Mechanical Compression of Cartilage Explants Induces Multiple Time-Dependent Gene Expression Patterns and Involves Intracellular Calcium and Cyclic AMP

Jonathan B. Fitzgerald¹, Moonsoo Jin², Delphine Dean³, David J. Wood⁴, Ming H. Zheng⁴,
Alan J. Grodzinsky¹,²,³

Biological Engineering Division¹, Center for Biomedical Engineering², and Department of Electrical Engineering and Computer Science³, MIT, NE47-377, 77 Massachusetts Avenue, Cambridge, MA 02139, USA

⁴Department of Orthopaedic Surgery, QEII Medical Centre, M-Block 2nd Floor, University of Western Australia, Nedlands, WA 6009, Australia

Correspondence to:
Alan J. Grodzinsky

Phone 617 253 4969

FAX 617 258 5239

Email alg@mit.edu

Current address: The CBR Institute for Biomedical Research, INC, Harvard Medical School, Boston, MA, 02115
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Summary

Chondrocytes are influenced by mechanical forces to remodel cartilage extracellular matrix (ECM). Previous studies have demonstrated the effects of mechanical forces on changes in biosynthesis and mRNA levels of particular ECM molecules, and have identified certain signaling pathways that may be involved. However, the broad extent and kinetics of mechano-regulation of gene transcription has not been studied in depth. We applied static compressive strains to bovine cartilage explants for periods between 1-24 hours, and measured the response of 28 genes using real-time PCR. Compression time-courses were also performed in the presence of an intracellular calcium chelator or an inhibitor of cyclic AMP activated protein kinase A. Cluster analysis of the data revealed four main expression patterns: two groups containing either transiently upregulated or duration-enhanced expression profiles could each be subdivided into genes that did or did not require intracellular calcium release and cyclic AMP activated protein kinase A for their mechano-regulation. Transcription levels for aggrecan, type II collagen, and link protein were upregulated approximately 2 to 3-fold during the first 8hrs of 50% compression and subsequently down-regulated to levels below that of free-swelling controls by 24hrs. Transcription levels of matrix metalloproteinases-3,9,13, aggrecanase-1 and the matrix protease regulator cyclooxygenase-2 increased with the duration of 50% compression 2 to 16-fold up to by 24hrs. Thus, transcription of proteins involved in matrix remodeling and catabolism dominated over anabolic matrix proteins as the duration of static compression increased. Immediate early genes c-fos and c-jun were dramatically upregulated 6 to 30-fold,
respectively, during the first 8hrs of 50% compression and remained upregulated after 24hrs.

**Introduction**

Articular cartilage is responsible for the smooth articulation of synovial joints during locomotion. Chondrocytes within cartilage constantly remodel the tissue’s extracellular matrix (ECM) throughout life. The major load-bearing constituents of the ECM are type II collagen and aggregates of the proteoglycan, aggregan, which provide the tissue’s tensile and compressive stiffness, respectively. Also present in the ECM are families of matrix proteinases, tissue inhibitors of matrix metalloproteinases (TIMPs)\(^1\), growth factors and cytokines that together regulate ECM remodeling and turnover in health and disease (1). It is known that mechanical exercise of the knee joint *in vivo* increases the density of aggregan in cartilage (2), whereas knee joint inactivity results in decreased aggregan deposition (3, 4). Traumatic injury to cartilage diminishes mechanical strength and leads to excessive catabolism of the ECM, increasing the risk of osteoarthritis later in life (5).

A number of model systems have been developed to simulate various aspects of the mechanical loading forces experienced by articular cartilage *in vivo*. Compressive and shear forces have been applied to cartilage explants and chondrocyte cultures *in vitro* to examine the transduction

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\(^1\) The abbreviations used are: TIMP, tissue inhibitor of matrix protease; MMP, matrix metalloproteinase; cAMP, cyclic adenosine monophosphorothioate; PLC, phospholipase C; ERK, extracellular signal-regulated kinase; AP-1, activating protein-1; PBS, phosphate buffer saline; FBS, fetal bovine serum; BAPTA-AM, bis-(aminophenoxy)ethane-tetraacetic acid acetoxymethyl; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; ADAMTS, a disintegrin and metalloprotease with thrombospondin motifs; HSP70, heat shock protein-70; IGF-1, insulin-like growth factor-1; NOS2, nitric oxide synthase-2; COX-2, cyclooxygenase-2; IL-1β, interleukin-1 beta; TNFα, tumor necrosis factor alpha; JNK, c-Jun N-terminal kinase.
of mechanical signals into biological responses. Application of 50% static compression to cartilage explants decreased synthesis of type II collagen and proteoglycans (PG) within the first 1-2 hours of loading, and synthesis remained suppressed throughout a 24 hour loading period (6-8). Dynamic compression (8, 9) and shear (10) increased type II collagen and PG synthesis at low amplitudes and frequencies (1-5% strain, 0.01-1Hz), however, compression also increased the activation of MMP-2 and MMP-9 (11). Injurious compression of cartilage explants results in increased PG loss to medium and damage to the collagen network (12, 13). Currently, many cartilage tissue engineering strategies employ some form of mechanical stimulation to enhance matrix production by chondrocytes during culture (14-18).

Recent studies of mechano-regulation of chondrocyte gene expression showed that application of 25-50% static compression to bovine cartilage explants caused a transient increase in expression of aggrecan (19, 20) and type II collagen (19) mRNA levels during the first 4 hours of loading (>1.5-fold), followed by a decrease in expression to levels below non-loaded controls by 24 hours. Intermittent hydrostatic pressure (IHP) applied to human chondrocytes in monolayer culture at 1 Hz frequency (4 hour/day for 4 days) increased aggrecan and type II collagen gene and protein expression (>1.4-fold) (21); IHP did not deform the chondrocytes. Millward-Sadler et al. applied hydrostatic pressure to chondrocyte monolayers at 0.33 Hz for 20 min in a manner that induced strain on the culture dish and plated cells (pressure-induced strain, PIS) (22). They observed an increase in aggrecan mRNA and a decrease in MMP-3 mRNA within 1 hour following stimulation, with a return to baseline levels by 24 hours. In a single
experiment applying hydrostatic pressure (IHP) to human chondrosarcoma cells, changes in the expression of 51 genes were measured by cDNA array technology without widespread change in RNA stability (23), indicating that many genes may be influenced by mechanical stimuli.

Studies have also focused on cellular mechanotransduction events that may initiate changes in gene expression. Application of intermittent PIS to chondrocytes induced $\alpha_5\beta_1$ integrin activation of interleukin-4, which caused cell hyperpolarization via intracellular calcium release (24). Inhibition of interleukin-4 suppressed the upregulation of aggrecan gene expression observed due to PIS (22). Intracellular calcium release, cAMP and the PLC pathway have been implicated for aggrecan gene upregulation in response to static compression in cartilage explants (25). Static compression also increased ERK1/2 phosphorylation within minutes of application, with sustained increases during 24 hours of compression (26). While such signaling pathways have been identified in the mechanical regulation of aggrecan gene expression, less is known about chondrocyte gene expression patterns of other ECM-related molecules or whether common upstream signaling pathways are responsible for their regulation.

Given these observations regarding the sensitivity of chondrocyte biosynthesis to mechanical forces in vivo and in the cartilage explant model, we hypothesized that mechanical loading would also induce widespread transcriptional changes, particularly for molecules involved in ECM maintenance. Our objective was to characterize the transcriptional response to static compression of chondrocytes within normal intact cartilage, focusing on a range of anabolic, catabolic, and signaling genes involved in tissue homeostasis. Temporal expression
profiles of 28 genes were measured to compare immediate and long-term changes in response to sustained compression. Molecular inhibitors of intracellular calcium, cAMP and AP-1, were used to identify possible upstream signaling pathways involved in the mechanotransduction of the genes studied here. Clustering analysis (27, 28) and principal component analysis (29, 30) were used to elucidate the main expression trends and to highlight genes that appeared to be co-regulated by mechanical compression. These computational techniques can help to classify groups of genes with common upstream signaling pathways, and may help to predict certain cell behavior (28, 30). We found that both anabolic and catabolic genes were induced by static compression, but with contrasting expression patterns. Intracellular calcium and cAMP were found to play a fundamental role in the mechanical regulation of gene transcription.

**Experimental Procedures**

*Cartilage Extraction & Mechanical Loading* -- Articular cartilage disks (3mm diameter, 1mm thick) were obtained from the middle zone of the patello-femoral groove of 1-4 week old calves as described previously (8). Disks were washed with PBS and maintained in low glucose Dulbecco’s modified essential medium supplemented with 10% FBS, 10mM Hepes Buffer, 0.1mM nonessential amino acids, 20µg/ml ascorbate, 100U/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml amphotericin B. Disks were allowed to equilibrate for 2-5 days before loading, with media changes every second day and 12hrs before loading. Anatomically matched disks (6 disks per timepoint per loading treatment) were transferred into polysulphone
loading chambers (8), slowly compressed over a ~3 minute period to 25% or 50% of cut thickness, and maintained at these static strain levels for 1, 2, 4, 8 or 24hrs (25% strain, n = 4; 50% strain, n = 11), with disks kept in free-swelling conditions as controls (see Fig. 1A,B).

Upon completion of loading disks were stored at -80°C in RNA-later solution (QIAGEN, CA).

Inhibitor Studies -- To investigate the role of intracellular signaling pathways during static compression, molecular inhibitors were added to media prior to application of 50% compression (Fig. 1C,D). Cartilage disks were pre-incubated for 1hr with 10µM BAPTA-AM, a chelator of intracellular calcium (A.G.Scientific, CA) or 50µM Rp-cAMP, an inhibitor of cyclic-AMP activated protein kinase A (PKA) (Sigma, MO), which have been previously shown to completely inhibit mechanically-induced aggrecan gene regulation (20, 25). For each timepoint six untreated and six treated disks were placed into a 12-well polysulphone chamber to allow identical compression (n = 4-5). In separate experiments, Paclitaxel, an inhibitor of transcription factor AP-1 binding to DNA, (10µM, Sigma, MO) was added 24hrs prior to 2hrs of 50% static compression (n = 5), at concentrations previously shown to inhibit AP-1 activity in bovine chondrocytes (31). To assess RNA stability, disks were either maintained in free-swelling conditions or loaded for 2hrs at 50% strain, and then released to free-swell for up to 6hrs before being stored at -80°C (n = 2). Actinomycin-D was added to media immediately upon completion of loading (Fig. 1E) at concentrations shown previously to inhibit transcription in bovine chondrocytes and cartilage explants (30µM, Sigma, MO) (23).

RNA Extraction, Primer Calibration & Real-time PCR -- For each timepoint and
loading treatment, six cartilage disks were pulverized using liquid-nitrogen cooled mortar and pestles, and homogenized in QIAshredder tubes (QIAGEN, CA) spun at 10,000rpm for 2 minutes. RNA was then extracted from the clear supernatant using the QIAGEN RNAeasy Mini kit protocol with the DNAse digest (QIAGEN, CA). RNA was stored in 40µl of RNase free water at -80°C until reverse transcription was performed using Applied Biosystems Reagents. Real-time PCR was performed on a 384-wells/plate ABI7900HT machine (2 min 50°C, 10 min 95°C, 50 cycles of 15 sec 94°C and 1 min 60°C) using SybrGreen MasterMix (Applied Biosystems, CA). The SybrGreen Master mix and H2O were combined, and aliquots dispersed into cDNA containing tubes. A multipipette was used to distribute 9µl aliquots into a 384-well plate, followed by 1µl of 10µM forward and reverse primer mix. Free-swelling controls, and inhibitor treated samples were always run on the same plate as untreated compressed samples. A total of 28 primer pairs for the 28 genes listed in Table 1 were designed with amplification product length 85-130bp and annealing temperature ~60°C. All primers were tested to produce proportional changes in threshold cycle with varying starting cDNA quantity. Measured threshold cycles (Cₜ) were converted to relative copy numbers using primer-specific standard curves.

Data Normalization & Statistical Analyses -- For each experiment and every loading condition, gene expression levels were normalized by the average levels of mean-centered housekeeping genes 18S and G3PDH. The time-course of each gene was then normalized by the
corresponding free-swelling expression level. For example, the BAPTA-AM treated time-
courses were normalized by BAPTA-AM treated free-swelling controls after normalizing by
housekeeping genes. Expression levels further than three standard deviations from the mean were
considered outliers and removed. Gene expression levels induced by compression were
compared to free-swelling expression levels using two-tailed Student’s t-tests. Comparison
between compression time-courses with and without the presence of inhibitors was performed
using comparison of means two-tailed t-tests, with Welch corrected degrees of freedom for
unequal variance. Games-Howell corrected standard deviations were used to account for
unequal sample sizes, as F-tests had revealed significant differences between untreated and
treated gene expression level variances (data not shown). Skew and Kurtosis criteria were used to
confirm that the data were normally distributed. A p-value of less than or equal to 0.05 was used
to assess statistical significance.

Clustering Analysis -- To further analyze the expression patterns and pathways activated
by compression, the expression levels for each gene from the 50% compression, BAPTA-AM
treated and Rp-cAMP treated time-courses were combined into fifteen point expression vectors
(23 genes had complete time-courses). These expression vectors were then standardized to have
equal variance, to emphasize expression patterns rather than amplitudes, and were then
iteratively clustered using two different k-means clustering techniques (see Appendix). First,
principal component analysis (PCA) (29, 30) was used to determine the principal components of
the data matrix comprised of the genes and timepoints. Each standardized expression vector was
then projected onto the three main principal components and the resulting three-dimensional coordinates were k-means clustered using a Euclidean distance metric. The second approach was to cluster the standardized expression vectors directly using correlation as a metric (27, 28). The main expression trends were then identified from comparison of the two techniques. Projection coordinates for each group centroid were calculated by averaging the projection coordinates of each gene within a group. Centroid vectors were formed by adding the three main principal components weighted by the centroid projection coordinates. Centroid variances were calculated, and the Euclidean distance between the projection coordinates of centroids was used to perform comparison of means Student’s t-tests to assess the distinctiveness of expression trends (see Appendix for details).

**Results**

*Effect of Static Compression on Gene Expression* -- Static compression was applied for 1-8hrs at 25% strain and 1-24hrs at 50% strain, and expression levels of 28 ECM maintenance genes were monitored. For ease of presentation and discussion, selected results for specific genes are reported in Figs. 2, 3, and 4 below; the complete results for all 28 genes in this study are given in the Appendix, Figs. A1,A2. Consistent with previous findings, aggrecan (19, 20) and type II collagen (19) were transiently upregulated in a strain-dependent manner during the first 8hrs of compression (up to 2.5-fold) before decreasing below free-swelling expression levels by 24hrs of 50% compression; link protein was similarly affected (Fig. 2). In contrast, type I collagen was
downregulated in response to 25% compression, and increasingly upregulated during 50% compression, greater than 3-fold after 24hrs (Fig. 2); however, comparison of C_T values showed that absolute mRNA abundance of type I collagen was two orders of magnitude lower than type II collagen (data not shown). MMP3 and ADAMTS4 were increasingly upregulated with 50% compression duration up to 16-fold and 4-fold respectively (Fig. 2), and TNFα, IL-1β, and COX-2 were similarly affected (Fig. A2). MMP9 and 13 were upregulated 2-fold and 8.6-fold by 24hrs of 50% compression, whereas MMP1 and ADAMTS5 were downregulated by 30% (Fig. A1). Transcription factor Sox9 was upregulated more by 25% compression (up to 1.7-fold) than 50% compression; in both cases the effect was transient lasting 4hrs or less (Fig. A2). c-fos and c-jun showed marked upregulation during the 25% compression time-course as well as peaked upregulation (6 & 35-fold respectively) during the 50% compression time-course (Fig. 2). The TIMPs were generally downregulated by 50% compression with upregulation only occurring at initial timepoints (Fig. A2). Fibromodulin, fibronectin, and ribosomal-6-phosphate were in general unaffected by static compression (Figs. A1,A2). HSP70 was slightly upregulated throughout the 50% compression time-course (Fig. A2). IGF1 and NOS2 were highly upregulated at certain timepoints but their expression levels were scarcely detectible even with real-time PCR (Fig. A2).

Effect of BAPTA-AM Treatment -- To determine if intracellular calcium release was a prevalent step in the mechanotransduction pathway, 10µM BAPTA-AM was added to the
medium 1 hour before application of the 50% compression time-course (Fig. 1C). The addition of BAPTA-AM did not appear to affect free-swell gene expression levels; however, genes which did show a greater than 70% or significant change are summarized in Figure 3. Aggrecan, link protein and TIMP1 free-swelling expression levels were significantly downregulated by 20%, 38% and 54% respectively. In general BAPTA-AM treatment suppressed the regulation induced by 50% compression (Figs. 4,A1,A2). In particular, aggrecan, link protein, and fibromodulin expression remained close to free-swelling expression levels throughout the BAPTA-AM treated time-course, while type II collagen was partially suppressed (Figs. 4,A1). The expression pattern of c-fos was unaffected by the presence of BAPTA-AM; however, c-jun levels, though upregulated compared to free-swelling controls, were reduced by approximately half compared to the untreated 50% compression time-course (Fig. 4). MMP1,9&13 were downregulated below free-swelling controls throughout the BAPTA-AM treated time-course, and the upregulation of MMP3 was largely suppressed, particularly at 24hrs (Figs. 4,A1). In contrast, ADAMTS-4 was mainly unaffected by the presence of BAPTA-AM during 50% static compression and HSP70 was actually increased (Figs. A1,A2). IL-1β was suppressed below free-swelling expression levels; however, COX-2 remained upregulated (Fig. A2).

Effect of Rp-cAMP Treatment -- To determine if cAMP activation of PKA was a prevalent step in the mechanotransduction pathway, Rp-cAMP was added 1 hour prior to application of the 50% compression time-course (Fig. 1C). Rp-cAMP had a more pronounced effect on free-swelling expression levels than BAPTA-AM, though still only affected a subset...
of genes (Fig. 3). Notably HSP70 was suppressed by 70%, IL-1β was upregulated 4.7-fold, and MMP1,9,13, ADAMTS4,5 were upregulated >2-fold. In general, Rp-cAMP suppressed gene induction by compression similar to BAPTA-AM, but also enhanced the regulation of a number of genes (Figs. 4,A1,A2). The initial timepoints of aggrecan and type II collagen were suppressed by Rp-cAMP; however, after 8hrs of loading, the expression levels of both genes were higher than corresponding untreated levels, and also after 24hrs for aggrecan (Fig. 4).

Although c-jun was still upregulated in the presence of Rp-cAMP, overall expression levels were much lower than in response to BAPTA-AM treatment or 50% compression alone, and increased with compression duration (Fig. 4). In contrast to the BAPTA-AM treated time-course, the upregulation of c-fos was mostly suppressed during the first 8hrs of Rp-cAMP treated compression (Fig. 4). Type I collagen and MMP expression were suppressed even below free-swelling expression levels for most timepoints, particularly MMP3 which was reduced to 0.30-fold after 24hrs compression (Fig. 4). In contrast, ADAMTS4 remained upregulated after 24hrs and COX-2, HSP70, and TIMP3 gene expression levels were higher with Rp-cAMP treatment during compression, peaking after 24hrs at 23-fold, 10.5-fold and 4-fold, respectively (Figs. A1,A2). Notably, Sox9 was downregulated to 0.06-fold after 24hrs (Fig. A2).

Main Expression Trends induced by Static Compression -- PCA revealed three main eigenvectors (principal components) that accounted for 60% of the variance in the data. The coordinates of each standardized gene expression vector when projected onto the three main principal components are shown in Fig. 5. Visual examination of the projection plot and varying
the number of groups while clustering revealed that the genes were best divided using 4 groups. K-means clustering of the projection coordinates using Euclidean distance produced four clusters with 4-7 genes each, shown in Fig. 5 and Table 2A. K-means clustering using a correlation metric and the standardized gene expression vectors produced almost identical results, with only aggrecan and ADAMTS4 swapping groups, indicating that the groupings were very robust. The optimal groupings produced by either method were significantly better than randomly assigning genes to groups (p << 0.001, see Appendix) and examination of the top five groupings showed only 1 to 2 gene placement variations from the optimal solutions produced by either clustering method. Comparing inter-centroid distances using comparison of means Student’s t-tests revealed that the four clusters were significantly separated and distinct (Table 2B).

The centroids of the four main expression patterns induced by compression with and without the presence of inhibitors are shown in Fig. 6. Group 1 paired aggrecan and type II collagen, which was expected from previous experiments, along with the AP-1 binding protein elements c-fos and c-jun. Both Centroids 1 and 2 transiently increased during the first 4 to 8hrs of static compression followed by a decrease towards free-swelling expression levels by 24hrs (Fig. 6). However, Centroid 2 was suppressed by the addition of either BAPTA-AM or Rp-cAMP, whereas Centroid 1 was only partially suppressed by BAPTA and only suppressed at initial timepoints by Rp-cAMP (Fig. 6). Group 3 contained mainly matrix metalloproteinases and type I collagen, and exhibited a 50% compression induced upregulation that peaked after
24hrs (Fig. 6). Group 4 had a similar pattern during 50% compression with a less pronounced peak at 24hrs. However, in contrast to Centroid 3 which was almost completely suppressed by either BAPTA-AM or Rp-cAMP, Centroid 4 mechano-induction was enhanced by BAPTA-AM and even more so by Rp-cAMP.

Effect of Paclitaxel Treatment -- To determine if the upregulation of c-fos and c-jun by compression, which may increase AP-1 signaling, was involved in regulating gene transcription during mechanical loading, paclitaxel was added 24 hrs before 50% compression (Fig. 1D). Genes in Group 1 remained upregulated after 2hrs of compression with paclitaxel present, and free-swelling controls remained unaffected (Fig. 7). Sox9 levels were reduced below controls, and c-fos upregulation was suppressed by 50% (Fig. 7), though c-jun expression was unaffected. MMP3 expression was reduced below free-swelling expression levels, MMP13 was upregulated by >2-fold, and COX-2 remained upregulated by 4-fold during paclitaxel treated compression (Fig. 7). MMP1 and MMP9 remained upregulated to the same extent with paclitaxel present; however, TIMP expression was further increased ~1.5-fold and type I collagen was further doubled by the presence of paclitaxel during compression (data not shown).

RNA Stability -- To determine if the changes in mRNA expression were due to changes in transcription or changes in RNA half-life, Actinomycin-D was added upon release of compression, and gene expression was monitored for up to 6hrs post-compression (Fig. 1E). Individual and group expression trends were examined for the genes listed in Table 2. However, transcript half-lives exceeded the 6hr timepoint and no clear differences between compressed
and free-swelling conditions could be determined (data not shown). Therefore, 2hrs of 50% static compression did not appear to modify RNA stability over the time-period examined.

**Discussion**

In this study, we demonstrated that most genes investigated responded to static compression, with expression profiles that were both strain and time dependent. The effects of static compression on transcription evolved with time, and after 24hrs the transcription of proteases and signaling molecules dominated over matrix molecules and early response genes. Cluster analysis revealed four group expression patterns induced by compression, with two groups requiring intracellular calcium and cAMP as common upstream mechano-regulators.

To further interpret the changes in gene expression in response to compression, we can compare our results to the kinetics of the intratissue mechanical forces and flows caused by compression. During joint loading *in vivo*, cartilage experiences a complex mixture of compressive and shear deformation having both static and dynamic components. For example, *in vivo* joint loading can result in high peak mechanical stresses (15-20 MPa) that occur over very short durations (< 1 sec) causing cartilage compressive strains of only 1-3% (32). In contrast, sustained (static) physiological stresses applied to knee joints for 5-30 minutes can result in compressive strains in certain knee cartilages as high as 40-45% (33). In the present study, application of a slow compression over ~3min to a final strain of 25% or 50% causes an initial transient intratissue pressurization and fluid flow within the matrix immediately following
compression and during a 15-30 minute period of stress relaxation (8, 34). After stress relaxation has ended, fluid flow ceases and intratissue pressure returns to zero (i.e., that of the medium) as the new equilibrium compressed state of the tissue is reached. Thus, the initial compression transient has certain physical attributes of slow dynamic compression, while the final compressed state mimics the static component of in vivo compression. Therefore, our objective was to explore the kinetics of changes in gene expression to both the initial transient loading and final static loading phases.

Static compression has been previously shown to decrease PG and type II collagen synthesis within 1-2hrs (8, 35). In our experiments, 25% compression transiently upregulated aggrecan gene expression and did not alter type II collagen during 8hrs of loading (Fig. 2). Consistent with previous studies that used Northern analyses (19), 50% compression caused transient upregulation of both aggrecan and type II collagen during the first 8hrs and a subsequent decrease in expression below free-swelling levels by 24hrs (Fig. 2). Thus, the temporal kinetics of transcriptional and biosynthetic responses to loading are considerably different, though they converge by 24hrs after application of static compression. Therefore, the initial transient upregulation of aggrecan and type II collagen genes may be more sensitive to the dynamic components of the applied compression. Recent experiments have shown that ERK1/2 and p38 phosphorylation levels peak within 10 minutes of static compression but only ERK1/2P levels remained upregulated after 24hrs (26). It was suggested that such an initial transient response was due to the dynamic components of static compression, consistent with the results of
Li et al. (36), which may similarly explain the transient transcriptional upregulation of matrix proteins observed here. In addition, loading may affect the apparatus for transcription and translation differently. Studies have shown that high pressure can cause changes in cell morphology and disorganization of the Golgi and microtubules in chondrocytes (37). Compression of cartilage explants also reduces cell volume and the volumes of several intracellular organelles; however, the volume of the Golgi remains unaffected by static compression of up to 50% strain (38). Thus, the synthesis of proteins which require significant post-translation modification may be affected by compression differently than transcription.

Dynamic compression, a known stimulator of matrix protein synthesis, was also found to induce MMP2&9 gene expression and activity (11). In our study, matrix protease gene expression followed a common trend of increasing upregulation with 50% compression duration (Figs. 2,A1). COX-2 and IL-1β were upregulated in a similar pattern to the matrix proteases and TNFα was highly upregulated after 24hrs (Fig. A2). IL-1β is a known modulator of COX-2 gene and protein expression (39) and IL-1β, TNFα and COX-2 are known to regulate the gene and protein expression of matrix proteases (40-45). Hence, the regulation profile of the MMPs may follow the regulation of IL-1β, TNFα and COX-2. IL-1β did not increase above control levels until after 8hrs of compression, and a similar delay of 3hrs was seen during 50% loading experiments by another group (46). Furthermore, blocking IL-1β signaling during compression suppressed the expected decrease in PG synthesis, but only at longer timepoints (6hrs) (46), confirming the involvement of IL-1β signaling during prolonged periods of static compression.
The early upregulation of transcription factors c-fos and c-jun (Fig. 2), which form the AP-1 binding protein, may be another signal for compression-induced matrix remodeling or catabolism. Increased AP-1 activity is a precursor to the IL-1β induction of matrix proteases, which can take up to several hours (41, 43, 47). ERK1/2, p38 and JNK phosphorylation is also upregulated as early as 10-60 minutes after compression (26), indicating activation of MAPK pathways possibly responsible for c-fos and c-jun upregulation. Therefore, the present study gives evidence for the temporal upregulation of transcription of matrix proteases in response to static compression.

ECM gene expression was unaffected by the presence of the AP-1 inhibitor, paclitaxel, during 2hrs of 50% compression (Fig. 7), demonstrating that AP-1 activation was not responsible for the early upregulation of aggrecan and type II collagen (Fig. 2). Another role for the pronounced upregulation of c-fos and c-jun is suggested by studies in which chondrocytes were transfected with c-fos, causing a decrease in PG synthesis (48) similar to static compression (6, 8). COX-2, which was upregulated in response to 50% compression (Fig. A2), and by PIS (49), is known to cause PG destruction via prostaglandin E-2 (PGE-2) (50). Interestingly, in chondrocyte cell lines, PGE-2 regulated cAMP and intracellular calcium pathways (51). Thus, the anti-anabolic effects of static compression may be mediated in part by mechanisms dependent on short-term c-fos/c-jun upregulation and long-term COX-2 upregulation.

When the 50% compression data were formed into gene expression vectors and clustered,
two main untreated 50% compression expression profiles were found. Groups 1 and 2 (Table 2) were characterized by a transient 4-8hr upregulation followed by a decline towards free-swell levels after 24hrs (Fig. 6) while Groups 3 and 4 showed increased upregulation with compression duration (Fig. 6). Each group behaved distinctly in response to BAPTA-AM or Rp-cAMP, further dividing the transcriptional responses induced by static compression into a total of four groups. Chelation of intracellular calcium using BAPTA-AM suppressed aggrecan gene upregulation in response to compression (Fig. 4) similar to previous findings (25). Calcium-dependent K+ channels have been implicated in the mechanotransduction pathway of isolated chondrocytes (24), suggesting intracellular calcium release is an initial event in mechanotransduction. We found that the presence of BAPTA-AM during mechanical loading suppressed the upregulation of many genes, including, aggrecan, type II collagen, link protein, c-jun, and many MMPs (Figs. 4,A1,A2). In particular Centroids 2 and 3 were completely suppressed when BAPTA-AM was present during loading, Centroid 1 was partially suppressed and Centroid 4 was mainly unaffected. The selective suppression by BAPTA-AM supports the idea that intracellular calcium is a common but not complete upstream signaling event controlling the mechano-regulation of anabolic, catabolic, and anti-catabolic genes. Interestingly, expression of stress protein HSP70 during compression was significantly greater when in the presence of BAPTA-AM (up to 3.8-fold) (Fig. A2). Hence, intracellular calcium release may also be required to elicit the stress-protective response seen in chondrocytes during
loading (52). IL-1β was downregulated below free-swelling controls and c-jun expression was significantly suppressed during BAPTA-AM treated compression, which may explain the downregulation and suppression of matrix proteases, even though COX-2 expression remained upregulated (Fig. A2).

The inhibition of cAMP activated PKA during 50% compression prevented aggrecan gene upregulation, although only during the first 4hrs of loading (Fig. 4), consistent with previous findings (20). Cluster analysis revealed that the effect of Rp-cAMP was distinct from that of intracellular calcium chelation. The presence of Rp-cAMP during loading suppressed Centroids 2 and 3 similar to BAPTA-AM; however, the upregulation of Centroid 4 was enhanced at later timepoints. In contrast to BAPTA-AM treatment, the early upregulation of both c-fos and c-jun was suppressed during Rp-cAMP treated compression (Fig. 4) which might prevent the AP-1 signaling necessary for MMP upregulation. The dominant change in Centroid 1 in response to Rp-cAMP treatment during loading was a shift from a transient initial upregulation to increasing upregulation during longer time periods. These results confirm cAMP activation as a prevalent upstream component of the intracellular mechanotransduction pathway. Both intracellular calcium and cAMP were necessary for Group 2 and 3 mechano-induction, suggesting that common downstream mechanisms may be involved. Suppression of c-fos by only Rp-cAMP, and the differing regulation of Groups 1 and 4 by the two inhibitors, suggests that cAMP is not simply downstream of intracellular calcium as was previously proposed (25). The failure of either BAPTA-AM or Rp-cAMP to suppress the mechano-induction of Group 4
genes suggests that additional upstream signaling mechanisms exist.

The widespread impact of mechanical loading on the transcription of genes involved in ECM maintenance has been demonstrated in this study. It is possible to speculate that gene mechano-regulation may play a role in maintaining a healthy cartilage ECM throughout life and that the inferior cartilage phenotype developed during OA may include improper gene mechano-regulation. Ongoing studies are examining gene mechano-regulation in response to the dynamic compression and shear components of in vivo mechanical loading. The exact order in which intracellular pathways are activated and the role of the transcribed signaling molecules cannot be directly inferred from the inhibitor studies and gene expression data presented. Further studies are required to determine what factors are responsible for the divergent anabolic and catabolic temporal expression profiles. Four main expression patterns were identified in response to static compression and could represent genes co-regulated by intracellular calcium and/or cAMP. The dramatic upregulation of c-fos and COX-2 by static compression suggests a possible role in mechanically-mediated cartilage remodeling and/or degradation, and it will be worthwhile to further examine these molecules in the presence of injurious mechanical compression of cartilage.

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Appendix – Statistical and Clustering Analysis Techniques

The relative expression levels induced by 25% and 50% compression in the presence and absence of BAPTA-AM and Rp-cAMP are given in Figs. A1 and A2 for all genes and timepoints.

*Gene Vector Standardization and PCA* -- The 23 genes containing complete 50% compression untreated, BAPTA-AM and Rp-cAMP treated time-courses represent an intermediate size dataset. It is possible to visually group certain genes together, but to do so in a mathematically rigorous manner requires the use of clustering techniques. Principal component analysis (PCA) is a tool commonly used to reduce large data matrices, in our case 23 genes x 15 timepoints, into a smaller number of essential characteristics. PCA requires solving the eigenvalue problem for the covariance matrix of the dataset and produces E eigenvectors (principal components) and corresponding eigenvalues, where E is the length of the smallest side of the data matrix, in our case 15. The reason PCA provides dimensional reduction is that many of the eigenvalues, which represent the variance accounted for by the corresponding eigenvector, are close to zero, and may be considered unimportant or simply noise and removed before clustering the data (53). A drawback of this technique when applied to gene expression data is that it is difficult to define noise in expression patterns since some of the lesser eigenvectors may represent important traits (54). However, the ability of PCA to produce meaningful insights has been shown when the data are properly pre-processed (29, 30).

Our initial attempts applying PCA to the data matrix revealed two main principal
components as shown in the Scree plot in Fig. A3. Projection of the gene expression vectors onto
the two principal components highlighted that PC1 was primarily composed of c-jun and PC2 of
COX-2 (Fig. A3). This was confirmed by performing Euclidean distance based clustering which
formed groups of COX-2 alone, c-jun alone, and then one large group containing the remainder
of the genes. Hence, our initial PCA-based clustering method was not able to distinguish the
distinct but lower amplitude trends of other genes such as aggrecan, MMP9 and ADAMTS4
(Fig. 2).

The main purpose of clustering was to establish the dominant expression patterns
activated by mechanical loading. Therefore, to improve our PCA-based clustering method the
data matrix was pre-processed by the following standardization equations to re-weight each
expression vector to have equal variance and thus remove the bias of the highly changing genes,
such as COX-2 and c-jun:

\[
Z = \frac{X - F}{S_X}, \tag{A1}
\]

\[
S_X^2 = \frac{||X - F||^2}{T}. \tag{A2}
\]

where \(X\) is a gene expression vector, \(F =\) free-swelling expression vector of 1’s, \(S_X\) is the
modified standard deviation of gene \(X\) with respect to the free-swelling expression level, \(T\) is the
number of timepoints in the expression vector, and \(Z\) is the resulting standardized expression
vector. Standardization with reference to the free-swelling expression level was chosen instead
of the mean of the gene expression vector to ensure that the direction of regulation was preserved
when averaging a group of genes. Subsequently, the standardized free-swelling expression level is equal to zero. When PCA was applied to the standardized dataset, the variance was spread over a larger number of principal components (Fig. A3). Methods for choosing a subset of principal components include keeping principal components with greater than 1/T variance, or sum to greater than 50% variance, or by observation of steps in the Scree plot (53). The three methods suggest choosing either three or five principal components, and the main three components, which accounted for 60% of the variance, were finally chosen to allow visualization to aid in the selection of the number of clusters.

The profile of principal component 1 (PC1) is increasingly upregulated with 50% compression duration with or without Rp-cAMP treatment, and is constantly upregulated during BAPTA-AM treated compression (Fig. A4). The profile of PC2 is similar to PC1 during 50% compression but remains close to free-swelling expression levels during BAPTA-AM and Rp-cAMP treated time-courses. PC3 contains a transient upregulation during 50% compression and to a lesser extent during BAPTA-AM treatment, and remains close to free-swelling expression levels during Rp-cAMP treated compression.

*Clustering using PCA* -- The coordinates of each standardized gene expression vector projected onto each main principal component were used to create the projection plot in Fig. 5. From examination of Fig. 5, four clusters were chosen as the minimum number capable to represent the data, though 3-6 clusters were tested for comparison. The following Euclidean distance metrics were used to cluster the data with 6000 repeated trials with random initial
seeding locations.

\[
d(P_a, P_b)^2 = \|P_a - P_b\| \tag{A3}
\]

\[
D^2 = \sum_{i=1}^{k} \sum_{j}^{} d(P_{ij}, P_{ic})^2 \tag{A4}
\]

where \(d(P_a, P_b)\) is the Euclidean distance between the three dimensional projection coordinates of gene \(a\) (\(P_a\)) and gene \(b\) (\(P_b\)), \(D\) is the sum over all genes of the distance between the projection coordinates of the \(j^{th}\) gene in group \(i\), \(P_{ij}\), from the projection coordinates of centroid \(i\), \(P_{ic}\), \(k\) is the number of groups, and \(N_k\) is the number of genes in each group.

The optimal clustering is chosen as the grouping with the minimum total distance after many repetitions and is shown in Fig. 5 and Table 2. The number of repetitions was chosen so that all initial combinations of starting genes are performed. It is the cluster centroids, not the principal components, that represent the main expression patterns of the dataset. Each centroid is the sum of the three principal components weighted by the centroid projection coordinates (see Table 2). Centroid 1 (Fig. 6) includes a transient increase during the 50% compression time-course, thus, contains PC3, but also was highly upregulated by Rp-cAMP treated compression and so contains PC1. In contrast, Centroid 2 which also includes a transient upregulation during 50% compression, was mainly composed of PC3 minus PC1, as the BAPTA-AM and Rp-cAMP treated time-courses are suppressed below control levels. Centroid 3 was mainly composed of PC2 reflecting the increasing with duration profile during 50% compression, and
suppressed expression with BAPTA-AM and Rp-cAMP. However, Centroid 4, which has a
similar 50% compression profile as Centroid 3, is mainly composed of PC1 due to the
upregulation seen during the BAPTA-AM and Rp-cAMP treated time-courses.

**Alternative Clustering Technique** -- To confirm the expression trends found by PCA-
based clustering, an alternative distance metric, correlation, was used to k-means cluster the
full-length standardized gene expression vectors. The correlation between genes was conserved
after standardization, using the following definitions:

\[
q_k = \sum_{j=1}^{N_k} \frac{Z_j}{N_k}
\]

\[
Q_k = q_k \frac{S_{q_k}}{S_{q_k}} + F \Rightarrow q_k = \frac{S_{q_k}}{S_{q_k}} (Q_k - F)
\]

\[
S(X_j, Q_k) = \frac{1}{T} \sum_{i=1}^{T} \frac{(X_{ij} - F_i)(Q_{ik} - F_i)}{S_{X_i} S_{Q_k}} = \frac{Z_j^T q_k}{T} S_{q_k}^{-1}
\]

\[
S(Z_j, q_k) = \frac{1}{T} \sum_{i=1}^{T} \frac{(Z_{ij} - 0)(q_{ik} - 0)}{S_{Z_j} S_{q_k}} = \frac{Z_j^T q_k}{T} S_{q_k}^{-1}
\]

where \(q_k\) is the standardized centroid of the \(k^{th}\) group, which is the average of the standardized
genes in a group. \(Q_k\) is the unstandardized centroid found from \(q_k\). \(S(a,b)\) represents the
correlation between two vectors, \(S_Z\) is equal to 1 by the standardization process and \(S_{Q_k}\) and \(S_q\)
are defined by Equation A2 with \(Z_j\) replacing \(X_j\) and zero replacing \(F\) for \(S_q\). The correlation
\(S(a,b)\) is not the standard form of Pearson’s correlation, as the reference point is taken from the free-
swelling expression level and not the mean for both the standardized and unstandardized vectors.
The optimal clustering was chosen by comparing the overall gene-centroid correlation, \( R \), over many repetitions:

\[
R = \frac{\sum_{i=1}^{k} \sum_{j=1}^{N_i} Z_{ij}^T q_i}{N} \tag{A9}
\]

where \( Z_{ij} \) is the expression vector for the \( j^{th} \) gene in group \( i \) and \( N \) is the total number of genes, in this case 23. The optimal grouping produced using the correlation metric and the full-length standardized expression vectors was different from the Euclidean distance based clustering of the gene projections by only the reversed placement of aggrecan and ADAMTS4, demonstrating that the four clusters were robust features of the dataset.

**Main Expression Trend Robustness and Distinctiveness** -- To further test the robustness of the optimal groupings, genes were randomly assigned into four groups, and the overall correlation was calculated. 100,000 repetitions were performed in an attempt to gain a cross-section of all possible combinations (>1 billion). The mean and standard deviation of the overall correlation was 0.49 \( \pm \) 0.03 (Fig. A5). The distribution of the overall correlation was not centered on zero indicating that the dataset contained positively correlated genes further suggesting the genes may be co-regulated. The optimal groupings found using PCA-based clustering and correlation-based clustering of gene expression vectors, had overall correlations of 0.7440 and 0.7466 respectively. Therefore, compared to random groupings, both solutions appear to be very optimal (p << 0.001). Examination of the top five correlation-based clustering groupings showed only 1 to 2 gene placement differences from either PCA-based or correlation-based solutions,
further demonstrating the robust nature of the main expression trends.

The variance of each group centroid was calculated using the distance between the projection coordinates of the centroid and each gene within a group.

\[
U_k = \sum_{j=1}^{N_k} \frac{d(P_{iC}, P_{jC})^2}{N_k} \quad (A10)
\]

where \( U_k \) is the variance of the \( k^{th} \) centroid, which is different from \( S_{qk} \) which is standard deviation along a centroid vector. The Euclidean distance between centroid projection coordinates \( d(P_{iC}, P_{jC}) \), and centroid variances \( U_i, U_j \) were used to perform comparison of means Student’s t-tests. All centroids were found to be significantly spatially separated (Table 2) indicating the expression patterns were actually distinct trends.
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Table 1: Genes examined using real-time PCR, categorized by function.
Primers were designed using Primer Express Software (Applied Biosystems) and Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), to have optimum annealing temperatures of 60°C and PCR product length between 85 & 130bp. Serial dilution standard curves comparing threshold cycle, Ct, and relative starting cDNA quantity, were created for all primers and had correlations R a 0.98, and slopes close to unity.

Table 2: Optimal groupings found by gene clustering.
The 50% compression time-course, and BAPTA-AM treated and Rp-cAMP treated time-courses were combined into gene expression vectors. A) Groupings found by k-means clustering the standardized gene expression vectors projected onto the three main principal components. Centroid coordinates represent the location of the average of all genes within a group projected onto the three main principal components. B) p-values for comparison of centroid locations. The Euclidean distance between the projection coordinates of two centroids was used for comparison of means Student’s t-test, with the number of genes within each group representing the degrees of freedom. Intra-centroid variance was calculated using Equation A6.

Fig. 1: Protocols for mechanical compression of cartilage explants with and without inhibitor treatment.
A) Free-swelling cartilage disks incubated for 4 or 24hrs with or without inhibitor treatment were used as controls for compression time-series. B) Continuous 25% or 50% compression was applied for 1-24hrs. C) No treatment, 10mM BAPTA-AM, or 50mM Rp-cAMP was added to media 1hr before commencement of a 50% compression time-series, with controls shown in (A). D) 10mM Paclitaxel was added 24hrs before application of 2hrs of 50% compression or a 2hr extended period of free-swelling. E) Cartilage discs were subject to 50% compression or kept free-swelling for 2hrs. Compression was released, 30mM Actinomycin-D was added to media, and disks were kept free-swelling for up to 4hrs. Six cartilage disks were used for every timepoint of each condition for all repeated experiments.

Fig. 2: Selected gene expression levels induced by static compression, measured by real-time PCR.
25% = 1-8hrs of 25% static compression (n = 4). 50% = 1-24hrs of 50% static compression (1-8hrs, n =11 & 24hrs, n = 4). Expression levels were normalized by 18S and G3PDH housekeeping genes and divided by free-swelling expression levels. Mean + SE. *= p<0.05 compared to free-swell control using Students two tailed t-test. 1hr, 2hr, 4hr, 8hr, 24hr, relative free-swelling expression level = 1.
Fig. 3: Expression levels of genes affected by the addition of BAPTA-AM or Rp-cAMP to media under free-swelling conditions.
Six cartilage discs were incubated in media with or without the addition of 10µM BAPTA-AM or 50µM Rp-cAMP for 4 hours or 24hrs. Most of the 28 genes examined were unaffected by the addition of either inhibitor under free-swell conditions, and only genes for which the inhibitor treated expression level changed by > 70% or were statistically significantly affected are shown.

Mean + SE (n = 3-6). *p < 0.05 Student’s t-test comparing inhibitor-treated expression to the untreated relative expression level = 1. Agg = aggrecan, AD4 = ADAMTS4, AD5 = ADAMTS5.

Fig. 4: Effect of BAPTA-AM and Rp-cAMP pretreatment on gene expression levels induced by 50% static compression for a selection of genes.
50% = untreated 50% compression time-course (1-8hrs, n = 11 & 24hrs, n = 4), BAP = BAPTA treated 50% compression time-course (n = 4), Rp = Rp-cAMP (n = 3) treated 50% compression time-course. Expression levels were normalized using 18S and G3PDH housekeeping genes, and divided by the appropriately treated free-swelling expression levels. Mean + SE. * = p<0.05 compared to untreated 50% compression time-course, using Welch and Games-Howell corrected, comparison of means, two-tailed t-tests. 1hr, 8hr, 24hr, relative free-swelling expression level = 1.

Fig. 5: Projection plot of the genes represented by the three main principal components.
The standardized gene expression vectors were projected onto the three main principal components found using PCA, with groupings found using k-means clustering. ● indicates PC3 projection coordinate of gene is > 0, ○ indicates PC3 coordinate < 0. ‘x’ marks the projection coordinates of the four cluster centroids. Abbreviations: a = aggrecan, c1 = type I collagen, c2 = type II collagen, L = link protein, Fm = Fibromodulin, Fbn = Fibronectin, ad4 = ADAMTS4, ad5 = ADAMTS5, m1 = MMP1, m3 = MMP3, m9 = MMP9, m13 = MMP13, t1 = TIMP1, t2 = TIMP2, t3 = TIMP3, s9 = Sox9, cf = c-fos, cj = c-jun, r6p = ribosomal-6-phosphate, mk = MAPk1, Tb = TGFβ, h7 = HSP70, X2 = COX2.

Fig. 6: Four main expression trends induced by 1-24hrs of 50% static compression with and without the presence of BAPTA-AM or Rp-cAMP.
Centroid-vectors were calculated from the average projection coordinates of genes within each group, re-constructed using the three main principal components. Optimal groups were found using k-means clustering of gene-projection coordinates using Euclidean distance. Standardized free-swelling expression level = 0. Mean + SE. Standardized amplitudes represent relative changes from control level within an expression vector. For example, the 1hr timepoint in Centroid 2’s 50% compression time-course is upregulated twice as much as the corresponding 4hr timepoint.
Fig. 7: Effect of paclitaxel on gene expression changes induced by 2hrs of 50% compression. Paclitaxel was added 24hrs before loading. Gene expression levels were normalized by housekeeping genes, repeated experiments were averaged, and then scaled by free-swelling expression levels. free-swell, \( \text{free-swell + 10\mu M paclitaxel} \), 2hrs 50% compression, 2hrs 50% compression + 10\mu M paclitaxel. Mean + SE. * = p< 0.05 using Games-Howell corrected comparison of means, t-test (n = 5). Abbreviations: Agg = aggrecan, Col2 = type II collagen and Link = link protein.

Fig. A1: Relative gene expression levels measured by real-time PCR in compressed cartilage explants.
A&B) Matrix Proteins and C&D) Matrix Proteinases. Static compression was applied for 1-24hrs in one of four loading conditions: 25% = 25% compression of cut thickness (n = 4), 50% = 50% compression of cut thickness (1-8hrs, n = 11 & 24hr, n = 4), BAP = pretreated with 10\mu M BAPTA-AM before 50% compression (n = 5), and Rp = pretreated with 50\mu M Rp-cAMP before 50% compression (n = 3). Expression levels were normalized using 18S and G3PDH housekeeping genes and divided by free-swelling expression levels. * = p < 0.05 compared to free-swelling controls using Students two tailed t-test. ‡ = p < 0.05 compared untreated 50% compressed expression levels, using Welch and Games-Howell corrected, comparison of means, two-tailed t-tests. 1hr, 2hr, 4hr, 8hr, 24hr, relative free-swelling expression level = 1.

Fig. A2: Relative gene expression levels measured by real-time PCR in compressed cartilage explants.
A) Tissue inhibitor of matrix proteinases, B) Transcription factors (TF), and C&D) Cytokines, growth factors and reference genes. Static compression was applied for 1-24hrs in one of four loading conditions: 25% = 25% compression of cut thickness (n = 4), 50% = 50% compression of cut thickness (1-8hrs: TIMPs & TFs, n = 4-11, others n = 4-7, 24hrs: all, n = 4), BAP = pretreated with 10\mu M BAPTA-AM before 50% compression (n = 4-5), and Rp = pretreated with 50\mu M Rp-cAMP before 50% compression (n = 3). Expression levels were normalized using 18S and G3PDH housekeeping genes and divided by free-swelling expression levels. * = p < 0.05 compared to free-swelling controls using Students two tailed t-test. ‡ = p < 0.05 compared untreated 50% compressed expression levels, using Welch and Games-Howell corrected, comparison of means, two-tailed t-tests. 1hr, 2hr, 4hr, 8hr, 24hr, relative free-swelling expression level = 1.

Fig. A3: Principal Component Analysis of expression levels induced by static compression before (A) and after (C) standardization of gene expression vector variance.
The 1-24hrs 50% compression untreated, BAPTA-AM and Rp-cAMP treated time-courses were combined to form a data matrix for PCA. A) Scree plot showing the variance accounted for
by each principal component of the unstandardized expression vectors, in descending order. B) Projection plot of unstandardized expression vectors onto first two principal components. C) Scree plot showing variance accounted for by each principal component of the standardized expression vectors, in descending order.

**Fig. A4: The three main principal components found by applying PCA to the standardized dataset.**
The 50% untreated, BAPTA-AM and Rp-cAMP treated time-courses were combined into 15-point expression vectors and standardized by gene-vector variance before application of PCA. The variance in expression patterns accounted for by PC1, PC2 & PC3 was 26.9%, 17.3%, & 15.8%, respectively. Standardized free-swelling expression level = 0. Standardized amplitudes represent relative changes from control level within an expression vector. For example, the 24hr timepoint in PC1’s 50% compression time-course is upregulated twice as much as the corresponding 8hr timepoint.

**Fig. A5: Distribution of the overall correlation metric from 100,000 random grouping trials.**
The twenty-three genes used for the clustering algorithm were each randomly assigned a group number from 1 to 4. Overall correlation for each trial was calculated using Equation A5. Mean ± SE = 0.49±0.03.
| Matrix Proteins      | Matrix Proteases | Protease Inhibitors | Transcription Factors | Cytokines/Growth Factors | Intracellular signaling | Reference Genes |
|---------------------|------------------|---------------------|-----------------------|--------------------------|-------------------------|------------------|
| Aggrecan            | MMP1             | TIMP1               | Sox9                  | IL-1β                    | COX-2                   | HSP70            |
| Type I Collagen     | MMP3             | TIMP2               | c-fos                 | TNFα                     | MAPK1                   | Ribosomal-6P     |
| Type II Collagen    | MMP9             | TIMP3               | c-jun                 | TGFβ                     | NOS2                    |                  |
| Type X Collagen     | MMP13            |                     |                       |                          |                         |                  |
| Fibromodulin        | ADAMTS4          |                     |                       |                          |                         | 18S              |
| Fibronectin         | ADAMTS5          |                     |                       |                          |                         | G3PDH            |
| Link Protein        |                  |                     |                       |                          |                         |                  |

Table 1
### A

| Group # | Grouped Genes                                                                 | Centroid Coordinates (PC1, PC2, PC3) |
|---------|-------------------------------------------------------------------------------|-------------------------------------|
| 1       | Aggrecan, Type II Collagen, c-fos, c-jun                                     | (1.45, 1.21, 2.45)                 |
| 2       | Link Protein, MMP1, TIMP2, Sox9, Fibromodulin, MAPk1                          | (-2.11, 0.77, 1.35)                |
| 3       | Type I Collagen, MMP3, MMP9, MMP13, TIMP1, Ribosomal 6-P                      | (-0.12, 2.71, -1.19)               |
| 4       | ADAMTS4, ADAMTS5, TIMP3, Fibronectin, HSP70, TGFβ, COX-2                     | (2.42, 0.18, 0.1)                  |

### B

| p-values for Centroid separation | Centroid 1 | Centroid 2 | Centroid 3 |
|----------------------------------|------------|------------|------------|
| Centroid 2                       | 1.3x10⁻³   |            |            |
| Centroid 3                       | 2.8x10⁻⁴   | 4.5x10⁻⁴   |            |
| Centroid 4                       | 4.8x10⁻³   | 4.7x10⁻⁵   | 1.7x10⁻⁴   |

Table 2
Fig. 1
Fig. 2
**Fig. 3**
Fig. 4
Fig. 6
Fig. 7
Fig. A3
Fig. A4
Fig. A5
Mechanical compression of cartilage explants induces multiple time-dependent gene expression patterns and involves intracellular calcium and cyclic AMP
Jonathan B. Fitzgerald, Moonsoo Jin, Delphine Dean, David J. Wood, Ming H. Zheng and Alan J. Grodzinsky

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