Biomechan Model Mechanobiol (2007) 6: 113–125
DOI 10.1007/s10237-006-0042-1

ORIGINAL PAPER

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Regulation of cartilaginous ECM gene transcription by chondrocytes and MSCs in 3D culture in response to dynamic loading

Received: 28 September 2005 / Accepted: 12 January 2006 / Published online: 12 May 2006

Abstract This study explored the biologic response of chondrocytes and mesenchymal stem cells (MSCs) to a dynamic mechanical loading regime. We developed a time-efficient methodology for monitoring regional changes in extracellular matrix gene transcription using reporter promoter constructs. Specifically, transfected cells were homogenously distributed throughout agarose hydrogel constructs, and spatial and temporal gene expression and the ability to form functional ECM were analyzed in response to dynamic mechanical stimuli. Theoretical analyses were used to predict the physical signals generated within the gel in response to these loading regimes. Using a custom compression bioreactor system, changes in aggrecan and type II collagen promoter activity in transfected chondrocyte-laden cylindrical constructs were evaluated in response to a range of loading frequencies and durations. In general, aggrecan promoter activity increased with increasing duration of loading, particularly in the outer annulus region. Interestingly, type II collagen promoter activity decreased in this annular region under identical loading conditions. In addition, we explored the role of mechanical compression in directing chondrogenic differentiation of MSCs by monitoring short-term aggrecan promoter activity. As an example of long-term utility, a specific loading protocol was applied to MSC-laden constructs for 5 days, and the resultant changes in glycosaminoglycan (GAG) production were evaluated over a 4-week period. This dynamic loading regime increased not only short-term aggrecan transcriptional activity but also GAG deposition in long-term culture. These results demonstrate the utility of a new reporter promoter system for optimizing loading protocols to improve the outcome of engineered chondrocyte- and MSC-laden cartilaginous constructs.

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1 Introduction

It has long been appreciated that the mechanical environment of musculoskeletal tissues modulates both their form and function. For hyaline cartilage, physiologic contact between two articulating layers generates a number of mechanical signals within the tissue that act directly (deformation and fluid flow) or indirectly (osmotic and hydrostatic pressure) on the chondrocytes embedded within (Mow and Wang 1999; Mow et al. 1999). These cells integrate numerous signals emanating from the extracellular matrix (ECM) and modulate their biosynthetic activities accordingly to maintain tissue homeostasis. In vitro studies using explant cultures have demonstrated that the method of application, as well as the duration, frequency, and duty cycle of loading can modify biosynthetic response (Buschmann et al. 1999; Sah et al. 1989; Gray et al. 1989; Guilak et al. 1994, 1997; Grodzinsky et al. 2000). By monitoring changes in proteoglycan (PG) or collagen synthesis, regional variation in biosynthetic response to applied loading has been observed. For example, rapid dynamic unconfined compression increased PG synthesis in regions of high fluid flow at the radial edge, while slower loading led to stimulation throughout the explant (Buschmann et al. 1999; Kim et al. 1994; Parkkinen et al. 1992). Alternatively, dynamic shearing of cartilage explants, which creates deformation with little associated fluid flow, increased collagen and PG synthesis uniformly (Jin et al. 2001). More recently, cartilage-specific ECM mRNA levels have been measured in response to a defined mechanical loading environment (Valhmu et al. 1998; Valhmu and Raia 2002; Blain et al. 2001, 2003; Fitzgerald et al. 2004) and signaling cascades that play a role in this process were evaluated using path-way specific inhibitors (Valhmu and Raia 2002; Fitzgerald et al. 2004).

Regardless of the complexity in distribution of these physical signals, the general finding that dynamic loading

improves matrix biosynthesis has been translated to the mechanical preconditioning of engineered cartilage constructs. In such studies, constructs subjected to both deformational loading and hydrostatic pressure respond with biosynthetic and gene expression changes similar to that observed for native explant cultures (Buschmann et al. 1995; Davisson et al. 2002; Lee and Bader 1995, 1997; Lee et al. 2000a; Ragan et al. 1999, 2000; Carver and Heath 1999a,b), with some studies showing long-term improvements in material properties with continued mechanical loading (Kisiday et al. 2004; Waldman et al. 2003; Mauck et al. 2000, 2003a,b). Apart from creating new tissues, these engineered matrices may also provide a simple and homogeneous environment for studies of chondrocyte mechanotransduction. Of the 3D matrices, hydrogels may prove most amenable for analysis of mechanical response as they are homogenous from the outset and fully surround cells embedded within. Such gels can be modeled as biphasic, having a solid and a fluid phase, and finite element formulations can be used to predict mechanical signals generated within the gel with different loading scenarios (Lima et al. 2004). Using this approach, Buschmann et al. (1995) demonstrated enhanced matrix biosynthesis by chondrocytes in agarose in regions that correspond with higher fluid flows. Other studies by Lee et al. (2000b) have shown alterations in matrix biosynthesis, cell shape, and cytoskeletal organization with specific deformation regimes (Knight et al. 2002, 2005).

While chondrocyte biosynthetic responses have been most studied in these systems to date, there is a growing interest in the response of mesenchymal stem cells (MSCs) to these same physical forces for cartilage tissue engineering applications. These multi-potential cells (Pittenger et al. 1999), typically isolated from the bone marrow, can be induced to undergo chondrogenesis and secrete cartilage-specific ECM in a variety of 3D hydrogels (Worster et al. 2001; Awad et al. 2004; Mauck et al. 2006; Angele et al. 2004; Erickson et al. 2002; Caterson et al. 2002). More recently, these cells have been shown to achieve functional cartilaginous properties in long-term free swelling agarose culture, although they do so to a lesser extent than primary chondrocytes (Mauck et al. 2006). It is generally believed that MSC differentiation is at least in part mechanically regulated, and that the same signals used to optimize construct growth may induce lineage-specific differentiation. For example, tensile stretching increased ligament differentiation of MSCs in collagen (Altman et al. 2002; Awad et al. 1999) while cyclic substrate strain in monolayer enhanced osteogenesis and mineralization (Simmons et al. 2003). Furthermore, both static and dynamic loading have been shown to increase the chondrogenic differentiation of limb bud mesenchymal cells in collagen and agarose (Takahashi et al. 1998; Elder et al. 2000, 2001, Elder 2002), depending on the frequency and duration of loading (Elder et al. 2001). More recently, studies in hyaluranon sponges (Angele et al. 2004) and agarose (Huang et al. 2004a) have demonstrated enhanced MSC chondrogenesis with dynamic compressive loading. In one study, increased aggrecan gene expression was observed with as few as three 4 h loading cycles (Huang et al. 2004a). Dynamic hydrostatic pressure has also been shown to enhance chondrogenesis of MSCs in pellet culture (Angele et al. 2003) and in alginate hydrogels (Mauck et al. 2003c).

Based on these previous findings, the purpose of this study was to develop a reporter promoter system for both chondrocytes and MSCs to examine the transcriptional regulation of cartilage-specific ECM molecules in a well-defined 3D culture system in response to variations in the mechanical environment. Furthermore, we sought to verify that this reporter system could be used to distinguish regional differences in molecular regulation, and to compare these differences to predicted mechanical signals using a custom biphasic finite element formulation. We also examined the persistence of signal within the construct to determine if studies could be carried out both before and after significant ECM deposition. Finally, we sought to demonstrate that short-term loading indicators of molecular regulation could be a useful metric for optimization of long-term matrix production and/or chondrogenic differentiation.

2 Materials and methods

2.1 Chondrocyte and MSC isolation and expansion

Bone marrow derived MSCs were harvested from the tibiae and femora of freshly slaughtered ($<$ 36 h) 3–6 month old calves (Fresh Farms Beef, Rutland, VT, USA). The marrow from four separate femur/tibia marrow isolations (minimum of four animals) was pooled and deposited into an equal volume of high glucose Dulbecco's Modified Eagle Medium (hgDMEM) supplemented with 1× penicillin/streptomycin/fungizone (PSF) and 300 U/mL heparin. After vortexing, the marrow was digested with 0.075% Trypsin–EDTA for 15 min at 37°C and the resulting suspension was plated onto 15 cm tissue culture plates (Falcon Becton Dickinson Labware, Franklin Lakes, NJ, USA). After 48 h to allow for cell attachment, cultures were washed vigorously with phosphate buffered saline (PBS) and then maintained in hgDMEM with 10% fetal bovine serum (FBS) and 1× PSF. The medium was exchanged twice weekly until cultures reached confluence. Subsequent sub-culturing was carried out at a 1:3 expansion ratio, with cultures up to passage three utilized for these studies.

Articular cartilage was harvested from the carpometacarpal (CMC) joints of matched donors. Cartilage was aseptically diced and removed from the joint, rinsed in DMEM containing 2×PSF, and incubated overnight at 37°C in a humidified 5% CO_2 incubator. The cartilage pieces were combined and digested in DMEM with 2.5 mg/mL Pronase (Calbiochem, San Diego, CA, USA) for 1 h at 37°C with stirring (180 rpm), followed by 0.5 mg/mL collagenase type IV (Sigma Chemicals, St. Louis, MO, USA) for 5 h at 37°C with stirring. The chondrocyte suspension was then filtered (40 μm cell strainer, BD Falcon, Bedford, MA, USA) to isolate individual cells. Chondrocytes were pelleted (5 min, 300g) and resuspended in 10 mL of DMEM, and viable cells

were counted using a hemocytometer. Chondrocytes were cultured in hgDMEM with 10% FBS and 1×PSF at 70,000 cells/cm^2 to achieve high initial confluency so as to limit cell division and maintain the chondrocyte phenotype.

2.2 Transfection and encapsulation of chondrocytes and MSCs

Chondrocytes (P1) and MSCs (P2, P3) were plated in T175s at 3.5×10^4 or 1.75×10^4 cells/cm^2, respectively, and transfected with 0.1 μg DNA/cm^2 using the Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN, USA) with 3 μL of reagent/microgram of DNA in DMEM with 10%FBS (without antibiotics). In preliminary studies, transfection efficiency was monitored using a constitutively active DsRed2 plasmid (pDsRed2-N1, Clontech, Mountain View, CA, USA), with pcDNA (Invitrogen, Carlsbad, CA, USA) used as a negative control. Three days after transfection, cell populations were imaged with an inverted fluorescent microscope and efficiency assayed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). For reporter studies, cells were co-transfected with 0.875 μg/10 cm^2 pAGC (−2.4 kb)-Luc (Aggrecan/firefly luciferase, courtesy of Wilmot Valhmu) or pCol2(−4.0 kb)-Luc (Collagen II/firefly luciferase, courtesy of Mary Goldring) promoter reporter plasmid and 0.125 μg/10 cm^2 of pRL-CMV (constitutive Renilla luciferase, Promega, Madison, WI, USA). Three days later, cells were trypsinized, counted, and seeded in 2% agarose at 20 million cells/mL as described previously (Mauck et al. 2003a), forming cylindrical disks of 4 mm diameter and 1.5 mm thickness. Chondrocyte-laden disks were cultured in a chemically defined medium (CDM) consisting of hgDMEM supplemented with 1× PSF, 50 μg/mL ascorbate 2-phosphate, 40 μg/mL L-proline, 100 μg/mL sodium pyruvate, and 1× ITS + pre-mix for 3 days to allow cells to acclimate to agarose encapsulation. The MSC-laden disks were cultured similarly, with further supplementation with 0.1 μM dexamethasone.

To assess the long-term stability of expression, chondrocyte-laden gels were formed as earlier with cells transfected with either CMV-Renilla or DsRed2 plasmid and cultured in CDM with (CDM+) and without (CDM-) the addition of 10 ng/mL TGF-β3 (R&D Systems, Minneapolis, MN, USA). Culture of chondrocytes and MSCs in CDM+ (containing TGF-β3) has been shown to expedite maturation and/or initiate differentiation in 3D agarose culture (Mauck et al. 2006). Disks from two replicate experiments with different cell isolations were cultured under free swelling conditions for 28 days, with medium changed every third day. At weekly intervals, three disks were assayed for Renilla luciferase activity and one was imaged in cross section to view fluorescent intensity and distribution.

2.3 Short-term loading studies with chondrocyte and MSC-laden constructs

Mechanical loading in unconfined compression was carried out using a custom dynamic compressive loading bioreactor (Fig. 1). Axial compression was applied using a stepper motor and linear stage (ATS0300, Aerotech, Pittsburgh, PA, USA), with resolution of 0.1 μm and maximum speed of 4 mm/s. Stage position was controlled using a Unidex U100 motor controller interfacing through a PC serial port and a LabVIEW GUI (National Instruments, Austin, TX, USA). Custom scripts were developed to define stage motion. Displacement was independently verified using an externally mounted linear variable displacement transducer (PR-812, Macro Sensors, Pennsauken, NJ, USA). Displacement profiles were sinusoidal (Fig. 1), though frequency response was slightly lower than nominal indications. Constructs were loaded in 10 cm Petri dishes with their lateral motion constrained by an agarose spacer (1 mm thick) with 5 mm diameter cores (Fig. 1). Flat Ultem indenters joined to the linear stage entered the Petri dishes to contact the underlying disks, while sterility was ensured with flanges that spanned the Petri dish edge. To delineate the pro-chondrogenic effects of dynamic stimulation, loading was carried out in 7 mL of CDM- (without TGF-β3), a sufficient volume to ensure that samples remained submerged. Control constructs were treated similarly, without deformational loading. To examine the effect of loading duration, dynamic loading was carried out for 60 or 180 min at 1 Hz and 10% deformation. To examine the effect of frequency, constructs were loaded at a nominal frequency of 0.33, 1.0, or 3 Hz for 60 min. After loading, constructs were transferred to non-tissue culture treated 6-well plates (6–8 constructs per well) containing 4 mL of the medium. Constructs were harvested 24 or 72 h after loading (Fig. 2), and separated into an inner core (2 mm) and outer annulus with a disposable dermal punch (Miltex, York, PA, USA). Positive controls were treated with CDM+ medium (containing TGF-β3) for 24 or 72 h. To increase luminescent signal, isolated regions of the two disks were combined, with a minimum of six disks (three independent samples) collected per condition per replicate experiment. Constructs were frozen in 100 μL of passive lysis buffer, thawed, and disrupted with a motorized pestle. After one further freeze–thaw cycle, 20 μL of supernatant was assayed for Firely and Renilla luciferase activity (Dual Luciferase Reporter Assay, Promega) using a luminometer. Luminescence from Firefly luciferase activity was integrated for 30 s, while Renilla luciferase was integrated for 10 s for each sample.

2.4 Long-term loading studies with MSC-laden constructs

In one pilot study examining the long-term consequence of a short period of loading on MSC-laden constructs, constructs containing non-transfected P2 bovine MSCs were formed as described earlier. The constructs (∼20 per group) were maintained in free-swelling culture or loaded at 10% deformation for 180 min per day at 1 Hz frequency each day for an initial 5 day period. The loading was carried out as mentioned earlier, in 7 mL of CDM-. After loading, cultures were replenished with 20 mL of fresh CDM- for overnight culture. Free-swelling constructs were maintained similarly.

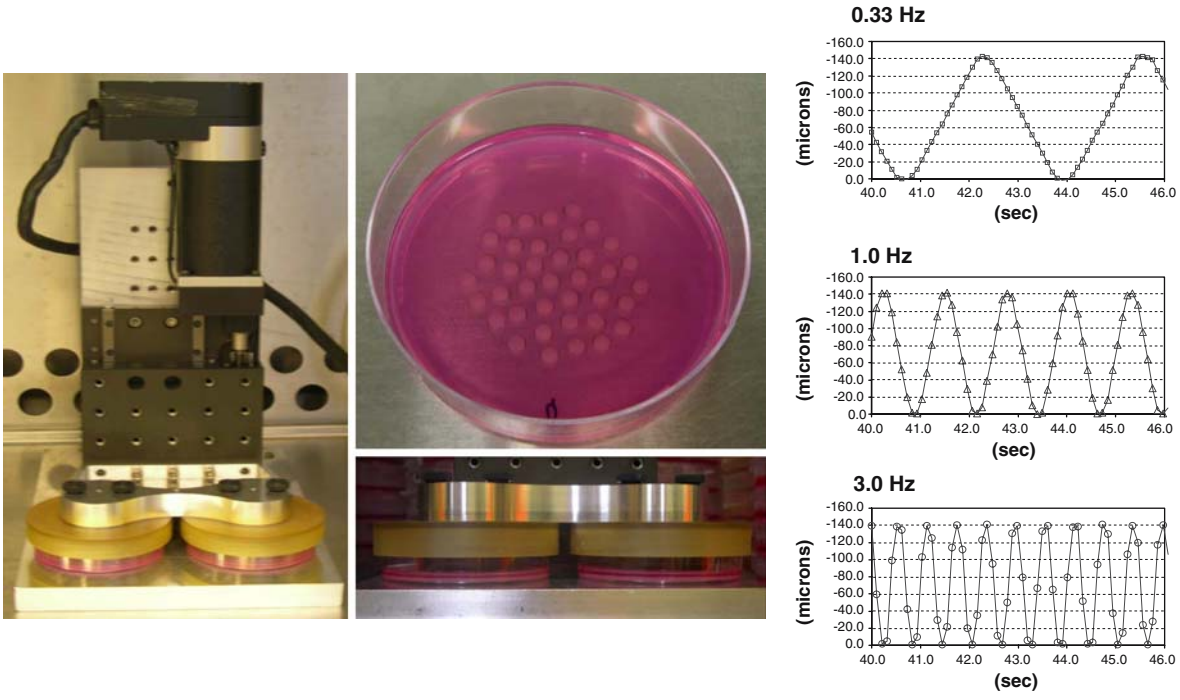

Fig. 1 A compressive loading bioreactor (*left*) was used to apply a defined cyclic compressive deformation to cell-seeded agarose constructs in a Petri dish (*top middle*). Simultaneous compression in two distinct chambers was applied via custom Ultem platens that enter the Petri dish with flanges to maintain sterility (*bottom middle*). Defined compressive loading regimes of 10% of specimen thickness at nominal frequencies of 0.33, 1.0, and 3.0 Hz were used, with true displacement for each loading regime visualized using an externally mounted LVDT (*right*)

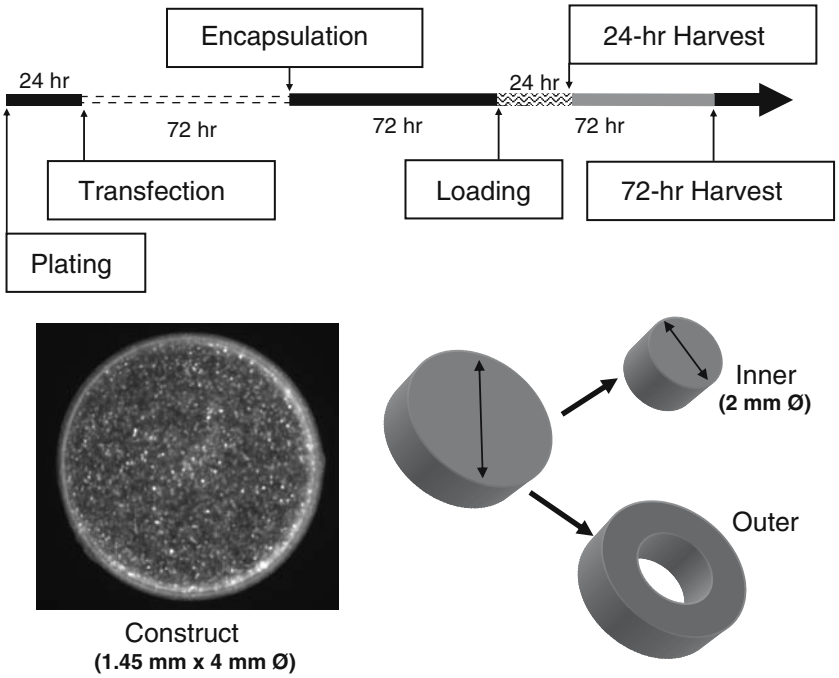

Fig. 2 Experimental design for reporter promoter studies. Bovine chondrocytes and MSCs were transfected with Aggrecan or type II collagen reporter promoter constructs, seeded in agarose, and exposed to a defined compressive loading protocol. At 24 and 72 h after loading, constructs were harvested and separated into a central core and an outer annular region for analysis

After this loading period, both groups of constructs were maintained in free-swelling culture in CDM-, with medium changed every third day. A separate group of constructs from the same MSC population was maintained in free-swelling conditions for the entire culture duration (28 days) in CDM+ (with TGF-β3) as a positive control. On days 0, 3, 7, 14,

and 28, the equilibrium Young's modulus was determined as described previously (Mauck et al. 2003a). After testing, samples were frozen for subsequent biochemical analyses. At each time point, one sample was fixed for histological analysis.

2.5 Real-time PCR

Real-time PCR was conducted to verify endogenous expression of cartilaginous ECM molecules by chondrocytes and MSCs in agarose hydrogels. Constructs containing non-transfected chondrocytes or MSCs were maintained in CDM- or CDM+ for 3 days. Five constructs from each condition were pooled and frozen at −80°C. Total RNA was extracted from cell-seeded agarose by grinding samples in 1 mL of TRIZOL reagent, followed by chloroform extraction. After phase separation, the aqueous supernatant from this first extraction was diluted with a further 1 mL of TRIZOL. After another chloroform extraction, precipitation in isopropanol and ethanol wash, dried pellets were resuspended in 50 μL of DEPC H_20 at 65°C for 5 min. Even after this dual-extraction, agarose contamination was observed as a small gel nodule at the base of each tube. The liquid phase above this gel remnant (∼ 30 μL) was removed and RNA yield and purity assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA yield ranged from 185–290 ng/μL, with 260/280 ratios of > 1.8 for all samples. Reverse transcription was performed using the First Strand cDNA Synthesis kit (Invitrogen) with 1 μg of total RNA using random hexamers according to the manufacturer's instructions. cDNA amplification was carried out using an iCycler real-time PCR system (Bio-Rad, Hercules, CA, USA) with primers for bovine type II collagen, aggrecan, and 18sRNA (Fitzgerald et al. 2004) using SYBR Green PCR Reaction Mix (Bio-Rad). Starting quantities of target gene transcripts in unknowns were derived from corresponding standard curves generated from bovine chondrocyte cDNA for each primer pair, with overall expression normalized to 18sRNA in each sample.

2.6 Biphasic finite element analysis

To quantify the regional variation in mechanical signals in dynamically loaded constructs, a custom biphasic finite element model was employed. Finite element meshes were defined with a commercial software package (I-DEAS, SDRC, Plano, TX, USA) to model a cylindrical agarose construct with an aspect ratio of 2.5 (diameter/height). Meshes were defined as axisymmetric, and contained 600 elements per mesh with eight nodes per element, with the distribution of elements biased towards the free (lateral) edge. The gel was defined as homogenous, with a linear isotropic elastic solid matrix having a compressive Young's modulus (E_Y) = 10 kPa, a poisson's ratio (ν) = 0.3, and a hydraulic permeability (k) = 1×10^{-12} m^4/Ns. A custom FEM program (as described in Park et al. 2003) incorporating biphasic theory (Mow et al. 1980) was used to solve the problem of unconfined deformational loading of constructs with an applied sinusoidal deformation of 10% of the gel thickness at frequencies of 3.0, 1.0, and 0.33 Hz. The spatial distribution of mechanical signals generated within the gel were output and analyzed at the point of maximal compressive axial deformation during the first cycle. For specific mechanical parameters, an average value was calculated by integrating across the radial expanse of the inner core and outer annulus regions.

2.7 Biochemical analysis

For long-term loading studies of MSC-laden constructs, samples were weighed wet, disrupted with a motorized pestle, and digested for 16 h in papain (9 U/mL in 0.1 M sodium acetate, 10 M cysteine HCl, 0.05 M EDTA, pH 6.0) at 60°C. Aliquots of digest were analyzed for sulfated glycosaminoglycan (sGAG) content using the 1,9-dimethylmethylene blue dye-binding assay (Farndale et al. 1986), against a standard curve of chondroitin-6-sulphate.

2.8 Histology

For long-term loading studies of MSC-laden constructs, samples for histology were fixed overnight at 4°C in paraformaldehyde, dehydrated in a graded series of ethanol, embedded in paraffin (Tissue Prep, Fisher Scientific), and sectioned at 8 μm thickness. The sections were stained with hematoxylin and eosin (H&E, Sigma, St. Louis, MO, USA) for general histology or with Alcian Blue (pH 1.0) to detect sulfated proteoglycans. Stained specimens were imaged with a color CCD camera and an inverted microscope.

2.9 Statistical analysis

Analysis of variance (ANOVA) was performed using STATISTICA (Statsoft Inc., Tulsa, OK, USA), with Fisher's LSD post hoc testing between groups. For short-term studies, independent variables were either duration (0, 60, 180 min) or frequency of loading (0.33, 1.0, and 3.0 Hz) with normalized reporter expression level (aggrecan or collagen II) as the dependent variable. For long-term studies, time in culture and loading condition were the independent variables, while GAG content or Young's modulus were dependent variables. All data are reported as the mean and standard deviation of 3–9 samples, with significance set at $p < 0.05$.

3 Results

In this study, we developed an economical, time-efficient approach to monitor the transcriptional activities of cartilage-matrix genes in 3D hydrogel culture following mechanical stimulation. In preliminary studies, transfection

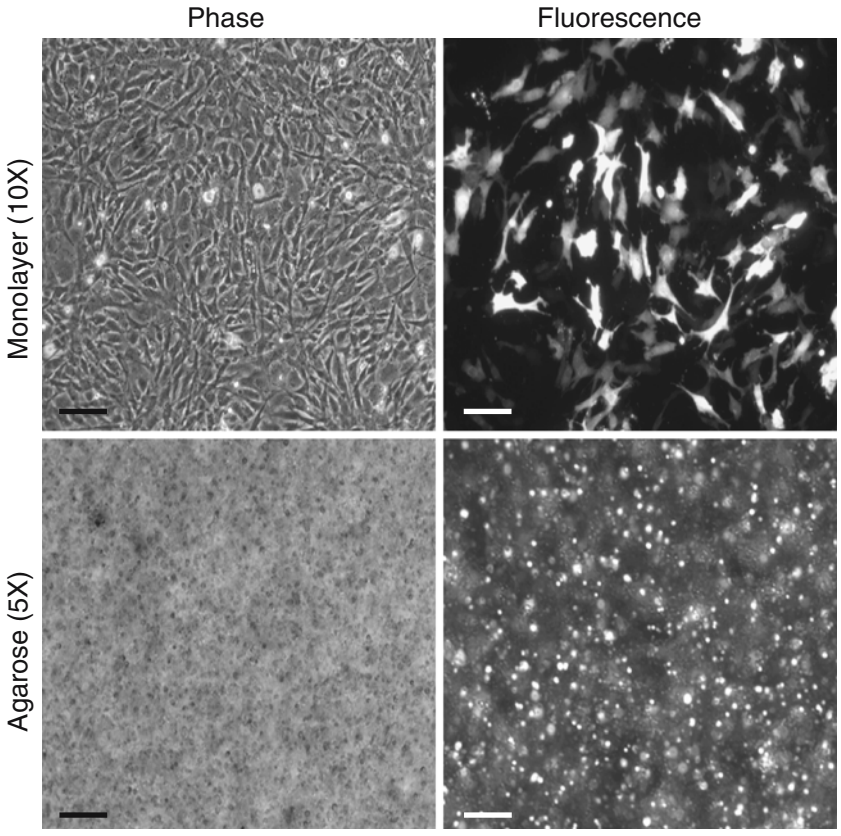

Fig. 3 Phase contrast (*left*) and fluorescent imaging (*right*) of chondrocytes in monolayer culture (*top*, scale bar: 100 μm) and 3D agarose culture (*bottom*, scale bar: 200 μm). Nearly half of all cells express DsRed2 in monolayer, and are well dispersed when seeded in agarose

efficiencies of chondrocytes and MSCs were assessed using Fugene transfection reagent. As determined by flow cytometric detection of DsRed2 3 days post-transfection, efficiencies were observed to be relatively high for this conventional liposome-mediated methodology, specifically $47 \pm 5\%$ for chondrocytes ($n = 4$) and $15 \pm 1\%$ for MSCs ($n = 7$). The DsRed2 expressing cells were visible with fluorescent microscopy both in monolayer and in 3D culture (Fig. 3). In 3D culture, encapsulated cells were well dispersed throughout the construct. In chondrocyte-laden constructs, quantitative analysis of Renilla expression revealed a rapid decrease in CDM- medium of $> 90\%$ over first week, with levels stabilizing at $\sim 3\%$ of starting values between days 14–28 ($n = 6$, Fig. 4). In chondrocyte-laden constructs cultured in CDM+ medium, the decline in expression levels was less abrupt, falling $\sim 25\%$ over the first week, and stabilizing at 20–30% of starting values between days 14–28 ($n = 6$, Fig. 4). Regardless of expression levels, signal from both culture conditions remained well above background levels and DsRed2 positive cells were readily observable after 14 days of culture in either media condition (Fig. 4). Furthermore, as evidenced by the phase microscopy, significant matrix deposition was observed in these transfected chondrocyte-laden gels following culture in CDM+ medium. Substantiating this observation, the compressive Young's modulus increased dramatically over the time course of the study for transfected chondrocyte-laden constructs maintained in CDM+ medium ($E_Y(\text{D14}) = 60 \pm 10\,\text{kPa}$, $E_Y(\text{D28}) = 170 \pm 20\,\text{kPa}$, $n = 6$). These values are similar to that found for non-transfected chondrocyte-laden constructs maintained similarly (Mauck et al. 2006), indicating that even following transfection, cells retain their phenotypic capacity to form functional matrix over time.

After verifying sustained reporter activity, homogeneous cell distribution, and cell phenotype retention, short-term dynamic loading experiments were performed on gels seeded with chondrocytes or MSCs transfected with either aggrecan or type II collagen promoter reporter constructs. To ensure that these genes were being endogenously transcribed in positive controls, real-time PCR was performed. Culture of MSC-laden gels in CDM+ with dexamethasone for 3 days led to an ~ 42- and ~ 125-fold increase in type II collagen and aggrecan mRNA levels, respectively, compared to CDM- controls. Increases of $\sim 2{,}160$- and ~ 85-fold, respectively, were observed for chondrocytes in CDM+ compared to CDM- controls (data not shown).

Finite element modeling of the various dynamic loading regimes revealed the anticipated heterogeneity of mechanical signals within the hydrogel construct. By applying the defined loading and boundary conditions to the finite element mesh, radial variation in mechanical signals was observed across the expanse of the construct (Fig. 5). At each

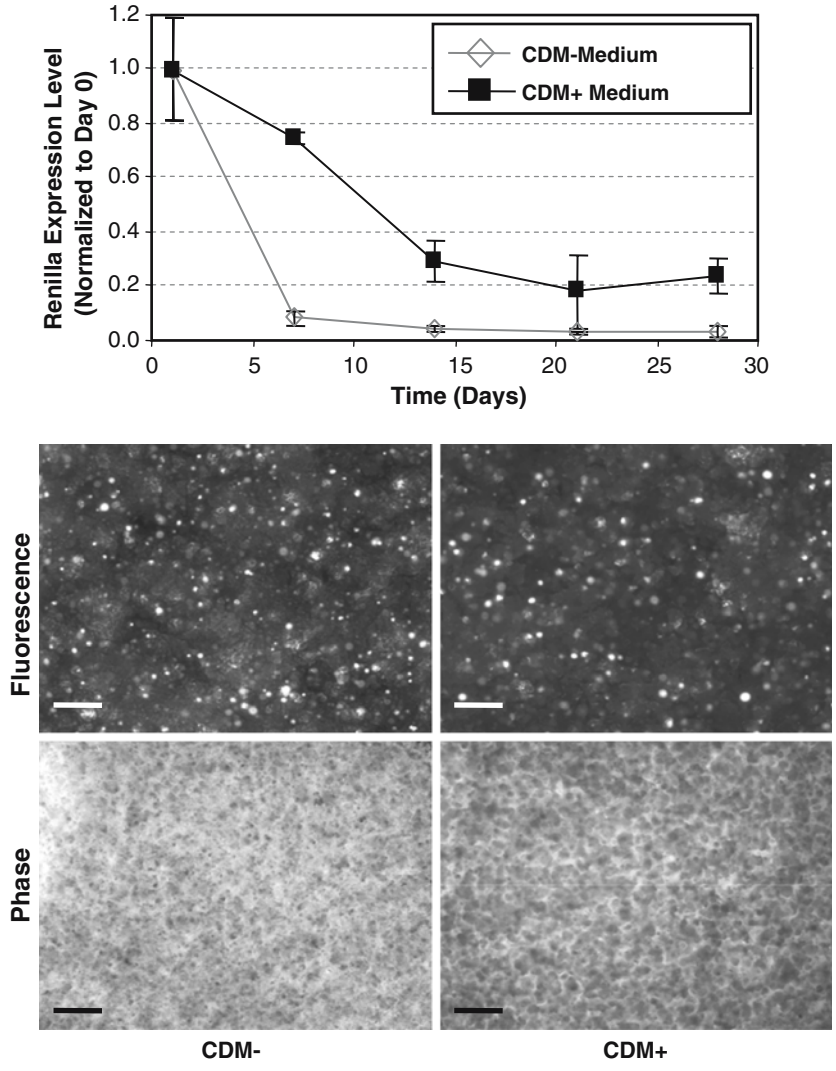

Fig. 4 (*Top*) Persistence of Renilla luciferase activity in chondrocyte-laden agarose disks with time in culture in CDM− and CDM+ medium ($n = 6$/time point). (*Bottom*) Fluorescent and phase microscopy of chondrocyte-seeded agarose cross-sections on Day 14 maintained in CDM+ or CDM−. Scale bar: 200 μm

loading frequency, hydrostatic pressure in the construct was maximal (∼400 Pa) at the center, and fell to zero at the free edge. Increasing frequency expanded the range of peak pressure, such that the average pressure in the outer annulus was slightly higher for 3.0 Hz compared to 0.33 Hz loading conditions (Table 1). Similarly, radial strain was highest at the center of the construct with rapid decreases occurring at the free edge. Fluid flux was nearly zero at the center and throughout the bulk of the construct, and quickly increased to peak values near the free border, with highest fluxes observed with the fastest loading regimes. It should be noted that on a gross level, loading at 1 Hz for 60 and 180 min results in a total of 3,600 or 10,800 deformation cycles, respectively, while loading for 60 min at 0.33 or 3.0 Hz results in 1,200 cycles or 10,800 cycles, respectively. Similarly, at higher loading frequencies, the average and maximal values calculated above are achieved over a shorter time span, resulting in a greater rate of change for these parameters.

Applying these dynamic loading protocols to chondrocyte-seeded gels using a custom bioreactor led to significant elevations in aggrecan promoter activity after 72 h (Fig. 6). In the inner core, increases of ∼60% were observed after 60 and 180 min of dynamic loading ($p < 0.02$ vs. free swelling (FS) inner for both). In the outer region, aggrecan promoter activity increased by ∼50% with 60 min of loading, and by ∼340% with 180 min of loading ($p < 0.01$ vs. FS outer for 180 min). Frequency of applied load also modulated aggrecan promoter activity in both the inner and outer regions. Loading for 60 min at 1.0 and 3.0 Hz led to significant elevations in aggrecan promoter activity in the inner core of ∼60% and ∼50%, respectively, after 72 h ($p < 0.03$ for both). In the outer region, loading for 60 min at 0.33, 1.0, and

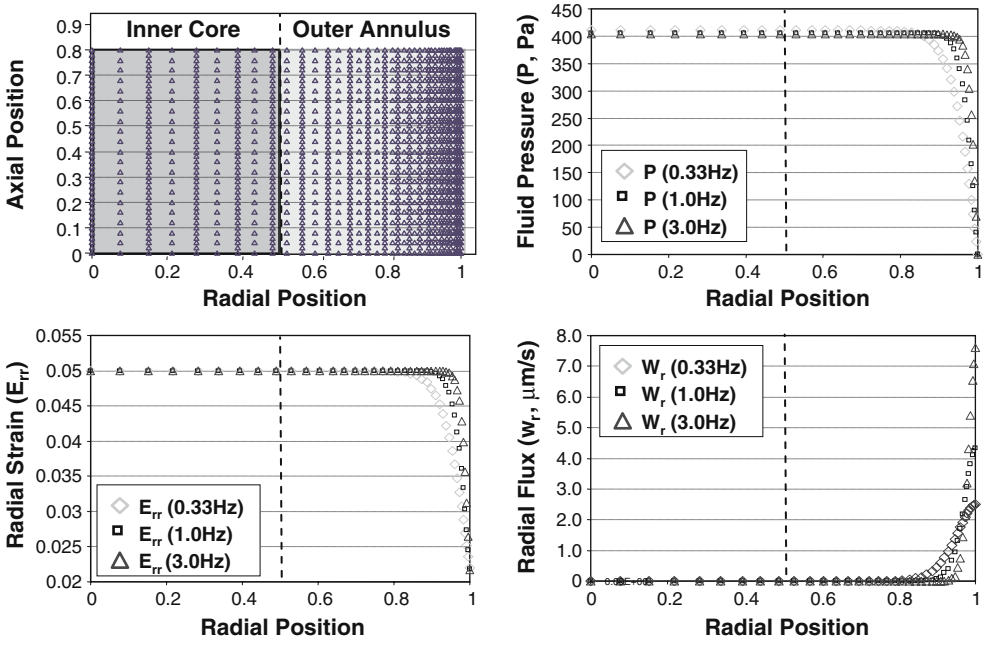

Fig. 5 Finite element modeling of mechanical response of cell-laden hydrogels to a defined mechanical loading regime. (*Top left*) Axisymmetric representation of the distribution of nodes in the finite element mesh, showing bias towards the free edge. Sample plots of hydrostatic pressure (*top right*), radial strain (*bottom left*), and fluid flow (*bottom right*) within constructs at the point of maximal compression for three different loading frequencies

Table 1 Summary of spatially averaged physical signals and maxima in the inner core and outer annulus regions of constructs loaded at three different frequencies

Measure	Frequency (Hz)	Inner core		Outer annulus	
		Average	Maximum	Average	Maximum
Radial flux (w_r, m/s)	0.33	7.91E-15	1.01E-13	3.21E-7	2.50E-6
	1.0	2.53E-16	1.54E-15	3.18E-7	4.34E-6
	3.0	2.79E-17	1.28E-16	3.16E-7	7.6E-6
Radial strain (E_{rr})	0.33	0.05	0.05	0.0475	0.05
	1.0	0.05	0.05	0.0485	0.05
	3.0	0.05	0.05	0.492	0.05
Pressure (P, Pa)	0.33	410	410	374	410
	1.0	406	406	385	406
	3.0	403	403	391	403
Axial stress (σ_{zz}, Pa)	0.33	−800	−800	−816	−976
	1.0	−800	−800	−809	−974
	3.0	−800	−800	−805	−972

3.0 Hz led to increase of ∼189, ∼50, and ∼328%, respectively ($p<0.05$ for 0.33 and 3 Hz). Positive control constructs treated with TGF-β3 for 72 h demonstrated increased aggrecan promoter activity in both the inner (∼267%, $p<0.003$) and outer (∼806%, $p<0.001$) regions, with the increases in the outer region higher than that of the inner core ($p<0.001$) (data not shown). Interestingly, type II collagen promoter activity showed an opposite response to dynamic loading (Fig. 7). Specifically, at both 24 and 72 h following 180 min of loading at 1 Hz, type II collagen promoter activity was decreased in the outer annulus relative to free swelling controls ($p<0.01$). The central core also showed a trend of decreasing type II collagen promoter activity with loading ($p<0.1$ at 24 h, $p<0.01$ at 72 h).

Dynamic loading of MSC-laden constructs also led to an increase in aggrecan promoter activity (Fig. 8). Twenty-four hours after loading, a similar pattern of promoter activity was observed in both the central core and the outer ring, with significant increases with 180 min of loading ($p<0.01$). Exposure to TGF-β3 for 24 h led to significant increases in aggrecan promoter activity in the outer region (775%, $p<0.05$), with smaller increases (180%, not significant) observed in the inner core (data not shown). To extend these short-term findings, MSC-laden constructs (with no prior transfection) were loaded for 180 min each day at 10% compression and 1.0 Hz for 5 days followed by culture under free-swelling conditions for a total culture time of 28 days. Histologic analyses revealed that even after 3 days of loading, increased Alcian blue staining of proteoglycans (PGs) was observed in dynamically-loaded (DL) constructs compared to FS controls maintained in CDM- with dexamethasone. However, addition of TGF-β3 to FS disks resulted in greater PG staining at this time point (Fig. 9). sGAG quantification revealed increases in DL constructs compared to FS controls (cultured in CDM-) by day 14 ($p<0.01$), with trends continuing over the 28-day culture period. On day 28, DL constructs contained 85 ± 13 μg sGAG/construct, while FS constructs contained 49±6 μg/construct ($p<0.01$). The constructs maintained in FS culture in the presence of TGF-β3 accumulated significantly more sGAG, however, reaching 512 ± 140 μg/construct on day 28 ($p<0.03$) (data not shown). In accordance to the relative sGAG content of the examined groups,

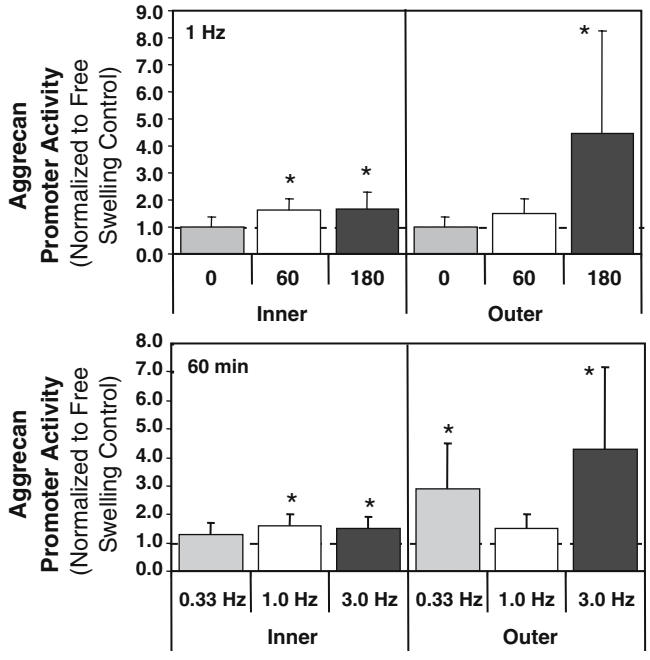

Fig. 6 Dynamic loading increases aggrecan promoter activity in both the inner core and outer annulus of chondrocyte-laden constructs. (*Top*) Aggrecan reporter promoter activity 72 h after loading as a function of duration of loading (0, 60, 180 min at 1 Hz). In each region, promoter activity is normalized to the corresponding free swelling (0 min of loading) control in that region. *Asterisk* indicates $p < 0.05$ versus control, $n = 7–8$. (*Bottom*) Aggrecan reporter promoter activity 72 h after being subjected to dynamic loading at different frequencies (0.33, 1.0, and 3.0 Hz for 60 min). In each region, promoter activity is normalized to the corresponding free-swelling control for that region. *Asterisk* indicates $p < 0.05$ versus 0 Hz (no load) control, $n = 7–8$

the mechanical properties of MSC-laden constructs in CDM- did not change over time with and without loading, while those maintained in free-swelling culture in the presence of TGF-β3 rose significantly, reaching 23 ± 6 kPa by day 28 ($p < 0.005$ vs. both FS and DL CDM-).

4 Discussion

This study explored the biologic response of chondrocytes and MSCs to a defined mechanical loading regime in 3D hydrogel culture. To evaluate the local mechanical environment generated with loading, a biphasic finite element formulation was used to predict physical signals generated with unconfined compressive loading across the radial expanse of the gel disks. With pre-defined dynamic loading regimes, distinct regions of mechanical signals were observed; signals were uniform in the central region (higher pressure, no flow), and changed rapidly at the free periphery (lower pressure, higher flow). Using a custom dynamic compression bioreactor system, the loading regimes used in the finite element formulations were applied to chondrocyte-laden constructs that had been transfected with promoter reporter constructs for aggrecan and type II collagen. At defined time points after cessation of loading (24 and 72 h), promoter activity was evaluated in both the central region (core) and outer annulus. In accordance with previous studies assaying sulfate incorporation (Buschmann et al. 1995, 1999), aggrecan promoter activity increased with increasing duration of loading, particularly in the outer region. Conversely, type II collagen promoter activity decreased in the outer region with these same loading conditions. This finding is counter to the previously reported measures of bulk collagen synthesis using radiolabeled proline (Buschmann et al. 1995), suggesting that collagen types may be differentially modulated by the higher fluid flow occurring at the periphery of constructs. Moreover, this finding suggests that optimization of expression of specific matrix elements may require fine-tuning of the loading regimes and boundary conditions to generate enhanced production throughout a dynamically loaded engineered construct.

In addition to the duration of loading, the number of loading cycles applied (Chowdhury et al. 2003) and the rate of onset (Bao et al. 2000) of mechanical signals play a role in the downstream biosynthetic response of cells. For example, studies by Lee and Bader (1999) and Buschmann et al. (1995) have suggested that chondrocytes in agarose increase sulfate incorporation with increasing loading frequencies to a certain level. In the present study, all frequencies examined in short-term studies resulted in increased aggrecan promoter activity, with fast (3.0 Hz) loading resulting in the highest activity. Additionally, highest aggrecan promoter activity was observed for groups experiencing the greatest number of cycles (10,800, either 60 min at 3 Hz or 180 min at 1 Hz). These findings necessitate further study to elucidate the specific mechanism and interaction of duration, time rate of change, and total number of applications of a physical stimulus to chondrocytes in 3D culture.

In addition to chondrocytes, we explored the role of mechanical compression in directing chondrogenic differentiation of MSCs in the short-term by monitoring aggrecan promoter activity. It has previously been reported that dynamic loading of rabbit MSCs in agarose leads to enhanced aggrecan gene expression within a period of 3 days (Huang et al. 2004b). Our findings confirm these results, with aggrecan promoter activity increasing with increasing loading duration when assayed after 24 h. Interestingly, and counter to what was observed for chondrocytes where effects in the annular region were more predominant, a similar pattern of increases in aggrecan promoter activity was observed in both the central core and the outer annulus in MSC-laden constructs. This finding suggests that mechanical signals that are persistent throughout the constructs (such as hydrostatic pressure and/or deformation) may be more important to direct chondrogenesis than the fluid flow that occurs at the periphery. To determine the long-term utility of these loading protocols for directing differentiation, one auspicious loading protocol was applied to MSC-laden constructs for 5 days, and the resultant changes in ECM production were evaluated over a 4-week period. PG deposition was enhanced as quickly as 3 days after loading onset, with quantitative differences

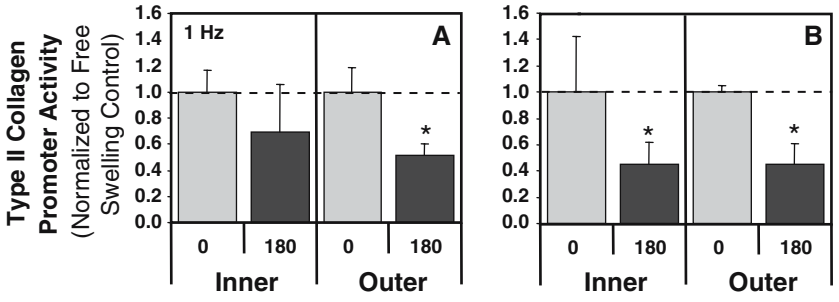

Fig. 7 Dynamic loading decreases type II collagen promoter activity in chondrocyte-laden constructs, particularly in the outer annulus. Type II collagen promoter activity 24 h (**a**) and 72 h (**b**) after dynamic loading at 1 Hz for 180 min. In each region, promoter activity is normalized to the corresponding free-swelling (0 min of loading) control in that region. $*p<0.01$, $n = 4$

persisting between loaded samples and non-loaded controls subsequently maintained in free-swelling culture for the remainder of a 28-day period. Notably, this lineage-specific differentiation and enhanced matrix formation occurred in the absence of TGF-β3, suggesting that mechanical signals alone can enhance chondrogenesis. It should be pointed out, however, that while loading increased both the appearance and quantity of PG in the constructs compared to non-loaded controls, dynamic loading alone did not influence the mechanical properties of MSC-laden disks. Moreover, both the biochemical content and mechanical properties of these gels (free-swelling and dynamically loaded) was significantly lower than that of constructs maintained in free-swelling culture in the presence of TGF-β3. While promising, these results suggest that continued optimization must be performed to improve the long-term growth and mechanical integrity of these MSC-laden hydrogel constructs.

In conducting these studies, we developed a time- and cost-efficient methodology for monitoring regional changes in ECM gene transcription using reporter promoter constructs. By embedding a transfected population of chondrocytes or MSCs in agarose, the cells themselves act as in situ indicators of the biologic consequence of the local mechanical environment. While this study focused on only two promoters of cartilaginous ECM elements, the system is sufficiently general that numerous other molecules could be similarly investigated. Furthermore, while this study examined time points reflective of the long-term effects (24 and 72 h), further explorations could be made of more transient phenomenon as the half-life of luciferase in mammalian cells is on the order of 3 h. Additionally, this methodology could be used to examine the interactions between biochemical signaling pathways, such as TGF-β superfamily molecules, and mechanical stimuli on chondrogenic gene transcriptional activity. Furthermore, signal transduction cascades thought to be involved in the processing of these mechanical signals may be tested with the inclusion of dominant negative constructs in conjunction

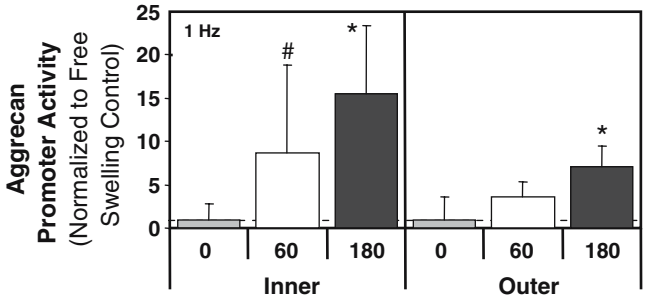

Fig. 8 Dynamic loading at 1 Hz increases aggrecan promoter activity in both the central core and outer annulus of MSC-laden constructs, with longer durations of loading trending toward higher activities. In each region, promoter activity is normalized to the corresponding free swelling (0 min of loading) control in that region. $\#p<0.10$, $*p<0.05$ vs. 0 min (no load) control, $n=3$–4

with reporter promoters of ECM constituents (Fortier et al. 2004). In the present study, reporter expression was observed to stabilize with time, suggesting that the system may be used to explore transcriptional activities over a broad time course of new tissue formation. Such a study, coupled with more refined finite element analysis (Guilak and Mow 2000) representing the heterogeneous forming ECM (Sengers et al. 2004) may be useful in tailoring loading regimes to optimize matrix deposition over the entire time course of construct growth. Taken together, these results demonstrate the utility of this reporter promoter system for optimizing loading protocols to improve the outcome of engineered chondrocyte- and MSC-laden cartilaginous constructs.

Acknowledgements This study was supported with funding from NIH ZO1 AR41131. The authors gratefully acknowledge Dr. Wilmot Valhmu (University of Wisconsin, Madison, WI, USA) and Dr. Mary Goldring (Beth Israel Deaconess Medical Center, Boston, MA, USA) for provision of the reporter promoter constructs for aggrecan and type II collagen.

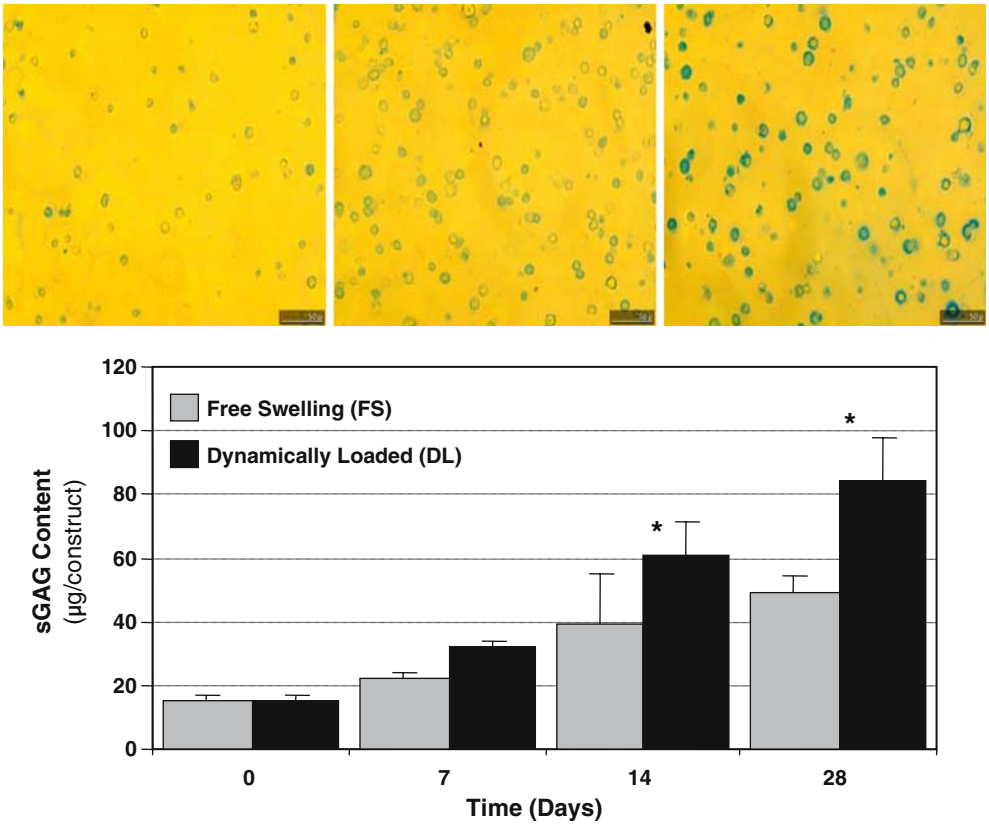

Fig. 9 Five days of dynamic loading (1 Hz, 180 min, 10% deformation) followed by 23 days of free-swelling culture (for a total of 28 days of culture) leads to both short-term and sustained increases in GAG deposition in MSC-laden hydrogels. (*Top*) *Alcian blue* staining of the central regions of constructs after 3 days of culture in FS CDM- (*left*), DL CDM- (*middle*) or FS CDM+ (*right*) conditions. Scale bar: 50 µm. (*Bottom*) sGAG content of constructs with time in culture in CDM- medium with (DL) and without (FS) dynamic loading over the first 5 days of culture. $^*p < 0.05$, $n = 3$/group

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