# LONG-TERM DYNAMIC LOADING IMPROVES THE MECHANICAL PROPERTIES OF CHONDROGENIC MESENCHYMAL STEM CELL-LADEN HYDROGELS

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## Abstract

Mesenchymal stem cells (MSCs) are an attractive cell source for cartilage tissue engineering given their ability to undergo chondrogenesis in 3D culture systems. Mechanical forces play an important role in regulating both cartilage development and MSC chondrogenic gene expression, however, mechanical stimulation has yet to enhance the mechanical properties of engineered constructs. In this study, we applied long-term dynamic compression to MSC-seeded constructs and assessed whether varying pre-culture duration, loading regimens and inclusion of TGF-β3 during loading would influence functional outcomes and these phenotypic transitions. Loading initiated before chondrogenesis decreased functional maturation, although chondrogenic gene expression increased. In contrast, loading initiated after chondrogenesis and matrix elaboration further improved the mechanical properties of MSC-based constructs, but only when TGF-β3 levels were maintained and under specific loading parameters. Although matrix quantity was not affected by dynamic compression, matrix distribution, assessed histologically and by FT-IRIS analysis, was significantly improved on the micro- (pericellular) and macro- (construct expanse) scales. Further, whole genome expression profiling revealed marked shifts in the molecular topography with dynamic loading. These results demonstrate, for the first time, that dynamic compressive loading initiated after a sufficient period of chondro-induction and with sustained TGF-β exposure enhances matrix distribution and the mechanical properties of MSC-seeded constructs.

**Keywords:** Cartilage, tissue engineering, mesenchymal stem cells, chondrogenesis, mechanical stimulation.

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## Introduction

Adult bone marrow derived mesenchymal stem cells (MSCs) are a multipotent cell type capable of differentiation along a number of tissue-specific pathways (Pittenger *et al.*, 1999). As MSCs are also easy to isolate and self-renewing, they have emerged as an attractive cell type for engineering tissue replacements. The ability of MSCs to undergo chondrogenesis and take on a 'chondrocyte-like' phenotype renders these cells especially useful for cartilage engineering (Pittenger *et al.*, 1999; Johnstone *et al.*, 1998; Prockop, 1997), and numerous studies have demonstrated successful induction of chondrogenesis in a variety of biomaterials (Chung and Burdick, 2009; Erickson *et al.*, 2009a; Li *et al.*, 2005; Hofmann *et al.*, 2006; Huang *et al.*, 2009a). In the presence of TGF-β superfamily members, MSCs in 3D culture express cartilage-specific markers and deposit a extracellular matrix (ECM) rich in proteoglycans and collagen type II (Erickson *et al.*, 2009a; Erickson *et al.*, 2009b). However, despite their initial promise, MSC-based constructs for cartilage regeneration have yet to achieve functional properties approaching that of the native tissue, or even that of chondrocyte-based constructs cultured identically (Huang *et al.*, 2009b; Mauck *et al.*, 2006). These limitations in functional chondrogenesis are not due to insufficient cell number within the constructs (Huang *et al.*, 2009b; Kavalkovich *et al.*, 2002), but rather, may be due to an intrinsic limitation in TGF-β mediated chondrogenesis, in the absence of additional stimuli. Given the mechanically demanding environment of articular cartilage, the ability of MSC-based constructs to function within this environment is an important consideration and will directly affect clinical success.

Mechanical stimulation may be one strategy for optimizing chondrogenesis and overcoming these functional limitations. The rationale for this strategy is self evident; mechanical loading plays a vital role in the development, remodeling and maintenance of normal articular cartilage. During development, inhibition of muscle contraction and forces acting on skeletal elements results in abnormal joint formation (Mikic *et al.*, 2004; Mikic *et al.*, 2000). After birth, loading-induced remodeling of articular cartilage leads to dramatic changes in tissue structure (particularly of the collagen network) and increases in mechanical properties; in the absence of loading, this remodeling is not observed (Williamson *et al.*, 2003a; Williamson *et al.*, 2001; Williamson *et al.*, 2003b). In addition, normal joint loading has also been implicated in the maintenance of the chondrogenic phenotype of articular chondrocytes within cartilage (Chen *et al.*, 2008).

For cartilage tissue engineering, dynamic compression has proven especially effective in improving the functional properties of chondrocyte-seeded constructs (Mauck *et al.*, 2000; Mauck *et al.*, 2003a; Lima *et al.*, 2006). While less is known regarding the mechanoresponsivity of MSC-based constructs, recent work with MSCs and related cell types show that dynamic compression modulates the chondrogenic phenotype of these cells (Elder, 2002; Elder *et al.*, 2001; Elder *et al.*, 2000). In general, loading of human or rabbit MSCs increases expression of aggrecan and collagen type II (Angele *et al.*, 2004; Huang *et al.*, 2004), and is mediated by induction of the TGF-β signaling pathway (Huang *et al.*, 2005). The presence/absence of TGF-β also dictates the response of MSCs to compressive loading. Loading in the absence of TGF-β improves proteoglycan synthesis levels of equine MSCs relative to free swelling controls while in cultures loaded in the presence of TGF-β, matrix synthesis levels diminish (Kisiday *et al.*, 2009). Taken together, these findings suggest that dynamic compression modulates MSC chondrogenic differentiation and that the presence/absence of TGF-β influences this process.

While promising, the majority of the studies noted above were limited to short-term loading durations and focused on changes in gene expression and instantaneous measures of ECM synthesis. These studies have not established a link between these transient events and functional outcomes related to matrix accumulation and mechanical properties of the forming tissue. Indeed, repeated exposure to physical stimuli is often required for transient perturbations in ECM synthesis to culminate in changes in functional properties (Angele *et al.*, 2003). In one recent study, dynamic compression (initiated immediately after porcine MSC encapsulation in agarose) was carried out over 42 days in the presence of TGF-β. Findings from this study showed a marked reduction in the mechanical properties of loaded constructs compared to free-swelling controls (Thorpe *et al.*, 2008). In this previous work, loading was initiated before chondrogenesis had developed or deposition of local ECM had occurred, however, others have suggested that these initial phenotypic transitions may regulate MSC response to physical signals. For example, Mouw *et al*. showed that a single application of dynamic compression improved both collagen type II and aggrecan expression when bovine MSC-seeded constructs were pre-cultured in TGF-β1 containing media for longer culture durations (Mouw *et al.*, 2007). Similarly, Terraciano *et al*. demonstrated that human embryonic stem cells in 3D culture responded adversely to compressive loading in the absence of TGF-β; but responded positively when loading was initiated after a period of chondrogenic pre-differentiation (Terraciano *et al.*, 2007).

Collectively, these foundational studies on MSC mechanobiology suggest that dynamic compressive loading can modulate MSC chondrogenesis, though it is important to consider species differences when interpreting these findings as a number of these studies utilized MSCs derived from various species. These same studies also indicate that loading-mediated improvements in functional maturation of MSC-seeded constructs with dynamic loading may require the establishment of a chondrogenic phenotype before exposure to mechanical perturbation. To test this hypothesis, we applied long-term dynamic compressive loading to MSC-seeded constructs and evaluated the resulting changes in construct mechanical properties over 6 weeks of culture. We assessed whether varying pre-culture duration, loading regimens and inclusion of TGF-β3 during loading would affect functional outcomes and phenotypic transitions. While long-term loading initiated soon after MSC encapsulation reduced the mechanical properties of constructs, loading initiated after a short period of chondrogenesis and matrix elaboration dramatically improved construct mechanical properties. These findings show, for the first time, that the mechanical properties of MSC-seeded constructs can be enhanced by dynamic compressive loading, and point towards improved clinical translation of MSC-based cartilage constructs.

## Materials and Methods

### Mesenchymal stem cell isolation and culture

Bovine bone marrow derived MSCs were isolated from the tibiae and femurs of 3-6 month old calves (Research 87, MA, USA). Trabecular regions were removed with a saw and agitated in a solution of high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2% penicillin/streptomycin/fungizone (PSF) and 300 U/mL of heparin. The resulting solution was centrifuged (5 min at 300×g) and plated onto 10 cm tissue culture plates. Cultures were expanded in DMEM supplemented with 1% PSF and 10% fetal bovine serum (Lot 462819, Gibco (Invitrogen), Carlsbad, CA) with medium changed twice weekly until confluence. Cultures were expanded at a ratio of 1:3 through passage two, and MSCs from at least two donors were combined for each study, with a total of 8 donors used (donor sets A, B, C and D).

### Dynamic compression of MSC-seeded constructs

MSCs were suspended in chemically defined, serum-free medium (CM) and combined 1:1 with sterile type VII agarose (49°C, 4% w/v, Sigma, St Louis, MO, USA). Constructs (∅4 mm x 2.25 mm) were formed with a final cell seeding density of 20 million cells/mL (Huang *et al.*, 2009b). CM consisted of DMEM supplemented with 1X PSF, 0.1 μM dexamethasone, 50 μg/mL ascorbate 2-phosphate, 40 μg/mL L-proline, 100 μg/mL sodium pyruvate, 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenous acid, 1.25 mg/ml bovine serum albumin, and 5.35 μg/ml linoleic acid. For all studies, dynamic unconfined compression was applied using a custom bioreactor with impermeable platens (Fig. 1) (Mauck *et al.*, 2007). The loading protocol consisted of a 10% dynamic strain superimposed on a static 2% tare strain, with constructs in unconfined conditions (i.e., allowed to expand radially with axial compression). For all studies, loading was carried out at 37°C in a humidified incubator for 5 days per week for 3 weeks with free-swelling controls cultured in parallel.

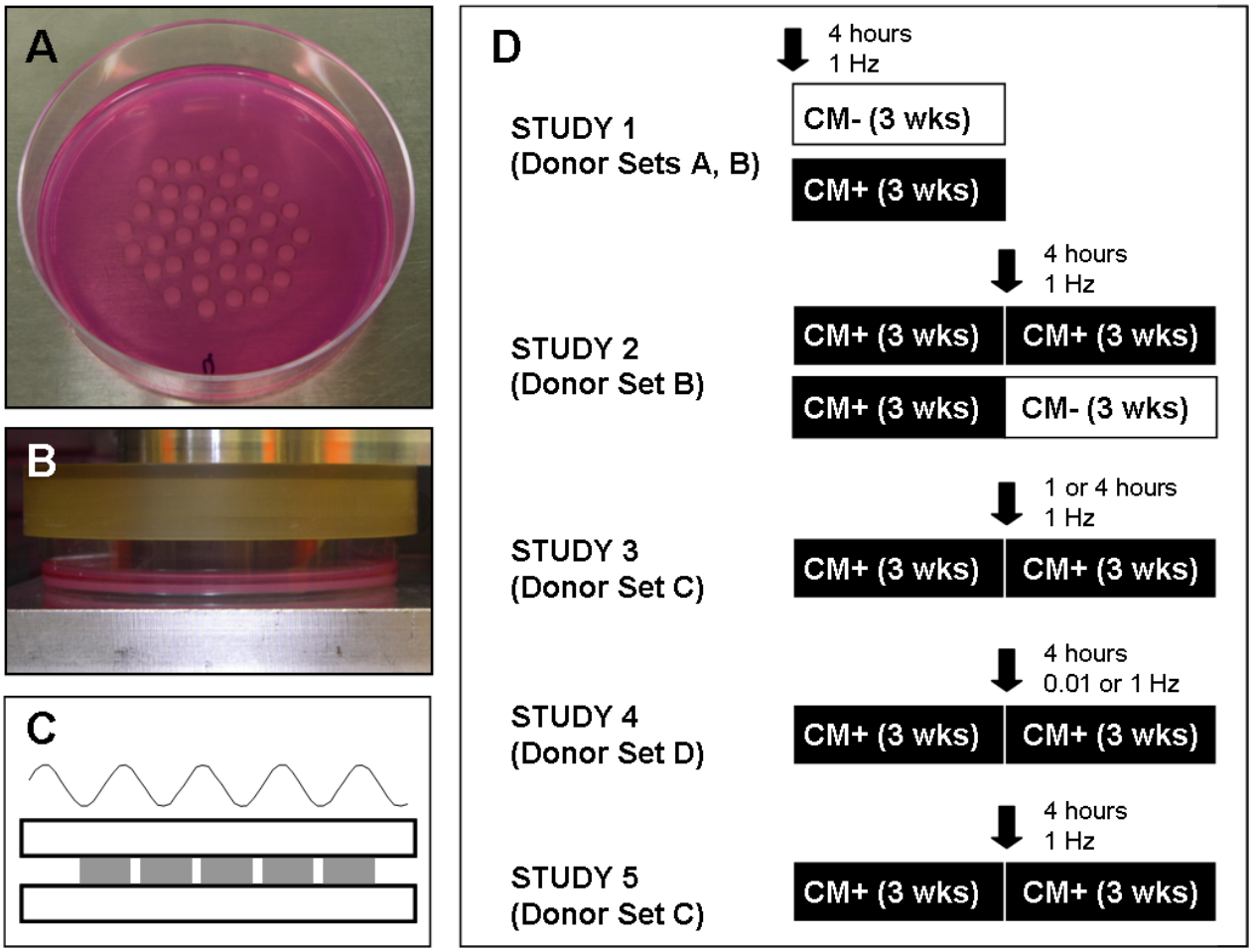


**Figure 1: Unconfined dynamic compressive loading of MSC-laden constructs in a custom bioreactor system.** **(A)** To hold constructs in place, molds were fabricated by casting a thin layer (~ 1.5 mm thickness) of sterile 4% agarose; ∅5 mm wells were made after gelation and MSC-seeded constructs (∅4 mm) were maintained in these wells throughout the culture duration. **(B)** Impermeable platens were used to apply a **(C)** sinusoidal displacement to MSC-seeded constructs. **(D)** Separate studies were carried out to examine the effects of pre-culture, loading duration, loading frequency, and dependence on TGF-β3. Arrow indicates timepoint (and conditions) at which loading was initiated.

A total of five independent studies were carried out as outlined in Fig. 1D, with cells combined from two donors for each study (each distinct donor set is indicated accordingly). In Study 1, dynamic compression was initiated 3 days after MSC encapsulation. Loading was applied at 1 Hz for 4 hours per day in CM supplemented with (CM+) or without (CM-) 10 ng/mL TGF-β3 (R&D Systems, Minneapolis, MN, USA). Gene expression was assessed weekly by real-time PCR. For Studies 2-5, dynamic compression was initiated after a 3-week pre-culture period in CM+. In Study 2, pre-cultured constructs were loaded in CM- or CM+ at 1 Hz for 4 hours per day. In Study 3, loading was applied for 1 hour or 4 hours per day at 1 Hz in CM+. In Study 4, dynamic compression was applied for 4 hours per day at 1 Hz or 0.01 Hz in CM+ medium. In Study 5, free-swelling constructs were cultured in CM- or CM+ for 6 weeks and loading was applied in CM+ at 1 Hz for 4 hours daily beginning at week 3. For all studies, mechanical and biochemical analyses were carried out at 3 and 6 weeks. For Study 5, global gene expression was assessed by microarray.

### Mechanical testing of constructs

To determine mechanical properties, constructs were tested in unconfined compression using a custom testing apparatus (Mauck *et al.*, 2006). Constructs were equilibrated in creep under a static load of two grams for 5 minutes. Following creep deformation, constructs were subjected to 10% strain applied at 0.05%/s followed by relaxation for 1000 seconds until equilibrium. Dynamic testing was carried out by applying an additional 1% sinusoidal deformation to equilibrated constructs at a frequency of 1.0 Hz. The equilibrium and dynamic moduli were determined (Mauck *et al.*, 2006). After mechanical testing, constructs were frozen at -20°C for biochemical evaluation.

### Biochemical analysis

For biochemical analyses, constructs were digested in papain (0.56U/ml in 0.1M sodium acetate, 10M cysteine HCl, 0.05M ethylenediaminetetraacetic acid (EDTA), pH 6.0) at 60°C for 16 hours. Following digestion, constructs were evaluated for sulfated glycosaminoglycan (GAG) content against a standard curve of chondroitin-6-sulphate

using the 1,9-dimethylmethylene blue dye-binding assay (Farndale *et al.*, 1986). Collagen content was assessed after acid hydrolysis using the orthohydroxyproline (OHP) assay (Stegemann and Stalder, 1967), with a 1:7.14 OHP:collagen ratio used (Neuman and Logan, 1950). DNA content was determined by means of the PicoGreen dsDNA assay (Molecular Probes, Eugene, OR). GAG and collagen are reported as percentage of construct wet weight while DNA is reported as quantity per construct.

**Real time polymerase chain reaction**

Total RNA was extracted by two sequential isolations in TRIZOL-chloroform and quantified (ND-1000, Nanodrop Technologies, Wilmington, DE, USA). Reverse transcription and amplification was carried out using an Applied Biosystems 7300 real-time PCR system with intron spanning primers and SYBR Green Reaction Mix (Applied Biosystems, Foster City, CA, USA) as in (Huang *et al.*, 2009b). For Study 1, collagen type II (COL2A1) and aggrecan (AGC1) expression levels were quantified and normalized to expression of a housekeeping gene (GAPDH).

**Microarray hybridization and data analysis**

Total RNA was extracted as described above and quality assessment was performed by the Agilent Bioanalyzer. All protocols were conducted as described in the NuGEN Ovation Manual and the Affymetrix GeneChip Expression Analysis Technical Manual. Briefly, 100 ng of total RNA was converted to first-strand cDNA using reverse transcriptase primed by a poly(T) oligomer that incorporated a synthetic RNA sequence. Following second-strand cDNA synthesis, ribo-SPIA (Single Primer Isothermal Amplification, NuGEN Technologies Inc. San Carlo, CA, USA) was carried out for linear amplification of each transcript and the resulting cDNA was fragmented and biotinylated. 5 μg of cDNA was added to hybridization cocktails, heated at 99ºC for 2 min and hybridized for 16 h at 45ºC to 5 Bovine GeneChips (Affymetrix Inc., Santa Clara, CA, USA). The microarrays were then washed at low (6X SSPE) and high (100 mM MES, 0.1 M NaCl) stringency and stained with streptavidin-phycoerythrin. To amplify fluorescence, biotinylated anti-streptavidin and streptavidin-phycoerythrin stain were added, and fluorescent signals were recorded after excitation at 570 nm by the Affymetrix Gene Chip Scanner 3000 (Affymetrix).

Output (.cel) files from scanning were processed using Microarray Suite (v.5, Affymetrix) and gene expression was assessed after normalization. Expression levels were evaluated for free-swelling week 6 MSCs in CM-, free-swelling week 6 MSCs in CM+ and week 6 dynamically loaded MSCs in CM+. Microarray data were analyzed using the Spotfire Software (Tibco, Somerville, MA, USA). All microarray data discussed in this manuscript have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE18879 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18879).

**Histology and immunohistochemistry**

Paraffin sections (8 μm) were stained with Hematoxylin and Eosin (H&E, Sigma,), Alcian Blue (pH 1.0), or Picrosirius Red for cell distribution, sulfated proteoglycans and collagens, respectively. For immunohistochemistry, antigen retrieval was performed by incubating sections in proteinase K (20 μg/mL in TE buffer, pH 8.0) at 37°C for 15 minutes, then at 25°C for 10 minutes. Collagens type I (MAB3391, Millipore, Billerica, MA) and type II (11-116B3, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) primary antibodies were used. Subsequent reaction and visualization with DAB chromagen reagent (DAB150 IHC Select, Millipore) was carried out according to manufacturer's instructions. Color images were captured at 2.5x or 10x magnification using a microscope equipped with a color CCD digital camera and the QCapturePro acquisition software.

**Fourier transform infrared imaging spectroscopy (FT-IRIS)**

FT-IRIS was carried out using a Spectrum Spotlight 300 spectrometer (Perkin-Elmer, Waltham, MA, USA) equipped with an optical microscope and an array detector. Sections (8 μm) were mounted onto barium fluoride windows and scanned with a spatial resolution of 25 μm and a spectral resolution of 4 $cm^{-1}$. The acquired spectra were analyzed using ISys software 5.0 (Malvern Instruments Ltd., Worcestershire, UK). Collagen and proteoglycan distributions were determined by molecular vibrations at specific frequencies (wavenumber, $cm^{-1}$); the amide I absorbance band (1720-1592 $cm^{-1}$; C=O stretch) was used to map collagen while proteoglycans were visualized using the 1176-960 $cm^{-1}$ band (C-O-C and C-OH ring vibrations)(Boskey and Pleshko Camacho, 2007; Kim *et al.*, 2005). FT-IRIS analysis was performed on three samples per group.

**Statistical analysis**

Statistics were performed on mechanical and biochemical data using analysis of variance (ANOVA). For Studies 1 and 2, a two way ANOVA was used with media and loading condition as independent variables. For Studies 3-5, a one way ANOVA was carried out. Where significance was indicated by ANOVA, Tukey's posthoc tests were performed. Significance was determined at $p \leq 0.05$ and a trend toward significance determined at $p < 0.1$. All values are reported as mean ± standard deviation.

## Results

**Dynamic compression initiated before chondrogenesis impairs functional maturation of MSC-seeded constructs**

To examine the effects of direct mechanical stimulation on functional chondrogenesis, MSC-seeded constructs were subjected to repeated dynamic compression over 3 weeks. In Study 1, dynamic loading (DL) was initiated 3 days after MSC encapsulation and carried out in the

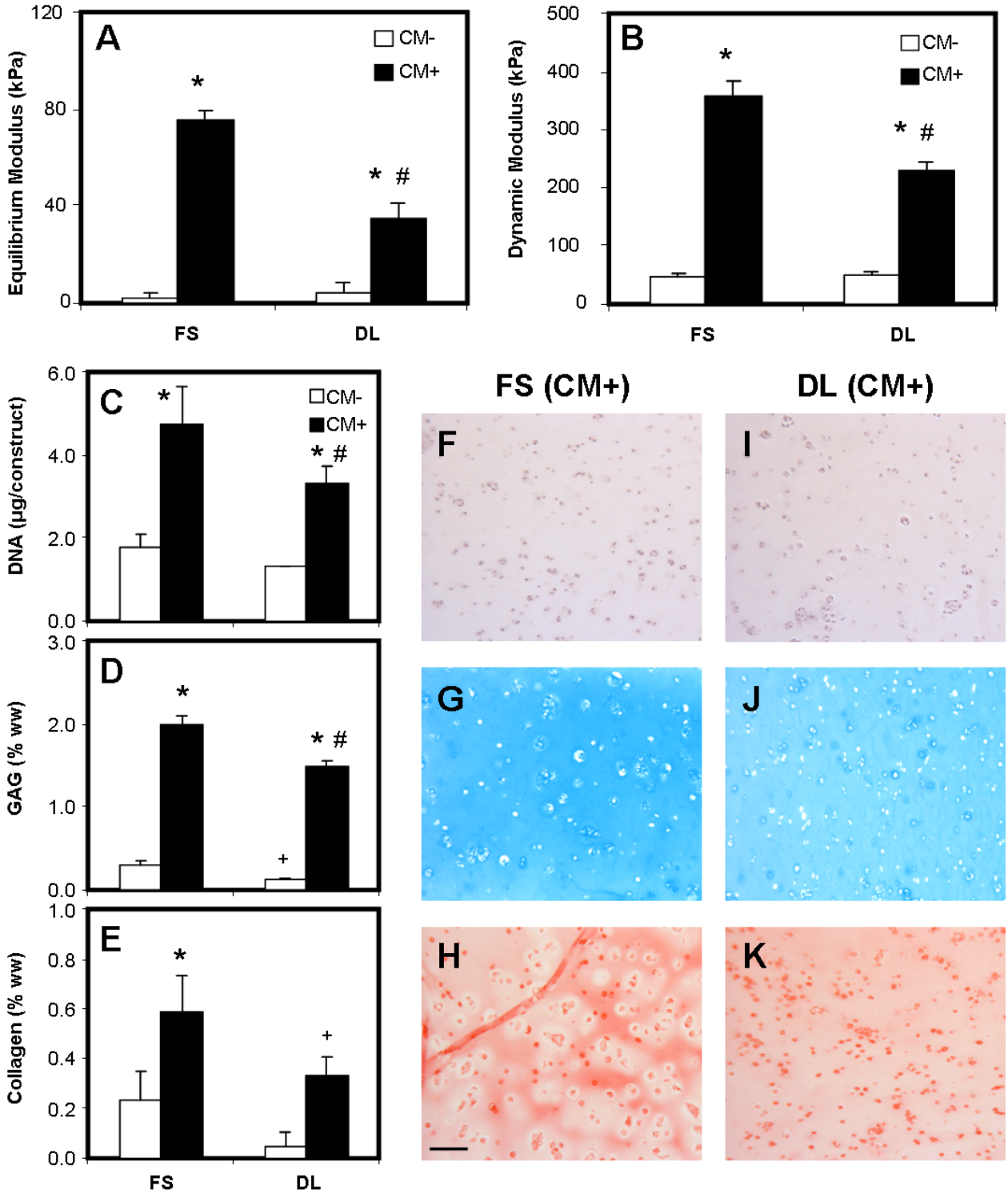


**Figure 2: Long-term dynamic compression initiated directly after construct fabrication blocks functional maturation. (A)** The equilibrium and **(B)** dynamic moduli of MSC-seeded constructs loaded in CM+ were impaired by 3 weeks of dynamic compression. **(D)** DNA, **(E)** GAG, and **(F)** collagen contents were similarly modulated. Histological analysis confirmed these findings with **(F, I)** H&E, **(G, J)** Alcian Blue, and **(H, K)** Picrosirius Red staining for cell content, proteoglycans and collagens, respectively. Scale bar: 100 µm. * indicates greater than CM- ($p<0.05$), # indicates lower than FS CM+ ($p<0.05$), + indicates lower than FS control within media condition ($p<0.1$). Data represent the mean and standard deviation of three samples per group per time point.

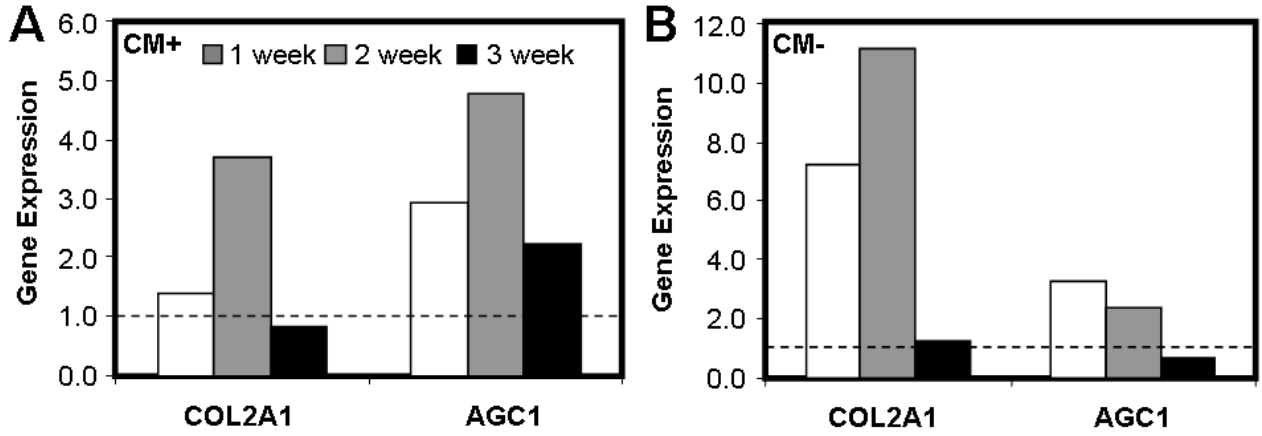


**Figure 3: Long-term dynamic compression initiated directly after construct fabrication improves chondrogenic gene expression.** After 1 and 2 weeks, COL2A1 and AGC1 expression improved with loading in **(A)** CM+ and **(B)** CM- media. Expression levels were normalized to free-swelling controls at each timepoint (indicated by the dashed line).

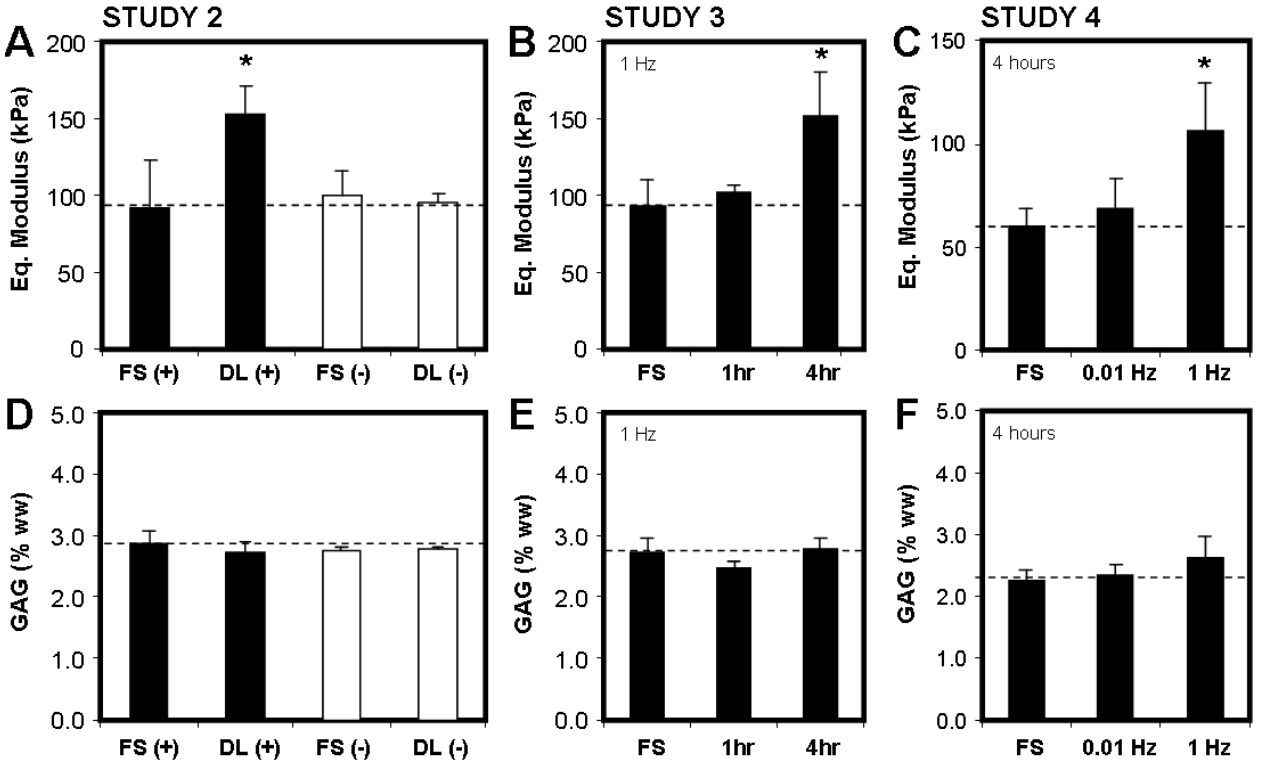


**Figure 4: Long-term dynamic loading initiated after 3 weeks of chondrogenic pre-culture improves mechanical properties. (A-C)** The equilibrium modulus of MSC-seeded constructs at week 6 improved only when loading was applied in CM+ for 4 hours per day at 1 Hz. No improvement in mechanical properties was observed when other loading regimens were employed. **(D-F)** GAG content at week 6 was not affected by loading. Black bars indicate CM+ media and white bars indicate CM- media. * indicates greater than control ($p$<0.05). Data represent the mean and standard deviation of three to five samples per group per time point.

presence (CM+) or absence (CM-) of TGF-β3. Free swelling (FS) controls were cultured and analyzed identically. The equilibrium and dynamic compressive moduli improved with time in culture for all groups cultured in CM+, regardless of loading ($p$<0.05). Consistent with our previous studies (Huang *et al.*, 2009b), the equilibrium and dynamic moduli of FS CM+ constructs reached ~80 kPa and ~350 kPa, respectively, by 3 weeks. Conversely, the modulus of FS CM- constructs remained similar to that of acellular 2% agarose (~2 kPa, Fig. 2A). Long-term DL initiated 3 days after construct fabrication significantly reduced the mechanical properties of CM+ constructs at 3 weeks relative to FS CM+ controls ($p$<0.05). At 3 weeks, the equilibrium and dynamic moduli of DL CM+ constructs were ~35 kPa and ~230 kPa, respectively (Figs. 2A, 2B). Although significantly greater than day 0 properties (equilibrium modulus: ~ 2 kPa, dynamic modulus: ~ 45 kPa), these values were less than two thirds that of FS CM+ constructs.

While DL did not change the mechanical properties of CM- constructs (Figs. 2A, 2B), GAG content was reduced with DL (trend, $p$=0.07), although DNA and collagen

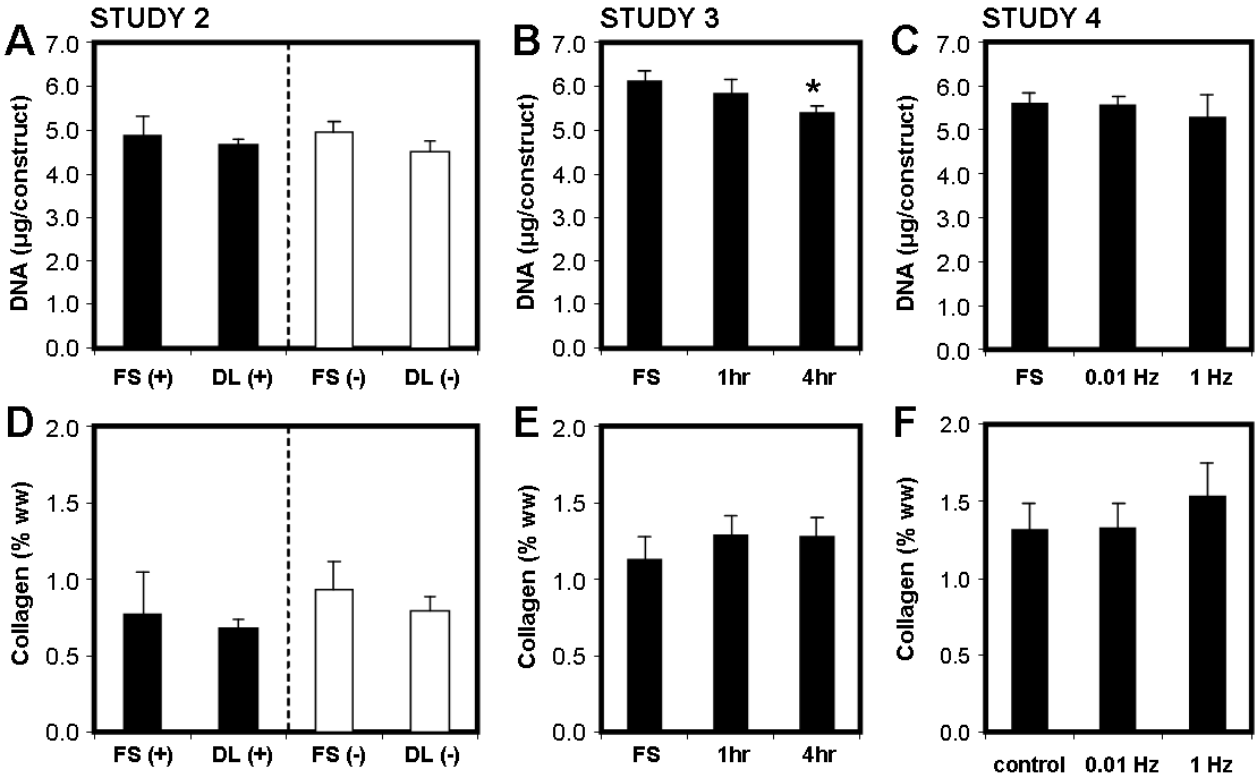


**Figure 5: Long-term dynamic loading initiated after 3 weeks of chondrogenic pre-culture does not improve biochemical content. (A-C)** The DNA and **(D-F)** collagen contents of MSC-seeded constructs were largely unchanged with dynamic compressive loading. Black bars indicate CM+ media and white bars indicate CM- media. * indicates greater than control ($p<0.05$). Data represent the mean and standard deviation of three to five samples per group per time point.

contents were not affected (Figs. 2C-2E). DL applied in CM+ reduced all biochemical measures relative to FS CM+. Despite this reduction, DNA and GAG contents remained significantly higher in DL CM+ compared to either FS CM- or DL CM- groups, indicating that successful chondrogenesis had occurred, but to a lesser extent. DNA content for both FS and DL groups at 3 weeks was also significantly higher than day 3 starting values, while GAG content on a per cell basis (GAG/DNA) was comparable between 3-week FS and DL CM+ groups (not shown). These quantitative biochemical findings were mirrored qualitatively in histological sections, with less intense staining for proteoglycans and collagens observed in DL CM+ compared to FS CM+ sections (Figs. 2F-2K). Real-time PCR analysis of these MSC-seeded constructs showed that while DL reduced mechanical and biochemical measures, DL increased expression of both AGC1 and COL2A1, particularly at weeks 1 and 2 in both CM- and CM+ media relative to FS controls (Fig. 3).

**Long-term dynamic compression initiated after chondrogenic pre-culture improves functional properties of MSC-seeded constructs**

To assess whether a period of chondrogenic pre-culture alters functional maturation in response to DL, MSC-seeded constructs were differentiated in TGF-β3 containing media prior to the initiation of loading (Study 2). Constructs were cultured in CM+ for 3 weeks (weeks 1-3) and then subjected to 3 weeks of dynamic loading (weeks 4-6) in CM+ or CM-. As we have previously observed (Huang *et al*., 2009b), the mechanical properties of MSC-seeded constructs increased with time, but plateaued after 3 weeks with no significant difference in mechanical properties between the 3 and 6 week FS groups in CM+ (not shown). In contrast to Study 1, the equilibrium and dynamic properties of pre-cultured constructs exposed to DL for weeks 4-6 in CM+ were significantly higher than FS controls in CM+ (Fig. 4A). DL CM+ constructs reached equilibrium and dynamic moduli at week 6 of ~150 kPa (65% increase vs. FS CM+) and ~800 kPa (38% increase *vs*. FS CM+), respectively. This increase in properties with DL was only observed when loading was applied in CM+. In the absence of TGF-β3 (CM- from week 4 to week 6), DL did not change mechanical properties. To better understand the threshold for DL-induced improvements in functional properties, Studies 3 and 4 examined the influence of loading duration (1 or 4 hours) and loading frequency (0.01 or 1 Hz) on construct properties. While DL in CM+ for 4 hours per day at 1 Hz always increased mechanical properties compared to FS controls, DL for 1 hour per day at 1 Hz or 4 hours per day at 0.01 Hz had no effect on these measures (Figs. 4B, 4C).

Despite increases in mechanical properties, GAG (Figs. 4D-4F) and collagen (Fig. 5) contents were not different between any of the CM+ groups. Similarly, GAG released into the media was not different when comparing FS to

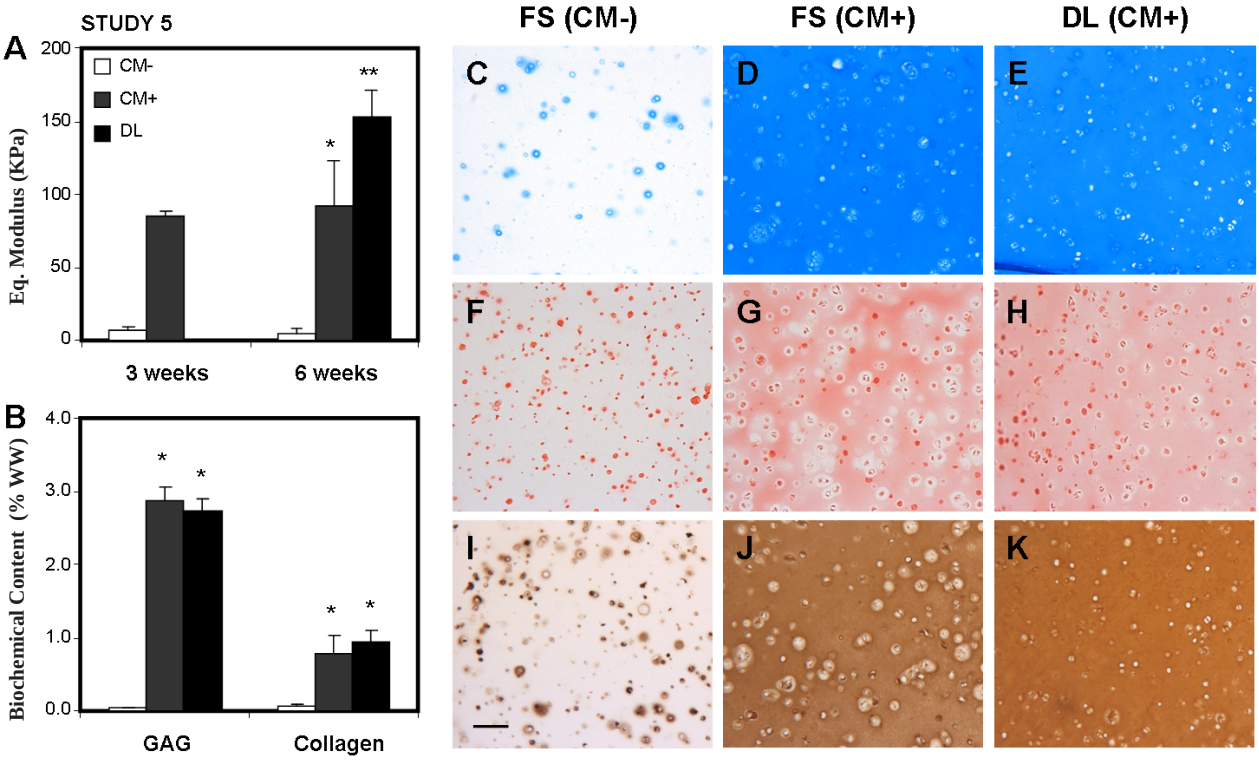


**Figure 6: Long-term dynamic loading initiated after 3 weeks of chondrogenic pre-culture improves bulk mechanical properties and microscopic ECM distribution. (A)** The equilibrium modulus of MSC-seeded constructs was higher in CM+ compared to CM- at 3 and 6 weeks; dynamic loading in CM+ for 3 weeks further improved mechanical properties. **(B)** Biochemical content of dynamically loaded constructs at week 6 was not different compared to CM+ controls. **(C-E)** Alcian Blue staining at week 6 showed equal distribution of proteoglycans between CM+ controls and loaded constructs with weak staining in CM- controls. **(F-H)** Picrosirius Red staining and **(I-K)** collagen type II immunostaining showed more homogeneous distribution of collagen in loaded constructs compared to controls, on the microscopic level. Scale bar: 100 μm. * indicates greater than CM- controls ($p<0.05$), ** indicates greater than CM+ controls ($p<0.05$). Data represent the mean and standard deviation of three samples per group per time point.

DL constructs (data not shown). DNA content was not affected by loading in Studies 2 and 4, but was slightly reduced in the 4 hour loaded samples compared to FS conditions in Study 3 (Fig. 5).

### Long-term dynamic compression enhances matrix distribution

Based on Studies 1-4, a dynamic compressive loading protocol of 4 hours per day at a frequency of 1 Hz, initiated after 3 weeks of pre-culture, was applied in a final study designed to further elucidate the mechanism of load-induced increases in mechanical properties of MSC-seeded constructs (Study 5). As before, loading was carried out for 5 days per week for 3 weeks. Consistent with these previous iterations, the equilibrium modulus improved with DL, but bulk GAG and collagen contents were not different compared to FS (Figs. 6A, 6B). FS CM- constructs did not deposit appreciable ECM with long-term culture; these findings were confirmed histologically with weak pericellular staining for proteoglycans and collagens observed. At the microscopic level, GAG distribution was similar between CM+ DL and FS constructs, while collagen content was more uniformly distributed in the CM+ DL groups (Fig. 6). At the final time point, all CM+ constructs stained strongly for type II collagen (Figs. 6J, 6K) and weakly for type I collagen (Fig. 7), regardless of loading condition . Type II collagen was more uniformly distributed with DL, consistent with the Picrosirius Red stains. Despite the absence of TGF-β3, by week 6, a subset of MSCs in CM- had undergone chondrogenesis to a limited extent, depositing a small amount of type II collagen in the immediate pericellular space (Fig. 6I). Finally, FT-IRIS was performed as a more sensitive measure of proteoglycan and collagen distribution across the construct expanse. Characteristic spectra for proteoglycans and collagens showed consistently improved distribution with DL, particularly in the construct central regions (Fig. 8).

### Expression profiles with chondrogenic induction and long term dynamic compression

In a final analysis, a preliminary microarray screen was performed to visualize shifts in molecular topography that might underlie the observed differences in mechanical properties with dynamic loading. This screen compared whole genome expression profiles for week 6 FS CM- samples, FS CM+ samples, and DL CM+ samples. CM- and CM+ FS groups were chosen as undifferentiated and differentiated controls, respectively, in order to identify

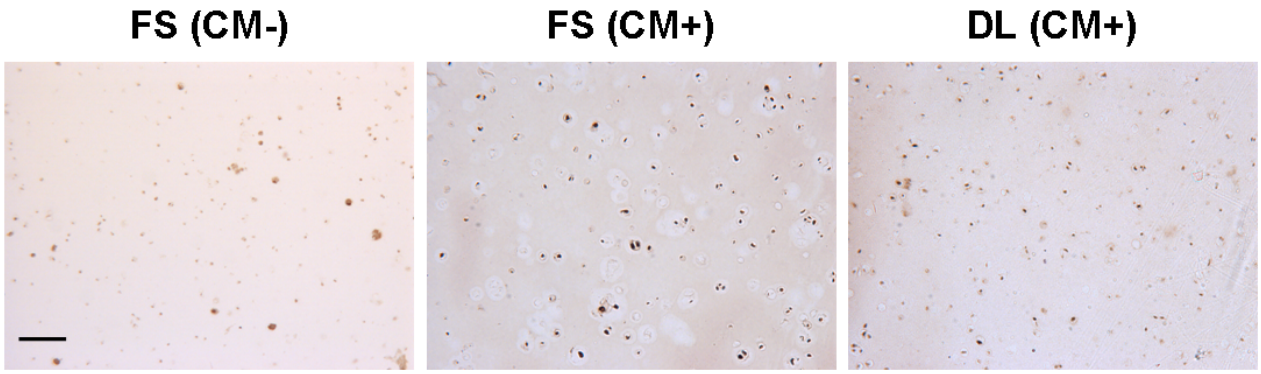


**Figure 7: Type I collagen staining of free-swelling and dynamically loaded constructs at week 6.** Weak, pericellular staining for type I collagen was observed for all constructs, regardless of loading. Scale bar: 100 μm.

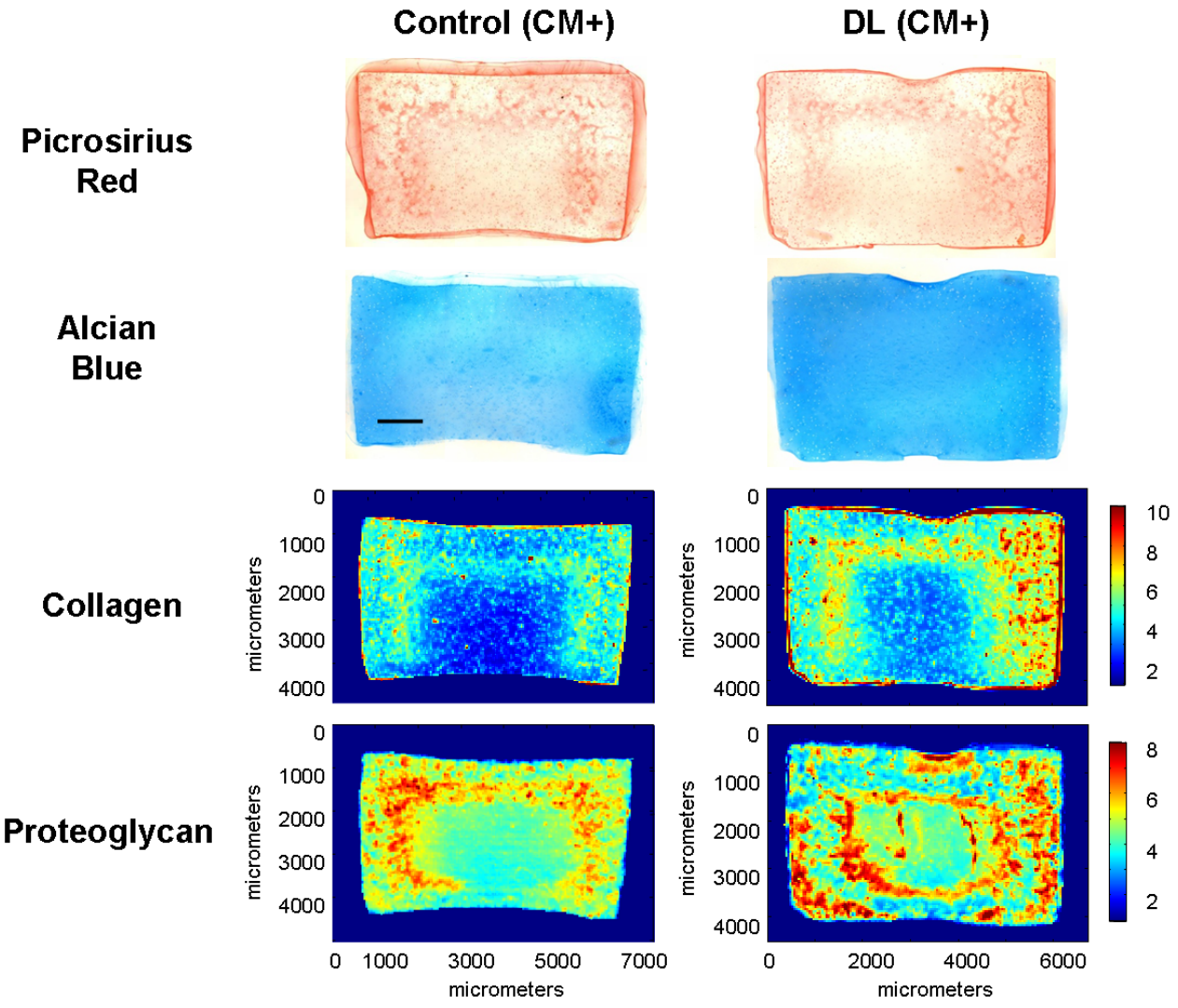


**Figure 8: FT-IRIS assessment of matrix distribution at 6 weeks.** Whole construct views of Picrosirius Red and Alcian Blue stained cross-sections showing distributions of collagen and proteoglycan within FS and DL constructs. Spectral data obtained from FT-IRIS analysis, a more sensitive and semi-quantitative measurement technique, showed improved collagen and proteoglycan distribution within MSC-seeded constructs with dynamic compressive loading. Scale bar: 1mm.

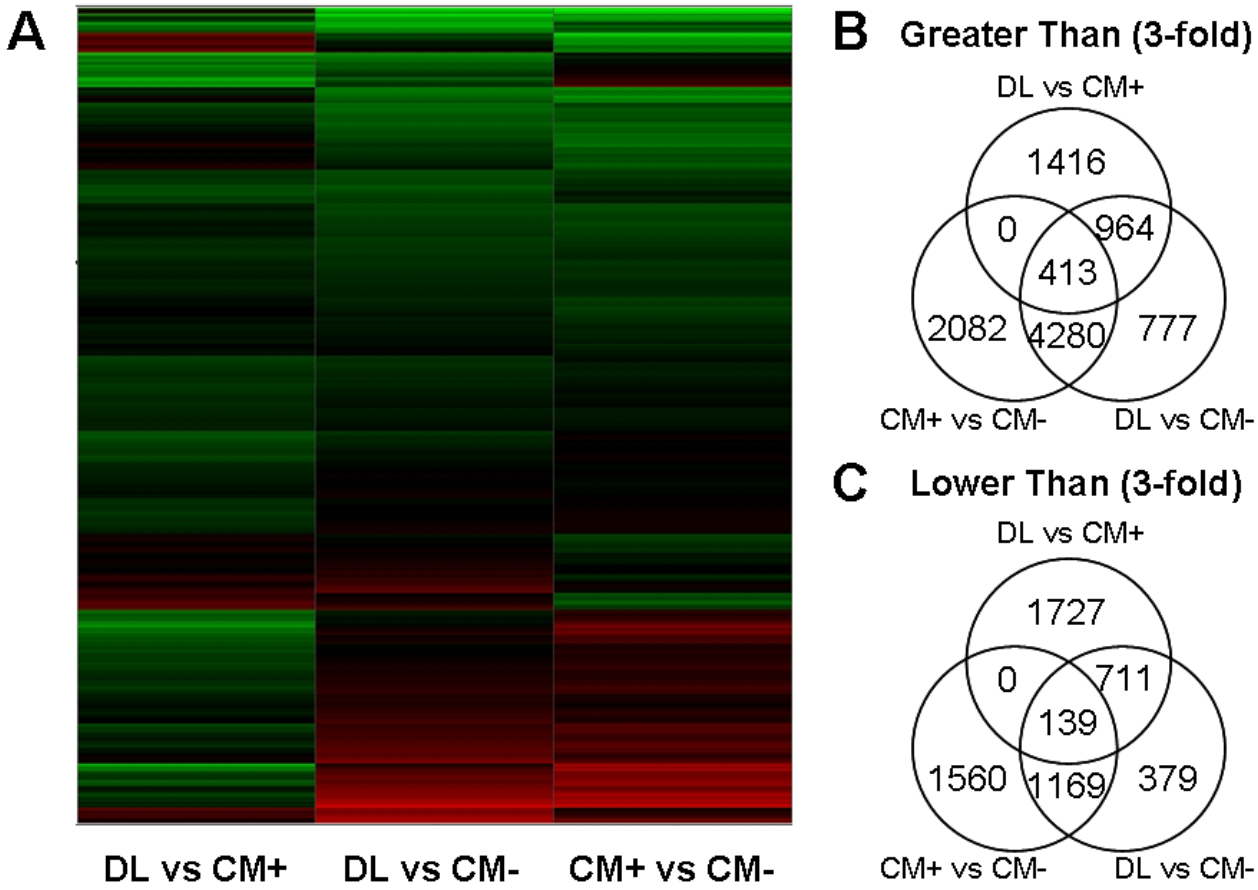


**Figure 9: Molecular topography of chondro-induction and mechanosensitivity. (A)** Heat map generated from microarray data showing differential gene expression (red = greater, green = lower) between CM- free-swelling (FS) controls (CM-), CM+ FS controls (CM+) and constructs dynamically loaded (DL) in CM+ at day 42. **(B, C)** Venn diagrams indicate a number of genes that are differentially regulated with chondrogenic induction (CM+) in 3D culture and with dynamic compressive loading (>3-fold).

**Tables:** These two tables are available separately as supplementary files and can be downloaded from the website (http://www.ecmjournal.org/journal/papers/vol019/vol019a08.php).

**Table 1:** (18 pages) Complete list of genes that were up-regulated (> 3-fold) at week 6 with dynamic compressive loading compared to CM+ free-swelling control.

**Table 2:** (22 pages) Complete list of genes that were down-regulated (> 3-fold) at week 6 with dynamic compressive loading compared to CM+ free-swelling control.

markers associated with functional chondrogenesis. Heat maps at week 6 showed changes in gene expression with chondrogenesis (CM+ *vs.* CM-), with higher and lower levels of expression for individual genes depicted in red and green, respectively (Fig. 9A). While the molecular fingerprints between FS CM+ and DL CM+ were more alike compared to FS CM-, DL modulated the expression of a number of genes (Fig. 9A). Venn diagrams showed 5449 genes that were chondrogenic, but not mechanically sensitive; of these genes, 4280 were up-regulated and 1169 were down-regulated during chondrogenesis (Figs. 9B, 9C). In addition, numerous genes associated with chondrogenesis were further modulated by DL (413 up-regulated, 139 down-regulated). A complete list of the genes modulated by DL can be found in Tables 1 and 2.

## Discussion

Mesenchymal stem cells (MSCs) are an ideal candidate for cartilage tissue engineering given their ability to undergo chondrogenesis in 3D culture. Under pro-chondrogenic conditions, MSCs deposit a cartilage-specific matrix and accrue increasingly robust mechanical properties with time. However, recent findings suggest that further optimization may be required to generate properties akin to that of the native tissue, or that of articular chondrocytes cultured similarly (Huang *et al.*, 2009b; Mauck *et al.*, 2006). As mechanical stimulation plays a unique role in both cartilage development and the maturation of chondrocyte-based engineered constructs, we examined long-term dynamic compressive loading as a means of modulating MSC-seeded construct properties and chondrogenesis

Consistent with previous findings, long-term dynamic loading initiated soon after MSC encapsulation in the presence of TGF-β3 reduced the mechanical properties of constructs compared to free-swelling controls (Thorpe *et al*., 2008). While biochemical content was lower in these dynamically loaded samples, GAG content per cell was not different from controls and chondrogenic gene expression was up-regulated with loading at each time point assayed. As loading did not affect cell viability, these data suggest that long-term loading in the presence of TGF-β3 reduced MSC proliferation with commensurate decreases in bulk biochemical and mechanical properties, but did not impair chondrogenic differentiation. Dynamic compression alone, in the absence of TGF-β3, failed to induce chondrogenesis; in fact, after three weeks of loading, GAG content decreased in loaded groups, though chondrogenic gene expression increased. Thus, while long-term dynamic compression, initiated prior to cell differentiation or matrix deposition, may improve the expression of AGC1 and COL2A1, mechanical properties and GAG content are inferior in these samples, regardless of TGF-β supplementation. Whether this lower GAG content in loaded constructs is due to poor retention of synthesized GAG (caused by differences in molecule size/assembly or possible upregulation of catabolic agents) or due to an actual reduction in GAG synthesis is currently unclear.

In contrast to these findings, loading initiated after chondrogenesis and matrix elaboration in the presence of TGF-β3 consistently improved the mechanical properties of MSC-seeded constructs. As all other factors remained constant, the timing of load initiation was crucial in determining functional outcomes. These divergent responses to loading can be attributed to changes in MSC phenotype and construct properties with maturation. Work in ligament tissue engineering support the notion that the timing of mechanical stimulation with MSC developmental stage may be a critical determinant of cell response to load (Chen *et al.*, 2006). Similarly, dynamic compression applied after an extended pre-culture time improved chondrogenic gene expression of MSCs, as well as embryonic stem cells (Mouw *et al.,* 2007; Terraciano *et al*., 2007). As the nuclei of undifferentiated stem cells deform more readily than that of differentiated cells (Pajerowski *et al.*, 2007), the differentiation status of MSCs may play an important role in how these cells perceive external mechanical stimulation. Cell-matrix interactions may also affect load-induced response; since agarose is an inert material, these interactions emerge only as MSCs differentiate and generate local ECM. With matrix elaboration, the physical environment of the cells under dynamic compression is also altered. As construct composition shifts from 2% agarose to a denser, cartilage-like matrix of proteoglycans and collagen and construct permeability decreases, the stresses induced by dynamic compression are higher and largely borne through fluid pressurization (Soltz and Ateshian, 1998); this is apparent from our mechanical testing results showing marked increases in the dynamic modulus with culture duration. These differences in fluid pressurization and fluid flow may underlie the profoundly disparate outcomes we observe with dynamic compressive loading, depending on construct maturity. This is consistent with several studies showing that application of hydrostatic pressure to human MSC aggregates improves cartilaginous matrix deposition and gene expression (Angele *et al*., 2003; Miyanishi *et al.*, 2006).

Interestingly, in the absence of TGF-β3, compressive loading initiated after chondrogenic pre-culture did not elicit any changes in functional properties, indicating that the load-induced increase in mechanics is dependent on TGF-β3. This is consistent with previous findings showing improved matrix synthesis and pSmad2/3 protein levels in pre-cultured bovine MSC-seeded agarose with a single application of loading, when loading was applied in the presence of TGF-β1 and dexamethasone (Mouw *et al*., 2007). In contrast to MSCs, repeated dynamic compression of pre-cultured chondrocyte-seeded agarose improved the mechanical properties of these constructs when loading was applied in the absence of TGF-β3 (Lima *et al.*, 2007). Long-term dynamic compression initiated prior to matrix elaboration also improved the functional properties of chondrocyte-based constructs, though these studies were conducted in the presence of serum (Mauck *et al*., 2000). Collectively, these studies suggest that the mechanotransduction pathways initiated by dynamic compression may be fundamentally different between chondrocytes and undifferentiated or chondrogenically differentiated MSCs and underscores the need for better characterization of these cell types relative to one another.

While it is unclear how dynamic compression may improve functional MSC chondrogenesis, one potential mechanism may be facilitated nutrient/growth factor transport with dynamic deformation. Theoretical models of dynamic compression of porous permeable materials indicate that solute transport into constructs may be improved by dynamic loading and that higher frequencies enhance this phenomenon (Mauck *et al.*, 2003b). Recent experimental findings validate these theoretical predictions and show that, in particular, the transport of large solutes (with molecular weights similar to that of growth factors such as TGF-β) is facilitated by dynamic compression and is dependent on loading duration (Albro *et al.*, 2008; Chahine *et al.*, 2009). In one set of experiments with acellular agarose gels of varying concentrations, solute uptake increased for higher concentration gels (Albro *et al*., 2008). These results parallel findings from the current study, where mechanical properties only increased when dynamic loading was initiated after pre-elaboration of matrix (denser construct), and applied at longer durations (4 hours *vs*. 1 hour) at a higher frequency (1 Hz *vs*. 0.01 Hz). Notably, loading in the absence of exogenous TGF-β3 (after chondrogenic pre-culture) failed to elicit any changes in mechanical properties.

Although enhanced transport of nutrients or TGF-β is one possible mechanism, MSC responsivity to mechanical stimulus may also be an important factor. To assess MSC response on the molecular level, we carried out microarray analysis of loaded and free-swelling constructs and saw marked overlap in chondrogenic gene expression, indicating successful induction and stability of the chondrogenic phenotype. Consistent with these findings,

all constructs stained strongly for collagen type II and weakly for collagen type I. As bulk GAG and collagen contents were not affected by loading (despite increases in mechanical properties), minor elements involved in matrix remodeling and refinement may be of consequence. Though preliminary, microarray analysis indicated modulation of several genes from the MMP/TIMP family, as well as specialized cross-linking molecules. Additional analyses will be necessary to validate these findings and determine the potential role of these genes under dynamic compression. Although measurement of gene expression is not necessarily a reliable predictor of functional chondrogenesis (i.e., Study 1), evidence for matrix remodeling was found by histology and FT-IRIS. These analyses showed improved matrix distribution after 3 weeks of repeated loading. Histological stains showed better pericellular distribution of collagen and FT-IRIS showed enhanced macroscopic distribution of proteoglycans and collagens throughout the construct expanse. This is consistent with previous studies of chondrocyte-based constructs showing improved collagen organization (assessed by polarized light microscopy) with dynamic compressive loading (Kelly *et al.*, 2006).

To our knowledge, this is the first study to demonstrate improved mechanical properties of MSC-based engineered cartilage through the long-term application of dynamic compressive loading. While the mechanism underlying these increased properties is not yet established, we show that improved matrix distribution, suggestive of matrix remodeling and refinement occurs with dynamic compression. Although we achieved a ~65% improvement in mechanical properties over 3 weeks of loading, these values remain lower than native cartilage. Future studies will optimize these parameters over longer culture durations to further explicate load-induced increases in mechanical properties. We will also evaluate MMP activity and the expression and distribution of minor elements indicated from microarray analysis, as well as assess proteoglycan size and collagen crosslinking with dynamic loading. The use of other biomaterials, including materials that mimic the native ECM or materials that include hydrolytic or MMP-cleavable components to allow better matrix distribution, may also aid matrix remodeling and further enhance functional outcomes (Erickson *et al.*, 2009a; Chung *et al.*, 2009; Park *et al.*, 2004; Lutolf *et al.*, 2003). The combination of these tunable materials with our optimized loading regime will generate clinically-relevant, mechanically robust MSC-based constructs for articular cartilage repair.

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