Three-Dimensional *In Vitro* Effects of Compression and Time in Culture on Aggregate Modulus and on Gene Expression and Protein Content of Collagen Type II in Murine Chondrocytes

Kumar Chokalingam, M.S.,¹ Shawn Hunter, Ph.D.,² Cynthia Gooch, B.S., L.V.T.,¹ Chris Frede, B.S.,³ Jane Florer, M.S.,³ Richard Wenstrup, M.D.,⁴ and David Butler, Ph.D.¹

The objectives of this study were to determine how culture time and dynamic compression, applied to murine chondrocyte–agarose constructs, influence construct stiffness, expression of col2 and type II collagen. Chondrocytes were harvested from the ribs of six newborn double transgenic mice carrying transgenes that use enhanced cyan fluorescent protein (ECFP) and green fluorescent protein (GFP-T) as reporters for expression from the col2a1 and col1a1 promoters, respectively. Sixty-three constructs (8 mm diameter $\times$ 3 mm thick) per animal were created by seeding chondrocytes (10×10^6 per mL) in agarose gel (2% w/v). Twenty-eight constructs from each animal were stimulated for 7, 14, 21, or 28 days in a custom bioreactor housed in an electromagnetic system. Twenty-eight constructs exposed to identical culture conditions but without mechanical stimulation served as nonstimulated controls for 7, 14, 21, and 28 days. The remaining seven constructs served as day 0 controls. Fluorescing cells with rounded morphology were present in all constructs at all five time points. Seven, 14, 21, and 28 days of stimulation significantly increased col2 expression according to ECFP fluorescence and messenger RNA expression according to quantitative reverse transcriptase polymerase chain reaction. Col2 gene expression in stimulated and nonstimulated constructs showed initial increases up to day 14 and then showed decreases by day 28. Stimulation significantly increased type II collagen content at 21 and 28 days and aggregate modulus only at 28 days. There was a significant increase in aggregate modulus in stimulated constructs between day 0 and 7 and between day 21 and day 28. This study reveals that compressive mechanical stimulation is a potent stimulator of col2 gene expression that leads to measurable but delayed increases in protein (type II collagen) and then bio-mechanical stiffness. Future studies will examine the effects of components of the mechanical signal in culture and address the question of whether such *in vitro* improvements in tissue-engineered constructs enhance repair outcomes after surgery.

Introduction

ARTICULAR CARTILAGE injuries are prevalent and, if left untreated, can lead to long-term osteoarthritis.^{1–4} In the United States alone, nearly 21% of the adult population suffers from osteoarthritis,⁵ resulting in more than \$128 billion in direct and indirect costs.⁶ Tissue engineering⁷ is an appealing conceptual alternative when conventional repair techniques (e.g., arthroscopic techniques, periosteal or perichondral grafts, autograft or allograft osteochondral transplantation, and prostheses)^{8–14} prove unsatisfactory.

Tissue engineering approaches have evolved as investigators recognize the importance of mechanical function. Researchers mix cells with scaffolds to create tissue-engineered “constructs” (TECs) that can be stimulated in culture before surgical implantation (e.g., to fill tissue defect sites),^{15–17} but these TECs often lack the inherent mechanical stiffness needed to tolerate large *in vivo* forces such as those acting on articular cartilage. To address this problem, investigators have been applying principles of functional tissue engineering^{18–20} to preconditioned constructs using aspects of *in vivo* tissue forces and deformations^{21,22} to create new generations

¹Department of Biomedical Engineering, University of Cincinnati, Cincinnati, Ohio.

²Community Tissue Services, Dayton, Ohio.

³Department of Human Genetics, Children's Hospital Medical Center of Cincinnati, Cincinnati, Ohio.

⁴Myriad Genetics Inc., Salt Lake City, Utah.

of reparative tissues.^{23–27} Such preconditioning improves construct material properties by increasing synthesis of extracellular matrix proteins like collagens I and II^{24–29} and repair biomechanics after implantation into defect sites.^{23,25}

Unfortunately, cartilage TECs often require months of intermittent mechanical conditioning to achieve mechanical properties and protein composition even remotely approaching those of native cartilage.²⁸ For example, Mauck *et al.*²⁹ determined that cartilage TECs required a full 8 weeks of dynamic loading in culture to achieve 75% and 25% of a normal tissue's Young's modulus and unconfined dynamic modulus, respectively. Even with these mechanical improvements, total collagen content was still only 12% of native tissue values.²⁹ Although such long development cycles may be satisfactory in a research setting, these intervals are not practical for patients in a clinical setting.

To shorten the development cycle and reduce production costs, new technologies are needed to rapidly and repeatedly assess the evolving TEC in culture. During the early development period, tissue engineers should be able to non-destructively assess the TEC in culture to avoid creating inappropriate collagen types (e.g. type I vs type II collagen in a TEC that could adversely affect the repair of articular cartilage). For example, if investigators could monitor how mechanical signaling influences col2 gene expression in near real time, they could dramatically improve their ability to synthesize type II collagen protein. Such technologies could also allow the tissue engineer to determine which mechanical signals most increase col2 gene expression and compressive stiffness.

To address these needs, we have developed two innovative technologies. We have bred specialized double transgenic (DT) mice that carry transgenes that use enhanced cyan fluorescent protein (ECFP) and green fluorescent protein (GFP-T) as reporters for col2a1 and col1a1 expression, respectively. ECFP and GFP-T each have a half life of 24 h and serve as visual reporters for cells that express the genes for types II and I collagen, respectively. ECFP linked to col2a1 offers the potential to ultimately track in near real time how various stimuli (mechanical and chemical) influence col2 gene activity in culture. We have also designed and fabricated a bioreactor to deliver compressive displacement profiles with micron precision to constructs in an incubator setting over 4-week intervals. This bioreactor is described elsewhere.³⁰

Using these two technologies, our objectives in this study were to test four hypotheses related to compressive stimulation of double transgenic chondrocyte–agarose constructs. We hypothesized that compressive stimulation significantly increases ECFP fluorescence and col2 messenger RNA (mRNA) expression, type II collagen production, and TEC aggregate modulus up to 28 days in culture. We also hypothesized that ECFP fluorescence and col2 mRNA expression are positively correlated.

Experimental Design

Rib chondrocytes were obtained from six newborn DT mice (one cell line per mouse). Sixty-three cell–agarose constructs (8 mm diameter × 3 mm thick) were created per animal using one cell density (10×10^6 cells/mL)³¹ in a 2% w/v ratio to maintain chondrocyte phenotype³² and to achieve a power of 85% to detect treatment-related differences if they existed. We compared the responses of 28 stimulated constructs and

28 nonstimulated controls at 7, 14, 21, and 28 days in culture (7 per time period). These results were also compared to findings from seven additional nonstimulated controls per cell line at day 0. Stimulated constructs all received the same compressive stimulation profile (sinusoidal displacement pattern at 1 Hz to 10% peak strain for 1 h followed by 1 h of rest repeated three times a day) adapted from Mauck *et al.*³¹ The seven stimulated and seven nonstimulated constructs at each time point were assigned as follows: one for ECFP fluorescence in a spectrophotometer (measured in relative fluorescence units (RFUs)),³³ one to evaluate changes in mRNA expression for col2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR),²⁴ one for confined compression to determine aggregate modulus,³⁴ one for cell viability using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay,³⁵ and three constructs for type II collagen content using enzyme-linked immunosorbent assay (ELISA).³⁶

Materials

Chondrocyte isolation and expansion

Creation of the murine DT model is described elsewhere. Newborn mice were euthanized using carbon dioxide (CO₂) using protocols approved by the Institutional Animal Care and Use Committee. Chondrocytes were harvested from the rib cages using previously published procedures.³⁷ Briefly, rib cages from each mouse were repeatedly washed in phosphate buffered saline (PBS) and digested in 2.5 mL of protease (Sigma P-8811; 2 mg/mL, St. Louis, MO) for 30 min in an incubator (Steri-Cult Model 3033, Forma Scientific, Marietta, OH). After incubating overnight in collagenase B solution (Sigma; 1 mg/mL), the digest was washed with medium (phenol red-free Dulbecco's modified Eagle medium, 10% fetal bovine serum (FBS), penicillin/streptomycin, filtered (70 μ m) to remove remaining bone pieces, sorted using flow cytometry to exclude non-ECFP cells, plated in a 100-mm tissue culture dish (Falcon, Bedford, MA), and incubated for 1 day at passage 0 (P0). Intracellular cyan fluorescence was evident in these chondrocytes when visualized under a fluorescence microscope (Axiovert 25, Carl Zeiss Inc., Göttingen, Germany) equipped with ECFP and GFP-T filter sets (Omega, Brattleboro, VT). The monolayer chondrocyte culture was then trypsinized using 0.25% EDTA, replated at 1×10^6 cells per 100-mm plate at P1, and cultured for 7 to 10 days. Chondrocytes were passaged until P3. No intracellular GFP-T expression was seen in cultured chondrocytes from P0 through P3.

Construct preparation

Chondrocytes at P3 were suspended at 20×10^6 cells/mL in medium (BGJB, Invitrogen-Gibco BRL/Life Technologies Inc., Gaithersburg, MD) containing 1% antibiotic/antimycotic and 10% FBS (Atlanta Biologicals, Lawrenceville, GA). Cell suspensions were mixed in equal volumes with 4% (w/v) agarose (type VII, low gelling temperature, Fisher Scientific, Pittsburgh, PA) and PBS to produce a cell–gel solution of 10×10^6 cells/mL in 2% (w/v) agarose. This solution was then cast for 20 min between sterile glass plates separated by 3-mm spacers. Disks (8 mm diameter) were cored using a sterile dermal punch and each cultured in 5 mL of medium in a multiwell plate for 2 days

(henceforth denoted as day 0) in a standard incubator (Steri-Cult Model 3033, Forma Scientific) to allow cells to adjust to their new culture environment. Medium was replaced daily.

Methods

Construct preparation for mechanical stimulation

All constructs were transferred from multiwell plates to individual 35-mm glass-bottom tissue culture dishes (P35G-0-10-C, MatTek Corp, Ashland, MA) covered with Teflon FEP film (100 Å; DuPont, Circleville, OH) using a custom Delrin ring. The film, permeable to oxygen (O₂) and CO₂ but not to water vapor, ensured sterility and prevented evaporation. The dish design is provided elsewhere (Fig. 3c³⁰).

Dynamic compressive stimulation

Constructs were subjected to dynamic compression in a custom-built bioreactor housed in an electromagnetic testing system (ElectroForce 3200; BOSE Corp., Eden Prairie, MN).³⁰ Briefly, this bioreactor consisted of 12 testing stations and a chamber to sustain a cell culture environment (37°C, 5% CO₂, ≥75% relative humidity). Six of the 12 stations were equipped with loading rods to stimulate the constructs; the remaining six were devoid of loading rods and housed the nonstimulated controls.

Before starting dynamic compression, the environmental chamber of the bioreactor was aseptically cleaned, and the system's culture environment was allowed to achieve steady-state culture conditions. Culture dishes were then placed into each holding station. Consistent with previous patterns,³¹ constructs were subjected to the compressive strain profile described above. Feeding medium was replaced daily. At specified time points, dishes containing treated and control constructs were removed from the bioreactor.

Fluorescence microscopy

At each time point after the end of stimulation, the constructs subjected to RFU analysis were washed in PBS for 1 h with gentle shaking to remove medium and then visualized for ECFP and GFP-T fluorescence under a fluorescence microscope (Axiovert 25, Carl Zeiss Inc.). To exclude cell autofluorescence, constructs were also visualized for rhodamine using a 11002VZ (Chroma, Rockingham, VT) filter set.

Spectrophotometric analysis

After imaging, constructs assigned for gene expression measurements were mechanically homogenized using a glass mortar and Teflon pestle. The homogenate was suspended in 1 mL of PBS and pipetted into a black-bottom microplate (200 µL per well in 5 wells). ECFP fluorescence in these digests was quantified in RFUs,³³ reading the microplate in a spectrophotometer (Spectra Max M2, Molecular Devices, Sunnyvale, CA) using excitation and emission wavelengths of 436 nm and 486 nm, respectively, with a cut off filter of 475 nm. ECFP RFU was normalized to day 0 values for all samples.

Real-time qRT-PCR

RNA extraction, conventional gene expression analysis, and real-time qRT-PCR were performed according to pub-

lished protocols.²⁴ Mouse-specific primers were used for type II collagen and GAPDH gene expression. Before use in the experiment, all primers were extensively tested under conventional and real-time qRT-PCR conditions to ensure that they were specific for each gene and that we obtained a single clean band when running the electrophoresis gel (with the associated base pair size for each gene). The absolute amount of the corresponding gene mRNA in each construct was obtained from the corresponding gene standard curve. Expression was normalized by calculating the ratio between type II collagen and GAPDH genes for each sample. GAPDH was used as the housekeeping gene, because it did not change with treatment conditions when we mechanically stimulated cell-scaffold constructs in tension-containing mesenchymal stem cells from 10-week-old double transgenic mice and from skeletally mature New Zealand white rabbits.²⁴

Determination of cell viability

At each time point, cell viability in constructs was determined using an MTT assay (Vybrant MTT Cell Proliferation Assay Kit, Invitrogen, Carlsbad, CA), in which live cells reduce MTT to a strongly pigmented formazan product to provide colorimetric indicators of cell viability, following the manufacturer's instructions. Briefly the cell-agarose constructs were homogenized using a mortar and pestle. The homogenate was incubated for 4 h in an incubator in 500 µL of 12-mM MTT solution. This was followed by incubation for 18 h in 5 mL sodium dodecyl sulfate-hydrochloric acid to completely dissolve the formazan. The absorbance of formazan was measured at 570 nm in a microplate spectrophotometer (Spectra Max M2, Molecular Devices).³⁵ The viability results were normalized to day 0 constructs.

Determination of type II collagen content

A native type II collagen detection kit (Catalog #6009; Chondrex Inc., Redmond, WA) was used to quantify type II collagen. The three constructs from each treatment group and animal were pooled. Each construct was washed in PBS for 1 h with gentle shaking to remove medium. The constructs were then lyophilized and their dry weight measured and then incubated in cold water at 4°C. Constructs were subjected to pepsin digestion and elastase digestion following the manufacturer's protocols. ELISA was measured according to these protocols.³⁶

Biomechanical evaluation of the agarose-cell constructs

Aggregate modulus of the cell-agarose constructs was determined using confined compression testing. Constructs were placed in cryovials and stored at -80°C. Before testing, each tissue specimen was thawed in room-temperature PBS for 10 min. A 6-mm-diameter core was created from the center of each construct and its height measured using a light-force micrometer (Model ID-C1012CE, Mitutoyo Corp., Kawasaki, Japan). Each sample was placed into a 6-mm-diameter×4.3-mm-deep well and subjected to a constant contact stress of 0.066 MPa until equilibrium displacement was achieved. During testing, specimen height and applied load were continuously measured using a fine-resolution Linear variable differential transformer (±0.25", Sensotec

FIG. 1. Rounded enhanced cyan fluorescent protein (ECFP)-fluorescing cells in nonstimulated (A, C, E, G) and stimulated (B, D, F, H) constructs at day 7 (A, B), day 14 (C, D), day 21 (E & F), and day 28 (G, H). Color images available online at www.liebertonline.com/ten.

Corp., Columbus, OH) and load cell (5 lb Sensotec Corp., Columbus, OH), respectively. Tissue aggregate modulus (H_A) was calculated using the formula $H_A = (F_0/\pi a^2)/(u/h)$, where F_0 is applied compressive force, a is specimen radius, h is specimen thickness, and u is equilibrium displacement.³⁴

Statistical analysis

We determined the presence of treatment-related differences between values for all response measures (ECFP RFUs, col2 mRNA, aggregate modulus, cell viability, and type II collagen content) using a two-way analysis of variance with Tukey's honestly significant difference post hoc testing (SAS Institute, Inc., Cary, NC). We chose animal as a random factor, culture time and compressive stimulation as fixed factors, and response measures as dependent variables.³⁸ Significance was set at $p < 0.05$.

Results

Qualitative inspection of constructs showed cells with rounded morphology distributed homogeneously in the con-

structs. Fluorescence microscopy revealed ECFP fluorescence at day 0 in all three-dimensional (3-D) constructs that was maintained in 7-, 14-, 21-, and 28-day constructs (Fig. 1). No qualitative differences were apparent in the amount or intensity of ECFP fluorescence between the images for non-stimulated and stimulated constructs at the four time points (Fig. 1A-G). Only cyan fluorescing cells were observed in the sections examined. No cells in any of the treatment groups expressed GFP-T and hence we did not measure RFUs or perform qRT-PCR for GFP-T and col1a1 expression, respectively. No autofluorescence was detected in samples using rhodamine filter sets.

Quantitative results showed that applying a dynamic compressive displacement significantly increased ECFP expression in the 3-D constructs. Compressive stimulation significantly increased RFU values by 1.34, 1.8, 1.27, and 1.24 times at 7, 14, 21, and 28 days, respectively ($p < 0.05$; Fig. 2A). There was a significant increase in RFUs in stimulated constructs between days 7 and 14 and a significant decrease between days 21 and 28 ($p < 0.05$; Fig. 2A). There was a significant decrease in RFUs in nonstimulated constructs between days 21 and 28 ($p < 0.05$; Fig. 2A).

Dynamic compressive displacement also significantly increased col2 mRNA according to qRT-PCR. Compressive stimulation significantly increased mRNA expression 1.5, 1.32, 1.36, and 1.45 times at 7, 14, 21, and 28 days, respectively ($p < 0.05$; Fig. 2B). There was a significant increase in col2 mRNA in stimulated constructs between days 7 and 14 and a significant decrease between days 21 and 28 ($p < 0.05$; Fig. 2B). There was a significant increase in Col2 mRNA in non-stimulated constructs between days 7 and 14 and a significant decrease between days 21 and 28 ($p < 0.05$; Fig. 2B). There were also no significant differences in GAPDH values between treatment conditions. The results from qRT-PCR and fluorescence analysis were also positively correlated (Fig. 3, coefficient of variation (r^2) = 0.92 with a slope for the linear regression curve of 2.09).

Compressive stimulation also increased type II collagen content of stimulated constructs but only after 21 and 28 days of stimulation. Stimulated constructs showed 1.40 and 1.24 times greater type II collagen content at 21 and 28 days,

FIG. 2. Dynamic stimulation increases (A) ECFP expression by relative fluorescence units (RFU) and (B) Col2 messenger RNA expression according to quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) at all four time periods of observation. *Significantly greater than nonstimulated control at same time point ($p < 0.05$). **Significant difference from same treatment at previous time point. Data represented as mean $\pm$ standard deviation. $N = 6$ for all groups.

FIG. 3. RFU fluorescence of ECFP expression and qRT-PCR are positively correlated over the four time periods of evaluation (days 7, 14, 21, and 28). (Slope = 2.09; $r^2 = 0.92$). Circle denotes day 0 constructs, triangles denote nonstimulated constructs, and squares denote stimulated constructs.

FIG. 4. Type II collagen content of constructs increased significantly after 21 and 28 days of dynamic compressive stimulation. *Significantly greater than nonstimulated controls at same time point ($p < 0.05$). **Significantly greater than same treatment at previous time point ($p < 0.05$). Data represented as mean $\pm$ standard deviation. $N = 6$ for all groups. w.w denotes wet weight.

respectively ($p < 0.05$; Fig. 4). There was a significant increase in type II collagen content in stimulated constructs between days 14 and 21 and between days 21 and 28 ($p < 0.05$; Fig. 4).

Compressive stimulation increased the aggregate modulus of the chondrocyte-agarose constructs but only after 28 days of stimulation. After this period of treatment, stimulated constructs showed a 1.67 times greater aggregate modulus than nonstimulated controls ($p < 0.05$; Fig. 5). There was a significant increase in aggregate modulus in stimulated constructs between days 0 and 7 and between day 21 and 28 ($p < 0.05$; Fig. 5).

No significant changes occurred in cell viability due to mechanical stimulation or increasing time in culture ($p > 0.05$; Fig. 6). Day 28 constructs showed 8% lower cell viability than day 0 constructs.

Discussion

Tissue engineers could benefit from new methodologies that rapidly and nondestructively assess the effects of vari-

FIG. 5. Aggregate modulus of constructs increased significantly after 28 days of dynamic stimulation. *Significantly greater than nonstimulated control at same time point ($p < 0.05$). **Significantly greater than same treatment at previous time point ($p < 0.05$). Data represented as mean $\pm$ standard deviation. $N = 6$ for all groups.

FIG. 6. No significant differences were measured in cell viability between stimulated and nonstimulated constructs after 0, 7, 14, 21, and 28 days ($p > 0.05$). Cell viability exceeded 90% at all time periods in culture. Data represented as mean $\pm$ standard deviation. $N = 6$ for all groups.

ous stimuli on expression of important structural genes. We chose to track changes in col2 gene expression because type II collagen, the primary structural protein found in articular cartilage, effectively resists ion-induced internal pressures as well as lateral strains created by compressive forces in cartilage.³⁹ We achieved this goal in the murine model by linking the col2 gene to a fluorescent protein with a 24-h half life that would allow us to detect near real-time changes in expression. Although a protein with a shorter half life might have provided a "transducer-like" biological response to changes in mechanical signal, we also wanted to ensure that we did not miss observing these changes when they occurred.

Effects of compressive stimulation and time on gene expression

This study using these transgenic chondrocytes was, in part, designed to examine the individual and combined effects of dynamic compression and time in culture on ECFP and col2 gene expression. Spectrophotometric analysis to measure ECFP RFUs and qRT-PCR to monitor col2 mRNA expression showed similar patterns over time in culture (Fig. 2A, B). Although nonstimulated constructs displayed modest temporal changes in RFUs (relative to day 0 values), mechanical stimulation resulted in significantly greater fluorescence both than in controls and between days 7 and 14 (Fig. 2A, 7A, B). Similar temporal and stimulation patterns were also observed using qRT-PCR (Fig. 2B), supporting our first hypothesis that compressive stimulation significantly increases ECFP fluorescence and col2 mRNA expression.

Our results mirror the findings of two shorter-term studies but directly contradict other published reports showing no changes in collagen gene expression after mechanical stimulation. De Croos *et al.*⁴⁰ found significant increases in col2 mRNA expression 12 h after exposing bovine chondrocyte-calcium phosphate constructs to 1 kPa of compressive pressure at 1 Hz for 30 min. In a similar way, Xie J *et al.*⁴¹ found that 24 h of continuous dynamic compression applied to rabbit chondrocytes seeded in microporous elastomeric scaffolds of poly(L-lactide-co-epsilon-caprolactone) significantly increased col2 gene expression. By contrast, Hunter *et al.*

FIG. 7. Fold increase from day 0 in (A) stimulated constructs and (B) nonstimulated constructs. Note the earlier increase in col2 expression, followed by delayed increase in type II collagen content, followed by increase in construct modulus. Data represented as mean $\pm$ standard deviation. N=6 for all groups.

found no change in col2 gene expression when constructs containing bovine chondrocytes seeded in collagen gels were subjected to dynamic compression ($\pm 4\%$ strain at 1 Hz for 24 h).⁴² Demarteau *et al.* also showed no alterations in col2 mRNA levels when human articular chondrocytes were seeded in polyethylene glycol terephthalate/polybutylene terephthalate foams and exposed to dynamic compression (6 cycles of sinusoidal deformation to 5% peak strain at 1 Hz followed by a 10-h rest period for 3 days).⁴³ Mauck *et al.*⁴⁴ observed decreases in type II collagen promoter activity when bovine chondrocytes (transfected with pcol2-LUC promoter reporter plasmid) were seeded in 2% w/v agarose. Mauck subjected these TECs to 10% cyclic compressive deformation at 1 Hz for 60 or 180 min, after which they observed decreases in type II collagen activity 24 and 72 h poststimulation. The differences seen in all of these studies could be attributed to different experimental conditions, such as the choice of biomaterial, cell source, stimulation profile, and time of evaluation. For example, our results are longer term, showing stimulation-induced increases in col2 mRNA expression and RFUs that persisted after 28 days of stimulation. Future studies could benefit from tracking changes over longer time periods, as well as systematically varying and controlling other factors to determine their importance in the tissue engineering fabrication process.

Numerous studies have sought to explain how dynamic compression increases col2 gene expression. One study found

that such increases occur through transcriptional activation, possibly through the Sp1 binding sites residing in the proximal region of the col2a1 gene promoter.⁴⁵ Others suggest that certain phenomena like cell-tissue strain, fluid pressurization and flow, electrokinetic phenomena, convective transport, and release of cytokines and growth factors can trigger the complex chain of events that modulate the production of extracellular matrix proteins such as type II collagen.^{5,6,46} Although not a specific objective of the current study, we plan to study several of these mechanisms and how they might increase col2 gene expression and ultimately type II collagen production.

Our results also support our fourth hypothesis that ECFP fluorescence and col2 mRNA expression are positively correlated ($r^2 = 0.92$; Fig. 3). These correlations are similar to GFP-T versus col1 mRNA results from a previous study in our lab in which mesenchymal stem cells from double transgenic mice were seeded in collagen sponge scaffolds and exposed to tensile stimulation. Both sets of findings are encouraging for tissue engineering applications and suggest that tracking fluorescence changes are a reasonable surrogate for monitoring changes in mRNA expression using qRT-PCR.

Effects of stimulation and time on type II collagen content and aggregate modulus

Our results support our second and third hypotheses that compressive stimulation significantly increases type II collagen production and TEC aggregate modulus. Although expression of type II collagen content occurs later than mRNA expression in both construct types, mechanical stimulation significantly enhances the amount of measured protein. Although col2 gene expression peaked between 7 and 14 days and then declined (Fig. 2B, 7A, B), type II collagen content showed significant increases at 21 days, with further enhancement at 28 days. The stimulated and nonstimulated constructs showed these significant effects (Fig. 4, 7A, B), with stimulated constructs showing further increases in protein content at these later time periods. The fact that changes in col2 expression occur before increased type II collagen content is not unexpected, although the fact that this delay may be as long as 14 days in culture and that mechanical stimulation can enhance protein production by as much as 25% to 33% are surprising findings and probably specific to the cells, scaffold, and medium conditions chosen for these experiments. It is also worth noting that greater type II collagen content at 21 and 28 days may have hampered diffusion of nutrients, inducing small decreases in cell viability (Fig. 6), and switched cells to more of a "maintenance mode," thereby decreasing col2 gene expression (Fig. 2B). These conclusions are only speculations at this time and will be the subject of further work by our group.

Our study also demonstrated further delays in biomechanical effects of mechanical stimulation. Although type II collagen content was elevated by 21 days, an additional 7 days of dynamic compression (28 days total) was required to significantly improve aggregate modulus (Fig. 5, 7A, B). Again, these effects are probably dependent on the cell, scaffold, and mechanical signals imposed. One might also question whether factors such as freezing the constructs before testing could have affected our biomechanical results. Freezing was required because multiple constructs had to be

tested each day, each test required 5 h to complete, and we had only two confined compression testers available. To directly examine potential freezing effects, we cultured two chondrocyte-agarose constructs from each of five cell lines (from 5 mice) for 7 days without mechanical stimulation and then froze one of each pair. Unconfined compression testing revealed no significant effect of freezing on aggregate modulus between groups ($p > 0.05$). Our laboratory has also previously shown that freezing does not affect mechanical properties of mesenchymal stem cell-collagen constructs.⁴⁷

Our improvements in construct aggregate modulus are similar to those of Mauck *et al.*,^{29,31} who stimulated bovine chondrocyte-agarose TECs to 10% cyclic compressive deformation. This stimulus profile resulted in significantly greater equilibrium aggregate modulus than in time-matched nonstimulated constructs, although these studies also showed that aggregate modulus of nonstimulated constructs decreased between days 28 and 55. These observations, taken together with our results, suggest that time in culture and dynamic stimulation can each significantly increase the matrix structure and biomechanics of chondrocyte-agarose constructs but that new strategies may be required to sustain these improvements in longer-term culture.

Overarching conclusions from the current study

Among the most important findings in our study are the temporal and stimulation-induced relationships between gene expression, protein expression, and biomechanics. Most studies in the literature have examined the effects of stimulation on only one or two of these response measures. Instead, we chose to simultaneously monitor treatment-induced effects on all three measures. The increases we observed in col2 gene expression at 7, 14, 21, and 28 days only culminated in greater type II collagen content after 21 and 28 days and greater aggregate modulus after 28 days. Gene expression must precede protein deposition, but any biomechanical benefits arise only after the protein is assembled into a more-functional matrix. Additional studies are needed to corroborate these temporal findings in the murine model and in other model systems and to determine whether gene and protein expression patterns might serve as predictors of biomechanical response *in vitro* and ultimately *in vivo*.

Using a murine chondrocyte source to develop TECs offers certain advantages and disadvantages. Although repairing cartilage defects in the mouse model using functional tissue engineering principles remains a distinct challenge, murine cells offer molecular tools that are not currently available in higher models such as rabbits, goats, and sheep. Such biological tools also exist in the human model, but human chondrocytes are not readily available. Performing *in vitro* experiments in mice that can be successfully correlated to results in higher models may someday permit translation to humans. Finally, the murine model is an ideal candidate to evaluate signaling pathways involved in mechanotransduction. For example, our group is already examining the effects of mechanical stimulation on bone morphogenetic protein and fibroblast growth factor signaling pathways with and without inhibitors.

Our study is not without limitations. We did not track changes in expression of ECFP with passage number. Reduction in ECFP levels might indicate that these chondrocytes

are dedifferentiating, hence prolonging any future increases in gene and protein expression and subsequent construct biomechanics. It has been shown that chondrocytes can rapidly dedifferentiate after even one passage and could exhibit more of a fibroblastic phenotype with greater col1 expression,^{48,49} although none of the cells in the current study showed any GFP-T fluorescence up to P3, a marker that would indicate col1a1 expression. In future studies, we plan to use our double transgenic cells to monitor the effects of cell passage number and loading type on col2a1 and col1a1 gene expression, type II collagen content, and construct biomechanics.

We did not track real-time changes in col2a1 expression in the intact construct. These structures are currently too thick and opaque to perform such nondestructive imaging. We continue to seek novel imaging technologies to observe such changes in cellular fluorescence that would permit us to perform longitudinal studies on the same constructs over time.

It was necessary to pool fluorescence from all cells extracted from constructs undergoing the same experimental condition. Thus, we can report only average rather than specific or local effects of compression on individual cells.

We used juvenile chondrocytes because they could be easily extracted from the ribs of the newborn mice and because of our interest in examining how developmental biology might be applied to adult tissue healing. We also plan to study how adult chondrocytes respond to these stimuli.

Autofluorescence of agarose gel fragments could increase overall RFU values. In this study, we assumed that these changes were constant across groups.

Although type II collagen content and biomechanics increased significantly after 21 to 28 days in culture, these response measures do not directly indicate the degree of collagen assembly or alignment.

We did not specifically track changes in the production of proteoglycans such as aggrecan. Increases in aggrecan content could reflect improvements in a construct's aggregate modulus. In the future, we intend to track the production of proteoglycans.

We performed only confined compression testing on the constructs. Future studies should apply other testing modes such as unconfined compression or indentation.

We applied only one compressive stimulus pattern to the constructs. We still need to systematically vary components of the mechanical signal so as to optimize gene and protein expression, as well as construct biomechanics in the shortest possible time interval.

Other forms of stimulation (e.g., chemical stimulation with transforming growth factor beta) may also induce increases in these response measures. In the future, we intend to contrast results using these methods with those from our current study.

In conclusion, our results reveal that compressive stimulation and time in culture increase col2 gene expression, type II collagen content, and aggregate modulus in constructs formed from murine chondrocytes seeded in agarose gels. The transgenic and bioreactor technologies that we report hold much promise in optimizing tissue engineering methodologies for replacement of damaged and diseased tissues like articular cartilage and fibrocartilage. In particular, we hope to soon identify which profiles of environmental stimulation (e.g., mechanical, chemical) result in the most-rapid and

greatest increases in col2 gene expression levels. Such innovative technologies will be needed to discover promising tissue engineering treatments to speed the repair of damaged and diseased tissues.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

David L. Butler, Ph.D.

Department of Biomedical Engineering

University of Cincinnati

2901 Woodside Drive

Cincinnati, OH 45221-0048

E-mail: david.butler@uc.edu

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