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Effects of Intermittent and Incremental Cyclic Stretch on ERK Signaling and Collagen Production in Engineered Tissue

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Abstract

Intermittent cyclic stretching and incrementally increasing strain amplitude cyclic stretching were explored to overcome the reported adaptation of fibroblasts in response to constant amplitude cyclic stretching, with the goals of accelerating collagen production and understanding the underlying cell signaling. The effects of constant amplitude, intermittent, and incremental cyclic stretching regimens were investigated for dermal fibroblasts entrapped in a fibrin gel by monitoring the extracellular signal-regulated kinase (ERK1/2) and p38 pathways, collagen transcription, and finally the deposited collagen protein. Activation of ERK1/2, which has been shown to be necessary for stretch-induced collagen transcription, was maximal at 15 min and decayed by 1 h. ERK1/2 was reactivated by an additional onset of stretching or by an increment in the strain amplitude 6 h after the initial stimulus, which was approximately the lifetime of activated p38, a known ERK1/2 inhibitor. While both intermittent and incremental regimens reactivated ERK1/2, only incremental stretching increased collagen production compared to samples stretched with constant amplitude, resulting in a 37% increase in collagen per cell after 2 weeks. This suggests that a regimen with small, frequent increments in strain amplitude is optimal for this system and should be used in bioreactors for engineered tissues requiring high collagen content.

Keywords

Fibrin; Fibroblast; Mitogen activated protein kinase; p38; Mechanical conditioning

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ELECTRONIC SUPPLEMENTARY MATERIAL

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CONFLICT OF INTEREST

Jillian B. Schmidt, Kelley Chen, and Robert T. Tranquillo declare that they have no conflicts of interest.

ETHICAL STANDARDS

No human or animal studies were carried out by the authors for this article.

INTRODUCTION

Mechanical conditioning is a widely employed method for obtaining the desired properties and function in engineered tissues. In engineered arteries, heart valves, ligaments, and skeletal muscle, where high mechanical strength is a requirement, cyclic mechanical stretching regimens have been used to induce cells to increase their production and maturation of collagen.^{5,9,14,16,19,22,24,25} Collagen is the major load bearing component in most native tissues, and increased collagen content is highly correlated with an increase in strength and stiffness in engineered tissues.^{10,16,19,21,22,25} While cyclic stretching regimens are a common step in many tissue engineering fabrication processes, much is still unknown about the mechanism through which stretching results in increased collagen content, and thus the optimal cyclic stretching protocol may not be employed. The need for optimized cyclic stretching protocols is particularly crucial in attaining suitable properties in engineered tissues based on a sacrificial fibrin gel scaffold. Although a variety of scaffold materials are used for tissue engineering applications including both synthetic polymer^{9,16,19,21} and biopolymer^{5,6,14,22–25} matrices, fibrin is an attractive scaffold biomaterial, as it is completely biological, degradable, can be cast in various geometries, and can be aligned and remodeled by entrapped cells.⁶ However, a fibrin gel has extremely low strength and stiffness compared to native collagenous tissues, and creating a fibrin-based tissue with sufficient mechanical stiffness and strength for *in vivo* function absolutely requires stimulating an increase in the cellular production of collagen.

The success of constant amplitude cyclic stretching protocols in increasing cellular collagen production is limited by adaptation of the cells to the applied mechanical stimulus.^{14,25} Previously, we demonstrated that biochemical inhibition of p38 during cyclic stretching increased collagen content in engineered tissues based on a dermal fibroblast-seeded sacrificial fibrin gel scaffolds.²⁷ Here, we extend our prior studies of mechanotransduction in this same model system for our engineered vascular graft²² and heart valve²³ tissues to assess the effects of optimized cyclic stretching regimens on collagen production. In order to combat the negative effects of adaptation, various stretching regimens have been employed including incrementally increasing strain amplitude approaches²⁵ as well as intermittent stretching, in which tissues are exposed to alternating periods of cyclic stretching and static culture.^{14,16} These perturbations to constant amplitude cyclic stretching allow the cells to be re-stimulated by either subsequent applications of stretching after a rest period or an increment in the applied stretch amplitude. Previous studies have focused on observing changes in response to stretching at one or two points in the collagen production pathway, including phosphorylation of two mitogen activated protein kinases: extracellular signal-regulated kinase 1/2 (ERK1/2), which is believed to be required for cyclic stretch induced collagen synthesis^{13,14,25,27} and p38, which has been found to have an inhibitory effect.^{13,27} Other responses investigated include transcription of genes involved in collagen synthesis, maturation, and remodeling,^{13,26} and collagen protein content.^{14,16,25}

While these studies have provided valuable insight into the response of cells to cyclic stretching, no single study has investigated the effects of constant amplitude, intermittent, and incrementally increasing strain amplitude cyclic stretching on fibroblasts in an engineered tissue environment throughout the entire collagen production process. The aim of

this study was to investigate the response and adaptation of fibroblasts entrapped in a fibrin gel to various stretching regimens at three key points in the collagen production pathway: ERK1/2 and p38 activation, transcription of collagen types I and III, and ultimately, deposition of insoluble collagen protein. This more comprehensive investigation of the collagen production pathway provides greater insight into the effects of cyclic stretching on collagen production and offers guidance in selecting a mechanical conditioning protocol to accelerate collagen production in fibrin-based engineered tissues.

METHODS

Cell Culture

Neonatal human dermal fibroblasts (Lonza) were expanded in 50:50 DMEM:F12 with 15% fetal bovine serum (Hyclone), 100 $\mu\text{g}/\text{mL}$ streptomycin, and 100 U/mL penicillin. Cells were grown to confluence and harvested for use at passage 7.

Fibrin-Based Tissue Construct Fabrication and Culture

Fibrin gels with entrapped cells were created by suspending neonatal human dermal fibroblasts in DMEM and then mixing the cells with a 20 mM HEPES-buffered saline solution containing bovine fibrinogen (Sigma). In order to form a fibrin gel, a solution of bovine thrombin (Sigma) and CaCl_2 in DMEM was added to the fibrinogen/cell solution. The solution was injected into 6-well circular tissue-train plates (Flexcell® International), which have a circular foam insert that anchors the gel around its circumference. The volume of gel in each well was 2 mL. The final composition of the gel was 6.7 mg/mL fibrinogen, 500,000 cells/mL, 0.8 U/mL thrombin, and 5 mM CaCl_2 . The discs were allowed to gel for 6 min in the tissue culture hood at 20 °C and an additional 24 min in a standard cell culture incubator at 37 °C. After the solutions had gelled, DMEM supplemented with 10% fetal bovine serum, 100 $\mu\text{g}/\text{mL}$ streptomycin, 100 U/mL penicillin, 0.25 $\mu\text{g}/\text{mL}$ amphotericin B, 50 $\mu\text{g}/\text{mL}$ ascorbic acid, and 2 $\mu\text{g}/\text{mL}$ insulin was added (5 mL per well). Medium was replaced three times weekly. All constructs were cultured statically for 1 week before cyclic stretching regimens were applied.

Cyclic Stretching of Tissue Constructs

Uniaxial cyclic stretching was applied to the constructs using the FX-5000 Tension System and Arctangle® loading station (Flexcell® International). The 6-well tissue-train plates have a flexible silicone bottom which was uniaxially stretched by the application of vacuum beneath the wells (Figs. 1a–1d). All cyclic stretching regimens were applied using a square waveform with a frequency of 0.5 Hz, and a duty cycle of 15%. The applied strain was validated for the fibrin constructs using digital image correlation as described by Raghupathy *et al.*¹⁵ Relatively uniform uniaxial strain was applied to a central rectangular area approximately 13 mm by 17 mm, and this strain was consistently applied throughout the duration of the experiments. While there was some heterogeneity in the strain field in this region, the strain was primarily uniaxial and, averaged over the region, was in good agreement with the set point. Four 6 mm biopsy punches were taken from this region for all characterization methods (Fig. 1e). The constructs remained in a standard cell culture incubator for the duration of the stretching regimens.

Three studies were performed to investigate cell signaling in response to cyclic stretching. First, the activation time courses for ERK1/2 and p38 were obtained by stretching samples continuously with 5% strain amplitude for 15 min, 1, 3, and 6 h. At each time point, stretched samples ($n = 3$) and static controls ($n = 3$) were harvested. Next, in order to determine how soon ERK1/2 could be reactivated by a second onset of cyclic stretching, samples were stretched with 5% strain amplitude for 15 min and allowed to rest for 15 min, 3 h, or 6 h. Following the rest period, the samples were stretched with 5% strain amplitude for an additional 15 min and harvested ($n = 3$ per group). Finally, we sought to investigate the effects of an increase in strain amplitude on ERK1/2 activation. Samples were stretched with 5% amplitude for 1 or 6 h, at which point the strain amplitude was increased to 6 or 10%. Samples were harvested ($n = 3$ per group) immediately before and 15 min after the increment in strain amplitude.

Forty eight-hour studies were performed to investigate collagen transcription for static control samples and three cyclic stretching regimens shown in Fig. 2. These cyclic stretching regimens were selected based on the results of the signaling studies. Constant strain amplitude ("constant") samples were stretched at 5% strain amplitude for 48 h. Incrementally increasing strain amplitude ("incremental") samples were stretched at 5% amplitude for 24 h followed by 6% amplitude for 24 h. Intermittently stretched samples ("intermittent") were stretched at 5% amplitude for 15 min, followed by a 6 h static period, and this regimen was repeated for the duration of the study. The intermittent samples were harvested at 49 h, which was exactly 24 h after the onset of a stimulation period. This was done for consistency with the incremental treatment group in which the samples were harvested 24 h after the final increment. Two repeats of this study were performed, and data were pooled ($n = 5$ per group).

Extensions of these cyclic stretching regimens were applied for 2 weeks in order to study collagen deposition. Samples were subjected to 2 weeks of constant amplitude (5 or 13.5% amplitude), intermittent (15 min at 5%, 6 h static, repeat), and incremental (5–13.5%, increment by ~1% every 24 h) cyclic stretching regimens. Due to limitations of the stretching system, the target 1% increment in strain every 24 h was not able to be achieved, and in the last several days of stretching, the increments decreased in magnitude. However, a measurable increase in strain was still applied every 24 h. Two full repeats of the experiment were performed, and data were pooled ($n = 6$ per group).

Protein Extraction

At harvest, constructs were excised from the wells, rinsed in phosphate buffered saline (PBS), and frozen at -80°C . Samples were lysed by sonication in Bicine/CHAPs buffer with 1X Aqueous and DMSO Inhibitor Cocktails (ProteinSimple) and placed in an end over end mixer at 4°C for 30 min. The lysates were purified by centrifugation at 13,000 rpm for 15 min at 4°C . The protein concentrations were determined using a bicinchoninic acid assay (Pierce).

Nano-Fluidic Proteomic Immunoassay for ERK1/2 Phosphorylation

Nano-fluidic immunoassay experiments were performed on the purified lysates using a Nanopro 1000 system (ProteinSimple).¹² Lysates were used at a final concentration of 100 $\mu\text{g/mL}$. Briefly, this system separates proteins by isoelectric point within a small capillary, and fixes them to the inner surface of the capillary after separation. This sensitive separation allowed differentiation between the isoforms of the ERK1/2 protein. Separated proteins were incubated with a pan-ERK1/2 antibody at a dilution of 1:200 for 2 h. This antibody binds to all isoforms of ERK1/2, so unphosphorylated, single phosphorylated, and double phosphorylated ERK1 and ERK2 could be probed using a single antibody (Supplemental Fig. 1). Proteins were incubated in HRP-conjugated secondary antibody at 1:100 for 1 h followed by chemiluminescence reagent. The emitted light was detected through the wall of the capillary with a 30 s exposure by a camera, outputting chemiluminescence intensity as a function of isoelectric point. Peak identification of the ERK1/2 isoforms was verified by registry with peaks from HeLa control samples and isoform specific antibodies. All antibodies and reagents were acquired from ProteinSimple. The results were quantified using Compass software (version 1.8.1) using the peak area function. Ratios of phosphorylated ERK1/2 (all phosphorylated isoforms) to total ERK1/2 (all isoforms) are reported and normalized as indicated in corresponding figure captions.

Western Blot for p38

Reducing SDS-PAGE was used to separate 20 μg of total protein per well. The proteins were transferred to a nitrocellulose membrane and blotted for phosphorylated p38 (Cell Signaling Technologies #9211) and total p38 (Cell Signaling Technologies #9212). The blots were blocked for 1 h in 5% dry milk, 0.1% Tween-20 in PBS, and then incubated for 1 h in primary antibody at 1:1000 in tris-buffered saline with 0.1% Tween-20 (TBS-T) and 5% bovine serum albumin. Blots were washed with TBS-T and incubated for 1 h in secondary antibody (1:2000 anti-rabbit IgG, GE Healthcare) in the blocking solution. Finally blots were washed in TBS-T and TBS and developed using Luminata Classico enhanced chemiluminescence (EMD Millipore). Films were scanned and digitized, and ImageJ (NIH) was used to quantify band density. The ratio of phosphorylated p38 to total p38 is reported and normalized as indicated in corresponding figure caption. The original Western blots with representative samples at each time point are included in Supplemental Fig. 2.

RNA Purification and cDNA Synthesis

Samples were harvested for real-time PCR 48 h after beginning the selected stretching regimens. For consistency, all tissues were harvested 24 h after a perturbation (onset of stretching or increment in amplitude). Tissues were excised from the wells and stored in RNeasy Lysis Buffer (RLT) (QIAGEN) at 4 °C. Samples were sonicated in RLT Plus, separated by centrifugation, and purified using the RNeasy Plus purification kit (QIAGEN) following the manufacturer's instructions. RNA concentration was measured by spectrometry (NanoDrop, University of Minnesota Biomedical Genomics Center). 275 ng of each RNA sample was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) and 100 ng of random hexamer primers. Controls without reverse transcriptase were also prepared for each sample.

Real-Time PCR

Real-time PCR was performed using the 7900HT Fast Real-time PCR system (Applied Biosystems, University of Minnesota Lillehei Heart Institute) using the cDNA equivalent of 14 ng RNA, forward and reverse primers at a concentration of 600 nM, and 1X SYBR Green reagent (KiCqStart, Sigma) in a total volume of 12.5 μ L. Primers were (5'–3'):

fGAPDH(CGCATCTTCTTTTGCCTCGCCAG),
rGAPDH(TGACAAGCTTCCCGTTCTCAGCC),
fCOL1A1(GCCAAGACGAAGACATCCCACCA),
rCOL1A1(CACGTCATCGCACAACACCTTGC),
fCOL3A1(AACGGTCTCAGTGGAGAACG), and
rCOL3A1(CCACTCTTGAGTTCAGGATGG).

Expression levels of COL1A1 and COL3A1 relative to static controls were determined by the comparative C_T method with GAPDH used as the reference gene.

Collagen and Cellularity Quantification

After 2 weeks of the applied stretching or static regimens, total collagen content was quantified using a hydroxyproline assay with a conversion factor of 7.46 mg of collagen for 1 mg 4-hydroxyproline.²⁰ A modified Hoescht assay was used to determine cellularity.²⁸

Statistics

All figures show mean $\pm$ standard deviation with at least $n = 3$ per group. Significance was determined using Student's t test for two groups and one-way ANOVA with the Tukey *post hoc* test for more than two groups in GraphPad Prism software for Windows with $p < 0.05$ reported as significant. Symbols or bars are used to indicate differences and are explained in the corresponding figure captions.

RESULTS

ERK1/2 and p38 MAPK Phosphorylation Time Course: Constant Amplitude Stretching

Phosphorylation time courses for ERK1/2 and p38 are shown in Fig. 3. Both ERK1/2 and p38 were transiently activated by 5% constant amplitude cyclic stretching with peak phosphorylation at 15 min. By 1 h, ERK 1/2 phosphorylation was only slightly elevated above static levels, while p38 phosphorylation remained elevated until the 6 h time point.

ERK 1/2 Pathway Reactivation: Intermittent Stretching

Figure 4 shows ERK1/2 reactivation due to a second onset of 5% amplitude cyclic stretching following a 15 min, 3 h, or 6 h rest period. The initial activation (5% amplitude for 15 min) is included for reference. ERK1/2 was unable to be reactivated fully after 15 min of rest, and there was a trend of increasing reactivation up to the 6 h time point where full reactivation was achieved. Note that each signaling experiment was carried out separately, so small discrepancies in the maximal ERK1/2 activation value are due to inter-experiment variation.

ERK1/2 Pathway Reactivation: Incrementally Increasing Strain Amplitude Stretching

Figure 5 shows ERK1/2 reactivation due to an increment in strain amplitude following 1 or 6 h of 5% constant amplitude cyclic stretching with the initial activation (5% amplitude for 15 min) included for reference. A 5% increment in strain amplitude was able to reactivate ERK1/2 after 6 h, but not after 1 h. Additionally, at the 6 h time point an increment in strain amplitude of only 1% (5% to 6% at 6 h) reactivated ERK1/2 to the same extent as the initial activation.

Transcription of Collagen Types I and III in Cyclically Stretched Samples

At 48 h there were no differences in transcription of collagen I (Fig. 6a) or collagen III (Fig. 6b) between the static, constant amplitude, intermittent, or incremental treatment groups ($p > 0.1$ assessed using one-way ANOVA with $n = 5$ per group). Data are pooled from two experimental repeats.

Collagen Deposition in Cyclically Stretched Samples

Total collagen per cell, based on cell number at harvest, was measured for tissues ($n = 6$ per group, pooled from two full experimental repeats) subjected to 2 weeks of constant amplitude, intermittent, and incremental cyclic stretching regimens. To account for variability between experiments, all collagen/cell values were normalized to the value for static controls cast and cultured at the same time. Across all castings, static control samples had collagen/cell values between 110 and 150 μg collagen/million cells. After 2 weeks, an increase in collagen per cell was measured in the 5% constant amplitude, intermittent (15 min at 5%, 6 h static, repeat), and incremental (5–13.5%) treatment groups compared to static controls (Fig. 7). The incremental treatment group showed the greatest increase in collagen per cell, 72% over static controls and 37% over the 5% constant amplitude samples. There was no difference in collagen per cell between the 5% constant amplitude and intermittent treatment groups.

DISCUSSION

In this model system for a fibrin-based engineered tissue, 5% strain amplitude cyclic stretching resulted in transient phosphorylation of both ERK1/2 and p38 MAPK, with peak phosphorylation at 15 min for both. Phosphorylation of ERK1/2 returned to near-static levels by 1 h, while p38 phosphorylation remained elevated for at least 3 h. Reactivation of ERK1/2 was achieved either by (i) the onset of an additional 5% amplitude stretching period after 6 h of static culture, or (ii) an increment of 1% in the strain amplitude after 6 h of constant amplitude cyclic stretching. Based on the ERK1/2 data, intermittent and incremental regimens were selected to repeatedly activate ERK1/2, based on the hypothesis that repeated activation would lead to increased collagen synthesis and deposition. After 48 h there were no differences in mRNA expression of collagen type I or III between any of the treatment groups. After 2 weeks, incrementally stretched tissues had the most collagen per cell, while intermittent samples showed no improvement over their continuously stretched counterparts. These data suggest that while ERK1/2 phosphorylation may be necessary for stretch-induced collagen transcription,¹³ it is not a sufficient predictor of final collagen content in this system.

The transient ERK1/2 activation in response to 5% continuous stretching noted above has been observed on a similar time scale for a wide variety of cell types in both 2D and 3D culture.^{7,11,13,14,18} As phosphorylation of ERK1/2 had been shown to be necessary for stretch-induced up-regulation of collagen transcription by cardiac fibroblasts,¹³ it was hypothesized that some perturbation to constant amplitude cyclic stretching may be able to repeatedly activate ERK1/2 and increase collagen synthesis for the dermal fibroblasts in our fibrin-based engineered tissues. After a 15 min period of 5% amplitude cyclic stretching, a 6 h static period was required before the fibroblasts were able to respond to a second onset of cyclic stretching and reactivate ERK1/2. Paxton *et al.* obtained a similar result in their engineered tendon system in which rat Achilles tendon fibroblasts also exhibited a 6 h refractory period before ERK1/2 could be reactivated by cyclic stretching.¹⁴ In addition, we found that an increment in strain amplitude of only 1% was also able to reactivate ERK1/2 after 6 h of constant amplitude cyclic stretching, but reactivation was not possible after only 1 h.

In order to provide additional insight into ERK1/2 dynamics, we also characterized the time course of p38 phosphorylation in our system. p38 activation has been implicated as having negative effects on stretch-induced collagen production. Papakrivopoulou *et al.* demonstrated that constitutively active p38 reduced promoter activity of procollagen1 $\alpha 1$, and that the combination of cyclic stretching and inhibition of p38 activation increased procollagen1 $\alpha 1$ transcription in cardiac fibroblasts.¹³ In our same fibrin-based engineered tissue, we showed that drug inhibition of p38 activation (SB 203580) increased stretching-induced ERK1/2 phosphorylation and collagen deposition.²⁷ In the present study, p38 phosphorylation was maximal after 15 min of 5% amplitude stretching, but its phosphorylation duration was longer than that of ERK1/2, returning to static levels only at the 6 h time point. Transient phosphorylation of p38 by cyclic stretching has been observed in other 2D and 3D systems as well.^{13,14} It is proposed that the cell's refractory period for ERK1/2 activation may be caused by the prolonged p38 phosphorylation and alleviated by the return of p38 to basal levels at 6 h.

Based on the dynamics of ERK1/2 phosphorylation, intermittent and incremental regimens were designed to maximize ERK1/2 activation throughout the culture period. Intermittently stretched samples were subjected to repeated periods of 5% amplitude cyclic stretching for 15 min followed by 6 h of static culture. The incrementally stretched samples were initially stretched with 5% strain amplitude, and the strain was increased by approximately 1% every 24 h. While an increment every 6 h would have been able to reactivate ERK1/2 more frequently, our goal was to apply the cyclic stretching regimen to long-term culture, and if the strain was incremented every 6 h for several weeks, the amplitude would soon have exceeded the capabilities of the Flexcell[®] system and likely damaged the tissue. The chosen intermittent and incremental regimens are within the capabilities of both this model system as well as our bioreactors for larger engineered cardiovascular tissues.^{22,23}

After 48 h of static culture, 5% constant amplitude cyclic stretching, or the chosen intermittent and incremental regimens, there were no differences in mRNA expression of collagen types I and III. The 48 h time point was chosen to allow differentiation between the regimens, and for consistency, intermittent and incremental samples were harvested 24 h

after a perturbation. In an engineered tissue strip made from polyglycolic acid seeded with vascular derived cells, Van Geemen *et al.* similarly observed only small differences in collagen type I and III mRNA after 24 h of continuous stretching or 3 h of stretching followed by 21 h rest.²⁶ However, in cyclic stretching studies where cells are simply adhered to the elastic substrate, significantly increased expression of collagen type I and/or III mRNA by fibroblasts has been observed on a similar time scale.^{8,13} It is unknown whether this discrepancy is due to the nature of our system, wherein cells may not be experiencing the applied strain due to strain-shielding by the fibrin, or if the increase in collagen transcription is transient and we have not yet identified the peak time point.

The selected intermittent and incremental regimens were applied to fibrin-based tissues for 2 weeks along with 5 and 13.5% constant strain amplitude regimens and a static control. Incremental stretching was the most beneficial in our system, resulting in a 37–72% increase in collagen per cell over the other regimens. The benefits of incremental stretching have been demonstrated previously in our lab, as Syedain *et al.* found that incremental stretching (5–15% with a 2.5% increment every 3–4 days) increased collagen deposition and tissue strength and stiffness in tubular engineered tissues similarly prepared from dermal fibroblasts in a sacrificial fibrin gel.²⁵ The current study suggests that this stretching regimen can be improved further by employing smaller, more frequent increments in strain amplitude.

While intermittent stretching did increase collagen per cell compared to static culture, it did not result in further improvement over 5% constant amplitude stretching. Rubbens *et al.* observed an increase in collagen per cell at 2 weeks in their intermittently stretched samples (3 h stretch, 3 h static) compared to static controls; however, a continuously stretched treatment group was not included in this study for comparison.¹⁶ In contrast to the present result, Paxton *et al.* found that intermittent stretching (10 min every 6 h) increased the percentage of collagen in their fibrin-based “sinews” approximately 20% over continuously stretched samples.¹⁴ However, there were several differences that may account for this discrepancy including the different cell type (tendon vs. dermal), fibrin gel composition, and strain amplitude (1 vs. 5%), as well as the sinew geometry in which the cells were allowed to compact the tissue strand, rather than being anchored on all sides in a circular well. Although the ERK1/2 dynamics appear to be favorable for the intermittent regimen in the present study, it may be that the prolonged static period has detrimental effects on collagen deposition in our system. Additionally, nutrient transport may be enhanced *via* convection during cyclic stretching of the highly hydrated fibrin gel and developing tissue,^{1,4} so it is possible that the intermittent samples did not experience this nutrient and metabolite transport enhancement during the 6 h static periods, offsetting any benefit of repeated ERK1/2 activation.

Although ERK1/2 was being repeatedly activated in both intermittent and incremental stretching, only incremental stretching resulted in an increase in collagen per cell over both static samples and samples stretched with constant strain amplitude. Clearly in our system, ERK1/2 phosphorylation alone is not a sufficient indicator of final collagen content. Additionally, transcription of collagen type I and III after 48 h does not seem to be predictive of collagen per cell at 2 weeks. It should be noted that the measured collagen content in a

tissue is not only a function of collagen transcription, but also translation and the many intervening collagen processing and transport steps prior to incorporation into the extracellular matrix,³ as well as collagen degradation,^{2,17} all of which may also be affected by the different cyclic stretching regimens.

In summary, the transient nature of ERK1/2 and p38 phosphorylation was characterized in a fibrin-based engineered tissue model, and the results were used to develop intermittent and incremental strain amplitude cyclic stretching regimens. While ERK1/2 was repeatedly activated in both regimens, incremental stretching resulted in a greater increase in collagen per cell, indicating that ERK1/2 phosphorylation alone is not a sufficient predictor of this long-term outcome of interest. The data suggest that an incremental stretching regimen with small, frequent increments is most beneficial for collagen production in this system, and motivate the use of a similar regimen in our engineered cardiovascular tissue bioreactor systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

ANOVA	Analysis of variance
COL1A1/COL3A1	Collagen I α 1/collagen III α 1
DMSO	Dimethylsulfoxide
ERK1/2	Extracellular signal-regulated kinase 1/2
MAPK	Mitogen-activated protein kinase
PBS	Phosphate buffered saline
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline

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FIGURE 1.

Schematic of Flexcell® tension system for uniaxial cyclic stretching. (a) and (b) depict the unstretched top and side views, respectively, for a single well. The stretched configuration, produced by the application of vacuum beneath the well, is shown in (c). A sample fibrin-based tissue construct is shown in (d), secured to the perimeter of the silicone membrane by a foam ring with the black bar indicating 1 cm. The strain field is shown for 5% applied strain (e). For analysis, four 6 mm biopsy punches are taken from the uniaxially stretched region (blue rectangle).

FIGURE 2. Schematics of strain vs. time for 48 h cyclic stretching regimens. (a) constant amplitude, (b) intermittent, and (c) incremental cyclic stretching. 0.5 Hz frequency and 15% duty cycle were used in all regimens.

FIGURE 3.

(a) ERK1/2 and (b) p38 phosphorylation time courses during continuous cyclic stretching with 5% strain amplitude. All data are normalized to the 15 min static value. Horizontal bars indicate differences ($p < 0.05$) using Student's t test between time-matched stretched and static samples ($n = 3$ per group).

FIGURE 4.

Reactivation of ERK1/2 15 min after onset of a second cyclic stretching period following rest durations of 15 min, 3 h, and 6 h. ERK1/2 activation at the end of the initial 15 min stretch is also shown. All data are normalized to the 15 min static value. Solid horizontal bars indicate differences ($p < 0.01$) and dashed horizontal bars indicate trends ($p < 0.09$) using one-way ANOVA with Tukey *post hoc* test ($n = 3$ per group).

FIGURE 5.

Activation of ERK1/2 15 min following an increment in the strain amplitude. All data are normalized to the pre-perturbation value immediately prior to the strain increment. Horizontal bars indicate differences between groups ($p < 0.01$) using one-way ANOVA with Tukey *post hoc* test ($n = 3$ per group).

FIGURE 6.

(a) Collagen I and (b) collagen III mRNA levels at 48 h in constant amplitude, intermittent, and incrementally stretched samples expressed relative to static control. GAPDH was used as the housekeeping gene. No significant differences were observed between groups ($p > 0.1$ using one-way ANOVA, $n = 5$ per group pooled from two experimental repeats).

FIGURE 7.

Total collagen per cell at harvest for static, constant amplitude, intermittent, and incremental regimens after 2 weeks. Data were pooled from two experimental repeats and normalized to respective static controls. \$Difference from static ($p < 0.01$), #difference from all other stretched treatment groups ($p < 0.01$) using one-way ANOVA with Tukey *post hoc* test ($n = 6$ per group).