Deficiency of hyaluronan synthase 1 (Has1) results in chronic joint inflammation and widespread intra-articular fibrosis in a murine model of knee joint cartilage damage

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SUMMARY

Objective: Articular cartilage defects commonly result from traumatic injury and predispose to degenerative joint diseases. To test the hypothesis that aberrant healing responses and chronic inflammation lead to osteoarthritis (OA), we examined spatiotemporal changes in joint tissues after cartilage injury in murine knees. Since intra-articular injection of hyaluronan (HA) can attenuate injury-induced osteoarthritis in wild-type (WT) mice, we investigated a role for HA in the response to cartilage injury in mice lacking HA synthase 1 (Has1−/−).

Design: Femoral groove cartilage of WT and Has1−/− mice was debrided to generate a non-bleeding wound. Macroscopic imaging, histology, and gene expression were used to evaluate naïve, sham-operated, and injured joints.

Results: Acute responses (1–2 weeks) in injured joints from WT mice included synovial hyperplasia with HA deposition and joint-wide increases in expression of genes associated with inflammation, fibrosis, and extracellular matrix (ECM) production. By 4 weeks, some resurfacing of damaged cartilage occurred, and early cell responses were normalized. Cartilage damage in Has1−/− mice also induced early responses; however, at 4 weeks, inflammation and fibrosis genes remained elevated with widespread cartilage degeneration and fibrotic scarring in the synovium and joint capsule.

Conclusions: We conclude that the ineffective repair of injured cartilage in Has1−/− joints can be at least partly explained by the markedly enhanced expression of particular genes in pathways linked to ECM turnover, IL-17/IL-6 cytokine signaling, and apoptosis. Notably, Has1 ablation does not alter gross HA content in the ECM, suggesting that HAS1 has a unique function in the metabolism of inflammatory HA matrices.

Introduction

Traumatic injuries to articular cartilage of the knee can result from excessive surface contact stresses after blunt impact or torsion, which occur frequently during sports and military training. Resulting patellar dislocation, joint incongruity, and instability can predispose to osteoarthritis (OA). Responses to cartilage injury share many features of wound healing, such as innate inflammation and activation of multipotent progenitor cells in the synovium or the articular surface. However, in many cases, the repair response leads to fibrotic remodeling and scarring of the joint lining tissues, subchondral bone sclerosis, and chondrophyte or osteophyte development at the articular margins.

 Whereas chronic inflammation is widely recognized as a driving factor in OA, the concept of a pathogenic role for fibrotic scarring is less well-studied. Evidence for focal scarring has been reported for synovial tissue and cartilages from both animal model and human OA, each of which exhibit activation of multiple genes associated with collagen production and deposition (CRLF1, PLOD2, LOX,
COL1A1, COL5A1, TIMP1). In addition, other laboratories also reported high expression of COL1A1, COL2A1, COL3A1, and COL5A1 in human OA cartilages. Moreover, mice deficient in genes that enhance collagen matrix formation and turnover in wound healing (Adamts5, Ddr2, Mmp13, Sdc4, and Tgm211–14) were variably protected from surgically induced OA.

In dermal wound healing, early inflammation is followed by formation of granulation tissue containing progenitor cells embedded in a provisional extracellular matrix (ECM) of collagens, fibronectin, hyaluronan (HA), and hyalectans.15 These cells, following re-epithelialization, mature into fibroblasts to generate the functional collagenous repair tissue. Correspondingly, following cartilage injury, proliferation of cells and ECM deposition in synovial lining and adjacent adipose or joint capsule tissues occurs. This response can progress into fibrotic remodeling, often reported in inflammatory arthritis. A similar response also develops in the meniscal destabilization mouse model of OA, where, at 2–4 weeks, an inflammatory period is followed by elevated expression of profibrotic mediators such as type III collagen (Col3a1), fibromodulin (Fmod, a catalyst for TGF-β1 signaling), and prolargin (Prelp, an inhibitor of osteoclastic activity).

To examine repair responses specifically in the context of articular cartilage injury, we have adapted a murine model induced by surgical excision of cartilage from the patellar groove. This allowed spatiotemporal macroscopic and microscopic evaluation of whole joint-responses and assay of gene expression in inflammatory, pro-fibrotic and ECM pathways, in both intact joints and separated tissue pools (meniscus and synovium (Men/Syn), cartilage and subchondral bone (CSCB), and patellar tendon (PT)).

We have also examined such injury responses in mice deficient in HA synthase 1 (Has1), which have previously been reported to exhibit an aberrant dermal healing phenotype.16 We show that Has1−/− mice, although not defective in overall HA production, are not able to control post-injury joint inflammation and develop extensive intra-articular scarring and severe OA-like symptoms.

Methods

Murine cartilage injury model

Wild-type (WT) and Has1−/− male C57Bl/6 mice (10–12 weeks old, ~30 g) were used under approval of the Rush University Institutional Animal Care and Use Committee. Routinely, four C57Bl/6 males were caged with one C57Bl/6 female littermate, to minimize male aggression and prevent wounding in the pre-op and post-op maintenance periods. After anesthesia, an ~8-mm medial para-patellar incision was made on the right knee, medial para-patellar arthroscopy was performed, and the patella laterally luxated. Cartilage was debrided along the distal groove with a #15 scalpel without penetration of the subchondral bone. Joint surfaces were lavaged with sterile saline, and the patella repositioned, defined with a scalpel without penetration of the subchondral bone. Joint surfaces were lavaged with sterile saline, and the patella repositioned, and lateral compartments, respectively. Slides 1/2, 22/23, 42/43, 62/63, 82/83, 102/103, 122/123, 142/143 and 182/183 were stained with Safranin O (SafO), and adjacent sections with hematoxylin and eosin (H&E) or biotinylated HA binding protein (bHABP) to localize HA. It should be noted that the histological analysis was not used in this study to generate a numerical scale for cartilage grading (as per OARSI guidelines) but evaluated, in combination with the macro-images, to describe structural alterations in multiple tissue types adjacent to the injury and throughout the whole joint.

Quantitative PCR (qPCR)

For gene expression in whole joints from naive, sham, and injured groups (n = 3–4, detailed in Table S-1), hind legs were harvested immediately after sacrifice, the skin and muscle removed, and knee joints isolated by sharp dissection through the growth plates, prior to storage at −20°C in RNAlater (Table S-1). To prepare separate tissue pools, twelve joints were used for Men/Syn or PT, and two for C/CSB. RNA purification, cDNA synthesis, and qPCR (3 technical replicates) with Taqman®-primers (Table S-2) was done as previously described.17 Transcript abundance was calculated as 1000 × 2−ΔΔCt, with ΔΔCt = [Ct(gene of interest) − Ct(Gapdh)] and Ct > 35 considered “non-detectable” (ND). RT2 Profiler PCR Arrays (Qiagen) were used for fibrosis (PAMM-1202A) and NF-κB signaling target (PAMM-2252A) genes. Injury-induced fold-change in expression was calculated as 2−ΔΔCt, where ΔΔCt = [ΔCt(post-injury time point) − ΔCt(naïve)]. Gene groupings indicated by Qiagen and Metacore™ software analysis of expression data were used to determine pathway associations.

Data and statistical analysis

For statistical comparisons across time points and between genotypes, qPCR assays were performed on whole joints (each joint an experimental unit), from naive, sham, and injury groups, because the large number of mice (12 per experimental unit) required for generating multiple pools of separated tissue types was outside the scope of this study. Gapdh Ct values from WT and Has1−/− samples were pooled to confirm normality of Ct values with the Shapiro-Wilk test, and analysis of variance (ANOVA) was used to compare Gapdh Ct values across groups to confirm selection of the housekeeping gene. For all combinations of WT/Has1−/− naïve/sham/injury joints (6 in total), Ct values were confirmed with the Shapiro-Wilk test to be normally distributed. For each gene, ANOVAs were performed on ΔCt values to compare expression in the following subgroups: 1-way ANOVA for WT naïve vs 12 or 28 day post-sham; 2-way ANOVAs for WT vs Has1−/−, naïve vs 12 or 28 day post-injury (main effects: experimental end point, genotype). ANOVAs were followed by post hoc analysis of statistically significant effects using unpaired, two-tailed Student’s t tests with Bonferroni correction of the P value for multiple comparisons against naïve. Since only two end points at biologically distinct phases of sham and injury response were compared to naïve, Bonferroni correction was chosen as the most conservative option for post hoc comparisons after ANOVA.

For each gene from the arrays, unpaired two-tailed Student’s t tests were used to compare between WT and Has1−/− ΔCt values at naïve. Similar analysis was performed to compare array results of WT naïve to WT sham. ANOVA was used to compare genotypes for ΔCt values after injury (to determine the overall effect of genotype), removal of skin and muscle) were fixed in 10% neutral-buffered formalin, decalciﬁed with 5% EDTA in PBS, parafﬁn-embedded, and microtome-cut into 6-μm sections across the entire joint20. Sections 1–60, 61–120, and 121–190 spanned medial, central groove, and lateral compartments, respectively. Slides 1/2, 22/23, 42/43, 62/63, 82/83, 102/103, 122/123, 142/143 and 182/183 were stained with Safranin O (SafO), and adjacent sections with hematoxylin and eosin (H&E) or biotinylated HA binding protein (bHABP) to localize HA. It should be noted that the histological analysis was not used in this study to generate a numerical scale for cartilage grading (as per OARSI guidelines) but evaluated, in combination with the macro-images, to describe structural alterations in multiple tissue types adjacent to the injury and throughout the whole joint.

Macroscopic joint imaging, histology, and HA staining

Joint-wide pathology was assessed in operated and contralateral joints as previously described. For histology, whole joints (after
followed by a post hoc unpaired, two-tailed Student’s t test with Bonferroni correction. The experiment-wide significance level was $\alpha = 0.05$.

Results

Macroscopic imaging of the murine model of cartilage injury in WT joints

Cartilage was debrided along the full length of the patellar groove without damaging the trochlear ridges [Fig. 1(A)] or subchondral bone. Typical images of joints at 4 weeks post-surgery [Fig. 1(B)] illustrate that the trochlear ridges adjacent to the injury-site were covered with a whitish tissue [Fig. 1(B), arrows] extending into the periosteum. Safranin O or H&E histology showed this to be dense fibrous tissue lacking chondroid staining and with few cells (data not shown). The damaged groove surface became covered with a thin layer of tissue, but the apposing patellar cartilage was markedly roughened, with evidence of tissue ingrowth from the margins [Fig. 1(B)]. Contralateral joints remained macroscopically unaffected [Fig. S-1(B)], and sham operated joint surfaces also showed no damage after 4 weeks [Fig. S-1(C)].

Histology of joint tissue responses to cartilage injury in WT mice

Typical images of Safranin-O-stained whole joint sections from naïve and 1- and 4-weeks post-injury WT mice [Fig. 1(C)] show the cartilage interfaces between the proximal groove and the center of the patella (top panels) and between the distal groove and the distal patellar tendon (bottom panels). In naïve mice, these showed full-depth patellar cartilage in apposition to the groove cartilage,
transitioning into a SafO-poor fibrous tissue at the proximal end. At 1 week post-injury, a thin layer of calcified cartilage, remaining after the debridement, covered the subchondral bone of the proximal groove [Fig. 1(C), upper panel]. The patellar cartilage apposing the injury site showed marked thinning relative to naïve, likely as a result of mechanical abrasion or degradative mediators released from the area of cartilage injury. Although subchondral bone was not penetrated, the cellularity in the marrow space of both bones was reduced at 1 week post-injury [Fig. 1(C)] and spaces filled with a disorganized ECM. A thick layer of hyperplastic synovium, extending between the patellar tendon and the distal patella was prominent at 1 week.

At 4 weeks, a continuous layer of SafO-stained “repair” cartilage covered the injured groove, and the cellularity of the marrow space had regenerated. The patellar cartilage surface was also restored, and all injured joints developed chondrophytic deposits with a fibrous covering [Fig. 1(C), lower panel] derived from the hyperplastic synovium adjacent to the patella. Hyperplasia of both peri-patellar and perimeniscal synovium and the loss of adipocytes by 1 week was confirmed by H&E histology [Fig. 2(A)]. By 4 weeks, normal cellularity was restored with minor thickening of the peri-vascular matrix. A transient influx of neutrophils to vascularized regions at the dermal incision site was evident at 3 days post-surgery (data not shown), but no infiltration of circulating inflammatory cells into the synovium or the joint space was detected, consistent with only innate inflammation.

When equivalent sections were examined for HA deposition, strong staining of synovium was seen at 1 week [Fig. 2(B), left panel], but at 4 weeks HA staining essentially returned to naïve levels. The HA accumulation early post-injury is consistent with the increased expression of Has1 \( (P < 0.01) \) and Has2 \( (P < 0.05) \) at those times. Has1 expression remained significantly elevated (12d: \( P < 0.01 \), 28d: \( P < 0.05 \)) at later times (Fig. S-3), suggesting that long-term ECM remodeling includes this prolonged expression response. Expression of Has1 and Has2 in contralateral joints was unaffected [Fig. S-3(A)]. Furthermore, sham-operated joints at 2 weeks showed only mild peri-patellar synovial hyperplasia, which resolved at 4 weeks (data not shown). Minor fibrotic/chondroid remodeling at the patellar margins was observed [Fig S-1(C)], but femoral or tibial articular and growth plate cartilage appeared normal.

### Post-injury expression of HA-matrix genes in WT joint tissues

Injury to skin\(^{21}\), intestine\(^{22}\), cartilage\(^{23}\), and other tissues is accompanied by inflammation and changes in HA accumulation, resulting from concurrent increased synthesis of HA itself and HA-associated proteins, such as TSG6 (encoded by Tnfaip6), the heavy chains of inter-alpha-trypsin inhibitor (encoded by Itih1-5 genes), and pentraxin.

Increased expression of some of these genes also occurred after cartilage injury (Table I). In naïve joints, all transcripts except for Tnfaip6 were most abundant in the Men/Syn tissue pools, followed by PT and C/SCB pools, consistent with the staining intensity of HA

Table I: Effect of cartilage injury on mRNA abundance relative to Gapdh of HA-network genes in isolated tissue pools from WT mice

<table>
<thead>
<tr>
<th></th>
<th>Has1</th>
<th>Has2</th>
<th>Tnfaip6</th>
<th>Itih2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cartilage and subchondral bone</td>
<td>0.36</td>
<td>0.06</td>
<td>0.12</td>
<td>0.11</td>
</tr>
<tr>
<td>Meniscus and synovium</td>
<td>24.4</td>
<td>0.41</td>
<td>1.28</td>
<td>1.03</td>
</tr>
<tr>
<td>Patellar tendon</td>
<td>2.58</td>
<td>0.27</td>
<td>2.32</td>
<td>0.51</td>
</tr>
<tr>
<td>1 week</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cartilage and subchondral bone</td>
<td>4.17 (12)*</td>
<td>0.06 (1.1)</td>
<td>1.75 (15)</td>
<td>0.04 (0.4)</td>
</tr>
<tr>
<td>Meniscus and synovium</td>
<td>78.5 (3.2)</td>
<td>2.83 (7.0)</td>
<td>55.6 (43)</td>
<td>1.91 (1.9)</td>
</tr>
<tr>
<td>Patellar tendon</td>
<td>33.9 (13)</td>
<td>2.68 (10)</td>
<td>51.2 (22)</td>
<td>0.65 (0.8)</td>
</tr>
<tr>
<td>4 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cartilage and subchondral bone</td>
<td>3.73 (10)</td>
<td>0.03 (0.5)</td>
<td>1.06 (8.9)</td>
<td>0.08 (0.8)</td>
</tr>
<tr>
<td>Meniscus and synovium</td>
<td>54.6 (2.2)</td>
<td>0.40 (1.0)</td>
<td>10.4 (8.1)</td>
<td>1.92 (1.9)</td>
</tr>
<tr>
<td>Patellar tendon</td>
<td>44.2 (17)</td>
<td>1.19 (4.4)</td>
<td>18.2 (7.9)</td>
<td>3.90 (7.7)</td>
</tr>
</tbody>
</table>

* Italicized numbers in parentheses are fold change relative to naïve levels.
Has1 expression increased markedly in all tissues and remained elevated at 4 weeks. Has2 was also increased at 1 week in the Men/Syn and PT, remaining elevated in PT up to 4 weeks but normalizing in the Men/Syn (Table I). Concurrent with the increased expression of Has1 and Has2, Tnfaip6 was activated at 1 week in all tissues, particularly in Men/Syn (43-fold) and PT (22-fold). Expression levels declined, but did not normalize, by 4 weeks. Itih2 expression was minimally affected in all tissues at both time points, except for a 7.7-fold increase in the PT at 4 weeks. Itih1 expression was virtually undetectable in any samples (data not shown).

**Post-injury changes to expression of ECM genes in WT tissues**

We also assayed for changes in several ECM-related genes known to be associated with matrix remodeling in cartilaginous and fibrous tissue in individual tissue pools (Table II). In naive tissues, transcripts for Acan, Vcan V1 (formerly named the V0 isoform), and Col2a1 were highest in the C/SCB, and for Col3a1 in the Men/Syn. Notably, Col1a1 transcripts were very high relative to other genes in the three tissue pools, and Vcan V2 (formerly V1) was barely detectable.

**Table II**

<table>
<thead>
<tr>
<th></th>
<th>Acan</th>
<th>Vcan V1</th>
<th>Vcan V2</th>
<th>Col1a1</th>
<th>Col2a1</th>
<th>Col3a1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Naïve</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cartilage and subchondral bone</td>
<td>1.87</td>
<td>26.9</td>
<td>ND</td>
<td>1720</td>
<td>1.22</td>
<td>1.6</td>
</tr>
<tr>
<td>Meniscus and synovium</td>
<td>0.85</td>
<td>5.21</td>
<td>0.16</td>
<td>1280</td>
<td>0.15</td>
<td>24.6</td>
</tr>
<tr>
<td>Patellar tendon</td>
<td>0.04</td>
<td>6.33</td>
<td>0.05</td>
<td>962</td>
<td>ND</td>
<td>2.6</td>
</tr>
<tr>
<td><strong>1 week</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cartilage and subchondral bone</td>
<td>10.1 (5.4)*</td>
<td>12.7 (0.5)</td>
<td>0.47 (–)</td>
<td>3550 (2.1)</td>
<td>1.87 (1.5)</td>
<td>62.9 (39)</td>
</tr>
<tr>
<td>Meniscus and synovium</td>
<td>61.8 (72.3)</td>
<td>15.2 (2.9)</td>
<td>15.2 (92)</td>
<td>10,300 (8.0)</td>
<td>0.38 (2.6)</td>
<td>4010 (163)</td>
</tr>
<tr>
<td>Patellar tendon</td>
<td>130 (3386)</td>
<td>11.1 (1.8)</td>
<td>17.2 (366)</td>
<td>10,900 (11)</td>
<td>0.42 (–)</td>
<td>4451 (1734)</td>
</tr>
<tr>
<td><strong>4 weeks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cartilage and subchondral bone</td>
<td>7.97 (4.3)</td>
<td>8.85 (0.2)</td>
<td>0.18 (–)</td>
<td>4880 (2.8)</td>
<td>1.38 (1.1)</td>
<td>54.8 (34)</td>
</tr>
<tr>
<td>Meniscus and synovium</td>
<td>8.12 (9.5)</td>
<td>2.80 (0.5)</td>
<td>1.75 (10.6)</td>
<td>2770 (2.2)</td>
<td>0.80 (5.4)</td>
<td>1080 (44)</td>
</tr>
<tr>
<td>Patellar tendon</td>
<td>83.7 (2180)</td>
<td>6.87 (1.1)</td>
<td>4.41 (94)</td>
<td>7380 (7.7)</td>
<td>17.7 (–)</td>
<td>3200 (1245)</td>
</tr>
</tbody>
</table>

*Italicized numbers in parentheses are fold change relative to naïve levels. ND = not detected (Ct > 35), with no fold change calculation possible (–).*

**Fig. 3.** Macroscopic and histological evaluation of joint tissue response to cartilage injury in Has1−/− mice. A) The macroscopic appearance of cartilage surfaces and adjacent soft tissues in the injured joint was examined at 4 weeks post-surgery. Extensive fibrotic deposits (solid arrow) were observed at the groove, and both the condylar and patellar cartilage showed signs of wear (dashed arrows). B) Sections from the femoral-patellar compartment of naïve joints and at 1 and 4 weeks post-injury were stained with Safranin-O. The approximate region of the groove and the patella taken for histology are indicated by circles in panel B. By 4 weeks post-injury, severe cartilage and bone loss (chevron arrowhead) combined with extensive fibrotic overgrowth (dotted arrow) results in a loss of joint space. (Pat = patella; FC = femoral condyle; PT = patellar tendon; Syn = synovium). Scale bar = 100 μm.
All genes were increased at 1 week post-injury, with the increases in both tissue- and gene-specific. Increases in expression were highest for Acan, Vcan V2, and Col3a1 in the PT, possibly induced by manipulation at the time of surgery and through its proximity to the reactive synovium (Figs. 1 and 2). In contrast, expression of assayed genes was minimally affected in C/SCB. At 4 weeks post-injury, expression in Men/Syn and C/SCB decreased markedly to near naïve levels, but there was continued elevation of Acan, Vcan V2, and Col3a1 in the PT.

Gene expression in contralateral and sham-operated joints

A selected group of genes was assayed in contralateral and sham operated joints (Fig. S-3). Expression was essentially unaffected in contralateral tissues, consistent with minimal systemic post-injury responses. In sham-operated joints, there was a significant increase in expression of Has1, Tnfaip6 and Col3a1 by 12 days, similar to that in fully injured joints. All genes returned to naïve levels in the sham by 28 days, except for Has1 and Col3a1, which both remained elevated, suggesting that these are specific indicators of joint damage itself.

Has1 ablation modifies joint tissue response to cartilage injury

Since Has1 was highly activated and sustained after cartilage injury (Table I) and given the importance of HA in connective tissue healing, we examined cartilage injury responses in Has1−/− joints. Macroscopic examination of Has1−/− joints at 4 weeks post-injury [Fig. 3(A)] showed a dense collagenous tissue covering of the trochlear ridges and extending to the adjacent periosteum, whereas this was not seen in the WT [Fig. 1(B)]. Instead of the partial regeneration of cartilage seen in WT joints, widespread damage to the cartilage and subchondral bone was seen [Fig. 3(B)] at 1 week, with extensive fibrotic overgrowth (devoid of any SaO staining) developing in these areas [Fig. 3(B)]. Most notable was the finding that, at 4 weeks, the fibrotic tissue expanded from the injury site [Fig. 3(B)] into all soft tissues and also filled and thus eliminated the joint space.

Has1 deficiency did not affect HA deposition at 1 week or its decrease at 4 weeks seen in the synovial lining, adherent adipose tissue, and SCB marrow (Fig. 4). This is consistent with the accepted notion that HAS2, but not HAS1 or HAS3, is the primary HA-producing synthase throughout all organs24, and therefore also responsible for the increased HA deposition seen in both the WT and Has1−/− joints.

The tissue-specific and temporal post-injury gene expression trends (Table S-3) were similar for WT and Has1−/− mice, with transcripts for Has3 and Itih1 undetectable in both strains; however, there were significant differences between genotypes with time (Fig. 5). In WTs, Col2a1 (P < 0.05) expression decreased at day 3 and returned to naïve levels by day 12, whereas expression of Has1 (P < 0.01), Has2 (P < 0.001), and Tnfaip6 (P < 0.01) increased at 3 days and remained increased at 12 days (Figs. 5 and S-4). Expression of Col1a1 (P < 0.05) and Col3a1 (P < 0.001) peaked at day 12, and Col1a1, but not Col3a1 (P < 0.05), returned to naïve levels by day 28 (Fig. 5). WT joints showed an increased expression of HA-matrix genes up to 12 days and a sustained increase of Col3a1 up to 28 days. In summary, whereas Has1−/− joints responded post-injury like WT for matrix genes, they did not show as clear a normalization of HA-matrix genes at later times.

Only in Has1−/− joints was Col1a1 (P < 0.01) inhibited at 3 days and Vcan V2 (P < 0.05) activated at 28 days (Fig. 5). Although...
temporal trends for HA-matrix genes were mostly similar to WT, Has1<sup>−/−</sup> joints showed no significant changes with injury in Has2 and Tnfaip6 expression (Fig. 5). Thus, Has1 ablation was accompanied by a lower trend in activation of Has2 expression at 3 days, a much greater stimulation of Tnfaip6 at 12 days (P < 0.01), and a significant activation of Itih2 expression (P < 0.05) at 12 days, indicating that its effects are not on HA production itself but on the genes associated with forming the HA matrix.

**Effect of Has1 ablation on gene pathway changes at different periods after injury**

The lack of cartilage regeneration, degenerative changes on adjacent cartilage surfaces, and extensive fibrotic remodeling of the synovium and joint capsule in Has1<sup>−/−</sup> mice are consistent with development of a chronic inflammatory environment in the post-injury joint. To establish a possible link between chronic inflammation and post-injury fibrotic scarring, we analyzed joints from both genotypes for NF-κB signaling target and fibrosis genes. Assays were done for naïve and 12- or 28-days post-injury in both genotypes, and data calculated as fold-change relative to genotype naïve (Tables S-6 and S-7). Notably, compared to WT, Has1<sup>−/−</sup> mice overexpressed a larger number (26 genes at >3 fold) of the NF-κB signaling target genes (Table S-4) and fibrosis genes (Table S-5).

Pathway associations were determined for genes that were modified at least 3-fold relative to naïve levels using Metacore™ software. For both genotypes, four functional gene groupings were identified: IL-17/IL-6 signaling, ECM remodeling, pro-apoptosis, and anti-apoptosis (Table III). Moreover, within those groups, there were differences between WT and Has1<sup>−/−</sup> in expression of many genes, with 27 showing 6-fold or greater differences between WT and Has1<sup>−/−</sup> (Table III): for IL-17/IL-6 signaling, Csf3, Map2k6 (12 days) and Cxcl1, Il6, Mmp1a, Ptgs2 (28 days); for ECM remodeling, Col1a2 (P < 0.01), Ctgf (P < 0.05), Timp2 (12 days) and Plat, Serpine1, Tgfβ3, Thbs2, Timp1 (28 days); for pro-apoptosis genes, Fasl, Ifng, Il4, Ins2 (P < 0.01), Lta (12 days) and Il4, Ins2, Tnfsf10, Traf2 (28 days); and, for anti-apoptosis genes, Birc3, Cd40, Csf2, Fas, Il2, Rela (12 days) and Csf2 (28 days).

**Discussion**

Repair of articular cartilage defects in human joints remains problematic despite extensive research, due in part to insufficient information on self-healing mechanisms after injury. To mimic the
focal cartilage defects commonly seen in highly active populations like athletes⁶ and the military⁵, we have used a non-bleeding, cartilage-only injury model to confine the repair response to factors from within the joint and minimize the role of cell infiltration from the circulation or the bone marrow.

Acute post-injury synovitis, peripatellar chondrophylisis, and joint capsule fibrosis seen in this model have not been reported for other widely used models of murine OA²⁶. This might be due to more severe responses to a cartilage injury or a reduced focus on the anterior joint compartment, where severe changes in the synovium and joint capsule were seen in the present study, using sagittal sections. A unique effect of cartilage injury on joint response is suggested by the finding that, with the exception of Col3a1 and Mmp3 activation, structural and metabolic responses in the DMM model¹⁷ are distinct from those in the current work, suggesting pathogenesis of murine and likely human OA is dependent on type and severity of tissue injury, as well as mechanical perturbations.

A mechanistic link among inflammation, intra-articular scarring, and poor joint repair is also evident in this study, as we identified 27 genes related to IL-17/IL-6, ECM remodeling, and apoptosis (Table III) with higher expression in Has1⁻/⁻ joints. To determine whether these are linked to the pathology of Has1⁻/⁻ joints, we researched whether they have previously been implicated in poor repair of connective tissue injury, using murine OA as reference. On the basis of this restricted search, we have concluded that the 27 genes identified are indeed good markers of ineffective joint repair.

With regard to genes related to IL-17/IL-6 pathways, IL-17 is highly linked to OA in a transcriptomic study of human hip cartilages¹⁰ and correlated with collagen expression in scleroderma and fibrosis²⁷. The IL-17/IL-6 pathway genes found differentially activated here are increased in inflammation and fibrosis. Csf3 and

### Table III

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**KEY:** Increase/decrease by <2([-1]), 2–4([-1]), 4–8([-1]), 8–16([-1]), or 16+([-1]) fold vs genotype naïve. No change (-). >6-fold differential between genotypes (*). Significant differences between genotypes (*P < 0.05, **P < 0.01).
Map2k6 promote inflammation in dermal repair and collagen-induced arthritis, and II-6 is central for development of keloid disease. Mmp1a is upregulated in inflammatory ischemia. Cxcl1 is a central inflammatory mediator in murine colitis, and Ptgs2 is highly expressed in the inflammatory phase of murine experimental OA.

Of the ECM-remodeling genes, the notably elevated Colla1 and Colla2 expression in injured Has1−/− joints supports the non-reparative fibrotic scarring response, which appears to arise in progenitor-cell rich tissues such as synovium and periosteum, and the histological resemblance to hypertrophic scar tissue seen in keloid disease. Tgfβ3 expression is high in murine dermal sclerosis, but requires activation by Il-17. The finding that Timp2 and Plat are upregulated in the Has1−/− joint appears to be inconsistent with the findings that they have shown protection in knockout studies. It is difficult to reconcile the Timp2 observations; however, the Plat difference could be explained by the simultaneous enhancement of Serpine1 (Table III), an inhibitor of Plat. Enhanced expression of Tgfβ3 occurs in experimental OA and is found in areas of osteophyte formation. In a similar fashion, increased Thsp2 expression is linked to chondrocyte formation, since it stimulates chondrogenesis in a rabbit osteochondral defect model. Finally, the increased expression of Timp2 expression in the Has1−/− mice is consistent with an increase in fibrous matrix deposition, since Timp1 activation also occurs in the anabolic phase of a murine model of OA when ECM genes, including Colla1 and Colla5, are also highly activated.

The distinct elevated expression of apoptosis genes in Has1−/− joints (relative to WT) is consistent with an altered stress-induced apoptotic response in fibroblasts from Has1−/− and Has3−/− mice. Moreover, Has1−/− joints showed elevated expression of Il4 (Table III), a known stimulator of synovial fibrosis and fibroblasts-to-myofibroblast transition. Elevated Lta (TNFβ) expression results in an increased inflammatory response, and increased Insf expression has been linked to enhanced collagen deposition in muscle repair after injury. Lastly, increases in Il6g and Il2 expression are consistent with the findings that their joint fluid levels increase with OA severity and that there is cross-talk between IFN-γ and TGF-β in dermal wound healing. Lastly, an increase in Rela would allow for a higher expression of Admacs5, which has been linked to fibrotic scarring in murine OA.

The apparently normal content of HA in naïve and injured Has1−/− joints suggests that HAS1 is not responsible for the synthesis of the “bulk” HA. However, a more limited activation of Has2 expression in Has1−/− post-injury joints indicates that HAS1 may regulate HAS2 levels during the wound healing process. Moreover the absence of Has1 could abrogate the activation of anti-inflammatory phagocytic cells, as evidenced by the decrease in pro-inflammatory cells and improvement of collagen fiber orientation observed with lentiviral over-expression of HAS1 in dermal repair. Further, since HAS1 activity has been shown to be regulated by glucose concentration and several cytokines during wound healing, it may synthesize a pericellular HA pool that contributes to the process of mesenchymal cell fate in a post-injury environment (Figs. 1, 2 and 4).

A link between inflammation and OA, particularly after injury, is well-documented. However, mechanisms by which inflammation becomes chronic without the obvious presence of inflammatory cells in the joint remains to be established. The linkage between persistent activation of the NF-κB pathway, the excessive deposition of fibrotic scar tissue, and the chronic cartilage damage in injured Has1−/− joints may provide a novel model in which to study the interplay of these pathways in the pathogenesis of injury-induced OA.

**Contributions**

DDC — contributed to experimental design and carried out murine surgeries, gene expression analyses, data evaluation, and manuscript preparation.

WFX — carried out murine surgeries, genotypic characterizing, and maintenance of Has1−/− mouse colony.

JL — carried out murine surgeries, maintenance of all mouse colonies, macroscopic imaging, and histology.

CdIM — consulted for experimental design and provided assistance with interpretation of HA histology and gene array data.

JDS — contributed to experimental design, data evaluation, and manuscript preparation.

AP — directed experimental design, data interpretation, and manuscript preparation.

AP (anna_plaas@rush.edu) takes responsibility for the integrity of the work as a whole, from inception to finished article.

**Role of the funding source**

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**Competing interest statement**

The authors have no conflicts of interest, perceived or actual, to declare.

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**Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2015.06.021.

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