c-Jun/Activator Protein-1 Mediates Interleukin-1β-induced Dedifferentiation but Not Cyclooxygenase-2 Expression in Articular Chondrocytes*

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Interleukin (IL)-1β is a major catabolic pro-inflammatory cytokine involved in cartilage destruction-associated processes, such as loss of the differentiated chondrocyte phenotype (dedifferentiation) and inflammation. Here, we investigated the role of c-Jun and activator protein-1 (AP-1) in IL-1β-induced dedifferentiation and cyclooxygenase (COX)-2 expression in primary cultured chondrocytes. IL-1β induced expression and transient phosphorylation of c-Jun in primary cultured chondrocytes. Ectopic expression of c-Jun was sufficient to cause dedifferentiation, whereas expression of dominant negative c-Jun blocked IL-1β-induced dedifferentiation. Interestingly, modulation of c-Jun expression did not affect IL-1β-induced COX-2 expression. Further experiments revealed that c-Jun phosphorylation was mediated by c-Jun N-terminal kinase and was required for IL-1β-induced dedifferentiation but not COX-2 expression. Consistent with its ability to induce phosphorylation of c-Jun, IL-1β caused transient activation of AP-1, which is necessary for IL-1β-induced dedifferentiation. IL-1β treatment suppressed expression of Sox-9, a major transcription factor that regulates type II collagen expression. Inhibition of c-Jun N-terminal kinase or AP-1 reversed IL-1β-induced suppression of Sox-9, and ectopic expression of c-Jun was sufficient to cause suppression of Sox-9. Our results collectively suggest that IL-1β suppresses type II collagen expression in articular chondrocytes by inducing expression and phosphorylation of c-Jun, AP-1 activation, and subsequent suppression of Sox-9.

The development of cartilage, which serves as a template for long bone formation, is initiated by the differentiation of mesenchymal cells into chondrocytes (1). During the process of differentiation, chondrocytes receive and process a complex array of signals from local and systemic factors. One such factor is the transcription factor, activator protein-1 (AP-1),1 which has an essential function in cartilage and bone development (2). AP-1 is a dimeric transcription factor formed by combinations of Jun (c-Jun, JunB, and JunD), Fos (c-Fos, Fra-1, Fra-2, and FosB), and ATF proteins (ATF-2 and ATF-3) and is involved in diverse biological processes such as differentiation, proliferation, cell survival, and transformation (3, 4). The various subunits of AP-1 can be induced at sites of active bone formation in vivo by transforming growth factor-β, parathyroid hormone, 1,25-dihydroxy vitamin D, and other factors (5).

Several lines of evidence indicate that AP-1 regulates cartilage and bone development. For example, AP-1 activation mediates Wnt regulation of chondrogenesis (6) and regulates hypertrophic maturation of chondrocytes (7). Among the components of AP-1, JunB plays a crucial role in endochondral ossification by regulating the proliferation and function of chondrocytes and osteoblasts (8). It has been shown that c-Jun mediates axial skeletogenesis by regulating notochord survival and intervertebral disc formation (9). Overexpression of c-Fos in ATDC5 cells inhibits chondrocyte differentiation in vitro (10). Ectopic expression of Fos in developing chicken limb buds by retroviral microinjection causes truncation of the cartilage in the long bones of the injected leg; this is due to chondrodysplasia caused by severely retarded differentiation of the proliferating chondrocytes into mature chondrocytes, hypertrophy of chondrocytes, and delays in prearticular ossification (11). AP-1 also plays an essential role in the regulation of chondrocyte differentiation by parathyroid hormone-related protein through induction of c-Fos protein expression (12). Genetic studies in mice have provided compelling evidence for the role of AP-1 family members in skeletal development in vivo. For example, transgenic mice overexpressing c-Fos develop chondro- and osteosarcomatous lesions (13, 14), whereas knock-out of c-Fos in mice causes osteopetrosis due to an early block of differentiation in the osteoclast lineage (15, 16). Another AP-1 family member, ATF-2, also contributes to endochondral ossification; chondrocyte proliferation is reduced in ATF-2-deficient mice, leading to dwarfism and skeletal deformities (17). Additionally, ectopic expression of Jun family members has been shown to perturb chondrocyte maturation (18).

In addition to the regulation of chondrocyte differentiation and cartilage development, it has been suggested that AP-1 may regulate destruction of arthritic cartilage. Arthritis is associated with perturbation of chondrocyte homeostasis and is characterized by loss of the differentiated phenotype (dedifferentiation), apoptotic cell death, stimulation of matrix metalloproteinases, signal-regulated protein kinase; IL-1β, interleukin-1β; JNK, c-Jun N-terminal kinase; NAC, N-acetylated-l-cysteine; NDGA, nordihydroguaiaretic acid; RA, retinoic acid; RT, reverse transcription; pc-Jun, phosphorylated c-Jun.
Western Blot Analysis—Chondrocytes were lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS supplemented with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, and 1 mM of 4-(2-aminoethyl)benzenesulfonyl fluoride) and phosphatase inhibitors (1 mM NaF and 1 mM Na3VO4). Thirty micrograms of proteins, unless otherwise indicated, were fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The following antibodies were employed to detect proteins: mouse monoclonal anti-type II collagen (Chemicon, MAB8887), mouse monoclonal anti-ERK-1/2 (554100, BD Transduction Laboratories), mouse monoclonal antibodies against pc-Jun (Santa Cruz, sc-822) and pJNK (Santa Cruz, sc-6254), rabbit polyclonal antibodies against p300 (Santa Cruz, sc-585), c-Jun (Santa Cruz, sc-1694), and Sox-9 (Santa Cruz, sc-20095), and rabbit polyclonal anti-COX-2 (Cayman Chemical).

Immunoprecipitation—Chondrocytes were lysed in Nonidet P-40 lysis buffer (50 mM Tris, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, containing inhibitors of proteases and phosphatases as described above. After incubation on ice for 30 min, the lysates were centrifuged at 13,000 × g for 10 min at 4 °C to remove cell debris. Supernatant containing 1000 μg of protein was pre-cleared by incubating with 25 μl of protein A-Sepharose for 1 h. After centrifugation, proteins in the supernatant were incubated with 2 μg of antibody against p300 (Santa Cruz, sc-656) and COX-2 (160106, Cayman Chemical). The immunocomplex was precipitated with 25 μl of protein A-Sepharose for 2 h at 4 °C. After washing with lysis buffer, the immunocomplex was fractionated by SDS-polyacrylamide gel electrophoresis. Immunoprecipitated p300 and associated Sox-9 or c-Jun was detected by Western blotting.

RT-PCR—Primary cultured chondrocytes were treated with IL-1β or various pharmacological agents as specified in each experiment. Total RNA was isolated using RNAstat-60 (Tel-Test B, Inc., Friendswood, TX) and reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen) as previously described (30). The following primers (based on the sequences of the human c-jun and Sox-9 genes) and conditions were used for PCR: type I collagen (COL1A1) (441-bp product, annealing temperature 60 °C, 27 cycle), sense 5'-GGC ACC CGT GGA GAA GAA GGA CCA CCA GCA ACC GGA GGA-3' and antisense 5'-ATG AAC GCA TGG GAC GGC TTT CCT-3'; type II collagen (COL2A1) (481-bp product, annealing temperature 60 °C, 27 cycle), sense 5'-GGC ACC CGT GGA GAA GGA GGA CCA CCA GCA ACC GGA GGA-3' and antisense 5'-ATG AAC GCA TGG GAC GGC TTT CCT-3'; Sox-9 (386-bp product, annealing temperature 62 °C, 27 cycle), sense 5'-GGC CGT GCT GCA CCA GAA CCA GCA GCC CCA GCC CCA GCC-3' and antisense 5'-ATG AAC GCA TGG GAC GGC TTT CCT-3'. Glyceraldehyde-3-phosphate dehydrogenase was amplified for control and normalization purposes using the following primers and conditions: 299-bp product annealing temperature 50 °C, 21 cycle, sense 5'-TCA ACC TCC TCC CCA ATT TGT AAG C-3' and antisense 5'-TCC AGG AAC GAC GAG GAC GAT CCA ACG-3'. The resulting PCR amplicons were subcloned into pBluescript II phagemid vector (Strategene). The resulting cDNA was cloned into the BamHI and EcoRI restriction sites of the vector, pDNA3.1 (+) (Invitrogen). Sox-9 expression vector was constructed in pDNA3.1 vector by using RT-PCR-amplified Sox-9 cDNA from newborn mouse rib chondrocytes with specific primers (sense 5'-CCG CGG CAA GGA AGC CAT CTT G-3' and antisense 5'-CCG AAT TCC AGG TCG TGT CGT G-3') designed to introduce BamHI and EcoRI restriction enzyme sequences at the 5' and 3' ends of the amplified fragment, respectively. The resulting cDNA was cloned into the BamHI and EcoRI sites of the vector, pDNA3.1 (+) (Invitrogen). Sox-9 expression vector was constructed in pDNA3.1 vector by using RT-PCR-amplified Sox-9 cDNA from newborn mouse rib chondrocytes with specific primers (sense 5'-CCG CGG CAA GGA AGC CAT CTT G-3' and antisense 5'-CCG AAT TCC AGG TCG TGT CGT G-3'). This clone contains promoter region, exon-1, intron-1, exon-2, and part of intron-2 from 0 to 3591 to +4639. The resulting DNA fragment was digested with Nael to produce a 3.5-kilobase fragment that contains promoter region, exon-1, intron-1, exon-2, and part of intron-2 from 3591 to +4639. The resulting DNA fragment was digested with Nael to produce a 3.5-kilobase fragment that contains promoter region, exon-1, intron-1, exon-2, and part of intron-2 from 0 to 3591 to +4639. The resulting DNA fragment was digested with Nael to produce a 3.5-kilobase fragment that contains promoter region, exon-1, intron-1, exon-2, and part of intron-2 from 0 to 3591 to +4639. The resulting DNA fragment was digested with Nael to produce a 3.5-kilobase fragment that contains promoter region, exon-1, intron-1, exon-2, and part of intron-2 from 0 to 3591 to +4639.
c-Jun Mediates IL-1β-induced Dedifferentiation but Not COX-2 Expression in Articular Chondrocytes—IL-1β is a major pro-inflammatory cytokine involved in cartilage destruction processes, such chondrocyte dedifferentiation and inflammatory responses. As shown in Fig. 1, RT-PCR analysis revealed that IL-1β treatment of primary cultured articular chondrocytes led to suppression of cartilage-specific type II collagen expression and induction of fibroblastic type I collagen expression (Fig. 1, A and B), two hallmarks of chondrocyte dedifferentiation. Immunofluorescence microscopy further indicated that IL-1β treatment dramatically increased the number of type I collagen-expressing cells with a concomitant decrease in type II collagen-expressing cells (Fig. 1C). Western blot analysis (Fig. 1, A and B) also revealed that IL-1β treatment induced expression of COX-2, a primary mediator of cartilage inflammation. Expression of COX-2 is localized mainly in perinuclear region (Fig. 1C), which is similar to the report by Kojima et al. (33). These results are consistent with the accepted function of IL-1β in dedifferentiation and induction of COX-2 expression in chondrocytes.

In an attempt to elucidate the role of c-Jun in IL-1β-induced dedifferentiation and COX-2 expression, we first determined the expression, phosphorylation, and subcellular localization of c-Jun in IL-1β-treated chondrocytes. As shown in Fig. 2, A and B, IL-1β treatment caused transient increases in the expression levels (both transcript and protein) and phosphorylation of c-Jun in a dose- and time-dependent manner. Induction of c-Jun expression and its phosphorylation were detectable within 30 min after IL-1β treatment and peaked at 1 h. Immunofluorescence microscopy revealed that the expressed c-Jun was localized mainly in the chondrocytic nuclei (Fig. 2C), which is consistent with its function as a component of the AP-1 transcription factor.

To examine whether increased c-Jun expression was required for IL-1β-induced dedifferentiation and COX-2 expression, we induced chondrocytes to express ectopic wild-type or dominant negative c-Jun and examined the effects of IL-1β treatment in these cells. As shown in Fig. 3A, overexpression of wild-type c-Jun was associated with a reduction in type II collagen expression level but did not appear to alter COX-2 expression. Double staining for type II collagen and c-Jun in chondrocytes transfected with wild-type c-Jun indicated that repression of cartilage-specific type II collagen expression and
IL-1β-induced suppression of type II collagen expression. Consistent with the above results, inhibition of JNK in IL-1β-treated chondrocytes inhibited the suppression of type II collagen expression but did not affect COX-2 expression (Fig. 5B). Immunofluorescence microscopy also demonstrated that pretreatment with SP600125 blocked IL-1β-induced JNK phosphorylation and inhibited suppression of type II collagen expression but did not block induction of COX-2 expression (Fig. 5C).

Because the above results suggest that JNK-mediated c-Jun phosphorylation is necessary for IL-1β-induced chondrocytic dedifferentiation and c-Jun is a component of the AP-1 transcription factor, we next examined whether AP-1 activity is involved in IL-1β-induced dedifferentiation of chondrocytes. AP-1 activity (i.e. the DNA binding activity of c-Jun) was determined using a highly specific and sensitive AP-1 enzyme-linked immunosorbent assay kit. We found that treatment with IL-1β caused a transient increase in the transcriptional activity of AP-1; this effect peaked at 1 h after IL-1β treatment (Fig. 6A) and was dose-dependent (Fig. 6B). As expected, inhibition of JNK by SP600125 treatment blocked IL-1β-induced AP-1 activation (Fig. 6C). To further examine the role of AP-1 activity, we pretreated chondrocytes with NAC or NDGA to inhibit AP-1 activity, NDGA inhibits lipoxygenase and AP-1 (29). NAC, an antioxidant and precursor of glutathione, is known to inhibit NFκB and AP-1 (28). Pretreatment with NAC or NDGA blocked IL-1β-induced activation of AP-1 transcriptional activity in a dose-dependent manner (Fig. 6D). NAC and NDGA also blocked c-Jun phosphorylation and reversed IL-1β-induced suppression of type II collagen expression but not induction of c-Jun phosphorylation by JNK and Activation of AP-1 Mediates IL-1β-induced Dedifferentiation of Chondrocytes—Having established that expression of c-Jun is necessary for IL-1β-induced dedifferentiation of chondrocytes, we next examined whether this process also requires the phosphorylation of c-Jun. We first examined the involvement of JNK in IL-1β-induced c-Jun phosphorylation, because JNK is one of the major protein kinases responsible for phosphorylating c-Jun. Western blot analysis of JNK phosphorylation (which represents JNK activation) revealed that IL-1β treatment caused a transient activation of JNK that apparently preceded the expression and phosphorylation of c-Jun (Fig. 5A). Pretreatment of cells with a JNK inhibitor (SP600125) blocked IL-1β-induced JNK activation, as evidenced by inhibition of its phosphorylation (Fig. 5B). More specifically, JNK inhibition by treatment with SP600125 did not affect IL-1β-induced c-Jun expression but dose-dependently blocked c-Jun phosphorylation (Fig. 5B). Consistent with the above results, inhibition of JNK in IL-1β-treated chondrocytes inhibited the suppression of type II collagen expression but did not affect COX-2 expression (Fig. 5B). Immunofluorescence microscopy also demonstrated that pretreatment with SP600125 blocked IL-1β-induced c-Jun phosphorylation and inhibited suppression of type II collagen expression but did not block induction of COX-2 expression (Fig. 5C).

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Taken together, the above results suggest that JNK-mediated c-Jun phosphorylation and stimulation of AP-1 transcriptional activity mediates IL-1β-induced suppression of type II collagen expression but not induction of COX-2 expression in articular chondrocytes.

**c-Jun/AP-1 Regulates Sox-9 Expression** — In an attempt to elucidate the regulatory mechanism of c-Jun/AP-1 in suppression of type II collagen expression, we examined the role of c-Jun/AP-1 in expression of Sox-9, a major transcription factor that regulates type II collagen (*COL2A1*) expression. IL-1β treatment reduced the transcript and protein levels of Sox-9 (Fig. 7A), and this effect was blocked by inhibition of JNK with SP600125 or by inhibition of AP-1 activity with NAC or NDGA (Fig. 7B). Additionally, ectopic expression of c-Jun was sufficient to reduce transcript and protein levels of Sox-9 (Fig. 7C), which is consistent with the reduction of type II collagen expression levels (Fig. 3A). To further clarify the relationship between c-Jun/AP-1 and Sox-9 in the regulation of type II collagen expression, we induced chondrocytes to express ectopic Sox-9 and examined the effects of IL-1β treatment in these cells on expression and promoter activity of *COL2A1*. We constructed two reporter genes for this purpose. One is Coll II-Pro/Enh gene, which contains promoter region, exon-1, and 60% of the first intron. The included intron-1 contains enhancer element that binds to Sox-9 transcription factor (31). The other is a Sox-9 reporter gene, which contains only the 48-bp Sox-9 binding site in the first intron of human *COL2A1* (Fig. 7E). As determined by reporter gene assay and Western blot analysis, IL-1β significantly reduced the promoter activity of *COL2A1* (Fig. 7F), transcriptional activity of Sox-9 (Fig. 7G), and expression of type II collagen transcript and protein (Fig. 7D). The effects of IL-1β were completely blocked by the ectopic expression of Sox-9 as shown in Fig. 7, D, F, and G. The results collectively suggest that expression and phosphorylation of c-Jun and subsequent stimulation of AP-1 transcriptional activity suppresses Sox-9 expression, which in turn leads to inhibi-
chondrocytes caused by IL-1α serial monolayer subculture. Similar to the dedifferentiation induced in other ways, including treatment with RA and EGF, or examining c-Jun and AP-1 during chondrocyte dedifferentiation of Chondrocytes—

**COL2A1** of human four independent experiments. Sox-9 (G) were determined by reporter gene assay. Data are presented as the results of a typical experiment or the mean values with S.D. from RA, and EGF but not by serial monolayer culture.

The results suggest that suppression of Sox-9 expression rather than regulation of Sox-9 activity by p300 is associated with inhibition of type II collagen expression.

**Involvement of c-Jun/AP-1 in RA- and EGF-induced Dedifferentiation of Chondrocytes**—Last, we examined whether c-Jun/AP-1 is a common mediator of chondrocyte dedifferentiation by examining c-Jun and AP-1 during chondrocyte dedifferentiation induced in other ways, including treatment with RA and EGF, or a serial monolayer subculture. Similar to the dedifferentiation of chondrocytes caused by IL-1β treatment, RA- or EGF-induced dedifferentiation, which was demonstrated by the suppression of type II collagen expression, was associated with increased expression of c-Jun, phosphorylation of c-Jun and JNK, and inhibition of Sox-9 expression (Fig. 9A and B). In contrast, subculture-induced dedifferentiation of chondrocytes was not associated with changes in the expression levels or phosphorylation of c-Jun and JNK (Fig. 9C), suggesting that c-Jun/AP-1 is involved in the dedifferentiation of chondrocytes caused by IL-1β, RA, and EGF but not by serial monolayer culture.

**DISCUSSION**

IL-1β is a pro-inflammatory cytokine that induces several mediators of cartilage degradation and plays a pivotal role in the pathogenesis of arthritis (20). IL-1β causes dedifferentiation of chondrocytes by suppressing type II collagen expression and inducing expression of types I and III collagen, which contribute to the destruction of articular cartilage. Articular chondrocytes express low levels of c-Jun, which are dramatically induced by IL-1β. Our gain-of-function and loss-of-function studies clearly indicated that increased expression of c-Jun is sufficient to abrogate type II collagen expression. Our results further indicated that IL-1β suppresses type II collagen expression in articular chondrocytes by inducing expression of c-Jun and its phosphorylation by JNK, AP-1 activation, and subsequent suppression of Sox-9, as depicted in Fig. 10. Therefore, unlike the requirement of Jun-containing AP-1 complexes in cartilage formation during embryonic development (8, 9), our current study suggests that low expression levels and activity of Jun are required for the maintenance of homeostasis in developed articular cartilage.

We also demonstrated that phosphorylation of c-Jun by JNK mediates IL-1β-induced cessation of type II collagen expression. It has been shown that in chondrocytes, IL-1β activates all subgroups of the mitogen-activated protein kinases, including extracellular signal-regulated protein kinase (ERK), p38 kinase, and JNK. Activation of these mitogen-activated protein kinase subgroups has been shown to activate the AP-1 transcription factor; activation of ERK and JNK was reported to stimulate c-Fos and c-Jun, respectively (23, 36–38). Here, we observed that treatment of chondrocytes with IL-1β led to activation of ERK, p38 kinase, and JNK (data not shown). Among the mitogen-activated protein kinase subtypes, inhibition of JNK blocked the IL-1β-induced effects on c-Jun phos-
phorylation and AP-1 activation (Figs. 5 and 6), indicating that IL-1β-induced JNK activation phosphorylates c-Jun in articular chondrocytes. It is well known that phosphorylation of c-Jun by JNK is required for the formation of a transcriptionally active c-Jun complex (4). Here, our observation that inhibition of JNK blocked the DNA binding activity of c-Jun further indicates that c-Jun phosphorylation is necessary for the formation of an active AP-1 complex (Fig. 6). The binding partner for phosphorylated c-Jun in the active AP-1 complex is currently unknown. AP-1 consists of a variety of dimers composed of Jun and Fos proteins. Members of the Fos protein family can only heterodimerize with members of the Jun family, whereas Jun proteins can both homodimerize and heterodimerize with other Jun or Fos family members to form transcriptionally active complexes. Based on the dimer protein pairings, transcription can be either positively or negatively modulated (3). Therefore, it is possible that phosphorylated c-Jun may homodimerize or heterodimerize with other Jun or Fos members to form an active AP-1 complex.

Although the mechanisms by which AP-1 activity abrogates type II collagen expression remain to be more clearly elucidated, our results suggest that suppression of Sox-9 expression by AP-1 activity contributes to the inhibition of type II collagen expression. This conclusion was further supported by the observation that ectopic expression of Sox-9 blocked IL-1β-induced inhibition of type II collagen expression. Sox-9 is a master transcription factor responsible for controlling the differentiation of mesenchymal cells into chondrocytes. Sox-9 binds to the chondrocyte-specific enhancer element in the gene for proalpha1(II) collagen (COL2A1) (39). Murakami et al. (40) revealed that IL-1β suppresses Sox-9 expression, prompting us to speculate that c-Jun/AP-1-mediated IL-1β-induced suppression of Sox-9 results in abrogation of type II collagen expression. One of interesting findings of this study is that c-Jun/AP-1 signaling appears to mediate dedifferentiation of chondrocytes caused by IL-1β, RA, or EGF, whereas dedifferentiation of chondrocytes caused by the serial monolayer subculture on plastic was found to be independent of c-Jun/AP-1. Indeed, it has been suggested that activation of the Sp-1 transcription factor is associated with subculture-induced dedifferentiation of chondrocytes (41), suggesting that Sp-1 and AP-1 may regulate different pathways of chondrocyte dedifferentiation.

In contrast to the regulation of type II collagen expression, our results indicate that IL-1β-induced induction of COX-2 expression is independent of c-Jun/AP-1 activity. However, a previous study showed that c-Jun/JNK regulates shear-induced COX-2 expression in the T/C28a2 chondrocytic cell line (25). This discrepancy may be due to differences in the cell...
types and/or the applied extracellular stimuli. Although inhibition of JNK did not affect IL-1β-induced COX-2 expression in this study, we observed that IL-1β-induced COX-2 expression was blocked by the inhibition of ERK with PD98059, inhibition of p38 mitogen-activated protein kinase with SB203580, or inhibition of nuclear factor κB with SN50 peptide treatment (data not shown). This suggests that the ERK, p38 kinase, and nuclear factor κB pathways regulate IL-1β-induced COX-2 expression, whereas IL-1β-induced inhibition of type II collagen expression is regulated by the JNK and c-Jun/AP-1 pathway.

In summary, our results collectively indicate that c-Jun expression and its phosphorylation by JNK as well as AP-1 activation and subsequent suppression of Sox-9 play essential roles in IL-1β-induced suppression of type II collagen expression in articular chondrocytes. Therefore, it appears that the c-Jun/AP-1 transcription factor regulates not only chondrocyte differentiation and cartilage formation during embryonic development but also cartilage destruction via chondrocyte dedifferentiation.

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