Progression of Chondrocyte Signaling Responses to Mechanical Stimulation in 3-D Gel Culture

by

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B.S. Bioengineering
University of California, Berkeley, 2002

Submitted to the Department of Biological Engineering
in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Bioengineering

at the

Massachusetts Institute of Technology

February, 2008

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Abstract

Mechanical stimulation of 3-D chondrocyte cultures increases extracellular matrix (ECM) production and mechanical stiffness in regenerating cartilage. The goal of this study was to examine the progression of chondrocyte signaling responses to mechanical stimulation in 3-D culture during tissue regeneration.

To investigate the role of integrins in chondrocyte mechanotransduction, function-blocking antibodies and small-molecule antagonists were used to disrupt integrin-matrix interactions during dynamic compression of chondrocytes in 3-D agarose culture. At early days in culture, blocking $\alpha v\beta 3$ integrin abolished dynamic compression stimulation of proteoglycan synthesis, independent of effects in free-swell culture, while blocking $\alpha 5\beta 1$ integrins abolished the effect of compression only when blocking in free-swell increased proteoglycan synthesis. This suggests that disrupting $\alpha v\beta 3$ and $\alpha 5\beta 1$ interactions with the ECM influences proteoglycan synthesis in distinct pathways and that $\alpha v\beta 3$ more directly influences the mechanical response.

To further distinguish individual mechanotransduction pathways, we investigated the temporal gene transcription response of chondrocytes to ramp-and-hold compression on Days 1, 10, and 28 in 3-D agarose culture. Clustered and individual gene expression profiles changed temporally and in magnitude over time in culture. Day 1 cultures differed from Days 10 and 28, reflecting changes in cell microenvironment with development of pericellular and extracellular matrices. Comparisons with the response of intact tissue to compression suggested similar regulatory mechanisms. We further investigated MAPkinase (ERK1/2, p38, JNK) and Akt activation on Days 1 and 28 in agarose culture through phosphorylation state-specific Western blotting. Compression induced transient ERK1/2 phosphorylation on both days, with Day 28 levels similar to intact tissue. Unique from tissue behavior, only slight transient p38 phosphorylation was observed on Day 28, and SEK phosphorylation was undetected. Akt was uniquely regulated in intact cartilage compared to MAPks, with decreased total Akt levels over time under static compression. In contrast, compression transiently decreased pAkt levels in agarose cultures, with no changes in total Akt.

Changes in the chondrocyte responses to compression with time in agarose culture suggest that cells sense different forces and respond differently with time; further studies may help optimize mechanical loading for tissue-engineering purposes. These studies provide a basis for further examination of mechanotransduction in cartilage.

Thesis Supervisor: Alan J. Grodzinsky, Prof. of Biological, Electrical, Mechanical Engineering

Acknowledgements

The pursuit of my PhD has been one of my most challenging and most rewarding experiences. For that I have to thank the Department of Biological Engineering, which initially attracted me with its collegial atmosphere and collaborate, high quality research environment. I can now confidently say that my initial impressions of the program were confirmed. The students in my entering class (2002) have become some of my closest friends as well as colleagues, for which I am extremely grateful.

I would like to thank my thesis advisor, Al, for his mentorship. He provided a comfortable environment in which I could explore my research interests. Under his guidance, I have grown a great deal as a scientist and engineer. Special thanks also goes out to my committee members, Rich Lee and Doug Lauffenburger, who provided many different perspectives on my work, challenged me to think, but also supported the decisions I made regarding my research direction.

Collaboration is essential in scientific research, and I am lucky to have worked with the Grodzinsky lab members, past and present. I would name them all, but I know that I would inadvertently forget someone. Instead, I would like to especially thank Anna Stevens for many helpful discussions, Rachel Miller for being a great listener, Han-Hwa Hung and Eliot Frank for being great technical advisors as well as friends, and Linda Bragman for all her help. Finally I would like to thank Jon S, John K, and Mike for getting me started in lab, and Cameron and Jon F for setting me up to do Real-time PCR. During the course of my research, I have also had the opportunity to work with some outstanding researchers outside of MIT. Drs. David Griggs and Elizabeth Arner from Pfizer, Inc. contributed greatly to my work involving integrins.

I also wish to thank my parents, who have always fostered my interests in math and science and have always been a constant source of comfort, and my sister, who is one of my best friends. Last, but not least, my boyfriend, Nick, whom I met in this program, has been tremendously loving and supportive throughout. He has also been a willing listener, ready to offer an objective outside opinion when needed.

My thesis research would not have been possible without the financial support of NDSEG and NSF Graduate Student Fellowships, as well as NIH Grant AR33236.

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Introduction

1.1 Cartilage Biology and Osteoarthritis

Articular cartilage provides a lubricated, wear-resistant, weight-bearing surface which distributes and transmits stresses in joints. *In vivo*, cartilage experiences a variety of mechanical stresses, including dynamic and static compressive, tensile, and shear forces. Peak dynamic stresses of 15-20 MPa result in low strains of 1-3%, while sustained forces of approximately 3.5 MPa result in peak strains up to 40-45% [1, 2]. The composition and structure of the hydrated extracellular matrix (ECM) of cartilage determines its ability to withstand these forces, and these forces help regulate the composition and maintenance of tissue structure.

The main ECM components are a network of cross-linked collagen II fibrils and aggregates of sulfated proteoglycans (aggrecan). The densely packed, negatively charged aggrecan gives the tissue compressive stiffness and promotes osmotic swelling, while the collagen fibrils provide tensile resistance, resists osmotic swelling and maintains tissue structure. The equilibrium compressive modulus of adult articular cartilage, as measured in uniaxial confined and unconfined compression, is on the order of 1 MPa; the dynamic compressive stiffness is approximately 10 times higher; the equilibrium shear modulus ranges from 0.2 to 0.5 MPa; and the equilibrium tensile modulus ranges from 10 to 50 MPa [3]. Other proteins and small glycoproteins make up the rest of the tissue.

Articular cartilage is an avascular, aneural, and alymphatic tissue with little capacity for regeneration. Chondrocytes, the main cell type in cartilage, are responsible for remodeling the ECM and maintaining tissue homeostasis, in part through regulating matrix production and secretion and activation of catabolic enzymes such as aggrecanases ADAMTS-4 and 5 and matrix metalloproteinases. Osteoarthritis (OA) is a degenerative joint disease predominantly in weight-bearing joints such as the knees and hips [4] . Risk factors include age, joint trauma, repetitive joint use, obesity, and gender [4]. Osteoarthritic cartilage is characterized by reduced mechanical properties and increased matrix degradation, including loss of aggrecan, damage to the collagen network, increased tissue swelling, and, ultimately, loss of normal chondrocyte phenotype [5].

Increased force transmission to cells due to loss of matrix integrity and alterations in mechanical responsiveness may play a role in the development and progression OA [6]. Expression of pericellular matrix proteins and the cell surface receptors with which they interact are altered with OA progression [7-11], which can result in altered responses to applied forces. In addition, isolated OA chondrocytes respond to direct mechanical stimulation differently than normal chondrocytes. While cyclic stretching of chondrocytes isolated from normal tissue caused membrane hyperpolarization and increased aggrecan and decreased MMP-3 transcription, OA chondrocyte membranes depolarized and mRNA levels remained unaltered [12].

1.2 Mechanical Fields and Forces in Cartilage Compression

Studies suggest that mechanical forces play a major role in regulating chondrocyte behavior (see recent reviews [13, 14]). However, the mechanotransduction pathways by which chondrocytes respond to mechanical forces are not well understood and may depend greatly on the specific mechanical stimuli (*e.g.*, fluid flow, deformation) transmitted to the cells. A detailed breakdown

of normal forces experiences by cartilage is reviewed in Grodzinsky *et al*., 2000 [15]. Cartilage is a non-homogeneous, anisotropic, poroelastic material. The alignment of collagen fibers in the matrix changes with depth, resulting in depth-dependent mechanical properties.

In vitro models of cartilage compression have been used to study and isolate specific mechanical stimuli. In general, cartilage compression can be broken into a time-varying dynamic component and a slowly evolving, time-averaged static component. Static compression of tissue explants results in high strains, characterized by fluid loss and compaction of the solid matrix. This compaction increases the negative fixed charge density, which attracts and concentrates positively charged ions (including H^+, Na^+, and K^+), causing a decrease in intratissue pH and increased osmotic pressure of the intratissue fluid, which can have effects on tissue maintenance [16-18]. Local tissue strain and cell deformation under static compression have been shown to be inhomogeneous and depth-dependent, with higher local strains in the superficial zone of cartilage and lower strains in the middle and deep zones [19, 20]. However, Choi *et al*. found that in the superficial zone, cell strain was consistently less than tissue strain, while in middle or deep zones, cell strains were amplified when tissue strain was under 25% but protected when tissue strain was over 25% [20]. This suggests that the pericellular matrix serves as a mechanical filter, causing cell deformation in response to static compression to be more homogeneous throughout the tissue depth than local tissue strain [20]. During the transient phase of low-strain compression, cell strain has also been observed to be amplified above tissue strains, suggesting that the pericellular matrix is transducing the mechanical deformation by propagating the strains to the cells [21]. Similar to the transient phase of ramp-and-hold (or static) compression, dynamic compression of cartilage induces hydrostatic pressure gradients, macroscopic fluid flow, streaming potentials and currents within the tissue [22-24], while shear deformation of

cartilage explants results in deformation of cells and matrix [25], but relatively little fluid flow [26].

Studies have also used hydrostatic pressure, fluid flow, and mechanical stretch in isolation on monolayers of chondrocytes [27-29]. In 3-D gel culture, the mechanical stimuli experienced by the encapsulated cells change with time in culture. Over 28 days in a 3-D agarose culture system similar to that used in this thesis research, the equilibrium stiffness of the tissue increased with matrix development, reaching approximately 1/5 of "parent" tissue stiffness by Day 28 [30]. The accumulation of fixed charges associated with glycosaminoglycans also resulted in streaming potentials under compression of approximately 1/5 of parent cartilage [30]. Finally, the permeability decreased to about twice that of cartilage [30]. The accumulation of glycosaminoglycans (GAGs) in agarose gel chondrocyte cultures correlated with decreased diffusivity of macromolecules, as measured by FRAP, through the construct, but were still 2-3 times greater than diffusivity in intact tissue after 28 days in culture [31, 32]. In static compression, cell strain reflected gel strain on Day 1 of culture, when little matrix has accumulated, but by Day 6 in culture, the developed pericellular matrix protected the cell from deformation [33]. Cell deformation was only partially restored by hyaluronidase treatment, suggesting that matrix components other than sulfated-GAGs were present [33].

1.3 Biosynthetic Effects of Mechanical Loading of Cartilage

1.3.1 Responses of cartilage tissue to mechanical loading The effects of mechanical stresses and strains on chondrocyte biosynthesis and homeostasis have been studied *in vivo* and in *in vitro* explant models. Static immobilization or reduced joint-loading leads to a loss of glycosaminoglycan (GAG) content and decreased proteoglycan synthesis *in vivo* [34-36], with only partial restoration of biomechanical properties after remobilization [35]. Large-

displacement static compression results in a decrease in biosynthesis in tissue explants [18, 37], and can lead to cell death [38]. *In vivo* and *in vitro* injurious loading, generally characterized by high strain rate and high stress, results in cell apoptosis [39, 40], loss of matrix integrity and mechanical properties [41-43], and increased degradation and decreased synthesis of matrix components [40, 42, 44]. In contrast, radially unconfined dynamic compression at frequencies greater than 0.001 Hz has been shown to increase proteoglycan and protein synthesis [17, 37]. At amplitudes up to 10% and stresses up to 0.5 MPa, these changes were related to fluid flow and/or changes in cell shape, but not hydrostatic pressure [37, 45], while tissue shear loading models show mechanical forces in absence of fluid flow can stimulate protein and proteoglycan synthesis by 50% and 25%, respectively [25].

Studies have also examined the effects of normal and injurious mechanical load on gene expression in cartilage [46-53]. These effects were highly dependent on loading methods and duration. While Chan *et al*. found little MMP upregulation 3 hours after traumatic injury [52], Lee *et al*. found large increases in MMP and aggrecanase expression over 24 hours post-injurious compression [51]. While Fehrenbacher *et al*. found short-term dynamic mechanical loading suppressed collagen mRNA expression but had little effect on matrix metalloproteinases or their inhibitors [50], Fitzgerald *et al*. found that different loading methods (static compression, dynamic compression, and dynamic shear) resulted in distinct temporal gene expression responses of many matrix proteins, proteases, protease inhibitors, transcription factors and signaling molecules, as well as cytokines and growth factors [46, 47]. Matrix genes were similarly upregulated by dynamic compression and dynamic shear, suggesting the importance of deformation. Also, many matrix proteases showed maximal stimulation at later timepoints in compression [47]. These studies highlight the need to study changes in gene expression over

time, instead of at specific timepoints, to fully characterize cartilage response to mechanical stimulation. Furthermore, by examining groups of co-expressed genes hypotheses about gene regulation and signaling pathways can be formed.

1.3.2 Mechanical loading in tissue engineered constructs Tissue engineered cartilage as a treatment for cartilage defects and injuries is of particular interest because of the lack of donor site morbidity and potential for maintaining chondrocyte growth and phenotype at the site of the defect. 3-D cultures of chondrocytes have been shown to respond to mechanical stimulation in a qualitatively similar manner as intact cartilage tissue. Static compression inhibits ECM biosynthesis [30, 54]. Dynamic compression has been shown to increase matrix synthesis and accumulation [30, 55-57], increase mechanical properties [30, 57, 58], counteract catabolic processes [59-61], and regulate ECM gene transcription [62-64]. However, the overall response of chondrocytes in 3-D culture is dependent on loading protocol and duration [57, 60] and can change with time in culture and ECM accumulation [30, 56]. Kisiday *et al*. found that continuous dynamic compression of chondrocytes in self-assembling peptide hydrogels inhibited matrix accumulation, while intermittent loading stimulated matrix accumulation [57]. Buschmann *et al*. found that 3-D agarose cultures of chondrocytes showed greater biosynthetic responses to dynamic and static compression after 28 days in culture, than on day 1 in culture, suggesting the importance of ECM accumulation in influencing chondrocyte response [30]. In addition, given the changing mechanical properties during 3-D agarose culture, "physiological" stresses and strains may be injurious to tissue engineered constructs, especially at early stages in gel culture. A clearer understanding of what mechanical stimuli are necessary for promoting regeneration of cartilage and how these signals are interpreted during tissue regeneration would aid in optimizing tissue engineering of cartilage.

1.4 Mechanotransduction Pathways

Much research has focused on investigating the signaling pathways that transduce mechanical stimuli in cartilage. In intact tissue, deformation of the rough endoplasmic reticulum and the Golgi apparatus can affect proteoglycan synthesis, GAG chain length, and sulfation observed during compression [65, 66]. Pericellular matrix deformation can also change the physicochemical environment of cells, altering transport of soluble factors to cell-surface receptors [67, 68]. Dynamic shear and static compression of cartilage explants stimulate phosphorylation of ERK1/2, p38 MAPK, and SAPK/ERK kinase-1 (SEK-1) of the JNK pathway with distinct time-varying responses [53, 69]. Akt phosphorylation has also been shown to be regulated by mechanical compression in a quasi-*in vivo* model [70]. Blocking MAPkinase signaling, cAMP, and intracellular calcium altered specific, but overlapping, gene expression responses of cartilage to mechanical compression [46, 47, 53]. These studies suggest that multiple, interacting pathways may be responsible for cartilage response to mechanical compression. Biochemical signals may also be involved in cartilage response to mechanical stimulation. Interleukin-1 (IL-1) signaling has been shown to play a role in static compression suppression of proteoglycan synthesis [71]. Other studies have suggested that extracellular stores of bFGF or FGF-2 bound to perlecan are involved in cartilage response to dynamic loading [72, 73].

Due to the complexities of studying mechanotransduction in intact cartilage, many studies have focused on monolayer or 3-D gel culture models. At the cellular level, chondrocytes have been shown to respond to mechanical stimulation with changes in cAMP levels, membrane potential, actin re-organization, and expression of genes that regulate cartilage content and turnover including aggrecan and matrix metalloproteases [28, 74-76]. Studies have also

identified many potential surface receptors, including stretch-activated ion channels [77, 78]; CD44, a hyaluronan (HA) receptor shown to participate in HA internalization [79]; annexin V (anchorin CII), a collagen binding protein with some calcium channel activity found to play a role in matrix vesicle-initiated cartilage calcification [80]; and integrins [77, 79, 81].

Several recent studies have focused on the role of integrin receptors in chondrocyte mechanotransduction [reviewed in [81]]. Integrins are heterodimeric transmembrane proteins with an α- and β- subunit. Their extracellular domain binds to various ECM components, such as collagen, fibronectin, laminin, vitronectin, and osteopontin. The intracellular domains can associate with a variety of cytoskeletal and signaling proteins, such as paxillin, tensin, and focal adhesion kinase (FAK). Chondrocytes express $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_{10}\beta_1$, $\alpha_V\beta_3$, and $\alpha_V\beta_5$ integrins [82, 83]. Integrin expression is affected by ECM composition and mechanical stresses [84, 85], and have been shown to mediate human chondrocyte adhesion to cartilage surfaces [82, 86], survival signaling [87, 88], and regulate matrix production and degradation [8, 12, 89]. Monolayer studies have also begun to elucidate downstream signaling events involving PKCα [90], membrane hyperpolarization and autocrine/paracrine IL-4 signaling [12], and MAPkinase pathway activation [91].

While these studies have provided insights into possible mechanotransduction mechanisms, it is important to note that subtle changes in chondrocytes due to monolayer culture may influence signaling pathways [92], and some studies have shown differences in gene expression and mechanotransduction events between monolayer and explant models [93], and even between monolayer and 3-D culture models [78]. In 3-D agarose culture, the roles of ion channel signaling in response to static and dynamic loading were more complicated than those identified in monolayer [78]. Additionally, while 3-D suspension cultures help maintain

chondrocyte phenotypes, the extracellular matrix is still developing in culture, and responses to mechanical stimuli may change with time [30, 87].

The goals of this project are therefore to study the progression of chondrocyte signaling responses to mechanical stimulation in 3-D agarose gel culture with ECM accumulation over time in culture and compare to responses in intact cartilage. By investigating similarities and differences in behavior, we can provide a basis for further examination of mechanotransduction of chondrocytes using a 3-D culture model as well as aid in optimizing loading for tissue engineering purposes.

1.5 Objectives

The objectives of this thesis were to investigate the role of integrin receptors in chondrocyte response to mechanical stimulation in a 3-D *in vitro* culture model, and to characterize chondrocyte signaling responses to mechanical stimulation in agarose culture and its evolution with culture duration.

In Chapter 2, we investigated the role of integrins $\alpha 5\beta 1$ and $\alpha v\beta 3$ in chondrocyte response to dynamic compression in 3-D agarose gel culture using function blocking antibodies and small-molecule antagonists.

In Chapter 3, we examined the temporal (2, 8, 24 hour) gene expression response of 3-D agarose cultures on Days 1, 10, 28 to a 25% ramp-and-hold compression through real-time RT-PCR. We clustered these gene expression profiles and compared them to previous observations in intact cartilage tissue to discover similarities and differences in regulation.

In Chapter 4, we measured MAPkinase (ERK1/2, p38, SEK) and Akt activation kinetics in response to 25% ramp-and-hold compression through phosphorylation state-specific Western

blotting at both a short (10-60 minute) and long (1-24 hour) time scale. We again compared these to previous studies in intact cartilage tissue.

The main findings and conclusions are summarized and interpreted in Chapter 5.

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Chapter 2

Blocking integrins avb3 and a5b1 suppresses dynamic compression – induced stimulation of proteoglycan synthesis in agarose gel culture through distinct pathways

2.1 Introduction

In vivo, cartilage experiences a combination of compressive, tensile, and shear forces, both dynamic and static in nature. These mechanical loads can induce a variety of macroscopic signals including changes in intratissue pH and osmotic pressure, hydrostatic pressure gradients, fluid flow, streaming potentials and current, and mechanical deformation [1-3], which are sensed by chondrocytes and serve to regulate cell behavior. *In vivo*, static immobilization or reduced joint-loading leads to a loss of glycosaminoglycan (GAG) content and decreased proteoglycan synthesis, which can be partially rescued by remobilization [4, 5]. *In vitro*, radially unconfined dynamic compression at frequencies greater than 0.001 Hz has been shown to increase chondrocyte biosynthesis of proteoglycans and protein in both explant and 3-D agarose gel culture models [6-9], while static compressive loading can lead to decreases in chondrocyte biosynthesis [10-13]. *In vitro*, static and dynamic loading cause activation of the MAPkinase pathway and ion channels, which result in time-varying changes in gene transcription of matrix proteins, catabolic enzymes, transcription factors [14-16].

Recent research on the transduction of mechanical signals into changes in cell behavior has begun to elucidate the role of integrin interactions with the extracellular matrix (ECM). Chondrocytes express α1β1,α5β1,αvβ5, αvβ3,α3β1 [17, 18]. This expression can change with local microenvironment, mechanical stimulation, and during osteoarthritis [19-21]. Integrins play a role in adhesion, cell survival, regulating matrix metabolism, and in chondrocyte response to mechanical stimuli [22-24] [reviews]. β1, α5β1, αvβ5 integrins have been shown to mediate chondrocyte adhesion to cut cartilage surfaces [17, 25]. In monolayer studies, blocking α5β1 integrin interactions can lead to increased MMP13 activation and cellular apoptosis [26, 27]. Mechanical stimulation of aggrecan mRNA and suppression of MMP3 mRNA by dynamic stretching of monolayer chondrocytes involves a β1-integrin dependent pathway as well as stretch-activated ion channels and autocrine/paracrine stimulation by interleukin-4 (IL-4) [28, 29]. Other studies have also identified a β1-integrin dependent translocation of PKCa to the cell membrane and increased association of RACK1 and PKCa with β1-integrin after mechanical stimulation as a possible downstream signaling cascade [30].

The use of physiologic dynamic compression as a stimulant for cartilage regeneration in tissue engineering has been demonstrated by several groups [7, 8, 16, 31-35] and has prompted studies on how cells transduce signals under physiological loading conditions. Some studies suggest the role of ion channels, especially calcium channels, in regulating sGAG synthesis in response to dynamic compression [36, 37]; however, the role of cell interactions with the extracellular matrix are less well understood. The goal of this study is to examine the role of integrins in chondrocyte response to dynamic compression by cells in 3D agarose gel culture at early times in culture, prior to significant accumulation of extracellular matrix.

2.2 Methods

2.2.1 Cell Harvest and Culture Chondrocytes were isolated from the femoral condyle cartilage of 2- to 3- week old bovine calves (Research 87, Marlborough, MA) by sequential digestion in 0.2% pronase (Protease type XIV, Sigma) and 0.025% Collagenase-P (Roche), as described previously [38]. Cells were counted by a hemocytometer and seeded in 2% agarose (low melting-temperature, Invitrogen) at concentrations of 15 million cells/mL using a stainless steel casting frame [32, 39], in a slab geometry approximately 1.6mm thick. 4mm diameter plugs were cored from the slab using a dermal punch and cultured in 1% ITS supplemented feed medium (high glucose DMEM, 0.1mM nonessential amino acids, 0.4mM proline, 100U/mL PSA – penicillin, streptomycin, amphoteracin, 10μg/mL ascorbate).

2.2.2 Integrin-blocking Compounds An array of integrin-blocking compounds was used including small-molecule peptidomimetics, function-blocking antibodies, and RGD-containing disintegrins. Small-molecule compounds have the advantage of rapid diffusion times, which allow for more spatial uniformity of treatment. PF001 (previously referenced as S247 [40]), PF002, PF003 are synthetic peptidomimetics (see Figure 2.1) of the conserved amino acid motif RGD (arginine-glycine-aspartic acid) and are potent *in vitro* antagonists of ligand interaction with specific integrins (generous gift of Drs. David Griggs and Elizabeth Arner, Pfizer, Inc.). Their molecular weights and IC50s (nM) are summarized in Table 2.1. Echistatin (Sigma) is a 5.4 kDa disintegrin peptide that non-selectively blocks the activities of several integrins including αvβ3, α5β1, αIIbβ3 [41]. Function blocking antibodies to αvβ3(MAB1976, clone LM609), αv(MAB1953, clone P3G8), α5(MAB1956, clone P1D6), β1(MAB1965, clone JB1A), and α5β1(MAB1969) integrins were obtained from Chemicon.

2.2.3 Activity and Toxicity of PF001, PF002, PF003 To test activity and toxicity of compounds, a cell-adhesion assay was performed using an RGD-conjugated comb copolymer surface which promote integrin-mediate adhesion and prevents non-specific adhesion. Prepared surfaces were obtained from Maria Ufret from the Griffith lab at MIT. Briefly, cover slips were spin-coated with PMPI polymer, activated, and conjugated with an RGD peptide+fibronectin synergy site. Cover slips were placed at the bottom of a 24-well plate and held in place by silicon rings. After isolation, 25,000 cells were seeded per well and given 2 hours to attach. 0, 50, 100, 200μM PF001, PF002, PF003 were then added to each well. Cells were cultured for 7 days, with medium changes every other day. Cells were imaged daily using an inverted microscope to qualitatively observe adhesion spreading. After 7 days, cells were live/dead stained with FDA/EtBR and imaged using an inverted UV microscope. Echistatin [27] and the blocking antibodies [17, 26, 30, 42, 43] have been previously used on chondrocytes with no reported cell death.

2.2.4 Mechanical Loading – Integrin Blocking Studies On day of casting, plugs were pre-incubated for 18 hours in one of the following conditions (3-4 plugs/condition): (1) untreated control (feed medium), (2) 200μM PF001, PF002, or PF003 (concentrations for PF003 was confirmed using a dose response of 0, 100, 150, 200μM), (3) 5 μg/mL integrin-blocking antibody, (4) 1 μM echistatin. Immediately prior to loading 5 μCi/mL 35S-sulfate, 10 μCi/mL 3H-proline was added to the medium. Plugs were then subjected to a 24-hour, 1 Hz continuous unconfined dynamic compression at 2.5% strain amplitude superimposed on a 7% static offset strain, with free-swell cultures as controls using an incubator-housed loading apparatus as described previously [44]. This low amplitude dynamic compression protocol is similar to previous studies in agarose gel culture [7] and self-assembling peptide hydrogel culture [32] in

which dynamic compression resulted in stimulation of chondrocyte biosynthesis. At the end of loading, plugs were removed and washed 3 x 20 minutes in PBS with 142 μg/mL sodium sulfate and 50 μg/mL L-proline to remove free radiolabel, and digested in 1mL Proteinase K (0.1 mg/mL, Roche) at 60^{o} C overnight (approx. 16 hours) for assays. Experiments were repeated for 1-6 animals.

2.2.5 Biochemical Assays Radiolabel incorporation rates were measured by scintillation counting [45, 46]. sGAG content in the digests and collected medium were assayed by DMMB dye binding [6]. DNA was quantified by Hoescht 33258 dye-binding assay [47].

2.2.6 Data Analysis Radiolabel incorporation and sGAG content data were normalized to DNA content to account for variations in cell number. Data were further normalized to averaged free-swell controls for each animal to count for baseline animal-to-animal variability. A Shapiro-Wilk test was used to test for normality of data. 1-way and 2-way ANOVA followed by post-hoc Bonferoni pairwise comparisons were used to analyze results. A p-value less than 0.05 was considered significant.

2.3 Results

2.3.1 Activity and Toxicity of PF001, PF002, PF003 RGD-conjugated comb copolymer surfaces that can promote binding by the αvβ3 or α5β1 integrins were used to confirm activity of PF001, PF002, and PF003 on chondrocytes. By day 6 in culture, untreated cells seeded onto RGD-conjugated surfaces showed a spread morphology, while cells treated with 100μM PF001, PF002, or PF003 remained rounded on the RGD-conjugated surfaces (Supplementary Figures, Figure 2S.1). At concentrations up to 200μM of each compound over 7 days, there was no qualitative increase in ethidium bromide stained cells compared to untreated controls, suggesting no adverse effects on cell viability (Figure 2S.1).

2.3.2 Effects of Small-Molecule Blockers on GAG Biosynthesis and Accumulation with Dynamic Compression of Agarose Gel Cultures To test the role of integrin-ECM interactions in chondrocyte biosynthesis response to dynamic compression, agarose cultures were incubated with small-molecule peptidomimetics of the RGD binding sequence recognized by integrins such as $\alpha v\beta 3$ and $\alpha 5\beta 1$ to block these interactions and subjected to a 24-hour continuous, unconfined dynamic compression. At the end of 24 hours in culture, untreated agarose cultures in free-swell incorporated an average of 192 pmol sulfate/μg DNA/hr, 113 pmol proline/μgDNA/hr and accumulated 7.38 μg GAG/μg DNA. Treatment with PF001, PF002, or PF003 resulted in no changes in DNA content, as measured by Hoescht dye-binding (Figure 2S.2). DNA content was used to normalize data for all subsequent analyses. In free-swell, PF001 and PF002 resulted in no significant changes in sulfate or proline incorporation, as measured by radiolabel incorporation, or in sulfated glycosaminoglycan (GAG) content, as measured by DMMB dye binding, while PF003 (200μM) resulted in a 27% decrease in sulfate incorporation ($p<0.0005$) and 17% decrease GAG content ($p<0.0005$), as well as a 26% decrease in proline incorporation ($p<0.0005$) (Figure 2.2A,B).

Consistent with previous studies [7, 8, 33], low amplitude (<10% strain amplitude) dynamic compression at 1Hz frequency increased sulfate incorporation rates by approximately 24% (to 243 pmol sulfate/μg DNA/hr, $p<0.0005$) after 24 hours of compression in untreated agarose gel plugs (Figure 2.2A). GAG loss to the medium was minimal; <10% of the total GAG content (in agarose plug and in the medium) or approximately 0.7μg GAG/μg DNA was lost to the medium over 24 hours in free-swell culture. While GAG loss to medium increased by approximately 75% to 1.2 μg GAG/μg DNA with dynamic compression, possibly due to increased transport, GAG accumulation in the samples after 24 hours of dynamic compression was increased by

approximately 14% (to 8.51 μg GAG/μg DNA, $p<0.0005$) (Figure 2.2B). Since the experiment was conducted during early times in culture and GAG loss was minimal, the majority of the GAG content was newly synthesized and GAG accumulation mirrored sulfate incorporation trends. PF001 treated samples responded to dynamic compression with similar increases in sulfate incorporation (27%, $p<0.0005$) and GAG accumulation (15%,$p=0.089$). PF002 treated samples responded to dynamic compression with slight increases in sulfate incorporation (12%, $p=0.16$) and GAG accumulation (6%, not statistically significant). In contrast, PF003 treated samples showed no stimulation of sulfate incorporation or GAG accumulation with dynamic compression (Figure 2.2). None of the treatments (PF001, PF002, or PF003) affected GAG loss to the medium. Markers of proteoglycan synthesis (sulfate incorporation and GAG content) were responsive to dynamic compression and sensitive to integrin blockers. A single dose response experiment to PF003 was conducted with 0, 100, 150, 200 μM PF003 treated agarose cultures. Samples showed a dose-dependent decrease in sulfate incorporation, proline incorporation and GAG accumulation in free-swell culture. Dynamic compression stimulation of proteoglycan synthesis (sulfate incorporation and GAG accumulation) was blocked dose-dependently with a slight (10%) but insignificant increase in sulfate incorporation and GAG accumulation at 100μM, and no stimulation by 150μM PF003 (Figure 2S.3).

2.3.3 Broad-spectrum blockers PF001 and Echistatin show little effects on dynamic compression Echistatin is a disintegrin containing the RGD-motif that has broad specificity to integrins [41]. Echistatin decreased sulfate incorporation by 43% ($p<0.0005$), proline incorporation by 42% ($p<0.0005$), and GAG content by 21% ($p<0.0005$) (Figure 2.3A,B), and increased GAG loss to medium by 77% in free swell culture. As with PF001, dynamic compression increased sulfate incorporation in treated samples by 20%, but not statistically

significant (p=0.125); however, dynamic compression did not stimulate GAG accumulation in echistatin treated samples (Figure 2.3C). This is possibly due to GAG loss to medium not seen in PF001 treated samples since the total GAG content (agarose plug + medium) was increased by approximately 9% (not statistically significant) after 24 hours dynamic compression with echistatin treatment.

2.3.4 In free-swell culture, αvβ3 blockers show differential effects from α5β1, β1 blockers, while αv and α5 blockers showed few effects To further test the hypothesis that blocking specific integrin-ECM interactions can disrupt chondrocyte response to dynamic compression, a series of blocking antibodies were used in parallel experiments. Antibodies are larger in size (approximately 150 kDa), but in early cultures of agarose can still diffuse in relatively easily [48, 49]. αvβ3 blocker PF003 decreased sulfate and proline incorporation by approximately 30% ($p<0.0005$) and GAG accumulation by 18% ($p<0.0005$) in free-swell culture (Figure 2.4A,B). αvβ3 blocking antibody clone LM609 at 10ug/mL concentration decreased sulfate incorporation by 10% ($p<0.05$) and slightly decreased GAG accumulation by 5% ($p=0.079$), while showing a slight but non-significant decrease in sulfate incorporation at 5 ug/mL (Figure 2.4A,B).
αv blocking antibodies showed no significant effects on sulfate or proline incorporation, GAG accumulation, or GAG loss in free-swell culture (Figure 2.4A,B). In contrast, α5β1 blocker PF002 showed a slight but statistically non-significant increase in sulfate incorporation (9%) and GAG accumulation (6%) (Figure 2.5). Blocking antibodies to α5β1 (5 ug/mL) and β1 (5 ug/mL, 10 ug/mL) integrins increased sulfate and proline incorporation rates, and GAG accumulation by 20%-40% ($p<0.0005$) (Figure 2.5A,B), while blocking antibody to α5 integrin showed only a 10% decrease in proline incorporation ($p<0.05$) (Figure 2.5A). None of the blocking antibodies had significant affects on GAG loss to the medium.

2.3.5 αvβ3 blocking antibodies abolish proteoglycan synthesis response to dynamic compression. β1 blocking antibodies, but not PF002 or α5 blocking antibodies abolish proteoglycan synthesis response to dynamic compression In untreated samples, dynamic compression increased sulfate incorporation rates by approximately 20% ($p<0.0005$) and GAG accumulation by 14% ($p<0.0005$). Treatment with PF003, αvβ3 blocking antibodies, and αv blocking antibodies abrogated the response to dynamic compression (Figure 2.4C). In PF002 and α5 blocking antibody treated samples, dynamic compression resulted in a 12-15% increase in sulfate incorporation ($p=0.079$, 0.023, respectively), while GAG accumulation increased by only 5-10% (statistically insignificant) (Figure 2.5C). β1 blocking antibody treated samples showed no stimulation of sulfate incorporation or GAG accumulation by dynamic compression (Figure 2.5C). None of the effects described were due to changes in GAG loss to medium.

While blockers of αvβ3 and α5β1 both appear to abrogate the response to dynamic compression, they appear to be acting through distinct and opposing mechanisms. Blockers of full integrin complexes or β subunits appear to have a greater effect that those of α subunits.

2.4 Discussion

Dynamic compression and other mechanical stimuli have been increasingly used in tissue engineering to promote development of cartilage constructs through increasing extracellular matrix content and mechanical properties [31, 32]. Even at early days in culture, when little pericellular matrix is present, chondrocyte cultures in agarose can respond to dynamic compression with increased sulfate incorporation and sGAG accumulation [7, 8, 33]. This response to 24 hours of continuous dynamic compression increased with number of days in free-swell culture [7]. In long-term studies of the effects of dynamic loading, the presence of a pre-elaborated pericellular matrix (either by seeding chondrons initially or culturing for 2 weeks

prior to loading) does not alter the stimulatory effects of extended dynamic loading, which increased over time [31]. This perhaps suggests that interactions between the cell and its surrounding matrix developed during dynamic compression play a greater role than pre-existing interactions. The goal of this study was to examine the role of integrin-ECM interactions in the response of chondrocyte cultures to dynamic compression at early time-points. Early times in culture have the added benefit of allowing comparison of multiple integrin blockers, including antibodies, without diffusion and penetration concerns of larger molecules into tissues. The results of this study suggest that multiple integrins ($\alpha5\beta1$, $\alpha v\beta3$) may play a role in mechanotransduction and chondrocyte's ability to sense its local microenvironment; however they may play opposing or complementary roles.

As previously shown [7, 8, 33], 24 hour continuous unconfined dynamic compression stimulated proteoglycan synthesis at days 1-2 in culture. Measureable amounts of sGAG were accumulated in constructs by the end of culture. Previous studies have shown that a pericellular matrix begins developing within 4 hours after isolation [50] and can be visualized at the cell surface on day 2 in agarose culture [51]. In the present study blocking $\alpha5\beta1$ integrins with blocking antibodies or $\alpha v\beta3$ integrins, either with small-molecule antagonists or blocking antibodies, abolished proteoglycan stimulation (as measured by sulfate incorporation or sGAG content) by dynamic compression. $\alpha v\beta3$ and $\alpha5\beta1$ are both RGD-recognizing integrins. While the main binding partner for $\alpha v\beta3$ and $\alpha5\beta1$ are vitronectin and fibronectin, respectively, $\alpha v\beta3$ has also been shown to bind to fibronectin, fibrinogen, osteopontin, and collagen [52]. In addition, $\alpha v\beta3$ and $\alpha5\beta1$ have been found in focal adhesions, while other integrins expressed by chondrocytes, $\alpha3\beta1$ and $\alpha v\beta5$, are more diffusely distributed [52]. Their proximity suggests possible functional overlaps or interactions.

While blocking both α5β1 and αvβ3 prevented dynamic compression stimulation of proteoglycan synthesis, they appeared to do so by independent and possibly opposing mechanisms. In this study, blocking α5β1 integrins in free-swell with α5β1 or β1 blocking antibodies result in a significant upregulation of sulfate incorporation, while blocking αvβ3 integrins with small-molecule compounds result in a downregulation, and blocking αvβ3 integrins with antibodies result in no detectable change in basal sulfate incorporation. Previous studies have suggested that αvβ3 and α5β1 have modulating roles in articular chondrocytes [43]. In that study, treatment with anti-α5β1 antibody JBS5 induced a pro-inflammatory response (upregulation of NO, PGE2, IL-6, IL-8, and IL-1b) in both normal and osteoarthritic cartilage as well as bovine articular chondrocytes, while treatment with αvβ3 blocking antibody LM609 decreased these pro-inflammatory signals and could regulate the α5β1 response in a dominant-negative fashion [43]. Other studies have demonstrated similar responses when blocking α5β1 antibodies or treating with fibronectin fragments [26]. While stimulation of proteoglycan synthesis by blocking α5β1 integrins in agarose culture appears to be in contrast to these previous studies, it is possible that the stimulation observed is part of increased turnover that has been described previously with hyaluronan (HA) oligosaccharide treatment of cartilage [53]. Also consistent with previous studies, treatment with different αvβ3 blockers can result in different free-swell responses [43]. Taken together, this study suggests that blocking α5β1 may decrease response to dynamic compression in a pro-inflammatory manner, while blocking αvβ3 may be preventing response to dynamic compression in a more direct manner. Supporting this hypothesis is the observation that treatment with broad-spectrum blockers, which bind to α5β1 and αvβ3 with similar affinities, did not affect dynamic compression stimulation of proteoglycan

synthesis. In addition, only blockers of α5β1 integrins that induced an increase in biosynthesis in free-swell resulted in abolishment of response to dynamic compression, while all blockers of αvβ3 integrins affected stimulation of chondrocytes by dynamic compression.

Previous studies in monolayer focus on the role of α5β1 integrins in chodrocyte mechanotransduction, while this study suggests a more complex view where multiple integrins may be playing a role in regulating chondrocyte response to mechanical stimuli. This is consistent with research on the role of ion channel signaling as well, where more complex interactions were observed in 3D compared to 2D culture [37]. Given the variety of mechanical signals present during physiological dynamic compression (fluid flow, streaming potentials, deformation), it is conceivable that individual ligand-ECM interactions and other surface receptors may be sensing different mechanical signals. Further studies on downstream signaling events can shed light on how these may interact, and allow for better understanding as to how mechanical stimulation affects tissue development for tissue engineering, and also how to preserve mechanical response in tissue.

2.5 Acknowledgements

Drs. Elizabeth Arner and David Griggs at Pfizer, Inc. for helpful discussions. Pfizer, Inc. for donating the small-molecule antagonists used in this study. NIH Grant AR33236, NSF and NDSEG Graduate Student Fellowships.

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2.7 Figures

Figure 2.1 Chemical structure for PF001 (S247).

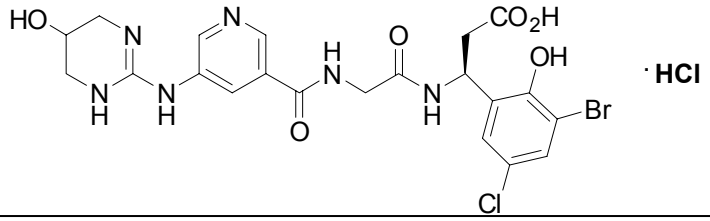

Figure 2.1 Chemical structure for PF001 (previously cited as S247 [40]). PF001 is a synthetic RGD peptidomimetic that acts as an integrin binding antagonist with broad specificity to αv and α5 integrins.

Table 2.1 Molecular weight and relative specificities for PF001, PF002, PF003

		IC50 (nM)		
	Mol Wt	**αvβ3**	**αvβ5**	**α5β1**
PF001 (S247[1,2])	569.8	0.40	1.50	64
PF002	388.9	179	1660	1.23
PF003	681.7	0.627	1.38	8940

Table 2.1. Molecular weights and relative specificities for PF001, PF002, PF003. IC50s were measured using cell adhesion assays. Data obtained from Pfizer, Inc. PF001 was previously cited as S247 [40].

Figure 2.2 Small-molecule RGD peptidomimetic compounds affect biosynthetic response of chondrocytes in agarose gel culture to dynamic compression

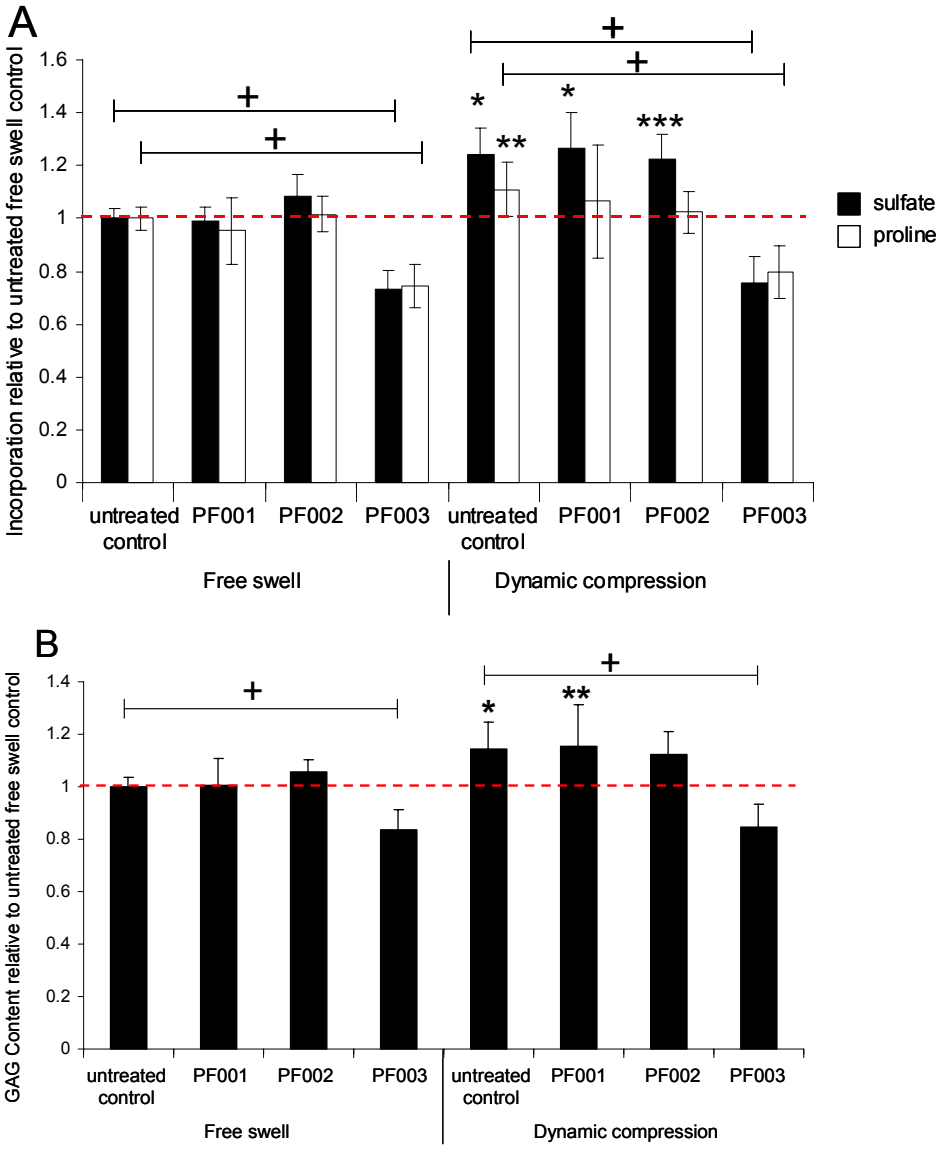

Figure 2.2 Biosynthetic response of chondrocytes in agarose culture to 24 hours of dynamic compression (1.0 Hz, 2.5% amplitude) in the presence of small-molecule integrin blockers PF001, PF002, PF003 (200uM). Data were normalized by the averaged untreated free-swell control for each animal.Data shown as mean ± SD, n = 6-19 samples from 2-6 animals. A) Sulfate and proline incorporation as measured by radiolabel incorporation. * $p<0.0005$,**$p<0.05$,***$p=0.16$ relative to free-swell, + $p<0.0005$ relative to untreated controls. B) glycosaminoglycan (GAG) accumulation as measured by DMMB dye binding assay. $p<0.0005$,**$p=0.089$ relative to free-swell, +$p<0.0005$ relative to untreated controls.

Figure 2.3 Effects of broad spectrum blockers in free swell and on dynamic compression response.

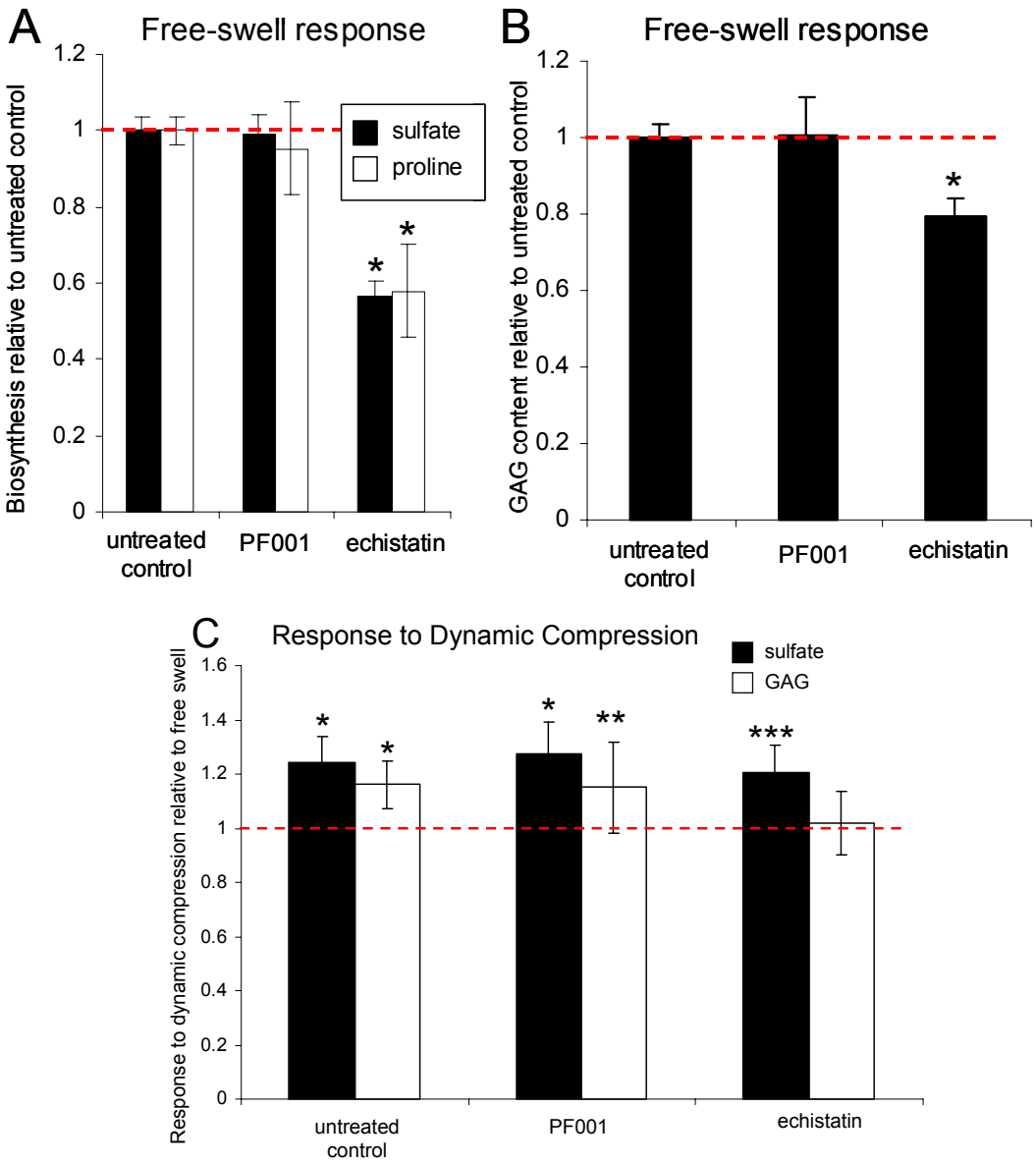

Figure 2.3 Effects of broad spectrum integrin antagonists PF001 and echistatin in free swell culture (A,B) and on dynamic compression stimulation of agarose cultures. Data shown as mean ± SD. A) Sulfate and proline incorporation in free swell relative to untreated controls. n = 6-51 samples from 2-11 animals, *$p<0.0005$ relative to untreated control. B) GAG content in free-swell cultured plugs relative to untreated controls. n = 6-51 samples from 2-11 animals, *$p<0.0005$ relative to untreated control. C) Proteoglycan synthesis as indicated by sulfate incorporation and GAG content with dynamic compression relative to treated free-swell controls. n = 6-13 samples from 2-4 animals, *$p<0.005$, **$p=0.071$,***$p=0.125$ relative to 1 (no change relative to free swell).

Figure 2.4 αvβ3 blockers in free swell and with dynamic compression

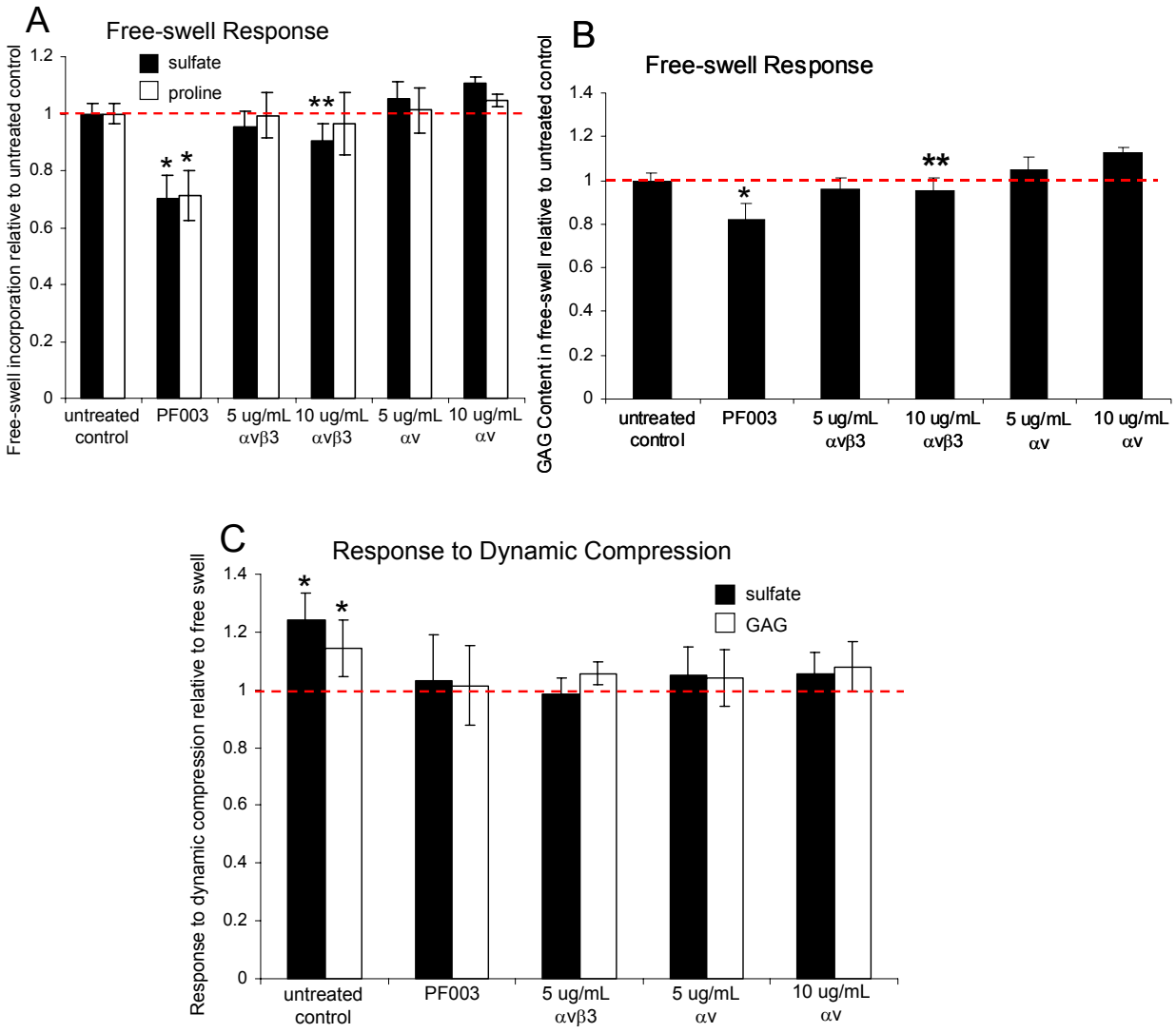

Figure 2.4 Effects of blocking αvβ3 integrins on biosynthetic behavior in free swell and in response to dynamic compression. Data shown as mean ± SD. A) Sulfate and proline incorporation in free swell agarose cultures relative to untreated controls. n = 3-51 samples from 1-11 animals, *$p<0.0005$, **$p<0.05$ relative to untreated control. B) GAG content in free-swell culture relative to untreated control. n = 3-51 samples from 1-11 animals, *$p<0.0005$, **$p=0.079$ relative to untreated controls. C) Proteoglycan synthesis (sulfate incorporation and GAG accumulation) after 24 hours of dynamic compression relative to respective treated free swell controls. n = 3-19 samples from 1-6 animals, *$p<0.0005$ relative to 1 (no change compared to free swell).

Figure 2.5 Effects of α5β1 blocking on free swell and dynamic compression

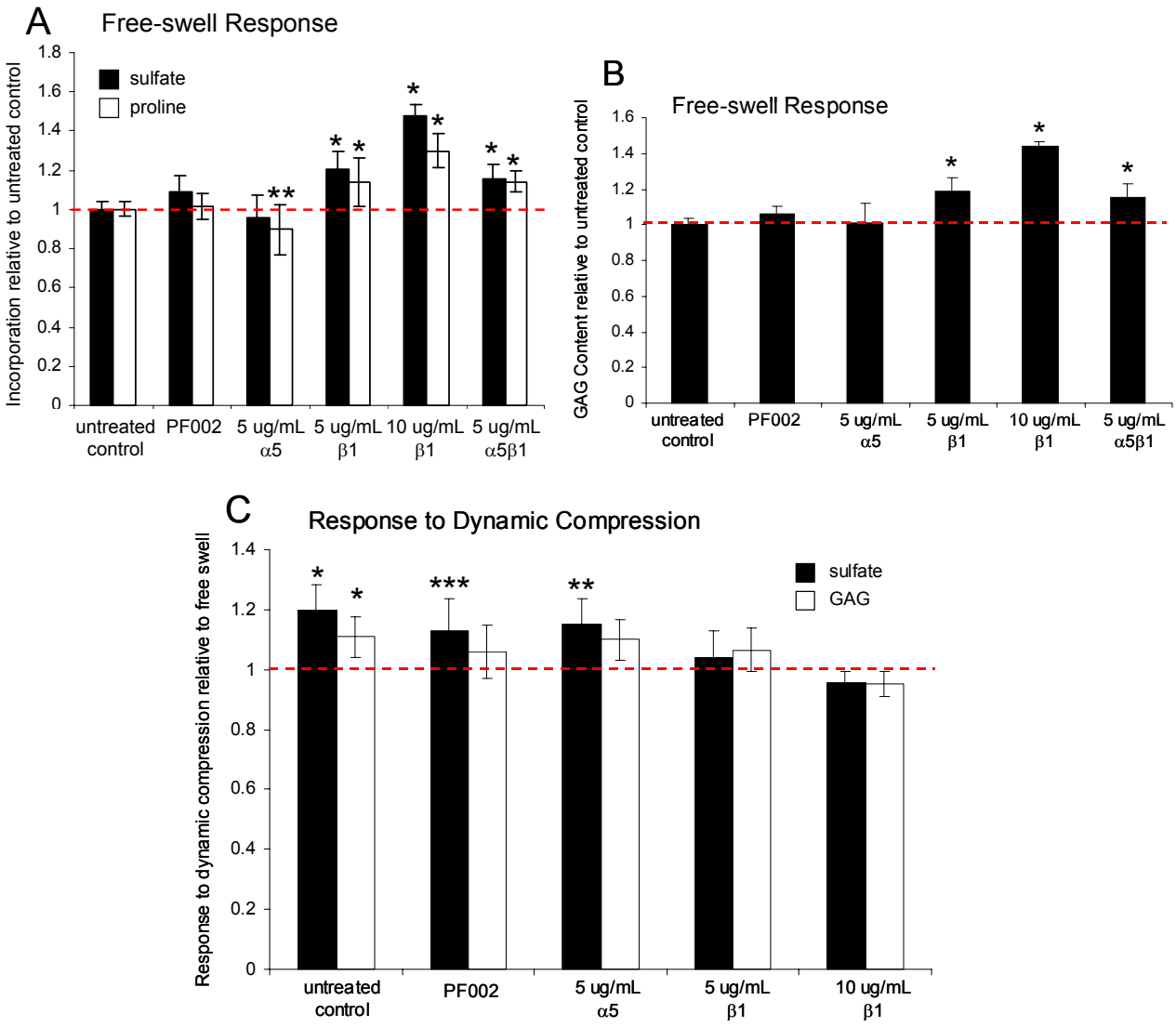

Figure 2.5 Effects of blocking α5β1 integrins on agarose cultures in free swell and in response to dynamic compression. Data shown as mean ± SD. A) Sulfate and proline incorporation in free-swell agarose cultures relative to untreated controls. n = 3-51 samples from 1-11 animals, *p<0.0005, **p<0.05 relative to untreated controls. B) GAG content in free-swell cultures relative to untreated controls. n = 3-51 samples from 1-11 animals, *p<0.0005 relative to untreated controls. C) Proteoglycan synthesis (sulfate incorporation and GAG content) response after 24 hours of dynamic compression relative to respective treated free-swell controls. n = 3-12 samples from 1-4 animals. *p<0.001, **p<0.05, ***p=0.079 relative to 1 (no change compared to free swell).

2.8 Supplementary Figures

Figure 2S.1 Activity and toxicity of echistatin, PF001, PF002, PF003

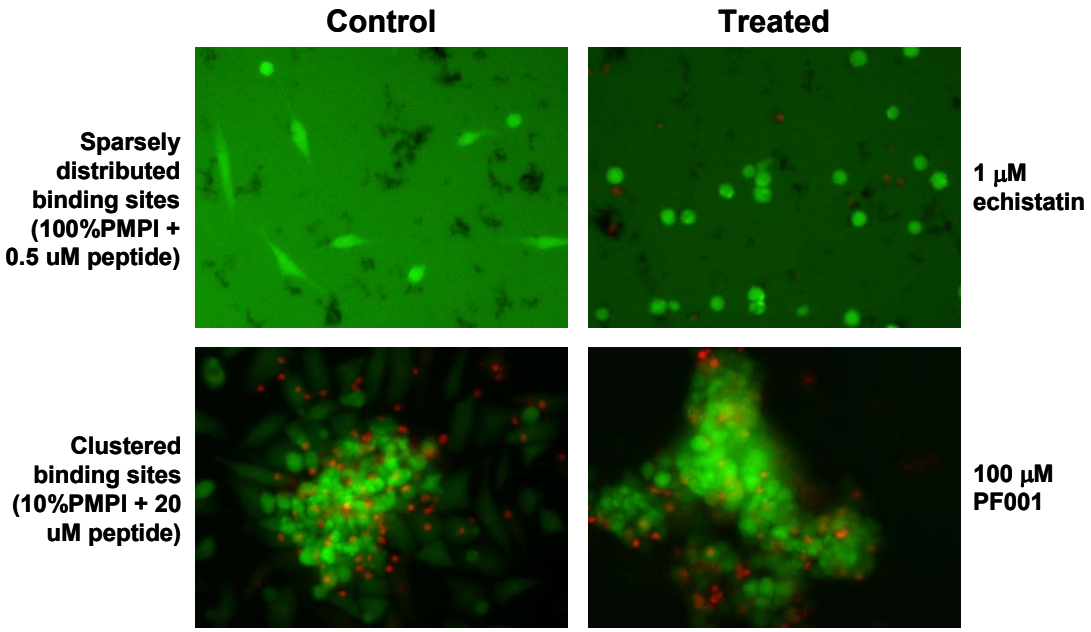

Figure 2S.1 Representative pictures of chondrocytes cultured in control (1%ITS supplemented medium) or treated (1 μM echistatin or up to 200 μM PF001, PF002, PF003) conditions on RGD-conjugated comb copolymer surfaces designed to present specific binding sites in sparsely distributed or clustered conformations. Cells were live/dead stained with FDA (green)/ethidium bromide (red) and imaged using an inverted UV microscope after 7 days in culture. Treated cells appeared rounded in comparison to control cells. No qualitatively changes in dead vs. live cells were observed with treatment.

Figure 2S.2 Treatment with PF001, PF002, PF003 did not significantly alter DNA content

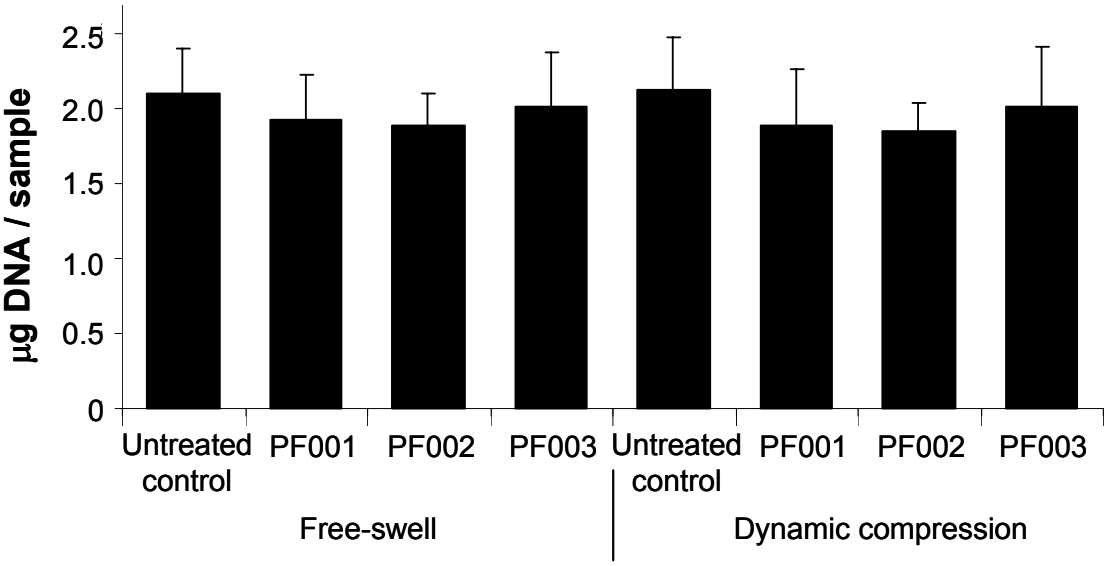

Figure 2S.2 DNA content, as measured by Hoescht 33258 dye-binding assay, for chondrocytes in agarose culture after 24 hours in free-swell or dynamic compression with or without the presence of integrin blockers PF001, PF002, PF003 (200 μM). Data shown as mean ± SD, n = 6-19 samples from 2-6 animals.

Figure 2S.3 Dynamic compression stimulation of proteoglycan synthesis decreases dose-dependently with PF003 treatment

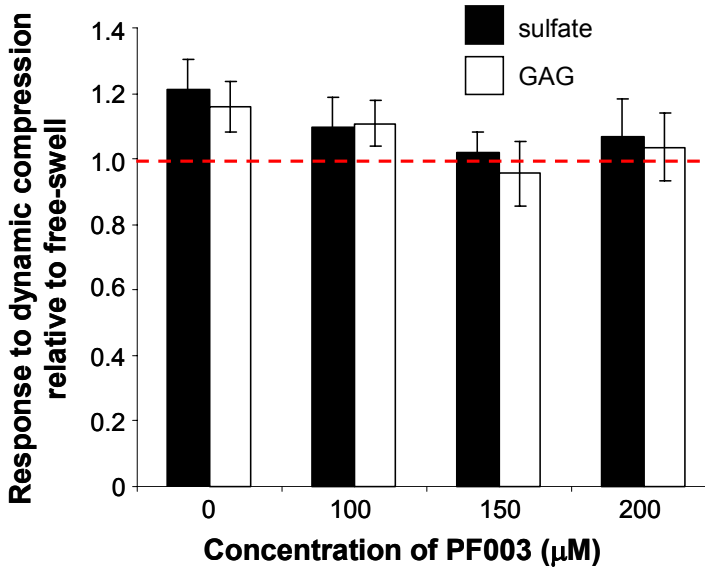

Figure 2S.3 Proteoglycan synthesis as indicated by sulfate incorporation and GAG content with dynamic compression relative to free-swell controls in samples treated with 0-200 μM PF003. n = 3 samples from 1 animal. Data shown as mean ± SD.

Chapter 3

Mechanical regulation of gene expression of chondrocytes in agarose culture evolves distinctly with matrix development over time in culture

3.1 Introduction

Articular cartilage functions as a lubricating surface that distributes and transmits stresses in joints. It is well accepted that mechanical stresses and strains can influence cartilage biosynthesis and helps to regulate cartilage biosynthesis. In cartilage explants, previous studies have shown that dynamic compression and dynamic shear increase proteoglycan synthesis, while static compression decreases biosynthesis [1, 2]. These mechanical loading protocols also affect gene expression of multiple matrix proteins, proteases, protease inhibitors, signaling molecules, transcription factors, growth factors, and cytokines in a distinct, time-dependent manner [3, 4]. By clustering the temporal gene expression response, Fitzgerald *et al*. were able to identify groups of co-expressed genes and possible mechanisms of regulation [3, 4].

In tissue-engineered constructs, dynamic compression has been used to stimulate the development of the extracellular matrix and enhance their mechanical properties [5-8]. The stimulatory effect of dynamic compression in tissue engineered constructs is dependent on load duration, with intermittent protocols generally providing stimulation of ECM production while

continuous loading suppressing ECM production [8, 9]. In addition, while the biosynthetic responses of chondrocytes in 3-D culture are qualitatively similar to that of intact tissue, the response of chondrocytes to mechanical compression can change with time in culture. Buschmann *et al*. found that chondrocytes in agarose gel culture responded to static and dynamic compression with greater alterations to sulfate and proline incorporation rate after 28 days in culture, than on initial days in culture [7].

The mechanical stimuli transmitted to the cell during compression changes with ECM development. Over 28 days in 3-D agarose culture, the equilibrium stiffness of the tissue increases with matrix development, reaching approximately 1/5 of "parent" tissue stiffness by Day 28, while the permeability decreases to about twice that of cartilage [7]. The accumulation of fixed charges associated with glycosaminoglycans also results in streaming potentials under compression of approximately 1/5 of parent cartilage by Day 28 [7]. In static compression, cell strain is reflective of gel strain on Day 1 of culture, when little matrix has accumulated, but by Day 6 in culture, the developed pericellular matrix protected the cell from deformation [10]. These changes may all contribute to altered downstream responses to mechanical stimulation.

The purpose of this study was to examine the temporal gene expression response of chondrocytes in 3-D agarose gel culture and how these responses change with ECM development with time in culture. By comparing these responses, we hope to identify similarities and differences in regulation between 3-D culture models and intact cartilage tissue and gain insights into important regulatory stimuli.

3.2 Materials and Methods

3.2.1 Cell Harvest and Culture Chondrocytes were isolated from the femoral condyle cartilage of 2- to 3- week old bovine calves (Research 87, Marlborough, MA) by sequential digestion in

0.2% pronase (Protease type XIV, Sigma) and 0.025% Collagenase-P (Roche), as described previously [11]. Cells were counted by a hemocytometer and seeded in 2% agarose (low melting-temperature, Invitrogen) at concentrations of 15 million cells/mL using a stainless steel casting frame [8, 12], in a slab geometry approximately 1.6mm thick. 4mm diameter plugs were cored from the slab using a dermal punch and cultured in 1% ITS supplemented feed medium (high glucose DMEM, 0.1mM nonessential amino acids, 0.4mM proline, 100U/mL PSA – penicillin, streptomycin, amphoteracin, 10μg/mL ascorbate) with medium changes every other day for up to 28 days.

3.2.2 Ramp-and-Hold Compression for Time Course Studies On days 1, 10, and 28 in culture, groups of 6 plugs were placed in fresh 1% ITS supplemented medium and compressed over a 2-minute interval to a final strain of 25% [13] and held for 2, 8, or 24 hours. Free-swell cultures for 2, 8, 24 hour time courses in fresh 1% ITS medium served as controls. At the end of incubation, plugs were pooled, gently blotted dry with sterile gauze, and flash frozen in liquid nitrogen. In parallel, 4 plugs were placed in 5 μCi/ml 35S-sulfate supplemented medium for 24 hours in free-swell or 25% compression. At the end of compression, plugs were washed with radiolabel-free PBS and individually frozen.

3.2.3 Biosynthesis and GAG Accumulation measurements Radiolabelled samples on each Day (1, 10, 28) were individually digested in proteinase K. Radiolabel incorporation was measured by scintillation counting to measure sulfate incorporation into glycosaminoglycans [14, 15]. DMMB dye binding was used to quantify sGAG accumulation [2]. DNA amounts were measured by Hoechst 33258 dye-binding [16].

3.2.4 RNA Extraction and Real-time RT-PCR For each pooled sample, RNA was extracted and reverse transcribed as previously described [3]. Briefly, samples were pulverized under

liquid nitrogen and homogenized (BioSpec Products Inc) in TRIzol lysis buffer (Invitrogen), then separated using phase-gel spin columns (Eppendorf). The supernatant was then further purified using the Qiagen RNeasy Minikit, according to manufacturer's protocol with optional DNase digest (Qiagen). Total RNA was quantified using a Nanodrop, and 1 μg RNA per sample was reverse transcribed (Applied Biosystems). Real-time PCR was performed using an Applied Biosystems 7900HT Fast Real-Time PCR System and SYBR Green Master Mix (SGMM, Applied Biosystems). Primers for the 32 gene examined (Table 3.1) were designed and calibrated as previously described [3].

3.2.5 Statistical Analysis Relative gene expression was obtained from the standard curve method. Gene expression in a given sample was normalized to 18S, as previous work suggests that mechanical loading can affect GAPDH and β actin expression [17]. For each gene, expression levels for loaded samples at each Day/Time point were then normalized to their respective free-swell controls for each experimental repeat. Mean gene expression vectors for loading at 2, 8, 24 hours on Days 1, 10, 28 from 4 experimental repeats were used for clustering analysis. Principle component based k-means clustering, a method previously established [3, 4], was used to examine expression patterns and trends. To emphasize expression patterns over magnitude, each expression vector was standardized to obtain equal variances. Principle component analysis was applied to the standardized gene expression vectors to optimize 3-D graphical representation of the data. Projection coordinates of the standardized gene expression vectors were clustered using Euclidean distance k-means clustering to group genes. Centroid vectors were calculated as the average expression profile from genes within a group. In addition to clustering the complete data, expression vectors (for 2, 8, 24 hour timecourses) for each

individual day of loading were clustered separately to examine the effect of culture duration on expression patterns.

3.3 Results

3.3.1 Biosynthesis response to mechanical compression and sGAG accumulation with time in culture is consistent with previous data sGAG content of cell-seeded plugs increased with time in free-swell culture to approximately 50 μg sGAG/μg DNA by Day 28, while proteoglycan synthesis rates, as measured by sulfate incorporation, decreased with time in culture (data in Supplementary Figures, Figure 3S.8); both are consistent with previous reports [7]. Proteoglycan synthesis was not affected by 25% compression over 24 hours at Day 1, 10, or 28, also consistent with [7], however GAG release to medium decreased by approximately 20%, possibly due to decreased transport.

3.3.2 Temporal gene expression trends in response to 25% compression on Days1, 10, and 28 Over the course of 28 days in culture, the biochemical and mechanical properties of agarose gel cultures of chondrocytes change dramatically. This results in an alteration of the forces and flows that the cells experience during loading [7]. To examine the effects of the evolving microenvironment on gene expression responses to mechanical loading, we measured the temporal response of 31 genes to 2, 8, and 24 hours of 25% ramp-and-hold compression on Days 1, 10 and 28 in culture. Looking at selected individual gene expression profiles (Figure 3.1), we found expression of aggrecan was slightly upregulated at 24 hours of compression on all Days in culture. In contrast, Collagen 2 showed a much higher magnitude of regulation by compression (not the logarithmic scale on the y-axis), with early upregulation (at 2 hours of compression) on Days 1 and 10 in culture, and downregulation by 24 hours on Day 28 in culture. Catabolic enzymes MMP3 and MMP13 showed upregulation at different times on different days in culture,

while ADAMTS-5 was upregulated in response to compression only on Day 1 of loading. Sox9, a transcription factor, showed little regulation by mechanical loading, with slight upregulation after 8 hours of loading on Day 10 and slight downregulation at 8 hours on Day 28. Remaining gene expression profiles can be found in the Supplementary Materials.

Clustering these gene expression vectors revealed 5 distinct groups (Figure 3.2). Gene groups are listed in Table 3.2A, and the centroid profiles are shown in Figure 3.3. Clusters were found to be statistically different from each other, except for Centroids 2 and 4 (Table 3.3). Further examination determined that this was likely due to the low number of genes in each group (low degrees of freedom) and the high variance of Centroid 2 (discussed below). Because of the biological significance of Centroid 4, we chose to maintain 5 clusters for all remaining analyses. Centroid 1 gene expression profile (Figure 3.3A) showed a temporal response to loading on Day 1, but little response on subsequent days in culture. Approximately half of the genes examined were in this centroid, suggesting that 25% compression has a maximal effect on Day 1 in culture. Centroid 2, which included ADAMTS-5, OP-1 (or BMP7), and TNFα showed a varied response to mechanical loading over time in culture, with upregulation on Day 1, downregulation on Day 10, and little response on Day 28 (Figure 3.3B). Looking at the individual gene expression profiles (Figure 3.4), we see that ADAMTS5 and OP-1 behave similarly on Day 1, with a bimodal upregulation at early (2 hours) and late (24 hours) times under compression. In contrast, gene expression of OP-1 and TNFα in response to loading in suppressed by approximately 50% by 24 hours under compression on Day 10 and only slightly suppressed on Day 28 in culture. Centroid 3 and Centroid 5 showed a shift in the temporal response over time in culture (Figure 3.3C, E), with early, transient upregulation of gene expression on Day 1 and increasingly delayed upregulation of gene expression on subsequent

days in culture. Centroid 3 genes showed higher peak stimulation, while Centroid 5 genes showed lower amplitude, but more sustained upregulation. Centroid 4 contained cFos and cJun, known early-response genes, which showed upregulation with 25% compression at the 2 hour timepoint on all days in culture (Figure 3.3D).

3.3.3 Temporal gene expression responses on each day in culture clustered separately In order to examine the temporal gene expression response on each day and how physical forces particular to each day in culture is affecting gene regulation, we clustered the temporal gene expression responses (at 2, 8, 24 hours) to 25% compression relative to free-swell controls separately for each day. We chose 5 groups for ease of comparisons with the full set of clustered data and to maximize the likelihood of identifying unique expression patterns. The genes in each cluster are listed in Table 3.2B-D for Days 1, 10, and 28, respectively. As expected, gene expression profiles and clusters differ greatly from day to day, suggesting that multiple mechanical and biochemical cues can influence gene expression, and that the balance of these cues changes with the development of the agarose gel constructs. On Day 1, the majority of genes examined were grouped in Centroid 2 (Table 3.2), which showed a transient upregulation at 2 hrs of loading with a rapid decay to free-swell levels by 8 hours (Figure 3.5A). Centroid 3, comprising of Aggrecan and ADAMTS5, was unique in showing upregulation at 24 hours of compression. On Day 10, the magnitude of expression changes decreased, with few genes or cluster centroids showing changes greater than 20% (Figure 3.5B). Centroid 5 genes (c-Fos, c-Jun, Collagen 2, and IGF1) maintained their early response to compression. On Day 28, most genes were transiently upregulated; Centroid 1 showed peak stimulation at 8 hours, Centroid 2 at 24 hours, and Centroid 3 at 2 hours of compression (Figure 3.5C).

3.3.4 Coexpression of genes in response to 25% compression on all days in agarose gel culture A few sets of genes were found clustered together on each day as well as in the clusters for the complete set of data: cFos and cJun; IL4 and TIMP2; IL6 and MMP1; ADAMTS-4 and Link; β-actin and bFGF; and TIMP3 and TGFβ. All sets of genes were upregulated at 2 hrs on compression on Day 1; however, their gene expression patterns on subsequent days diverged (Figure 3.6). cFos and cJun were upregulated at 2 hours by compression with a rapid decay on each day of culture. IL4 and TIMP2 were transiently downregulated at 8 hours of compression on Day 28. IL6 and MMP1 showed a minimal response after Day 1 in culture. The transient upregulation of ADAMTS-4 and Link shifted to 8 hrs on Day 10 and 24hrs on Day 28. β-actin and bFGF upregulation was delayed to 24 hrs by Day 10. TIMP3 and TGFβ showed a transient upregulation at 8 hours of compression on Days 10 and 28.

3.4 Discussion

Gene expression in response to different forms of mechanical loading have been investigated in intact cartilage tissue [3, 4] and in monolayer [18, 19]. Gene expression in response to dynamic compression has also been used to evaluate its use in stimulating matrix accumulation in various tissue engineered constructs [9, 20-23]. However, the mechanical and biochemical environment of chondrocytes in tissue engineered constructs change with culture duration, which can greatly alter mechanical signaling and response of the cells. To examine how this evolving microenvironment can affect chondrocyte mechanotransduction, we investigated the temporal gene expression response of chondrocytes in agarose gel culture to a 25% ramp-and-hold mechanical compression on Days 1, 10, 28 in culture. Ramp-and-hold compression was chosen because of its well characterized effects on intact cartilage tissue. On Day 1 in agarose culture, cell strain is expected to be similar to total construct strain, but cell deformation in

agarose gel constructs decreases with development of the pericellular matrix [10]. In contrast, modulus, streaming potential, permeability, and the development of cell-matrix interactions become more similar to intact tissue over time in culture [7]. By comparing the gene expression response of chondrocytes in agarose gel culture to the previously studied responses in intact tissue, we hope to get insights into common mechanotransduction mechanisms as well as differences remaining between tissue engineered constructs and native tissue.

In our study, we examined the temporal gene expression levels of 31 genes over the course of 2, 8, 24 hours of 25% ramp-and-hold compression on Days 1, 10, and 28 in agarose gel culture. As expected, we found distinct changes in the gene expression profiles on each day in culture. Matrix molecules aggrecan and collagen 2 showed contrasting behavior, with aggrecan gene upregulation at 24 hours of compression on all Days in culture while collagen 2 was transiently upregulated at 2 hours of compression on Days 1 and 10, and downregulated by 24 hours of compression on Day 28. This opposing behavior is consistent with previous studies showing that aggrecan and collagen 2 promoter activity were opposite of one another in response to dynamic compression of agarose gel constructs [21]. Catabolic enzymes MMP3, 9, and 13 showed transient upregulation in response to loading, with peak upregulation shifting from 2 hrs on Day 1 to 8 or 24 hours by Day 28.

To further examine trends in gene expression responses to 25% compression, we performed clustering analysis to group co-expressing genes with an emphasis on expression patterns as opposed to magnitudes. By clustering gene expression behavior on all days in culture together, as well as each day separately, we hoped to gather insights into how changes in mechanical and biochemical signals are affecting gene expression responses. When clustering the gene expression responses on all days in culture together, we found 5 distinct groups.

Interestingly, the majority of genes we examined were clustered into Centroid 1, which showed a transient upregulation at 2 hours of loading on Day 1 in culture, but minimal responses on Days 10 or 28 in culture. This suggests that 25% compression has a maximal effect on Day 1 in gel culture and that cell deformation, which is unique to day 1, may play a large role in regulating gene expression. It is possible that larger strains would result in higher responses at later times in culture; however, due to agarose construct failure at higher strains, it was not possible to apply higher strains consistently. In contrast, Centroid 3 and 5 genes, which included catabolic enzymes MMP3, 9, 13 as well as inhibitors of metalloproteinases TIMP1 and TIMP3, showed a temporal shift in upregulation of gene expression, while Centroid 4 genes, c-Fos and c-Jun, is consistently transiently upregulated at 2 hours of compression, with maximal stimulation on Day 28 in culture. The correlation between the increase in c-Fos and c-Jun stimulation by compression with the shift in peak upregulation of MMPs 3, 9, and 13 is consistent with previous studies in cartilage, which suggested that MMP3, 9, and 13 are all regulated by AP-1, a heterodimer of c-Fos and c-Jun [4], and studies in tissue engineered cartilage which showed a mechanical stimulation of MMP3 and 13 through AP-1 [23].

To isolate the effects of mechanical stimulation on each day in culture on gene expression, we clustered the temporal (2, 8, 24 hour) response to 25% compression on each day separately. Predictably, the expression profiles in response to compression and groups of genes differed greatly with time in culture, emphasizing the idea that multiple mechanical and biochemical cues regulate gene expression, and that these are evolving with matrix development and changing biomechanical of the agarose gel construct. Only a few genes were consistently grouped together, of which 2 pairings stood out. The first was c-Fos and c-Jun, components of the AP-1 complex, and shown to be upregulated transiently in response to mechanical loading in

intact tissue [4]. The second was TIMP3 and TGFβ. TIMP3 is an inhibitor of ADAMTS-4 and ADAMTS-5 in cartilage [24, 25]. TGFβ is a growth factor implicated in chondrocyte differentiation, and has been shown to regulate TIMP3 in cartilage [26]. Interestingly, both were shown to be similarly regulated by intracellular calcium and cAMP in 50% compressed cartilage [3, 4].

Looking closer at the groupings on each day in culture, we found that on Day 1, the majority of gene clustered into one group, Centroid 2, which showed a transient upregulation at 2 hours, with a rapid decay to levels just below free-swell. The transient nature of the gene expression behavior suggests that the transient phase of the ramp-and-hold compression is dominating the response to loading on Day 1 in culture. This phase is characterized on Day 1 by cell deformation and fluid flow. In studies with cartilage tissue, Fitgerald *et al*. [4] identified a group of genes (M1: aggrecan, collagen 2, link protein, MMP1, and TIMP3) that showed a similar transient response to 50% static compression as well as similar responses to dynamic compression and dynamic shear stimulation, which suggested deformation as a dominant regulatory signal. All of these genes except for aggrecan are found in Centroid 2 on Day 1, further supporting the hypothesis that deformation is a dominant signal in regulating these genes. In contrast, aggrecan showed upregulation at 24 hours on all days in culture, suggesting a different mode of mechanical regulation. On Day 10, the magnitude of gene expression changes in response to mechanical compression decreased dramatically, with most genes up- or down-regulated by less than 20%. Only Centroid 5, containing c-Fos, c-Jun, Collagen 2, and IGF-1, showed a robust transient upregulation in response to compression. While it is possible that the limited sample times (2, 8, and 24 hours) were not sufficient to detect maximum gene transcriptional changes, it appears improbable that this would occur with the majority of genes

investigated. Instead, it is likely that the development of the pericellular matrix is protecting the cells from deformation [10], but is still insufficient for transmitting mechanical signals. This is consistent with studies suggesting that FGF-2 plays a role as a chondrocyte mechanotransducer, and accumulation in the matrix during development is necessary for functional response [27]. On Day 28, gene expression was more strongly regulated by mechanical compression. In most cases, gene expression was transiently upregulated, but the time of peak upregulation was different between different sets of genes. Centroid 5 genes, which included c-Fos and c-Jun were still upregulated transiently during the first 2 hours of compression, while Centroid 1 genes were upregulated at 8 hours, and Centroid 2 genes were upregulated at 24 hours under compression. The distribution of behavior and complexity of gene expression responses on Day 28 are more similar to those seen in cartilage [3]; however, unlike in tissue, clusters of co-expressed genes were not dominated by genes with particular functions. Instead, catabolic enzymes, cytokines, growth factors, and matrix molecules were often co-clustered, suggesting that the balance of signaling is still altered from functional, intact tissue.

Through our investigation of gene expression regulation by 25% ramp-and-hold compression on Days 1, 10, and 28 in agarose gel culture, we have found that while similar gene expression patterns between chondrocytes in agarose gel culture and in intact cartilage tissue exist, the disparity of mechanical cues between the two systems result in dramatically different gene expression responses. In addition, the presence of functional matrix, as seen when comparing behavior between Day 1 and Day 28 agarose gel culture, results in more complex temporal signaling responses, which are still unique from behavior in intact cartilage tissue. Considering the increasing use of loading for the purpose of enhancing tissue engineered constructs, and the fact that these constructs will be subjected to many loads when implanted, it

is important to better understand how long-term effects of loading may differ between engineered and native tissue.

3.5 Acknowledgements

Dr. Rebecca Fry, Center for Environmental Health Sciences at MIT, for helpful discussions regarding self-organizing maps. NIH Grant AR33236 , NSF and NDSEG Graduate Student Fellowships for funding.

3.6 References

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3.7 Figures

Table 3.1 Genes examined and their primers for real-time PCR

Gene	Forward Primer	Reverse Primer
18S	TCGAGGCCCTGTAATTGGAA	GCTATTGGAGCTGGAATTACCG
ADAMTS-4	CTGGGCCATGTCTTCAGCAT	GGCGGGAGGTGCTCTCA
ADAMTS-5	CTCCCATGACGATTCCAA	AATGCTGGTGAGGATGGAAG
Aggrecan	CCTGAACGACAAGACCATCGA	TGGCAAAGAAGTTGTCAGGCT
β-actin	GATGAGATTGGCATGGCTTT	GTCACCTTCACCGTTCCAGT
bFGF or FGF-2	GCCATAATCGGAACAGCACT	AGGAATGCACTGTGGAGCTT
c-Fos	CTCCACTTCATCCTAGCGGC	GCCCCCACTCAGATCAAGAG
c-Jun	AGCTGGAGCGCCTAATCATACA	CCTCCTGCTCATCTGTCACGTT
CD44	GTCCTATGCGGAAACCTCAA	CTGCCCACACCTTCTCCTAC
Collagen 1	AATTCCAAGGCCAAGAAGCATG	GGTAGCCATTTCCTTGGTGGTT
Collagen 2	AAGAAGGCTCTGCTCATCCAGG	TAGTCTTGCCCCACTTACCGGT
Fibronectin	ACTGCCCACTCCTACAACCA	CAAAGGCATGAAGCACTCAA
HA-synthase	GCACATCTGGAAGGAAAACC	AAAATCACACCACCCAGGAG
IGF-1	CAGCAGTCTTCCAACCCAAT	GAAGAGATGCGAGGAGGATG
IGF-2	TTCTACTTCAGCCGACCATCC	TGGCACAGTAAGTCTCCAGCA
IL-1β	GAAGAGCTGCATCCAACACC	ATGCAGAACACCACTTCTCG
IL-4	GCGGACTTGACAGGAATCTC	TTCAGCGTACTTGTGCTCGT
IL-6	TGAGTGTGAAAGCAGCAAGG	AGCAAATCGCCTGATTGAAC
Integrin α5	ACCAGGGTCACAGGACTCAG	AGGAACATCCGTCTTTGCAG
Integrin αv	CTCATCGTTTCCATCCCACT	ACGCACAGGAAAGTCTTGCT
Link	AAGCTGACCTACGACGAAGCG	CGCAACGGTCATATCCCAGA
MMP1	GGACTGTCCGGAAATGAGGATCT	TTGGAATGCTCAAGGCCCA
MMP13	TCTTGTTGCTGCCCATGAGT	GGCTTTTGCCAGTGTAGGTGTA

Table 3.1 continued

Gene	Forward Primer	Reverse Primer
MMP3	CACTCAACCGAACGTGAAGCT	CGTACAGGAACTGAATGCCGT
MMP9	TCCCTTCCTTGTCAAGAGCAA	TACTTGGCGCCCAGAGAAGAA
OP-1	GGGCTTTTCCTACCCCTA	CACGAGATTGACGAAGCTCA
Sox9	TGAAGAAGGAGAGCGAGGAG	GTCCAGTCGTAGCCCTTGAG
TGFβ	CACGTGGAGCTGTACCAGAA	ACGTCAAAGGACAGCCACTC
TIMP1	TCCCTGGAACAGCATGAGTTC	TGTCGCTCTGCAGTTTGCA
TIMP2	CCAGAAGAAGAGCCTGAACCA	TGATGTTCTTCTCCGTGACCC
TIMP3	TTTGGCACGATGGTCTACACC	CCTCAAGCTTAAGGCCACAGA
TNFα	ACGGTGTGAAGCTGGAAGAC	CCCTGAAGAGGACCTGTGAG

Figure 3.1 Gene expression responses for select genes

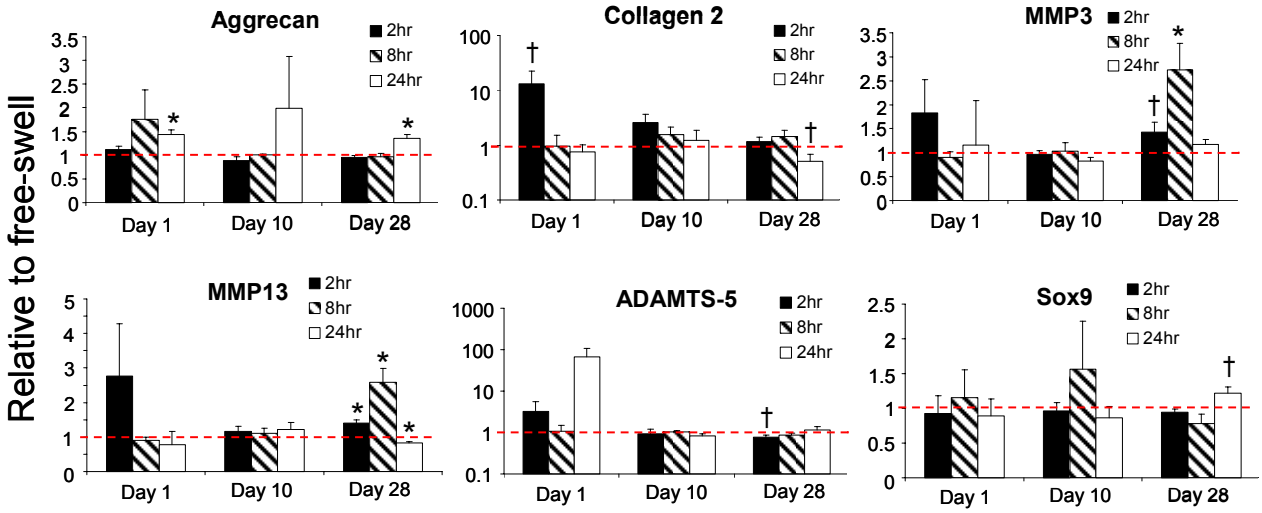

Figure 3.1 Gene expression responses for select genes, measured by real-time RT-PCR in response to 2, 8, and 24 hours of 25% compression on Days 1, 10, and 28 of agarose gel culture normalized to 18S, relative to respective free-swell controls. Free-swell levels correspond to a value of 1. Data shown as mean ± S.E for 4 complete replicates. * $p<0.05$, † $p<0.1$ by t-test of log-transformed ratios compared to free-swell levels of log(1) = 0.

Figure 3.2 Projection plot of individual genes along the three main principle components

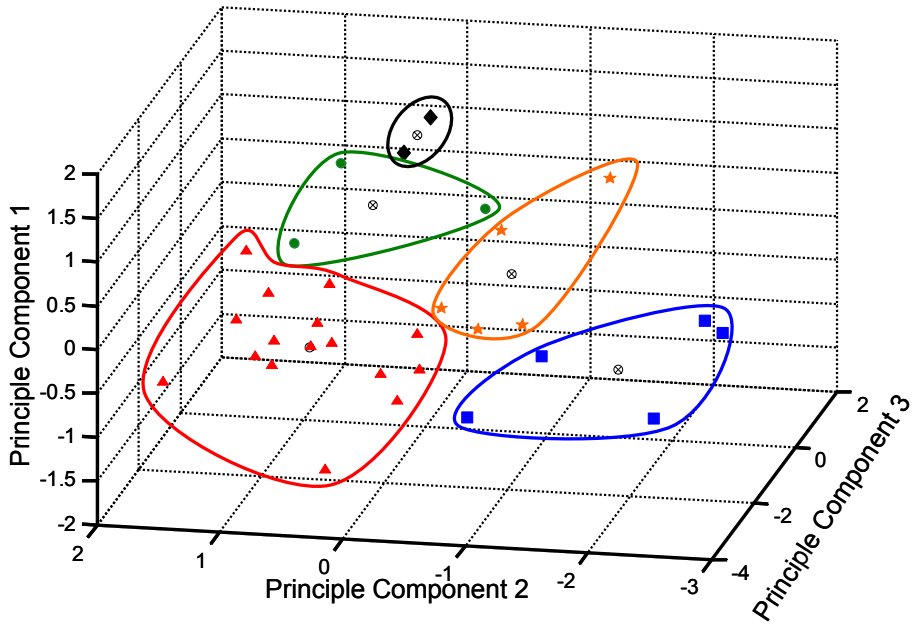

Figure 3.2 Projection plot of individual gene expression profiles represented by the three main principle components derived from principle component analysis of standardized gene expression vectors. Genes are visually grouped into 5 clusters found by k-means clustering.

Table 3.2 Centroid components for k-means clustering of complete data set as well as of gene responses on individual days

Conditions Used in Clustering	Groups	Genes Clustered
A. Complete Timecourse (2,8,24 hours) for Days 1, 10, 28	C1	ADAMTS4, β-actin, bFGF, CD44, Collagen 1, Collagen 2, HA-Synthase, IGF-1, IGF-2, IL-1β, IL-4, IL-6, αv integrin, Link, MMP1, TIMP2
	C2	ADAMTS5, OP-1, TNFα
	C3	α5 integrin, MMP13, MMP3, TGFβ, TIMP3
	C4	c-Fos, c-Jun
	C5	Aggrecan, Fibronectin, MMP9, Sox9, TIMP1
B. Day 1 Timecourse (2, 8, 24 hours)	D1-1	Sox9
	D1-2	ADAMTS4, β-actin, bFGF, CD44, c-Fos, c-Jun, Collagen 2, HA-Synthase, IGF-1, IGF-2, IL-4, IL-6, α5 integrin, αv integrin, Link, MMP1, MMP13, MMP9, TGFβ, TIMP2, TIMP3
	D1-3	ADAMTS5, Aggrecan
	D1-4	Collagen 1, IL-1β, TIMP1, TNFα
	D1-5	Fibronectin, MMP3, OP-1
C. Day 10 Timecourse (2, 8, 24 hours)	D10-1	IL-1β, IL-4, IL-6, MMP1, TIMP2
	D10-2	ADAMTS5, CD44, Collagen 1, αv integrin, MMP3, OP-1, TNFα
	D10-3	ADAMTS4, Fibronectin, HA-Synthase, α5 integrin, Link, Sox8, TGFβ, TIMP3
	D10-4	Aggrecan, β-actin, bFGF, IGF-2, MMP13, MMP9, TIMP1
	D10-5	c-Fos, c-Jun, Collagen 2, IGF-1
D. Day 28 Timecourse (2, 8, 24 hours)	D28-1	CD44, Collagen 2, MMP3, MMP13, TGFβ, TIMP3, TNFα
	D28-2	ADAMTS4, β-actin, bFGF, Fibronectin, α5 integrin, αv integrin, Link, MMP9, TIMP1
	D28-3	ADAMTS5, Aggrecan, HA-Synthase, IGF-2, IL-6, MMP1, Sox9
	D28-4	Collagen 1, IGF-1, IL-4, OP-1, TIMP2
	D28-5	c-Fos, c-Jun, IL-1β

Table 3.3 p-values for centroid separation determined by student's t-test as described in [3]

	Centroid 1	Centroid 2	Centroid 3	Centroid 4
Centroid 2	<0.01			
Centroid 3	<0.01	<0.05		
Centroid 4	<0.01	0.27	<0.05	
Centroid 5	<0.01	<0.05	<0.01	<0.02

Figure 3.3 Centroid profiles for k-means clustering of complete data set

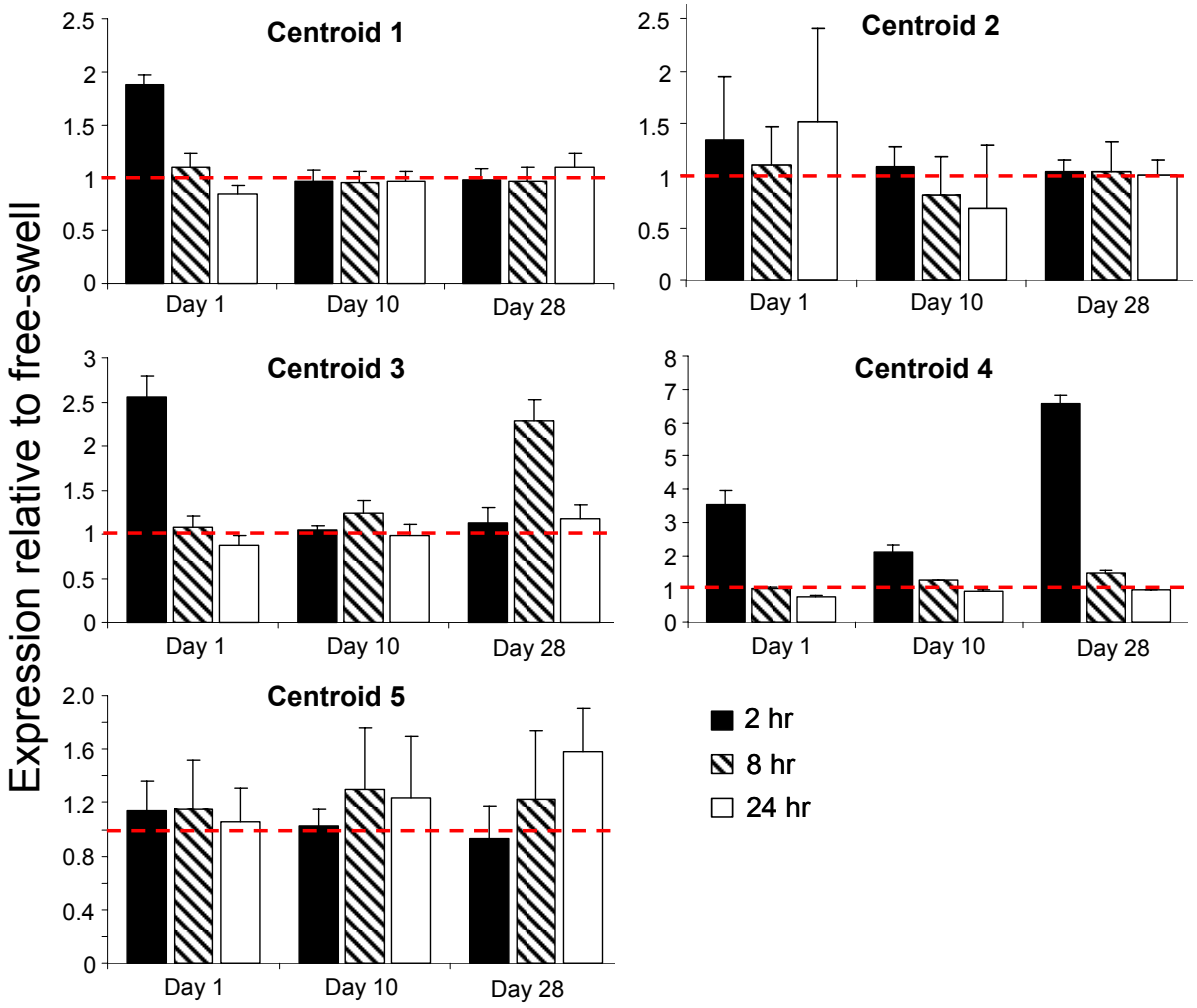

Figure 3.3 Centroid profiles for 5 clusters found by k-means clustering. Centroid profiles were calculated as the average expression profile (in response to 25% ramp-and-hold compression relative to free-swell) of genes within each cluster. Data shown as mean ± S.E.

Figure 3.4 Individual gene expression responses for genes in Cluster C2 show large variation

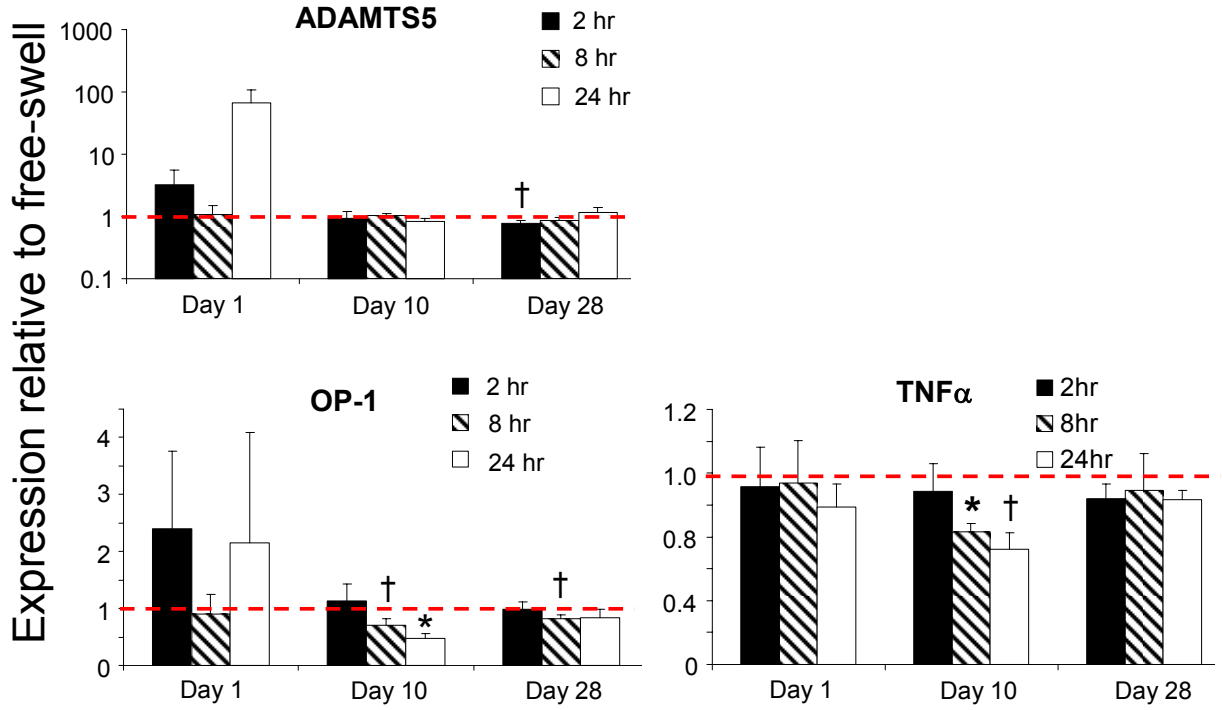

Figure 3.4 Individual gene expression responses of Cluster C2 genes ADAMTS-5, OP-1, and TNFα to 25% compression relative to free swell. Data shown as mean ± S.E. for 4 complete replicates. ADAMTS-5 and OP-1 displayed similar behavior on Day 1 in culture, while OP-1 and TNFα showed similar responses to compression on Days 10, 28. * $p<0.05$, † $p<0.1$ by t-test of log-transformed ratios compared to free-swell levels of log(1) = 0.

Figure 3.5 Centroid profiles for k-means clustering of gene expression responses on individual days in culture

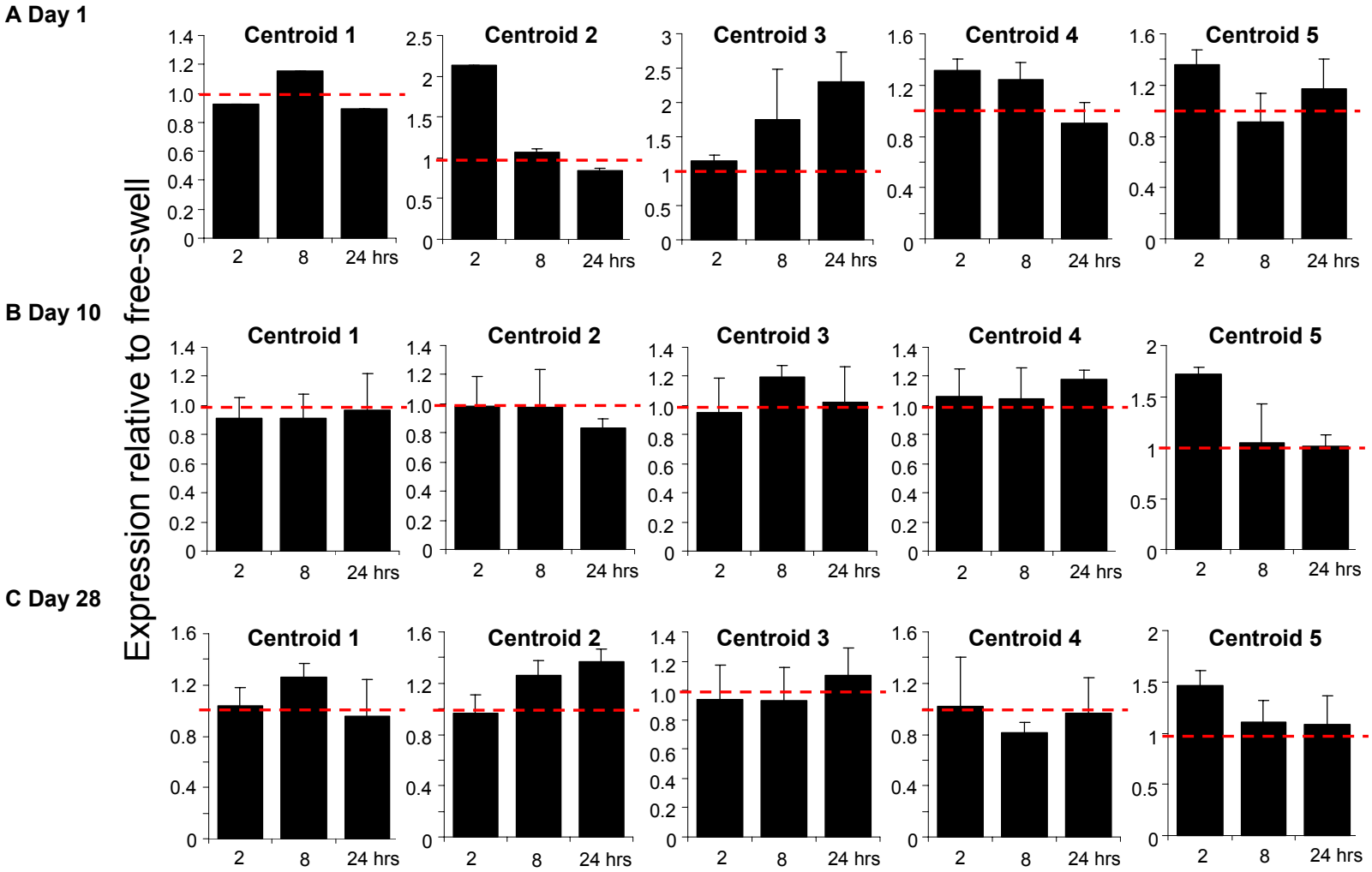

Figure 3.5 Centroid profiles for k-means clustering of gene expression responses to 2, 8, 24 hours of 25% compression relative to free-swell on A) Day 1, B) Day 10, C) Day 28 in agarose gel culture. Centroids profiles are calculated as the average gene expression profile for genes within a cluster. Data shown as mean ± S.E.

Figure 3.6 Individual gene expression profiles for co-clustering genes

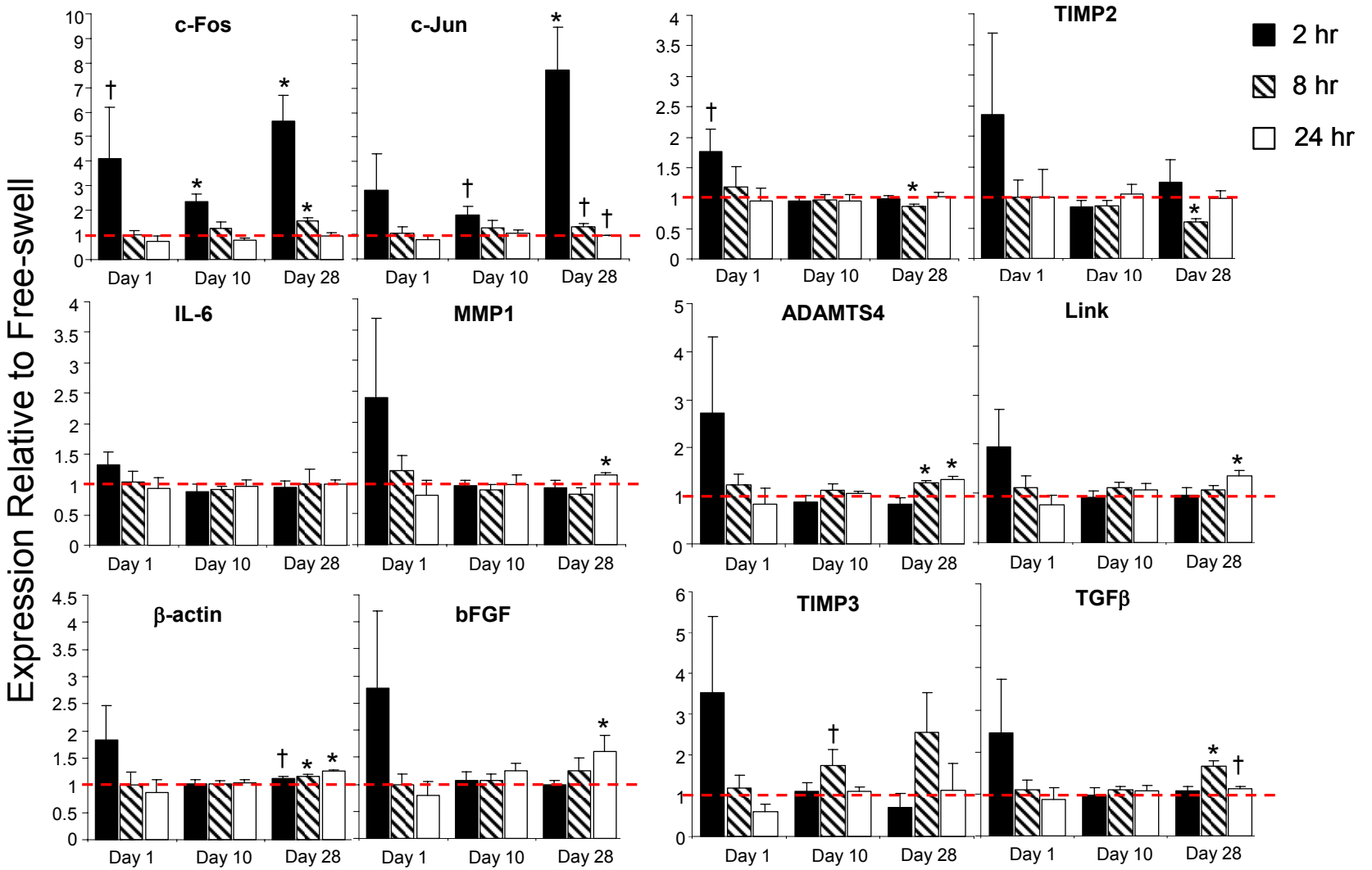

Figure 3.6 Individual gene expression responses for 6 pairs of co-clustering genes. Data shown as mean ± S.E. for 4 complete replicates. * $p<0.05$, † $p<0.1$ by t-test of log-transformed ratios compared to free-swell levels of log(1) = 0.

3.8 Supplementary Analysis – Self-organizing Maps

In addition to k-means clustering, we also examined the use of self-organizing maps (SOMs) to group co-expressed genes in our experimental system. The self-organizing map is an unsupervised neural network learning algorithm first developed by Kohonen (1984). An SOM starts with a set of nodes with a simple topology (e.g., two-dimensional grid) and a distance function. During the learning process, the nodes are iteratively mapped into "gene expression space," with nodes moving towards gene clusters. When training is complete, the clusters are identified by mapping the data to the closest node (reviewed in [1, 2]). Both k-means clustering and SOMs require pre-determining the initial number of clusters; in addition, SOMs require an initial topology [1]. SOMs are appealing in that they simplify and reduce the dimensionality of the expression data. In addition, they are more robust than k-means to noisy data. However, when clustering gene expression data, the SOM results are more dependent on the size of the clusters than on actual differences among gene profiles [1]. SOMs have been previously applied to DNA microarray data to examine yeast gene expression [3] and hematopoietic differentiation [4].

To create the self-organizing maps, we first standardized our gene expression vectors and then used principle component analysis as described above. We then applied the Self-Organizing map (SOM) clustering algorithm available in the neural network toolbox of MATLAB. The "newsom" function creates a Self-Organizing map network which can then be trained with the "train" function. Clusters were assigned by finding the nearest node to each point in the data set (see demo in MATLAB Help section). We found that a 2x3 hexagonal topology gave the most robust results. A 2-D plot of the self-organizing map is shown in Figure 3S.1, and the cluster constituents are listed in Table 3S.1. Unlike in k-means clustering, where all the closely related

genes were grouped into a single group of similarly behaving genes, the SOM nodes tended to congregate in areas more densely populated with genes. This predisposition of SOMs to depend more on cluster size [1] combined with our limited gene set (in comparison to microarray data) resulted in less than optimal grouping of genes that had more extreme behaviors. Overall, the average gene expression profiles for the clusters found by the SOM (Figure 3S.2) were similar to the centroid profiles found by k-means clustering (Figure 3.3); however, the individual behavior of genes within a cluster was not necessarily representative of the cluster as a whole (for example, TNF-α gene expression behavior is distinct from Cluster 3, although it is grouped in that cluster).

When clustering the temporal gene expression responses to compression (at 2, 8, 24 hours) on each day in culture (Day 1, 10, 28) separately, we found that as the data spread more evenly across the principle component space (Figure3S.3), the cluster components from SOM analysis became more similar to those found by k-means clustering. In the Day 28 clustering, taking into account 6 groups found in SOMs vs. 5 groups by k-means clustering, the groups of genes were almost identical, with the exception of only a few genes (Centroid profiles shown in Figure 3S.4).

To further investigate the biological significance of the clusters, we separated out two groups of strongly clustered genes observed in both clustering techniques: (1) those that showed upregulation at 2 hrs of compression on Day 1 in culture but little subsequent mechanical responses (ADAMTS4, CD44, Collagen 1, Collagen 2, HA-synthase, IGF-1, IGF-2, IL-1β, IL-4, IL-6, αv integrin, Link, MMP1, TIMP2) , and (2) those that showed a transition from early upregulation on Day 1 in culture to late (after 8 hrs of compression) upregulation on subsequent days in culture (α5 integrin, fibronectin, MMP13, MMP3, MMP9, TGFβ, TIMP1, TIMP3). We

used the Ingenuity knowledge base (http://ingenuity.com with the kind assistance of Rebecca Fry from Center for Environmental Health Sciences at MIT, see [5] for other applications of this program) to search for known interactions among these groups of genes. Out of the first group of genes, Ingenuity found 10 of 15 genes, 8 of which were in a single network (Figure 3S.5). This network is associated functionally most highly with cancer and cell cycle processes. This is an interesting finding, especially since chondrocyte proliferation and biosynthesis rates in agarose gel culture are higher on Day 1 in culture, and further distinguishes the Day 1 response to mechanical compression from that during subsequent days in culture. The probability of finding this network given the input list is less than 10^{-12}, calculated by a Fisher's exact test.

Although the genes we examined were pre-selected for their relevance to cartilage biology, there was no prior assumption of interactions between the individual genes. Out of the second group of genes, Ingenuity found 6 of the 9 genes, all of which were part of a single network (Figure S3.6, $p<10^{-17}$), whose function was associated with matrix development. This network is similar to what we described previously upon visual comparison with cartilage tissue response to mechanical compression, and includes AP-1 and NF-κB, further supporting our conclusion that this set of genes is similarly regulated in both cartilage tissue and agarose gel culture.

In conclusion, while SOMs can be a powerful tool for visually mapping large sets of gene expression data obtained from microarray analysis, because of our limited data set, k-means clustering appeared to find more relevant gene expression trends. This was especially pronounced given that a large portion of the genes examined behaved similarly with upregulation at 2 hours of compression on Day 1 in culture, but little response to compression on subsequent days in culture. When applying the SOMs to our data on individual days in culture, we found

that with the increased spread of the data across the "gene expression space," there was better agreement between both clustering techniques. Finally, by searching the Ingenuity knowledge base with groups of gene observed with both clustering algorithms, we were able to identify potential protein networks from the groups of co-expressed genes which further supported our visual observations and conclusions.

References

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3. Toronen, P., M. Kolehmainen, G. Wong, and E. Castren, *Analysis of gene expression data using self-organizing maps.* FEBS Lett, 1999. **451**(2): p. 142-6.
4. Tamayo, P., D. Slonim, J. Mesirov, Q. Zhu, S. Kitareewan, E. Dmitrovsky, E.S. Lander, and T.R. Golub, *Interpreting patterns of gene expression with self-organizing maps: methods and application to hematopoietic differentiation.* Proc Natl Acad Sci U S A, 1999. **96**(6): p. 2907-12.
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3.9 Supplementary Figures

Figure 3S.1 2-D Projection plot of self-organizing map and standardized gene expression vectors represented the two main principle components

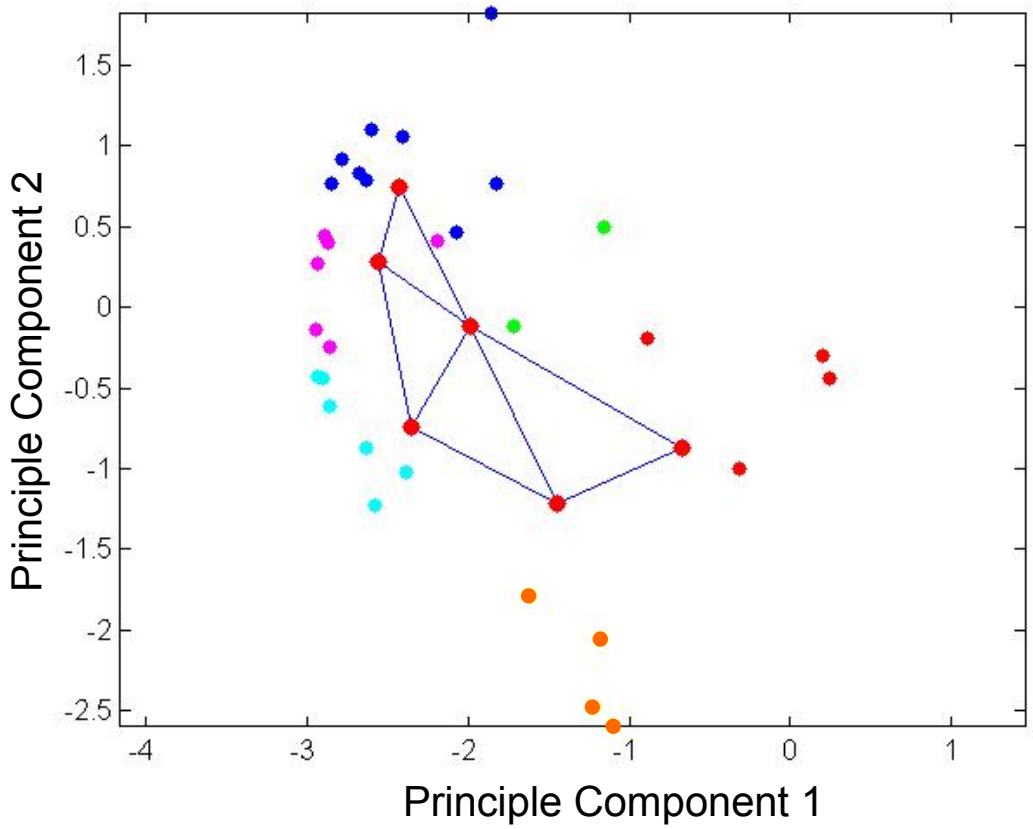

Figure 3S.1 2-D projection plot of self-organizing map generated from MATLAB, with 2 x 3 hexagonal initial topology and corresponding genes represented by the two main principle components obtained from PCA analysis of the standardized gene expression vectors.

Table 3S.1 Cluster constituents for self-organizing map clustering of complete data set as well as 2, 8, 24 hour timecourse on individual days in culture

Conditions Used in Clustering	Groups	Genes Clustered
A. Complete Timecourse (2,8,24 hrs)	CS1	ADAMTS5, aggrecan, cJun, Sox9
for Days 1, 10, 28	CS2	fibronectin, MMP3, MMP9, TIMP1
	CS3	cFos, TNFα
	CS4	β-actin, bFGF, α5 integrin, MMP13, TGFβ, TIMP3
	CS5	CD44, Collagen 1, HA-Synthase, IGF-1, IL-4, IL-6, MMP1, OP-1, TIMP2
	CS6	ADAMTS4, Collagen 2, IGF-2, IL1β, αv integrin, Link
B. Day 1 Timecourse (2, 8, 24 hrs)	DS1-1	Aggrecan, Sox9
	DS1-2	Collagen 1, IL1β, TIMP1, TNFα
	DS1-3	ADAMTS4, HA-Synthase, IL-4, IL-6, α5 integrin, Link, MMP1, MMP9, TIMP3
	DS1-4	ADAMTS5, fibronectin, MMP3, OP-1
	DS1-5	β-actin, bFGF, CD44, cFos, cJun, Collagen 2, IGF-1, IGF-2, αv integrin, MMP13, TGFβ, TIMP2
C. Day 10 Timecourse (2, 8, 24 hrs)	DS10-1	Aggrecan, β-actin, bFGF, cFos, cJun, Collagen 2, IGF-1, IGF-2, MMP9, MMP13
	DS10-2	Fibronectin, TGFβ, TIMP1
	DS10-3	ADAMTS4, HA-Synthase, α5 integrin, Link, Sox9, TIMP3
	DS10-4	CD44, Collagen 1, IL1β, IL-4, IL-6, MMP1, OP-1, TIMP2, TNFα
	DS10-5	ADAMTS5, αv integrin, MMP3
D. Day 28 Timecourse (2, 8, 24 hrs)	DS28-1	ADAMTS5, HA-synthase, IGF-2, IL-4, MMP1, Sox9
	DS28-2	Collagen 1, IGF-1, OP-1, TIMP2
	DS28-3	Aggrecan, IL-6
	DS28-4	CD44, cFos, cJun, Collagen 2
	DS28-5	ADAMTS4, β-actin, bFGF, fibronectin, αv integrin, Link, MMP9, TIMP1
	DS28-6	IL1β, α5 integrin, MMP13, MMP3, TGFβ, TIMP3, TNFα

Figure 3S.2 Average expression profile for clusters found by self-organizing maps

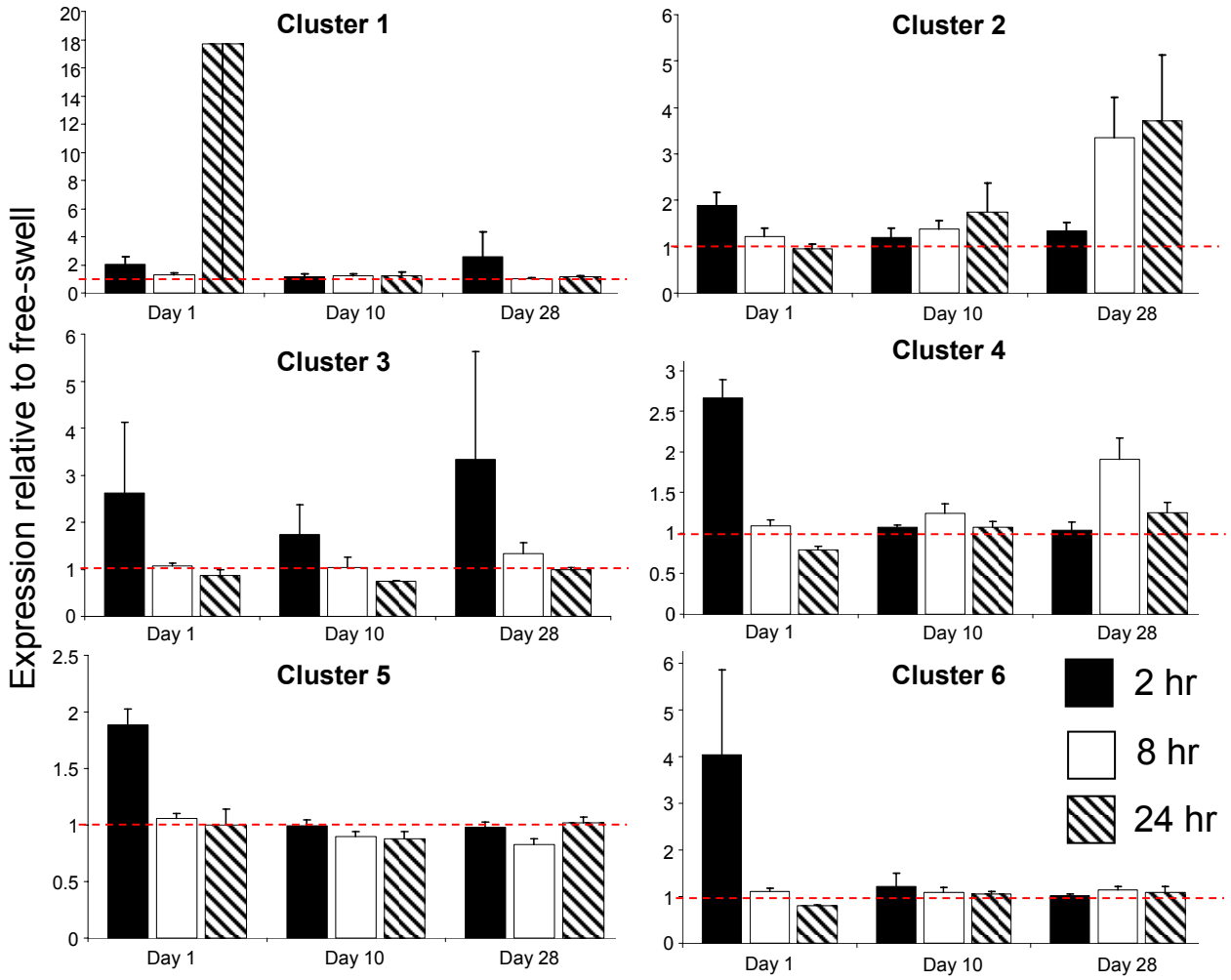

Figure 3S.2 Average gene expression profile in response to 25% compression for cluster found by self-organizing maps applied to complete data set. Data shown as mean ± S.E. of genes within each cluster.

Figure 3S.3 2-D Projection plots of self-organizing map and gene expression vectors for Days 1, 10, and 28 clustered separately represented by the two main principle components

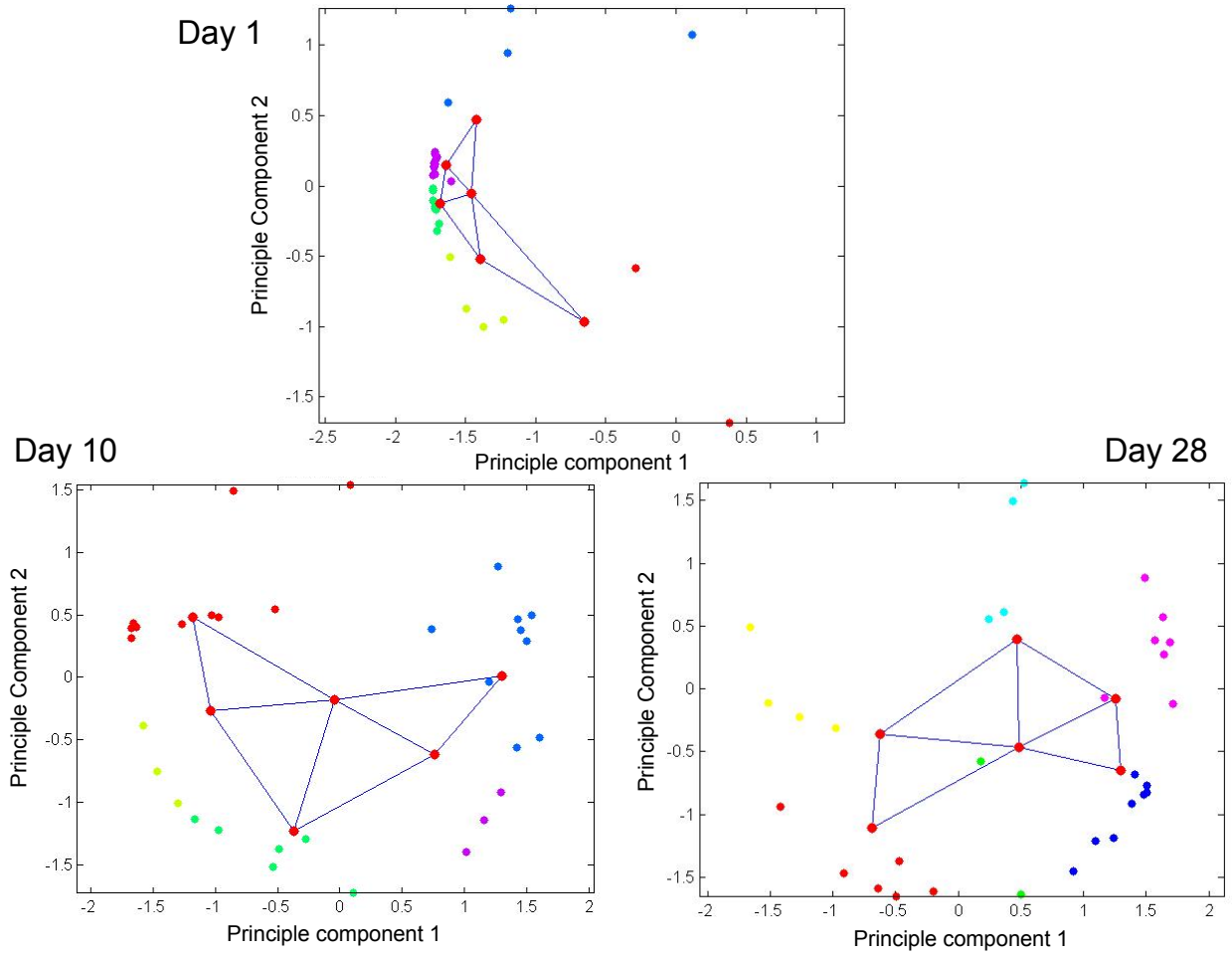

Figure 3S.3 2-D projection plot of self-organizing maps generated from MATLAB, with 2 x 3 hexagonal initial topology and corresponding genes represented by the two main principle components obtained from PCA analysis of the standardized gene expression vectors on Days 1, 10, and 28, clustered separately.

Figure 3S.4 Average expression profile for clusters found by self-organizing maps of gene expression on Days 1, 10, and 28 individually

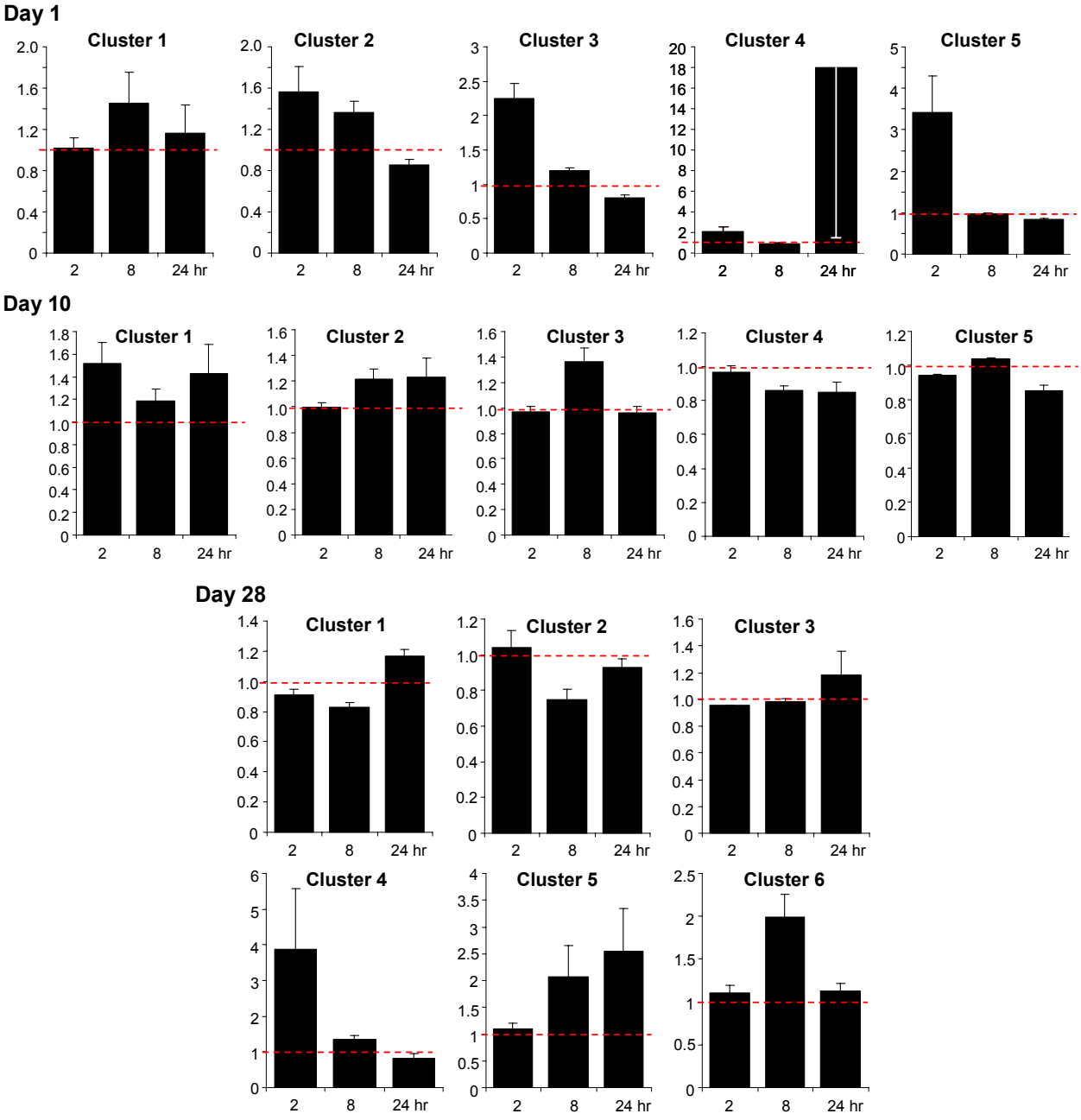

Figure 3S.4 Average gene expression profile in response to 2, 8, and 24 hours of 25% compression for clusters found by self-organizing maps applied to Day 1, 10, and 28 separately. Data shown as mean ± S.E. of genes within each cluster.

Figure 3S.5 Protein network identified by search using Ingenuity knowledge base

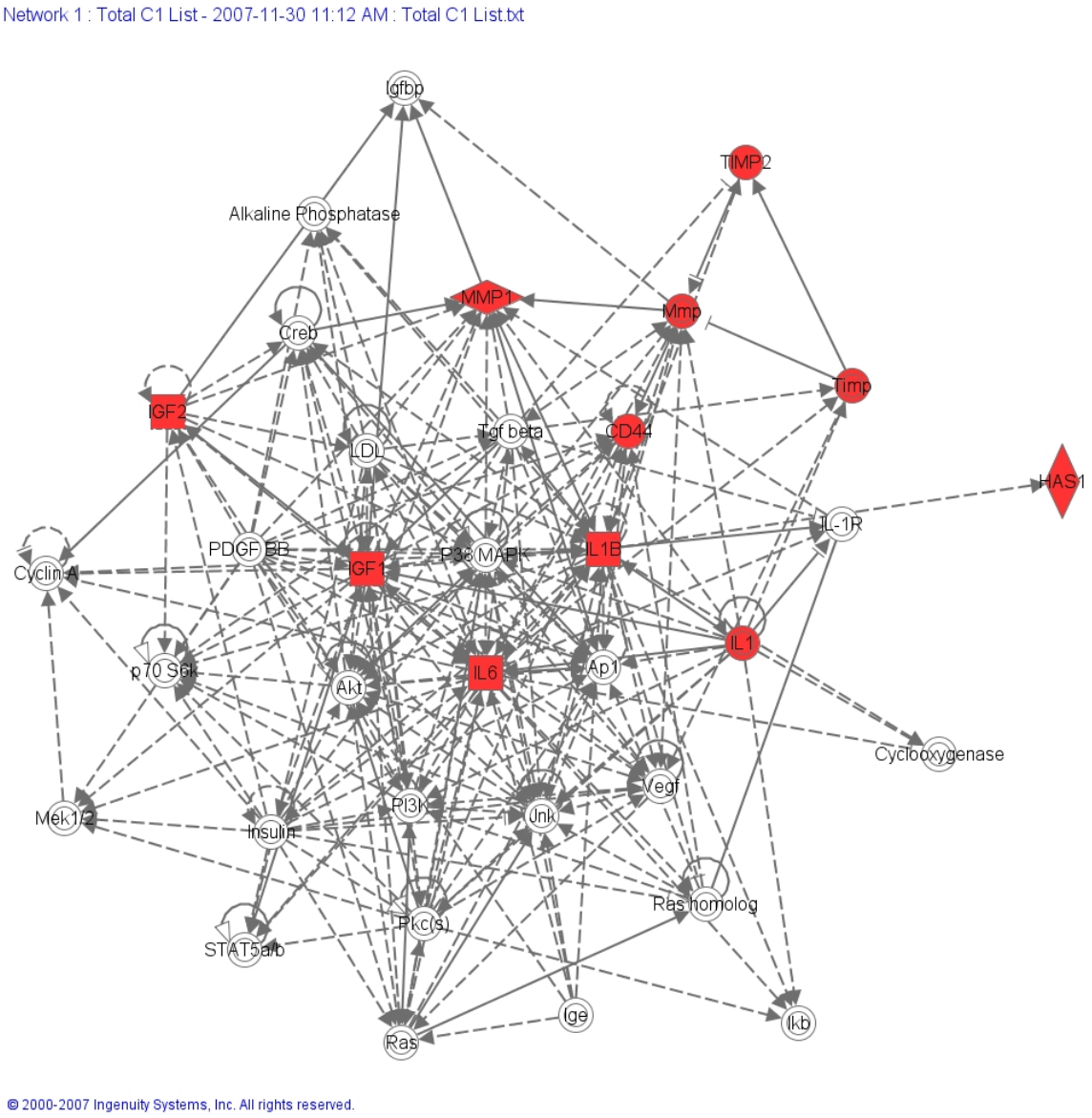

Figure 3S.5 Protein network identified by search using Ingenuity knowledge base. Out of genes that showed upregulation at 2 hrs of compression on Day 1 in culture but little subsequent mechanical responses (ADAMTS4, CD44, Collagen 1, Collagen 2, HA-synthase, IGF-1, IGF-2, IL-1β, IL-4, IL-6, αv integrin, Link, MMP1, TIMP2), Ingenuity found 10 of 15 gene products, 8 of which were in the network above. Probability of finding this network given the input list is less than 10^{-12}, by a Fisher's exact test.

Figure 3S.6 Protein network identified by search using Ingenuity knowledge base

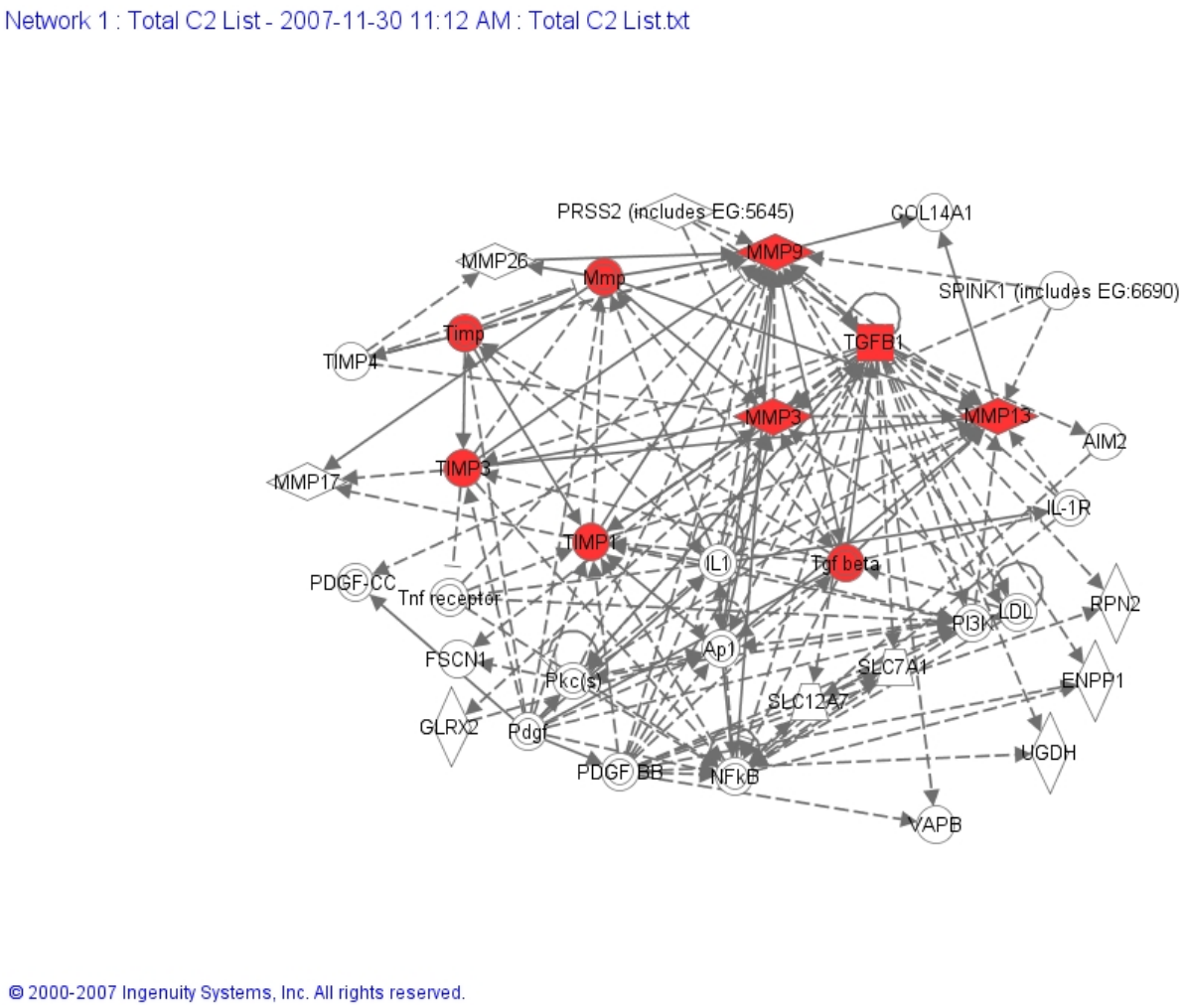

Figure 3S.6 Protein network identified by search using Ingenuity knowledge base. Out of the genes that showed a transition from early upregulation on Day 1 in culture to late (after 8 hrs of compression) upregulation on subsequent days in culture (α5 integrin, fibronectin, MMP13, MMP3, MMP9, TGFβ, TIMP1, TIMP3), Ingenuity found 6 of 9 gene products, all of which were in the network above. Probability of finding this network given the input list is less than 10^{-17}, by a Fisher's exact test

Figure 3S.7 Individual gene expression responses to 2, 8, 24 hours of 25% compression on Days 1, 10, and 28 in agarose culture

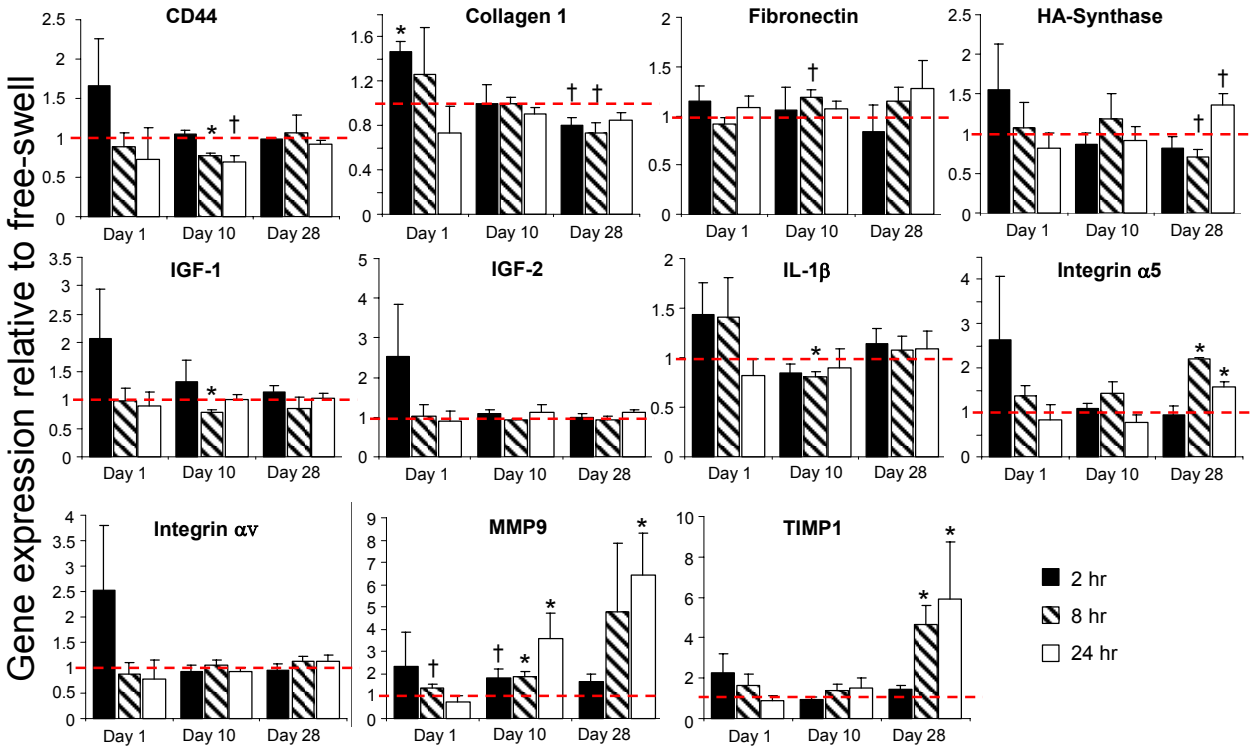

Figure 3S.7 Individual gene expression responses, measured by real-time RT-PCR, to 2, 8, 24 hours of 25% compression on Days 1, 10, and 28 in agarose culture. Data shown as mean ± S.E. for 4 complete replicates. * $p<0.05$, † $p<0.1$ by t-test of log-transformed ratios compared to free-swell levels of log(1) = 0.

Figure 3S.8 Biosynthesis measurements for Days 1, 10, 28 in agarose gel culture

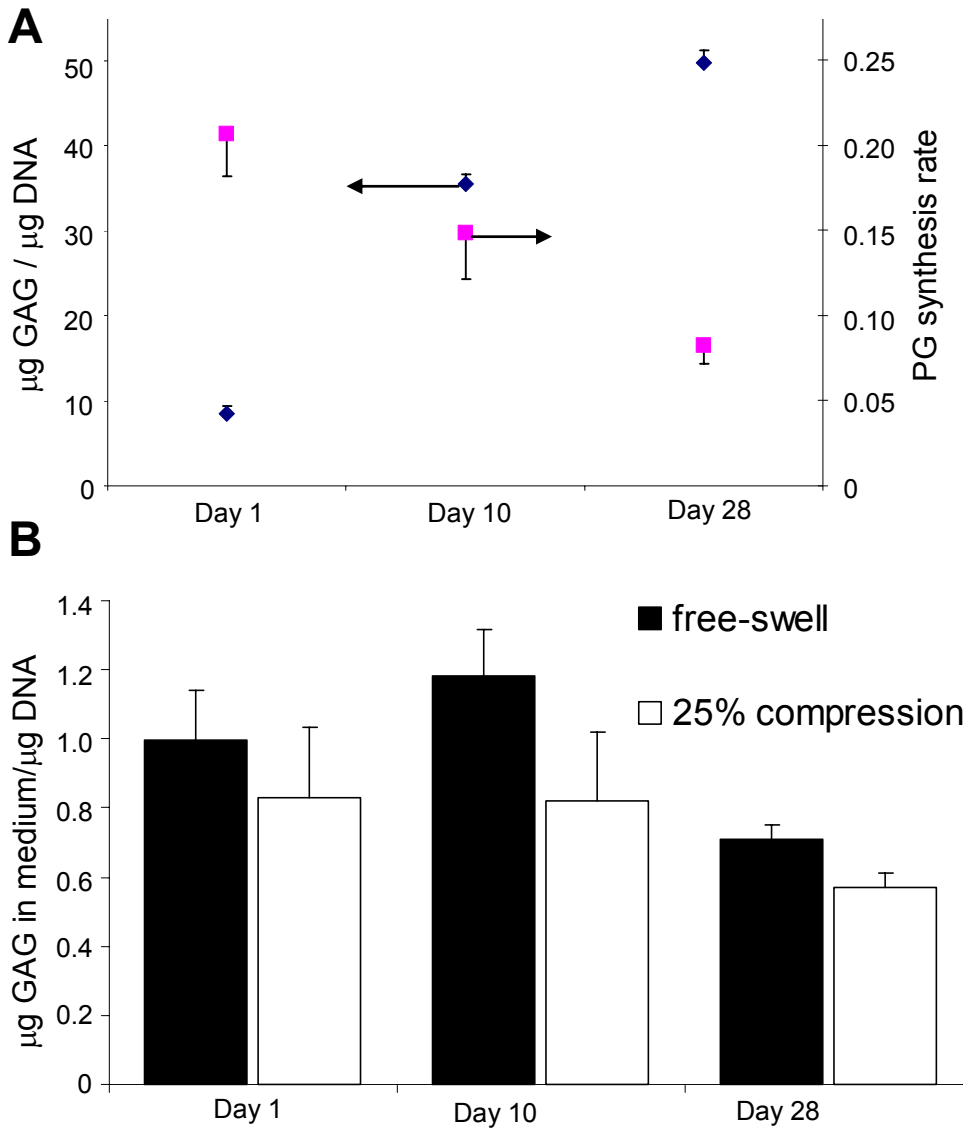

Figure 3S.8 Biosynthesis measurements for Days 1, 10, 28 in agarose gel culture. A) Glycosaminoglycan (GAG) content and proteoglycan synthesis rate, as measured by average sulfate incorporation rate over 24 hours, of free-swell samples on Days 1, 10, and 28 in agarose gel culture. B) GAG loss to medium over 24 hours under free-swell or 25% compression on Days 1, 10, 28 in agarose gel culture. Data shown a mean ± S.D. for 4 complete replicates.

Chapter 4

Mechanical regulation of MAPkinase (ERK1/2, p38, SEK) and Akt signaling pathways in chondrocytes in agarose cultures evolve distinctly with matrix development over time in culture

4.1 Introduction

Articular cartilage is an avascular, aneural, and alymphatic tissue with little capacity for regeneration. *In vivo*, cartilage experiences a variety of mechanical stresses, including dynamic and static compressive, tensile, and shear forces. Peak dynamic stresses of 15-20 MPa result in low strains of 1-3%, while sustained forces of approximately 3.5 MPa result in peak strains up to 40-45% [1, 2]. Osteoarthritis is a degenerative disease, found primarily in weight-bearing joints [3], and is characterized by the loss of matrix integrity, mechanical properties, and mechanical responsiveness of the tissue [4, 5]. Tissue engineering as a means of repairing cartilage defects has been extensively studied. While many approaches have shown promise in promoting chondrocyte phenotype, matrix development, and increased mechanical properties, given the mechanical environment and functionality of the tissue, the response of tissue engineered cartilage to mechanical loads can be important for long-term success.

In Chapter 3, we have shown that while gene expression responses of 3-D agarose culture of chondrocytes change with ECM accumulation and show similarities with responses of intact

cartilage tissue, the overall responses to mechanical stimulation at Day 28 in culture are still distinct from that of "parent" cartilage tissue. MAPkinase signaling has been shown to be important in cartilage response to mechanical stimulation. *In vivo*, rats fed a hard diet showed ERK and JNK activation which led to AP-1 activation [6]. Static compression and dynamic shear of cartilage explants *in vitro* have been shown to activate the MAPkinases in a distinct, time-dependent manner [7, 8]. Blocking MAPkinases ERK1/2 and p38 using small-molecule inhibitors altered downstream gene expression of the majority of genes examined, including matrix proteins and proteases, in these explant models, demonstrating the regulatory importance of MAPks in mechanotransduction [8]. Fluid flow has also been shown to decrease aggrecan promoter activity in monolayer chondrocytes in an ERK-dependent pathway [9]. While Akt signaling in response to mechanical stimulation has been studied in other tissue systems, only recently, in a quasi-*in vivo* model, Akt phosphorylation was shown to be differentially regulated by static and dynamic compression in a frequency-dependent manner in cartilage [10].

To further examine the mechanical signaling responses of chondrocytes in 3-D agarose culture, we investigated the MAPkinase (ERK1/2, p38, SEK) and Akt signaling kinetics of chondrocytes in agarose culture on Days 1 and 28 in response to ramp-and-hold compression. We then compared these responses to previous observations in intact cartilage to gain insights into similarities and differences of regulation.

4.2 Materials and Methods

4.2.1 Cell Harvest and Culture Chondrocytes were isolated from the femoral condyle cartilage of 2- to 3- week old bovine calves (Research 87, Marlborough, MA) by sequential digestion in 0.2% pronase (Protease type XIV, Sigma) and 0.025% Collagenase-P (Roche), as described previously [11]. Cells were counted by a hemocytometer and seeded in 2% agarose (low

melting-temperature, Invitrogen) at concentrations of 15 million cells/mL using a stainless steel casting frame [12, 13], in a slab geometry approximately 1.6mm thick. 4mm diameter plugs were cored from the slab using a dermal punch and cultured in 1% ITS supplemented feed medium (high glucose DMEM, 0.1mM nonessential amino acids, 0.4mM proline, 100U/mL PSA – penicillin, streptomycin, amphoteracin, 10μg/mL ascorbate) with medium changes every other day for up to 28 days.

For positive controls and to study the effects of compression on Akt signaling in intact cartilage, articular cartilage was harvested from the femoropatellar groove of 2- to 3- week old bovine calves (Research87), as previously described [7]. 3mm-diameter x 1mm-thich cartilage plugs were harvested from the middle zone of the cartilage and cultured for 2-3 days in 1% ITS supplemented feed medium (low glucose DMEM, 0.1mM nonessential amino acids, 0.4mM praline, 100U/mL PSA, 10μg/mL ascorbate) with medium changes every other day to recover.

4.2.2 Ramp-and-Hold Compression for Time Course Studies On days 1 and 28 in culture, groups of 8 plugs were placed in fresh 1% ITS supplemented medium and compressed over a 2-minute interval to a final strain of 25% [14] and held for either a short-term (10, 20, 40, 60 minute) or long-term (1, 2, 4, 8, 24 hour) time course. Free-swell cultures for short-term (10, 20, 40, 60 minute) or long-term (1, 2, 8, 24 hour) time courses in fresh 1% ITS medium served as controls. At the end of incubation, plugs were pooled, gently blotted dry with sterile gauze, and flash frozen in liquid nitrogen. For cartilage controls, groups of 8 cartilage plugs were placed in fresh 1% ITS supplemented medium and compressed over a 2-minute interval to a final strain of 50% [14] and held for 1, 2, 8, or 24 hours, with free-swell cultures as controls. At the end of incubation, plugs were pooled, gently blotted dry with sterile gauze, and flash frozen in liquid nitrogen.

4.2.3 Protein Isolation and Immunoblotting For each pooled sample, protein was extracted as previously described [7]. Briefly, samples were pulverized under liquid nitrogen and homogenized (BioSpec Products Inc) in 400 uL NP-40 type homogenization buffer with protease inhibitors (20mM Tris (pH7.6), 120mM NaCl, 10mM EDTA, 10% glycerol, 1% Nonidet P-40, 2mM Na3VO4, 100mM NaF, 10mM Na4P2O7, 1mM phenymethylsulfonyl fluoride, 40 ug/mL leupeptin). Homogenates were then extracted by end-over-end rotation at 4C for 1 hour and clarified by centrifugation (13,000g, 4C) for 1 hour. Total protein in the supernatant was quantified using the BCA Assay (Pierce). *For probing ERK1/2 and Akt phosphorylation state* – Aliquots of 30ug total protein suspended in Laemmli buffer were resolved by 10% SDS-PAGE (BioRad), transferred to Trans-Blot® nitrocellulose membranes (BioRad), and blocked in 5% BSA (ERK1/2) or 5% Nonfat milk (Akt) in PBST (phosphate-buffered saline +0.05% tween-20) for 2 hours at 37C (phospho-ERK1/2) or room temperature(RT) (total ERK1/2, phospho- and total Akt). Membranes were then incubated with phosphorylation state-specific antibodies (Cell Signaling Technology) overnight at 4C; washed with PBST (3x10 minutes); incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary (Cell Signaling Technology) for 1 hour at RT; and washed again with PBST (3x10minutes). *For probing p38 and SEK phosphorylation state* – Aliquots of 40ug total protein suspended in Laemmli buffer were resolved by 10% SDS-PAGE (BioRad), transferred to Immuno-Blot™ PVDF membranes (BioRad), and blocked in 5% milk in TBST (Tris-buffered saline +0.05% tween-20) for 2 hours at RT. Membranes were then incubated with phosphorylation state-specific (Cell Signaling Technology) overnight at 4C; washed with TBST (3x10minutes); incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary (Cell Signaling Technology) for 1 hour at RT; and washed again with TBST (3x10minutes). For the ECL reaction, immunoblots were

developed for 5 minutes using Amersham ECL Plus Western Blotting Reagent and visualized using a gel imaging system (Alpha Innotech). After imaging, immunoblots were washed 2x5minutes in wash buffer, stripped with Restore™ Plus Western Blot Stripping Buffer (Pierce), washed again 2x5minutes and reprobed with their respective phosphorylation state-independent antibodies (Cell Signaling Technology) to account for loading variabilities.

4.2.4 Statistical Analysis Band intensities were quantified using the AlphaEase™ FC v4.0 software. For analysis, phosphorylation-state specific band intensities were normalized to phosphorylation state independent bands. Then, the band densities within a given blot were normalized to the most common test condition between experiments. Four complete replicates (four different animals) were performed. Two-way ANOVAs (Systat12 software), with dependent values created by normalization removed, were used to determine whether significant changes ($p<0.05$) occurred.

4.3 Results

4.3.1 Time course of ERK1/2 phosphorylation in response to ramp-and-hold compression show distinct responses on days 1, 28 in agarose gel culture On Days 1, 28 in agarose gel culture, groups of plugs were subjected to 25% ramp-and-hold compression for a short-term (0-60 minute) or long-term (1-24 hour) time course. ERK1/2 phosphorylation was probed by Western blotting. On Day 1, ERK1/2 phosphorylation varied significantly with load duration (ANOVA, $p<0.001$ for ERK1, $p<0.05$ for ERK2). In both short-term (<60min) long-term (1-24hr) loading time courses, phospho-ERK1/2 levels under compression were highest at the earliest timepoints (Figure 4.1), with levels dropping to below that of free-swell samples by 24 hours. On Day 28, ERK1/2 phosphorylation also varied significantly with load duration (ANOVA, $p<0.001$) and showed highest levels of phospho-ERK1/2 at the earliest timepoints

(Figure 4.2). However, ERK1/2 activation remained elevated in compressed samples even at 24 hours (Figure 4.2). On both Days 1 and 28, ERK1/2 activation showed a bimodal decay, with rapid decrease in phospho-ERK1/2 levels between 1 to 4 hours of compression followed by a leveling out after 8 hours of compression (Figure 4.3). 25% compression induced a greater response in ERK1/2 phosphorylation relative to free-swell levels on Day 28 compared to Day 1 (ANOVA, $p<0.001$), with phospho-ERK1/2 levels peaking at an approximately 8-10 fold increase in compressed samples on Day 28 and 3-fold increase on Day 1 (Figure 4.3). In addition, phospho-ERK1/2 levels remained elevated under compression at 24 hours on Day 28, while phospho-ERK1/2 levels under compression drop to levels at or below that of free-swell by 24 hours on Day 1 (Figure 4.3). Phosphorylation kinetics of ERK1/2 of chondrocytes in agarose gel culture in response to 25% compression appear similar on Days 1, 28, but the overall magnitude of stimulation differ as the chondrocyte-gel construct develops.

4.3.2 Akt phosphorylation response to 50% compression in cartilage explants Akt is a signaling molecule in the PI3K pathway that has been implicated in cell growth, differentiation, survival, and motility. Previous studies on signaling cascades in response to mechanical compression in cartilage have focused on MAPKinase signaling. We examined Akt phosphorylation in response to 50% compression of intact cartilage explants over 1-24 hours by Western blotting with phosphorylation state-specific antibodies. In free-swell, Akt was weakly phosphorylated, with little variation for 24 hours after placement in fresh medium (Figure 4.4A). Under 50% compression, phospho-Akt levels were suppressed at all times observed, with no detectable levels of phospho-Akt after 2 hours of loading (Figure 4.4A). Trends were consistent across all four complete replicates. Interestingly, total Akt levels decreased significantly over the

load duration (ANOVA, $p<0.01$), with levels dropping to approximately 50% of free-swell levels after 8 hours of compression (Figure 4.4B).

4.3.3 Akt phosphorylation in response to 25% ramp-and-hold compression of agarose gel chondrocyte cultures similar on Days 1, 28 in culture To examine the effects of 25% ramp-and-hold compression on Akt activation in agarose gel culture, we examined Akt phosphorylation over both a short-term (10-60 minute) and long-term (1-24 hours) compression time course by Western blotting, as we did with ERK1/2, on Days 1 and 28 in culture. On Day 1, phospho-Akt levels varied significantly with load duration (ANOVA, $p<0.01$). Over the short-term time course, phospho-Akt levels were suppressed in compressed samples ($p<0.01$) (Figure 4.5A,C). Akt phosphorylation was similar in compressed and free-swell levels over the long-term time course, with slight upregulation (approximately 40%) at 24 hours (Figure 4.5B,D). On Day 28, phospho-Akt levels were similarly suppressed by compression over the short-term time course ($p<0.001$, Figure 4.6A,C). Over the long-term time course, Akt phosphorylation still showed slight downregulation ($p<0.05$, Figure 4.6B,D), with levels rebounding by 24 hours. Comparing the response of phospho-Akt relative to free-swell controls over both time scales on Day 1 v. Day 28, we saw no significant differences over the short-term time course, with suppression of phospho-Akt levels by approximately 60% at the earliest timepoint (Figure 4.7A). However, phospho-Akt levels appeared to recover more slowly at later times on Day 28 relative to Day 1, although this trend is not significant ($p=0.074$, Figure 4.7B). Total Akt levels on Days 1 and 28 showed no systematic changes over the loading duration. Overall, we observed similar levels of phospho-Akt downregulation in the initial response to 25% compression in agarose gel culture on both Day 1 and 28 of culture. Responses were similar between Days 1 and 28 in culture.

4.3.4 p38 and SEK showed minimal activation in response to 25% ramp-and-hold compression on Days 1 and 28 in culture We also examined the role of p38 and JNK MAPkinase pathways in agarose gel cultures of chondrocytes to 25% compression. Both pathways were transiently activated in a strain-dependent manner in cartilage tissue [7]. However, both were minimally phosphorylated in response to 25% compression of agarose gel cultures at all times examined. Low levels of phospho-p38 were detected at 10 and 20 minutes of compression on Day 28 in agarose gel culture (Figure 4.8C), but not on Day 1 in agarose gel culture (Figure 4.8B). No detectable levels of phospho-SEK (upstream activator of JNK) were found (Westerns shown in 4.8 Supplementary figures). To determine whether the pathways were intact, agarose cultures were treated with 10ng/mL IL1β+100 ng/mL TNFα on Days 1 and 28 [15]. Cartilage tissue compressed to 50% strain for 1 hour was used as an additional positive control [7]. Protein was extracted from each sample and immunoblotted for phospho-p38 and phospho-SEK (Figure 4.8A,D). phospho-p38 and phospho-SEK were found in response to 50% compression in cartilage, as well as in response to IL1+TNFα treatment, suggesting that both pathways are intact in agarose culture, but not stimulated by 25% compression.

4.4 Discussion

As previously discussed, many physical and biochemical signals can contribute to chondrocyte mechanical signaling. Cell deformation in agarose gel constructs decreases with development of the pericellular matrix [16]; however, modulus, streaming potential, permeability, and the development of cell-matrix interactions become more similar to intact tissue over time in culture [17]. Given the complexity of signals, we sought to study the role of MAPkinase signaling and Akt signaling in agarose cultures of chondrocytes and its dependence on matrix development over time in culture by examining temporal phosphorylation of these

signaling molecules in response to ramp-and-hold compression. By comparing the transient activation of MAPkinases ERK1/2, p38, SEK as well as Akt in agarose cultures to that in intact tissue, we hope to gain insights into relevant mechanotransduction pathways, and how tissue engineered constructs may behave under load.

Previous studies in cartilage have found that ERK1/2 is strongly phosphorylated in response to compression in a strain-dependent manner. The decay in phospho-ERK1/2 showed a bimodal temporal response, with a rapid decay from maximum levels of phosphorylation at early times ($t<2$hours) followed by a sustained level of ERK1/2 activation at later times (through 24 hours) in response to 50% compression [7]. Similarly, in agarose gel culture, ERK1/2 was phosphorylated transiently in response to 25% compression on both Days 1 and 28. However, sustained levels of phospho-ERK1/2 in response to compression were seen only on Day 28. In addition, peak levels of phospho-ERK1/2 were higher on Day 28 than Day 1.

In contrast to observations in intact cartilage [7], p38 and pSEK showed minimal responses to ramp-and-hold compression at all times observed. 50% compressed cartilage controls showed that this was a real observation and not a result of changes in immunoblotting technique. In addition, treatment of agarose cultures on both Days 1 and 28 with IL-1β and TNFα stimulated p38 and SEK phosphorylation as expected [15], suggesting that the signaling pathways were intact. While low levels of phospho-p38 were detected at 10 and 20 minutes of compression in Day 28 agarose cultures, they were much lower than stimulated by cytokines IL-1β and TNFα, possibly suggesting little functional relevance. It is possible that peak stimulation under compression occurred at times under 10 minutes; however, this would be technically difficult to confirm given experimental conditions. It is also possible that compression levels higher than 25% are required to stimulate p38 and SEK phosphorylation in agarose constructs;

once again, loss of agarose construct integrity and cracking at higher strains prevented testing this possibility. In addition, ERK1/2 responses at 25% ramp-and-hold compression in agarose culture were similar to that of 50% compression of cartilage, suggesting that this may be a functionally similar level of loading given the different material properties between agarose culture and intact cartilage tissue.

The increase in magnitude of ERK1/2 phosphorylation in response to mechanical loading with time in agarose gel culture is consistent with studies suggesting that accumulation of FGF-2 (bFGF) was necessary for loading-induced ERK1/2 activation [18]. In contrast with that study, we observed transient, but not sustained ERK1/2 activation, on Day 1 of agarose gel culture, when little to no FGF-2 would have accumulated in the pericellular matrix, suggesting that other mechanical signals are contributing to this ERK1/2 response. This discrepancy could also be due in part to the different loading or hydrogel culture systems used. Fluid flow has been shown to activate ERK1/2 signaling in monolayer chondrocytes [9]. Although fluid velocities during the transient phase of ramp-and-hold loading would be much lower than those used in the monolayer study, fluid flow could contribute to the transient upregulation of ERK1/2 observed at early points in loading on both Day 1 and 28 in agarose culture.

We also examined the role of Akt signaling in response to mechanical compression in intact cartilage as well as over time in agarose gel culture. In cartilage Akt has been implicated MMP2 linked actin reorganization during development [19], IGF regulation of chondrocyte differentiation [20], as well as OncostatinM and TGFβ signaling [21, 22]. In chondrocytes isolated from human ankle cartilage, Akt was shown to be stimulated by α5β1 integrins in response to dynamic mechanical stretch in monolayer [23]. However, its role in tissue-level mechanotransduction is not well understood. Recently, in a quasi-*in vivo* porcine model, Akt

phosphorylation was shown to be transiently downregulated by static and dynamic compression in a frequency-dependent manner in cartilage [10]. In our study, we found low basal levels of phospho-Akt in free-swell culture of cartilage explants. 50% compression over 2 hours in duration decreased phospho-Akt levels to below detection level. In addition, we observed a significant downregulation of total-Akt levels over 24 hours of 50% compression in intact cartilage tissue. This is in contrast to MAPkinase signaling, where no significant changes in total-MAPkinase levels were seen in response to loading [7], and to the studies in porcine patellofemoral joints where no changes in total Akt were described [10]. Akt degradation by caspases during apoptosis has been observed in other cell systems, and TNFα has been shown to induce caspase-dependent ubiquitination and subsequent degradation of Akt, impairing insulin signaling in adipocytes [24]. It is possible that a similar mechanism involving cytokine signaling is involved in Akt responses to mechanical compression. In cartilage IL-1 has been shown to at least partially contribute to static compression inhibition of proteoglycan synthesis. Blocking with IL-1 receptor antagonist increases sulfate incorporation in compressed, but not in free-swell, cartilage [25]. It is interesting to speculate that IL-1 may also be involved in changes in Akt, although IL-1 treatment of monolayer chondrocytes has only been shown to decrease Akt phosphorylation [26]. Further studies are necessary to elucidate the mechanism by which total-Akt levels are diminished in cartilage.

Unlike in cartilage, 25% compression of agarose gel cultures on Day1 and 28 of culture transiently decreases phospho-Akt levels during early times of loading (under 1 hour) with no observed effects on total Akt levels. There is also no significant effect of culture duration on the response to loading, although a slight delay in recovery of phospho-Akt levels is seen on Day 28.

This suggests that the mechanism of Akt signaling is unique to intact tissue, and that by Day 28 in agarose gel culture, this mechanism is not present in agarose gel culture.

One possible downstream effect of Akt signaling in cartilage is regulation of proteoglycan synthesis. IGF stimulation of proteoglycan synthesis in human chondrocytes in monolayer or alginate culture has been shown to require Akt, but not ERK1/2 signaling. Blocking Akt inhibits proteoglycan synthesis, in part through translation regulation [27]. In cartilage, 50% static compression greatly inhibits proteoglycan synthesis, while 25% compression in agarose up to Day 28 has little effect on proteoglycan synthesis rates [17]. Given the disparity in Akt signaling behavior in cartilage and agarose gel cultures in response to ramp-and-hold compression, it is likely that this plays a role in the biosynthetic response. Further examination is warranted.

4.5 Acknowledgements

NIH Grant AR33236 , NSF and NDSEG Graduate Student Fellowships for funding.

4.6 References

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4.7 Figures

Figure 4.1 ERK1/2 phosphorylation in response to 25% ramp-and-hold compression on Day 1 in agarose culture

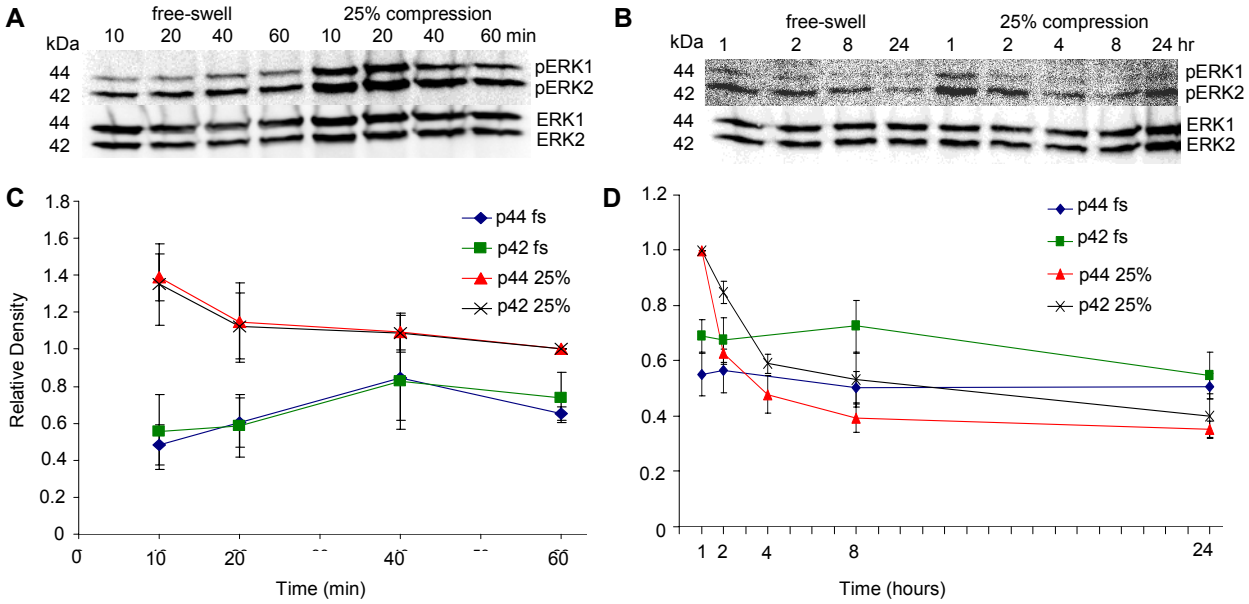


Figure 4.1 ERK1/2 phosphorylation in response to 25% ramp-and-hold compression on Day 1 in agarose gel culture. Representative phosphorylation state-specific and state-independent Western blots for A) short (10-60 minute) and B) long (1-24 hour) time courses. Equal amounts of protein were loaded for each condition. Optical density was used to quantify band intensities (C,D). Phosphorylation-state specific band intensities were normalized to phosphorylation state independent bands. Then, the band densities within a given blot were normalized to the most common test condition between experiments (1 hr 25% compression). Data shown as mean ± S.E. for 4 complete replicates.

Figure 4.2 ERK1/2 phosphorylation in response to 25% ramp-and-hold compression on Day 28 in agarose gel culture

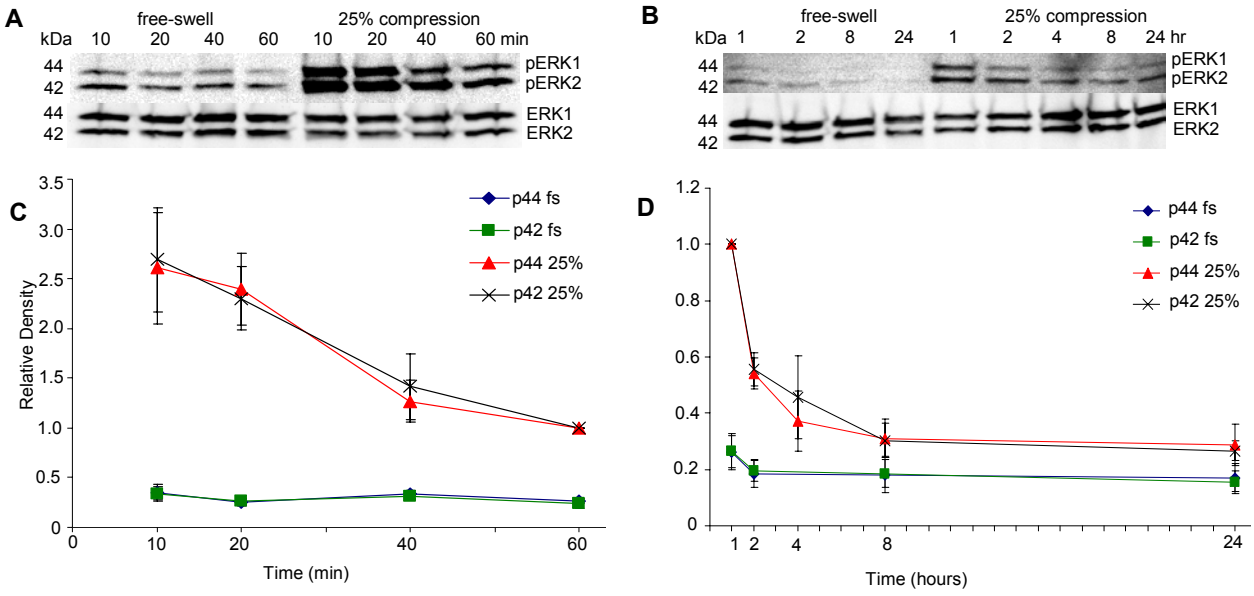


Figure 4.2 ERK1/2 phosphorylation in response to 25% ramp-and-hold compression on Day 28 in agarose gel culture. Representative phosphorylation state-specific and state-independent Western blots for A) short (10-60 minute) and B) long (1-24 hour) time courses. Equal amounts of protein were loaded for each condition. Optical density was used to quantify band intensities (C,D). Phosphorylation-state specific band intensities were normalized to phosphorylation state independent bands. Then, the band densities within a given blot were normalized to the most common test condition between experiments (1 hr 25% compression). Data shown as mean ± S.E. for 4 complete replicates.

Figure 4.3 ERK1/2 phosphorylation under 25% compression relative to free-swell on Days 1 and 28 in agarose gel culture

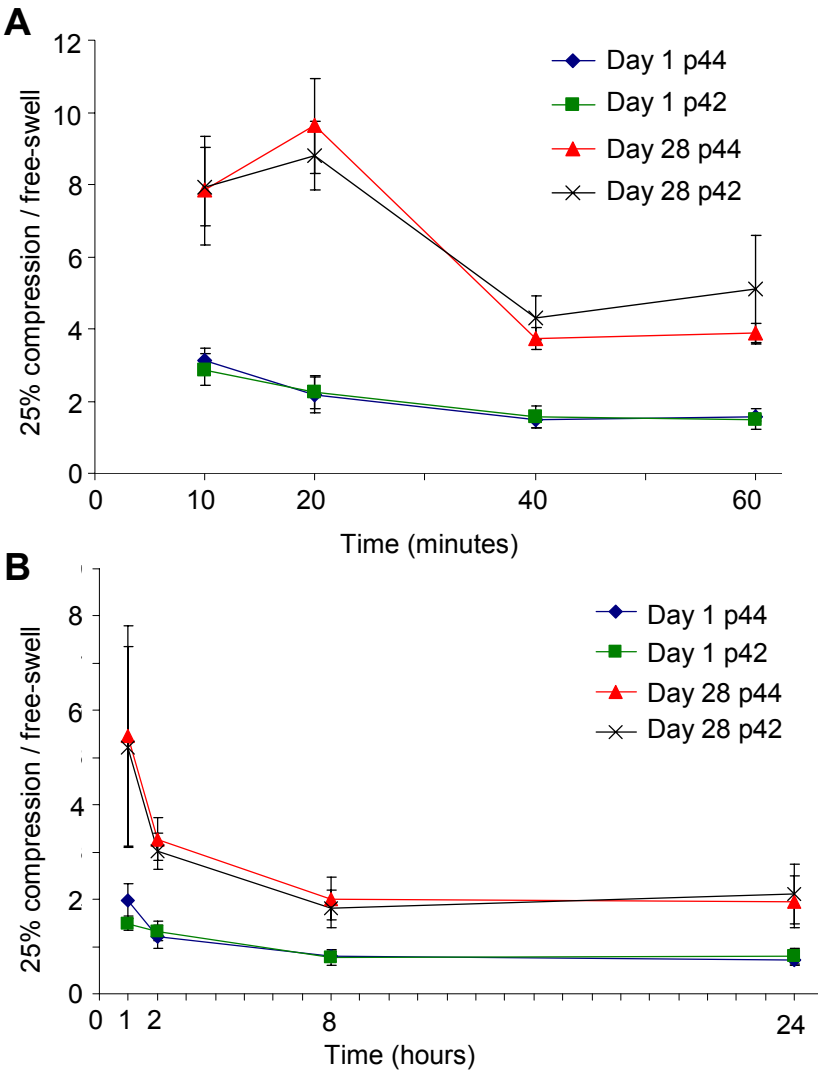


Figure 4.3 ERK1/2 phosphorylation under 25% compression relative to free-swell on Days 1 and 28 in agarose gel culture. Optical density was used to quantify band intensities. Phosphorylation-state specific band intensities were normalized to phosphorylation state independent bands. Then, the band densities for sample under compression within a given blot were normalized to their respective free-swell control. Data shown as mean ± S.E. for 4 complete replicates. Day 28 response is significantly different from Day 1 response, as determined by ANOVA, $p<0.001$.

Figure 4.4 Akt regulation by chondrocytes in intact cartilage in response to 50% compression

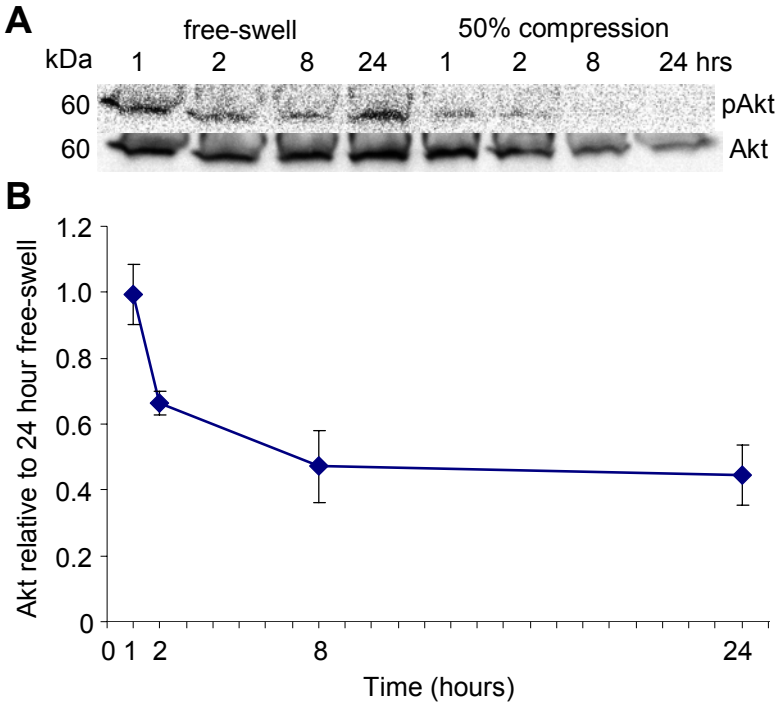


Figure 4.4 Akt regulation by chondrocytes in intact cartilage in response to 1, 2, 8, 24 hours of 50% compression. A) Representative blot of 1, 2, 8, 24 hour timecourse for free-swell and 50% compressed cartilage. Equal amounts of protein were loaded for each sample. B) Total Akt levels under 50% compression, as measured by optical densitometry, normalized to 24 hour free-swell controls. Data shown as mean ± S.E. for 4 complete replicates. Akt levels were significantly decreased in comparison to free-swell by ANOVA, $p<0.01$.

Figure 4.5 Akt phosphorylation in response to 25% ramp-and-hold compression on Day 1 in agarose culture

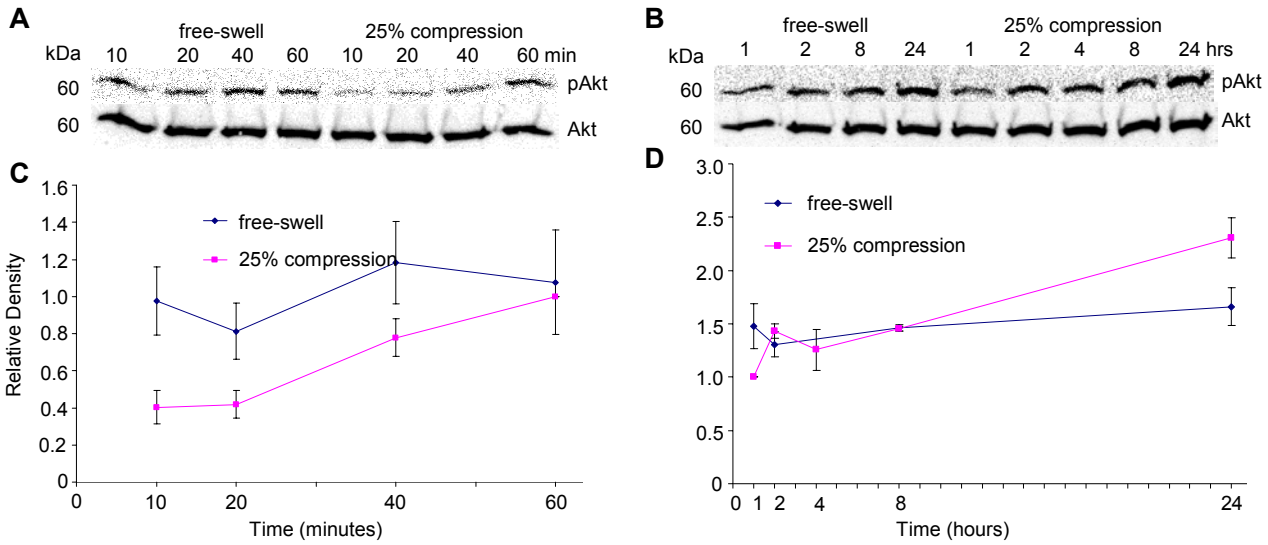


Figure 4.5 Akt phosphorylation in response to 25% ramp-and-hold compression on Day 1 in agarose culture. Representative phosphorylation state-specific and state-independent Western blots for A) short (10-60 minute) and B) long (1-24 hour) time courses. Equal amounts of protein were loaded for each condition. Optical density was used to quantify band intensities (C, D). Phosphorylation-state specific band intensities were normalized to phosphorylation state independent bands. Then, the band densities within a given blot were normalized to the most common test condition between experiments (1 hr 25% compression). Data shown as mean ± S.E. for 4 complete replicates.

Figure 4.6 Akt phosphorylation in response to 25% ramp-and-hold compression on Day 28 in agarose culture

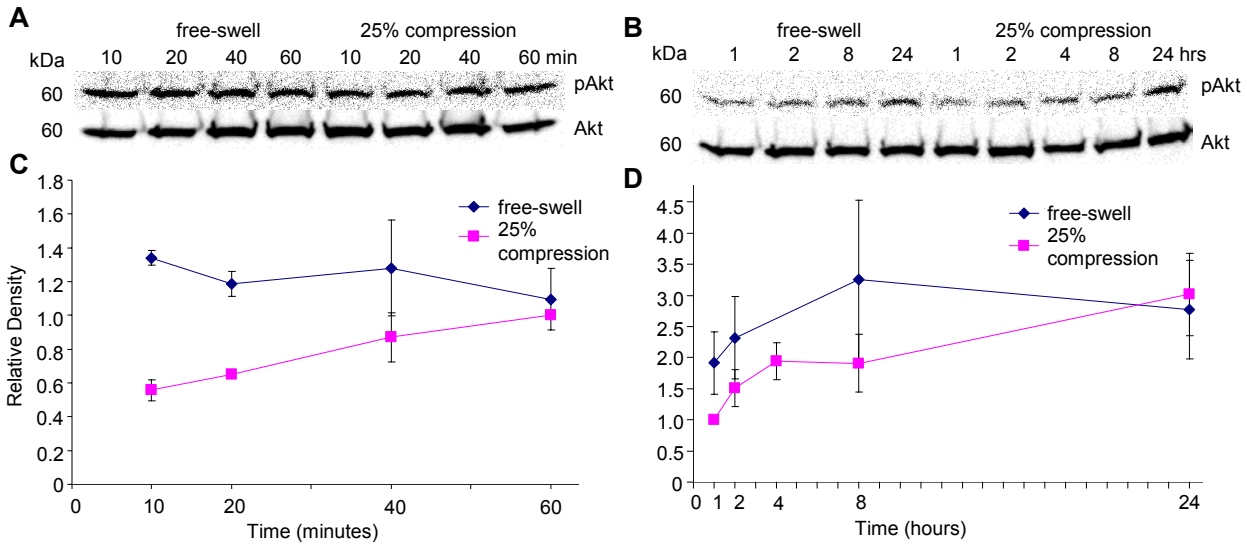


Figure 4.6 Akt phosphorylation in response to 25% ramp-and-hold compression on Day 28 in agarose culture. Representative phosphorylation state-specific and state-independent Western blots for A) short (10-60 minute) and B) long (1-24 hour) time courses. Equal amounts of protein were loaded for each condition. Optical density was used to quantify band intensities (C, D). Phosphorylation-state specific band intensities were normalized to phosphorylation state independent bands. Then, the band densities within a given blot were normalized to the most common test condition between experiments (1 hr 25% compression). Data shown as mean ± S.E. for 4 complete replicates.

Figure 4.7 Akt phosphorylation under 25% compression relative to free-swell on Days 1 and 28 in agarose gel culture

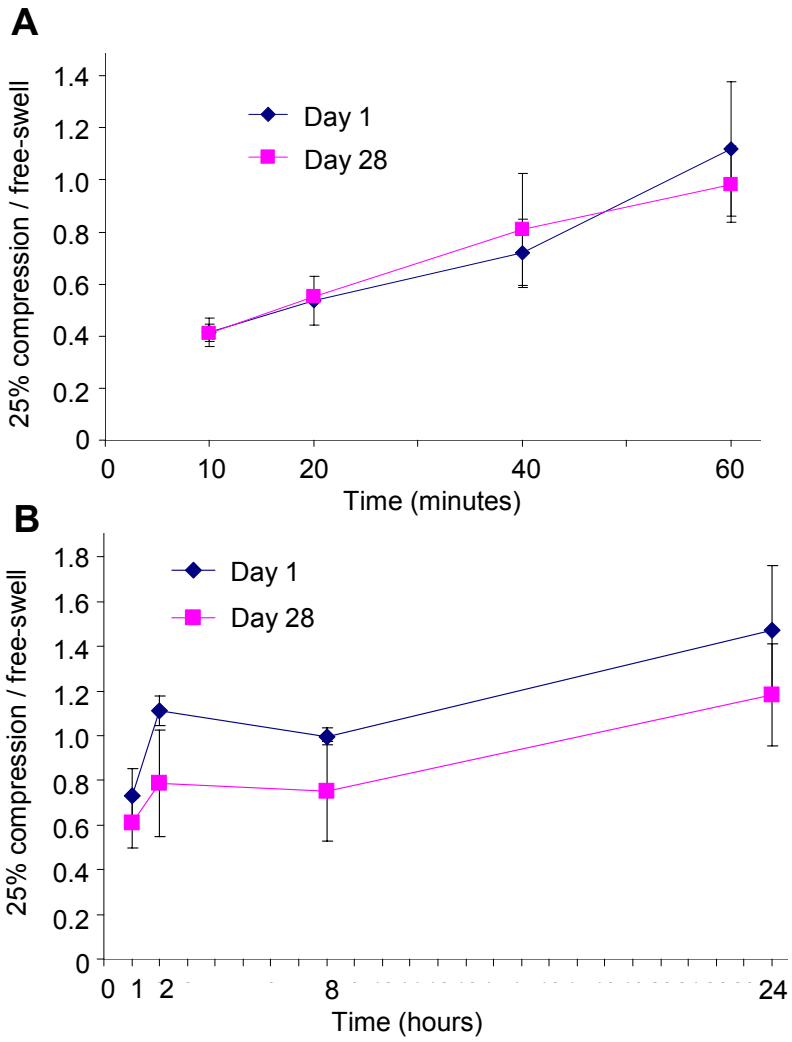


Figure 4.7 Akt phosphorylation under 25% compression relative to free-swell on Days 1 and 28 in agarose gel culture. Optical density was used to quantify band intensities. Phosphorylation-state specific band intensities were normalized to phosphorylation state independent bands. Then, the band densities for sample under compression within a given blot were normalized to their respective free-swell control. Unlike intact cartilage, no changes in total Akt were observed. Data shown as mean ± S.E. for 4 complete replicates. Day 1 and 28 responses are not significantly different.

Figure 4.8 Representative blots for p38 and SEK phosphorylation positive controls

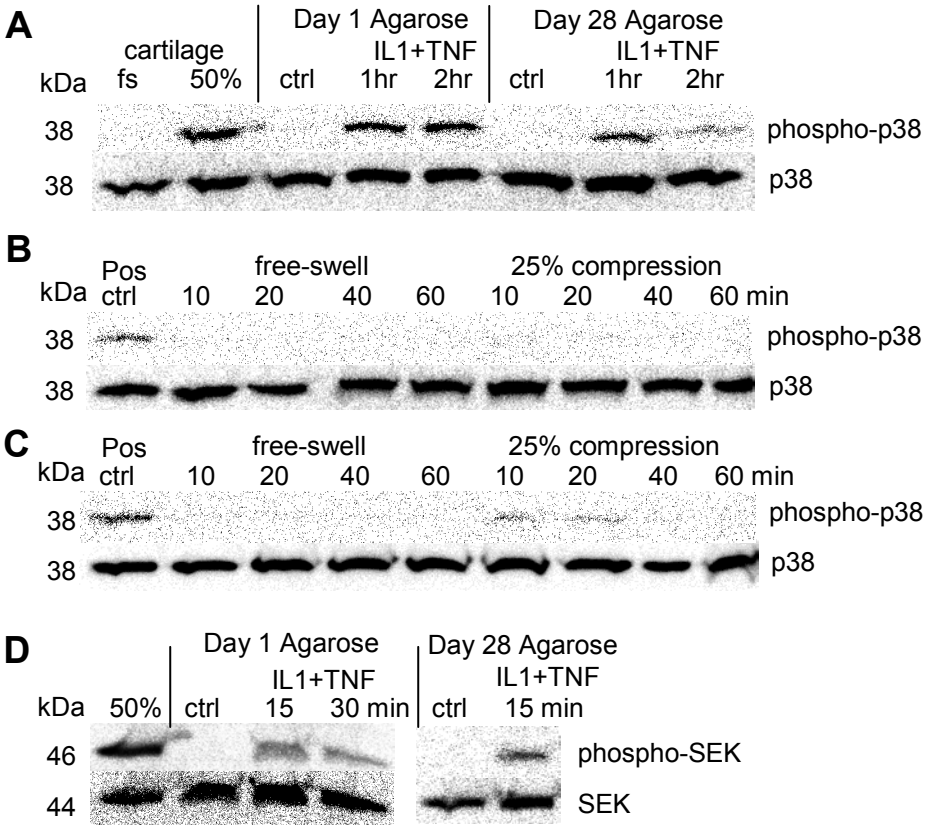


Figure 4.8 Representative blots for p38 and SEK phosphorylation positive controls and in response to 25% compression. Equal amounts of protein were loaded for each sample. A) Intact tissue and IL1+TNFα treated agarose cultures on Days 1, 28 show p38 phosphorylation. p38 in response to short-term (10-60 minute) compression on B) Day 1 and C) Day 28 in agarose culture shows little phosphorylation in comparison with positive controls. D) Intact tissue and IL1+TNFα treated agarose cultures on Days 1, 28 show SEK phosphorylation.

4.8 Supplemental Figures

Figure 4S.1 Western blot replicates for phospho-ERK1/2

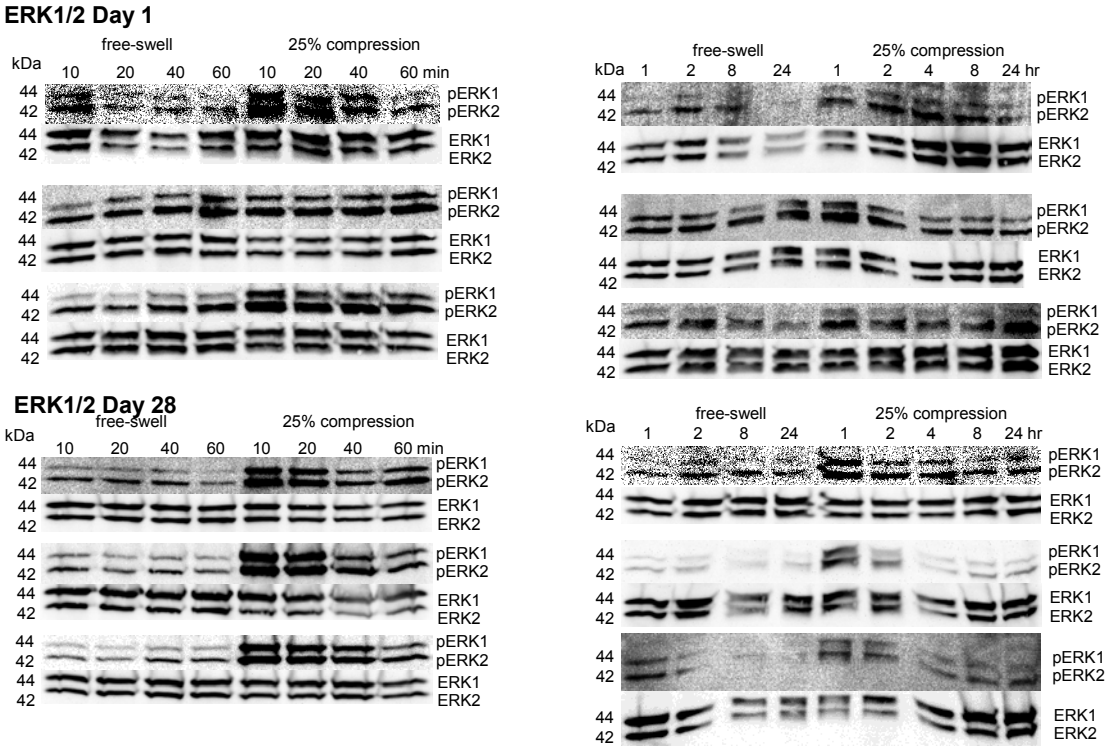


Figure 4S.2 Western blot replicates for phospho-Akt

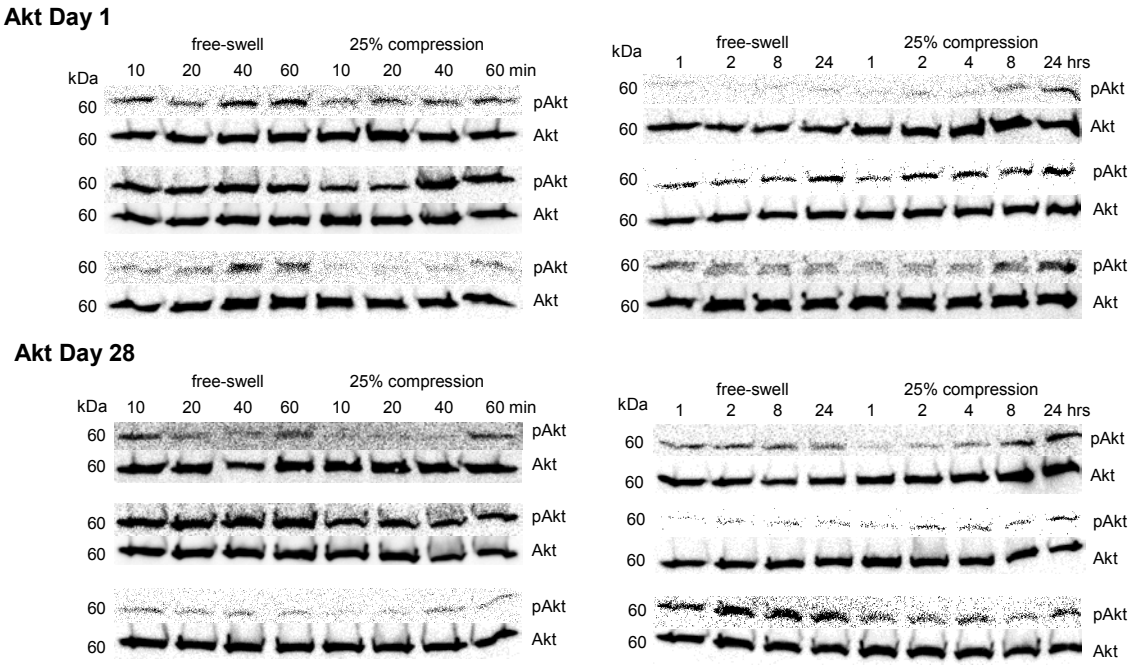


Figure 4S.3 Western blot replicates for phospho- and total- Akt in intact cartilage

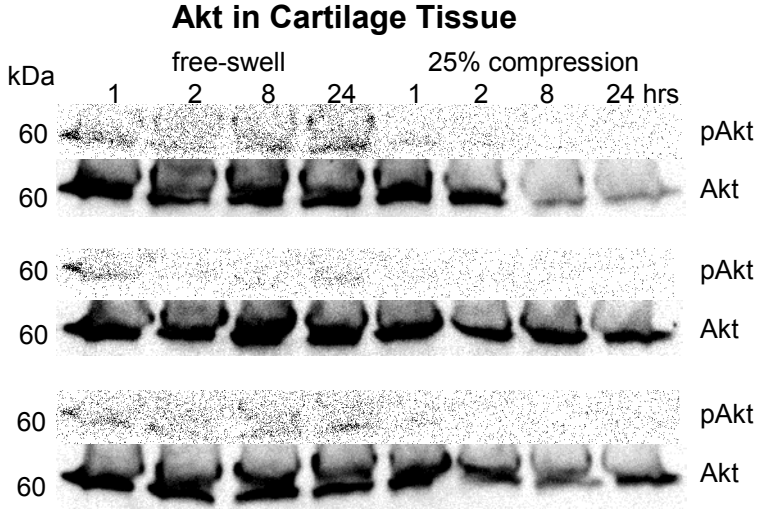
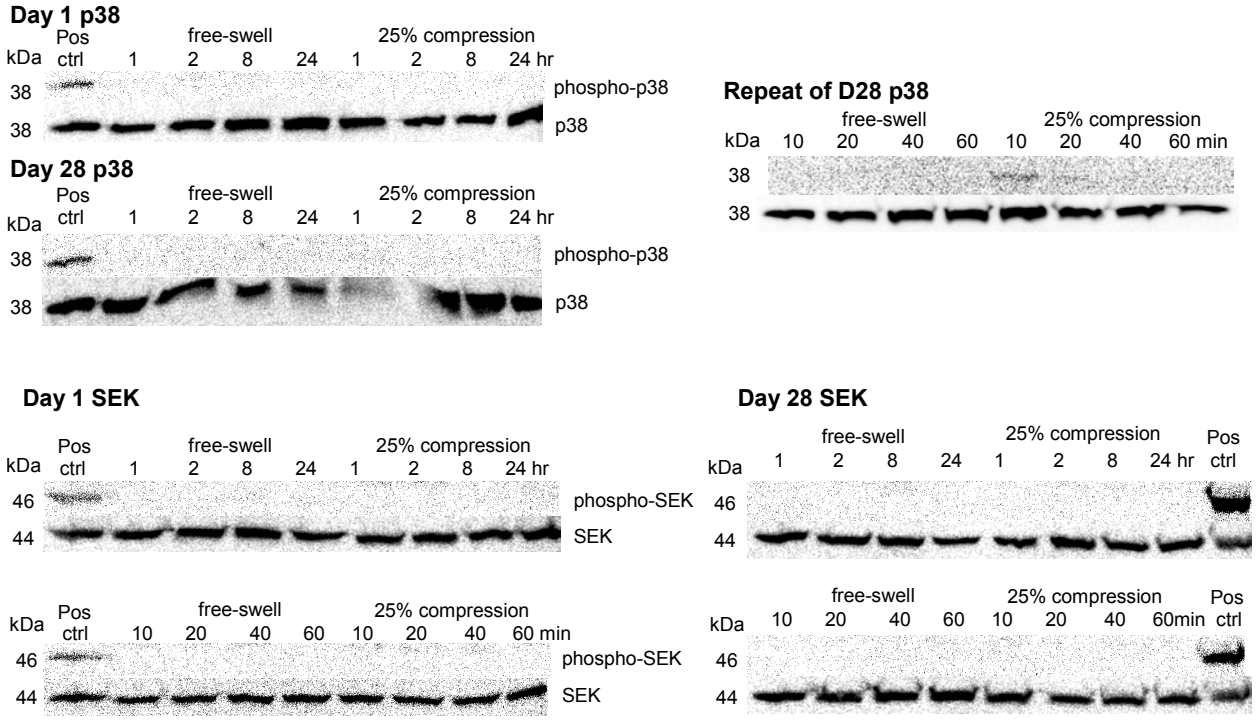


Figure 4S.4 Western blots for phospho-p38 and phospho-SEK in agarose cultures



Chapter 5

Conclusion

Studies have shown that mechanical loading of intact cartilage and chondrocytes in 3-D culture models can change chondrocyte biosynthesis, protein expression, intracellular signaling, and gene expression. Mechanical stimulation is increasingly used to stimulate extracellular matrix production and accumulation in tissue engineering [1-6]; however, the strains and forces that the cells experience during tissue growth changes with time in culture. Few studies have investigated how the evolving mechanical and biochemical microenvironment affects cell mechanical signaling with time in culture. The purpose of this thesis was to study chondrocyte mechanotransduction in 3-D agarose gel culture, and how mechanical signaling progresses with extracellular matrix accumulation over time in gel culture.

In Chapter 2, we investigated the role of integrin receptors in chondrocyte response to dynamic compression in agarose gel culture. In contrast to previous studies in monolayer, we found that multiple integrin receptors may play a role in regulating proteoglycan synthesis in response to dynamic compression. When blocking $\alpha 5\beta 1$ increases proteoglycan synthesis in free-swell cultures, dynamic compression does not increase synthesis above this level. In contrast, blocking $\alpha v\beta 3$ prevents dynamic compression stimulation of proteoglycan synthesis, independent of the effects of blocking $\alpha v\beta 3$ on free-swell cultures. This finding is consistent

with other studies on the role of ion channel signaling in chondrocytes, where more complex interactions were observed in 3-D compared to 2-D culture [7]. The complexity of response to mechanical stimulation can be reflective of both the more physiological configuration of 3-D gel culture as well as the greater variety of mechanical stimuli associated. In order to study the interplay of these mechanical stimuli and how they may differ from native tissue, we investigated the temporal gene expression regulation and MAPkinase and Akt activation in response to a ramp-and-hold compression and compared these results to those of native tissue.

In Chapter 3 we examined the temporal gene expression response of chondrocytes in agarose gel culture to 2, 8, and 24 hours of 25% ramp-and-hold compression on Days 1, 10, and 28 in culture and then compared this response to that of native tissue [8, 9]. We found that several genes were regulated similarly in agarose gel culture and in intact tissue. For example, in cartilage, the behavior of aggrecan, collagen 2, link protein, MMP1, and TIMP3 suggested that it was regulated largely by deformation. Similarly, these genes show similar temporal responses in agarose culture as in intact tissue on Day 1 of loading, where cell deformation is a dominant mechanical signal, as opposed to later days in culture, where cell deformation is prevented by the formation of the pericellular matrix [10]. In addition, we found that the temporal expression of c-fos and c-jun and its progression with day in culture correlated well with the progression of MMP3, 9, and 13 upregulation, suggesting a similar regulatory link involving AP-1 as found in native tissue [8, 9]. Through comparisons of expression profiles and gene groupings on Day 1 and Day 28, we found that the presence of a pericellular matrix greatly altered gene expression responses to mechanical stimulation. While on Day 1, most genes were upregulated during the transient phase of the ramp-and-hold compression, by Day 28, the temporal responses were more diverse. Despite the development of a pericellular matrix and mechanical properties more similar

to intact cartilage, expression response to mechanical compression still differed greatly from native tissue.

In order to further examine the changes in mechanical signaling over the course of agarose gel culture, and differences between gel culture and intact tissue, we examined the temporal activation of MAPkinase (ERK1/2, p38, SEK) and Akt signaling pathways in response to 25% compression on Days 1 and 28 in agarose gel culture and compared them to intact tissue [11]. Consistent with previous studies suggesting that FGF-2 accumulation in the pericellular matrix was necessary for ERK activation in response to load [12], we found that ERK1/2 activation in response to compression behaved more similarly to intact tissue after 28 days in culture. In contrast, p38 and SEK showed little activation in response to mechanical compression on both Day 1 and 28 in culture, although transient activation of p38 after 10 and 20 minutes of compression was observed on Day 28 in culture.

The shift from ERK1/2 dominated MAPk signaling on Day 1 to participation of p38 on Day 28 agrees with the changes in MMP gene transcription. MMP13 transcription has been shown to be induced by bFGF [13], IL-1β [14], TNFα [15], fibronectin fragments [16], mediated by MAPkinases ERK1/2, p38, and JNK, as well as the AP-1 transcription factor. Based on this and previous studies suggesting the importance of bFGF in ERK1/2 mediated signaling in 3-D cultures of chondrocytes [12], it is interesting to speculate that bFGF signaling, in part through IL-1β, transiently activates MAPkinases ERK1/2 and p38, which leads to transient upregulation of MMP13 under compression. In contrast, the transient upregulation of ERK1/2 on Day 1 of culture may be due to a different mechanism, which would explain the different kinetics for MMP13 expression in response to compression. This mechanism is further supported by studies in intact tissue that have shown that blocking MAPkinases p38 or ERK blocks transcription of

the matrix metalloproteinases, while blocking p38 only partially suppresses transcription of c-Jun and c-Fos [17], as well as a role for IL-1β in static compression suppression of proteoglycan synthesis in cartilage [18]. Further studies into blocking different aspects of this pathway would be necessary to confirm this regulatory link.

In addition, previous studies in intact cartilage has shown that blocking MAPkinases ERK1/2 and p38 affects the gene transcription response of many cartilage-associated genes [17]. Given the altered balance of p38 and ERK signaling in agarose gel culture in comparison to intact cartilage, it is not surprising that gene expression profiles are unique from cartilage even at Day 28 in culture. Also, the dominance of ERK signaling in response to mechanical compression on Day 1 in culture explains why many genes were coexpressed in response to compression on Day 1 in contrast to Day 28 in culture.

We also examined the role of Akt signaling in cartilage and in chondrocytes in agarose gel culture in response to compression. Akt signaling has roles in development and as a downstream effector of growth factors such as IGF and TGFβ, but its role in mechanotransduction is not well understood. We found that 50% compression in cartilage abolished the low basal levels of Akt phosphorylation found in free-swell cartilage. In addition, we found significant downregulation of total Akt levels in cartilage over 24 hours of compression, which had not been previously described in cartilage. A potential role of Akt signaling is regulation of aggrecan syntheses; however, further studies into the mechanism of Akt regulation and the implications of Akt downregulation are warranted.

Unlike in cartilage, we did not observe changes in total Akt levels in agarose gel cultures of chondrocytes. Phospho-Akt levels were transiently suppressed by compression, but recovered to levels at or above free-swell levels by 1 hour of loading. Akt signaling response was similar

on both Day 1 and 28 in cultures. This suggests that the mechanical and/or biochemical cues regulating Akt signaling are similar on both days in culture, and that these are unique from cartilage. In chondrocytes, Akt signaling can influence regulation of ADAMTS-4, MMP3 [19], TIMP3 [20] gene expression. Further studies are necessary to determine the role they play in chondrocyte mechanotransduction.

In conclusion, we have found that 3-D agarose gel culture can be a good model for studying chondrocyte mechanotransduction in a more native environment than monolayer culture. However, differences in the local mechanical and biochemical environment of chondrocytes result in different overall responses to load than intact cartilage, although similar pathways can be identified in both cartilage and agarose gel cultures. Finally, by examining the gene expression and intracellular signaling responses of agarose gel culture in comparison to native tissue, we have provided a base of information for understanding how tissue engineered constructs compare to native tissue and for determining which mechanical cues will result in optimal tissue development.

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Appendix A: Western Blotting for Signaling Protein Phosphorylation

Protein Purification:

Homogenisation Buffer (HB) – make every 2 weeks

Material	Concentration	MW (Da)	Amount/100 mL
TrisHCl (pH7.6)	20mM	121.1	0.2422 g
NaCl	120mM	58.44	0.70128 g
EDTA (disodium salt)	10mM	372.24	0.37224 g
Glycerol	10%	---	10 mL
NP-40	1%	---	1 mL

Add fresh each time:

Na3VO4	2mM	183.9 g/mol	0.7356 g/20mL dH2O pH10 for 20 vials of 100x – see product insert
NaF	100mM	41.99	0.8398 g/20mL dH2O for 20 vials of 10x
Na4P2O7	10mM	446.1	0.8922 g/20mL dH2O for 20 vials 10x
PMSF	1mM	174.2	0.03484 g/2mL isopropanol for 20 vials of 100x
Leupeptin	40 ug/mL	---	0.008g/2mL dH2O for 20 vials of 100x

Store at –20 degrees Cm PMSF is stable for less than 1 hr at room temp. EDTA inhibits activity of metalloproteases, PMSF and leupeptin against serine and cystein proteases, NaF, sodium orthovanadate, sodium pyrophosphate inhibit activity of diphosphatases. *can also use pepstatin A, an acid protease inhibitor, or aprotinin, a serine protease inhibitor*

1. Prepare HB + protease inhibitors
2. Immerse pulverizers in liquid nitrogen until cold.
3. Pulverize 6-8 samples (4mmx1.6mm disks) by pounding 12 times
4. Transfer pulverized sample to 5mL tube
5. Add 400uL HB per tube
6. Repeat steps 2-5 for all samples, keeping samples on ice.
7. Homogenize samples using tissue tearor for about 15s per sample
8. Transfer to 1.5mL eppendorf tubes and sonicate for 10 seconds
9. Rotate end-over-end for 1 hour at 4 degrees C.

10. Clarify by centrifugation at 13000g for 1 hour at 4 degrees C.
11. Transfer to clean eppendorf tube. Quantify by BCA assay using full 20uL sample.
12. Concentrate samples by lyophilization:
    a. 30μg protein for ERK and Akt blots
    b. 40μg protein for p38 and SEK blots

Running SDS-PAGE Gel:

Materials:
10x Running buffer – 30g Tris Base (25mM), 144g Glycine (192mM), 10g SDS (0.01%), pH 8.3 in 1 Liter

Loading buffer (LB) – BioRad Laemmli buffer + 5% β-Mercaptoethanol

10% SDS-PAGE Gel

1. Heat water to boil in glass container
2. Add 25uL LB to each concentrated sample, vortex, and boil for 5 min.
3. Set up gels and add 1x running buffer
4. Remove boiling samples, vortex, and spin down briefly
5. Apply 10uL low-range standard and up to 30uL sample to each well.
6. Run gel at 90-100V for approx 2-2.5 hours at Room Temp

Transfer:

Materials:
Transfer buffer – 3g Tris Base (25mM), 14.4g Glycine (192mM), pH8.3, 0.5g SDS (0.05%) in 800mL. Add 200mL Methanol (20%)

Nitrocellulose membrane for Akt/ERK; PVDF membrane for p38/SEK

Transfer setup – sponges, blotting paper, ice block, stir-bar

1. Cut nitrocellulose to size of gel and soak in water for 1 min. If using PVDF, wet with 100% methanol for 5 seconds before soaking in water.
2. Pour transfer buffer into metal dish over ice. Soak sponges, blotting paper, and allow gel to equilibrate in transfer buffer for approx 15 min. Separately soak nitrocellulose/PVDF in transfer buffer for 15 minutes.
3. Assemble in correct order (bottom to top):
    a. Black panel, sponge, blotting paper, gel, nitrocellulose/PVDF, paper, fiber pad, clear panel
    b. Use tweezers with nitrocellulose/PVDF
    c. Use 15 mL conical tube to roll out bubbles after last blotting paper and fiber pad
    d. Avoid overhang with nitrocellulose/PVDF
4. Close cassette and place black on black
5. Transfer at 90V for 45 minutes at room temp (with ice block).

Western Blotting:

Materials:
1x PBS
1xPBS + 0.05% Tween-20 (PBST)
1xTBS+0.05% Tween-20 (TBST)
5% Milk in PBST/ 5% BSA in PBST/5% milk in TBST
Stripping Buffer
Antibodies (from Cell Signaling Technology): 9102 – ERK1/2 (p44/p42), 9101 – pERK1/2 (pp44/42), 9217 – pAkt, 9272 – Akt, 9212 – p38, 9211 – pp38, 9152 – SEK, 9151 – pSEK

1. Remove nitrocellulose/PVDF and rinse briefly with PBST/TBST
2. Block the blot in 10-15mL:
    a. For pERK: 5% BSA in PBST 2hr at 37C
    b. For pAkt: 5% milk in PBST 2 hr RT
    c. For pp38: 5% milk in TBST 2 hr RT
    d. For pSEK: 5% milk in TBST 2 hr RT
3. Incubate in primary antibody overnight at 4 degrees C in 10 mL blocking buffer
    a. For pERK: 1:1000, for pAkt: 1:500, for pp38: 1:500, for pSEK: 1:500
    ERK, Akt, SEK antibodies can be re-used, p38 antibodies should not be.
4. Rinse briefly, then wash with PBST/TBST 3 x 10 min
5. Incubate in 1:2000 secondary (anti-rabbit) in blocking solution for 1 hour at room temp.
6. Rinse and wash with PBST/TBST as before
7. Visualize with Amersham ECL kit and image
8. Rinse, then wash 2 x 5 minutes with PBST/TBST
9. Incubate with stripping buffer at room temperature
    a. 15 minutes RT for all but pERK which requires 30 minutes RT
10. Rinse, then wash 2 x 5 minutes with PBST/TBST
11. Block again for total protein western:
    a. For ERK: 5% BSA in PBST 2 hr RT
    b. For Akt: 5% milk in PBST 2 hr RT
    c. For p38 5% milk in TBST 1 hr RT
    d. For SEK: 5% milk in TBST 1 hr RT
12. Incubate in primary antibody overnight at 4 degrees C in 10 mL blocking buffer
    a. For ERK: 1:1000, for Akt 1:1000, for p38: 1:500, for SEK: 1:1000
13. Rinse and wash 3 x 10 minutes with PBST/TBST
14. Incubate in secondary antibody (1:2000) for 1 hr at RT
15. Rinse and wash 3 x 10 minutes with PBST/TBST
16. Store in PBST/TBST until imaging.
17. Visualize with Amersham ECL kit and image. Save membranes wrapped in saran wrap at 4C.

Appendix B: Mechanical Gene Regulation of Normal and OA Human Tissue

The purpose of this study was to investigate the gene transcriptional response to mechanical loading of human cartilage tissue from normal joints and macroscopically-intact looking tissue from osteoarthritic joints. Our hypothesis was that normal-looking tissue in osteoarthritic joints may respond to mechanical loading in a different manner than tissue from normal joints, signifying a change early in the disease process. This project was done in collaboration with Pfizer, Inc.

Methods

Harvest and culture of cartilage

Upon opening of joint, visual Collins grading with supporting photography was recorded for each surface (tibial plateau, groove, condyles) and a Collins grade for the whole joint was determined (highest grade of all articulating surfaces).

grade 0 - no cartilage degeneration
grade 1 – minor surface roughening
grade 2 - fibrillations and fissuring, no full thickness defects
grade 3 – full defects covering less than 30% of the articular surface
grade 4 – full defects covering more than 30% of the articular surface

Full thickness, 3-mm plugs was harvested from 4 regions of the joint, with a target of at least 17 plugs per region (12 for loading studies, 2 for histology, 3 for biomechanics testing) + scraps (for biochemical testing). The four regions investigated were the medial condyle, medial tibial plateau (from tissue covered by meniscus), medial groove, lateral groove.

Cartilage was allowed to equilibrate for two-four days and cultured in low-glucose DMEM (supplemented with ascorbic acid, praline, non-essential amino acids, HEPES buffer, PSA – penicillin, streptomycin, amphoteracin) + 10% FBS. Medium was changed daily; final medium change occurred approximately 18 hours prior to loading.

Immediately prior to loading, thickness of the plugs were cut to uniform dimension (approximately 1 mm thick), with retention of articular surface for normal joints and the remaining macroscopically-intact looking surface in OA joints.

2 x 3-mm, full thickness plugs per region were fixed overnight in 10% formalin at 4C for histology then stored in 70% ethanol.

Mechanical loading of tissue

<u>4 mechanical loading conditions were used:</u>

Free swell – plugs were cultured in 48-well plate with 500 uL medium per plug for 24 hours
Static compression – Plugs were loaded in 12-well polysulfone chambers with 500 uL medium per plug. A slow ramp was applied over 3 minutes to 50% strain, which was held for 24 hours.

Dynamic compression – Plugs were loaded in 12-well polysulfone chambers with 500 uL medium per plug. 0.1 Hz, 3% amplitude, 5% static offset, displacement controlled dynamic compression was applied for 24 hours.

Injurious loading – Plugs were injured individually to 65% cut thickness, at a strain rate of 200%/s. They were then cultured in 48-well plates with 500 uL medium per plug for 24 hours.

3 plugs per loading condition per region = 12 plugs per joint were used for each loading condition. Medium from each well was frozen and saved. Immediately after loading, each plug was frozen in liquid nitrogen and kept at –80 C and sent to Pfizer for RNA extraction and microarray analysis.

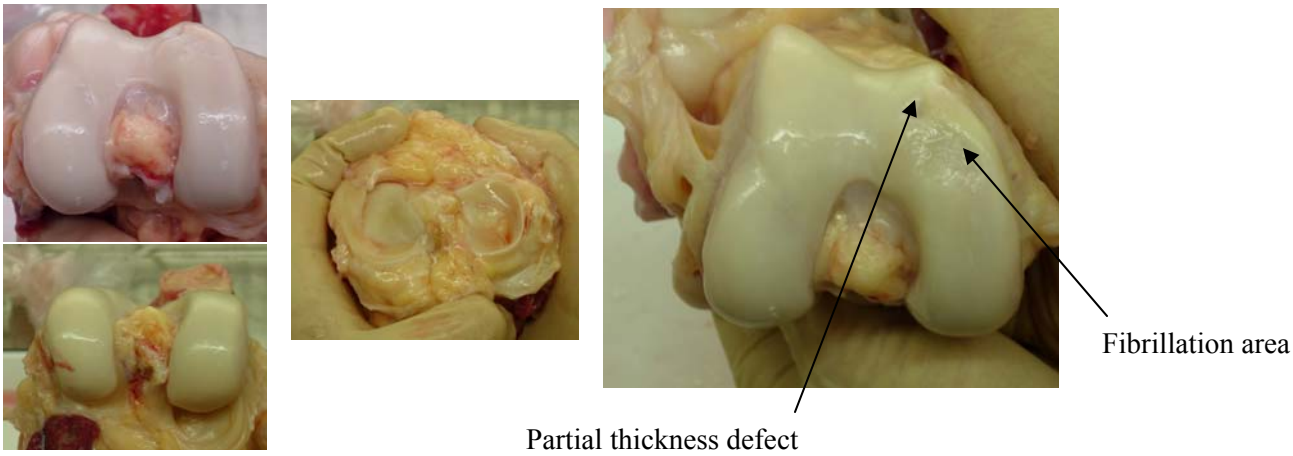
Subsequent Analyses – Biochemical and Biomechanical testing

Scraps from each region were proteinase K digested. Digests were analyzed for DNA content and GAG content. 3 x 3-mm diameter, 1-mm thick plugs per region were mechanically tested to obtain equilibrium modulus and dynamic stiffness.

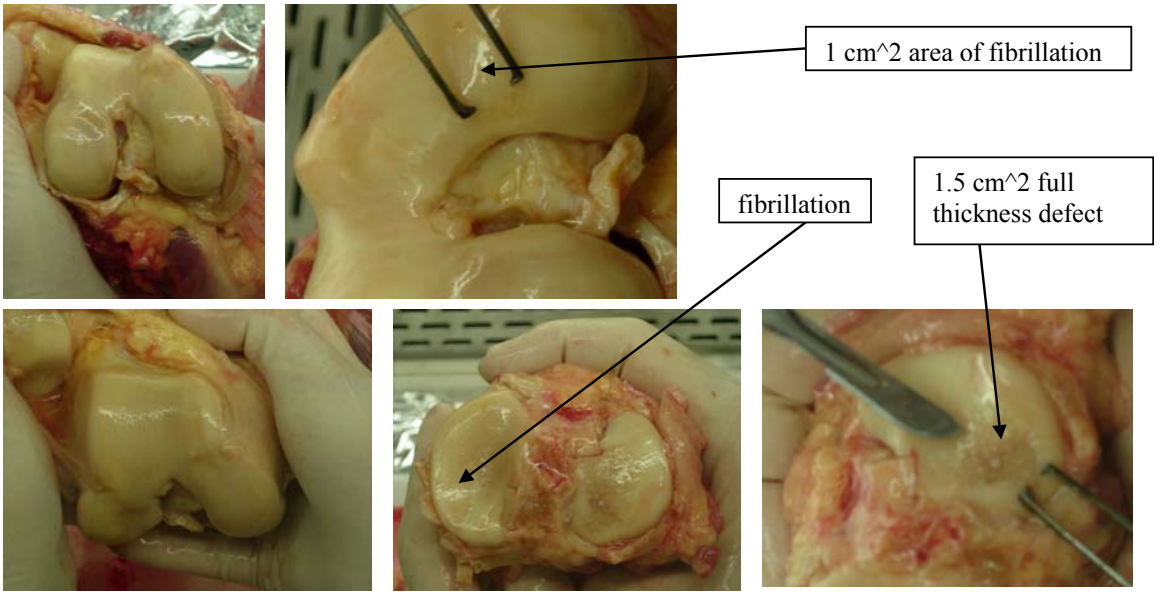
Results – Joint Harvest and Collins Grading

Tissue from 6 joints were harvested for this study – 4 OA, 2 normal.

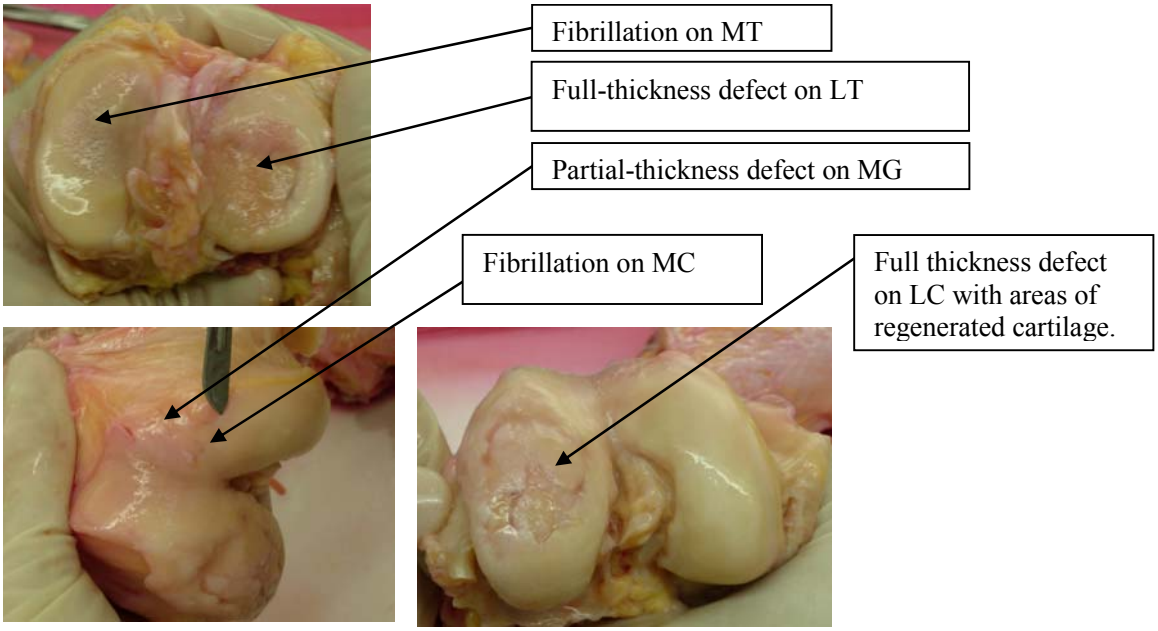
BDC01 – Donor was 44 year old female. Joint was determined to be Collins grade 2. There was a 2 cm fibrillation area at the transition between the lateral condyle and the groove. An 8 mm partial thickness defect was also present. All other surfaces appeared normal and intact. Accompanying photography shown below:



BDC02 – Donor was 65 year old female. Joint was determined to be Collins grade 3. There was a 1 cm^2 area of fibrillation on the inner aspect of the medial condyle (grade 1); 1.5 cm^2 full thickness defect on the front, inner aspect of the medial tibial plateau (grade 3); fibrillation over the lateral tibial plateau surface. Accompanying photography shown below:

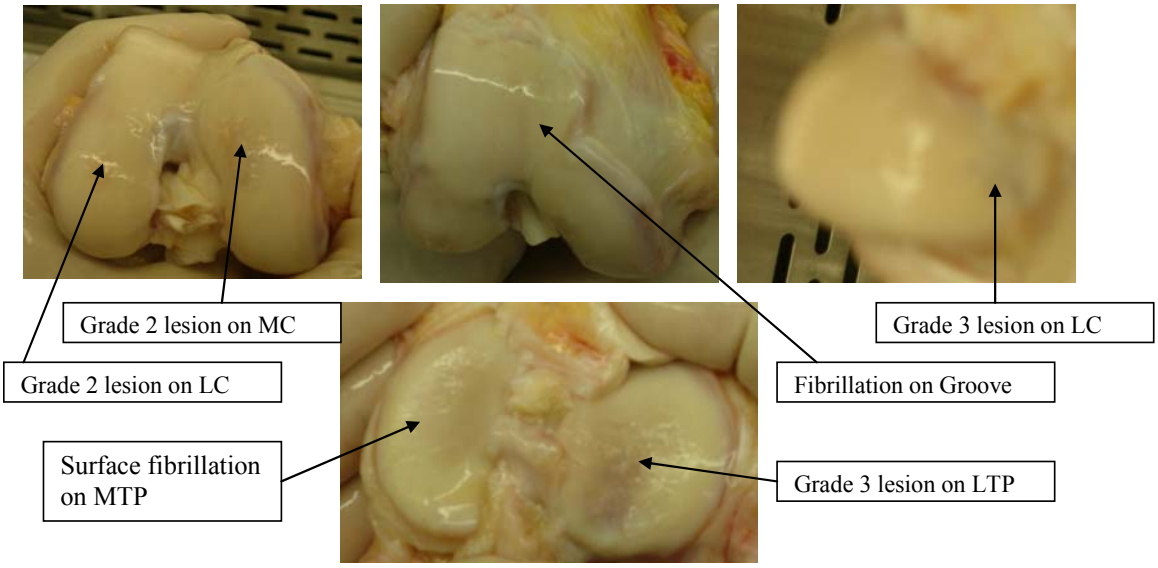


BDC03 – Donor was 65 year old female. Joint was determined to be Collins grade 3. The medial condyle (MC) was grade 1 with fibrillation at the interface with the medial groove. The lateral condyle (LC) was grade 3 with a 3cm x 2cm full and partial-thickness defect with areas of regenerated fibril cartilage. The medial groove (MG) had a triangular shaped 1.5 cm x 1cm partial thickness defect, grade 2. The laterial groove (LG) with grade 1 with fibrillation. The lateral tibial plateau (LT) had a 2cm x 2cm circular full-thickness defect, grade 3. The remaining carilage was fibrillated, Grade 1, with 5mm left in the meniscus covered region. The medial tibial plateau (MT) was grade 1 with fibrillation at the inner aspect that covered approx 25% of surface. Accompanying photography shown below:

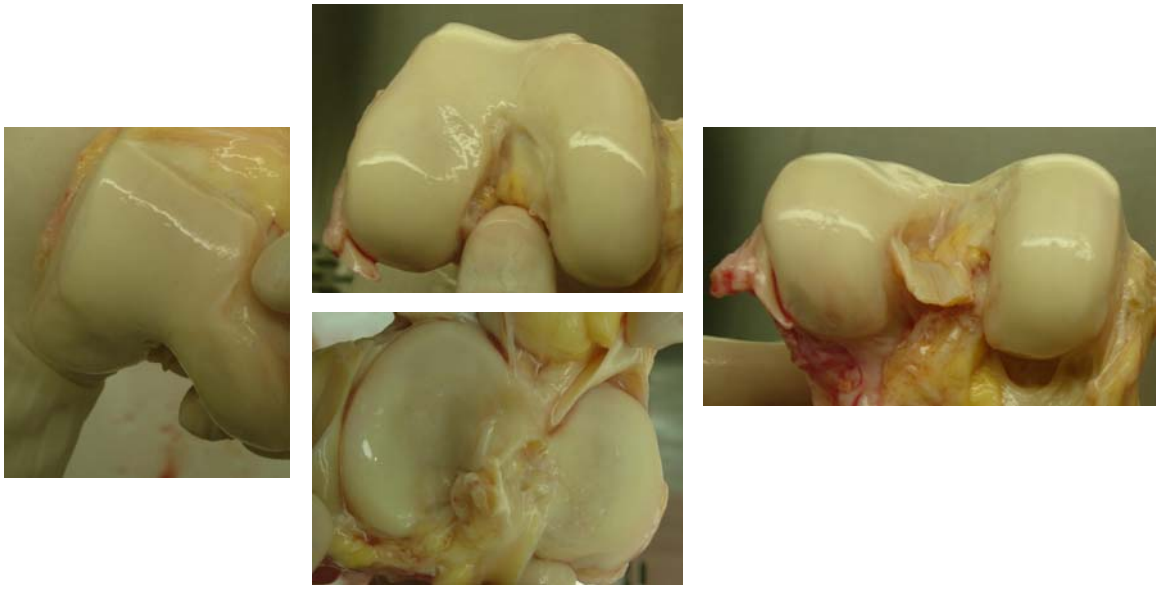


BDC04 – Donor was 52 year old female. Overall joint (right knee) is a Collins grade 3. On the inner aspect of the medial condyle (MC), 1 cm below the transition between the condyle and the groove, there was a 2cm x 1.5cm Grade 2 lesion with fibrillation and surface loss but no visible

bone. On the lateral condyle (LC), 2cm below the transition between the condyle and groove, there was a 0.5cm x 0.5cm area of surface loss and surrounding fibrillation. On the posterior aspect of the lateral condyle, there was a 1cm x 1cm full thickness defect, Collins grade 3, partially covered by repair tissue. The groove showed areas of fibrillation. The medial tibial plateau (MTP) showed surface fibrillation. The lateral tibial plateau (LTP) had a 2cm x 1cm full-thickness defect covered by repair tissue, Collins grade 3. Accompanying photography shown below:

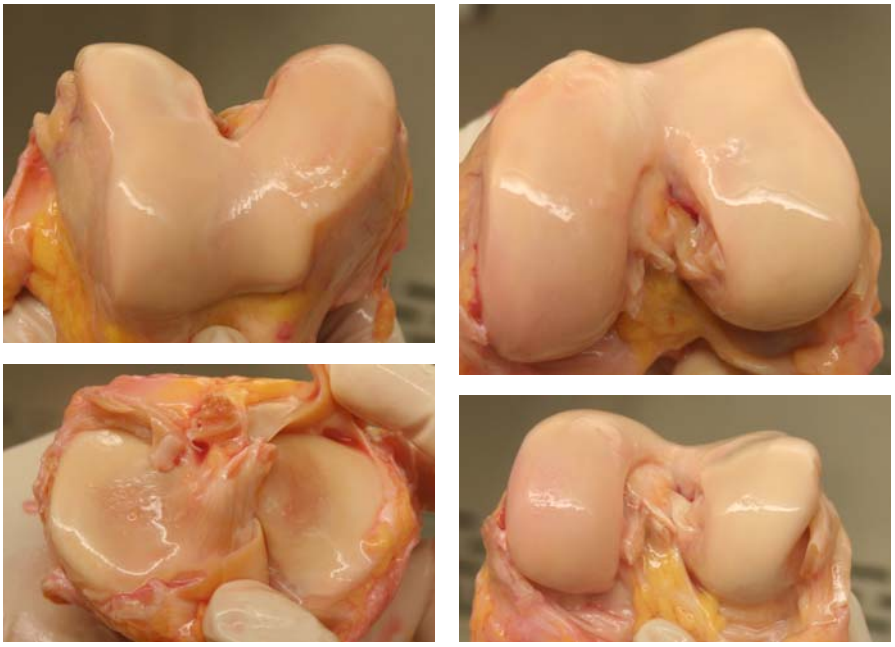


BDC05 – Donor was 56 year old female. Joint is Collins grade 0-1. Slight fibrillation on the medial condyle near transition between groove and condyle. Fibrillation on the inner aspect of tibial plateaus uncovered by the meniscus – as normally observed. All other surfaces appear normal and intact.



BDC06 – Donor was 67 year old female. Joint is Collins grade 0-1. Small areas of fibrillation on the medial condyle near transition between groove and condyle. Fibrillation on the inner aspect

of tibial plateaus uncovered by the meniscus – as normally observed. All other surfaces appear normal and intact.



Discussion

The bulk properties of tissue samples varied between individuals and location within the joint. There were no systematic changes in GAG content, hydroxyproline content, DNA content, or GAG-to-hydroxyproline ratio with respect to locations within a joint, disease state, or proximity to lesions. High variability between individuals was observed. Mechanical properties varied similarly, with no correlations to GAG content, hydroxyproline content, or GAG-to-hydroxyproline ratio.

Microarray data showed high individual variability in the gene expression response to mechanical loading, with no correlations to disease state. There also appeared to be an effect of culture on basal gene expression levels. Additional data would be required to determine whether systematic changes in gene expression response to mechanical loading is affected by disease state of the joint in macroscopically normal-looking tissue. It is likely that many additional joints would be needed at each condition (*i.e.*, age, OA grade) in order to achieve clear results. This project was discontinued after 6 joints.

Appendix C: Biomechanical Aspects: Joint Injury and Osteoarthritis*

C.1 Introduction

Osteoarthritis (OA) is a degenerative joint disease of mechanical wear-and-tear, often localized in weight-bearing joints such as the knees and hips [1]. While age is the most important risk factor for OA, joint trauma, repetitive joint use, obesity, and gender are also risk factors for the disease [1]. Arthritis is associated with a progressive wearing away of the articular cartilage of the joint surface caused by mechanical injury, by joint instability, or by an inappropriate response to normal mechanical stimuli [4]. The molecular pathogenesis of the disease is associated with loss of aggrecan, damage to the collagen network and, ultimately, loss of normal chondrocyte phenotype and a limited degree of chondrocyte cell death [4]. Chondrocyte dedifferentiation, marked by the decrease in type II collagen and aggrecan and an increase in collagen type I and type X, occurs as the disease progresses and marks the beginning of the inevitable end as dedifferentiated cells can no longer synthesize useful matrix material [4]. Although the mechanism of matrix loss and the driving force for the disease progression are not completely understood, acute mechanical injury and prolonged inflammatory insult increase the risk of developing osteoarthritis [7].

Cartilage degeneration is driven by the entire synovial joint; however, the chondrocyte, by virtue of its location within cartilage, plays a primary degenerative role by producing matrix degrading proteases, altering the synthesis of matrix molecules, and producing inflammatory cytokines and inappropriate levels of morphogenetic or growth factors [4, 8]. In response to joint injury or cytokine stimulation, latent matrix proteases or newly secreted proteases may rapidly degrade aggrecan [9-12], with loss of aggrecan significantly altering the mechanical

* Appeared in Chai DH, AL Stevens, AJ Grodzinsky, *Biomechanical Aspects: Joint Injury and Osteoarthritis*, in *Bone and Osteoarthritis*, F Bronner, MC Farach-Carson *Editors*. 2007, Springer-Verlag: London. p.165-179.

properties of the tissue [13]. Chondrocytes may, however, synthesize and replace lost aggrecan, causing the tissue moduli and function to return to normal, without long term damage [14, 15]. Collagen network damage, on the other hand, seems to be an irreversible step in the pathogenesis of osteoarthritis [4]. Protease-induced collagen degradation often occurs after aggrecan depletion; this suggests that aggrecan may protect collagen fibrils from proteolytic degradation [16].

Joint injury can lead to loss of chondrocyte viability and to damage of cartilage matrix and other joint tissues, *i.e.*, ligaments, tendons, and synovium. The resulting change in cartilage matrix composition and mechanical properties may be partly responsible for increasing the risk of arthritis, inasmuch as chondrocytes can rarely repair the damage [17]. Thus, human knee injuries including ACL or meniscal tears may significantly increase the relative risk of developing OA, a risk that increases with age at the time of injury and with the time that has elapsed since the injury [18-20]. Joint instability may contribute to secondary disease development, yet ACL correction does not seem to decrease the risk of OA development [21-24]. The acute traumatic event may therefore have been sufficient to trigger a cascade of irreversible effects that initiate arthritis and cause its progression.

Osteoarthritis can have an inflammatory component, which involves the production of cytokines, continued local, low-level inflammation without inflammatory cell migration, and accompanying systemic immune responses. *In vivo*, mechanical joint injury occurs with concomitant inflammation, characterized by an increase in pro-inflammatory cytokines, TNF-α and IL-1β [25-27] , as well as by an increase in MMP-3, COMP fragments, collagen II cross-links and aggrecan fragments [28-32] . Pro-inflammatory cytokines IL-1β and, to a lesser extent, TNF-α cause extracellular matrix breakdown in cartilage and may be present in OA.

C.2 Joint Loading and Cartilage Biomechanics: Changes with OA

Articular cartilage functions as a weight-bearing and lubricating layer in joints that absorb and distribute loads to underlying bone. Cartilage is exposed to a variety of mechanical forces during normal joint loading in vivo [33-35]. Loading across joint surfaces can be highly nonuniform [33, 35]. Whereas local stresses in the hip rise to 10-20 MPa during activities such as stair-climbing [33-35], forces of 2-5 times body weight in the hip during walking [36]cause peak stresses on the cartilage surface of 2-3 MPa [33]. These forces cause changes in cartilage thickness in vivo as high as 20%, though long-term (static) loading causes even greater compressive deformation [33-35].

The structure of the extracellular matrix of cartilage is designed to withstand the dynamic range of forces. The main structural components are densely packed, negatively charged aggregating proteoglycans (aggrecan), water (~80%), and a network of collagen fibrils. Aggrecan contributes to the compressive stiffness of the tissue, while collagen provides tensile and shear resistance. Tissue biomechanical properties vary with depth and are strain-dependent [37-39]. The equilibrium compressive stiffness measured in uniaxial confined or unconfined compression is on the order of 1MPa, and the frequency-dependent dynamic compressive stiffness is approximately 5-10 times greater (reviewed in [40]). Alterations in the matrix structure, due either to mechanical injury or pathologic degradation, can greatly affect the mechanical properties and therefore the deformations experienced by the tissue.

In osteoarthritis, changes in matrix content and structure weaken the tissue and lead to changes in the subchondral bone [41-43]. Loss of collagen integrity, increased water content, decreased aggrecan content and alterations in GAG sulfation (charge) patterns contribute to decreases in mechanical stiffness of the tissue [41, 44, 45]. Kleeman et al. [46] found that

stiffness decreases significantly with disease progression from 0.5MPa to 0.28MPa, with this decrease reflected in the grade assigned to the tissue when the the ICRS Grade Scale is used. The ICRS Grade Scale is based on gross examination of fissures, cracks, lesion extent and depth, and Mankin score.

C.3 Clinical Findings

Injurious joint loading results in peak stresses and deformations that can be significantly higher than the normal ranges quoted above and can lead to significant tissue and joint damage. Insights concerning the molecular mechanisms of cartilage degeneration *in vivo* have come from analyses of synovial fluid samples taken from Swedish patients after an ACL or meniscal tear [7, 47, 48]. The concentration of proteoglycan fragments in the synovial fluid was elevated 2-3 fold after injury, and these levels were similar to those found in patients with primary OA [47]. ELISA analysis of the synovial fluid revealed the presence of matrix metalloproteinase-3, (MMP-3), a protease linked to pathways of matrix degradation. MMP-3 levels in synovial fluid were markedly increased at presentation and remained elevated for many years [7]. Joint fluid also showed an initial and persistent elevation of the neoepitope Col2CTx in the C-telopeptide cross-linking domain of type II collagen. This indicates digestion of mature, cross-linked collagen by a matrix metalloproteinase. Taken together, these clinical studies suggest that proteoglycan and collagen degradation rates are significantly altered within days of the injury and remain altered for years. It thus seems that the acute joint tissue response to the original mechanical insult initiates an unbalanced degradative process that can significantly increase the risk of OA.

C.4 *In vitro* Models of Acute Mechanical Injury to Cartilage

C.4.1 Biomechanical parameters For more than a decade, *in vitro* model systems have been developed and specialized to study the effects of acute mechanical trauma on articular cartilage. For example, the incubator-housed instrument shown in Figure C.1a can apply compressive loads or displacements to individual or multiple geometrically defined cartilage explant disks held in specially designed autoclavable loading chambers that are mounted within the instrument [49]. Direct mechanical compression of tissue can be performed by applying a known force ("load control") or a known displacement ("displacement control") to one surface of the specimen via a solid platen, while the opposite platen is held fixed. One-dimensional ("uniaxial") *unconfined* compression [50, 51] typically employs a non-porous compression platen, and the sample is allowed to bulge slightly and exude fluid in the radial direction (Figure C.2a). In radially *confined* compression, a barrier is placed around the circumference of the sample and a porous compression platen is used; the sample is not allowed to bulge radially, and fluid flow occurs in the axial direction emulating an articular surface geometry [52]. Joint loading *in vivo* produces within the tissue a complex, nonuniform 3-dimensional distribution of stresses and strains that has attributes of both the idealized confined and unconfined loading configurations.

The choice between confined and unconfined loading for *in vitro* studies may depend on assumptions underlying the experimental hypotheses, as well as on practical considerations concerning maintenance of the organ culture with adequate nutrient supply. Whether loading is confined or unconfined, it is essential to understand the spatial distribution of physical forces and flows within the explant (*i.e.* intratissue strain, fluid flow, fluid pressure gradients, etc.). These fields and flows can be highly nonuniform in either configuration, and the resulting cellular responses will therefore be equally nonuniform. For example, a compressive strain applied

rapidly (at a high "strain rate") to a cylindrical cartilage disk will result in high peak stresses that may damage the tissue matrix (Figure C.2b). Chondrocyte biosynthesis changes and GAG is lost in response to such injurious compression. However, quantitative autoradiography at the tissue and cell level has shown that cellular biosynthesis [53, 54] and GAG loss can vary markedly across the tissue cross-section. Corresponding theoretical analyses of the biomechanical and biophysical forces and flows have shown, likewise, that intratissue fluid flow, hydrostatic pressure, and even electrical streaming currents induced by compression will vary within the explant disk with radius and height (Figure C.2c). It may therefore be possible to correlate the resulting spatially nonuniform matrix and cellular responses with the spatially varying biomechanical stimuli, thereby enabling the investigator to better understand which biomechanical parameter(s) is responsible for the biological response [55].

In general, the *rate of loading*, the *peak stress*, and the *final strain* must be clearly specified to define an injurious loading scheme (e.g., Figure C.2b). Any two of these parameters can be independently imposed experimentally; the third must then be measured. Without knowledge of all three parameters, it is difficult to compare results to those in the literature. For example, two investigators [56, 57] loaded cartilage explants at different loading rates to a pre-specified peak stress (in load control). Comparisons of their data show the counterintuitive result that cell death was higher in more slowly loaded cartilage. However, compressing cartilage more quickly in these circumstances means that the pre-specified peak stress was reached more quickly, and less total compression (strain) was produced. Thus, studies in which two different loading parameters are varied independently in a parametric fashion are most helpful in understanding the fundamental mechanical variables that cause injury to cartilage cells and matrix.

For example, Morel and Quinn [58] subjected young adult bovine cartilage disks to uniaxial unconfined compression at 5 different strain rates (over five orders of magnitude) and 3 different peak stresses (between 3.5 to 14 MPa). The strain rate was defined relative to the intrinsic gel swelling [59] or poroelastic relaxation time, $\tau \sim [\delta^2/(Hk)]$, where τ is related to the swelling (or deswelling) time for a disk having equilibrium compressive modulus H, hydraulic permeability k, and characteristic distance δ through which fluid flows (*e.g.*, the disk radius for the case of unconfined compression with fluid-impermeable platens [60]). This relaxation time is also related to the characteristic mechanical stress relaxation or creep time to within a numerical constant that depends on the mechanical boundary conditions for each configuration. At the lowest strain rates (at or below the relaxation time), resulting in highest final strains, no cracks in the matrix occurred, but cells died throughout the tissue depth. In contrast, the highest strain rates resulted in high intratissue pressurization, causing impact-like surface cracking with cell death isolated near the superficial zone. Consistent with these trends, Kurz et al. [3] also found increased cell death and decreased mechanical stiffness with increasing strain rate.

It has been proposed that threshold levels of either strain rate or peak stress determine the threshold of tissue damage. Tissue age and species also appear to be strong determinants of the ability of cartilage to withstand overload. Torzilli *et al*. compressed mature bovine occipital cartilage in a load-controlled apparatus at a constant stress rate of 35 MPa/s to reach final stress values in a range of 0.5 to 65 MPa [61]. Cell death was significant at a stress of ~17.5 MPa; cartilage damage may therefore occur when a threshold is reached in peak stress. Similarly, cell death increased with peak stress in immature bovine knee cartilage [45]. Patwari et al. [62] studied the relation between peak stress and GAG loss after injury to disks of normal human knee and ankle cartilages. Compression was applied to 65% final strain at 400%/sec strain rate.

At these loading conditions, knee cartilage suffered significantly more damage than ankle cartilage, but peak stress was not an important correlate of GAG loss.

C.4.2 Damage to matrix and cells Using a variety of instruments as in Figure C.1a, investigators have demonstrated a range of events that can occur immediately following cartilage traumatic injury. These include loss of proteoglycan constituents to the culture medium [50, 53, 56, 61, 63], increased tissue swelling [50, 61], and increased levels of denatured collagen neoepitopes [64, 65], indicative of damage to the collagen network. At the same time, the tissue's biomechanical properties become degraded [66]. Loening et al. observed decreased equilibrium and dynamic stiffness associated with increased tissue swelling in hypotonic saline, which was suggestive of collagen network damage [50]. Mechanical injury can also cause cell death by apoptosis [50, 67, 68]. The possibility that apoptosis can be induced at levels of mechanical loading below those that cause macroscopic damage [50], as assessed by tissue swelling and GAG loss, suggests that apoptosis can be triggered by direct loading injury to the chondrocytes.

Chen et al. and Thibault et al. tested the effects of mechanical injury on the production of collagenase-generated neoepitope of type II collagen [64, 65, 69]. In both confined and unconfined compression injury models, injury caused an increase in collagenase-generated collagen fragments. Thibault et al. [65] postulate that this is the result of mechanical denaturation of the collagen fibril, which enables the fibril to become susceptible to cleavage by metalloproteinases. A study focusing on repair secondary to mechanical compression injury suggested that both fibronectin and proteoglycan synthesis were increased over control levels in the course of the ten days that follow the injury. This situation is similar to that seen in early OA [64]. DiMicco et al. analyzed the release of sulfated GAGs into the medium over a period of 7

days following injury [70]. A small, but statistically significant, increase in sGAG release occurred during the first 24 hours after injury. This was attributed to mechanical disruption of the matrix, inasmuch as it was not reversed by inhibitors of biosynthesis (cyclohexamide) or degradative enzymes. However, a broad spectrum hydroxamate MMP inhibitor reduced the cumulative GAG loss from injured disks in the course of the 7-day postinjury period [70].

Studies using a drop-tower loading system to apply impact loads on cartilage-bone cylinders have documented decreases in cell viability in a canine model [71], in the porcine patella [72] and in bovine articular cartilage [73]. These studies have shown that bone plays a major role in mediating the effects of an impact load on cartilage [73]. With bone attached to cartilage, the cartilage was damaged much less [73]; higher-energy impacts damaged the bone rather than the cartilage. This finding is consistent with the importance of subchondral fracture in animal impact models [74] and with the early work of Radin *et al*., who emphasized the involvement of subchondral bone, having found that impact trauma to the patellofemoral joint led to OA in animal models [74, 75]. The response of bone to impact and subsequent changes in joint stresses are clearly important in OA pathology. For this reason, impact models using cartilage that has been removed from underlying bone do not simulate *in vivo* loading magnitude and distribution. Nevertheless, injury to the cartilage alone may suffice to initiate OA processes *in vivo* [76, 77].

Moreover, using cartilage explants without underlying bone makes it possible to impose controlled mechanical loading, allowing specific loading parameters to be more easily quantified and correlated with cellular and matrix changes. Thus, studies regarding the mechanism of response to injurious loads by cells and matrix in cartilage explants are therefore meaningful.

C.4.3 Apoptosis versus necrosis Recently, it has been proposed that cell death by apoptosis may be an important event in osteoarthritic cartilage. Most compression injury models suggest that chondrocytes may undergo apoptosis in response to injury [50, 51, 78, 79]. High stress repetitive loading can also cause chondrocyte necrosis, which occurs soon after the injury as visualized by TEM [80] (see also [66] for a recent review). We and others have used TUNEL staining, along with nuclear morphology on light microscopy, to demonstrate induction of apoptosis by mechanical injury [50, 51]. Investigators have since emphasized that false-positive staining by the TUNEL assay can be a major limitation [4, 81]. Chen et al. [68] found that cells in cartilage subjected to freeze-thaw cycles were 90% TUNEL positive after three days of culture; this suggests that TUNEL staining does not reliably distinguish apoptotic from necrotic cell death. In addition, it is increasingly clear that there exist modes of cell death with features of both necrosis and apoptosis.

To investigate further, Patwari et al. [79] subjected newborn bovine cartilage disks to compression injury (50% strain, 100%/sec) and performed a quantitative analysis of cell morphology by electron microscopy (EM), with comparison to the TUNEL assay. By TUNEL, the cell apoptosis rate increased significantly from 7 ± 2% in unloaded controls to 33 ± 6% after injury (N = 8 animals) and, by EM, the apoptosis rate increased from 5 ± 1% in unloaded controls to 62 ± 10% in injured cartilage. Analysis by EM also indicated that 97% of the dead cells in injured disks were apoptotic by morphology. These results confirm that cell death increases significantly after injurious compression and suggest that in the injury protocol used in these experiments most of the observed cell death involved an apoptotic process.

To further relate the apoptotic response to the biomechanical parameters of injurious compression, we note that high strain rates causes tissue pressurization in the center of the

explants (Figure C.2c) with the periphery experiencing more strain and fluid flow [70, 82]. Most reports have focused on the central pressurized region of the explant to avoid cutting artifacts [50] which cause apoptosis independent of injury. Patwari et al. suggested that compression injury may alter cell-matrix interactions sufficiently to initiate apoptosis [79]. While injury-induced cell death and accompanying damage to the extracellular matrix are clearly demonstrable, the contribution of cell death to arthritis is still controversial [4].

C.4.4 Effects of cartilage injury on chondrocyte gene expression: recent discoveries Several studies have provided evidence of marked changes in chondrocyte expression of MMPs and other selected genes following mechanical injury to cartilage *in vitro*. Techniques used for these studies include Northern analysis, RT-PCR, and *in situ* hybridization [66]. Recent technological advances involving real time qPCR and gene array technologies have made it possible to use systems level genomic approaches to study changes in chondrocyte transcription in response to mechanical injury of the cartilage. Chan et al. [83] used a bovine cDNA microarray and real time PCR to characterize changes in transcription three hours after unconfined compression injury to metacarpophalangeal cartilage explants from 18-24 month steers. They loaded to a peak stress of 30 MPa at a stress rate of 600 MPa/s, and found 19 genes that were differentially expressed. Up-regulated genes included chemokine (CCR10, HMGB2, neurogranin, and ezrin) and cytokine receptors, enzymes, and molecules involved in signal transduction. In contrast, ICAM-3, NCAM, N-cadherin, VCAM-1, and IGF-1 were down-regulated [83].

In complementary studies using immature bovine cartilage explants, Lee et al. [2] used real time qPCR to measure levels of mRNA encoding selected matrix molecules, proteases, their natural inhibitors, transcription factors, growth factors and cytokines. Expression levels were assessed in free swelling culture (4 and 24 hours) and at 1, 2, 3, 6, 12, and 24 hours after

application of a single injurious compression to 50% strain at 100%/s strain rate. Expression levels measured in non-injured free swelling cartilage varied over five orders of magnitude with matrix molecules being the most highly expressed, and cytokines, MMPs, aggrecanases (ADAMTSs), and transcription factors showing lower levels of expression. Changes in expression levels after mechanical injury were gene specific and time dependent. While the matrix molecules showed little change in expression after injury, MMP-3 increased ~250-fold, ADAMTS-5 increased ~40-fold, and TIMP-1 increased ~12-fold over free swelling levels by 12 hours after injury. Genes typically used as internal controls, GAPDH and β-actin, increased expression levels ~4-fold after injury; this makes them unsuitable for use as normalization genes in this and similar studies. TNF-α and IL-1β did not change expression levels in the chondrocytes after injury. Group expression profiles, using k-means clustering techniques, showed the main temporal gene expression patterns that were induced by injurious compression of the cartilage explants (Figure C.3). Interestingly, one of the group profiles was associated exclusively with the immediate response genes c-fos and c-jun, which showed increased transcription within the first hour of injury. This suggests that the AP-1 pathway may be an important response pathway in injury [2]. The authors [81] concluded that changes in expression may alter the quantity of specific proteins that lead to degradation of the tissue structure and function.

C.4.5 Mechanical injury compromises chondrocyte biosynthesis and mechanoresponsiveness Several studies have shown that chondrocyte biosynthesis in cartilage explants decreases after an injury [61, 63, 64, 84, 85]. Quinn et al. [86] examined changes in biosynthesis and increased cell death in osteochondral explants from 18-month-old steer shoulder joints subjected by unconfined compression to peak stresses between 3.5 and 14 MPa,

at strain rates between 3 x 10^{-5}/s to 0.7/s. With higher strain rates, these authors observed that matrix damage occurred primarily in the superficial zone [86]. Furthermore, proteoglycan synthesis was suppressed at low strain rates throughout the cartilage depth in a radially dependent manner. Kurz et al. [3] showed that increasing the strain rate of injurious mechanical compression significantly decreased recovery from injury in chondrocytes. Recovery was identified by the ability of the cells after injury to respond to subsequent stimulation by low amplitude cyclic compression, considered to be an anabolic stimulus in normal cartilage explants. At higher strain rate injury (*i.e.*, 1/sec in Figure C.2b, but not 0.01/sec), there was a dramatic decrease in ^{35}S-sulfate and ^{3}H-proline incorporation three days after the injury. Even more striking, chondrocytes in tissues subjected to high strain rate injury lost their the ability to be stimulated by dynamic compression in a dose-dependent manner with increasing strain rate of injury [3]. These results had been normalized to the surviving viable cells and therefore were not simply the result of loss of cell viability. Although these studies had focused only on early time points following injury, the findings suggest that mechanical injury causes a decrease in extracellular matrix production by chondrocytes. This in turn may contribute to further degeneration. A critically important unanswered question is whether the remaining cells can respond by increasing biosynthetic activity in an attempt to repair the cartilage matrix. If mammalian chondrocytes were able to respond to anabolic factors, both biological and mechanical, this would be of great therapeutic interest.

C.5 Osteoarthritic Changes in Mechanoresponsiveness of Chondrocytes

Mechanical forces are widely thought to play a major role in regulating chondrocyte behavior (see recent reviews [87, 88]). However, the mechanotransduction pathways by which mechanical injury alters long term activity of the surviving chondrocytes are not understood. There is

increasing focus on the effect of injury on intracellular signaling and regulation of gene expression. Microtubules and comparable connections between the cell surface and the ECM transmit deformations of the pericellular matrix to the cell membrane and from there to intracellular organelles via cytoskeletal elements [87, 89, 90]. Within the cell, changes in nuclear morphology can lead to compaction of chromatin and altered molecular transport through nuclear pore complexes, processes important to cellular metabolism. Deformation of the rough endoplasmic reticulum and the Golgi apparatus can affect proteoglycan synthesis, GAG chain length, and sulfation observed during compression [91]. Deformations of the pericellular matrix can also change the physicochemical environment of cells, altering transport of soluble factors to cell-surface receptors [55, 92, 93]. Mechanical activation of chondrocyte surface receptors, such as the α5β1 fibronectin-binding integrin, induces multiple intracellular signaling pathways involving tyrosine protein kinases, cytoskeletal proteins, ion channels, and second-messenger signaling cascades [94]. Mitogen-activated protein kinase signaling, involving ERK-1 and -2, JNK, and p38 is activated in immature bovine cartilage explants that have been subjected to static, dynamic, and shear loading [95-97], and by fluid shear across isolated chondrocytes [98].

Osteoarthritic cartilage is characterized by reduced mechanical properties, increased matrix degradation, and altered responses to mechanical stimuli. Increased force transmission to cells and changes in mechanoresponsiveness may play a role in the development and progression OA [99]. The ratios of collagen II to collagen I, and of aggrecan to versican, are defined as measures of chondrocyte dedifferentiation and decrease in human OA cartilage [100, 101]. Expression of matrix proteins and cell surface receptors are altered with OA progression [101-105]. Integrin receptors known to be involved in mechanotransduction have altered expression patterns in OA cartilage, with increased α1 and decreased β1 expression [103]. These changes

can result in altered responses to applied forces. Enhanced expression of fibronectin and osteopontin, two matrix components that bind and signal through integrin receptors, has also been observed [104]. In addition, isolated OA chondrocytes respond differently than normal chondrocytes to direct mechanical stimulation [106]. While cyclic stretching of normal chondrocytes increased aggrecan and decreased MMP-3 transcription, mRNA levels in OA chondrocytes remained unaltered. In addition, normal chondrocyte membranes hyperpolarize in response to cyclic stretch, whereas OA chondrocytes depolarize. These altered responses to mechanical stimulation may represent adaptive responses to the altered mechanical environment that prevails in OA tissue [106]. OA chondrocytes also respond to intermittent hydrostatic pressure with increased aggrecan and type II collagen mRNA levels, and to fluid shear stress with increased nitric oxide release and decreased expression of matrix proteins [107].

C.6 *In Vitro* Models Emulating Injury to the Joint

C.6.1 Injury plus cytokine treatment Certain aspects of OA have led to the development of new in vitro models of the mechanically injured joint. It is widely accepted that OA is a disease of the whole joint [108, 109] that, in addition to cartilage, also involves the synovium, bone, muscles and the nervous system. Inflammatory processes associated with cytokine-induced activity are increasingly acknowledged to play a role in the pathogenesis of OA [109, 110]. As a result, research using in vitro injury models has broadened to account for interactions with other tissues, such as factors secreted by the joint capsule [111]. These novel models include (1) incubation of normal or injured cartilage in the presence of exogenous cytokines, and (2) co-culture of normal or injured cartilage in the presence of intact joint capsule tissue (Figure C.4). The direct use of an exogenous cytokine allows focus on the role played by that specific cytokine following injury, and its subsequent effects on mechanically injured cartilage.

In a recent study involving exogenous cytokines, Patwari et al. [6, 111] examined proteoglycan loss from mechanically injured bovine knee cartilage explants that had been cultured in the presence of IL-1α or TNF-α. Similar experiments were performed with injured knee and ankle cartilage obtained from the same human donor whose injured tissue had been cultured with IL-1. The cytokines caused a synergistic loss of proteoglycan [86] from the injured cartilage of both bovine and human tissue. In the case of the bovine tissue, the PG loss was significantly increased after mechanical injury. However, the loss amounted to only 2% of the total PG content and occurred only in the first 3 days following injury. However, the addition of 1 and 10 ng/ml IL-1α and 100 ng/ml TNF increased the PG loss over that due to injury or cytokine treatment alone. This interaction between cytokine treatment and injury was statistically significant. In human knee cartilage, the interaction was also significant for both IL-1α (Figure C.1b) and TNF-α, although the relative increase in PG loss was less than in bovine cartilage. Importantly, there was no significant interaction between injury and IL-1α in human ankle cartilage (Figure C.1b). This study suggests that mechanical injury and cytokine-induced chondrocyte mediated degradation cause changes in tissue behavior and properties in complementary and synergistic ways, even though the human ankle cartilage was relatively impervious to either injury and/or cytokine treatment.

C.6.2 Co-culture of joint capsule tissue with injured cartilage The role played in cartilage matrix degradation and remodeling by synovial tissues and the release of cytokines and other mediators can be studied *in vitr*o. Jubb and Fell [112] have studied how joint capsule tissue, when co-cultured with normal cartilage, affect chondrocyte biosynthesis. Ilic et al. [112] have shown that bovine joint capsule and its fibroblasts express soluble aggrecanase activity. Patwari et al. [6, 111] developed a model in which mechanically injured bovine or human cartilage was

co-cultured with bovine or human joint capsule tissue (Figure C.4) and observed that coincubation of human joint capsule tissue with normal human knee cartilage explants inhibited chondrocyte biosynthesis, similar to that which had been observed in animal models. When this was studied in a newborn bovine model, blockade of IL-1 had no palliative effect on the inhibition ^{35}S-sulfate incorporation. It was therefore concluded that the effect was mediated by an interleukin-1-independent signaling pathway. Using this model, Lee et al. [113] have reported that co-culture of bovine joint capsule tissue with normal or injured immature bovine cartilage caused dramatic upregulation of chondrocyte gene expression (via qPCR) and increased the expression of ADAMTS5, but not of ADAMTS4. The mediating factor(s), presumably released from the joint capsule tissue have not yet been identified. This model system may well lead to new insights of soluble factors that may become therapeutic targets acting on synovial tissues that do contribute to the initiation and progression of OA.

C.7 New Directions: Proteomic Approaches to Biomarkers of Joint Injury and OA

Biomarkers of OA typically target indicators of inflammation or indicators of matrix degradation. Markers of interest include hyaluronic acid, type II collagen N-propeptide, type II collagen C-propeptide (CTX-II), cross-linked collagen II peptides from the C-telopeptide, collagen III N-terminal propeptide (PIIINP), COMP, osteocalcin, pyridinoline, AgKS (keratan sulfate containing aggrecan fragment), ykl-40 [114]. Recent studies have demonstrated the utility of proteomics in the study of normal versus OA human cartilage. An example is 2-D gel electrophoresis followed by mass spectroscopy [115, 116]. Proteomics permits following groups of proteins, as well as specific target biomarkers. Stevens et al. [117] used mass spectrometry applied to an entire system to quantify the effect of mechanical compressive injury of cartilage and how treatment with TNF-α and IL-1β affected the joint injury (Figure C.1a). Protein

profiling together with clustering analyses revealed distinguishing treatment features that may be specific markers for a given degradative process.

C.8 New Engineering Directions: Molecular Nanomechanics and Chondrocyte Response

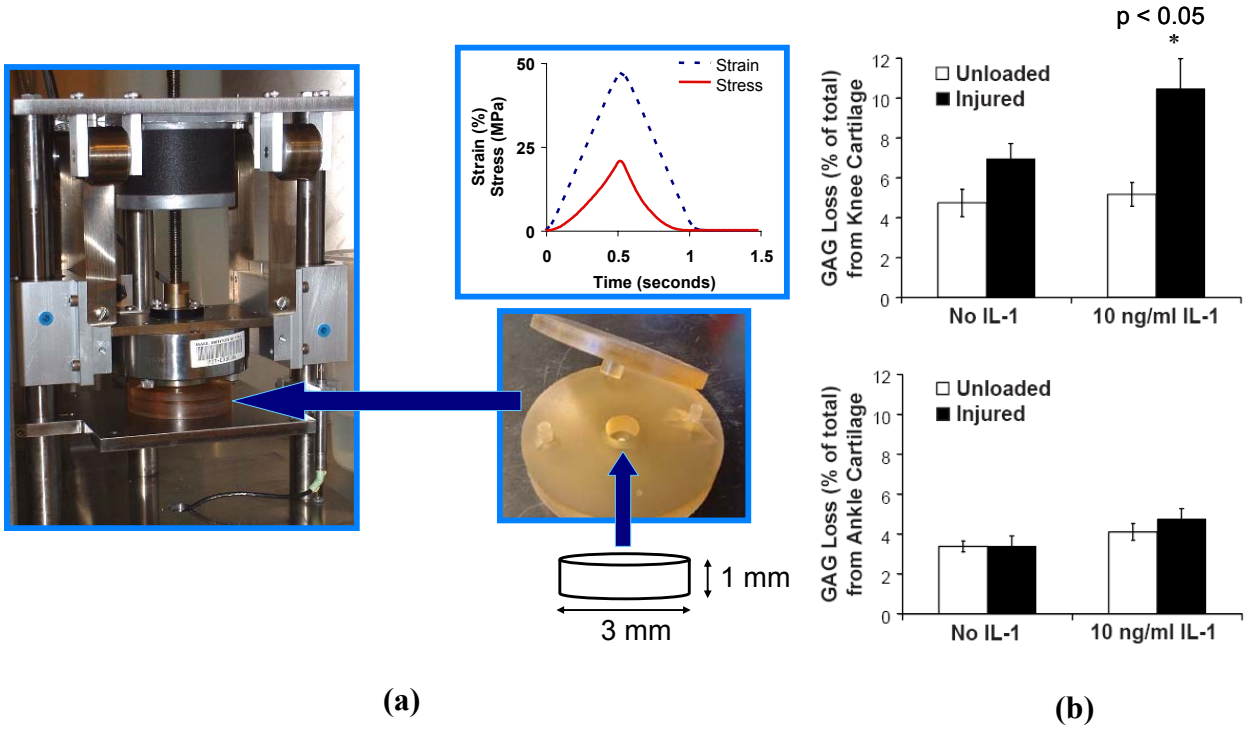
The functional properties of normal and injured cartilage tissue are determined, in part, by the mechanical properties of the various ECM molecules that are synthesized by chondrocytes. Functionally inferior matrix macromolecules that cannot properly contribute to or assemble into a functional ECM may be a hallmark of the progression of posttraumatic cartilage degradation. Using the newer techniques of optical tweezers, atomic force microscopy, and high resolution force spectroscopy, recent studies isolated matrix molecules have focused on the tensile properties of hyaluronan and collagen [118, 119] and the repulsion between chondroitin sulfate GAG chains [120] and aggrecan [120]. This is an exciting new area that emphasizes the connection between tissue-level mechanical properties, molecular mechanical properties of the matrix PGs, GAGs, and collagens. It also points to the importance of chondrocyte mechanotransduction as the glue between the tissue, cell, and molecular constituents in cartilage remodeling.

C.9 Acknowledgements

Supported by NIH Grants AR45779 and AR33236.

C.10 Figures

Figure C.1 Injurious mechanical compression of individual explant disks



(a) **(b)**

Figure C.1 (a) Example of an apparatus for applying injurious mechanical compression to individual cartilage explant disks. A triangle wave of displacement reaching 50% compression in 0.5 sec is applied, resulting in a peak stress of ~20 MPa. **(b) GAG loss 3 days after IL-1 treatment of normal and mechanically injured human knee and ankle cartilage.** Unloaded and injured cartilage from adult human donors was incubated with 0 or 10 ng/ml IL-1α, and GAG content of the medium was measured after 3 days of culture. In the knee tissue, incubation of injured tissue with IL-1 caused a synergistic increase in loss of GAG ($n = 8$, $p < 0.05$ for interaction). In ankle tissue from the talar dome, the interaction between injury and IL-1 treatment was not significant ($n = 6$, $p = 0.50$ by 2-way ANOVA). (a) from [2]; (b) from [6].

Figure C.2 Mechanical forces and flows associated with compression of cartilage

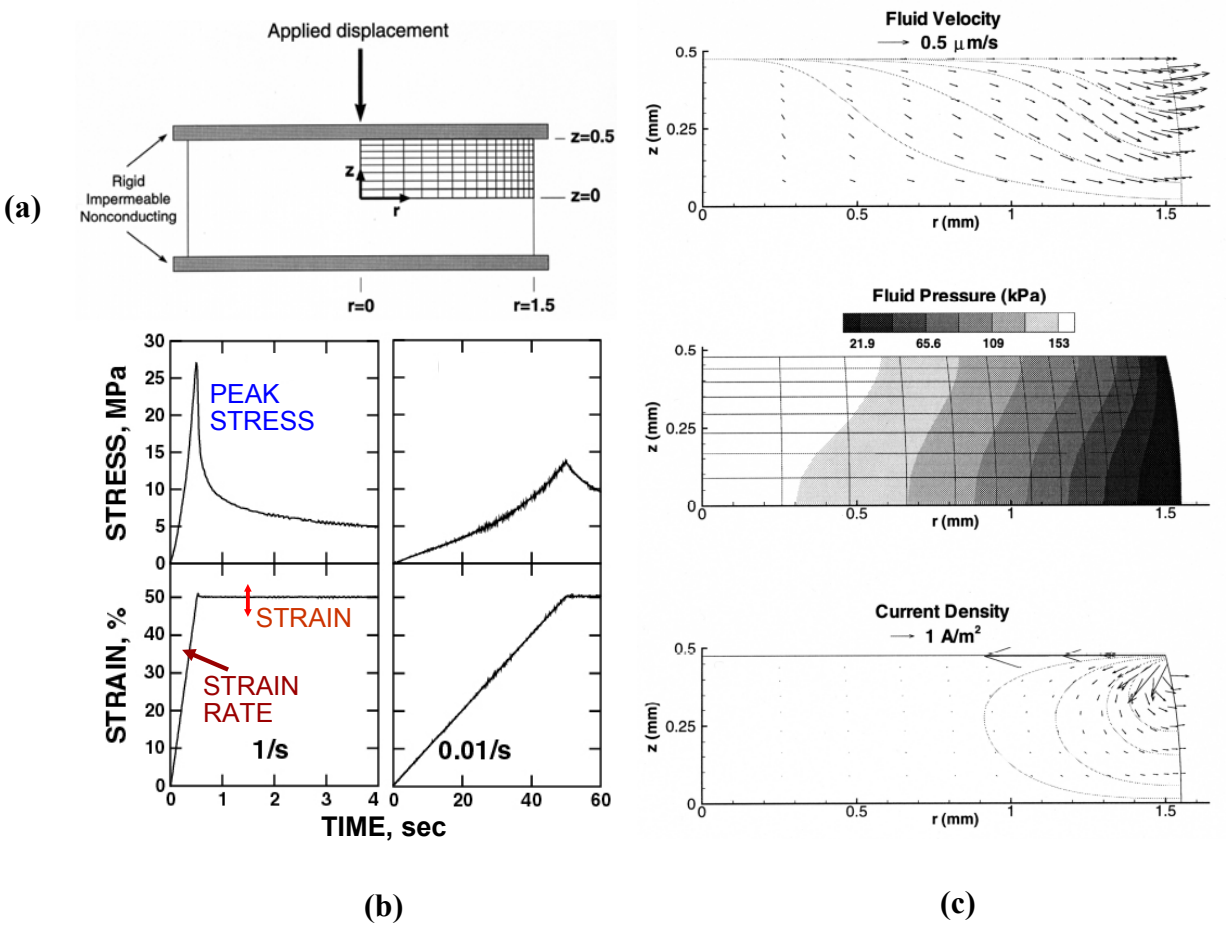


Figure C.2 (a) Schematic of radially unconfined axial compression geometry to apply injurious compression to a cylindrical disk cartilage specimen. **(b)** Application of a "ramp-and-hold" compression in the configuration of (a) to 50% strain at strain rates of 1% /sec and 100% /sec; higher strain rates produce higher peak stress. **(c)** Spatial profiles of intratissue fluid velocity, fluid pressure, and current density in the top quadrant of (a) induced at the end of a 60 sec ramp, similar to that in (b), but employing only a 5% (non-injurious) compression. The fluid flow is predominantly radial with maximal flow near the radial edge. Radial and axial pressure gradients develop, with maximal pressure near the center of the disk beneath the loading platens. Interestingly, an electrical streaming current is also induced with relatively high magnitude and inwardly directed beneath the loading platens, along with lower, more dispersed, outwardly directed currents toward the midplane. In principle, each of these physical forces affects cell behavior. ((b) Adapted from [3]; (a,c) adapted from [5]).

Figure C.3 Group expression profiles in response to injury generated by k-means clustering

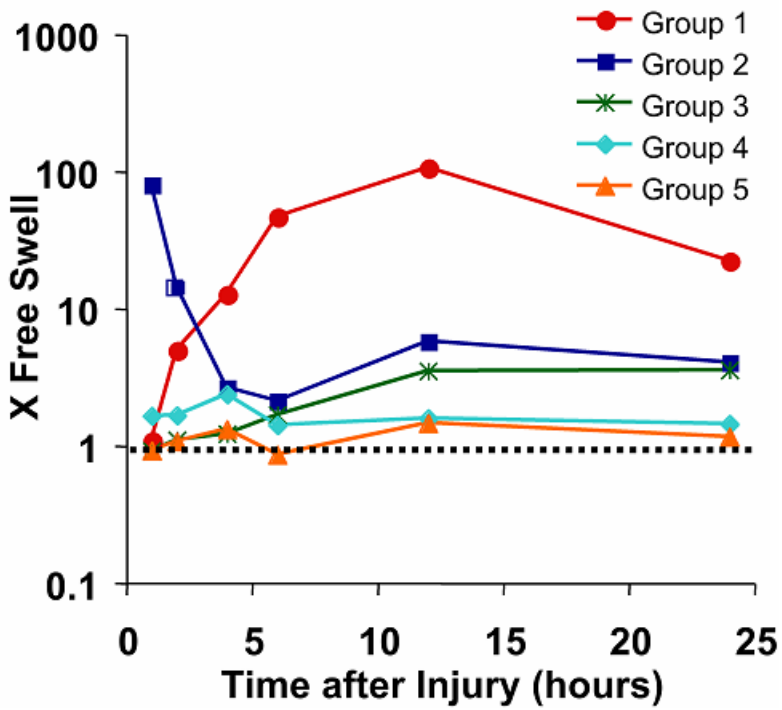


Figure C.3 Group expression profiles generated by k-means clustering, showing the principal gene-expression patterns induced initially by injury of cartilage explants. Group profiles were calculated by averaging the expression profiles of genes within each group. Results are the mean change from free swelling levels, with a value of 1 (broken line) indicating similar expression after injury to the level measured in free swelling conditions. *Group 1*: MMP-3, ADAMTS5, TGF-β; *Group 2*: c-fos, c-jun; *Group 3*: MMP-1, 9, 13, collagen 1, TIMP-1, TIMP-2, fibronectin, sox 9, GAPDH, β-actin, TNF-α; *Group 4*: IGF-1, IGF-2, ADAMTS4; *Group 5*: aggrecan, fibromodulin, link protein, IL-1β. From [2].

Figure C.4 Schematic of *in vitro* models of joint injury

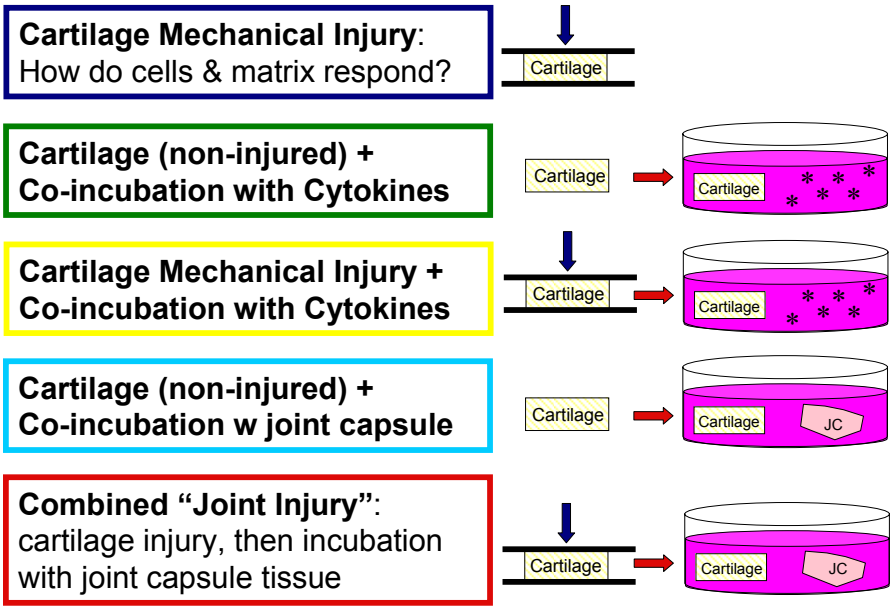


Figure C.4 Schematic of *in vitro* models of joint injury, included injurious compression of cartilage alone, treatment of compressed and non-compressed cartilage with cytokines, and co-culture of compressed and non-compressed cartilage with explants of joint capsule tissue (synovium). (Adapted from [2].)

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