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Mechanical loading regimes affect the anabolic and catabolic activities by chondrocytes encapsulated in PEG hydrogels

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Summary

Objective: Mechanical loading of cell-laden synthetic hydrogels is one strategy for regenerating functional cartilage. This work tests the hypothesis that type of loading (continuous vs intermittent) and timing when loading is applied (immediate vs delayed) influence anabolic and catabolic activities of chondrocytes when encapsulated in poly(ethylene glycol) (PEG) hydrogels.

Methods: Primary bovine chondrocytes encapsulated in PEG hydrogels were subjected to unconfined dynamic compressive strains applied continuously or intermittently for 1 week (i.e., immediate) or intermittently for 1 week but after a 1 week free-swelling (FS) period (i.e., delayed). Anabolic activities were assessed by gene expression for collagen II and aggrecan (AGC) and extracellular matrix (ECM) deposition by (immuno)histochemistry. Catabolic activities were assessed by gene expression for matrix metalloproteinases, MMP-1, 3, and 13.

Results: Intermittent loading (IL) upregulated ECM and MMP expressions, e.g., 2-fold, 16-fold and 8-fold for collagen II, MMP-1, MMP-3, respectively. Continuous loading upregulated AGC expression 1.5-fold but down-regulated MMP-1 (3-fold) and -3 (2-fold) expressions. For delayed loading, chondrocytes responded to FS conditions by down-regulating MMP expressions ($P < 0.01$), but were less sensitive to loading when applied during week 2. Spatially, deposition of ECM molecules was dependent on the timing of loading, where immediate loading favored enhanced collagen II deposition.

Conclusions: The type and timing of dynamic loading dramatically influenced ECM and MMP gene expression and to a lesser degree matrix deposition. Our findings suggest that early applications of IL is necessary to stimulate both anabolic and catabolic activities, which may be important in regenerating and restructuring the engineered tissue long-term.
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Key words: Cartilage, Dynamic loading, Poly(ethylene glycol) hydrogels, Matrix metalloproteinases, Gene expression.

Introduction

In designing strategies to regenerate functional cartilage tissue, emulating the physiological loading environment will likely be important in enhancing extracellular matrix (ECM) deposition and mechanical properties of engineered cartilage. However, selecting appropriate loading regimes yielding functional cartilage will be dependent on a combination of factors including the scaffold structure and chemistry, the presence of biochemical cues, and cell source[1–4]. Although there may not be a ubiquitous loading scheme that stimulates cartilage regeneration, improving our understanding of the impact different loading regimes have on chondrocyte response will aid in designing strategies for tissue development.

Natively, chondrocytes maintain a homeostatic balance between ECM synthesis and degradation, leading to the turnover of new tissue. A shift in this balance favoring catabolic processes can lead to cartilage degeneration. Matrix metalloproteinases (MMPs), including MMP-1 and MMP-13, are involved in the degradation of collagen fibrils while MMP-3 is involved in the degradation of aggrecan (AGC)[5]. MMP-1 and MMP-3 are involved in cartilage homeostasis[6]. MMP-13 has been associated with cartilage degeneration, where several studies have reported increased MMP-13 expressions, but decreased MMP-3 expression in osteoarthritic cartilage[7,8]. Although many tissue engineering strategies focus on anabolic activity, several studies have suggested that mechanical loading induces catabolic activity and may be necessary for remodeling cartilage. For example, De Croos *et al.*[9] highlighted a cascading effect after a 30 min dynamic loading application, where catabolic expression initially increased followed by increased anabolic expression and ECM synthesis.

To study the effects of loading on anabolic and catabolic activities of chondrocytes in a controlled 3D environment, poly(ethylene glycol) (PEG) hydrogels are particularly attractive due to their ability to maintain the chondrocyte phenotype and promote cartilage-specific ECM deposition[10–12]. Recently, we characterized the role of hydrogel crosslinking density on chondrocyte proliferation, ECM gene expression, and ECM deposition when subjected to continuous dynamic loading during early culture times[13,14]. While our previous studies have provided insight into the combined role of scaffold architecture and loading, continuous loading (CL) is not representative of the physiological environment.

Various intermittent loading (IL) regimes have been studied in an effort to mimic the physiological environment of cartilage. For example, Chowdhury *et al.*[15] reported improved proteoglycan synthesis within 48 h under IL in agarose constructs over continuous regimes. Other studies have also employed IL, whereby loading improved

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mechanical properties and/or matrix synthesis when compared to free-swelling (FS) constructs[16–19]. A few studies have also highlighted that the timing of when loading is applied dramatically influences the developing tissue. For example, a single application of dynamic loading was sufficient to enhance long-term ECM synthesis for chondrocyte-seeded, calcium polyphosphate, porous scaffolds, but was dependent on the loading duration and when it was applied[20]. In a separate study, supplementing the culture medium with transforming growth factor, beta 3 (TGF-β3) prior to applying a dynamic load enhanced mechanical properties of chondrocyte-laden agarose constructs after 4 weeks compared to when loading was applied concurrently with TGF-β3 supplementation[21]. Interestingly, no differences in glycosaminoglycan (GAG) or collagen contents were observed, suggesting that loading improved tissue quality. Collectively, these studies and others demonstrate that chondrocyte response and the developing tissue are dependent on the loading profile (i.e., continuous vs intermittent) and timing of when loading is applied.

The focus of this study was to examine the temporal effects of mechanical stimulation on chondrocyte response when encapsulated in PEG hydrogels. These scaffolds have shown success in cartilage tissue engineering[12,22–24], but have not been explored under temporal mechanical stimulations. Specifically, we assessed the loading pattern (continuous vs intermittent) and timing of the initial loading application (applied immediately or after 1 week) on the expression of anabolic and catabolc ECM genes and tissue synthesis. We present here information that will enhance our understanding of the role of mechanical stimulation in mediating chondrocyte anabolic and catabolic activities within PEG hydrogels, which will ultimately aide in selecting appropriate loading regimes for use with chondrocyte-laden PEG hydrogels towards regenerating functional cartilage.

Materials and methods

EXPERIMENTAL DESIGN

Study 1 investigated the influence of loading pattern (continuously vs intermittently) on chondrocyte response. Cell-laden hydrogels were subjected to unconfined, dynamic compression applied sinusoidally from 5 to 20% strain at 0.3 Hz. CL applied 25,920 loading cycles/day while IL applied 6 sessions of 1 h on/1 h off, resulting in 6480 cycles/day.

Study 2 investigated the timing of the initial application of loading on chondrocyte response. Cell-laden hydrogels were cultured under: (1) FS conditions for 1 week followed by intermittent dynamic loading for 1 week described by Study 1 (referred to as delayed intermittent loading, DIL), or (2) intermittent dynamic loading for 1 week followed by FS culture for 1 week (referred to as immediate intermittent loading, IIL). Under these schemes, total number of loading cycles is constant.

The four loading schemes are described in [Fig. 1(a)]. The dynamically loaded gels were individually placed in the bioreactor between a permeable base and platen (Porex 40–70 μm) that imparted a 5% tare strain on the constructs prior to mechanical stimulation [Fig. 1(b)] (referred to as *loaded constructs*). Each experiment was carried out on separate occasions but performed under identical isolation and culturing procedures as described below. All experiments were performed in tandem with control constructs, which were placed in the bioreactor and subjected to the same 5% tare strain imparted by the platens [Fig. 1(c)], but did not experience any dynamic deformation (referred to as *control constructs*). FS constructs were also included in part of Study 1 for biochemical analysis and as an additional culture condition for Study 2 [Fig. 1(d)] (referred to as *FS constructs*).

CHONDROCYTE ISOLATION

Full depth articular cartilage was harvested from the patellar-femoral groove of 1–3 week old calves ($n = 2$) (Research 87) within 24 h of slaughter

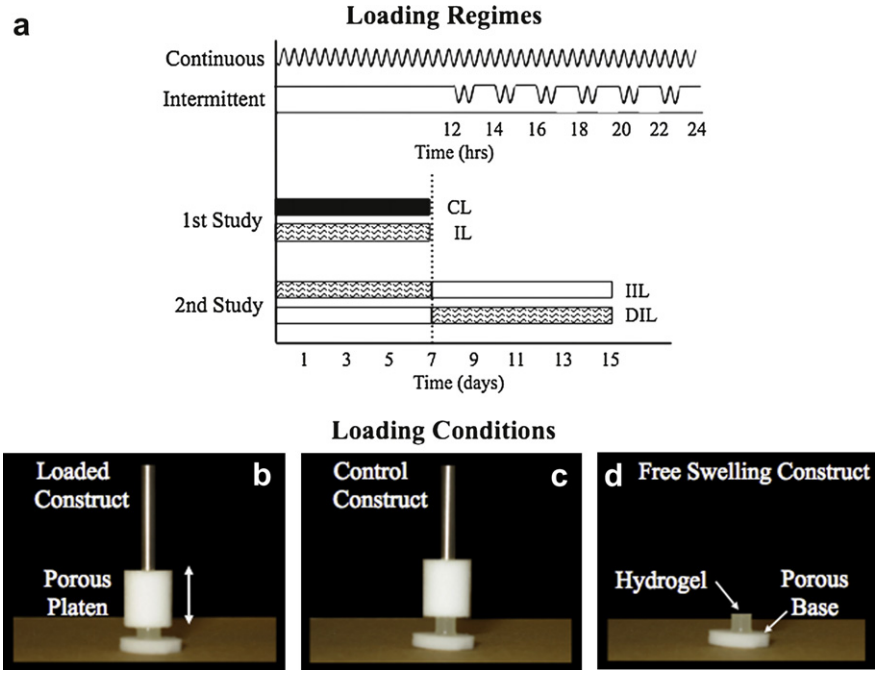

Fig. 1. (a) Schematic of the dynamic loading profiles and experimental conditions employed in this study to mechanically stimulate chondrocytes encapsulated into PEG hydrogels. The sinusoidal waveform pictured at the top of the figure indicates dynamic compressive loading whereas horizontal lines indicate down-time. Hydrogels were subject to unconfined dynamic compressive strains applied either continuously (CL) or intermittently (IL) in a sinusoidal waveform from 5 to 20% strain at 0.3 Hz. IIL and DIL were used in the second study. For all loading conditions, a porous disc, which is placed in the base of a tissue culture well, and a porous platen that applies dynamic compressive strains axially confine the gel. Three culture conditions were employed and are referenced throughout the manuscript as follows: (b) dynamically loaded constructs (hydrogels were subject to the tare strain and then dynamically stimulated between 5 and 20% strain), (c) control constructs (hydrogels were subject to the tare strain imparted by the platen, 5%), and (d) FS constructs.

Table I
Real-time RT-PCR primers

Gene (Abr.) (accession #)	Primers (sense/*anti-sense*)	Melt temp.	Amplicon length
L30	GGCCATAACTGGTGCTTCTCTTG	63	100
(AF063243)	*cgagaaAGCCCTGATAGCTTGTTCT[c-FAM]G*	64	
Collagen II (COL2)	cgatcaAGTGGGGCAAGACTATGAT[c-JOE]G	64	73
(X02420)	*GCAATGTCAATGATGGGCAGAC*	65	
AGC	GCCACTGTTACCGCCACTTC	66	84
(BTU76615)	*cggaaAGGTGTGACTGCTGCTTC[c-AF546]G*	63	
MMP-1	cggtcAGAAGTGATGTTCTTCAAAGAC[c-FAM]G	63	68
(NM_174112)	*TCCACTTCTGGGTACAAGGGATTT*	64	
MMP-3	cgttcCTCCAGCACTCAACCGAA[c-JOE]G	67	67
(AF135232)	*AGGTCTGTGCGAGGGTCGTAG*	64	
MMP-13	cgggtCATGTGATGGATAAAGACTACC[c-AF546]G	64	69
(NM_174389)	*CTTTATCACCAATTCCTGGGAAGA*	63	

and digested in 500 units/mL collagenase II (BD Worthington) in high glucose DMEM (Invitrogen) supplemented with 5% FBS (Invitrogen) for 16 h at 37°C. Isolated cells were resuspended in chondrocyte medium (DMEM supplemented with 10% FBS (v/v), 0.04 mM L-proline, 50 mg/L L-ascorbic acid, 10 mM HEPES buffer, 0.1 M MEM-nonessential amino acids, 1% penicillin–streptomycin, 0.5 μg/mL fungizone, and 20 μg/mL gentamicin (Invitrogen)). Cell viability was determined by trypan blue exclusion and was >95% prior to encapsulation.

HYDROGEL FORMATION

Poly(ethylene glycol) dimethacrylate (PEGDM) was synthesized by reacting PEG (3000 Da, Fluka) with methacryloyl chloride in the presence of triethylamine for 24 h at 4°C[25]. PEGDM was purified by precipitation in ethyl ether and analyzed by ^{1}H-NMR (Varian YVR-500S), which indicated 90% methacrylation. PEGDM was dissolved in PBS at 10% (w/w) containing 0.05% (w/w) photoinitiator Irgacure 2959 (Ciba Specialty Chemical). Sterile macromer solution was mixed with 50 million cells/mL. Hydrogel cylinders (5 × 5 mm) were fabricated by exposing cell/macromer solution to 365 nm light (6 mW/cm^2) for 10 min[26]. Hydrogel constructs were equilibrated under FS conditions for 24 h in media prior to loading.

MECHANICAL STIMULATION

After pre-conditioning, samples were either removed from culture and analyzed (time = 0 days) or placed into custom-built bioreactors[14]. Hydrogels were subject to dynamic strains as described above. Strain was verified by direct measurements using the onboard linear variable displacement transducer (LVDT). Cell viability was qualitatively assessed using a live/dead assay (Invitrogen), which indicated high cell viability and no observable differences among culture conditions (data not shown).

GENE EXPRESSION

At specified times, samples ($n = 3$) were removed, snap-frozen under liquid nitrogen, and processed with TRI Reagent (Sigma) to isolate RNA[27]. The purity and quantity of RNA was determined (Nanodrop, ND-1000, ThermoFisher). Pure RNA (100 ng, A260/280 > 1.90) was treated with Turbo DNA-free (Ambion) and transcribed to cDNA using the High Capacity cDNA Kit (Applied Biosystems). Real-time RT-PCR (ABI 7500 Fast) was performed using custom designed and validated D-LUX primers (Invitrogen) for the housekeeping gene (mitochondrial ribosomal protein L30), anabolic genes for collagen type II (COL2) and AGC, and catabolic genes for MMP-1, -3 (MMP-3), and -13 (MMP-13) with the Taqman® Fast Universal PCR Master Mix (Applied Biosystems) (Table I). Gene expression was normalized to a calibrator (e.g., 0 days, controls, or FS constructs) described by Pfaffl[28].

BIOCHEMICAL ANALYSIS

At specified times, samples ($n = 3$) were removed from culture, wet weights obtained, homogenized and enzymatically digested by papain for 16 h at 60°C. GAG content was assessed by 1,9-dimethylmethylene blue dye method[29] and normalized to gel wet weight. Additional constructs were removed from culture, fixed in 4% paraformaldehyde, dehydrated, paraffin-embedded, sectioned (10 μm), and stained for negatively charged GAGs using Safranin-O/Fast Green or for collagen using Masson's Trichrome. Cell nuclei were counterstained by hematoxylin. Sections were also treated with hyaluronidase (5000 U/mL) and chondroitinase ABC (500 mU/mL), blocked in 1% bovine serum albumin (BSA) and dual-labeled with rabbit anti-collagen type II (Chemicon, 1:50) and mouse anti-aggrecan (US Biological, 1:2). Detection was achieved using secondary goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 546 antibodies (Invitrogen, 1:400), respectively. Sections were mounted and counterstained using VectaMount w/DAPI (Vector). Images were acquired by laser scanning confocal microscopy (Zeiss LSM 5 Pascal).

STATISTICAL ANALYSIS

Data are represented as a mean and 95% confidence interval of the mean ($n = 3$). Normalized gene expression values as a function of culture time and loading conditions were analyzed by two-way analysis of variance (ANOVA) and significant differences due to loading factors were analyzed *post-hoc* using Tukey's HSD with $\alpha = 0.05$ considered significant.

Results

Type and timing of dynamic loading significantly affected gene expression of anabolic and catabolic markers. In Study 1, the combination of the 3D PEG environment and IL conditions resulted in an upregulation in all genes with culture time ($P < 0.05$) (Fig. 2). After 7 days of loading, there was a 2-fold increase in AGC and COL2 expressions, 10-fold increase in MMP-1 and -3 expressions, and 100-fold increase in MMP-13 expression when compared to day 0 levels. In contrast, the same gel environment under CL caused a ~3-fold increase in AGC expression, while significantly down-regulating MMP-1 by 5-fold and MMP-3 by 10-fold ($P < 0.05$). No changes were observed for COL2 or MMP-13 as a function of culture time under CL. Although the 3D culture environment was identical, expression levels for all genes were significantly higher under IL conditions compared to CL, with the exception of AGC (Fig. 2). Control constructs exhibited no observable changes in expression as a function of culture time, except for AGC which was slightly upregulated after 1 week.

To isolate the effects of dynamic loading from the gel environment, loaded constructs were normalized to their respective controls at the same time point (Fig. 3). Dynamic strains under IL did not affect expression levels until day 7, where loading resulted in higher expressions by 2-fold for COL2, 21-fold for MMP-1, 9-fold for MMP-3, and 8-fold for MMP-13 ($P < 0.05$) with no change in AGC expression. Under CL, dynamic strains down-regulated COL2 and MMP-1 at day 3 and MMP-3 at day 5. ACG expression was upregulated by 1.5-fold due to CL ($P < 0.05$).

GAG production was assessed for CL, IL and FS culture conditions (Fig. 4). Total GAG production increased with culture time for FS and CL conditions. However, GAG

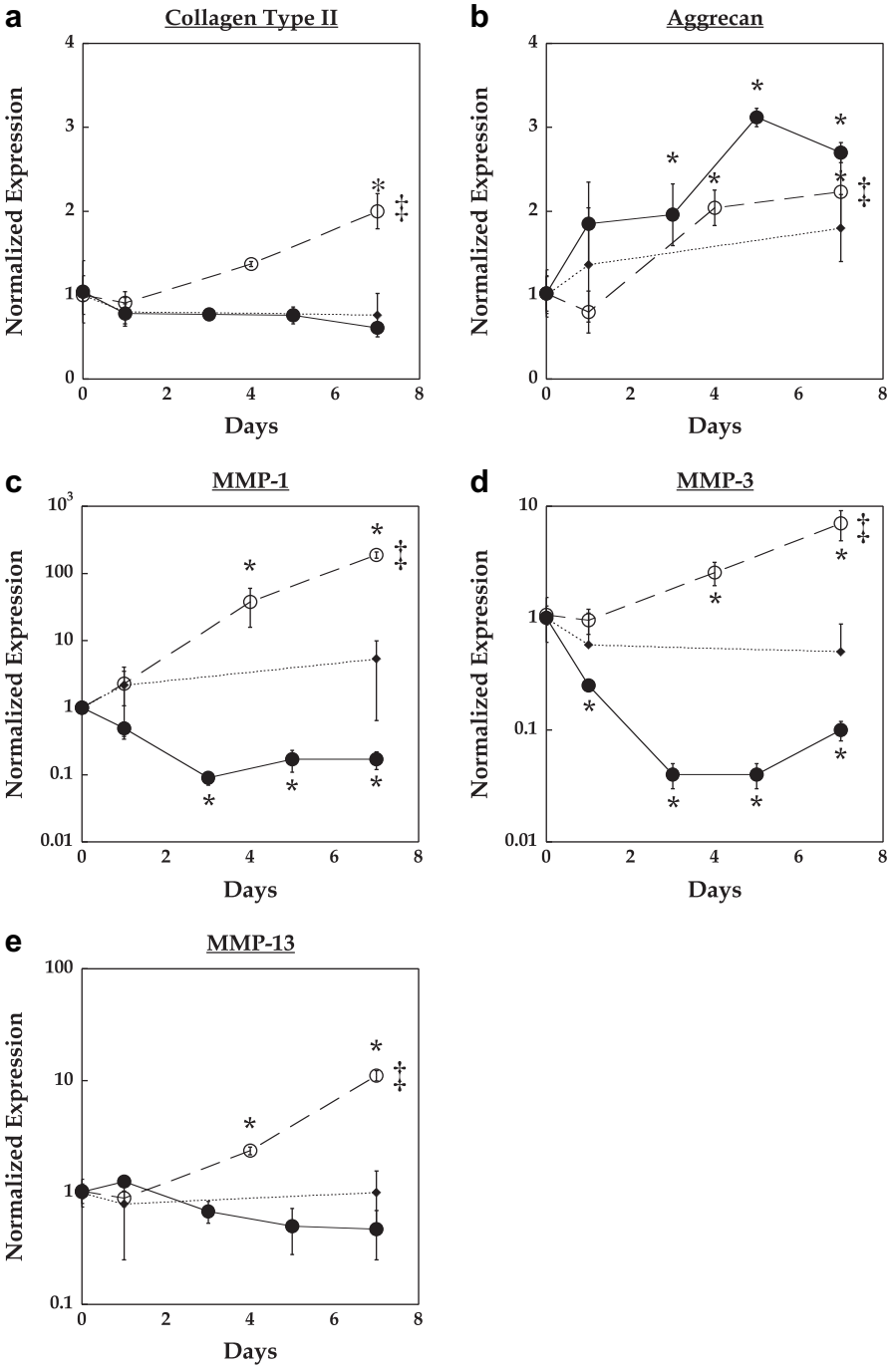

Fig. 2. The effects of continuous (CL, ●) vs intermittent (IL, ○) loading as a function of culture time for the normalized expression for COL2 (a), AGC (b), MMPs-1 (c), -3 (d) and -13 (e). Normalized expression is defined as the relative expression (gene of interest relative to housekeeping gene expression) for the dynamically loaded gels and normalized to the relative expression for the respective day 0 expression levels. Controls (♦) represent an average from the two separate runs performed at different times ('loading controls' were run with CL samples and a separate set of 'controls' were run with IL samples). * indicates significant difference from day 0 ($P < 0.05$), ‡ indicates a significant difference between CL and IL conditions for overall expression profile ($P < 0.05$).

content remained constant in the constructs under IL conditions and was statistically lower than CL constructs at day 7.

In study 2, the influence of the 3D gel environment and loading was examined with respect to timing of the initial load application. Under IIL conditions, COL2 expression increased in gels immediately loaded, but the removal of loading after 7 days resulted in a 4-fold down-regulation of COL2 by day 15 ($P < 0.05$) falling to levels 2-fold lower than day 0 ($P < 0.05$) [Fig. 5(a)]. There was no significant effect on AGC expression [Fig. 5(c)], although mean levels dropped upon removal of loading. When cultured under DIL conditions, no change in COL2 expression was observed over the 15-day culture regardless of loading. AGC expression was not significantly affected ($P > 0.05$) [Fig. 5(d)].

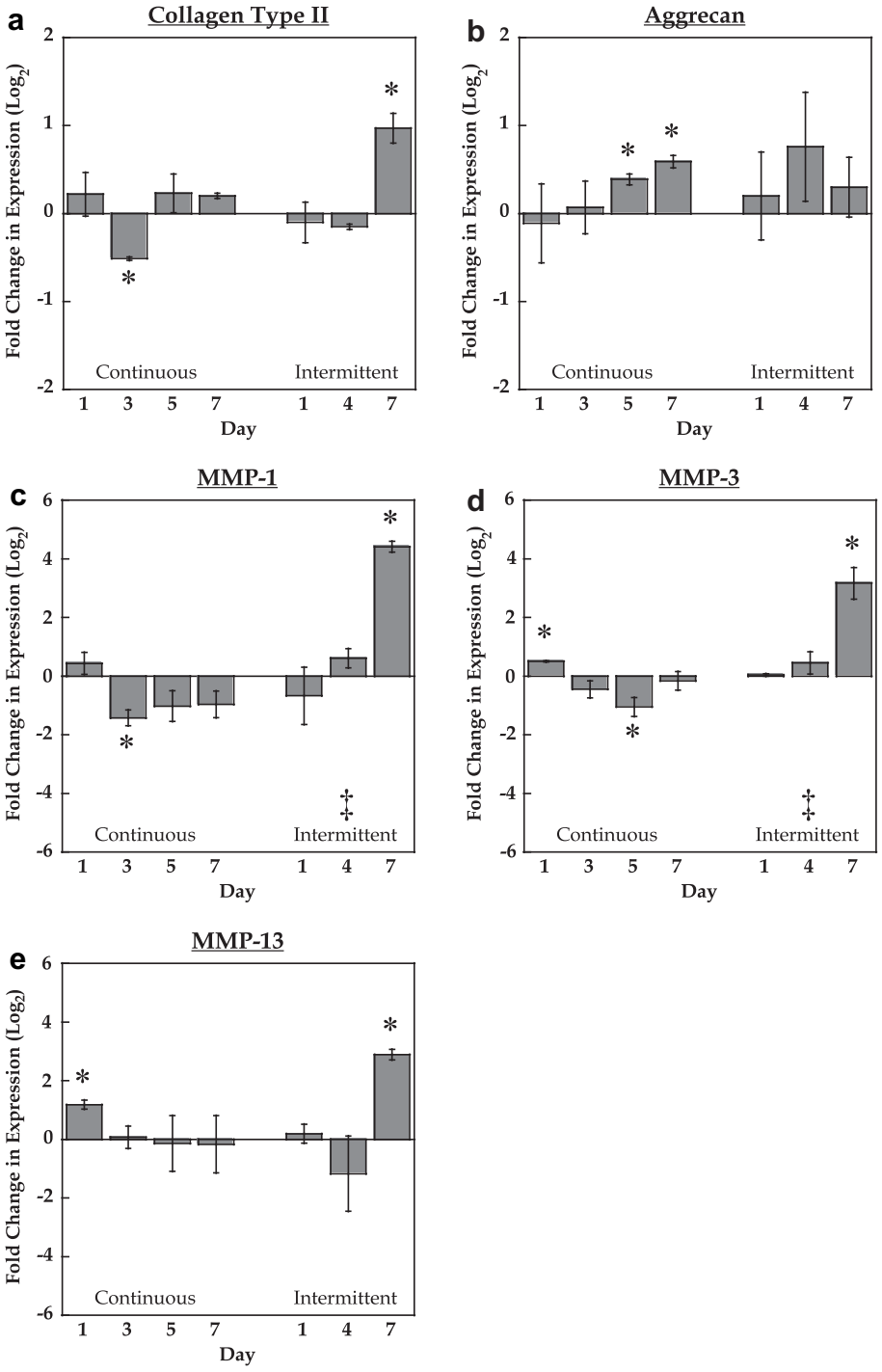

Fig. 3. The influence of dynamic loading on gene expression. The responses of dynamically loaded gels were normalized to that of their internal control gels for COL2 (a), AGC (b), MMPs-1 (c), -3 (d) and -13 (e). Data is represented as $\log_2$ of the mean fold change, where a value of 0 indicates no difference from control, values of 1, 2, or 3 indicate a 2-, 4- or 8-fold upregulation, respectively, and negative values indicate a down-regulation. * indicates a significant difference from control gels at the same time point and loading condition ($P < 0.05$), ‡ indicates a significant difference between CL and IL conditions for overall profile ($P < 0.05$).

In gels subjected to IIL conditions, MMP-1, -3, and -13 were upregulated during the initial 7 days of loading (184-fold, 7-fold, and 11-fold, respectively). Upon removal of loading, expression levels decreased for all three genes ($P < 0.05$) [Fig. 6(a, c, e)] with the most significant drop occurring after 1 day. By day 15, MMP-3 levels were below day 0 values, while MMP-1 and -13 returned to day 0 levels. In contrast, culturing cells initially in PEG gels under FS conditions (DIL) down-regulated MMP-1 (10-fold) and -3 (10-fold) by day 7 [Fig. 6(b, d)]. Upon loading the gels after

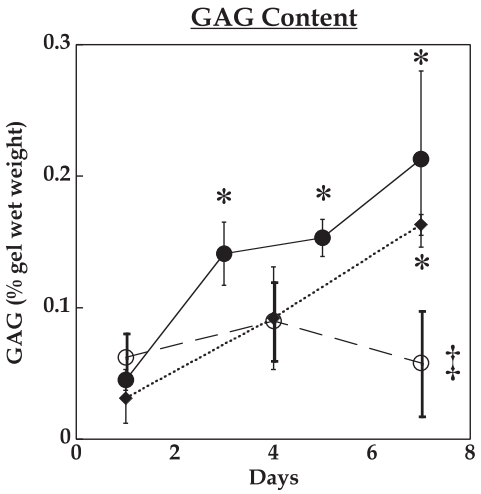

Fig. 4. The total amount of GAG accumulated in the hydrogel construct per wet weight hydrogel and subject to either FS (♦), CL, ●, or IL (IL, ○) conditions. * indicates significant difference from day 0 ($P < 0.05$), ‡ indicates a significant difference between CL and IL conditions ($P < 0.05$).

the initial FS period, MMP-1 and -3 were upregulated. At the end of the delayed loading phase, MMP-1 was upregulated by 7-fold to levels higher than day 0 values, whereas MMP-3 expression returned to similar day 0 levels. Differences in MMP-13 expression were observed as a function of the gel environment and timing of loading (IIL vs DIL). When cultured in the PEG gels and initially dynamically loaded (IIL), MMP-13 was upregulated 11-fold. However, if loading was applied after 7 days in the hydrogel environment (DIL), MMP-13 expression was unaltered [Fig. 6(e, f)]. Table II summarizes the relative fold changes in expression as function of culture time and their loading and gel environment.

To isolate the effects of dynamic loading under DIL conditions, expression levels were normalized to their respective controls at same time point (Fig. 7). Delayed loading had no effect on anabolic expression, which differed from IIL conditions. Surprisingly, loading also had no impact on catabolic gene expression [Fig. 7(a)]. To better assess the impact of the bioreactor set-up [described by Fig. 1(b, c)], control and loaded constructs were normalized to FS constructs. Normalized expressions are reported at the end of each loading period (Fig. 8). The increase observed for COL2 after 7 days of IIL was strictly due to dynamic loading, while AGC was unaltered by loading. Gels subject to control conditions elicited catabolic responses which were unique from the FS constructs. For IIL, MMP-1 and -3 levels were

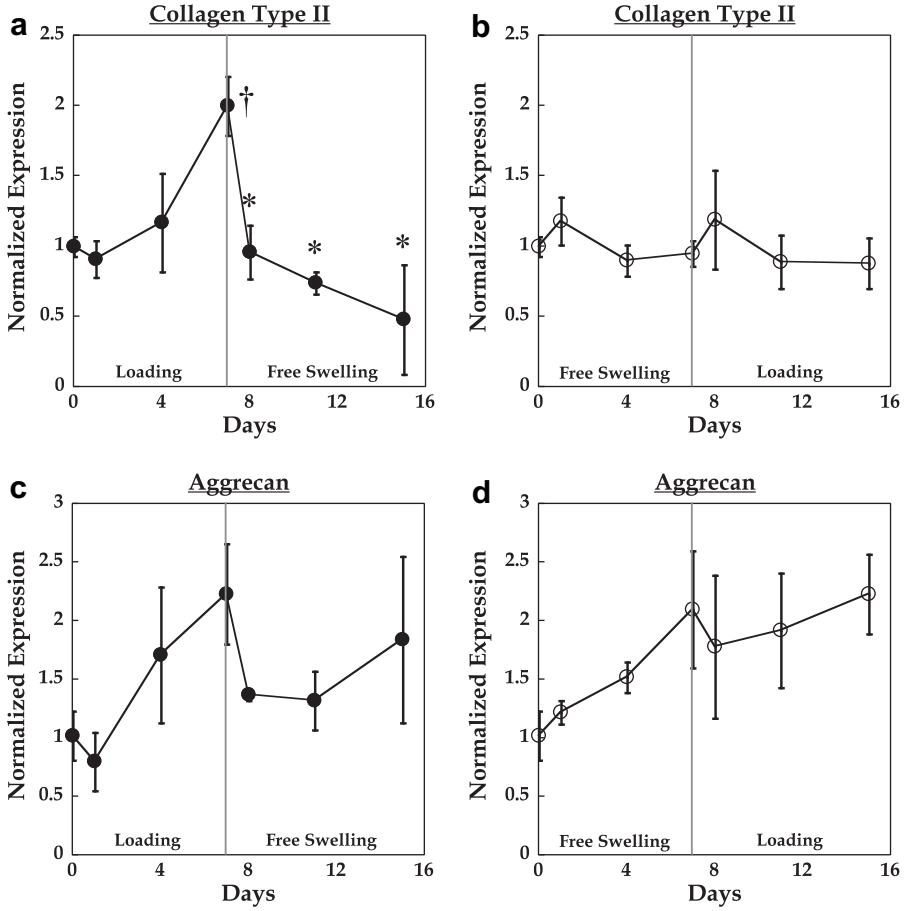

Fig. 5. The effects of the timing at which loading was applied, immediately (IIL) (a, c, (●)) or delayed (DIL) (b, d, ○), on gene expression for COL2 (a-b) and AGC (c-d). Data are presented as normalized expression relative to day 0. Vertical line at day 7 represents the time at which the gels were switched from either IL to FS (IIL) or FS to IL (DIL). † indicates a significant difference between day 7 and day 0 ($P < 0.05$), * indicates significant difference from levels measured at day 7 ($P < 0.05$).

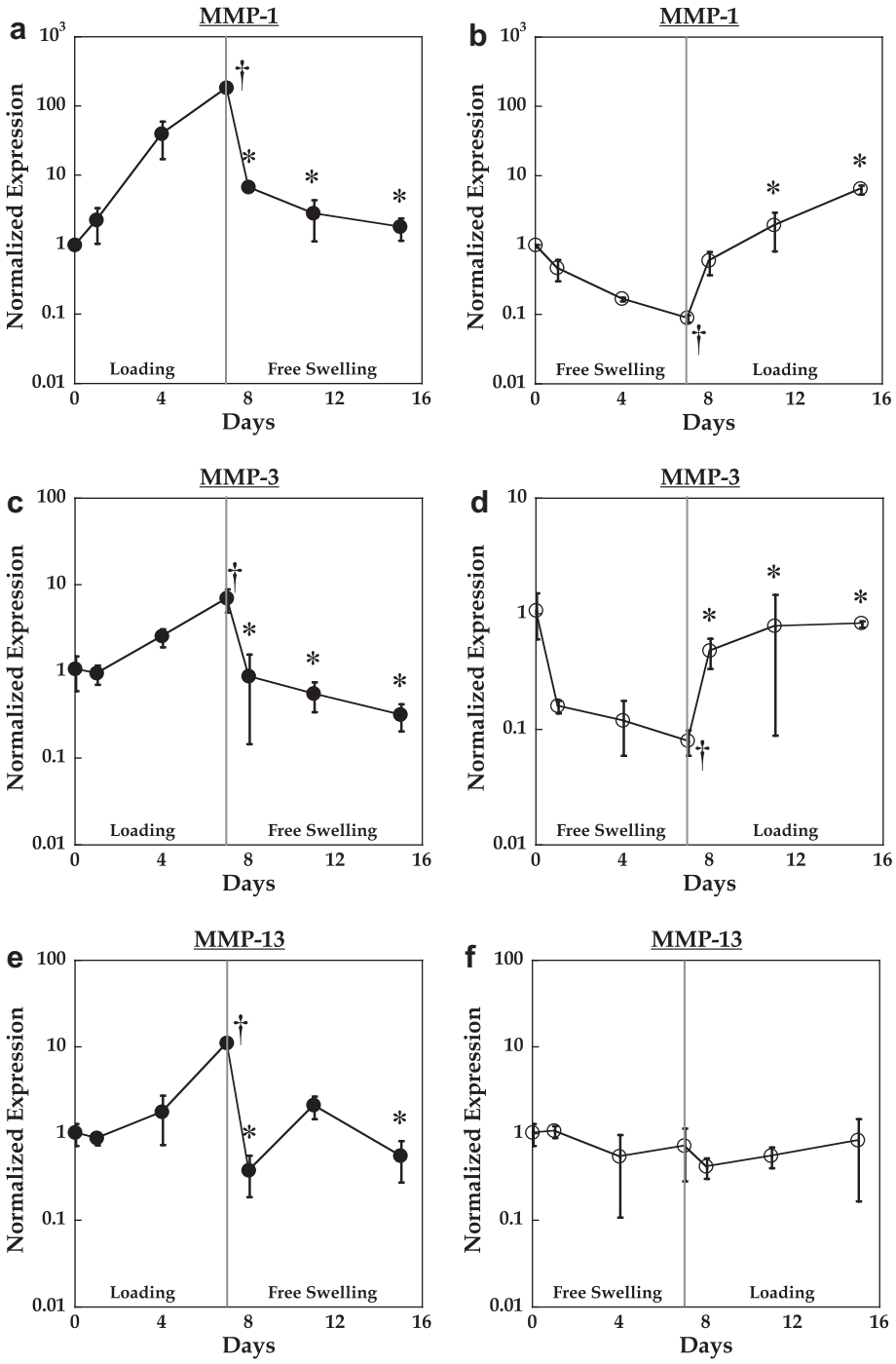

Fig. 6. The effects of the timing at which loading was applied, immediately, (IIL) (a, c, (●)) or delayed (DIL) (b, d, ○), on gene expression for MMP-1 (a, b), MMP-3 (c, d) and MMP-13 (e, f). Data are presented as normalized expression relative to day 0. Vertical line at day 7 represents the time at which the gels were switched from either IL to FS (IIL) or FS to IL (DIL). † indicates a significant difference between day 7 and day 0 ($P < 0.05$), * indicates significant difference from levels measured at day 7 ($P < 0.05$).

upregulated at day 7 in controls, but were heightened by dynamic loading ($P < 0.05$). MMP-13 was only upregulated by loading for the IIL conditions. During week 2, both control and loaded samples elicited upregulations of MMP-1 and -3, although no changes in MMP-13 expression were observed.

GAG content (Fig. 9) did not significantly change during loading under either loading scheme. However, GAG content increased ($P < 0.05$) under FS conditions for both loading schemes. DIL conditions resulted in higher GAG content after 1 week compared to IIL conditions ($P < 0.05$), but by 2 weeks GAG content was similar for both loading conditions.

Spatial deposition for cartilage-specific ECM components, collagen and proteoglycans, were assessed histologically (Fig. 10). GAG staining appeared densest in the

Table II
Fold changes in expression

Condition	Fold change† (Day 0—Day 7)		Fold change† (Day 7—Day 15)	
	IIL	FS	FS	DIL
Gene				
COL2	2.0 ± 0.2	-0.4 ± 1.3	-8.1 ± 8.2	$-0.4 \pm 1.4^{*}$
AGC	2.2 ± 0.4	2.1 ± 0.5	-0.1 ± 1.7	0.4 ± 1.3
MMP-1	180 ± 20	-12 ± 2.0	$-110 \pm 40^{*}$	$75 \pm 11^{*}$
MMP-3	7.0 ± 2.1	-13 ± 3.1	-24 ± 8.5	10 ± 0.6
MMP-13	11 ± 1.2	-1.1 ± 2.1	$-23 \pm 11^{*}$	$-0.5 \pm 2.5^{*}$

*Indicates a significant difference from respective initial week values (FS vs FS and IIL vs DIL) ($P < 0.05$).
†Upregulation (+) and down-regulation (−) of genes are represented as mean ± standard deviation.

pericellular region after 7 days, with sparse staining in extracellular regions. By day 15, GAG staining was more pronounced in extracellular regions for both IIL and DIL conditions. However, under DIL, GAG staining was heavier in the extracellular space with less staining intracellularly compared to IIL. Collagen staining was most pronounced in the pericellular space. No observable differences were present as a function of loading regime.

The organization and presence of collagen II and AGC were assessed by immunohistochemistry (Fig. 11). By day 7, collagen II and AGC were primarily localized in the cell vicinity. Although, immediate loading appeared to promote some AGC deposition in the extracellular regions. By day 15 under IIL, greater collagen II deposition was observed surrounding the cells and throughout the gel with heavier staining in the extracellular regions. Collagen II deposition mirrored the gross staining for non-specific collagens in Fig. 10. However, sulfated GAG staining by Safranin-O (Fig. 10) was markedly different from the AGC staining (Fig. 11). By day 15, positive staining for AGC was limited to pericellular regions even though GAG staining was present throughout the gel.

Discussion

This study highlights that the loading pattern (continuous vs intermittent) and timing at which loading is applied dramatically influences chondrocyte response when cultured in PEG hydrogels. In Study 1, chondrocyte function was assessed in response to CL vs IL. IL was selected to better mimic the physiological loading environment of cartilage while CL for long periods may be interpreted as over-loading. Interestingly, our findings indicate that IL conditions up-regulate genes for major cartilage proteins, COL2 and AGC, as well as major enzymes responsible for ECM degradation, MMP-1, -3, and -13 during the first week of culture. Gels subject to CL conditions responded in an opposite manner, down-regulating COL2, MMP-1 and -3. In constructs which were subjected to IL, there was no change in GAG accumulation from days 1 through 7 even though there were significant increases in AGC expression. Previous studies have shown that dynamic loading leads to release of ECM molecules into the medium[9,17,20,30]. However, under CL, increases in aggrecan expression and GAG accumulation were significant with culture time. Increased MMP-3 expressions under IL conditions may

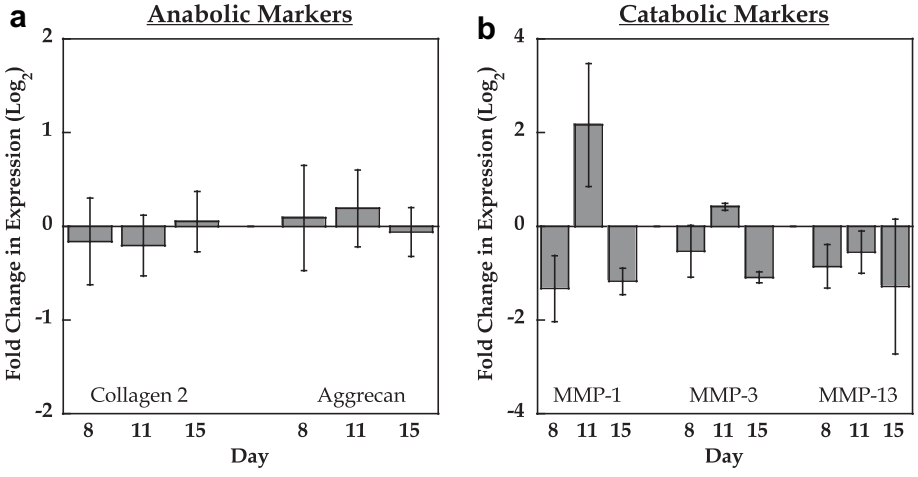

Fig. 7. Isolating the effects of dynamic loading on gene expression for the delayed intermitting loading (DIL) scheme. The responses of dynamically loaded gels were normalized to that of the control gels for anabolic genes (a) and catabolic genes (b). Data is represented as $\log_2$ of the mean fold change, where a value of 0 indicates no difference from control, values of 1, 2, or 3 indicate a 2-, 4- or 8-fold upregulation, respectively, and negative values indicate a down-regulation. * indicates a significant difference from control gels at the same time point and loading condition ($P < 0.05$), ‡ indicates a significant difference between CL and IL conditions for overall profile ($P < 0.05$).

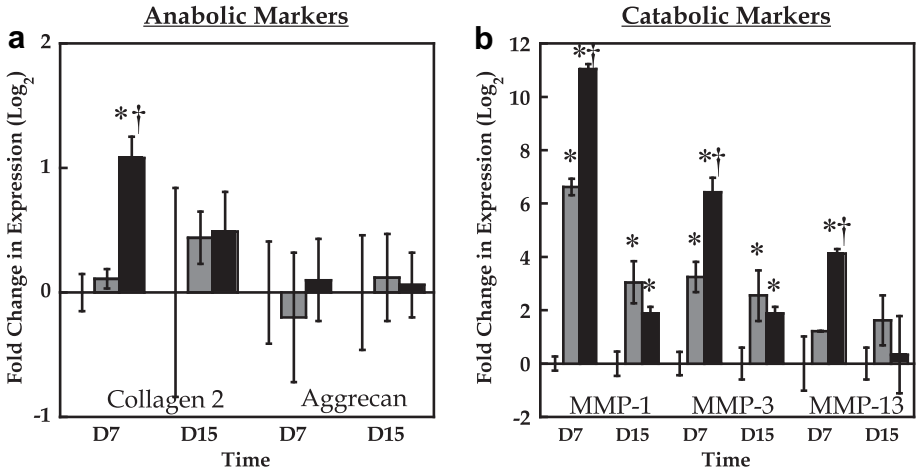

Fig. 8. The effects of the culture environment where gene expression for control gels (gray) and dynamically loaded gels (black) at the end of each loading period (day 7 for the IIL condition and day 15 for the DIL condition) were normalized to FS gels at same time point (no bar, but standard deviation is provided). Anabolic (a) and catabolic (b) expression data are represented as $\log_2$ of the mean fold change, where a value of 0 indicates no difference from control, values of 1, 2, or 3 indicate a 2-, 4- or 8-fold upregulation, respectively, and negative values indicate a down-regulation. * indicates a significant difference from FS gels ($P < 0.05$), † indicates a significant difference between dynamically loaded and control gels ($P < 0.05$).

have stimulated catabolic activity leading to AGC degradation and its release into the culture medium. However, additional studies are necessary to confirm MMP activity. Several studies have suggested mechanical loading may be important in facilitating a normal "remodeling" process of the ECM where both anabolic and catabolic activities are upregulated. De Croos *et al.*[9] showed that when cells received a single application of dynamic loading, MMP-3 and -13 expression levels were upregulated within 2 h, resulting in active MMPs and subsequent release of proteoglycans and collagen by 6 h. This catabolic response was followed by up-regulating COL2 and AGC genes suggesting a rebuilding phase. Kisiday *et al.*[31] recently showed the upregulation of MMPs and aggrecanases in dynamically loaded peptide hydrogels, resulting in increased PG degradation and release from the scaffold. This response was attributed to "over-loading" and an injurious response, however the authors did suggest a rebuilding process could be occurring to support the applied load. Blain[32] also reviewed this idea that mechanical loading induces a classical ECM "turnover" response.

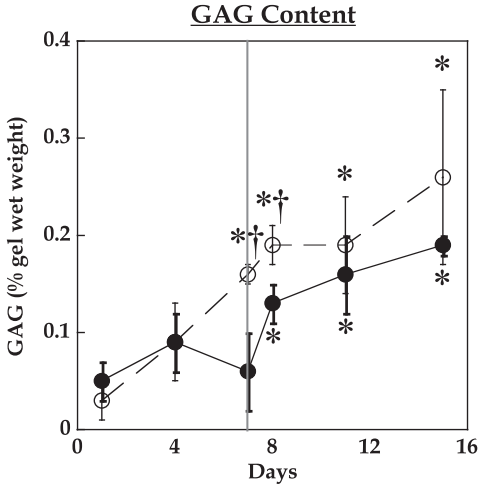

Fig. 9. The total amount of GAG accumulated in the hydrogel construct per wet weight hydrogel and subject to IIL (●), or DIL (○) conditions. * indicates significant difference from day 0 ($P < 0.05$), † indicates a significant difference between IIL and DIL samples at same time point ($P < 0.05$).

When chondrocytes are isolated from enzymatically digested cartilage, they are stripped away of their extracellular and pericellular matrices. Upon being placed into their new environment, one of their innate functions appears to be reforming their pericellular matrix (PCM)[33,34]. Although the exact functions of the PCM are not well known, it is thought to act as a mechanical barrier protecting the cells from the large deformations experienced by the tissue under physiological loading. Previous studies have reported that the PCM begins to form 3 days post-encapsulation for chondrocytes encapsulated in PEG hydrogels[13,35]. By day 6, a mechanically functional PCM develops where cell deformation was markedly reduced even though large 15% strains were applied grossly to PEG hydrogels[36]. In Study 1, chondrocytes were subjected to dynamic loading one day post-encapsulation, where cells initially experience large compressive strains similar to the gross strains applied to the construct. Previous studies from our group have shown that CL at 1 Hz delays the formation of a PCM[13]. It is possible that the off-periods associated with the IL conditions may have permitted more down-time to produce a functional PCM compared to CL, leading to an enhanced anabolic response. Any differences in the PCM development will certainly influence how the cells perceive their biomechanical environment.

The major goal of Study 2 was to examine chondrocyte response to loading after the cells had time to rebuild their own PCM. Interestingly, hydrogel constructs cultured under DIL conditions exhibited no change in anabolic or catabolic expression upon the application of a dynamic load whereas initial loading of the constructs resulted in upregulation in both anabolic and catabolic expressions. These findings suggest that once a mechanically functional PCM has developed within the PEG hydrogels, chondrocytes are less sensitive to dynamic loading. Waldman *et al.*[20] suggested

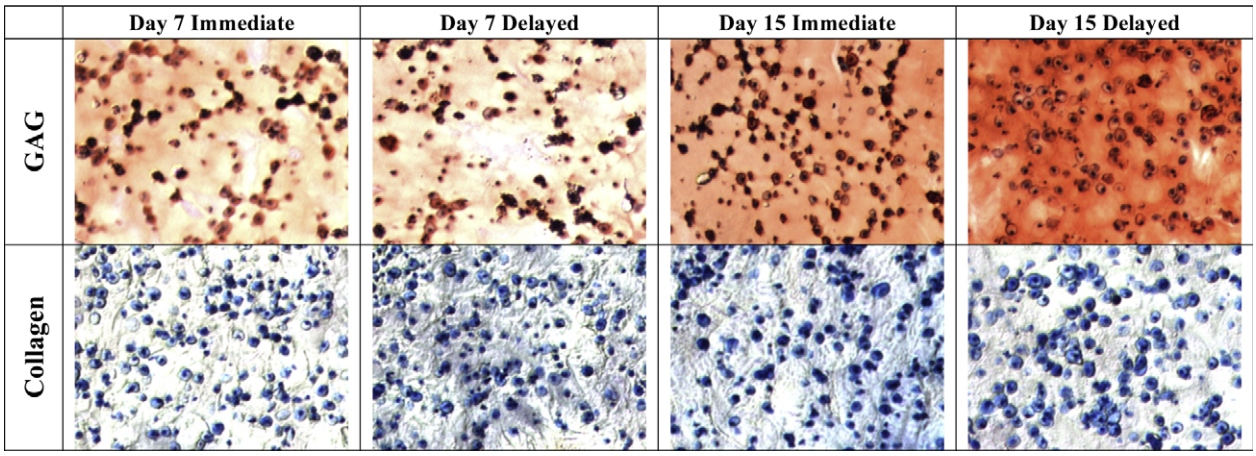

Fig. 10. Gross examination of matrix deposition by histological evaluation for chondrocytes encapsulated in PEG hydrogels subject to either IIL or DIL after 7 or 15 days. Sections were stained for GAGs (red) using Safranin-O/Fast green or collagen (blue) using Masson's Trichrome. Cell nuclei (dark purple) were counterstained using hematoxylin. Original magnification is 100×.

that chondrocytes were most sensitive to mechanical stimulation during early culture. However, Lima *et al.*[21] reported that delaying dynamic loading until after 2 weeks of TGF-β3 treatment compared to immediate loading improved the overall mechanical properties for agarose constructs after 4 weeks. The novel findings reported here are that within PEG hydrogels: (1) applying dynamic stimulation intermittently, but within 1 day post-encapsulation, induces catabolic *and* anabolic activity while still supporting ECM deposition and (2) dynamic, IL applied after the cells have had time to deposit their own matrix has no effect on chondrocyte anabolic and catabolic activity. Our findings underscore the importance of the timing when mechanical loading is applied and its dramatic impact on how cells sense and respond to mechanical stimulation. Longer culture times, however, are needed to assess the long-term impact of timing on tissue development within PEG hydrogels.

Surprisingly, placing constructs in the bioreactor with a 5% tare strain [Fig. 1(c)] was sufficient to upregulate MMP-1 and 3 expressions when compared to FS conditions regardless of the timing (i.e., IIL or DIL conditions) (Fig. 8). However, MMP-13 expression, which is typically associated with early stages of osteoarthritis, was not affected by the tare strain. It is well known that static loading suppresses ECM biosynthesis in chondrocytes[37–39] and has been used to induce osteoarthritic behavior in cartilage explants[40]. Therefore, in this study it is possible that the "down-times" programmed into the intermittent cycles, which impose tare strains, upregulated MMP expressions. A number of studies, which employ dynamic loading, impose tare strains to prevent impact loading on the samples[16–18]. However, these studies have generally observed increased ECM deposition and mechanical properties, suggesting that small tare strains are not derimental. Lima *et al.*[21] reported an initial 10% tare strain, which increased to 25% by conclusion of their experiment due to the growing construct thickness, yet increases in gross mechanical properties were still observed. Our findings indicate that the control conditions may impact the interpretation of loading effects on cell response. In our study, the use of controls (i.e., which are placed in the bioreactor) serves to isolate the role of *dynamic* stimulation while FS constructs allow us to probe the impact the tare strain has on cell responses.

Our results demonstrate that there are marked differences in the gene expression profiles over the course of 15 days depending on the timing at which loading was applied (i.e., immediately or delayed). These differences also led to spatial disparities in matrix deposition. In general, collagen II deposition was primarily localized pericellularly due to its large size and limited diffusivity in PEG hydrogels[10]. Interestingly under IIL, extracellular deposition of collagen II was enhanced over DIL, suggesting that diffusion is possible. This observation agrees well with the gene expression profiles where early applications of loading upregulated COL2 expression while delayed loading did not affect COL2 gene expression.

There was also a marked difference in GAG deposition. Delayed loading led to heavier staining of GAGs extracellularly by day 15 while an immediate application of loading resulted in stronger staining pericellularly, although the total GAG content was similar in both constructs. This observation may suggest that loading permits better transport of GAGs into the extracellular regions[38,41–43]. However, positive staining for AGC was limited to the pericellular regions suggesting that the GAGs observed by Safranin-O staining may be AGC degradation products. With the high MMP-3 expressions observed under DIL conditions, it is possible that AGC is being degraded. AGC transport through the hydrogel may also be restricted due to its large size, reaching molecular weights upwards of 200×10^6 Da[44]. Overall, differences observed in spatial deposition of ECM suggest that the local environment surrounding the cell is different depending on the loading environment, which will ultimately impact the biomechanical signals perceived by the cells.

This relationship between anabolic and catabolic processes is crucial in maintaining a homeostatic balance in native cartilage. Although many cartilage tissue engineering approaches have focused on anabolic activities, recent evidence suggests that both anabolic and catabolic processes may be important in regenerating functional tissue. The application of dynamic mechanical stimulation may play an important role in initiating both processes. Our findings demonstrate that the type of loading and the timing of when loading is applied dramatically influence the anabolic and catabolic activity for chondrocytes encapsulated in PEG gels. The immediate application of an IL regime appears to be essential for up-regulating both anabolic and

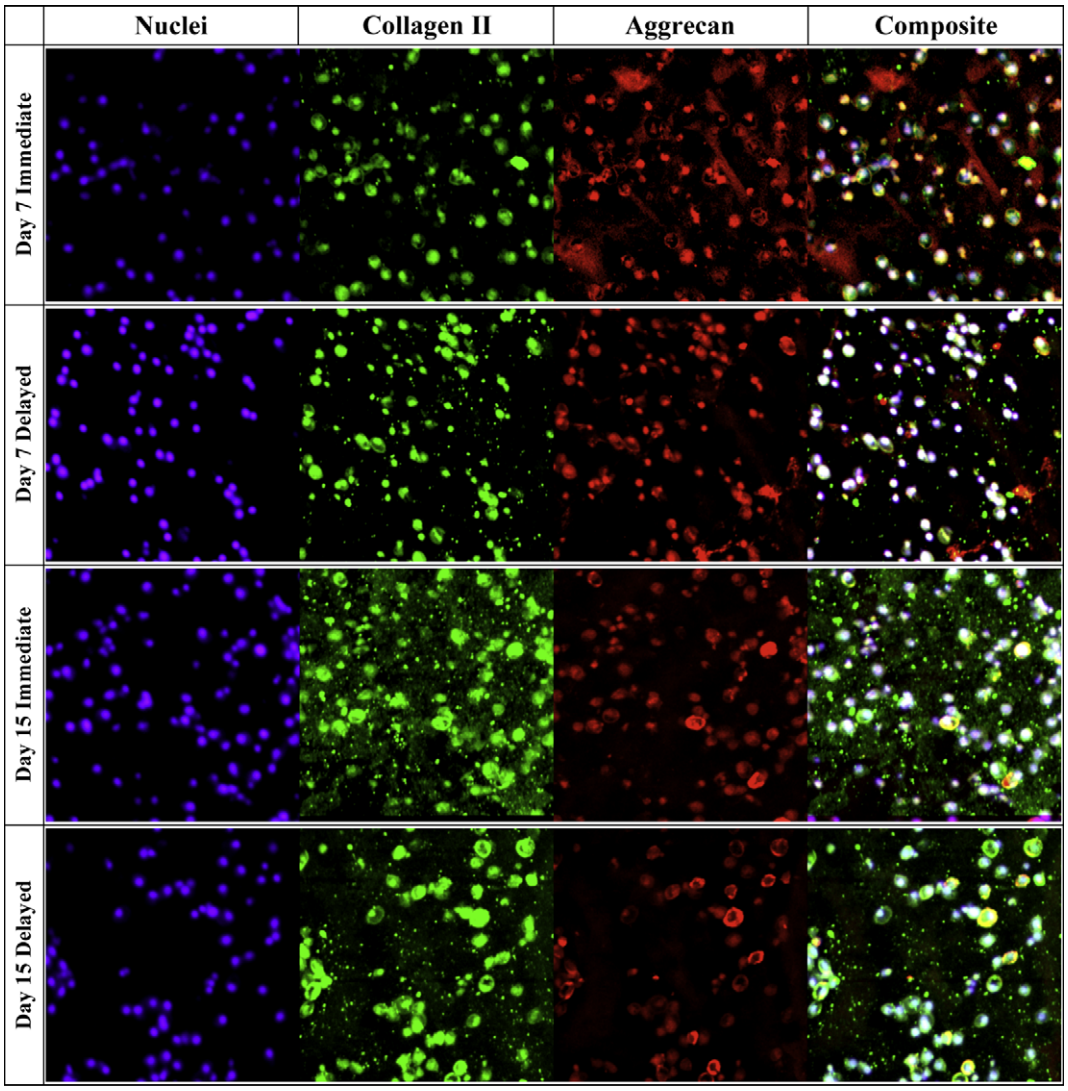

Fig. 11. Gross examination of cartilage-matrix deposition by immunohistochemical evaluation for chondrocytes encapsulated in PEG hydrogels subject to either IIL or DIL after 7 or 15 days. Sections were stained for cell nuclei (purple), COL2 (green), and AGC (red) and imaged by laser scanning confocal microscopy. Composite images are also shown from the single image captured. Original magnification is 100×.

catabolic activity while for a delayed loading regime, loading has less of an effect on gene regulation. However, long-term cultures are necessary to assess the effect of loading schemes on functional tissue development.

Conflict of interest

There are no conflicts of interest or sources of bias in the research contained in this manuscript.

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