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A novel device to stretch multiple tissue samples with variable patterns

Application for mRNA regulation in tissue-engineered constructs

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A broad range of cells are subjected to irregular time varying mechanical stimuli within the body, particularly in the respiratory and circulatory systems. Mechanical stretch is an important factor in determining cell function; however, the effects of variable stretch remain unexplored. In order to investigate the effects of variable stretch, we designed, built and tested a uniaxial stretching device that can stretch three-dimensional tissue constructs while varying the strain amplitude from cycle to cycle. The device is the first to apply variable stretching signals to cells in tissues or three dimensional tissue constructs. Following device validation, we applied 20% uniaxial strain to Gelfoam samples seeded with neonatal rat lung fibroblasts with different levels of variability (0%, 25%, 50% and 75%). RT-PCR was then performed to measure the effects of variable stretch on key molecules involved in cell-matrix interactions including: collagen 1α, lysyl oxidase, α-actin, β1 integrin, β3 integrin, syndecan-4, and vascular endothelial growth factor-A. Adding variability to the stretching signal upregulated, downregulated or had no effect on mRNA production depending on the molecule and the amount of variability. In particular, syndecan-4 showed a statistically significant peak at 25% variability, suggesting that an optimal variability of strain may exist for production of this molecule. We conclude that cycle-by-cycle variability in strain influences the expression of molecules related to cell-matrix interactions and hence may be used to selectively tune the composition of tissue constructs.

Introduction

Many cell types are sensitive to their mechanical environment. Hence, essential cell functions are influenced by the physiological levels of mechanical forces cells experience within the body.[1] Primary examples of mechanical stimuli on cells are found in the respiratory and circulatory systems, where pressures due to tidal breathing and cardiac output, respectively, impart stretches on adherent cells. Most studies related to how cells respond to mechanical forces, mechanotransduction, have examined cells in isolation and focused on static and simple sinusoidal stretching patterns.[2-6] However, cells in the body are subject to irregular and varying mechanical stimuli. In the respiratory system in particular, tidal breathing varies considerably in both frequency and amplitude,[7] resulting in cell stretches that simple sine waves of constant amplitude and frequency do not reproduce.

Recently, there has been interest in the effects of irregularly varying mechanical stimuli on cell function. In particular, it has been shown in type II alveolar epithelial cells in culture that adding variability to the stretching stimulus can result in an increase in surfactant secretion.[8] The stretch pattern applied to the epithelial cells randomly varied from cycle to cycle in both frequency and amplitude, but had the same mean frequency and amplitude as a constant sinusoidal pattern. Different levels of variability, defined as the interval around the mean from which random amplitudes were chosen, showed distinct levels of surfactant secretion. However, a limitation of this study was that these experiments were performed in two dimensional cell culture rather than with a three dimensional tissue or construct. While various stretching devices have been designed,[9,10] to our knowledge, devices that can accommodate tissues have not been able to provide variable stretch patterns such as those found in the body. In order to further explore the effects of variable stretch on the function of a broader range of cells it is necessary to develop the tools to apply this type of stretch to three dimensional tissues and tissue-engineered constructs.

The primary purpose of this study was to design, build and test a uniaxial stretching device capable of delivering cyclic variable stretch to tissues and constructs in a controlled environment. Furthermore, we aimed to develop a multi-well tissue stretcher

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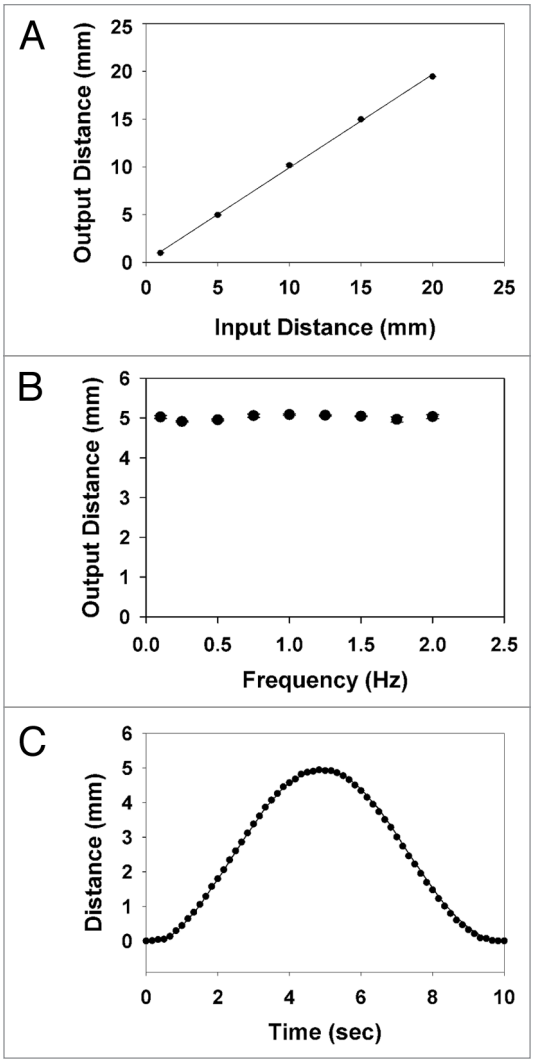

Figure 1. The distance traveled by the linear guide is plotted against the prescribed travel distance and fit with a linear relationship (**A**). The frequency dependence of travel is shown for the maximum travel distance of the linear guide (**B**). The sinusoidal travel of the linear guide is shown before (solid line) and after (circles) 3 h of stretch (**C**).

in order to be able to simultaneously stretch several samples that would provide for rapid experimental testing of novel hypotheses related to mechanotransduction. To this end, we developed such a system and used it for preliminary testing of the effects of variable stretch on mRNA expression of several intracellular and extracellular matrix (ECM)-related molecules in a collagen-based construct (Gelfoam) seeded with neonatal rat lung fibroblasts.

Results

Device characterization. **Figure 1A** shows the relationship between the input and output distances for cyclic stretches performed at 0.5 Hz. The linear fit has a slope of 0.977 and an R^2 of 0.999 thus showing that the output distance was consistent and linearly related to the input distance over the entire range of the actuator stroke. **Figure 1B** shows the dependence of the output distance on frequency, ranging from 0.1 to 2 Hz. The output distance remained within 2% of the desired distance (5 mm) for all tested frequencies. The repeatability of the stretch is shown in **Figure 1C**, where the solid line represents the stretch waveform at the beginning of a three hour stretch while the stars indicate the waveform at the conclusion of the stretch. The desired cyclic stretch had a frequency of 0.1 Hz and amplitude of 5 mm. The two curves are nearly identical, showing that the motion of the actuator was reproducible over the length of the experiment.

mRNA expressions. Mechanotransduction involves the translation of forces from the extracellular space into the cell leading to cellular response. To determine how variable stretch might modulate cell function we evaluated the relative expression of important ECM components (type 1 collagen, and lysyl oxidase (LOX)), cell surface ECM receptors (integrins and syndecan 4), a critical cytoskeletal component (α-smooth muscle actin) and vascular endothelial growth factor-A (VEGF) involved in maintaining cell viability and stimulating tissue repair. The expression level of each mRNA was evaluated at 0, 25, 50, and 75% variability. The monotonous sinusoidal (0% variability) condition had a sample size of 9, while all other conditions had a sample size of 3. **Figure 2** shows the relative mRNA expressions of collagen 1α and LOX for increasing levels of variability. Both collagen 1α and LOX mRNA levels were elevated with increased variability and this was statistically significant among the groups with $p = 0.033$ for collagen 1α and $p = 0.034$ for LOX.

The relative expression of mRNA for α-actin and VEGF are shown in **Figure 3**. Both molecules exhibited a decreasing trend in expression at 25% variability in strain. The dependence of α-actin on variability was statistically significant among the group ($p = 0.039$), whereas VEGF nearly reached statistical significance ($p = 0.066$).

The expression of cell surface ECM receptors syndecan-4, β1 and β3 integrins is shown in **Figure 4**. Although β1 and β3 integrins did not show a statistically significant difference among groups ($p = 0.075$ and 0.260 respectively), syndecan-4 displayed a strong difference among groups ($p = < 0.001$). Furthermore, the mRNA level at 25% variability was different from the 0%, 50% and 75% variability conditions and the 0% variability condition was also statistically different from the 50% and 75% variability groups.

Discussion

Cells in the body receive mechanical stimuli caused by the stresses and strains of locomotion, breathing, and due to various other physiological activities such as the heart pumping blood. The deformations that cells are exposed to are always irregular. For example, both the tidal volume of breathing[7] and the ejection volume by the heart vary from cycle to cycle.[11] Consequently, the mechanical stimuli that cells are exposed to within the ECM of the lung and the blood vessels also vary from cycle to cycle. Thus, it is likely that cells are tuned to recognize and respond to these variable mechanical inputs. In order to determine how such variability in strain might affect the expression of key ECM and cell related molecules, we designed, built and tested a uniaxial

stretching device that can mechanically stimulate up to six tissue pieces or constructs at a time, delivering cycle to cycle variability. Although similar uniaxial stretchers exist in the literature,[9,10] this is the first study to apply cycle-by-cycle variability in strain and frequency. In this study, we developed a device that is robust and can deliver reliable strains for long durations in the controlled environment of a cell incubator. Additionally, our preliminary exploration with the device suggests that variable stretch patterns can influence the expression of mRNA for several key molecules known to play important roles in cell-ECM interactions.

Device characteristics. Although several uniaxial stretchers have been introduced some of which are also commercially available, this is the first device to allow the user to apply variability of strain on a cycle-by-cycle basis. Our stretcher is designed as a high throughput system that can stretch up to six samples under the same mechanical conditions while allowing for different biochemical conditions in different wells. Our system is ideal for biochemical analysis of cells and ECM in mechanically stimulated tissues and constructs. Commercially available systems such as LigaGen by Tissue Growth Technologies or ElectroForce BioDynamic Test Instruments by Bose cannot accommodate as many samples simultaneously and therefore require multiple experiments to achieve the same sample number. Such repeated experiments can increase the inter-sample variability of the data potentially requiring a further increase in sample number. The stretcher developed by Kluge et al. features multiple wells but also uses multiple actuators to deliver the strain.[10] A disadvantage of our current system is the lack of force transducers, which prevents us from acquiring stress and stiffness data. The purpose of this device, however, was to be able to measure the biochemical response of multiple samples to mechanical stimulation. Moreover, the addition of force transducers to each well is still possible if deemed necessary. Nevertheless, it is not expected that significant deposition of the major structural molecules such as the collagens would occur during our short-term stretching and hence the stiffness of the samples is not likely to increase by the end of the protocol used in this study.

Our system features the following important design considerations: (1) the stretcher size and material composition allow it to be placed inside the controllable environment of an incubator; (2) the system can be easily sterilized using ethanol or UV light; (3) having six wells increases the sample number over current systems and also allows for simultaneous mechanical stimulation under different biochemical treatment conditions; (4) the system is flexible and can also be set up such that any of the samples receive static or no stretch while the rest of the samples undergo cyclic stretch; (5) the actuator can stretch up to 2 cm allowing a large range of strains; (6) the mechanism for clamping the samples allows easy and reliable mounting of tissues and constructs; and (7) the well size is minimized in order to concentrate any proteins released by the cells as well as to reduce the amount of reagents used in certain conditions.

mRNA expression. The molecules chosen for mRNA analysis are integral to controlling the mechanical environment of cells in the ECM as well as the cells' own mechanical properties. For example, cells can regulate the stiffness of their environment by depositing type I collagen, an important structural protein of the ECM.[12,13] LOX catalyzes the crosslinking of collagen and therefore can also regulate the stiffness of the ECM.[14] Syndecan-4, β1 integrin and β3 integrin are membrane-bound receptors that function as ECM receptors transducing signals from the ECM into the cell[15,16] whereas α-actin is a protein that is required for contractile force generation and also contributes to the stiffness of the cell.[17] Finally, VEGF is a signaling protein that cells produce to enhance local cell viability and to promote vasculogenesis and

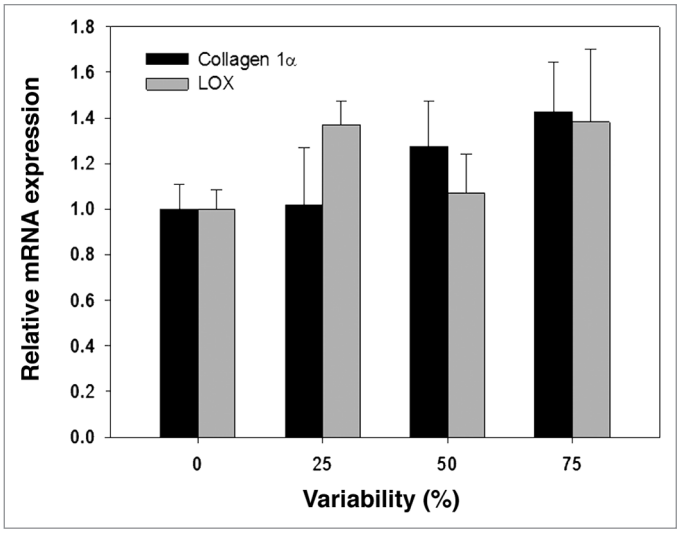

Figure 2. Relative mRNA expression for collagen 1α and lysyl oxidase at 25%, 50% and 75% variability, normalized to the 0% variability condition. Both collagen 1α and lysyl oxidase show a statistically significant difference among the groups with p = 0.033 and p = 0.034 respectively.

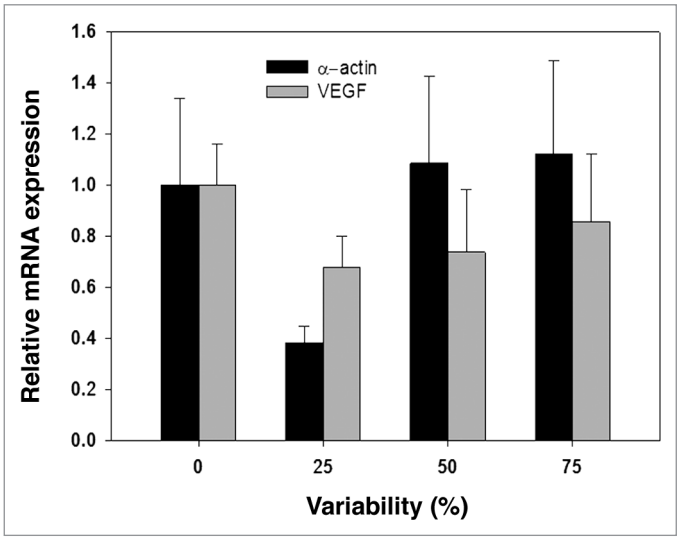

Figure 3. Relative mRNA expression for α-actin and VEGF at 25%, 50% and 75% variability, normalized to the 0% variability condition. α-actin shows a statistically significant difference among the treatment groups (p = 0.039), while VEGF does not (p = 0.066).

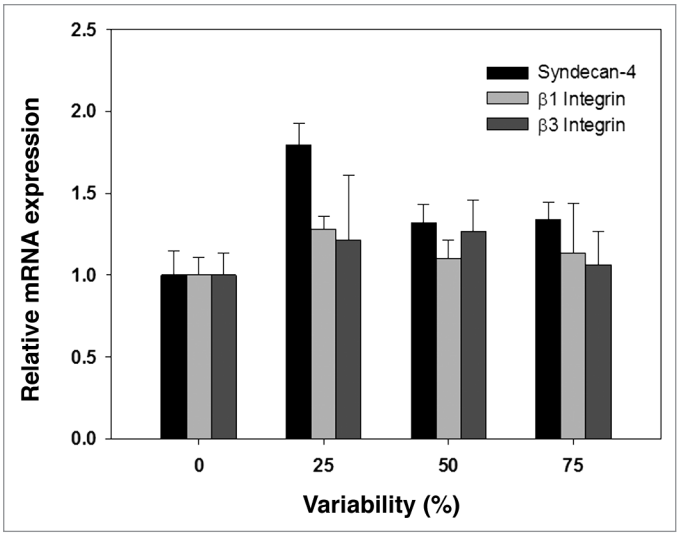

Figure 4. Relative mRNA expression for syndecan-4, β1 integrin and β3 integrin at 25%, 50% and 75% variability, normalized to the 0% variability condition. Syndecan-4 mRNA levels are statistically significant ($p < 0.001$), while β1 integrin and β3 integrin do not show a significant difference ($p = 0.075$ and $p = 0.260$ respectively).

angiogenesis.[18] Mechanical stretch delivered as a cyclic monotonous waveform has been found to influence many molecules.[19] For example, the mRNA expression of α1 procollagen was shown to be upregulated by stretch on elastin and laminin coated membranes but not on fibronectin.[3] Mechanical stresses were shown to dynamically regulate syndecan-4 expression and relocation on cell surface.[20] Additionally, 4 h of uniaxial cyclic stretch was found to upregulate the expression of integrin β3 in human umbilical endothelial cells, which may enhance their ability to adhere to the vessel wall.[21]

Our data obtained using the device in this preliminary investigation, however, suggest that mRNA expression of most of studied molecules is also sensitive to the introduction of variability with some molecules being upregulated while others downregulated. In particular, in fibroblasts seeded in collagen-based gelfoam constructs, actin mRNA production decreased at 25% variability, but recovered at larger levels of variability whereas syndecan-4 showed peak expression at 25% variability. The mRNA expression of collagen and LOX increased above control levels for larger levels of variability. The mRNA of VEGF and β1 integrin nearly reached significance with variability that may be achieved with a larger sample size. The only molecule whose mRNA expression did not show signs of sensitivity to variability was β3 integrin. Thus, there appear to be multiple and sophisticated mechanisms in place for cells to sense and respond to variable stretch-induced mechanical stimulation.

The most interesting result was observed with syndecan-4 mRNA, which also showed the strongest statistically significant differences among the different levels of variability. Syndecan-4 mRNA displayed a distinct peak in production at 25% variability, which then decreased at increasing levels of variability. In their paper on surfactant secretion, Arold et al. also showed a peak in surfactant secretion at an optimal level of variability.[8] We note that actin also exhibited a similar behavior in that there was a specific level of variability or noise at which its mRNA production was maximally reduced. Such variability- or noise-enhanced signaling is similar to the phenomenon known as stochastic resonance that occurs in nonlinear systems where the addition of noise to the input signal can result in the amplification of the output.[22,23] Stochastic resonance has been implicated in various biological phenomena.[8,24-27] We speculate that physiological levels of variability must be optimal in some sense for the functioning of the cell or its components within its native mechanical and biochemical environment. Since the biochemical environment includes the composition of the ECM whereas the mechanical environment is a function of the organization of the ECM, our results are likely specific to the cell type and construct composition used.

With regard to the possible mechanisms of how variability around a mean level of stretch can influence signaling, we note the following. Even though this preliminary investigation did not allow us to reveal the detailed signaling mechanism by which variability in stretch regulates mRNA production of the proteins tested here, it is certainly true that signal transduction pathways are invariably nonlinear due to feedbacks in the reaction networks and Michaelis-Menten kinetics.[28] As cells adhere to the ECM via cell surface receptors such as integrins, the stretch of the ECM fibers generates force in the integrins which is transmitted through various focal adhesion proteins to the actin cytoskeleton.[29] Because of the complex structure of the focal adhesion and the network organization of the actin, this mechanical transmission pathway is nonlinear. While it is not known exactly how and where these mechanical signals are transduced into chemical signals, upregulation or downregulation of mRNA requires a certain threshold stimulus mostly due to feedback from the output to some components of the pathway itself perhaps through the NFκB pathway.[30] Such threshold phenomena are inherently nonlinear. Depending on the specific pathway nonlinearities for a given molecule, the occasional larger than average stretch amplitudes can overcome the thresholds associated with that pathway and if this occurs a sufficient number of times, the baseline signal transduction generated by the monotonous sinusoidal stretching will be altered. Thus, the specific responses that we see in the various signal transduction pathways should depend on the specific nonlinearities in these pathways, the mean level and the variability of stretch as well as the total time of stretching.

It is also possible that due to the viscoelastic nature of both the cell and the ECM, different strain rates could generate different tensile forces on the focal adhesions that in turn could affect the final mRNA expression through the nonlinearities of the pathway. In our case, the time period of a given sinusoidal was proportional to the amplitude and hence the strain rate was constant for different cycles and across all variabilities. The strain rate was kept constant in order to avoid possible effects of increasing strain rate on mRNA expression, which we did not study specifically.

Considering that a broad range of cell types experience irregular mechanical stimuli, it is important to investigate the effects of variability on various cell functions. Our results demonstrate that the addition of variability can cause cells to alter their mRNA

production for key molecules when compared with regular cyclic stretch. Furthermore, the specific effects of variability appear to differ among the different molecules studied. Depending on how lasting these effects are, it may thus be possible to differentially control cell function simply by adding variability to the stretching stimulus. This finding can have implications for future studies in mechanotransduction as well as tissue engineering. If, for example, cells can be stimulated to produce more collagen and LOX at different variabilities it may be possible to control the stiffness of tissue engineered constructs while other signal transduction pathways are not influenced. Likewise, physiological levels of variability may influence many other cell functions that warrants exploring in future experiments related to mechanotransduction.

Methods

Tissue stretching device. A uniaxial stretching device was designed and built to deliver mechanical stimuli to native tissue or construct samples (**Fig. 5A**). The device, which measures 20 × 24 × 14 cm, is compact and can be placed inside a cell incubator so as to provide the appropriate environmental conditions such as temperature, humidity and CO_2. The device consists of six wells, each of which can accommodate one sample. The wells measure 5 × 2 × 1 cm, and can accommodate up to 9 ml of media depending on the size of the sample. The six samples can be stretched simultaneously providing identical stretch to each sample or several samples can be held at a fixed strain and serve as controls. The tissue samples are secured within the wells on both sides by stainless steel clamps (**Fig. 5B**). The clamps consist of two parts: the top part snap fits onto the rods while the bottom part is joined to the top part by a stainless steel screw, securing the tissue between the two parts. One side of the clamps remains fixed, while the other can travel on a linear motion guide. The travel of the clamps, and thus the stretch of the tissue sample, is controlled by a linear actuator (Size 14 Linear Actuator, Haydon Kerk Motion Solutions) powered by a stepper motor that has a minimum step size of 25.4 μm. The stroke of the actuator is 2.54 cm, which is the maximum stretch that can be applied to any sample.

The linear actuator is controlled by custom built Labview software that delivers step and direction pulses to the actuator via a Labview DAQ (Labview DAQ 6221, National Instruments). The amplitude and frequency of stretch are controlled by the input of the corresponding step and direction signals delivered via the DAQ. The device can deliver static as well as cyclic stretching of the tissue sample. Furthermore, by delivering appropriate stretching signals, the device can stretch samples with cycle by cycle variability.

Calibration of the device. The device was calibrated by ensuring that the linear actuator traveled the specified distance, and therefore applied the correct strain to the tissue sample. The distance between a stationary point on the device and the linear motion guide was imaged during stretch (DSC-T70, Sony) and the travel distance was measured via MATLAB software developed in the laboratory. The device was imaged under different amplitudes and frequencies to verify the ability of the actuator to deliver the correct travel distances. Likewise, the ability of the actuator to be consistent over time was measured by comparing the initial and final cycles of a three hour long stretch.

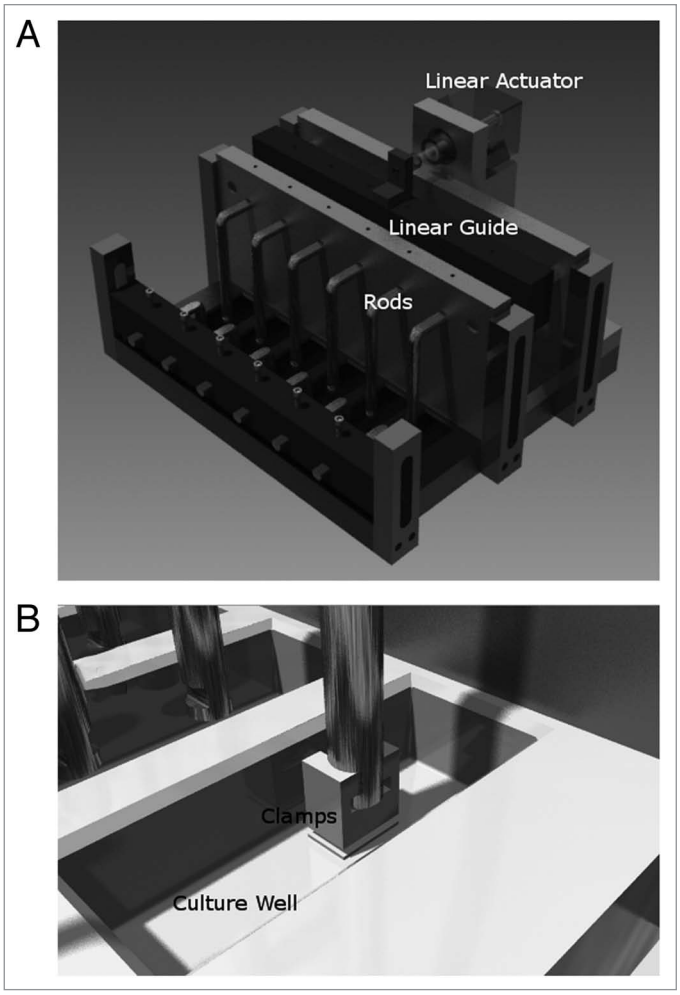

Figure 5. Top view (**A**) of the stretcher design in a CAD program. The device consists of a linear actuator attached to a linear guide and a plate containing six wells. The tissue sample is clamped on both sides using stainless steel clamps and is placed inside the wells (**B**). On one side the clamps attach to stationary rods while on the other the rods are attached to the linear guide which controls their position. The position of the rods is computer controlled via the linear actuator and a custom built Labview program.

Cell culture and Gelfoam seeding. The protocol was approved by the Boston University IACUC. Neonatal rat pulmonary fibroblasts (NNRLF) were isolated from 2–3 d old Sprague-Dawley rats and were cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin antibiotic cocktail, 1% sodium pyruvate and 1% nonessential amino acids. The cells were cultured in flasks for one week before being seeded on Gelfoam constructs.

Gelfoam constructs (Pharmacia and Upjohn) were cut into 4 cm × 1 cm × 0.3 cm pieces the day before seeding and were equilibrated in NNRLF medium overnight prior to seeding. The constructs were seeded by spot pipetting 250,000 cells over the entire 4 cm × 1 cm surface. Samples were then placed in an

incubator for 2–3 h and then into centrifuge tubes containing NNRLF medium. The medium was changed 24 h after seeding and at 3 d intervals thereafter. The constructs were cultured for 8 d before stretching.

Stretching protocol. Gelfoam constructs were stretched on the 8th day following seeding without changing the medium. Samples were attached to the device using the special clamps and the stretching was performed on six samples simultaneously. Two unstretched constructs were maintained as controls for each day. The two types of stimuli delivered were monotonous cyclic and variable cyclic stretch. Variable stretch delivers a sequence of sinusoids in which each cycle has a different strain amplitude (ε) and frequency (f). Here we defined the variability of the stretch signal to be an interval described by the percentage of the mean strain; thus, a signal with a mean strain of 20% and an imposed variability of 25% would deliver peak strains in each cycle in the range of 15 to 25% with equal probability. The corresponding strain and frequency were fixed by the equation $\varepsilon f = C$, such that a larger peak strain would have a smaller frequency resulting in a constant strain rate.

All stretched conditions received an average strain amplitude of 20% at an average frequency of 0.2 Hz. In total, there were four conditions: (1) monotonous stretch, which corresponds to 0% variability, and consists of a single repeated sinusoidal stretch at 20% strain (2) 25% variability, (3) 50% variability and (4) 75% variability. All stretches were applied for three hours. Following stretching, the samples were removed from the wells and cut from the clamps. Only the portion of the sample that experienced stretch was collected for analysis.

mRNA expression. All Gelfoam samples were collected for total RNA purification immediately following stretching. The samples were washed using PBS and then cut into smaller pieces with scissors. Two samples of the same condition were combined into one to obtain enough RNA for analysis. A previously described protocol was used to extract the total RNA from the cut pieces.[31]

To remove genomic DNA, the RNA samples were incubated with RNase-free DNase I (New England BioLabs, M0303S) in conjunction with the use of an RNase inhibitor (Life Technologies, N808–0119). The cDNA was prepared by annealing the RNA with random hexamer and oligo dT primers and allowing the first strand synthesis to be performed with MuLV reverse transcriptase (Life Technologies, N808-0234). No reverse transcriptase was used in the negative controls. An Applied Biosystems 7300 Real-Time PCR system was used to carry out real-time PCR analysis. ABI TaqMan gene expression assays for rat collagen 1α (Rn00801649-gl), elastin (Rn01499782-m1), lysyl oxidase (Rn00566984-m1), α-smooth muscle actin (Mn01546133-m1), Vegf (Rn01511605-m1), syndecan-4 (Rn00561900-m1), β1 integrin (Mn01253227-m1) and β3 integrin (Rn00596601-m1) were used as target probes. Eukaryotic 18 S rRNA (4308329) was used as an endogenous control. Standard cycling parameters of 50°C for 10 min, 95°C for 2 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min were completed. Data were analyzed with the $\Delta\Delta C_T$ method with 18 S rRNA as the endogenous control.

Statistical analysis. Data are presented as mean ± standard deviation for each group. Data were analyzed using one-way Anova and differences between groups were considered statistically different for $p < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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