-seeded membranes were also incubated for 48 h prior to stretching.

Stretching device design and characterization. The cell-stretching device employed here is based on circular membrane deformation analysis and has been previously described in detail (11, 38). Briefly, a computer-controlled stepper motor drives a rack-and-pinion mechanism that radially deforms circular constructs by vertically displacing the clamped outer circumference past a stationary circular platen (Teflon). The mechanism is housed within a culture chamber (Lexan), allowing the construct to remain submerged in medium during stretching.

The loading action generates uniform radial displacement of the outer circumference of the construct, resulting in unequal and nonuniform radial and circumferential stretch components. Stretching membranes or gels containing a circular central defect results in gradients in these stretch components, with low radial stretch (λᵣ) at the outer edge increasing to higher λᵣ near the outer edge and, conversely, high circumferential stretch (λₗ) at the inner edge decreasing to lower λₗ at the outer edge. This nonuniform stretch field yields a uniaxial environment at the inner region and a near-equibiaxial environment at the outer region.

Elastomeric membrane deformations on this device have previously been characterized (38). PEGDA hydrogel deformation was characterized by tracking fiduciary markers before and after various levels of stretch. Radial positions were measured for markers arranged in eight lines extending radially from the gel’s inner edge. Stretch ratios were then calculated based on the changes in the radial positions of these markers: circumferential stretch = r/R and radial stretch = ∆r/AR, where r is deformed radial position and R is original radial position. Assuming incompressibility, the out-of-plane thinning of gels was calculated as 1/(λᵣ × λₗ). Markers with similar original positions were grouped, and stretch components were averaged within each group. Deformation theory was then solved to fit the Mooney-Rivlin strain energy constitutive equation to the experimentally measured stretch data. Fitting this constitutive model allowed for the theoretical estimation of hydrogel deformation due to any prescribed loading, so that selecting experimental regimens would not be restricted to the limited levels of loading at which measurements were taken.

Stretching experiments. After 48 h of incubation, cell-seeded hydrogels and membranes were mounted on the above-described stretching device. Rings of sandpaper (180 grit) were placed between the gels and outer circumference clamps to ensure adequate gripping. On the basis of the hydrogel constitutive modeling results, motor gear
displacement was selected to achieve ~10% λ_R at the inner edge of the hydrogels. Cell-seeded membrane stretches were preselected with λ_R varying from λ_R^inner = 1.11 to λ_R^outer = 1.03 and λ_R varying from λ_R^inner = 0.95 to λ_R^outer = 1.02 (Fig. 1). Both constructs were cyclically stretched for 24 h at 1 Hz. During this duration, the stretching device chamber was filled with culture medium and enclosed within an incubator at 37°C and 5% CO₂.

Real-time RT-PCR analysis of mRNA expression. To assess changes in phenotype-related gene expression due to stretch, real-time RT-PCR was employed. After 24 h of stretching, disk-shaped samples were taken from two regions of the gel: an inner ring (7.5 mm < R < 11.5 mm) and an outer ring (28.6 mm < R < 30 mm) encompassing the locations of extreme mechanical conditions. Samples were also taken from a nonstretched control gel. For nucleic acid collection, samples were submerged in TRIzol reagent (Invitrogen) and homogenized with a power tissue homogenizer (POLYTRON PT2100, Melville, NY). The PureLink RNA Mini Kit was then used according to manufacturer’s instructions to isolate mRNA, which was reverse transcribed into cDNA with the SuperScript First-Strand Synthesis System (Invitrogen).

To measure relative amounts of gene expression for various phenotype-related proteins, quantitative real-time RT-PCR was carried out with 2× SYBR Green dye (Applied Biosystems, Grand Island, NY). Primers used for genes associated with the contractile and synthetic/proliferative phenotypes were previously generated with Primer Express Software (Applied Biosystems) and have been published (38). Expression levels were quantified with the ΔΔCT method, with GAPDH as a reference gene for normalization: ΔΔCT = (C_{Tstretch} - C_{Tnonstretch})_{gene} - (C_{Tstretch} - C_{Tnonstretch})_{GAPDH}. CT represents the PCR cycle number at which the gene’s fluorescent signal crosses a threshold value. The mRNA fold expression reported for each gene equals 2^-ΔΔCT and represents the amount of that gene’s mRNA collected within a sample relative to a nonstretched control sample, both normalized by GAPDH mRNA levels.

Cell imaging. A limitation of PEGDA hydrogels is that cells are not able to alter morphology because of encapsulation within the small polymer mesh size. However, their intracellular structure could potentially be altered in response to mechanical loading. To examine overall morphology as well as intracellular changes in cytoskeletal organization, actin filaments were labeled and cells were imaged subsequent to stretching experiments. After 24 h of cyclic stretching, a small wedge encompassing all radial positions was cut from the gel and washed with several changes of PBS. Cells were then fixed by submerging the gel in a 4% paraformaldehyde solution for 30 min, washed three times with PBS for 5 min each, and permeabilized for 15 min with Triton X (Sigma-Aldrich) diluted in water to a concentration of 0.5%. After permeabilization, the gel was washed again (PBS, 3 × 5 min) and then submerged in Alexa Fluor 488 phalloidin dye (Invitrogen) for 8 h. The durations of these steps were longer than the prescribed protocol, extended to allow sufficient time for chemicals to diffuse through the hydrogel. After staining, gels were briefly washed and imaged at ×10 and ×60 magnifications with a Nikon TE-2000 inverted fluorescent microscope equipped with confocal capability and a Nikon C-FL FITC by HYQ filter set (Nikon Instruments, Melville, NY).

Proliferation assessment. Cellular proliferation was quantified with the BrdU incorporation technique, a commonly used method for proliferation studies. A uridine group, BrdU can function as a thymidine substitute and is thereby incorporated into DNA during the synthesis phase of the cell cycle. By subsequent immunostaining, a BrdU-positive reading preferentially indicates those cells that have undergone division while cultured in the presence of the molecule. The proliferation assay was conducted on cell monolayers stretched on the membrane construct rather than the hydrogel setup to ensure uniform uptake of BrdU (i.e., not limited by diffusion through the gel thickness).

BrdU (Invitrogen) was diluted 1:100 in culture medium and used to replace plain culture medium in the stretching chamber after the membrane had been stretched for 16 h. After an additional 8 h of stretching (total of 24 h stretching), membranes were removed from the device. Cells were then washed with PBS several times and fixed in 70% EtOH at 4°C for 30 min. After fixation, cells were washed with PBS three times for 2 min each, and then a BrdU immunostaining protocol (biotinylated monoclonal anti-BrdU antibody technique, Invitrogen) was carried out according to the manufacturer’s instructions. After staining, cells that incorporated BrdU (i.e., proliferative cells) displayed darkened nuclei compared with those that did not (i.e., nonproliferative cells). Cells were then imaged with bright-field microscopy at ×10 magnification with the microscope listed above. Between 6 and 10 images were taken within three different radial regions upon the membrane: uniaxial stretch from 7.5 mm < R < 15 mm, transition biaxial stretch from 15 mm < R < 22.5 mm, and near-equibaxial stretch from 22.5 mm < R < 30 mm. Cells from a nonstretched control membrane were also imaged at the same positions. Within all images, proliferation was quantified as the ratio of the number of darkened cells to nondarkened cells.

Statistical analysis. For proliferation experiments, cell images were divided into six groups: three radial regions from both stretched and nonstretched samples. The nonstretched control proliferation ratios were averaged for each of the three nonstretched groups, and proliferation ratios for all individual images were then normalized by the average control ratio for the corresponding radial region. The normalized proliferation ratios for all three stretched groups and unstretched controls were averaged, and their distributions were compared with one-way ANOVA followed by post hoc Tukey-Kramer multiple-comparison test. Significance level was set at P = 0.05, and data are reported as means ± SE.

For RT-PCR data, four stretching experiments were conducted along with four nonstretched control gels. For each experiment, 3 or 4 real-time runs were performed on the collected mRNA samples, resulting in 13 total measurements for each gene’s expression within each of the 3 experiment regions: no stretch, uniaxial stretch, or biaxial stretch. Means and SEs of ΔΔCT data were calculated for each, and the expression levels are reported as 2^{-ΔΔCT} with intervals [2^{-ΔΔCT - SE}, 2^{-ΔΔCT + SE}]. For statistical comparisons, one-way ANOVA followed by post hoc Tukey-Kramer multiple-comparison tests was performed, with significance level set at P = 0.05.
RESULTS

Hydrogel deformation characterization. Radially deforming circular PEGDA hydrogels with a central defect generates nonuniform distributions of both circumferential and radial stretch components (Fig. 2). A Mooney-Rivlin strain energy function fit the experimental data appropriately for both these stretch components as well as the out-of-plane thinning stretch. As predicted by finite deformation theory, circumferential stretch is maximal at the inner edge and monotonically decreases toward the outer edge, while radial stretch is minimal at the inner edge and monotonically increases toward the outer edge. This produces a high-magnitude uniaxial stretching environment in the inner region that transitions to a lower-magnitude near-equi-biaxial environment toward the outer edge. Furthermore, by increasing the vertical displacement of the clamped outer circumference of the gel, the degree of nonuniformity (i.e., steepness of the gradients) is increased. For each case, the out-of-plane thinning remained relatively uniform across radial direction. On the basis of the stretching characterization, a moderate stretching regimen was selected for experimental cases (Fig. 3). The stretch distribution used for experiments subjected the hydrogels to $\lambda_{\text{inner}} = 1.10$, $\lambda_{\text{outer}} = 1.04$, $\lambda_{r, \text{inner}} = 0.98$, and $\lambda_{r, \text{outer}} = 1.037$. The out-of-plane thinning remained uniform at 0.93.

mRNA expression. Quantitative real-time RT-PCR yielded expression values of phenotype-related genes for cells taken from inner and outer regions of hydrogels, corresponding to uniaxial stretch and near-equi-biaxial stretch environments, respectively (henceforth referred to as uniaxial and biaxial, respectively). ANOVA with post hoc comparisons identified significant differences in mRNA expression (relative to GAPDH reference gene) between stretched gels and unstretched gels and in some instances between uniaxial and biaxial stretch regions (Fig. 4). However, of the 17 mRNA levels analyzed the majority did not show statistically significant differences, although some tendencies were evident (Table 1).

In the case of cFos, cells from the uniaxial stretch region showed significantly elevated expression of 1.82 (1.66, 2.00) fold compared with both nonstretched expression of 1 (0.84, 1.19) fold and biaxial stretch region expression of 1.05 (0.95, 1.16) fold (Fig. 4A). Similarly, expression of caldesmon was significantly highest in the uniaxial region: 9.73 (5.64, 16.79) fold compared with 1 (0.56, 1.77) fold for nonstretched and 0.60 (0.40, 0.88) fold for biaxial (Fig. 4B). Expression of Smad5 was also significantly elevated in the uniaxial region [3.84 (2.66, 5.55) fold] compared with nonstretched [1 (0.85, 1.18) fold] (Fig. 4C). The expression of Smad5 in the biaxial region was elevated at 2.05 (1.54, 2.72) fold, though not statistically different from the nonstretched expression.

Fig. 2. Hydrogel stretching characterization. A circular polyethylene glycol diacrylate (PEGDA) hydrogel with a central defect was radially deformed by displacement over a stationary platen, generating nonuniform distributions within the 3 principal stretch components. Experimental data (shown as means ± SE) were fit with a Mooney-Rivlin strain energy constitutive function.

Fig. 3. Stretch distributions for hydrogel experiments. PEGDA hydrogels with central defects were radially deformed. The resulting circumferential and radial stretch components were highly nonuniform across radial position, while out-of-plane thinning remained relatively uniform.
Several mRNAs also showed significant downregulation compared with nonstretched controls. Early growth response factor 1 showed a significant decrease in expression in both uniaxial [0.22 (0.19, 0.26) fold] and biaxial [0.18 (0.16, 0.21) fold] regions compared with nonstretched regions [1 (0.60, 1.68) fold] (Fig. 4D). Levels of Id2 were also decreased in uniaxial [0.54 (0.41, 0.72) fold] and biaxial [0.44 (0.39, 0.5) fold] compared with nonstretched [1 (0.92, 1.08) fold] regions, although only the biaxial region decrease was statistically significant (Fig. 4E). In similar fashion, PAI-1 levels were decreased for both uniaxial [0.15 (0.06, 0.36) fold] and biaxial [0.05 (0.02, 0.1) fold] regions, but again only the biaxial region was significantly different from the nonstretched region [1 (0.73, 1.37) fold] (Fig. 4F).

Actin cytoskeleton. After staining with Alexa Fluor 488 phalloidin, actin filaments within 10T1/2 cells were clearly visible in samples from nonstretched gels and both uniaxial and biaxial regions of stretched gels (Fig. 5). Low magnification revealed low cell distribution, most often in singles or small groupings (2–4 cells), and relatively uniform throughout gel regions. Cells also demonstrated a rounded, spherical morphology with no spreading or indications of filopodial extension. Higher magnification was able to capture cytoskeletal organization. In planar cross-section views, filaments appear punctate and not well connected. Taking a vertical stack of images, short fiber segments appear to run in the out-of-plane direction, i.e., through the gel thickness, although still not well developed or well connected. In fact, it is possible that these apparent fibers
are image artifacts due to the z-resolution limit of the confocal microscopy setup. In any case, there appear to be no significant differences of actin organization between cells from nonstretched and stretched gel regions.

**Cellular proliferation.** After cyclic stretching for 24 h, BrdU incorporation and staining demonstrated significantly different proliferation rates depending on cell location upon an elastomeric membrane, with a graded increase in the ratio of BrdU-positive cells to BrdU-negative cells from near equibiaxial, to transition biaxial, to high uniaxial regions of the membrane (Fig. 6). Relative to a nonstretched control, cells subjected to uniaxial stretch exhibited elevated proliferation of 2.65 ± 0.34 fold. Interestingly, cells subjected to near-equibiaxial stretch exhibited a decrease in proliferation (0.49 ± 0.05 fold) compared with nonstretched control. The proliferation ratio within the transition biaxial region (0.83 ± 0.07 fold) measured higher than that of the biaxial region and lower than that of the uniaxial region but was not statistically different from the nonstretched control or the biaxial region.

**DISCUSSION**

Numerous reviews have highlighted the seeming disparity among stretch-dependent SMC phenotype studies, revealing a lack of complete understanding of the intricacies involved in this process (17, 18, 44, 52). It appears that many stretch conditions induce cells to take on a “hybrid” phenotype expressing both contractile and synthetic markers (52). In the present study, 10T1/2 cells were subjected to a complex, nonuniform stretching environment with an inner region of high-magnitude, uniaxial stretch and an outer region of lower-magnitude, near-equibiaxial stretch. Measured proliferation rates and mRNA expression values further demonstrate the complexity of phenotype modulation response, with these parameters varying in cells taken from different positions on elastomeric membranes or PEGDA hydrogels.

We have previously measured mRNA expression of these same genes from 2D monolayers of 10T1/2 cells stretched on elastomeric membranes with the same device used here (38). Interestingly, when stretched in 2D caldesmon and Id2 mRNA levels were decreased while egr1, cFos, and pai1 mRNA levels were increased (SMAD5 mRNA levels were insignificantly changed). The disparity between the 2D and 3D cases can potentially be attributable to several factors. Most simply, cells of most types are known to exhibit different adhesion behaviors and morphologies (e.g., spread and rounded vs. stellate) when cultured on any 2D vs. 3D environment (35). These fundamental cell behaviors can drastically impact how stretch stimuli are transduced and subsequently affect many different biological responses such as gene expression. In addition, it should be noted that 10T1/2 monolayers were near-confluent when mRNA was collected, whereas cells within PEGDA gels were sparse and isolated (as shown above). Cell-cell contacts have been suspected to play a significant role in several phenotype-related behaviors (52). Furthermore, cells within PEGDA hydrogels are tightly encapsulated within the polymer matrix. This encapsulation potentially interferes with actin cytoskeletal remodeling and thereby potentially interferes with intracellular force transduction into phenotype-related expression changes. It is important to keep all of these factors in mind when comparing expression data from 2D and 3D cases, and therefore great care should be taken to isolate and verify all such factors for thorough understanding of specific genes and proteins. These discrepancies also highlight the motivation here to design in vitro studies to recreate 3D geometry as closely as possible since it is clear that differences in dimensionality can produce major differences in cell mechanobiology, complicating the application of 2D experimental results to in vivo conditions.

Here, real-time RT-PCR-quantified phenotype-related mRNA expression levels were varied between the uniaxial and biaxial stretch environments within a PEGDA hydrogel. Although most of the genes analyzed yielded insignificant results, several mRNAs were significantly upregulated or downregulated. cFos expression was significantly elevated in the uniaxial region but not significantly changed in the biaxial region. An intermediate-early gene, cFos is known to mediate cell growth and has been shown to increase expression in as little as 0.5–1 h in rat mesenchymal cells and SMCs exposed to equibiaxial stretch (41, 43). However, in one study, this heightened expression reduced back to normal levels after ~2 h (41).

Similarly, the mRNA coding for caldesmon was significantly upregulated within the uniaxial region but insignificantly changed in the biaxial region. Caldesmon is a calmodulin- and actin-binding protein that is involved in the regulation of acto-miosin contraction within SMCs, and thereby associated with their more contractile phenotype (42). Birukov et al. (5) also reported increased expression of caldesmon by rabbit aortic SMCs subjected to stretch, although their study subjected cells to 8 days of 15% biaxial stretch. Smad5, a receptor-regulated SMAD involved in TGF-β signaling and cell prolif-
eration, showed slightly (but nonsignificant) elevated expression in biaxial stretching region and significantly increased expression in the uniaxial stretching region. To our knowledge, no other study has examined Smad5 mRNA expression in response to stretch, although another member of the Smad family (Smad2) was shown to be increasingly phosphorylated when human umbilical cord perivascular cells were stretched equibiaxially in the presence of an adipogenic medium (50).

Early growth response factor 1 (egr-1) is a DNA binding cofactor involved in transcription regulation and related to cell proliferation (54). Several studies have shown that egr-1 levels increase with stretch but with a very quick response of 1–4 h, after which levels begin to decrease (31, 54). Interestingly, our measured egr-1 levels showed reduced expression in both stretch regions compared with the nonstretch control. It is possible that the stretching duration employed here (24 h) was long enough for egr-1 to quickly increase but then decrease over time, even past the initial control level. Id2 (inhibitor of DNA binding) is a transcription regulator that contributes to SMC proliferation after vascular injury (28). Levels of Id2 mRNA were marginally decreased in both stretch regions. Finally, plasminogen activator inhibitor 1 (PAI-1), an inhibitor of fibrinolysis and thrombin activity, showed reduced mRNA levels as well. PAI-1 has been shown to play a significant role in intimal hyperplasia, with PAI-1-deficient mice producing greater neointimal formation, and treatments of recombinant PAI-1 inhibiting neointimal formation in rat carotid injuries (21, 53).

Fig. 5. Actin filament organization. Alexa Fluor 488 phalloidin dye was used to stain actin filaments within 10T1/2 cells cultured within PEGDA hydrogels. Cells were imaged within nonstretched gels (A and B) and within the uniaxial and biaxial regions of stretched gels (C and D and E and F, respectively). Scale bars, 100 μm (×10 images) or 20 μm (×60 images).
The sum of our gene expression results clearly reveal that stretch induces a sort of “hybrid” phenotype, leading to the up- and downregulation of both synthetic and contractile proteins. It is important to mention that the resulting protein expression changes due to altered mRNA levels were not quantified here, and thus we cannot conclude which protein levels are ultimately modulated under nonuniform stretch. It is also important to highlight (as mentioned above) that many gene and protein changes vary with time after a stimulus. Since we only collected samples after 24 h of stretch, there are likely other expression changes that were missed in the present measurements. Future studies should seek to explore a more complete time course distribution, while also following up PCR results with more detailed protein level analysis for those species that show marked changes in gene expression.

The actin cytoskeleton plays a large role in mediating many stretch-dependent responses of cells including differentiation and dedifferentiation (18). To assess changes in 10T1/2 cytoskeleton due to stretch, actin filaments were fluorescently labeled and imaged with confocal microscopy. Although stretch conditions typically result in marked reorganization of actin fibers, there were no apparent differences between cytoskeletons of stretched and nonstretched 10T1/2 cells within PEGDA hydrogels. In fact, actin was mostly punctate, with short fiber segments running in the out-of-plane direction. This lack of actin organization and stress fiber formation is potentially attributable to the encapsulation of cells within PEGDA hydrogels, which restricts cells to a spherical morphology.

Quantified by BrdU incorporation, 10T1/2 proliferation was graded across the radial position of cells stretched on a circular membrane, with increased proliferation (relative to cells from a nonstretched membrane) within the region of uniaxial stretch, decreased proliferation within the region of biaxial stretch, and statistically unchanged proliferation within the middle transition region. Many studies have shown that cells of several types (including mesenchymal cells) tend to upregulate proliferation in the presence of stretch (5, 13, 15, 46, 49). Also, fibroblasts have been shown to proliferate more rapidly in uniaxial than biaxial stretch, supporting the graded response result found here (15). However, it is unclear why the proliferation rate was presently decreased in the biaxial stretch region. Such a disparate result between stretch conditions has been seen in other mechanobiological responses such as the α-actin expression of bone marrow mesenchymal cells, which was increased in response to uniaxial stretch but decreased by biaxial stretch (34). Potentially, such a disparity is due to the ability of cells to reorient themselves to a mechanically preferred position by aligning perpendicular to the direction of stretch in uniaxial environments, whereas they are unable to “escape” the stretch components of a biaxial environment.

Some primary limitations of this study are related to the use of PEGDA hydrogels as 3D constructs. Besides the aforementioned issue of encapsulation, isolating quality mRNA after stretching experiments is made difficult in PEGDA hydrogels because of the abundance of polymer chains (51). Low yields of nucleic acids greatly increase the amount of error in RT-
stress-strain data, which yielded a value of 19 kPa for 20% PEGDA hydrogel rings and calculated a secant modulus of 10–16 kPa. Browning et al. (7) uniaxially strained gels, the mismatch of cell and hydrogel stiffnesses is probably greater than those of the surrounding fibrin cap. Within our mechanical stiffness properties of calcifications were 10 times stiffer than those of the surrounding fibrin gels (37, 47, 48). However, in that case the stiffnesses of 10T1/2 cells and PEGDA hydrogels are certainly not identical, they are probably within the same order of magnitude. These findings, taken with the very low cell density in our gels, lead us to suspect that the inclusion of cells had minimal perturbation on neighboring substrate deformations.

A final limitation of this work arises from the complexity of the nonuniform stretch environment in that multiple mechanical metrics are coincidentally varying with position in the construct: stretch component magnitudes, gradient magnitude, gradient direction, biaxial stretch ratio, biaxial stretch product (area stretch), etc. Thus, for ultimate elucidation of cell mechanobiological processes, future studies should employ more thorough and robust stretching regimens of various combinations and controls for these mechanical variables. The device used here is well-suited for independent control of many of these parameters as previously described (35).

In summary, a novel cell-stretching device has been employed to stretch cell-seeded membranes and PEGDA hydrogels in order to subject cultures to nonuniform stretch environments and investigate SMC phenotype modulation therein. Proliferation measured by BrdU and mRNA expression measured by quantitative real-time RT-PCR revealed differential responses of 10T1/2 cells depending on their location within the stretching environment, which ranged from a high-magnitude uniaxial stretch to a lower-magnitude near-equibiaxial stretch. These results reveal the complexity of phenotype modulation, while establishing the capability of this device to study this process in the presence of stretch conditions representative of in vivo mechanical stimuli.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: W.J.R., E.W., and J.E.M. conception and design of research; W.J.R. performed experiments; W.J.R. analyzed data; W.J.R., E.W., and J.E.M. interpreted results of experiments; W.J.R. prepared figures; W.J.R. drafted manuscript; W.J.R., E.W., and J.E.M. edited and revised manuscript; W.J.R., E.W., and J.E.M. approved final version of manuscript.

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