Progression of Gene Expression Changes following a Mechanical Injury to Articular Cartilage as a Model of Early Stage Osteoarthritis

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1. Introduction

Osteoarthritis is estimated to affect 27 million Americans and this number is predicted to rise over the coming years [1]. While the causes and progression of OA are not completely understood, a prior joint injury is a known predisposing factor for the development of OA [2]. Therefore, in a laboratory setting, an injury model may be used to study the early stage progression of cartilage degeneration.

One common method of modeling OA in a laboratory setting is that of an impact injury. In this scenario, a controlled impact is delivered to the joint surface and induced changes are evaluated. The impacts can be done in vivo [3–5] or in vitro [6–9]. However, an in vivo impact injury may prove difficult to evaluate in terms of ongoing loading following the discrete loading event. Thus, an in vitro model allows for much more accurate quantification of the mechanical forces delivered to the articular surface. Most impact studies have utilized loading normal to the cartilage surface [3, 5–11]; however a real physiologic loading event likely has loading along multiple axes. Therefore one of our aims was to employ a more complex impact model with elevated shear loading.

Identifying differences in gene expression related to OA progression may aid in the identification of pathways of early stage disease development. Combining an impact injury model with an evaluation of gene expression changes may help to identify future targets for intervention during OA progression. Previous studies have utilized cyclical loading [12], constant strain [13], dynamic loading [14], and impact loading [15] in order to evaluate gene expression changes. Most of the previous work has utilized cartilage explants. With our model, a patella is removed from the knee and
the articular cartilage is maintained intact on the underlying bone. This avoids any potential changes produced by cutting the tissue free from the surface. We have used porcine articular cartilage in our model to study the progression of OA. Animal tissue is frequently used for the study of OA progression [3, 5, 7, 10, 16–18]. More specifically, porcine tissue is readily available and has often been used for both gene expression and impact studies [8, 19–22], making it an appropriate tissue for our use as a model of impact injuries and early stage OA progression.

In our previous work developing this impact injury model of OA, we maintained intact patellae in culture for up to two weeks [8]. We evaluated loading normal to the surface at only the day 14 time point. In this study we aim to evaluate the progression of early OA symptoms by measuring gene expression changes on the day of impaction and at 3, 7, and 14 days following the impaction event. In addition to the axial impaction model we utilized previously, we also evaluated a model with increased shear forces. Eighteen genes were selected including those associated with cartilage matrix, degradative enzymes and inhibitors, inflammatory response and signaling, and cell proliferation and apoptosis and evaluated in the “traditional” impaction model and the shear model, which we believe is more indicative of a clinical injury.

2. Materials and Methods

2.1. Tissue Acquisition and Preparation. Porcine patellae were sterilely removed from knee joints obtained fresh from a local slaughterhouse. A total of 72 paired patellae were included (36 right, 36 left). The patellae were cleaned of soft tissue and the cartilage was maintained intact on the surface for testing. Throughout the testing the patellae were kept immersed in PBS with antibiotics to minimize chance of infection and prevent drying of the articular surface.

2.2. Impaction, Culture, and Specimen Collection. The patellae were randomized to one of three treatments: axial impaction, shear impaction, or nonimpacted control. A custom mold was used to position each patella in a test fixture on the base of a servo-hydraulic load frame for testing of the impact specimens. This allowed the patellar facet to be aligned perpendicular to the loading direction. The impactor tip was hemicylindrical and was 10 mm long by 10 mm in diameter. It was also pinned along one axis to allow rotation to accommodate any unevenness in the patellar surface. The impactor tip was attached to a piezoelectric load cell that allowed for measurement of forces in three dimensions. The axial impaction delivered a targeted load of 2000 N at 25 mm/sec [8]. The shear impaction type delivered both normal loads and an elevated shear force. This was achieved by slowly loading the articular surface to 500 N at 0.05 mm/sec. When the targeted normal loading was reached, the patella was displaced tangentially 10 mm at 200 mm/sec (via a cable and pulley system attached to a second hydraulic load frame) to induce larger shear forces.

Upon completion of the impaction, the patellae were placed into culture (Delbecco’s MEM/Ham’s F12 with 10% fetal calf serum, ascorbic acid (25 μg/mL) with penn. 100 units/mL, strep. 100 μg/mL, and amphotericin B 25 μg/mL) at 37 °C with 5% CO₂ in dishes that allowed complete immersion of the patella. Culture media were changed daily to minimize chance of infection.

After culture for 0, 3, 7, or 14 days, a full-thickness cartilage specimen was harvested from the patella directly below the location of the impact and immediately flash-frozen in liquid N₂ and stored at −80 °C. The day 0 sample was collected at approximately 2 hours after impaction.

2.3. Gene Expression Analysis. The cartilage specimens were ground to a fine powder in a liquid nitrogen cooled mortar and pestle, and total RNA was extracted via Tri Reagent (Molecular Research Center Inc., Cincinnati, OH) following the method previously described [23]. The purity of the RNA was measured and quantitated on a Nanodrop-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). A High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA) was used for reverse transcription of 250 ng of total RNA. A panel of 18 genes related to the progression of early stage OA was evaluated in the extracted RNA. The selected genes were as follows: (1) cartilage matrix: Coll1a1, Col2a1, Acan, Sox9, Opn, and Comp; (2) degradative enzymes and inhibitors: Mmp1, Mmp3, Mmp13, Timp1, Timp2, and Adamts5; (3) inflammatory response and signaling: Il1b, Tgfb, Inos, and Chi3l1; and (4) apoptosis: Casp8, Fas (full gene names in Table 1).

Primer pairs for quantitative real-time PCR (qPCR) were designed with Beacon Designer software (Premier Biosoft International, Palo Alto, CA) for compatibility with SYBR Green I Master Mix. When possible, primers were designed from porcine gene sequences. If not available, they were designed from conserved regions of human, bovine, or canine sequences. The primers were designed to cross an intron-exon boundary (Table 1). qPCR was performed in a 20 μL reaction, consisting of 1 μL of diluted cDNA, 400 nM of forward and reverse primers, 10 nM of fluorescein (as a reference dye), and 0.5 μL of 1x Power SYBR Green I Master Mix. A three-step amplification protocol was performed in an iCycler iQ (Bio-Rad, Hercules, CA) with the following steps: denaturation with one cycle at 95 °C for 7 minutes followed by 40 cycles of 30 sec at 95 °C for denaturation, 30 sec at 50–62 °C for annealing, extension for 30 sec at 72 °C, and a product melting cycle of 5 min at 72 °C, 1 min at 95 °C, and 1 min at 55 °C. Samples were amplified in triplicate, and reaction efficiency for each primer set was assessed using standard curves via a dilution series using iCycler iQ Real-Time PCR Detection System Software. The gene target specificity of the reactions was evaluated with a melt curve generated at the end of the PCR amplification cycle. Additionally, one cDNA product from each primer pair was sequenced to verify that the PCR product corresponded to the intended gene [25]. The expressions for the genes of interest were normalized to the geometric mean of 4 reference genes identified as being the most stable in our tissue subjected to our treatment regimen [23]: Actb, Gapdh, Sdhb, and Ppia [23].
Table 1: OA related genes. Full gene names, abbreviations, forward and reverse primer sequences, annealing temperatures, amplicon lengths, and NCBI numbers.

| Gene name                                | Gene abbreviation | Sequence (5' → 3')              | Annaling temp. | Amplicon length | NCBI Number   |
|------------------------------------------|-------------------|---------------------------------|----------------|-----------------|---------------|
| **Cartilage matrix**                     |                   |                                 |                |                 |               |
| Collagen, Type I, Alpha 1                | **Col1a**         | F: CAACCGCTTCACCTACAGC          | 60             | 101             | AK236626      |
|                                          |                   | R: TTTGTATTTGATCTGCTTGGCC       |                |                 |               |
| Collagen, Type II, Alpha 1               | **Col2a1**        | F: GAGAGGTCTTTCTGGCAAGAG      | 60             | 118             | AF201724.1    |
|                                          |                   | R: AAGTCCCTTGGAAGCCAGAT        |                |                 |               |
| Aggrecan                                 | **Acan**          | F: TGCAGGTCACAGTCAGGGG         | 60             | 79              | AF201722b     |
|                                          |                   | R: CGGTATGGACACACACCGGGG       |                |                 |               |
| SRY (sex determining gene region Y) box-9| **Sox9**          | F: CAGGGCTCTGTGCTCTACTCC       | 60             | 230             | NM_213843.1   |
|                                          |                   | R: GGGTACGCTCTGCTCTTGCGT       |                |                 |               |
| Osteopontin                              | **Opn**           | F: CCGAGGCGAGAGAGGAGACC       | 55             | 214             | NM_214023.1   |
|                                          |                   | R: GTTGATCTCAGAGGACGACTCTC     |                |                 |               |
| Cartilage oligometric matrix protein     | **Comp**          | F: GGCTGGAAGGACACGACATC        | 55             | 82              | XM_003123529.1|
|                                          |                   | R: CCTCATAGAAGCGCAGCTG         |                |                 |               |
| **Degradative enzymes & inhibitors**     |                   |                                 |                |                 |               |
| Matrix metalloprotease-1                 | **Mmp1**          | F: TGATGGACCTGGAGAAACC         | 59             | 131             | NM_001166229  |
|                                          |                   | R: GGCAGCCACAGGACGATACAAG      |                |                 |               |
| Matrix metalloprotease-3                 | **Mmp3**          | F: GATGTTGTTACCTGAGACG         | 50             | 197             | NM_001166308.1|
|                                          |                   | R: ATCAATATCCGCTCTCTCC         |                |                 |               |
| Matrix metalloprotease-13                | **Mmp13**         | F: CCAGGGTTGCTGCTGCTGTG        | 60             | 77              | AF069643      |
|                                          |                   | R: TGGGTCCTGCTGAGTGGGTC         |                |                 |               |
| TIMP metallopeptidase inhibitor-1        | **Timp1**         | F: CCTGTAACCAGGTTATG           | 59             | 177             | NM_213857.1   |
|                                          |                   | R: CGTTCCACAGTGGCTCCAG         |                |                 |               |
| TIMP metallopeptidase inhibitor-2        | **Timp2**         | F: ATATACGAGAACACACGACC        | 59             | 152             | AK237154.1    |
|                                          |                   | R: GGAATGATTACAAAGGATGC        |                |                 |               |
| ADAM metallopeptidase with thrombospondin| **Adams5**        | F: CGCTGGCACCACACACTCA         | 60             | 80              | NM_007038.3   |
| Type 1 motif 5                           |                   | R: CGTATGGCTCTCCTGATCATC       |                |                 |               |
| **Inflammatory response**                |                   |                                 |                |                 |               |
| Indian hedgehog                          | **Ihh**           | F: CAGCGGGCCCTATGGAAGGCA       | 60             | 140             | XM_001925486.1|
|                                          |                   | R: GGTCTCTTGAGCGCTGGGTC        |                |                 |               |
| Transforming growth factor β              | **Tgfb**          | F: GGAGTGGCTGTCTTGTATG          | 60             | 117             | NM_214015.1   |
|                                          |                   | R: AGTGGTATACTGCTGTCTG         |                |                 |               |
| Nitric oxide synthase 2, inducible       | **inos**          | F: TGAATTTGTGCAACGTTGAT        | 53             | 82              | NM_00143690.1 |
|                                          |                   | R: CTTGGTTACCGCTCCAC           |                |                 |               |
| Chitinase-3-like protein 1               | **Chi3l1**        | F: TGACGCTCTATGACACACT          | 62             | 194             | NM_001001540  |
|                                          |                   | R: GGCTAGGTCCAGCTTCA           |                |                 |               |
| **Cell proliferation & apoptosis**       |                   |                                 |                |                 |               |
| Caspase-8                                | **Casp8**         | F: TGGGAAACACAGTGGCACCACCT     | 60             | 153             | NM_001031779.2|
|                                          |                   | R: CCCCCTCTAATGCTGCCCCCC       |                |                 |               |
| Fas (TNF receptor superfamily, member 6) | **Fas**           | F: TAGAGTTTGAGGAGAA            | 53             | 107             | NM_213839.1   |
|                                          |                   | R: ATTTGAGGTCTGAGCAGA          |                |                 |               |
2.4. Analysis of Results. qPCR data for the genes were evaluated by comparing the relative gene expression levels (Ct values) across treatments and across time. A linear mixed model was used for analysis following the methods proposed by Steibel et al. [24]. Differences for comparisons of interest were evaluated for statistical significance using PROC MIXED in SAS 9.2 statistical software (SAS Institute Inc., Cary, NC). The raw P values were adjusted for multiple comparisons using the false discovery rate (FDR) method [25]. Due to the relatively small number of samples in each combination of treatment and time in this experiment, the threshold for a significant FDR adjusted P value (q-value) was set at q < 0.2. This threshold allows for an appropriate sensitivity for the analyses being conducted and insures that the interpretation of the data is not overly restrictive in eliminating potentially valuable findings that may not achieve a higher level of significance. Due to the fundamental difference in how FDR controls for a type I error rate within results already deemed significant, a higher threshold may be acceptable, up to even 0.5 [26].

For each comparison of groups (example: comparing day 0 shear specimens to day 0 control specimens) the fold changes were calculated using the method of Steibel et al. [24]. Fold changes for the targeted genes of interest were normalized to the geometric mean of the previously identified four housekeeping genes. Differential gene expression between groups was first evaluated by comparing each treatment (axial versus control, shear versus control, and shear versus axial) at each time point. Differential gene expression was next evaluated within each treatment (control, axial, and shear) by comparing each time point (days 0, 3, 7, and 14) to day 0 control specimens. Day 0 control specimens were used as the reference for temporal changes as they most closely represent a cartilage surface in its natural state that has not been impacted.

3. Results

There were 72 patellae included in the analysis (36 right and 36 left). The patellae were randomized for treatment and collection time point. Therefore there were 6 patellae at each combination of treatment and time point (3 treatments x 4 time points x 6 patellae = 72 total patellae). RNA was extracted from one facet of each of the 72 patellae for a total of 72 specimens.

Fold changes (FC; Table 2) were evaluated within each treatment for all 18 genes over time. All genes showed significantly different (q < 0.2) expression within the control treatment at d14 compared to d0 with the exception of Fas. The most highly upregulated genes at d14 for control
specimens were Col1a1, Mmp1, and Mmp13. The genes that have the greatest decrease in expression at d14 were Col2a1, Sox9, Comp, and Casp8. The axial specimens compared to d0 control all showed significantly different expression at d14 with the exception of Opn, Ihh, and Inos. Again, Col1a1, Mmp1, and Mmp13 had the highest increase in expression, and Col2a1, Sox9, Comp, and Mmp13 demonstrated the largest decreases in expression. Similar changes were observed for the shear specimens over time, with the exception that Chi3l1 also demonstrated a large increase in expression (FC = 13.30).

Expression changes were also evaluated between treatments at each time point. The comparison of most interest was the shear impact treatment compared to the axial impact treatment (Table 2 and Figure 1). Col1a1 had significantly higher expression at both d0 and d3 in shear versus axial specimens; however, by d14 expression was lower in shear specimens (Figure 1(a)). Col2a1 expression was lower in shear specimens at all time points with the exception of d3 where it was 2.46-fold higher (Figure 1(a)). Both Acan and Sox9 demonstrated significantly higher expression at
### Table 3: Differential gene expression for shear compared to control specimens at each time point. Fold changes are shown on the left, and the corresponding *q*-values are shown on the right. Significant *q*-values (*q* < 0.2) and the associated fold changes are in bold.

| Genes grouped by functional type | Comparing treatments within time point | q-values (FDR) |
|---------------------------------|---------------------------------------|---------------|
|                                | **Fold changes**                      | **Shear versus control** | Day 0 | Day 3 | Day 7 | Day 14 | Day 3 | Day 7 | Day 14 |
| Cartilage matrix               | **Col1a1**                            |                | 1.37  | 5.36  | 0.17  | 0.35  | 0.70  | **0.09** | **0.09** | 0.24 |
|                                | **Col2a1**                            |                | 1.41  | 0.97  | 1.13  | 1.96  | 0.94  | 0.94  | 0.94  | 0.62 |
|                                | **Acan**                              |                | 0.82  | 1.29  | 1.14  | 1.53  | 0.70  | 0.70  | 0.70  | 0.70 |
|                                | **Sox9**                              |                | 1.00  | 0.71  | 0.62  | 1.33  | 0.99  | 0.56  | 0.56  | 0.56 |
|                                | **Opn**                               |                | 1.02  | 2.26  | 0.78  | 0.58  | 0.97  | 0.40  | 0.83  | 0.53 |
|                                | **Comp**                              |                | 0.60  | 0.87  | 0.93  | 0.81  | 0.47  | 0.83  | 0.83  | 0.83 |
| Degradative enzymes & inhibitors | **Mmp1**                              |                | 1.55  | 0.67  | 0.39  | **0.17** | 0.60  | 0.60  | 0.25  | **0.01** |
|                                | **Mmp3**                              |                | **3.69** | 0.61  | 3.58  | **0.26** | **0.10** | 0.49  | **0.10** | **0.10** |
|                                | **Mmp13**                             |                | 1.05  | **3.56** | 0.54  | **0.19** | 0.94  | 0.12  | 0.47  | **0.05** |
|                                | **Timp1**                             |                | 1.24  | 0.85  | 0.96  | 0.84  | 0.84  | 0.84  | 0.90  | 0.84 |
|                                | **Timp2**                             |                | 0.96  | 1.34  | 0.49  | 1.03  | 0.93  | 0.93  | 0.28  | 0.93 |
|                                | **Adamts5**                           |                | 0.52  | 0.87  | **0.37** | **0.22** | 0.39  | 0.81  | **0.18** | **0.04** |
| Inflammatory response & signaling | **Ihh**                               |                | 2.33  | 0.83  | 2.05  | 1.27  | 0.42  | 0.74  | 0.42  | 0.74 |
|                                | **Tgfb**                              |                | 0.72  | 1.22  | 0.86  | 0.68  | 0.57  | 0.63  | 0.63  | 0.57 |
|                                | **Inos**                              |                | 0.77  | 0.92  | 0.94  | **0.31** | 0.91  | 0.91  | 0.91  | **0.13** |
|                                | **Chi3l1**                            |                | **2.00** | **0.46** | 1.20  | **0.51** | **0.17** | **0.17** | 0.69  | 0.17 |
| Cell proliferation & apoptosis  | **Casp8**                             |                | 0.98  | 0.70  | 0.63  | 1.31  | 0.95  | 0.58  | 0.58  | 0.58 |
|                                | **Fas**                               |                | **0.35** | 1.20  | 0.84  | 1.45  | **0.06** | 0.69  | 0.69  | 0.69 |

### 4. Discussion

The aim of this study was to identify differential gene expression changes in a porcine model of early stage cartilage degeneration in an impact injury OA model. An analysis of temporal changes in specimens over the 14 days following impact showed that *Col1a1*, *Mmp1*, and *Mmp13* were generally upregulated over time in all treatments. The genes that have the lowest expression over time for all treatments were *Col2a1*, *Sox9*, *Comp*, and *Casp8*. The general trend of the temporal changes was similar for all treatments, including the nonimpacted control. It is therefore possible that removing the patella from the body and from its normal loading and subsequently placing it in culture affected gene expression similarly in all patellae. Therefore, to identify the effects of an individual treatment it was necessary to compare the treatments to each other within the time points.

#### 4.1. Cartilage Matrix

*Col1a1* expression was elevated in shear compared to axial impacted specimens on both d0 and d3. The rise in expression of *Col1a1* may indicate that the chondrocytes are reverting to a more fibroblastic phenotype indicative of their attempt to initiate repairs, albeit with the incorrect collagen. This correlates with other work, where more damaging impacts resulted in elevated *Col1a1* expression [15], and with theories of dedifferentiated chondrocytes in OA progression [11, 27–30]. The overexpression of *Col1a1* has been correlated with chondrocyte hypertrophy in OA [31] and even focal chondrocyte cluster formation [32]. Sanchez-Adams et al. propose that impact or overload conditions may result in persisting dysfunctional chondrocyte responses to further loading, even after the injurious load is removed, and
these changes may be a prelude to early OA [33]. This has
been further suggested to result in a positive feedback loop
where the proliferation of chondrocytes amplifies growth
factors, bone cysts, and resulting damage to neighboring
chondrocytes and the extracellular matrix [34]. Col2a1, the
most abundant collagen in articular cartilage, was also more
highly expressed in the shear specimens on d3. Both Acan
and Sox9, a transcription factor for Acan and Col2a1, were
more highly expressed in shear compared to axial impact
specimens on d14. The early downward trend of Sox9 agrees
with results found with a mechanical strain model [13], and
the increased expression of both Sox9 and Acan at d14 is
indicative of repair efforts underway. The lower expression of
Comp in shear compared to axial specimens may be indicative
of matrix degradation [10, 35], possibly because the shear
specimens experienced more damage during the loading
event.

4.2. Degradative Enzymes. Mmp expression was generally
elevated in shear compared to axial specimens at the earlier
time points and then was downregulated at d14 for all
Mmps. In particular, Mmp13 was significantly higher in
shear specimens at d3 and then significantly lower in shear
versus axial specimens by d7. The increase in degradative
enzyme transcript levels is likely a response on the part of
the chondrocytes to the damaging nature of the mechanical
trauma. Early increase in Mmp levels is consistent with both
high load models [15, 36] and early stage OA progression
[9, 34]. Timp2 levels were elevated in shear compared to
axial specimens and were significantly higher at d0 and d3;
however Timp1 showed minimal differences between the
treatments. Lee et al., however, found Timp1 levels increased
in the 24 hours following an injurious compression, with
minimal changes in Timp2, though the general trends were
similar [37]. Adams5, an aggregcanase, was elevated early and
then showed significantly lower expression in shear versus
axial specimens by d14. The findings for the degradative
enzymes suggest that there is early, relatively higher matrix
breakdown in the shear specimens following injury that
tapers by the later time points.

4.3. Inflammatory Response and Signaling. Ihh, a signaling
molecule associated with chondrocyte proliferation, was gen-
erally elevated in shear specimens in line with other studies
of early stage OA [38, 39]. Tgfb showed significantly lower
expression at d0 and d3 and then higher expression at d14 in
shear versus axial specimens. Tgfb may be a critical part of
the inflammatory process for initiating repairs of the cartilage
matrix and aiding cell proliferation, and lack of its expression
may coincide with OA development [40].

4.4. Apoptosis. Both Fas and Casp8 showed similar trends at
each time point. Each gene had lower expression in shear
versus axial specimens at d0; however Fas expression was
significantly lower. At d14, expression of both genes was
increased, but Casp8 was significantly higher for shear versus
axial specimens. In a study of aged rabbits with normal
cartilage, Allen et al. found increases in Casp8 and Fas
expression believed to be a prelude to the development of OA
[41]. Furthermore, Casp8 was found to be upregulated in an
OA transection model [42], while Fas expression has been
found to have increased expression in the immediate vicinity
of OA lesions [43]. The elevated level of apoptosis genes at d14
could indicate higher levels of apoptosis and may mean later
loss of chondrocytes in the tissue.

The panel of 18 genes evaluated in this study was chosen
based on their anticipated relationship to early stage OA
progression. In previous work where we developed our
impact injury model, we created a SAGE library to identify
differentially expressed genes [8] and have used the find-
ings to explore OA related gene expression changes in an
axial impact injury model alone [44]. We correlated genes
identified in our previous work with published literature to
identify the most relevant genes to explore for early stage OA
progression using qPCR. This work demonstrates that
multiple genes in our panel have altered expression in our
model of early stage OA. New technologies, such as RNA-Seq,
may provide enhanced capability for detecting other genes
related to OA progression [29, 45–48]. For example, Peffers
et al. have completed recent work to identify nearly 400
genes that are differentially expressed between young and old
equine cartilage with naturally occurring OA [29, 47]. This
evolving technology promises to provide additional targets
for further detailed exploration with qPCR.

5. Conclusions

The results presented here show a successful implementa-
tion of a porcine impact injury model for evaluating early stage
OA progression. We generated a more complex loading
model that incorporated elevated shear forces that may have
more physiological relevance. In comparing this model to
a standard normal loading model we found elevated levels
of degradative enzymes and matrix constituents, similar to
those found in naturally occurring cartilage degeneration.
However one of those was Col1a1, an abnormal collagen for
articular cartilage. It appears that the chondrocytes in the
shear specimens are attempting repairs but are unable to
mount a successful effort.

Conflict of Interests

The authors have no conflict of interests that has any bearing
on this paper.

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