

# Tensile Stimulation of Murine Stem Cell–Collagen Sponge Constructs Increases Collagen Type I Gene Expression and Linear Stiffness

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The objectives of this study were to determine how tensile stimulation delivered up to 14 days in culture influenced type I collagen gene expression in stem cells cultured in collagen sponges, and to establish if gene expression, measured using a fluorescence method, correlates with an established method, real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Using a novel model system, mesenchymal stem cells were harvested from six double transgenic mice in which the type I and type II collagen promoters were linked to green fluorescent protein-topaz and enhanced cyan fluorescent protein, respectively. Tissue-engineered constructs were created by seeding  $0.5 \times 10^6$  mesenchymal stem cells onto type I collagen sponge scaffolds in a silicone dish. Constructs were then transferred to a custom pneumatic mechanical stimulation system housed in a standard incubator and stimulated for 5 h/day in tension for either 7 or 14 days using a repeated profile (2.4% peak strain for 20 s at 1 Hz followed by a rest period at 0% strain for 100 s). Control specimens were exposed to identical culture conditions but without mechanical stimulation. At three time points (0, 7, and 14 days), constructs were then prepared for evaluation of gene expression using fluorescence analysis and qRT-PCR, and the remaining constructs were failed in tension. Both analytical methods showed that constructs stimulated for 7 and 14 days showed significantly higher collagen type I gene expression than nonstimulated controls at the same time interval. Gene expression measured using qRT-PCR and fluorescence analysis was positively correlated ( $r = 0.9$ ). Linear stiffness of stimulated constructs was significantly higher at both 7 and 14 days than that of nonstimulated controls at the same time intervals. Linear stiffness of the stimulated constructs at day 14 was significantly different from that of day 7. Future studies will vary the mechanical signal to optimize type I collagen gene expression to improve construct biomechanics and *in vivo* tendon repair.

## Introduction

**I**NJURIES TO SOFT TISSUES (tendon, ligament, and meniscus) represent almost half of the 33 million musculoskeletal injuries occurring in the United States each year<sup>1–6</sup> and often lead to surgery.<sup>7,8</sup> Inadequate healing of these injuries places patients at risk to dysfunction and disability. Tissue engineering<sup>9</sup> is an appealing conceptual alternative when conventional repair techniques (autografts, allografts, xenografts, and prostheses<sup>10–18</sup>) prove unsatisfactory.

Tissue-engineered constructs made by seeding mesenchymal stem cells (MSCs) in scaffolds are being used to repair soft

tissue defects,<sup>19–24</sup> but these are usually vulnerable early after surgery because they lack the stiffness and strength of native tissue structures.<sup>22,24</sup> To address this problem, investigators have been applying principles of functional tissue engineering<sup>25,26</sup> to use recorded *in vivo* tissue forces<sup>27,28</sup> as design parameters for new generations of reparative tissue constructs. Some in functional tissue engineering<sup>25,26</sup> have also delivered aspects of these *in vivo* mechanical signals to precondition constructs while still in culture. Such preconditioning improves the material properties of constructs for soft tissue engineering by increasing the synthesis of extracellular matrix proteins such as collagen.<sup>29–32</sup>

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Unfortunately, any mechanical and biological benefits of mechanical stimulation are usually not assessed until the end of mechanical stimulation in culture.<sup>23,33,34</sup> Typically, 4 or more weeks may be required before a destructive test is performed to judge whether a stimulus upregulates gene expression or increases protein accumulation. This delay hampers the ability of the investigator to quickly optimize the stimulus in culture. A strategy that permits investigators to monitor near real-time gene expression throughout the tissue engineering process could allow them to either modify the stimulus or terminate an experiment leading to an undesirable outcome. Unfortunately, no such method currently exists for tissue engineers to rapidly assess how mechanical (chemical, etc.) stimuli affect near real-time gene expression.

To address this need, we bred double transgenic mice having type I and type II collagen promoters linked to green fluorescent protein-topaz (GFP-T) and enhanced cyan fluorescent proteins (ECFP), respectively. These intracellular proteins are expressed when the type I and type II collagen genes are activated, respectively. Such fluorescent proteins have recently served as visual reporters for transgene activity and can be viewed in real-time in living tissues.<sup>35–39</sup> Investigators have used these promoter-fluorescent protein reporters to (1) examine early embryonic development, (2) conduct cell culture studies, and (3) identify cells within a defined lineage in primary cell culture.<sup>35–39</sup> Another value of this technology, when applied to tissue engineering, could be the ability to track how mechanical stimulation affects near real-time collagen gene expression during maturation of constructs in culture.

The first objective of this study was to determine how a controlled mechanical stimulus applied to a stem cell–collagen sponge construct in culture influences the expression of the type I collagen gene as well as linear stiffness. The second objective was to establish if gene expression, measured using a fluorescence method, correlates with an established method, real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). We hypothesized that (1) mechanical stimulation would increase type I collagen gene expression as well as linear stiffness and (2) the fluorescence data and qRT-PCR data would be positively correlated.

## Experimental Design

Bone marrow was harvested from both the femur and tibia of six 6–8-week-old double transgenic mice as previously described.<sup>40,41</sup> After isolating and expanding the MSCs to second or third passage using a previously published protocol,<sup>40</sup> 15 constructs were created from each animal by seeding the cells at a concentration of  $0.5 \times 10^6$  cells/construct in a type I collagen sponge (P1076; Kensey Nash Corporation, Exton, PA). Hence, a total of 90 samples were created using cells from six animals. For each animal, the resulting 15 constructs were assigned to five treatment groups: day 0 nonstimulated, day 7 stimulated, day 7 nonstimulated, day 14 stimulated, and day 14 nonstimulated (Table 1). Thus, three constructs were available per animal from each treatment group. One construct was assigned to evaluate GFP-T fluorescence in a spectrophotometer (measured in relative fluorescence units [RFU]),<sup>42</sup> one construct was assigned to evaluate changes in type I collagen and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene expression using real-time qRT-PCR,<sup>43</sup> and the remaining construct was failed in tension to determine its linear stiffness.<sup>44</sup> Stimulated (S) constructs were stretched between 0% and 2.4% peak strain<sup>27</sup> at 1 Hz for 20 s followed by a 100-s rest period. This pattern was repeated for 5 h/day. Peak amplitude, frequency, and duty cycle were chosen based on studies performed in our lab on the effects of tensile stimulation on rabbit MSCs seeded in collagen scaffolds.<sup>43,44</sup> The nonstimulated (NS) constructs served as controls. The sample size of six animals was sufficient to detect a 30% treatment effect with an 80% power. Differences were considered significant for  $p < 0.05$ .

## Materials

### Double transgenic mice

The mouse containing the transgene pOBCol3.6GFPtpz was acquired courtesy of David Rowe, University of Connecticut Health Center. This transgene contains a 3.6 kb fragment of the rat *col1a1* promoter, enhancer sequence, and GFP-T. GFP-T expression in these transgenic mice is evident in skin, tendon, and osseous tissues.<sup>37</sup>

TABLE 1. TREATMENT CONDITIONS, RESPONSE MEASURES, AND ASSIGNMENT OF CONSTRUCTS

Treatment conditions	Constructs/treatment condition/animal	Response measures (assignment of constructs)
Day 0 nonstimulated	3	Gene expression by qRT-PCR ( $n = 1$ ) Gene expression by RFUs ( $n = 1$ ) Linear stiffness ( $n = 1$ )
Day 7 stimulated	3	Gene expression by qRT-PCR ( $n = 1$ ) Gene expression by RFUs ( $n = 1$ ) Linear stiffness ( $n = 1$ )
Day 7 nonstimulated	3	Gene expression by qRT-PCR ( $n = 1$ ) Gene expression by RFUs ( $n = 1$ ) Linear stiffness ( $n = 1$ )
Day 14 stimulated	3	Gene expression by qRT-PCR ( $n = 1$ ) Gene expression by RFUs ( $n = 1$ ) Linear stiffness ( $n = 1$ )
Day 14 nonstimulated	3	Gene expression by qRT-PCR ( $n = 1$ ) Gene expression by RFUs ( $n = 1$ ) Linear stiffness ( $n = 1$ )

Plasmid *pCol2-ECFP* was derived by replacing the  $\beta$ -gal gene with ECFP (Clontech, Palo Alto, CA), in the expression gene containing the mouse type II collagen promoter and enhancer<sup>45</sup> (provided by W. Horton, Northeastern Ohio College of Medicine). This *pCol2-ECFP* construct was injected into mice blastocysts. Founder mice showed high levels of ECFP expression in cartilaginous tissue.

Mice transgenic for either *pOBCol3.6GFPtpz* or *pCol2-ECFP* were then bred to produce double transgenic animals. No apparent phenotypic differences were observed between the double and nontransgenic mice. Both GFP-T and ECFP have a 24-h half-life, which indicates that fluorescence decays to half of its original by 24 h.

#### *Mesenchymal stem cells*

Mice were euthanized by CO<sub>2</sub> according to Institutional Animal Care and Use Committee (IACUC) protocols. Their long bones were excised, transected, and placed in adapted centrifuge tubes.<sup>40</sup> Marrow was extracted by centrifugation for 1 min at 400 g. Extracted cells were plated at 7.5×10<sup>6</sup> cells/100 mm dishes and fed media with supplements (MesenCult™ Basal Medium for Mouse Mesenchymal Stem Cells and Mouse Mesenchymal Stem Cell Stimulatory Supplements; Stem Cell Technologies, Vancouver, Canada). Cells were allowed to attach for 2 days, washed with phosphate-buffered saline (PBS; Gibco BRL/Life Technologies, Gaithersburg, MD) to remove nonadherent cells, and fed fresh media. Adherent cells were allowed to grow for 7–10 days before first passage. Cells were then trypsinized and replated at 1×10<sup>6</sup> cells/100 mm dishes and cultured for 1 additional week. Cells at P2 and P3 were then subcultured at a density of 1×10<sup>6</sup> cells/100 mm dish and cultured for another week.

#### *Scaffold and construct preparation*

Sterilized collagen type I sponges (94% pore volume; 62  $\mu$ m mean pore diameter) were provided by Kensey Nash Corporation. Scaffolds were cut from these sponges such that they fit in the wells of a silicone dish.<sup>44</sup> Before seeding with cells, each scaffold was soaked overnight in PBS and then placed in each well of the dish. MSCs were suspended in media at a concentration of 2×10<sup>6</sup> cells/mL. Two hundred fifty microliters of this cell suspension was pipetted on top of each scaffold. All constructs were placed in an incubator (Steri-Cult Model 3033; Forma Scientific, Marietta, OH; 37°C, 5% CO<sub>2</sub>, and 95% relative humidity) for 2 weeks and fed three times weekly with advanced DMEM, 1% antibiotic/antimycotic, 1% glutamax, and 10% FBS.

## Methods

#### *Mechanical stimulation*

After 2 days of incubation, the silicone dishes containing the constructs to be stimulated were placed into a computer-controlled five-station pneumatic mechanical stimulation system housed within an incubator (Steri-Cult Model 3033; Forma Scientific)<sup>44</sup> and stretched using the pattern described in Experimental Design section. Dishes were removed from the incubator after either 7 or 14 days, and constructs were then prepared for either evaluation of gene expression using fluorescence analysis and qRT-PCR or failure testing in tension.

#### *Fluorescence microscopy*

Each construct was washed in PBS for 1 h with gentle shaking to remove media and then visualized for GFP-T fluorescence in a fluorescence microscope (Axiovert 25; Carl Zeiss, Göttingen, Germany) equipped with filter sets for visualizing GFP-T and ECFP (XF104-2 and XF114-2, respectively; Omega, Brattleboro, VT). To rule out cell auto-fluorescence, each construct was also visualized for rhodamine using a specific filter set (11002VZ; Chroma, Rockingham, VT).

#### *Spectrophotometric analysis*

After imaging, constructs were digested for 40 min in 4 mL of 100 U/mL type I collagenase (Sigma Chemical, St. Louis, MO) in an incubator with gentle shaking. The resulting collagen fragments were further digested for 20 min in 2 mL of trypsin (Invitrogen–Gibco BRL/Life Technologies, Gaithersburg, MD). Digests were then centrifuged at 2000 rpm for 6 min, and the supernatant was discarded. The remaining pellets were re-suspended in 1 mL of PBS and filtered using a 100  $\mu$ m nylon mesh (BD Falcon, Bedford, MA). These digests were then pipetted into a black-bottom microplate (200  $\mu$ L per well in three wells). GFP-T fluorescence in these digests was quantified in RFUs<sup>42</sup> by reading the microplate in a spectrophotometer (Spectra Max M2; Molecular Devices, Sunnyvale, CA) using an excitation wavelength of 491 nm and an emission wavelength of 529 nm, with a cut off filter of 519 nm. Pilot studies on GFP-T expressing fibroblasts harvested from the ribs of new born mice did not show any qualitative differences in GFP-T fluorescence when subjected to the above-mentioned collagenase, trypsin digestion and filtration steps.

#### *RNA extraction and conventional gene expression analysis*

RNA extraction and conventional gene expression analysis were performed according to previously published protocols.<sup>43</sup> Briefly, the constructs were stored in RNAlater (Qiagen, Valencia, CA) for 2 days at –4°C. The RNAlater was aspirated carefully, and the constructs were frozen in liquid nitrogen to prevent RNA degradation. RNA from each construct was extracted using an RNeasy mini kit (Qiagen). Conventional RT reaction (MuLV reverse transcriptase; Applied Biosystems, Foster City, CA) was performed<sup>43</sup> to create first-strand complementary deoxyribonucleic acid (cDNA). Mouse-specific primers were used for type I collagen and *GAPDH* gene expression. The forward and reverse primer sequences and the resultant products are summarized in Table 2. Before use in the experiment, all primers were tested under conventional and real-time qRT-PCR conditions to ensure specificity with only one band by electrophoresis. The conventional PCR of the reverse-transcribed RNA was performed according to previously published protocols.<sup>43</sup> The amplified products were verified by 2% agarose gel electrophoresis in Tris-acetate-EDTA and SYBR safe DNA gel stain (Invitrogen–Molecular Probes, Eugene, OR).

#### *Real-time qRT-PCR*

Real-time qRT-PCR was performed to quantify mRNA levels of the genes according to previously published protocols.<sup>43</sup> qRT-PCR was performed by monitoring SYBR Green fluorescent dye (SYBR Green PCR master mix; Applied Biosystems)

TABLE 2. SEQUENCE OF PRIMERS USED FOR GENE EXPRESSION ANALYSIS AND PRODUCT SIZE IN BASE PAIRS

Gene	Primer sequence	Product size (bp)	Annealing temperature (°C)
Collagen I	TGT GTG CGA TGA CGT GCA AT GGG TCC CTC GAC TCC TAC A	132	58
GAPDH	AAT GGT GAA GGT CGG TGT G CCT TCG GGT AGT GGT AGA AG	200	55

bound to double-strand DNA with a continuous fluorescence detector (DNA Engine Opticon 2 System; MJ Research Incorporated, Waltham, MA). All samples were run in duplicate since differences in cycle number values between samples were less than 0.3 cycles. Standard curves were created for each target gene to quantify gene expression for each cell–sponge construct. The absolute amount of the corresponding gene mRNA in each construct was obtained from the corresponding gene standard curve. Gene expression was normalized by calculating the ratio between type I collagen and *GAPDH* genes for each sample.

#### Biomechanical evaluation of the constructs

After 0, 7, or 14 days in culture, constructs for biomechanical analysis were placed in cryovials and frozen at  $-80^{\circ}\text{C}$ . On the day of testing, constructs were removed from the freezer and thawed to room temperature. Small squares of gauze were used to cover the postholes in each end of the constructs. These gauzes provided a surface that would minimize slippage and the premature failure of the specimens in the grips. Constructs were inserted into custom grips in a materials testing system (100R6; Testresources, Shakopee, MN) and fixed at a gauge length of 12 mm to minimize Saint Venant's gripping effects on specimen properties.<sup>46</sup> The specimens were then failed under displacement control at a rate of 10%/s in a PBS bath at room temperature.<sup>20</sup> Linear stiffness was calculated from the linear region of the force–elongation curve generated by the constructs during failure testing.

#### Statistical analysis

The duplicate gene expression measures and triplicate RFU measures for each cell line from each of the six mice were averaged before statistical analysis. Gene expression and mechanical properties of the stimulated versus nonstimulated stem cell–collagen sponge constructs were compared using a mixed-effects model (SAS proc mixed) with culture time and stimulation as fixed factors and animal as a random factor.<sup>47</sup> All conclusions regarding the significance of mechanical stimulation on gene expression and mechanical properties were made for  $p < 0.05$ .

#### Results

Cells did not fluoresce in any of the constructs at day 0. However, GFP-T fluorescent cells were found in all 7- and 14-day constructs. No fluorescence signal was evident when using the ECFP and rhodamine filter sets, indicating the specificity of the GFP-T signal and the lack of detectable levels of type II collagen gene expression, even in the end regions where the construct attaches to the posts. The fluorescing cells appeared elongated in all constructs at both time periods of stimulation (Fig. 1A–D). Elongated cells in both the controls

(Fig. 1A, C) and the S constructs (Fig. 1B, D) were randomly oriented with respect to the direction of applied tensile strain.

Tensile stimulation significantly increased measured RFU values at both time points. At day 7, RFU values in the S constructs increased by 2.2-fold compared to those for the NS constructs ( $p = 0.0002$ ) (Fig. 2). At day 14, RFU values in the S constructs increased by 1.5-fold compared to those for the NS constructs ( $p = 0.01$ ) (Fig. 2). RFU values for the S versus NS construct groups averaged  $6.3 \pm 0.22$  versus  $2.8 \pm 0.69$  at day 7 and  $4.63 \pm 0.16$  versus  $3.04 \pm 0.97$  at day 14 (mean  $\pm$  SD). No difference was seen between NS constructs at days 7 and 14 ( $p > 0.5$ ) (Fig. 2). There was a significant decrease in RFUs in S constructs between days 7 and 14 ( $p = 0.0001$ ) (Fig. 2).

Seven and 14 days of *in vitro* mechanical stimulation significantly increased type I collagen gene expression in stem cell–collagen sponge constructs measured using qRT-PCR. S constructs showed a 12-fold increase in type I collagen gene expression ( $p = 0.001$ ) relative to NS controls at day 7 and a 5-fold increase at day 14 ( $p = 0.01$ ) (Fig. 3). Type I collagen gene expression as measured by qRT-PCR for the S versus NS construct groups averaged  $7.8 \pm 2.6$  versus  $0.6 \pm 0.5$  at day 7 and  $2.8 \pm 1.1$  versus  $0.5 \pm 0.4$  at day 14 (mean  $\pm$  SD). No difference was seen between NS constructs at days 7 and 14 ( $p > 0.5$ ) (Fig. 3), but there was a significant decrease in type I collagen gene expression in the S constructs between days 7 and 14 ( $p = 0.005$ ) (Fig. 3). Mechanical stimulation did not significantly increase *GAPDH* gene expression ( $p > 0.5$ ) at day 7 or 14 in culture. *GAPDH* values for the S versus NS construct groups averaged  $5.8 \text{ E-}07 \pm 1.7 \text{ E-}07$  versus  $5.6 \text{ E-}07 \pm 1.3 \text{ E-}07$  at day 7 and  $5.4 \text{ E-}07 \pm 1.4 \text{ E-}07$  versus  $5.3 \text{ E-}07 \pm 1.2 \text{ E-}07$  at day 14 (mean  $\pm$  SD). The *GAPDH* values for day 0 constructs were  $5.8 \text{ E-}07 \pm 1.7 \text{ E-}07$ . The results from qRT-PCR and fluorescence analysis were positively correlated ( $R^2 = 0.79$ ; Fig. 4). The slope of the linear regression curve was 0.56.

Both 7 and 14 days of tensile stimulation increased the construct's linear stiffness compared to NS constructs, but had no effect on dimensions ( $p > 0.05$ ; Table 3). S constructs showed a 2.3-fold increase in linear stiffness ( $p = 0.0006$ ) relative to NS controls at day 7 and a 1.6-fold increase at day 14 ( $p = 0.003$ ) (Fig. 5). Linear stiffness for the S versus NS construct groups averaged  $0.035 \pm 0.006 \text{ N/mm}$  versus  $0.015 \pm 0.003 \text{ N/mm}$  at day 7 and  $0.029 \pm 0.004 \text{ N/mm}$  versus  $0.0175 \pm 0.004 \text{ N/mm}$  at day 14 (mean  $\pm$  SD). No difference was seen between NS constructs at days 7 and 14 ( $p = 0.1$ ) (Fig. 5). Tensile stiffness values of both 7- and 14-day constructs were significantly less than the corresponding stiffness values of day 0 constructs ( $p = 0.005$ ) (Fig. 5).

#### Discussion

Understanding how to control matrix production and assembly during culture is one of the primary objectives in developing functional tissue-engineered replacements for

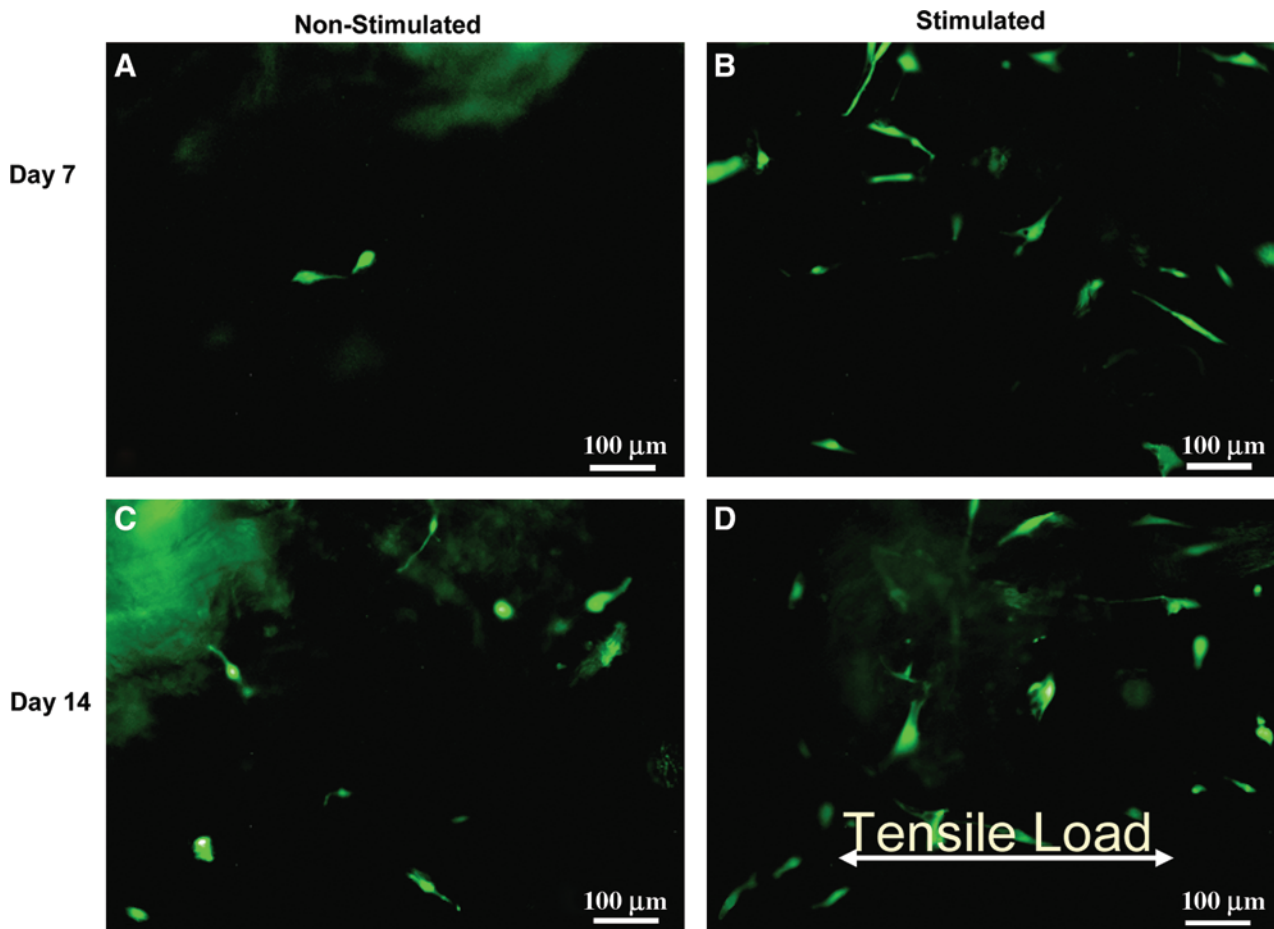


FIG. 1. Elongated GFP-T fluorescent cells in (A) nonstimulated constructs and (B) stimulated constructs at day 7 as well as (C) nonstimulated constructs and (D) stimulated constructs at day 14. Original magnification,  $\times 10$ . Greater fluorescence was observed in the stimulated versus nonstimulated control constructs at 7 days. Color images available online at [www.liebertonline.com/ten](http://www.liebertonline.com/ten).

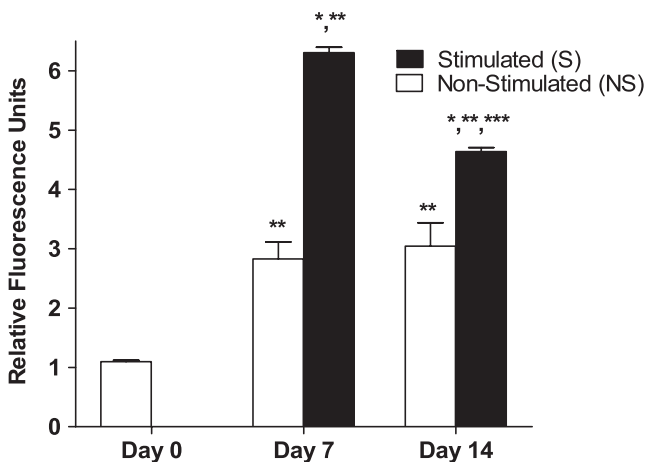


FIG. 2. Tensile stimulation increased GFP-T expression by RFUs. \*Significantly different from nonstimulated (NS) controls at same time interval ( $p < 0.02$ ). \*\*Significantly different from day 0 ( $p < 0.00001$ ). \*\*\*Significantly different from day 7 stimulated (S) constructs ( $p < 0.00002$ ). Data represented as mean  $\pm$  standard deviations;  $n = 6$  for all groups.

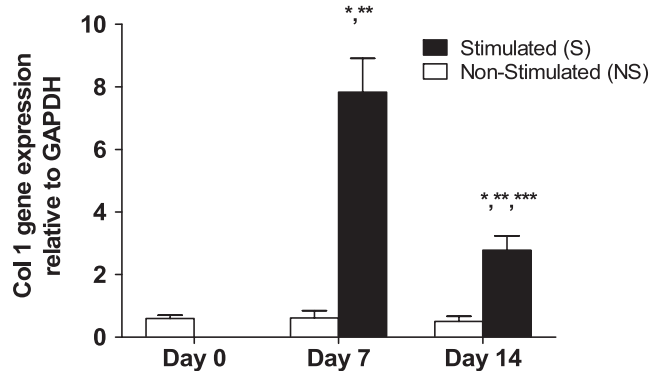
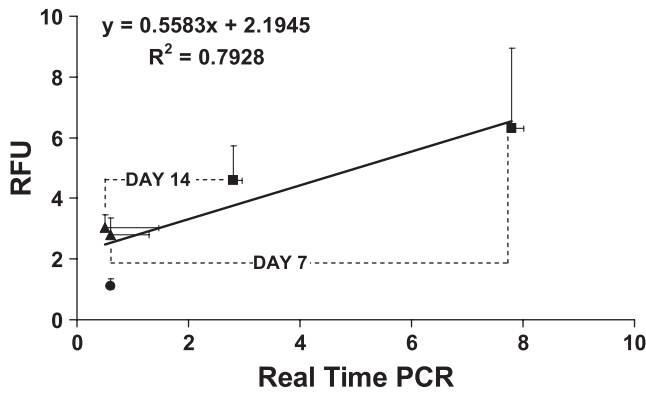


FIG. 3. Tensile stimulation increased type I collagen gene expression by qRT-PCR. \*Significantly different from non-stimulated (NS) controls at same time interval ( $p < 0.02$ ). \*\*Significantly different from day 0 ( $p < 0.0003$ ). \*\*\*Significantly different from day 7 stimulated (S) constructs ( $p < 0.006$ ). Data represented as means  $\pm$  standard deviations;  $n = 6$  for all groups.



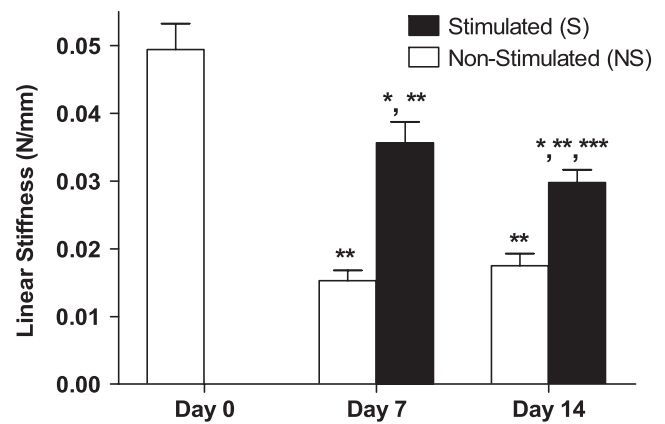
**FIG. 4.** Type I collagen gene expression as measured by RFUs and by qRT-PCR were positively correlated ( $R^2 = 0.79$ ). Each point represents the average of six samples (means  $\pm$  standard deviations). Circle denotes day-0 constructs, triangles denote nonstimulated constructs, and squares denote stimulated constructs.

load-bearing connective tissues.<sup>25</sup> Although externally applied deformations have been shown to strengthen tissue-engineered constructs,<sup>29–32,48</sup> long culture times are typically required for cells to synthesize enough matrix to improve resulting mechanical properties that can remotely approach the levels of the native tissue. This slow process could reflect the fact that mechanical stimuli have yet to be optimized for inducing such cellular-based benefits. In particular, we still know little about how various components of the stimulation profile (e.g., strain amplitude, frequency, and duty cycle) affect construct quality and functionality in culture and after surgery. We do know, however, that determining the importance of each component can be a very expensive and time-consuming process. Therefore, a strategy to evaluate how these factors influence the production of extracellular matrix components like type I (tendon and ligament mid-substance) or type II collagen (soft tissue insertions, articular cartilage, meniscus, and intervertebral disc) would appear to be a very beneficial step in this development effort. It was for these reasons that we bred the double transgenic mouse model for the current study. By using this fluorescence technology, we could repeatedly track changes in gene expression during rest periods between bouts of mechanical stimulation in culture rather than use destructive microarray or qRT-PCR techniques that could only detect final changes after stimulation. Also, this technology provides a unique way to detect near real-time changes in expression in culture by one or both of these genes and to readjust the mechanical signal applied to

**TABLE 3. TENSILE STIMULATION DID NOT SIGNIFICANTLY AFFECT CONSTRUCT DIMENSIONS ( $P > 0.05$ )**

Evaluation time points	Length (mm)	Width (mm)	Thickness (mm)
Day 0	23.2 $\pm$ 0.5	8.5 $\pm$ 0.7	2.7 $\pm$ 0.1
Day 7 stimulated	23.8 $\pm$ 0.4	8.3 $\pm$ 0.5	2.6 $\pm$ 0.4
Day 7 nonstimulated	23.1 $\pm$ 0.6	8.1 $\pm$ 0.5	2.5 $\pm$ 0.5
Day 14 stimulated	23.6 $\pm$ 0.3	8.0 $\pm$ 0.6	2.5 $\pm$ 0.5
Day 14 nonstimulated	23.2 $\pm$ 0.4	8.1 $\pm$ 0.9	2.4 $\pm$ 0.5

Shown are means  $\pm$  standard deviations for all three parameters.



**FIG. 5.** Tensile stimulation increased linear stiffness. \*Significantly different from nonstimulated controls at same time interval ( $p < 0.004$ ). \*\*Significantly different from day 0 ( $p < 0.0005$ ). \*\*\*Significantly different from day 7 stimulated ( $p < 0.05$ ). Data represented as means  $\pm$  standard deviations;  $n = 6$  for all groups.

the evolving construct to obtain the desired spatial and temporal gene expression patterns.

This study utilizing MSCs harvested from these mice was designed to evaluate the effects of uniaxial tension on gene expression and biomechanics in cell–collagen constructs. We hypothesized that (1) mechanical stimulation would increase type I collagen gene expression as well as linear stiffness and (2) the fluorescence data and qRT-PCR data would be positively correlated. In our study, we found that tensile stimulation increased GFP-T fluorescence and type I collagen gene expression compared to controls, thus validating the first part of our hypothesis. The increases in both measures (Figs. 2 and 3) that we observed in S constructs reinforce the importance of stimulating a cell–scaffold construct with a defined mechanical signal in a cell culture system. The fact that the tensile stimulus chosen in this study significantly increased GFP-T fluorescence in the construct's cells without affecting ECFP activity suggests that tensile stimulation did not affect collagen type II gene expression.

Several reasons could explain the increases in GFP-T fluorescence (type I collagen gene expression) we observed after 7 and 14 days of tensile stimulation. Tensile strains are known to trigger the creation of cell–surface stretch receptors and integrins, activating a cascade of genes responsible for the synthesis and secretion of extracellular matrix components.<sup>49</sup> Of course, the stimulus we applied may have also produced other cellular changes (cell proliferation, mass transfer rates of nutrients, metabolites, and waste materials,<sup>50</sup> and expression and synthesis of various growth factors and cytokines), some of which are known to be sensitive to mechanical stimulation.<sup>51–54</sup> Several of these factors will be examined in greater detail now that we have demonstrated a significant positive effect of mechanical stimulation on gene expression in this model system.

Our findings are generally consistent with previous reports examining how tensile stimulation affects gene expression of cells and cell-based constructs, although the specific treatments, conditions, and response measures vary greatly among studies. Investigators using human fibroblasts<sup>32,55,56</sup> as

well as human mesenchymal<sup>23,57</sup> and human and bovine stromal cells<sup>50</sup> have demonstrated positive effects of mechanical stimulation on type I collagen gene expression and protein synthesis. However, these studies were conducted either using cells on monolayer<sup>57</sup> or after placing cells in three-dimensional polyurethanes<sup>32</sup> and collagen gels.<sup>23,50</sup> These differences in local matrix environment can be important to driving cell phenotype and gene expression patterns. The 2.4% peak strain that we delivered in the current study is far less than the 5% and 10%<sup>32,55–57</sup> peak strain treatments used by other groups and not as complex as reported biaxial strain patterns.<sup>50</sup> While the studies by Park *et al.*<sup>57</sup> and Noth *et al.*<sup>23</sup> matched the frequency (1 Hz) delivered in our study and the 14-day treatment assessment<sup>23,50</sup> was identical to our time interval, other aspects of these studies differed markedly. In fact, direct comparison of our results to those used by other investigators was difficult given the broad range of frequencies (0.0167 Hz,<sup>50</sup> 0.25 Hz,<sup>55</sup> and 0.167 Hz<sup>56</sup>) and time assessment periods (from 6 to 24 h)<sup>32,55–57</sup> reported in the literature. Studies will ultimately need to either limit these stimulation conditions across studies or examine the interactive effects of these treatment components if we are to understand how to control and optimize gene and protein expression in tissue engineering.

Our findings are also different from a previous study in the literature.<sup>50</sup> Although Altman *et al.*<sup>50</sup> examined mechanically induced changes in gene expression using stem cells over the same time interval used in the current study, they used collagen gels rather than sponges, and found that tensile stimulation significantly increased type I collagen gene expression at day 14 but not at day 7. Such differences between our two outcomes could be due to different cell sources (bovine vs. murine) or mechanical strain patterns (combined axial and torsional strains vs. uniaxial tensile strains), although other differences may have also contributed. For example, a cell's biosynthetic activity is strongly related to its ability to attach to its collagen scaffold.<sup>58–63</sup> Once accomplished, subsequent collagen synthesis within these three-dimensional collagen lattices is then regulated by transcriptional and posttranscriptional mechanisms mediated by  $\alpha$  and  $\beta$  integrins.<sup>61</sup> Similarly, synthetic scaffolds can also provide a conducive environment, as shown by Chastain *et al.*,<sup>64</sup> who found increases in  $\alpha 6$  and  $\beta 6$  gene expression over a 5-week culture period using MSCs deposited in PLGA scaffolds. Regardless of scaffold type, increases in type I collagen gene expression will clearly require the appropriate signals and microenvironment for effective cell-based therapy.

We also found in this study that collagen type I gene expression measured using real-time qRT-PCR and fluorescence analysis were positively correlated (Fig. 4), thus validating our second hypothesis. This correlation serves as a validation for using fluorescence as an indicator of average gene expression within tissue-engineered constructs. Additional correlations of this type may soon allow investigators to use fluorescence as a reasonable estimate of expression of genes like type II collagen than using more traditional approaches like qRT-PCR. These correlations may also be possible at even finer levels in the tissue-engineered constructs, including expression by groups of cells or even individual cells in the future.

We also found that applying both 7 and 14 days of tensile stimulation increased the construct's linear stiffness compared to NS constructs in culture (Fig. 5) validating the last part of our first hypothesis. Other investigators have

noted positive effects of stimulating collagen-based constructs on biomechanics as well as gene and protein expression using different cell types (tendon fibroblasts,<sup>65</sup> smooth muscle cells,<sup>53</sup> ligament fibroblasts,<sup>66</sup> and MSCs<sup>50,57</sup>) and methods of stimulation.<sup>50,53,55,57,65,66</sup> Our results are also similar to another study in our laboratory where mechanical stimulation of rabbit MSCs in collagen sponge constructs not only increased construct stiffness but also produced increases in type I and III collagen mRNA expression using qRT-PCR.<sup>43</sup>

Day-7 and day-14 constructs showed significantly lower stiffness than day-0 constructs (Fig. 5). It has been shown that when chondrocytes<sup>67</sup> and fibroblasts<sup>68</sup> are seeded in collagen-based constructs, they produce matrix metallo proteinase (MMPs) that degrade the collagen scaffold. It is plausible that in our study the mouse MSCs also produced MMPs that degraded the collagen scaffold and, in turn, decreased its stiffness. Such decreases in stiffness are likely offset by tensile stimulation. In the future, we plan also to monitor MMP production in our stimulated and control constructs.

The stiffness of our constructs is still orders of magnitude less than that of that of native tendons<sup>44</sup> and hence cannot currently replace the entire ruptured tendon. Instead, we have been using these constructs for biological augmentation in a load-protected regime to repair central defect injuries to the rabbit patellar tendon. Implanting such stimulated cell-scaffolds *in vivo* can speed up the repair process as the cells are already acclimatized *in vitro* to some of the deformations that they might experience *in vivo*. Further, these cells are already synthesizing type I collagen *in vitro* and might fill the repair site much faster. We have shown that such compliant constructs produce rabbit patellar tendon repairs whose average stiffness is three orders of magnitude greater than the stiffness of initial constructs at surgery and capable of matching the stiffness of normal patellar tendon up to 150% of peak *in vivo* force values.<sup>44</sup>

The results obtained from this study have the potential to be translated to higher species such as humans. If we can demonstrate through *in vitro* studies that murine cell-based constructs respond to mechanical stimuli (as well as chemical stimuli) in ways similar to rabbit cell-based constructs by a range of response measures, it is then our hope to be able to apply the results of genetically based experiments in the mouse to new therapies in larger animals where such tools do not yet exist. The benefits that we formulate and test in lower species and then validate in larger preclinical models can then be translated to improved repair in humans.

Our study has limitations. (1) In this study we recorded nondestructively the average effects of uniaxial tension on GFP-T expression in MSCs. We are currently examining techniques to measure fluorescence without destroying the constructs. (2) We did not quantify the number of fluorescing cells. This prevented us from distinguishing whether the increases in fluorescence were due to increased numbers of fluorescing cells and/or increased production of GFP-T by already fluorescing cells. Determining which of these effects predominates will require more local recordings of gene expression at the individual cell level combined with *in situ* hybridization. Fluorescing cells could also be counted in a nondestructive way by applying statistical methods if we had the optical systems that could scan through the entire depth of the opaque constructs. (3) We selected each

fluorescent protein to have a 24-h half-life, thus providing near, rather than actual, real-time gene expression. While it would have been ideal to have instantaneous feedback of gene upregulation much like a conventional transducer, the longer period ensured that we could detect measurable changes in fluorescence before signal dissipation. (4) Cell viability within the constructs was not determined. Diminished cell viability in individual constructs could have adversely affected our RFU results for GFP-T fluorescence and thus increased interspecimen variability. (5) This method of fluorescence analysis is limited to the specific transgenic species described with two modified fluorescent proteins. (6) We chose to measure only the construct's linear stiffness because we were concerned that the lower aspect ratio of 2:1 (less than the ideal ratio of 3:1 to avoid St. Venant effects) might alter the structure's failure properties. (7) These constructs can only be used to repair tendons in a load-protected regime. We are currently investigating ways to further improve the stiffness of our constructs.

In conclusion, this study has demonstrated that short-term tensile stimulation increases type I collagen gene expression and linear stiffness of murine stem cell–collagen sponge constructs, and that two measures used to track changes in type I collagen gene expression are correlated. Future studies will focus on optimizing the effects of these *in vitro* signal components on expression of the type I collagen gene and the degree to which they affect construct and then repair biomechanics. Given that construct stiffness is positively correlated with repair stiffness after implanting rabbit autologous MSC–collagen constructs in patellar tendon defects,<sup>44</sup> our efforts to optimize stiffness in culture might also positively impact tendon repair biomechanics and gene expression across multiple species and injury scenarios. We also envision that once optical technologies have been developed to visualize fluorescence in opaque constructs in a bioreactor at magnifications consistent with light microscopy, we and others should be capable of tracking real-time, local changes in gene fluorescence. Ultimately, we hope to speed tendon repair and match the tangent stiffness of normal tendons to levels well above peak *in vivo* force levels.<sup>44</sup>

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### Disclosure Statement

No competing financial interests exist.

### References

- Huang, H.H., Qureshi, A.A., and Biundo, J.J., Jr. Sports and other soft tissue injuries, tendinitis, bursitis, and occupation-related syndromes. *Curr Opin Rheumatol* **12**, 150, 2000.
- Liu, S., and Nguyen, T. Ankle sprains and other soft tissue injuries. *Curr Opin Rheumatol* **11**, 132, 1999.
- Bennett, W.F. Arthroscopic repair of anterosuperior (supraspinatus/subscapularis) rotator cuff tears: a prospective cohort with 2- to 4-year follow-up. Classification of biceps subluxation/instability. *Arthroscopy* **19**, 21, 2003.
- Praemer, A., Furner, S., and Rice, D.P. *Musculoskeletal Condition in the United States*. Parke Ridge, IL: American Academy of Orthopaedic Surgeons, 1999.
- Bey, M.J., Ramsey, M.L., and Soslowky, L.J. Intratendinous strain fields of the supraspinatus tendon: effect of a surgically created articular-surface rotator cuff tear. *J Shoulder Elbow Surg* **11**, 562, 2002.
- Soslowky, L.J., Thomopoulos, S., Tun, S., Flanagan, C.L., Keefer, C.C., Mastaw, J., and Carpenter, J.E. Neer Award 1999. Overuse activity injures the supraspinatus tendon in an animal model: a histologic and biomechanical study. *J Shoulder Elbow Surg* **9**, 79, 2000.
- Albright, J.C., Carpenter, J.E., Graf, B.K., and Richmond, J.C. Knee and leg: soft-tissue trauma. *Orthop Knowledge Update*, **20**, 281, 1999.
- McNicholas, M., Rowley, D., McGurty, D., Adalberth, T., Abdon, P., Lindstrand, A., and Lohmander, L. Total meniscectomy in adolescence. A thirty-year follow-up. *J Bone Joint Surg Br* **82**, 217, 2000.
- Langer, R., and Vacanti, J.P. Tissue engineering. *Science* **260**, 920, 1993.
- Richmond, J.C., Manseau, C.J., Patz, R., and McConville, O. Anterior cruciate reconstruction using a Dacron ligament prosthesis: a long-term study. *Am J Sports Med* **20**, 24, 1992.
- Moyen, B.J.L., Jenny, J.-Y., Mandrino, A.H., and Lerat, J.-L. Comparison of reconstruction of the anterior cruciate ligament with and without a Kennedy ligament-augmentation device. A randomized, prospective study. *J Bone Joint Surg A* **74**, 1313, 1992.
- Amiel, D., Billings, E.J., and Akeson, W.H. Ligament structure, chemistry and physiology. In: Daniel, D., Akeson, W., O'Connor, J., eds. *Knee Ligaments: Structure, Function, Injury and Repair*. New York: Raven Press, 1990, p. 77.
- Jorgensen, U., Bak, K., Ekstrand, J., and Scavenius, M. Reconstruction of the anterior cruciate ligament with the iliotibial band autograft in patients with chronic knee instability. *Knee Surg Sports Traumatol Arthrosc* **9**, 137, 2001.
- Steenbrugge, F., Verdonk, R., Vorlat, P., Mortier, F., and Verstraete, K. Repair of chronic ruptures of the anterior cruciate ligament using allograft reconstruction and a ligament augmentation device. *Acta Orthop Belg* **67**, 252, 2001.
- Stone, K.R. Meniscus replacement. *Clin Sports Med* **15**, 557, 1996.
- Bruns, J., Kahrs, J., Kampen, J., Behrens, P., and Plitz, W. Autologous perichondral tissue for meniscal replacement. *J Bone Joint Surg Br* **80B**, 918, 1998.
- Kohn, D., Wirth, C.J., Reiss, G., Plitz, W., Maschek, H., Erhardt, W., and Wulker, N. Medial meniscus replacement by a tendon autograft—experiments in sheep. *J Bone Joint Surg Br* **74**, 910, 1992.
- Messner, K. Meniscal substitution with a Teflon periosteal composite graft—a rabbit experiment. *Biomaterials* **15**, 223, 1994.
- Ouyang, H.W., Goh, J.C., Thambyah, A., Teoh, S.H., and Lee, E.H. Knitted poly-lactide-co-glycolide scaffold loaded with bone marrow stromal cells in repair and regeneration of rabbit Achilles tendon. *Tissue Eng* **9**, 431, 2003.
- Awad, H.A., Boivin, G.P., Dressler, M.R., Smith, F.N., Young, R.G., and Butler, D.L. Repair of patellar tendon injuries using a cell-collagen composite. *J Orthop Res* **21**, 420, 2003.
- Young, R.G., Butler, D.L., Weber, W., Caplan, A.I., Gordon, S.L., and Fink, D.J. Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. *J Orthop Res* **16**, 406, 1998.
- Juncosa-Melvin, N., Boivin, G.P., Galloway, M.T., Gooch, C., West, J.R., Sklenka, A.M., and Butler, D.L. Effects of cell to collagen ratio in mesenchymal stem cell-seeded implants on



- tendon repair biomechanics and histology. *Tissue Eng* **11**, 448, 2005.
23. Noth, U., Schupp, K., Heymer, A., Kall, S., Jakob, F., Schutze, N., Baumann, B., Barthel, T., Eulert, J., and Hendrich, C. Anterior cruciate ligament constructs fabricated from human mesenchymal stem cells in a collagen type I hydrogel. *Cytotherapy* **7**, 447, 2005.
  24. Walsh, C.J., Goodman, D., Caplan, A.I., and Goldberg, V.M. Meniscus regeneration in a rabbit partial meniscectomy model. *Tissue Eng* **5**, 327, 1999.
  25. Butler, D.L., Goldstein, S.A., and Guilak, F. Functional tissue engineering: the role of biomechanics. *J Biomech Eng* **122**, 570, 2000.
  26. Guilak, F., Butler, D.L., Goldstein, S.A., and Mooney, D. *Functional Tissue Engineering*. New York: Springer-Verlag, 2003.
  27. Juncosa, N., West, J.R., Galloway, M.T., Boivin, G.P., and Butler, D.L. *In vivo* forces used to develop design parameters for tissue engineered implants for rabbit patellar tendon repair. *J Biomech* **36**, 483, 2003.
  28. West, J., Juncosa, N., Galloway, M., Boivin, G., and Butler, D. Characterization of *in-vivo* Achilles tendon forces in rabbits during treadmill locomotion at varying speeds and inclinations. *J Biomech* **37**, 1647, 2004.
  29. Mauck, R.L., Seyhan, S.L., Ateshian, G.A., and Hung, C.T. Influence of seeding density and dynamic deformational loading on the developing structure/function relationships of chondrocyte-seeded agarose hydrogels. *Ann Biomed Eng* **30**, 1046, 2002.
  30. Lo, S.S., Mauck, R.L., Seyhan, S.L., Palmer, G.D., Mow, V.C., and Hung, C.T. Mechanical Loading Modulates Gene Expression in Chondrocyte-Seeded Agarose Hydrogels. American Society of Mechanical Engineers, Bioengineering Division (Publication) BED, NY, 2001, pp. 291–292.
  31. Shearn, J.T., Juncosa-Melvin, N., Butler, D.L., Boivin, G.P., Galloway, M.T., Goodwin, W., and Gooch, C. Mechanical stimulation of tendon tissue engineered constructs: effects on construct stiffness, repair biomechanics, and their correlation. *J Biomech Eng* **129**, 848, 2007.
  32. Webb, K., Hitchcock, R.W., Smeal, R.M., Li, W., Gray, S.D., and Tresco, P.A. Cyclic strain increases fibroblast proliferation, matrix accumulation, and elastic modulus of fibroblast-seeded polyurethane constructs. *J Biomech* **39**, 1136, 2006.
  33. Butler, D.L., Shearn, J.T., Juncosa, N., Dressler, M.R., and Hunter, S.A. Functional tissue engineering parameters toward designing repair and replacement strategies. *Clin Orthop Relat Res* **427S**, S190, 2004.
  34. Butler, D.L., Juncosa, N., and Dressler, M.R. Functional efficacy of tendon repair processes. *Annu Rev Biomed Eng* **6**, 303, 2004.
  35. Grant, T.D., Cho, J., Ariail, K.S., Weksler, N.B., Smith, R.W., and Horton, W.A. Col2-GFP reporter marks chondrocyte lineage and chondrogenesis during mouse skeletal development. *Dev Dyn* **218**, 394, 2000.
  36. Harvey, K.J., Lukovic, D., and Ucker, D.S. Membrane-targeted green fluorescent protein reliably and uniquely marks cells through apoptotic death. *Cytometry* **43**, 273, 2001.
  37. Kalajzic, I., Kalajzic, Z., Hurley, M.M., Lichtler, A.C., and Rowe, D.W. Stage specific inhibition of osteoblast lineage differentiation by FGF2 and noggin. *J Cell Biochem* **88**, 1168, 2003.
  38. Ma, X., Robin, C., Ottersbach, K., and Dzierzak, E. The Ly-6A (Sca-1) GFP transgene is expressed in all adult mouse hematopoietic stem cells. *Stem Cells* **20**, 514, 2002.
  39. Mignone, J.L., Kukekov, V., Chiang, A.-S., Steindler, D., and Enikolopov, G. Neural stem and progenitor cells in Nestin-GFP transgenic mice. *J Comp Neurol* **469**, 311, 2004.
  40. Peister, A., Mellad, J.A., Larson, B.L., Hall, B.M., Gibson, L.F., and Prockop, D.J. Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. *Blood* **103**, 1662, 2004.
  41. Dobson, K.R., Reading, L., Haberey, M., Marine, X., and Scutt, A. Centrifugal isolation of bone marrow from bone: an improved method for the recovery and quantitation of bone marrow osteoprogenitor cells from rat tibiae and femur. *Calcif Tissue Int* **65**, 411, 1999.
  42. Molecular Devices Spectramax Gemini-XS Application Note 44. [http://www.moleculardevices.com/product\\_literature/family\\_links.php?prodid=63](http://www.moleculardevices.com/product_literature/family_links.php?prodid=63). Product Literature from Molecular Devices, June 1 2008.
  43. Juncosa-Melvin, N., Matlin, K.S., Holdcraft, R.W., Nirmalanandhan, V.S., and Butler, D.L. Mechanical stimulation increases collagen type I and collagen type III gene expression of stem cell-collagen sponge constructs for patellar tendon repair. *Tissue Eng* **13**, 1219, 2007.
  44. Juncosa-Melvin, N., Shearn, J.T., Boivin, G.P., Gooch, C., Galloway, M.T., West, J.R., Nirmalanandhan, V.S., Bradica, G., and Butler, D.L. Effects of mechanical stimulation on the biomechanics and histology of stem cell-collagen sponge constructs for rabbit patellar tendon repair. *Tissue Eng* **12**, 2291, 2006.
  45. Tsumaki, N., Tanaka, K., Arikawa-Hirasawa, E., Nakase, T., Kimura, T., Terrig Thomas, J., Ochi, T., Luyten, F.P., and Yamada, Y. Role of CDMP-1 in skeletal morphogenesis: promotion of mesenchymal cell recruitment and chondrocyte differentiation. *J Cell Biol* **144**, 161, 1999.
  46. Popov, E.P. *Engineering Mechanics of Solids*. Englewood Cliffs, NJ: Prentice Hall, 1990.
  47. Montgomery, D. *Design and Analysis of Experiments*, fifth edition. New York: John Wiley and Sons, 2001.
  48. Juncosa-Melvin, N., Boivin, G.P., Gooch, C., Galloway, M.T., West, J.R., Dunn, M.G., and Butler, D.L. The effect of autologous mesenchymal stem cells on the biomechanics and histology of gel-collagen sponge constructs used for rabbit patellar tendon repair. *Tissue Eng* **12**, 369, 2006.
  49. Toyoda, T., Matsumoto, H., Fujikawa, K., Saito, S., and Inoue, K. Tensile load and the metabolism of anterior cruciate ligament cells. *Clin Orthop Relat Res* **353**, 247, 1998.
  50. Altman, G.H., Horan, R.L., Martin, I., Farhadi, J., Stark, P.R., Volloch, V., Richmond, J.C., Vunjak-Novakovic, G., and Kaplan, D.L. Cell differentiation by mechanical stress. *FASEB J* **16**, 270, 2002.
  51. Archambault, J., Tsuzaki, M., Herzog, W., and Banes, A.J. Stretch and interleukin-1 $\beta$  induce matrix metalloproteinases in rabbit tendon cells *in vitro*. *J Orthop Res* **20**, 36, 2002.
  52. Riser, B.L., Cortes, P., Heilig, C., Grondin, J., Ladson-Wofford, S., Patterson, D., and Narins, R.G. Cyclic stretching force selectively up-regulates transforming growth factor- $\beta$  isoforms in cultured rat mesangial cells. *Am J Pathol* **148**, 1915, 1996.
  53. Sumpio, B.E., Banes, A.J., Link, W.G., and Johnson, G., Jr. Enhanced collagen production by smooth muscle cells during repetitive mechanical stretching. *Arch Surg* **123**, 1233, 1988.
  54. Zeichen, J., van Griensven, M., and Bosch, U. The proliferative response of isolated human tendon fibroblasts to cyclic biaxial mechanical strain. *Am J Sports Med* **28**, 888, 2000.
  55. Howard, P.S., Kucich, U., Taliwal, R., and Korostoff, J.M. Mechanical forces alter extracellular matrix synthesis by

- human periodontal ligament fibroblasts. *J Periodontal Res* **33**, 500, 1998.
56. Kim, S.-G., Akaike, T., Sasagawa, T., Atomi, Y., and Kurosawa, H. Gene expression of type I and type III collagen by mechanical stretch in anterior cruciate ligament cells. *Cell Struct Funct* **27**, 139, 2002.
  57. Park, J.S., Chu, J.S.F., Cheng, C., Chen, F., Chen, D., and Li, S. Differential effects of equiaxial and uniaxial strain on mesenchymal stem cells. *Biotechnol Bioeng* **88**, 359, 2004.
  58. Paye, M., Nusgens, B.V., and Lapiere, C.M. Modulation of cellular biosynthetic activity in the retracting collagen lattice. *Eur J Cell Biol* **45**, 44, 1987.
  59. Nakagawa, S., Pawelek, P., and Grinnell, F. Long-term culture of fibroblasts in contracted collagen gels: effects on cell growth and biosynthetic activity. *J Investig Dermatol* **93**, 792, 1989.
  60. Thie, M., Schlumberger, W., Rauterberg, J., and Robenek, H. Mechanical confinement inhibits collagen synthesis in gel-cultured fibroblasts. *Eur J Cell Biol* **48**, 294, 1989.
  61. Langholz, O., Rockel, D., Mauch, C., Kozłowska, E., Bank, I., Krieg, T., and Eckes, B. Collagen and collagenase gene expression in three-dimensional collagen lattices are differentially regulated by  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  integrins. *J Cell Biol* **131**, 1903, 1995.
  62. Rikonen, T., Westermarck, J., Koivisto, L., Broberg, A., Kahari, V.-M., and Heino, J. Integrin  $\alpha 2\beta 1$  is a positive regulator of collagenase (MMP-1) and collagen  $\alpha 1(I)$  gene expression. *J Biol Chem* **270**, 13548, 1995.
  63. Xu, J., and Clark, R.A.F. A three-dimensional collagen lattice induces protein kinase C-zeta activity: role in  $\beta 2$  integrin and collagenase mRNA expression. *J Cell Biol* **136**, 473, 1997.
  64. Chastain, S.R., Kundu, A.K., Dhar, S., Calvert, J.W., and Putnam, A.J. Adhesion of mesenchymal stem cells to polymer scaffolds occurs via distinct ECM ligands and controls their osteogenic differentiation. *J Biomed Mater Res A* **78**, 73, 2006.
  65. Zeichen, J., van Griensven, M., and Bosch, U. The proliferative response of isolated human tendon fibroblasts to cyclic biaxial mechanical strain. *Am J Sports Med* **28**, 888, 2000.
  66. Kim, S.G., Akaike, T., Sasagawa, T., Atomi, Y., and Kurosawa, H. Gene expression of type I and type III collagen by mechanical stretch in anterior cruciate ligament cells. *Cell Struct Funct* **27**, 139, 2002.
  67. Ronziere, M.C., Roche, S., Gouttenoire, J., Demarteau, O., Herbage, D., and Freyria, A.M. Ascorbate modulation of bovine chondrocyte growth, matrix protein gene expression and synthesis in three-dimensional collagen sponges. *Biomaterials* **24**, 851, 2003.
  68. Karamichos, D., Brown, R.A., and Mudera, V. Collagen stiffness regulates cellular contraction and matrix remodeling gene expression. *J Biomed Mater Res A* **3**, 887, 2007.

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