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**Citation:** Lee, J.H. et al. "Co-culture of mechanically injured cartilage with joint capsule tissue alters chondrocyte expression patterns and increases ADAMTS5 production." Archives of Biochemistry and Biophysics 489 (2009): 118-126. Web. 26 Oct. 2011. © 2009 Elsevier Inc.

**As Published:** <http://dx.doi.org/10.1016/j.abb.2009.07.006>

**Publisher:** Elsevier Ltd.

**Persistent URL:** <http://hdl.handle.net/1721.1/66582>

**Version:** Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

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Published in final edited form as:

*Arch Biochem Biophys.* 2009 September ; 489(1-2): 118–126. doi:10.1016/j.abb.2009.07.006.

## Co-culture of Mechanically Injured Cartilage with Joint Capsule Tissue Alters Chondrocyte Expression Patterns and Increases ADAMTS5 Production

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### Abstract

We studied changes in chondrocyte gene expression, aggrecan degradation, and aggrecanase production and activity in normal and mechanically injured cartilage co-cultured with joint capsule tissue. Chondrocyte expression of 21 genes was measured at 1,2,4,6,12, and 24 hours after treatment; clustering analysis enabled identification of co-expression profiles. Aggrecan fragments retained in cartilage and released to medium and loss of cartilage sGAG were quantified. Increased expression of MMP-13 and ADAMTS4 clustered with effects of co-culture, while increased expression of ADAMTS5, MMP-3, TGF- $\beta$ , c-fos, c-jun clustered with cartilage injury. ADAMTS5 protein within cartilage (immunohistochemistry) increased following injury and with co-culture. Cartilage sGAG decreased over 16-days, most severely following injury plus co-culture. Cartilage aggrecan was cleaved at aggrecanase sites in the interglobular and C-terminal domains, resulting in loss of the G3 domain, especially after injury plus co-culture. Together, these results support the hypothesis that interactions between injured cartilage and other joint tissues are important in matrix catabolism after joint injury.

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## Introduction

Joint injury in young adults leads to increased risk for development of osteoarthritis (OA) [1-3] irrespective of surgical intervention to stabilize the joint[4,5]. Joint injury initiates a cascade of responses which can result in cartilage degeneration, pain, and compromised joint function. Following human knee joint injuries which involve damage to the anterior cruciate ligament (ACL) or meniscus, analysis of synovial fluid has revealed elevated levels of collagen II and aggrecan fragments[6-8], inflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$ , IL-6, and IL-8)[9], suppressors of inflammation (IL-1Ra and IL-10)[9], matrix degrading enzymes[6] and markers of matrix turnover and tissue repair (TIMP-1 and aggrecan synthesis)[6] compared to uninjured controls. These proteins and proteolytic fragments could originate from cartilage, joint capsule tissue, meniscus, ligament, tendon, or bone, and may participate in the initiation of cartilage degradation leading to OA.

Animal models of joint injury, such as transection of the ACL (ACLT), show changes in cartilage, bone, synovium, and joint capsule. These changes include cartilage fibrillation and full-thickness loss, chondrocyte cloning, osteophyte formation, hyperplasia of synovial lining, mononuclear cell infiltration into the synovium, and joint capsule fibrosis[10]. In rabbits, ACLT increased chondrocyte gene expression of collagen II, aggrecan, MMP-1,3,13, and decreased expression of decorin and fibromodulin[11,12], while increasing synovial cell expression of MMP-3 and IL-1 $\beta$ [12]. Together, human and animal studies highlight the complexity of events following joint injury and motivate the need for complementary *in vitro* studies to isolate particular factors and identify mechanisms of cartilage degradation.

*In vitro* models of mechanical injury to cartilage, alone, demonstrate damage to cartilage matrix resulting in increased water content[13-15], decreased stiffness[13,16], increased hydraulic permeability[17], sGAG loss to the culture medium[13,18-20], and damage to collagen[14, 15,17]. Additionally, chondrocytes show decreased biosynthesis[13] and undergo apoptosis and necrosis[15,16,19,21-23]. Recently, we found that within 24 hours following a single injurious compression of bovine cartilage explants, gene expression of MMP-1,3,9,13, ADAMTS4,5 and TIMP-1 increased from 4-250-fold, while expression of matrix molecules such as aggrecan and type II collagen remained essentially unchanged compared to that in uninjured cartilage[24].

Directly relevant to the present study, models using normal or mechanically injured cartilage co-cultured with joint capsule have focused on multiple tissue involvement and tissue interactions that naturally occur during joint injury. Excision of tissues from the joint for these studies is considered a model of injury and has been shown to cause cell death at the cut surface [25,26]. For example, co-culture of normal cartilage with joint capsule tissue caused decreased chondrocyte biosynthesis[27,28] and loss of cartilage aggrecan and collagen[29]. Mechanical injury of cartilage followed by co-culture with joint capsule resulted in further reduction in chondrocyte biosynthesis than that occurring after injury or co-culture alone[28]. Capsular tissue released soluble factor(s) that caused aggrecanase cleavage of aggrecan in the interglobular domain[30], and conditioned medium from capsule tissue caused sGAG release from cartilage[31]. ADAMTS5 (aggrecanase-2) is expressed and active in normal bovine and osteoarthritic human synovium[32].

While previous co-culture studies showed degradative capacity of synovium, the combined effects of mechanical injury and co-culture with synovial tissue are not well characterized. The objectives of this study were (1) to determine if co-culture of normal or mechanically injured cartilage with excised joint capsule tissue (including synovial lining cell layers) affects gene co-expression profiles of matrix molecules, matrix degrading enzymes, inhibitors, growth factors, and inflammatory cytokines, and (2) to identify the kinetics of sGAG loss, the presence

and tissue localization of ADAMTS4 and ADAMTS5 in the cartilage, and the specific cleavage of aggrecan observed in these injury models.

## Materials and Methods

### Tissue harvest and culture

1mm-thick by 3mm-diameter cartilage disks were harvested from the middle zone of femoropatellar grooves of 1-2 week old bovine calves (48 disks per joint) as described[24]. Joint capsule tissue was cut from the medial side in the joint immediately proximal to the articular cartilage; full thickness samples consisted of fibrous tissue with a single layer of synovium (visualized in histological cross-section, not shown). Joint capsule tissue was cut into 36 pieces (~5mm × 5mm) using a razor. Cartilage and joint capsule explants were equilibrated separately in medium (low glucose DMEM supplemented with 10% fetal bovine serum, 10mM HEPES buffer, 0.1mM nonessential amino acids, 0.4mM proline, 20µg/ml ascorbic acid, 100U/ml penicillin G, 100µg/ml streptomycin, and 0.25µg/ml amphotericinB) for 2 days in a 37°C, 5% CO<sub>2</sub> environment. Serum was replaced with 1% ITS for samples used in Western analyses.

### Injurious compression, co-culture, and exogenous IL-1α treatment

Injurious compression of cartilage disks was performed using a custom-designed incubator-housed loading apparatus[33] as described previously[13,18,20,24]. A single unconfined compression displacement ramp was applied to a final strain of 50% at a velocity of 1mm/s (strain rate 100%/s) in displacement control, followed by immediate release at the same rate. This strain waveform resulted in an average measured peak stress of ~20MPa, shown previously to produce damage to the ECM, decreased cell viability, decreased biosynthesis by the remaining viable cells, increased sGAG loss to the medium, and altered chondrocyte gene expression when applied to similar bovine cartilage explants in the absence of co-culture with joint capsule tissue[13,16,18,20,24,34].

In co-culture studies, normal or injured cartilage disks were cultured in the same well with a single joint capsule explant after the time of cartilage injury (but not before). Normal cartilage maintained free swelling without joint capsule or exogenous cytokine was the negative control, and normal cartilage treated with 10ng/ml rhIL-1α (R&D Systems, MN), added at the time of medium change every two days throughout the test, was the positive control. Cartilage disks in each treatment or control group were matched across depth and location along the joint surface to prevent bias based on anatomical location.

For analysis of time dependent changes in gene expression, groups of six cartilage disks from each condition were removed from culture at 1,2,4,6,12, and 24 hours, flash frozen in liquid nitrogen, and stored at -80°C. Groups of six non-injured control disks from the same animal were frozen at 4 and 24 hours. This experiment was repeated to give 3 replicates in total, using one joint from each of 3 different animals (data are mean ± SE, n=3). For Western analyses, medium was collected at days 2,4,6, and 8 after initiation of treatment (injurious compression, co-culture, compression plus co-culture, or IL-1α) at the time of medium change and stored at -20°C. Thus, these medium samples contained released molecules from day 0-2, 3-4, 5-6, and 7-8 respectively. Medium collected concurrently from free swelling cartilage served as controls.

### RNA extraction

RNA was extracted from the 6 pooled cartilage disks by pulverizing the tissue and homogenizing in Trizol reagent (Invitrogen, CA) to lyse the cells. Extracts were transferred to Phase Gel Tubes (Eppendorf AG, Germany) with 10% v/v chloroform, spun at 13,000g for 10

min, and the clear liquid removed from above the phase gel. RNA was isolated using the RNeasy Mini Kit (Qiagen, CA). Genomic DNA was removed by DNase digestion (Qiagen, CA) during purification. Absorbance measurements at 260nm and 280nm gave the concentration of RNA extracted from the tissue and the purity of the extract. The average 260/280 absorbance ratio was  $1.91 \pm 0.10$ . Reverse transcription (RT) of equal quantities of RNA (25 ng per  $\mu$ l RT volume) from each sample was performed using the Amplitaq-Gold RT kit (Applied Biosystems, CA).

### Real-time PCR

Expression levels were quantified using the MJ Research Opticon2 instrument and SYBR Green Master Mix (Applied Biosystems, CA). Bovine primers were designed for matrix molecules (collagen II, aggrecan, link protein, fibronectin, fibromodulin, and collagen I), proteases (MMP-1,-3,-9,-13,ADAMTS4,5), protease inhibitors (TIMP-1,-2), cytokines (TNF- $\alpha$ , IL-1 $\beta$ ), housekeeping ( $\beta$ -actin, GAPDH), transcription factors (c-fos, c-jun), and growth factor (TGF- $\beta$ ) using Primer Express software (Applied Biosystems, CA). All bovine primer sequences are as published [35]. Standard curves for amplification showed that all primers demonstrated approximately equal efficiency with standard curve slopes  $\sim 1$ , indicating a doubling in cDNA quantity each cycle.

### Immunostaining of cartilage explants for ADAMTS4 and ADAMTS5

On days 4 and 16 following mechanical injury or co-culture of normal or injured cartilage with joint capsule, cartilage samples were fixed in formalin, paraffin embedded, and sectioned. For each analysis, 40 consecutive 5  $\mu$ m sections were cut from the mid-region of each explant. Adjacent sections were used for immunostaining of ADAMTS4 (JSCVMA[36]), ADAMTS5 (JSCKNG[36]), and non-immune rabbit IgG at 1,5, and 10 $\mu$ g/ml. Primary antibody binding was detected with horseradish peroxidase labeled secondary antibody and 3,3'-diaminobenzidine (DAB) substrate. All sections were counterstained with methyl green. Four sections from each condition were scored on a 4-point scale for staining with each antibody. Briefly, scoring representations were, **0**: no to sparse cellular staining, **1**: cellular staining with no pericellular or matrix staining, **2**: pericellular staining in the absence of matrix staining, and **3**: >50% cells stained + pericellular and interterritorial matrix staining. The sections were read without knowledge of treatment. Images corresponding to the average score for each group are shown to represent the data.

### sGAG measurement

Cartilage disks were digested in 1 ml proteinase K solution (100 $\mu$ g/ml in 50mM Tris-HCl, 1mM CaCl<sub>2</sub>, pH8) at 60°C for 18 hours. Sulfated glycosaminoglycan (sGAG) content of tissue digests was measured using the dimethylmethylene blue (DMMB) dye-binding assay.

### Western analysis of aggrecan from cartilage and medium

Aggrecan was extracted from cartilage in 4M guanidine hydrochloride (in 50mM sodium acetate buffer with protease inhibitors 5mM EDTA, 15mM benzamidine, 1 $\mu$ g/ml pepstatin A, 0.3M aminohexanoic acid, and 0.1mM PMSF) by rocking for 48 hours at 4°C, and then ethanol precipitated (3 volumes ethanol with 5mM sodium acetate) overnight at -20°C. After addition of inhibitors (5mM EDTA, 0.1mM PMSF, and 5mM NEM), culture medium was ethanol precipitated (3 volumes ethanol with 5mM sodium acetate) overnight at -20°C. Samples were spun at 13,000g for 30min at 4°C. Supernatant was removed, and the pellet was dried in a Speedvac, dissolved in digestion buffer (50mM sodium acetate, 50mM Tris, 10mM EDTA, pH7.6), and treated with protease-free chondroitinase-ABC, keratanase II, and endo- $\beta$ -galactosidase (Seikagaku, Japan) to remove sGAG chains from the core protein. Samples were dried, resuspended in Tris-Gly SDS buffer (BioRad, CA) with reducing agent (DTT), boiled,

run on 4-15% gradient Tris-HCl gels (BioRad, CA), and transferred to nitrocellulose membranes at 4°C. Membranes were then blocked with non-fat milk and incubated with primary antibody (one of anti-G1, anti-G3, or anti-NITEGE diluted 1:2000 in 5% non-fat milk; please see Supplementary Appendix A for a detailed description of the antibodies and associated references) overnight. Anti-mouse or anti-goat (depending on primary used) secondary antibody incubation for 1 hour was followed by development using Western Lightning kit (PerkinElmer, MA). Membranes were imaged for chemiluminescence with Fluorochem (Alpha Innotech, CA).

### Statistical analyses

Gene expression levels measured for each gene in treated groups were normalized to those of free swelling controls; expression data are mean ( $\pm$  SE) of three replicate experiments, each utilizing 6 pooled cartilage disks at each time point. Differences in log transformed gene expression levels for treatment samples versus free swelling controls (taken from the same animal) were compared using a paired t-test at the 4 hour and 24 hours. Adjustment for multiple comparisons was made using the false discovery rate approach[37]. Scores for immunohistochemistry sections are summarized as mean ( $\pm$  SE) for each condition. Paired t-tests were performed to compare the effects of each experimental condition to control.

### Clustering and principle component analyses

To distinguish the main co-expression trends in genes measured by real-time PCR, a k-means clustering algorithm[38,39] was applied to the combined time course expression data from experiments involving (1) co-culture of normal cartilage with joint capsule, (2) cartilage injury plus co-culture, and (3) data from our previous study[24] of changes in expression caused by injurious compression of cartilage with no co-culture (utilizing the same identical gene set, injury protocol, and same-aged bovine cartilage explants). Each gene was grouped based on the correlation of the time course expression profile in the three injury models to a set of randomly chosen starting genes. Group profiles were calculated as the average of the expression profiles of the genes in each group. The correlation between each gene and group profile was calculated, and the genes regrouped in an iterative fashion until the groupings settled. To ensure that an optimal clustering solution for the 21 genes was found, the algorithm was run ~20,000 times to cover every possible selection of starting genes. Each set of randomly chosen starting genes produced a deterministic grouping of the genes, with each gene paired with the highest correlating group profile. The number of deterministic groupings found by the clustering algorithm is much smaller than the number of starting gene combinations so the best solutions are found multiple times. The optimal solution was chosen as the grouping that had the highest overall correlation of genes to group profiles, by averaging over all the genes[39]. The number of groups was varied from three to six, and five groups were chosen to best represent the trends.

## Results

### Gene expression of matrix molecules, enzymes and inhibitors, soluble and transcription factors, and housekeeping genes

Co-culture of normal cartilage with joint capsule tissue caused chondrocyte expression of GAPDH to increase 12-fold (Figure 1A) while expression in cartilage that was mechanically injured and co-cultured increased by 7-fold for GAPDH ( $p=0.007$ ) and 4-fold for  $\beta$ -actin (Figure 1A). Among the 21 genes evaluated, those that were significant using a false discovery rate of 0.05 were GAPDH, MMP-3, MMP-9, and c-fos. Because GAPDH and  $\beta$ -actin responded to co-culture with or without injury, we did not normalize expression levels of other genes to either of these molecules; instead, all data were normalized to the total quantity of RNA in each sample as described in Methods.



In normal cartilage co-cultured for 24 hours with joint capsule tissue, collagen II and aggrecan expression levels remained at or near levels in control tissue (Figure 1A). In contrast, after injury plus co-culture, collagen II and aggrecan expression decreased to 40% and 53% of control levels by 24 hours (Figure 1A). Additionally, fibromodulin responded to injury plus co-culture with decreased expression at all times to 30-60% of control levels, while co-culture alone did not significantly affect expression (data not shown). Link protein and fibronectin expression were not significantly affected by co-culture in injured or normal cartilage (data not shown).

In contrast to the generally stable or decreased expression seen for matrix molecules, enzymes able to cleave matrix molecules showed increases in expression during co-culture with joint capsule tissue. MMP-3 (stromelysin) showed the largest increase (16-fold) during 24 hours of co-culture, while injury plus co-culture caused a 140-fold increase in MMP-3 expression by 12 hours (Figure 1B,  $p=0.009$ ). ADAMTS5 (aggrecanase-2) expression increased 6-fold over controls during co-culture, while cartilage injury plus co-culture resulted in an 85-fold increase by 12 hours (Figure 1B). ADAMTS4 (aggrecanase-1) increased to 6-fold by 6 hours of co-culture and 10-fold in injured plus co-cultured cartilage, both returning to control levels by 24 hours (Figure 1B). MMP-13 (collagenase-3) first increased to 7-fold above controls by 6 hours and then decreased to 4-fold over control by 24 hours of co-culture; however, no change was observed when cartilage was first mechanically injured (Figure 1B). MMP-1 (collagenase-1) expression remained within 2-fold of control during co-culture of normal or injured cartilage (Figure 1B). MMP-9 (gelatinase B) decreased to 30% of control levels after injury plus co-culture ( $p=0.004$ ), but showed no response to co-culture alone (data not shown). Tissue inhibitors of metalloproteinase (TIMPs) can bind to and inactivate MMPs. TIMP-1 expression did not change with co-culture in either normal or injured cartilage, while TIMP-2 increased ~2-fold from 4 hours to 24 hours after co-culture in both normal and injured cartilage (data not shown).

The inflammatory cytokines, interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), were differentially regulated compared to transforming growth factor  $\beta$  (TGF- $\beta$ ), during co-culture of normal or injured cartilage. Expression of IL-1 $\beta$  increased gradually during co-culture from 0.5 to 2-fold above the control levels between 1 and 24 hours (Figure 1C); injury plus co-culture caused an immediate decrease in expression to 15% of controls and then a return to baseline by 6 hours (Figure 1C). TNF- $\alpha$  expression did not change significantly with time in either model (Figure 1C). TGF- $\beta$  expression did not respond to co-culture alone, but showed a 5-fold increase in expression between 4 and 12 hours after injury plus co-culture, then decreased to 2-fold above controls by 24 hours (Figure 1C). Expression of transcription factors c-fos and c-jun increased 2-5-fold during 24 hours of co-culture (Figure 1D). In contrast to these relatively low magnitude changes with co-culture, by 1 hour after injury plus co-culture, c-fos and c-jun increased to 110-fold and 40-fold of controls, respectively, and then decreased to 1.5-5.5-fold over controls by 24 hours of culture (Figure 1D,  $p=0.006$  for c-fos).

### Gene clustering profiles: comparison of injury models

The individual experimental treatments of Figure 1 sometimes led to reproducible, high magnitude changes for a particular gene with trends that did not reach statistical significance. Nevertheless, principle component analysis and k-means clustering techniques are powerful statistical tools that can identify patterns of gene co-expression in the large data set represented by the 3 $\times$ 6 $\times$ 21 matrix corresponding to the 3 experimental conditions, 6 time points, and 21 genes studied here (see ref. 38 for a detailed discussion). Thus, clustering analyses formed gene groups (Figure 2) based on the temporal expression profile of each gene in response to the two models involving co-culture (i.e., Figure 1) as well as the response to cartilage injury alone with no co-culture (reported previously[24]). This analysis using the combined data sets for

the three injury models enabled the identification of trends in gene expression changes across injury, co-culture, and the combined model of mechanical injury followed by co-culture for 24 hours.

Group 1 (Figure 2A) contains matrix molecules aggrecan, collagen II, and fibromodulin, and showed relatively invariant expression following either injury alone or co-culture alone, but decreased expression to 50% of control following injury plus co-culture. Group 2 (Figure 2B) contains genes with a variety of functions (MMP-1, MMP-9, TIMP-1, TIMP-2, IL-1 $\beta$ , TNF- $\alpha$ , collagen I, link protein, fibronectin). In contrast to Group 1, Group 2 expression increased 3.5-fold by 12 hours after injury, but remained relatively unchanged in the two models involving co-culture. Group 3 (Figure 2C) contains the proteases MMP-13 and ADAMTS4 as well as GAPDH, and showed increased expression over 24 hours after injury compared to free swelling controls. Co-culture of either normal or injured cartilage caused an immediate decrease in expression of Group 3 genes at 1 and 2 hours, followed by increased expression to 6-fold and 5-fold, respectively, above controls. Group 3 genes showed a larger magnitude response in co-culture models compared to injury without co-culture. This may indicate that expression of Group 3 genes is more sensitive to factors released from joint capsule tissue.

Group 4 genes showed increased expression in response to all three injury models (Figure 2D). In contrast to Group 3, which was more responsive in the two co-culture models, Group 4 showed the largest increases in the two models that included mechanical injury, with all members showing a peak in expression 12 hours after injury. Thus, Group 4 genes appear more sensitive to injurious compression than co-culture with joint capsule tissue. Transcription factors c-fos and c-jun (Group 5, Figure 2E) showed an immediate transient upregulation followed by rapid decrease within 4 hours in response to injury alone or injury plus co-culture. However, in normal cartilage plus co-culture, Group 5 showed only a moderate (2-4-fold) upregulation at all time points. Thus, c-fos and c-jun expression also appeared more sensitive to injurious compression than co-culture.

### Immunostaining of Cartilage Explants for ADAMTS4 and ADAMTS5

Because the ADAMTS enzymes have been identified as critical to the destruction of cartilage in mouse models[40,41], we sought to determine if increased gene expression of aggrecanases resulted in a concomitant increase in aggrecanase abundance in cartilage. Additionally, we extended the analysis of these three injury models from 24 hours used in the gene expression experiments to 4-16 days for protein analyses. IHC was performed on cartilage disks 4 and 16 days after injury alone and after co-culture of normal and injured cartilage with joint capsule tissue. Increased immunostaining of ADAMTS5 was observed in cartilage at 4 days in all three treatments (Figure 3) with average scores, however the difference was only statistically significant in the comparison between injury and control:  $0.3 \pm 0.3$  for control,  $1.8 \pm 0.2$  ( $p=0.04$ ) for injury,  $1 \pm 0$  (n.s.) for co-culture, and  $1.8 \pm 0.2$  ( $p=0.10$ ) for injury followed by co-culture. A trend for elevated staining for ADAMTS5 continued through 16 days following injury alone ( $1.8 \pm 0.4$ ) and injury plus co-culture ( $2.0 \pm 0$ ) compared to control ( $1.2 \pm 0.4$ ); co-culture alone appeared reduced ( $0.2 \pm 0.2$ ) however no differences were statistically significant (Figure 3). Notably, the immunoreactive protein was predominantly associated with chondrocytes with essentially undetectable signal in the matrix. Staining for ADAMTS4 was low or absent at all time points with no discernible differences between experimental conditions (data not shown). Minimal ADAMTS4 staining was observed in hypertrophic areas in the cartilage originating from the remnants of the secondary centers of ossification in these immature joints.

### Aggrecan fragments released to the medium

To determine if increased ADAMTS5 protein levels observed by immunohistochemistry was accompanied by changes in the release of aggrecan fragments from the tissue, medium from



the different models was analyzed by Western blotting using antibodies to the G1 and G3 globular domains of aggrecan (anti-G1 and anti-G3) and the aggrecan G1-NITEGE fragment resulting from cleavage by aggrecanase at amino acid 373 (anti-NITEGE). Medium samples collected on day 4 stained positively for anti-NITEGE in all conditions that included joint capsule tissue (Figure 4, lanes 4-8) and for cartilage treated with IL-1 $\alpha$  as a positive control for aggrecanase-mediated aggrecan catabolism (Figure 4, lane 2). Results were confirmed with anti-G1 and anti-G3 antibodies (data not shown). Importantly, because medium from joint capsule tissue without cartilage contained measurable aggrecan cleavage products, it was not possible from analysis of the culture media to determine if co-culture resulted in increased damage to cartilage matrix through cleavage at this aggrecanase site. We therefore analyzed cartilage extracts directly to determine the effect that each injury model had on cartilage protein abundance and enzymatic activity.

### Tissue sGAG content and aggrecan cleavage in cartilage

Cartilage sGAG content decreased slightly by 4 days after injurious compression alone compared to free-swell control but was significantly decreased in all three injury models by 16 days (Figure 5A). We therefore analyzed aggrecan processing within cartilage disks at day 16 by Western blotting (anti-G1 and anti-G3). All three injury models had increased levels of aggrecan cleavage at the interglobular domain aggrecanase site (Figure 5B). Increased abundance of this band was confirmed by staining with the specific anti-NITEGE antibody that recognized this cleavage site (data not shown). In addition, the combination of injury plus co-culture led to a decrease in cartilage G3 domain, measured as anti-G3 reactivity in Western blots (Figure 5C). While an extensive study by Western analyses was not performed here at earlier time points, results from a parallel study [42] comparing control and injury-alone at day 6 showed no appearance of NITEGE bands caused by injury- alone by day 6 (via anti-G1 blotting), and a similar result to that seen on day 16 in Figure 5C for injury-alone versus free swelling controls.

## Discussion

### Gene expression trends in injury models

Clustering analyses revealed specific temporal patterns of chondrocyte gene expression that highlighted changes associated with co-culture (using either normal or injured cartilage) versus expression trends associated with cartilage mechanical injury (alone or co-cultured with joint capsule tissue). The co-culture model caused maximal increases in ADAMTS4 and MMP-13 expression (Figure 2C). In marked contrast, mechanical injury alone or injury plus co-culture caused maximal increases in ADAMTS5, MMP-3, and TGF- $\beta$  (Figure 2D), and maximal transient increases in c-fos and c-jun (Figure 2E). The role of increased TGF- $\beta$  in these models may be consistent with a potential role for an initial attempt at enhancing anabolic repair after mechanical injury or, alternatively, increased TGF- $\beta$  may accelerate matrix remodeling and degradation through its reported ability to activate aggrecanases [43], MMP-3, and MMP-13 [44] in human chondrocytes and cartilage explants. TGF- $\beta$  is also elevated in joint fluid in vivo, driving synovial hyperplasia and proteoglycan loss from cartilage [45]. c-fos and c-jun, members of the activating protein-1 (AP-1) family of genes, were shown previously to lead to transcription of MMPs in a chondrocyte cell line following IL-1 $\beta$  treatment [46]. Together, these changes in expression highlight the shift toward pro-catabolic processes in the injury models, especially those including mechanical overload injury. In light of our recent finding that the MAPK pathway further upstream of c-fos/c-jun appears to be a central conduit for transducing *non-injurious* dynamic compression and dynamic shear forces into biological responses in cartilage [47] it will be of great interest to elucidate the role of MAPK pathway members in the responses to *injury and co-culture* reported here. In addition to pro-catabolic changes, matrix molecules collagen II and aggrecan decreased expression in mechanically

injured cartilage co-cultured with joint capsule; a change that further contributes to the overall weakening of the tissue over time. These characteristics of the models are consistent with the increased risk for joint tissue damage and OA following injury in patients and provide some specific mechanisms for further investigation in *in vivo* injury models.

### Enzyme abundance and aggrecan degradation in cartilage and joint capsule

In agreement with the high levels of ADAMTS5 gene expression induction observed in these injury models, ADAMTS5, but not ADAMTS4 protein was increased in all three models (Figure 3). The apparent accumulation of ADAMTS5 in the injured cartilage may indicate its involvement in the observed increase in aggrecan catabolism. Aggrecan fragments were released from full-thickness joint capsule tissue cultured without cartilage, consistent with previous observations of aggrecan production by joint capsule tissue [48,49] as well as the recent observation of gene transcription of aggrecan in joint capsule explants from similar immature bovine knees[50]. Degraded aggrecan was released from live and dead joint capsule (Figure 4), indicating that such release was not a cell dependent process. Thus, the presence of aggrecan fragments in the medium of co-culture systems does not conclusively reveal pathways of *cartilage* aggrecan degradation. Importantly, this finding suggests that aggrecan fragments found in synovial fluids of injured or arthritic joints may also be released by a variety of joint tissues. Thus, direct analysis of aggrecan within cartilage was performed in all three *in vitro* injury models, revealing aggrecan cleavage in the cartilage and loss from the tissue continuing for up to 16 days (Figure 5).

### Limitations

Our study is limited by inclusion of only two tissue types (joint capsule and cartilage) present in the joint environment. It is likely that other tissues and fluids have a significant effect on disease progression following joint injury. The effects of mechanical injury on chondrocyte cell death are well documented for the case of isolated cartilage explants; however, it will be also important to assess the possible effects of co-culture on long-term cell viability in both cartilage and joint capsule tissues. While the young age of the tissue used in this study may be considered a limitation, we found previously that the synergistic effects on GAG loss of injurious compression combined with IL-1 or TNF- $\alpha$  treatment using this same immature bovine knee cartilage were also observed in adult human knee cartilage[18]. Indeed, the present study is motivated by the increasing importance of joint injury in young individuals. Nevertheless, it is possible that tissue from mature animals will behave differently in these injury models and this should be considered when making comparisons to other studies.

### Degradative capacity of joint capsule

Both synovium and joint capsule tissue generate a soluble aggrecan-degrading activity *in vitro* [30,31]. Higher aggrecanase-mediated cleavage potency was measured in medium from co-culture of synovium with either live or dead cartilage than that seen from synovium alone [30]. This result could be explained by release of latent aggrecanase activity from cartilage and synovium; alternatively, the presence of cartilage may stimulate new aggrecan production and aggrecanase activity in synovium leading to increased cleavage of cartilage aggrecan. ADAMTS5 protein is present in arthritic human and normal bovine synovium[32,51] where it is concentrated in the synovial lining and around blood vessels[32]. IL-1 $\alpha$  and retinoic acid increase activity of ADAMTS5 in cartilage without a concomitant change at the gene expression level[52]. TGF- $\beta$  (but not IL-1 or TNF- $\alpha$ ) increase mRNA and protein expression of ADAMTS4 in synoviocytes[51]. TGF- $\beta$  also causes an increase in expression of ADAMTS4 (not ADAMTS5) in cartilage from normal and OA joints[43]. Taken together, these studies demonstrate synovial cell production of degradative factors in the joint environment. Further

study is required to determine mechanisms involved in the regulation and downstream effects of these factors following joint injury that may lead to progression of OA.

### Comparison to previous *in vitro* co-culture studies

Co-culture of cartilage with cut synovium resulted in nearly complete depletion of collagen II and aggrecan from the cartilage by 14 days[29]. Matrix damage was most severe when live cartilage and synovium were in contact, and less severe, though still elevated, when cartilage was at a distance from live synovium. However, these studies did not identify the specific catabolic factors involved and whether these factors originated from the synovium or cartilage. In the present study, MMP-3, MMP-13, ADAMTS4, and ADAMTS5 expression in normal cartilage were upregulated by co-culture (Figure 1B). Injury plus co-culture additionally upregulated TGF- $\beta$  expression (Figure 1C) and ADAMTS5 protein production (Figure 3). Ongoing studies focus on identifying which factors may regulate matrix degradation in these models. Previous studies also showed decreased proteoglycan synthesis when cartilage was cultured in contact with or at a distance from synovium for 8 days[27]. Injurious compression plus 6 days of co-culture caused a further decrease in biosynthesis compared to 6 days of co-culture without initial injury[28]. Here, we found a significant decrease in collagen expression by 24 hours of co-culture, and both collagen II and aggrecan expression were substantially decreased to ~50% control levels by 24 hours after injury plus co-culture. Importantly, injurious compression alone did not decrease expression of matrix molecules within 24 hours[24].

### Comparison to *in vivo* injury models

In the rabbit, ACLT induced changes in gene expression in both cartilage and synovium. MMP-13 increased ~15-30-fold by 3 weeks and was ~7-16-fold above controls 8 weeks post-ACLT, while MMP-1 and MMP-3 changed at most only ~2-3-fold in cartilage from different sites[11]. In another study, MMP-3 expression was elevated 9 weeks after ACLT in both the cartilage and synovium; IL-1 $\beta$  was elevated in synovium but below the level of detection in cartilage[12]. Though the models are quite different, we also observed increased chondrocyte expression of MMP-3 and MMP-13, but not IL-1, during co-culture of cartilage with joint capsule tissue. Together, these studies suggest that chondrocyte MMP expression is increased in both *in vivo* and *in vitro* models of joint injury. Additionally, they suggested that IL-1 present in joint fluid following injury and during OA progression is likely produced by tissues other than cartilage, possibly synovium. 3-8 weeks post ACLT, expression of collagen II and aggrecan was elevated ~2-4-fold in cartilage, while decorin and fibromodulin decreased[11]. We measured either no change or decreased expression of these molecules in normal or mechanically injured cartilage after 24 hours of capsule co-culture *in vitro*. This difference in response of matrix gene expression may be due to the difference in time points or alternatively may be mediated by a cell type that is present *in vivo* but absent in our culture system.

Interestingly, ADAMTS5 was identified as the primary aggrecanase responsible for cartilage degradation in murine joint instability[40] and inflammatory[41] models of arthritis. Both these ADAMTS5 deficient murine models showed reduction in pathology, proteoglycan release, and aggrecanase cleavage in the interglobular domain, while no protection was seen for ADAMTS4 deficiency. In the present study, ADAMTS5 gene expression and protein levels increased far more than ADAMTS4 in all three *in vitro* models. Together, these studies suggest that ADAMTS5 may be a key degradative enzyme in aggrecanolysis following joint injury, though differences in species and the role of additional ADAMTSs and MMPs may still be important to consider[8].

In summary, *in vitro* model systems such as that described here may be very useful in testing the efficacy of therapeutic agents. Our results strongly suggest that mechanical injury to cartilage in the presence of cut joint capsule tissue shifts chondrocyte metabolism towards pro-

catabolic pathways that may result in matrix degradation. While the efficacy of aggrecanase inhibitors remains to be demonstrated in human clinical trials, our study suggests that such in vitro models can provide a useful test bed for mechanistic understanding complex multi-joint tissue interactions that may underlie pathology. Similar in vitro injury models involving the combination of cartilage injury and the presence of exogenous cytokines [18] can also be used to test the efficacy of selected cytokine blockers, e.g., that for IL-6 [42]. Interestingly, the present study showed that neither IL-1 $\beta$  nor TNF- $\alpha$  expression was upregulated in chondrocytes by co-culture of normal or injured cartilage with joint capsule tissue. This is consistent with the recent finding by [53] that inhibition of proteoglycan biosynthesis by co-incubation with joint capsule could not be reversed by blockers of IL-1 $\beta$  or TNF- $\alpha$ , again suggesting the utility of our in vitro systems for exploration of therapeutic interventions.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

Supported NIH NIAMS Grant AR45779. Dr. Lee supported by a National Science Foundation pre-doctoral fellowship.

NIH NIAMS Grant AR45779

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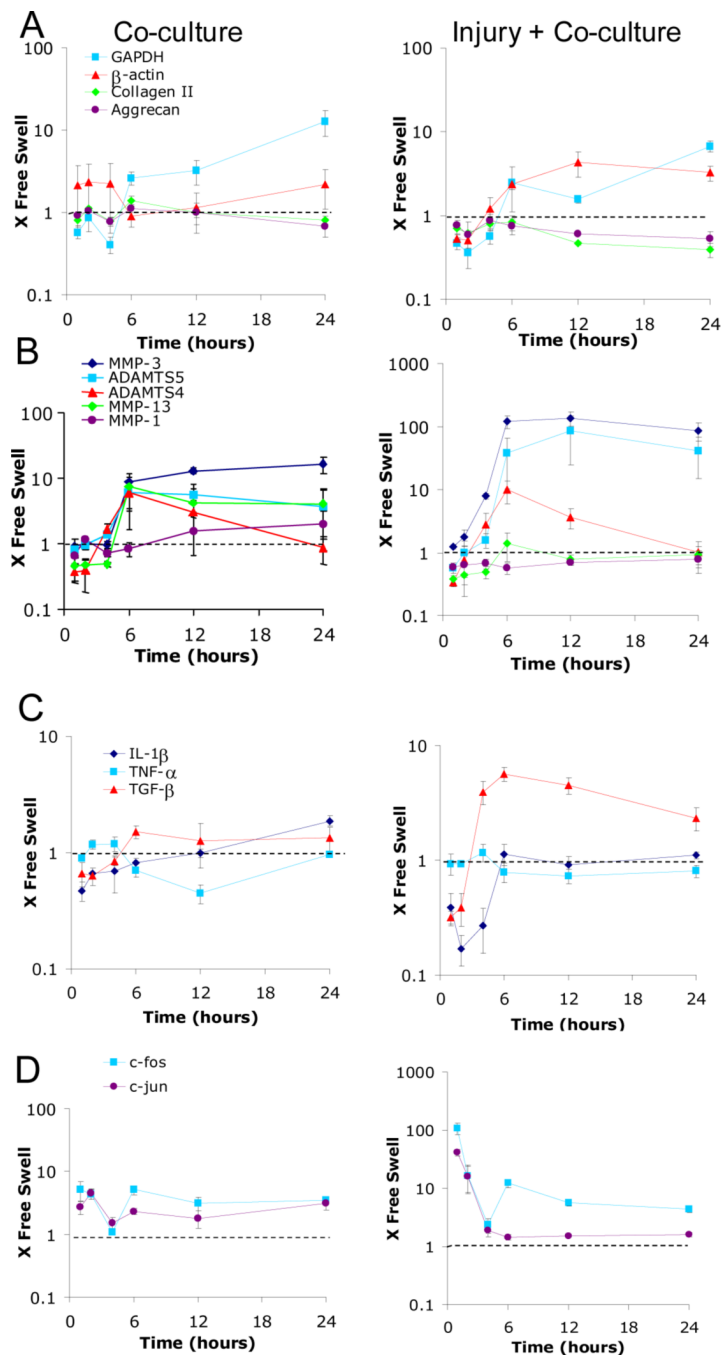
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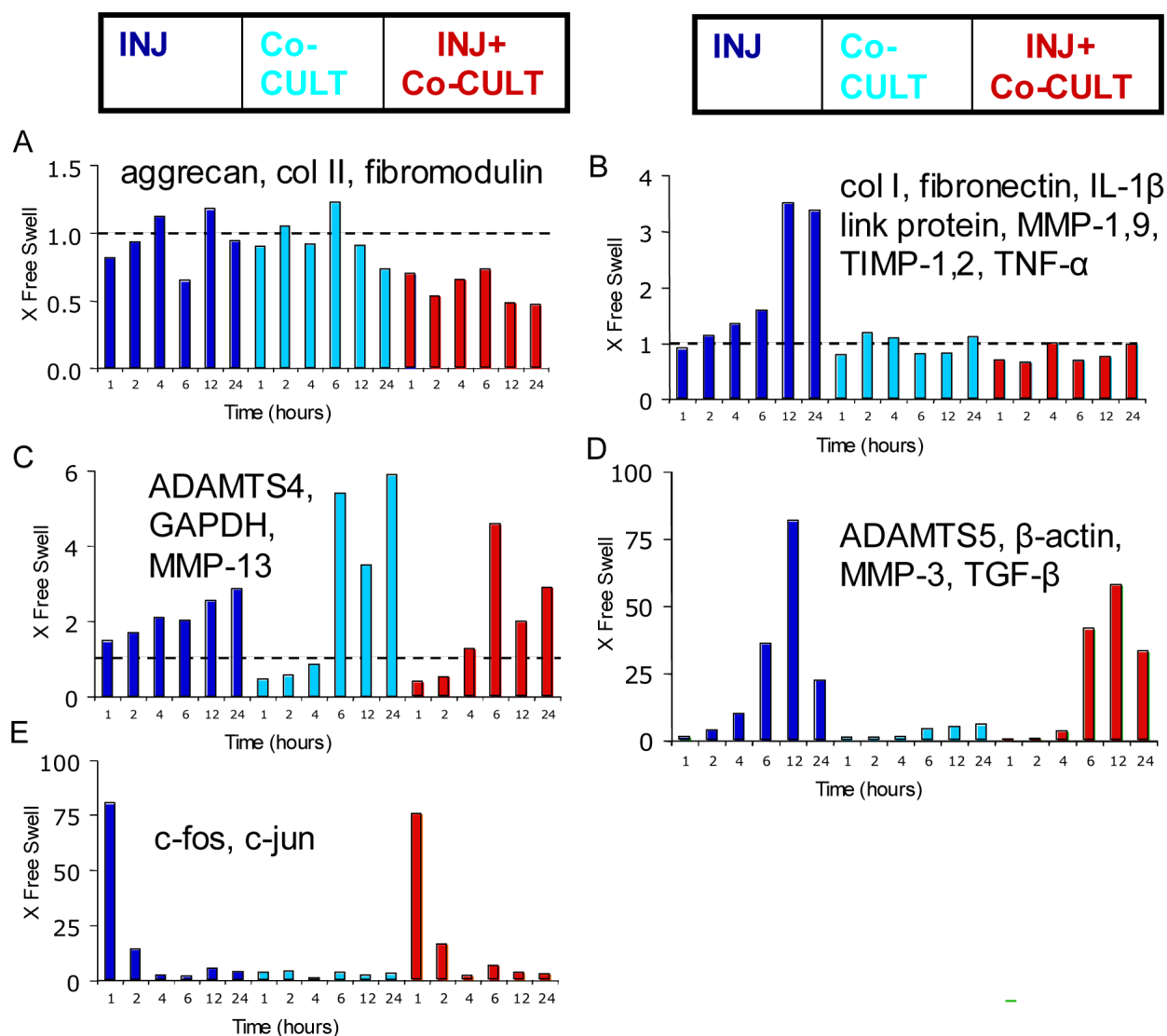


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**Figure 1. Changes in expression level during co-culture of uninjured and injured cartilage with joint capsule tissue**

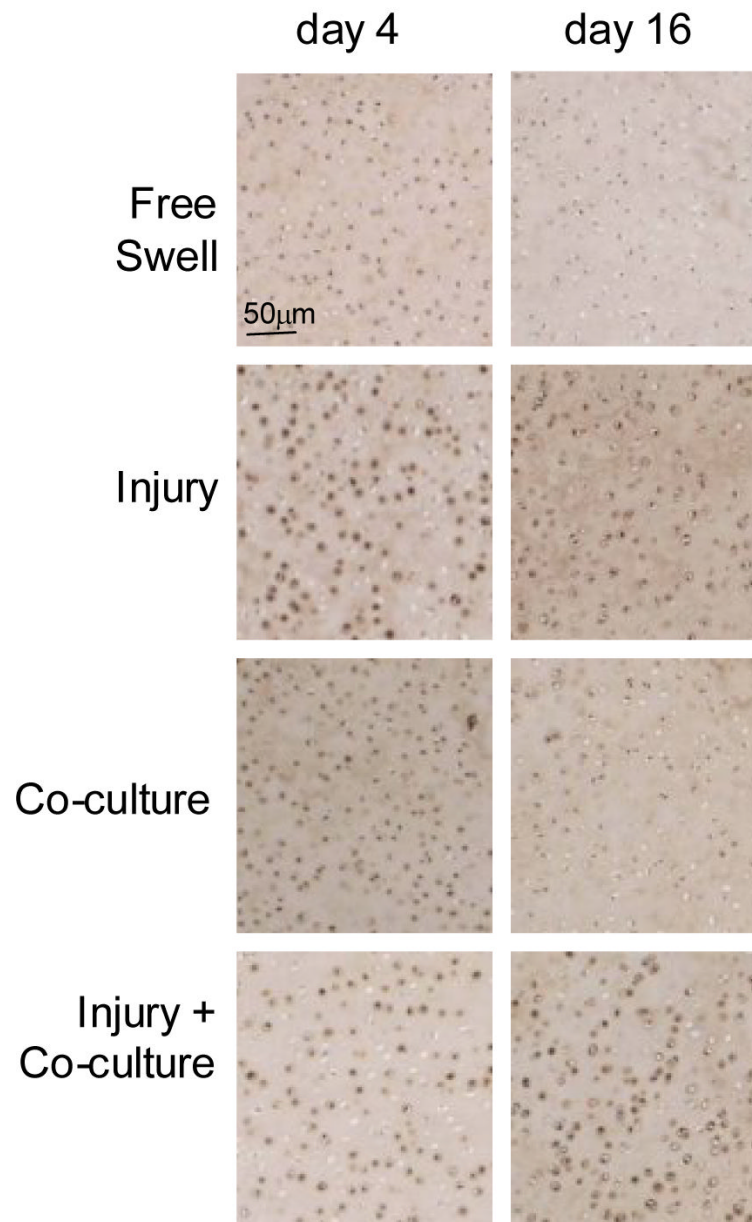
Values on the y-axis represent fold change from free swelling control levels with a value of 1 indicating similar expression during co-culture of uninjured cartilage (left panels) or mechanically injured cartilage (right panels) to the level measured in free swelling, non-co-cultured conditions. Six cartilage disks were pooled for each time point; this experiment was repeated to give 3 replicates in total, using one joint from each of 3 different animals (data are mean  $\pm$  SE, n=3). All samples were normalized to total RNA at the reverse transcription step.



**Figure 2. Group expression profiles generated by k-means clustering**

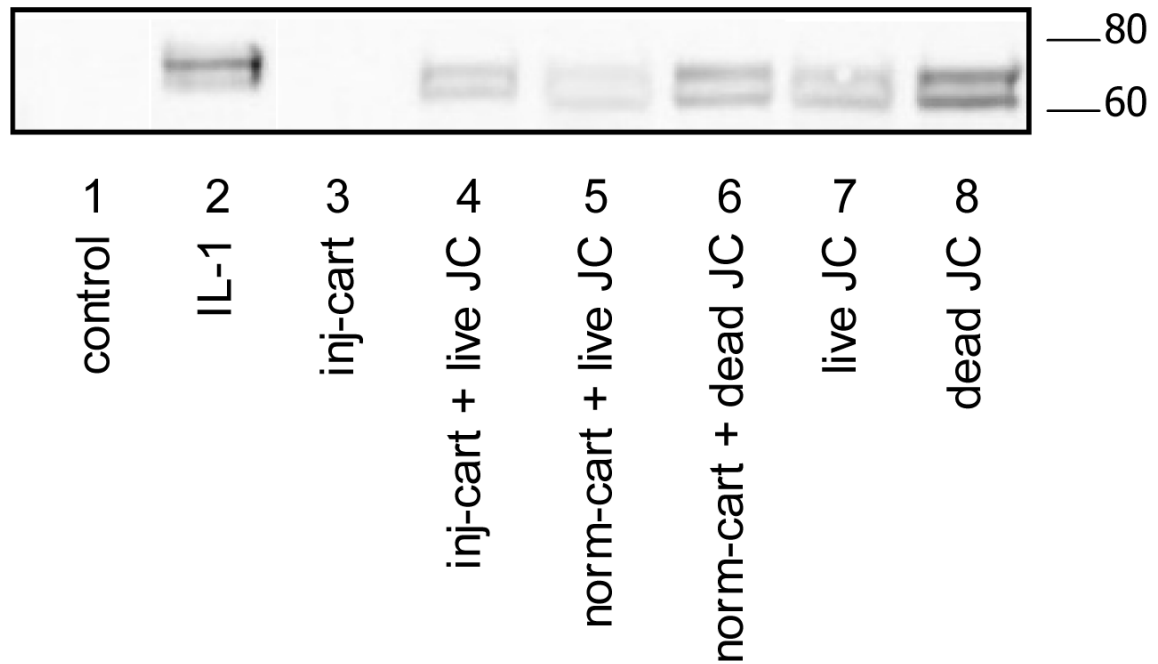
The temporal gene expression patterns in cartilage explants induced by injurious compression, co-culture with joint capsule tissue, and injurious compression followed by co-culture with joint capsule tissue. The gene expression data set for clustering consisted of expression levels at six time points each after mechanical injury (dark blue), during co-culture with joint capsule (light blue) and after mechanical injury followed by co-culture with joint capsule tissue (red). Expression data were clustered into five groups. A) Group 1, B) Group 2, C) Group 3, D) Group 4, and E) Group 5 with all members listed in the Figure. Group profiles were calculated by averaging the expression profiles of genes within each group.

# anti-ADAMTS5



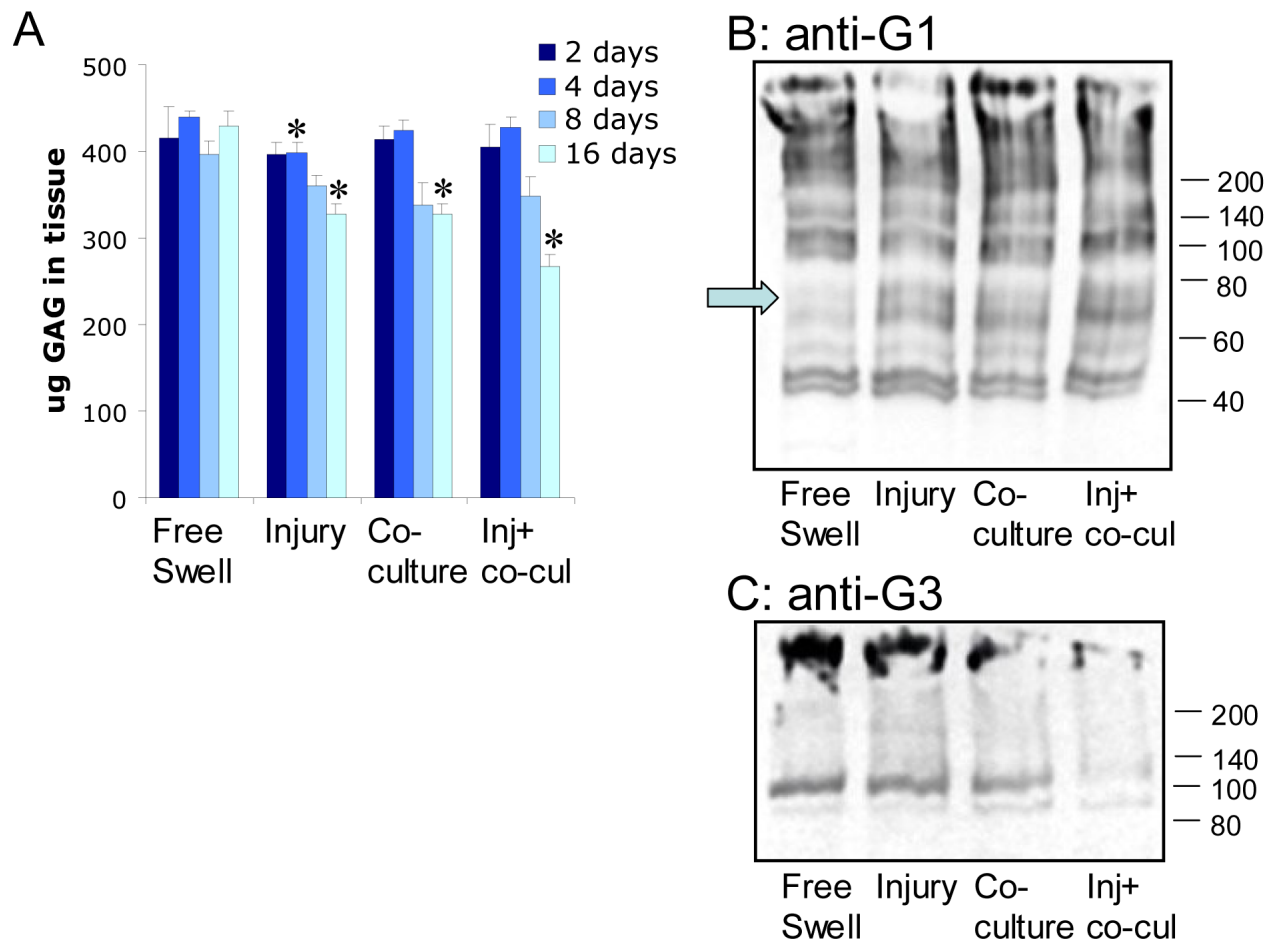
**Figure 3. Immunostaining of ADAMTS5 in cartilage tissue sections following injury and during co-culture of uninjured and injured cartilage with joint capsule tissue**

Cartilage tissue was fixed in formalin, paraffin embedded, sectioned, and stained with anti-ADAMTS5 antibody. Images are representative of 6 images scored for each group (see Materials and Methods). Scale bar of 50  $\mu$ m is the same for all images.



**Figure 4. Western blot for aggrecanase fragments in *in vitro* models of joint injury**

Anti-NITEGE antibody blot of conditioned medium collected on day 4 from free-swell control (lane 1), IL-1-treated cartilage (lane 2), mechanically injured cartilage (lane 3), mechanically injured cartilage co-cultured with joint capsule tissue (lane 4), uninjured cartilage co-cultured with live (lane 5) or dead (lane 6) joint capsule tissue, live (lane 7) or dead (lane 8) joint capsule tissue cultured alone. Dead joint capsule tissue was subjected to three cycles of freeze-thaw. The portion of the gel shown includes all reactive bands.



**Figure 5. Analysis of cartilage tissue subjected to injurious compression and co-culture with joint capsule tissue**

(A) Cartilage tissue explants were subjected to injurious compression, co-culture with joint capsule tissue, or the combination of injurious compression followed by co-culture with joint capsule tissue. After 2, 4, 8, or 16 days in culture, tissue was digested and sGAG content of the digest was measured by DMMB dye binding assay. \* indicates  $p \leq 0.05$  compared to free swell control. Mean  $\pm$  SE (n=4). (B) Cartilage tissue extract samples from each of the injury models at day 16 were prepared in parallel and probed with anti-G1 primary antibody or (C) anti-G3 antibody. Sample extracts are from free swell control cartilage (lane 1), injured cartilage (lane 2), cartilage co-cultured with joint capsule tissue (lane 3), and injured cartilage co-cultured with joint capsule (lane 4). Molecular weight standards (kDa) shown at the right. Arrow in (B) identifies the band known from previous studies to be that of the G1-NITEGE fragment; Arrow in (C) corresponds to full length aggrecan. The portion of the gels shown include all reactive bands.