Deletion of Runx2 in Articular Chondrocytes Decelerates the Progression of DMM-Induced Osteoarthritis in Adult Mice

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Runx2 may play an important role in development of osteoarthritis (OA). However, the specific role of Runx2 in articular chondrocyte function and in OA development in adult mice has not been fully defined. In this study, we performed the destabilization of the medial meniscus (DMM) surgery at 12-week-old mice to induce OA in adult Runx2Agc1CreER mice, in which Runx2 was specifically deleted in Aggrecan-expressing chondrocytes by administering tamoxifen at 8-weeks of age. Knee joint samples were collected 8- and 12-weeks post-surgery and analyzed through histology, histomorphometry and micro-computed tomography (μCT). Our results showed that severe OA-like defects were observed after DMM surgery in Cre-negative control mice, including articular cartilage degradation and subchondral sclerosis, while the defects were significantly ameliorated in Runx2Agc1CreER KO mice. Immunohistochemical (IHC) results showed significantly reduced expression of MMP13 in Runx2Agc1CreER KO mice compared to that in Cre-negative control mice. Results of quantitative reverse-transcription PCR (qRT-PCR) demonstrated that expression of the genes encoding for matrix degradation enzymes was significantly decreased in Runx2Agc1CreER KO mice. Thus, our findings suggest that inhibition of Runx2 in chondrocytes could at least partially rescue DMM-induced OA-like defects in adult mice.

Osteoarthritis (OA) is the most common degenerative joint disease, affecting close to 27 million Americans. The major pathological features of OA include progressive loss of articular cartilage, osteophyte formation, the increases in subchondral bone mass and synovial tissue inflammation and hyperplasia1,2. Multiple animal models have been established to mimic the development of OA. Among them, DMM-induced OA is the most widely used OA animal model3.

During OA progression, articular chondrocytes undergo hypertrophic differentiation. Runx2 plays a pivotal role in regulation of genes important for chondrocyte differentiation, matrix degradation and osteoblast differentiation4,5. Several studies reported that Runx2 expression levels are high in human OA cartilage6–8. To explore the role of Runx2 in OA progression, genetic animal models have been used. Mice with Runx2 overexpression (Runx2-Tg) display an increased number of cartilage protease expression in chondrocytes9. Overexpression of Runx2 also activates matrix degradation enzymes (MMP13 and ADAMTS5) through mitogen-activated protein kinase (MAPK) pathways10 and through direct regulation of Mmp13 gene transcription11. Previous work

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were decreased in articular cartilage of Cre-negative control mice (Fig. 1b). Consistent with this result, the IHC analysis showed that Runx2 protein levels (and green after recombination) after excision. Also, mG labeling is Cre-dependent, compared with the Cre reporter mice that express membrane-targeted tandem dimer Tomato (mT) prior to Cre-mediated excision with the Cre reporter mice. The Mmp13 proximal promoter contains an OSE2 site conserved among different species, such as human, rabbit, mouse and rats. The Mmp13 promoter in osteosarcoma UMR 106–01 cells has been shown to enhance Mmp13 promoter activity. It has been shown that 148 bp upstream of Mmp13 transcription start site is sufficient and necessary for Mmp13 gene expression in bone, teeth and skin. Moreover, transgenic mice with constitutively active MMP13 expression in the hyaline cartilage have chondro-protective effect on DMM-induced OA development.

Matrix metalloproteinase 13 (MMP13) is a potent enzyme that targets cartilage for degradation. MMP13 expression was low in normal and early degenerative cartilage but was strongly up-regulated in late-stage OA specimens. Moreover, transgenic mice with constitutively active MMP13 expression in the hyaline cartilages and joints developed pathologic changes in articular cartilage of mouse joints similar to those observed in human OA. Clinical investigation suggests that MMP13 may be associated with cartilage degradation during OA development. This clinical observation was further confirmed by the study with Mmp13 KO mice. Pharmacological inhibition of MMP13 activity has been demonstrated to be an effective strategy to decelerate articular cartilage loss in a murine model of injury-induced knee OA.

The relationship of Runx2 and Mmp13 has been studied in the developing skeleton during the process of endochondral ossification. The DNA sequence of Runx2 binding site was originally described as osteoblast-specific element-2 (OSE2), which is essential for expression of osteoblast-specific gene osteocalcin. The Mmp13 proximal promoter contains an OSE2 site conserved among different species, such as human, rabbit, mouse and rats. Runx2 binds to the OSE2 site in the Mmp13 promoter and increases Mmp13 gene transcription in cooperation with c-Fos and c-Jun binding to a neighboring AP-1 site. Moreover, co-transfection of Runx2 with the Mmp13 promoter in osteosarcoma UMR 106–01 cells has been shown to enhance Mmp13 promoter activity. It has been shown that 148 bp upstream of Mmp13 transcription start site is sufficient and necessary for Mmp13 gene expression in bone, teeth and skin in vivo and the AP-1 and Runx2 binding sites are likely to regulate this Mmp13 proximal promoter activity. Runx2 also regulates Mmp13 during chondrocytes differentiation. A recent study reported that the interaction of Runx2 and Osterix, a downstream molecule of Runx2, cooperatively induces Mmp13 expression during chondrocyte differentiation. In recent studies, we demonstrated, through mutation analysis and ChIP assays, that Runx2 activates Mmp13 expression by binding to the OSE2 site located in the proximal region of the human Mmp13 promoter in chondrocytes.

Results

Agc1-CreER mice drive Cre recombination in articular chondrocytes. To efficiently target articular chondrocytes in adult mice, we used Agc1-CreER transgenic mice. We evaluated the targeting efficiency and specificity of these mice by breeding them with ROSAmT/mG reporter mice. ROSAmT/mG mice are double-fluorescent Cre reporter mice that express membrane-targeted tandem dimer Tomato (mT) prior to Cre-mediated excision and membrane-targeted green fluorescent protein (mG) after excision. Also, mG labeling is Cre-dependent, complementary to mT at single cell resolution, and distinguishable by fluorescence-activated cell sorting: red before and green after recombination. Analysis of histologic frozen sections from 3-month-old mice (tamoxifen was given to the mice at 2-month-old) using fluorescence microscopy showed that Agc1-CreER targeting cells are located in growth plate, articular cartilage and meniscus in Agc1-CreER; ROSAmT/mG mice (Fig. 1a).

The efficiency of Runx2 KO in the articular cartilage in Runx2\(^{Agc1CreER}\) mice. To assess the efficiency of Runx2 KO in the articular cartilage in adult Runx2\(^{Agc1CreER}\) KO mice, we administrated tamoxifen to 2-month-old Runx2\(^{Agc1CreER}\) mice and their Cre-negative littermates. We then sacrificed them at the 5-months of age. Subsequently, the q RT-PCR and IHC staining were performed. The q RT-PCR analysis showed that Runx2 mRNA levels were significantly decreased by 60% in articular cartilage of Runx2\(^{Agc1CreER}\) KO mice compared to Cre-negative control mice (Fig. 1b). Consistent with this result, the IHC analysis showed that Runx2 protein levels were decreased in articular cartilage of Runx2\(^{Agc1CreER}\) KO mice (Fig. 1c).

OA progression was decelerated in Runx2\(^{Agc1CreER}\) mice. To investigate if Runx2 deletion could prevent or decelerate DMM-induced OA-like defects, we crossed Runx2\(^{Agc1CreER}\) mice with Agc1-CreER transgenic mice to generate the Runx2\(^{Agc1CreER}\) conditional KO mice. Tamoxifen was administered into 8-week-old Runx2\(^{Agc1CreER}\) mice. Deletion of Runx2 had no significant effect on chondrocyte morphology (Figs 2a and 3a). To create an OA mouse model, DMM surgery was performed when the mice were 12-week-old. Knee joint samples were harvested 8- and 12-weeks post-surgery (n = 6–8 mice per group). Results of histological analysis showed that the OA-like phenotype, including fibration, clefting and cartilage degradation, was observed 8-weeks after DMM surgery and worsened at the 12-week time point in Cre-negative control mice. In contrast, much less articular cartilage excavation (but not statistical significant) was observed in 8-week-old Runx2\(^{Agc1CreER}\) KO mice (Fig. 2a). In contrast, deletion of Runx2 significantly protected DMM-induced OA development at the time point 12-weeks after DMM surgery (Fig. 3a).

The evaluation using OARSI scoring system revealed that there was no significant difference, rather a decreased tendency of cartilage degeneration in the mice 8-weeks after DMM surgery in Runx2\(^{Agc1CreER}\) KO mice (Fig. 2b). With time progression, significantly reduced cartilage degeneration was observed in Runx2\(^{Agc1CreER}\) KO mice 12-weeks after DMM surgery (Fig. 3b). We then quantify the OA progression by performing histomorphometry using the OsteoMeasure system. The results showed that at the time point 8-weeks after DMM surgery,
although there was no significant difference between the Runx2Agc1CreER KO mice and Cre-negative controls, the tendency of increased articular cartilage area was observed in Runx2Agc1CreER KO mice (Fig. 2c). At the time point of 12-weeks after DMM surgery, deletion of Runx2 significantly protected DMM-induced cartilage degradation (Fig. 3c). The subchondral sclerosis was observed 8- and 12-weeks after DMM surgery in Cre-negative control mice and this phenotype was also rescued in the Runx2 Agc1CreER KO mice (Figs 2a and 3a). Consistent with this result, the data of μCT analysis showed that subchondral bone mass was increased in Cre-negative control mice 12-weeks after DMM surgery and this effect was rescued in the Runx2 Agc1CreER KO mice (Fig. 4).

The increased MMP13 protein levels by DMM surgery were rescued by Runx2 deletion. IHC results showed that MMP13 expression was weak in articular cartilage and was restricted mainly in deep zone (below the tidemark) and adjacent to subchondral bone in Sham operated mice (Fig. 5). However, MMP13 expression was increased in articular cartilage in Cre-negative control mice 8- and 12-weeks after DMM surgery (Fig. 5). MMP13 was expressed not only in deep zone but also in middle and superficial zones of articular cartilage in the mice after DMM surgery (Fig. 5). Since superficial and mid zones of cartilage of Cre-negative mice were no longer present in the mice 12 weeks after DMM surgery, the MMP13 expression at this time point were only observed in deep zone of articular cartilage. The increased MMP13 expression was reduced in both time points 8- and 12-weeks after DMM surgery in Runx2Agc1CreER KO mice (Fig. 5).

The expression of chondrocyte marker genes was reduced in articular chondrocytes derived from Runx2Agc1CreER KO mice. Primary articular chondrocytes were isolated from 4-day-old Runx2Agc1CreER mice and control littermates and were treated with 4-hydroxy tamoxifen (1μM) for 24 hours, followed by real-time PCR assay. The results showed that there was 56% reduction in Runx2 mRNA expression in articular chondrocytes derived from Runx2Agc1CreER mice (Fig. 6a). Expression of Mmp9 and Mmp13 was decreased by 56 and 59% in Runx2 deficient cells (Fig. 6b and c). Similar to Mmp, expression of Adamts family members was also regulated by Runx2. Expression of Adamts4 and Adamts5 was decreased by 62 and 19%, respectively (Fig. 6d and f), and expression of Adamts7 and Adamts12 was decreased by 23 and 67%, respectively (Fig. 6g and h) in Runx2 deficient chondrocytes. To further determine Runx2 regulation of gene expression in articular cartilage in vivo, we administrated tamoxifen to 2-month-old Runx2Agc1CreER mice and their Cre-negative littermates and isolated mRNA from articular cartilage of Cre-negative and Runx2Agc1CreER KO mice. The qRT-PCR data showed the...
similar gene expression patterns as those observed in the primary articular chondrocytes isolated from 4-day-old Runx2Agc1CreER mice treated with 4-OH tamoxifen (Fig. 7a–h).

Discussion

OA is the most common degenerative joint disorder and a major cause of disability. To investigate the mechanism of the development of OA, several mouse models mimicking human OA were reported in recent years. Among them the well-accepted OA mouse model is DMM-induced OA model. Compared to anterior cruciate ligament transaction (ACLT) model, the OA severity and location of the lesion in DMM model are similar to the lesions observed in aging-related spontaneous mouse model of OA. In addition, DMM model has sufficient sensitivity to show disease modification3. The major symptom of OA is the progressive cartilage breakdown and eventually complete loss of articular cartilage 1, 2, 21. Chondrocytes are the sole cell type in articular cartilage. To better understand the function of specific gene, such as Runx2, in articular cartilage

in vivo

, it is desirable to delete this gene specifically in joint tissues in adult mice 16.

Aggrecan is a major extracellular matrix (ECM) protein in both growth plate and articular cartilage. A previous study15 showed that there was no difference in the localization of Col2a1 and Aggrecan expression in young mice (4–9 weeks old). They express throughout the entire articular cartilage of both medial and lateral tibiae. In contrast to young mice (4–9 weeks old), Col2a1 expression was not detected in older mice (36–50 weeks old). However, there was strong signal for Aggrecan mRNA expression throughout the entire articular cartilage of the medial and lateral tibiae in STR/ort mouse strain. In this study we have used the Agc1-CreER mice to delete Runx2 in articular chondrocytes. The results of frozen sections of Agc1-CreER; ROSA mT/mG mice showed that articular chondrocytes can be efficiently targeted by Agc1-CreER mice. Furthermore, the results of qRT-PCR and IHC staining showed significantly decreased mRNA and protein levels of Runx2 in Runx2Agc1CreER KO mice.
compared to Cre-negative mice. These findings suggest that Runx2 could be efficiently deleted in articular cartilage in Runx2^{Agc1CreER} KO mice.

A recent study showed that Mmp13 and Adamts5 up-regulation may be mediated by Runx2^{Agc1CreER}. Studies of mutation analysis in the Mmp13 promoter and the ChIP assays further demonstrated that Runx2 directly binds to its blinding site at the Mmp13 promoter in chondrocytes. Recent study also showed that expression of Adamts7 and Adamts12 could also be regulated by Runx2 in human chondrocytes.

Runx2 is a critical transcription factor for chondrocyte maturation and its role in OA development has not been fully defined. Runx2 expression levels increase in human OA cartilage. Additionally, Runx2 overexpression activated ECM-degrading enzymes (MMP13 and ADAMTS5) through direct interaction with the Runx2 binding sites at the promoters of these genes and through mitogen-activated protein kinase (MAPK) pathway in chondrocytes. A previous study showed that after induction of knee joint instability, Runx2 global heterozygous KO mice exhibited decreased cartilage destruction and osteophyte formation. The study using Runx2 heterozygous KO mice did provide important information about the role of Runx2 in OA pathogenesis. However, the Runx2 deficiency in the heterozygous Runx2 global KO mice is not chondrocyte-specific and Runx2 deletion in those mice is not specific at adult stage so the possibilities of embryonic effect (carrying-over to the adult stage) and indirect effect of Runx2 on articular cartilage could not be ruled out in those studies. In the present studies, we have deleted Runx2 in Aggrecan-expressing chondrocytes and investigated the specific effect of Runx2 in articular chondrocytes at the adult stage. Our studies provided additional and critical information about the role of Runx2 in OA development. Since many genetic OA mouse models showed Runx2 up-regulation, suggesting that Runx2 may be a central molecule mediating downstream target gene expression during OA development, it would be very important to clearly define if Runx2 is indeed required in OA development and progression.

A recent study in our laboratory showed that Mmp13 and Adamts5 up-regulation may be mediated by Runx2^{Agc1CreER}. Studies of mutation analysis in Mmp13 promoter and the ChIP assays further demonstrated that Runx2
directly binds to the OSE2 site at the Mmp13 promoter11, 12. Recent study also showed that expression of Adamts7 and Adamts12 could also be regulated by Runx2 in human chondrocytes13.

To better understand the role of Runx2 in adult mice during the initiation and progression of OA, in this study we generated Runx2Agc1CreER mice by crossing Runx2flox/flox mice with Agc1-CreER transgenic mice. Tamoxifen was administered to the mice at 8-week-old when growth plate development is basically completed. DMM surgery was performed in both Runx2 Agc1CreER KO mice and Cre-negative controls at 12-weeks-old. Our results demonstrated that a progressive OA-like phenotype was observed 8- and 12-weeks after DMM surgery in Cre-negative control mice. The evaluation of histology results using the OARSI scoring system33 showed that Runx2 deletion has a protective effect on the DMM-induce OA. Consistently, the histomorphometric analysis of changes in cartilage area at the proximal tibiae showed the similar results. These findings provide evidence showing that Runx2 deletion has a protective role in the DMM-induced OA.

The critical event in OA development is the progressive loss of articular cartilage. Two major structural components of articular cartilage are collagen and aggrecan. Accordingly, enzymes mainly targeting collagen and aggrecan degradation are MMPs and ADAMTS2, 34, 35. A previous study showed that increased expression of Mmp9 and Mmp13 was found in the cartilage during OA development35. In the present studies, the inhibition of Mmp9 and Mmp13 expression was found in Runx2 deficient chondrocytes, suggesting that Runx2 is the upstream regulator of Mmp9 and Mmp13 expression.

MMP13 is the most potent enzyme among collagenases for degradation of type II collagen36, 37. In addition to cleaves type II collagen, it also targets the degradation of aggrecan, collagen types IV and type IX, gelatin, osteonectin, and perlecan in cartilage38. The recent studies demonstrated that Mmp13 may be a downstream target gene of Runx211, 12. In the present studies, we observed the significant reduction of Mmp13 expression in primary articular chondrocytes of Runx2Agc1CreER KO mice. IHC result showed that MMP13 expression was reduced in deep zone of articular cartilage, adjacent to subchondral bone and below the tidemark in the Sham operated mice. In mice with DMM surgery, an obvious increase in MMP13 expression was observed in deep zone, as well as an obvious increase in MMP13 expression at this time points were only observed in the deep zone of the articular cartilage. In contrast, the increased MMP13 expression was rescued in Runx2Agc1CreER KO mice after DMM surgery.

MMP13 is also a maker of the chondrocyte hypertrophy39 and Runx2 could regulate the Mmp13 expression in hypertrophic chondrocytes11, 40. During OA development, articular chondrocytes undergo hypertrophy leading to extracellular matrix degradation and articular cartilage breakdown11, 40. Consistent with these findings, in
the present studies, we found that the expanded expression of MMP13 was observed in middle and superficial zones of the cartilage in Cre-negative control mice after DMM surgery, suggesting that chondrocyte hypertrophy is increased in this OA mouse model. Deletion of Runx2 could significantly inhibit MMP13 expression in Runx2Agc1CreER KO mice, even after DMM surgery. The ADAMTS family consists of large members of aggreganases and they share several distinct modules. Both Adamts4 and Adamts5 are responsible for aggregcan degradation in a human model of OA. The previous study showed that Adamts5 up-regulation may be mediated by Runx2 in Tgfbr2 Col2CreER KO mice. The most recent study demonstrated that Adamts7 and Adamts12 are also regulated by Runx2. In the present studies, we observed down-regulation of Adamts 4, 5, 7, 12 mRNA expression in Runx2 deficient chondrocytes derived from new born Runx2Agc1CreER mice treated with 4-OH TM and in articular cartilage of adult Runx2Agc1CreER KO mice. Col10a1 is the most specific hypertrophic chondrocyte maker. The previous studies showed that Runx2 directly targets Col10a1 transcription through interaction with the cis-enhancing elements. Consistent with this finding, we found a significant down-regulation of Col10a1 expression in Runx2 deficient articular chondrocytes.

Runx2 plays a critical role in osteoblast and chondrocyte differentiation in mice and in humans and is a key transcription factor for chondrocyte hypertrophy and osteoblast differentiation. In chondrocyte-specific Runx2 KO mice, DMM-induced cartilage degradation was inhibited. A close relationship and a cross-talk may exist between the articular cartilage and subchondral bone. Moreover, cartilage and subchondral bone are in close proximity and soluble proteins produced in the cartilage are likely to be able to move from one compartment to the other. Perturbing cartilage is expected to preferentially affect subchondral bone. This may explain why chondrocyte-specific Runx2 KO contributes to the inhibition of subchondral bone sclerosis.

In addition to articular cartilage degeneration, subchondral bone sclerosis is also a characteristic of OA. Runx2 plays a critical role in osteoblast and chondrocyte differentiation in mice and in humans and is a key transcription factor for chondrocyte and osteoblast differentiation. In chondrocyte-specific Runx2 KO mice, DMM-induced cartilage degradation was inhibited. A close relationship and a cross-talk may exist between the articular cartilage and subchondral bone. Moreover, cartilage and subchondral bone are in close proximity and soluble proteins produced in the cartilage are likely to be able to move from one compartment to the other. Perturbing cartilage is expected to preferentially affect subchondral bone. This may explain why chondrocyte-specific Runx2 KO contributes to the inhibition of subchondral bone sclerosis.

In summary, we performed chondrocyte-specific Runx2 deletion in adult Runx2 KO mice and demonstrated that Runx2 deletion has chondro-protective effect on DMM-induced OA development and progression. Since we have targeted chondrocytes at adult stage, the potential effects of Runx2 deletion during embryonic development and indirect effect of Runx2 on growth plate cartilage development have been ruled out in the current studies. Our studies indicate that deletion of Runx2 in Aggrecan-expressing mature articular chondrocytes prevents DMM-induced OA development. The chondro-protective effect of Runx2 deletion could be due to the inhibition of genes encoding for multiple matrix degradation enzymes. Our studies suggest that Runx2 could serve as a molecular target for drug development for OA treatment.
Methods

DMM-induced OA model. Tamoxifen (Sigma, St. Louis, MO, USA) was administered into 2-month-old Runx2<sup>Agc1CreER</sup> mice and Cre-negative littermates by intraperitoneal (i. p.) injection (1 mg/10 g body weight) for 5 consecutive days. DMM surgery was performed on the right knee of mice to induce knee OA in 12-week-old mice. Sham operation was performed by opening and exposing the structures of the right knee and then closing the skin incision without manipulating the joint tissue on 3-month-old Runx2<sup>Agc1CreER</sup> mice and Cre-negative control mice. Pre- and post-surgery, mice were provided with analgesia (2.5 mg/kg banamine, i. p. injection) every 24 hours for 72 hours and the sutures were removed 10 days after surgery. The right legs were harvested 8 and 12 weeks post-surgery (n = 6–8 in each group), processed, sectioned and stained. The animal protocol of this study has been approved by the IACUC of the Rush University Medical Center and all experimental methods and procedures were carried out in accordance with the approved guidelines.

The generation of Runx2<sup>Agc1CreER</sup> conditional KO mice. Runx2<sup>floxed</sup> (Runx2<sup>flox-neo</sup>) mice were provided by Dr. Takeshi Takarada (Okayama University, Japan). Runx2 floxed mice were generated mice carrying a Conditional Runx2 allele with exon 4, which encodes the Runt domain, flanked by loxP sites. Runx2<sup>flox-neo</sup> mice were crossed with FLPe transgenic mice to eliminate the neomycin cassette. To generate Runx2<sup>Agc1CreER</sup> conditional KO mice, Runx2<sup>flox-neo</sup> mice were crossed with Agc1-CreER transgenic mice. Agc1-CreER transgenic mice were obtained from Jackson laboratories. The resulting Agc1-CreER; Runx2<sup>flox-neo</sup> (Runx2<sup>Agc1CreER</sup>) mice were administered with tamoxifen (1 mg/10 g body weight/day; i. p. injection, for 5 days) at age two-month-old and were sacrificed at ages 5 or 6 months (8 and 12 weeks post DMM surgery) for histologic analysis. Cre-negative littermates were used as controls.

Cre recombination efficiency. To determine whether Agc1-CreER mice could target articular chondrocytes efficiently in adult mice, Agc1-CreER transgenic mice were bred with ROSA<sup>mT/mG</sup> reporter mice (obtained from Jackson Laboratories). Tamoxifen was administered into 2-month-old mice. Mice were sacrificed at age of 3 months. Histologic sections were analyzed using a fluorescence microscope.
Micro-computed tomography (μCT). Prior to histologic processing, we evaluated formalin-fixed mouse legs by μCT using a μCT-35 cone-beam scanner (Scanco Medical) with a 55 kVp source and a 145 μAmp current. We scanned the mouse legs at a resolution of 12 μm. Morphometric analysis was performed on 50 slices extending proximally, beginning with the first slice in which the tibia condyles had fully merged. The subchondral bone was segmented from the cortical shell manually on key slices using a contouring tool, and the contours were morphed automatically to segment the trabecular bone on all slices. The morphometry was reconstructed and analyzed.

Histology and immunohistochemistry. Knee joint tissues were fixed in 4% paraformaldehyde for 48 hours, decalcified with 10% formic acid (commercially-available decalcification solution) for 10 days, dehydrated with graded ethanol, and embedded in paraffin. Serial mid-sagittal sections (3-μm thick) were cut and stained with Alcian blue/hematoxylin and eosin (AB/H&E) for morphologic analysis. Histomorphometric measurements were performed with OsteoMeasure software (OsteoMetrics, Inc., Atlanta, GA, USA). AB/H&E-stained areas were outlined on projected images of each histologic section to determine articular cartilage area. IHC was performed on the 3-μm thick tissue sections and sections were baked at 60 °C overnight. Slides were then deparaffinized, rehydrated, and washed twice in dH2O for 5 minutes each. The antigen retrieval was performed with Antigen Unmasking solution (Vector Laboratories, H-3300) in 95 °C for 10 minutes. Slides were then quenched in 3% hydrogen peroxide for 10 minutes at room temperature. Slides were then incubated with primary antibodies against MMP13 (Mouse anti-Human, MAB 13424, 1:100 dilution) or Runx2 (Mouse IgG, MBL, D130-3, 1:200 dilution) at 4 °C overnight. On the second day, secondary biotinylated goat anti-mouse antibody (Vector Laboratories, BA-9200) was added for 30 minutes, followed by incubation with VECTASTAIN Elite ABC HRP Kit (Vector Laboratories, PK-6100) for 30 minutes. Positive staining was detected by ImmPACT DAB Peroxidase (HRP) Substrate (Vector Laboratories, PK-6100). Slides were then counterstained with CAT Hematoxylin (Biocare Medical, CATHE-GL), dehydrated with graded ethanol and cleared with 3 changes of Xylene and then coverslipped.
Grading of cartilage structure. Histology sections of knee joint (tibia, sagittal view) were stained with Alcian blue/Orange G and graded by two blinded observers based on the scoring system developed by Glasson et al.33 In brief, each section was assigned a grade 0–6: 0, normal cartilage; 0.5, loss of Safranin O staining without structural changes; 1, small fibrillations without loss of cartilage; 2, vertical clefts down to the layer below the superficial layer; 3–6, vertical clefts or erosion to the calcified cartilage (<25% (grade 3), 25–50% (grade 4), 50–75% (grade 5) and >75% (grade 6) of the articular surface is affected)33. The maximal score was used to represent severity of the OA progression of each mouse.

Cell culture and real-time polymerase chain reaction (PCR) analysis. Primary articular chondrocytes were isolated from articular cartilage of 4-day-old neonatal mice, as described previously35. The isolated cells were treated with 4-OH tamoxifen (1 μM) for 24 hours. Total mRNA was extracted with Trizol (Invitrogen Life Technologies, CA, USA). 1 μg total RNA was used to synthesize complementary DNA (cDNA) using the iScript cDNA Synthesis kit (Quanta Biosciences, MD, USA). Real-time PCR amplification was performed using specific primers of genes encoding for matrix degradation enzymes and a SYBR Green real-time PCR kit (Quanta Biosciences, MD, USA). The primer names and sequences were listed in Table 1. Data were collected from cells of 3 independent mice (n = 3).

Statistical analysis. Data are presented as the mean ± SD. For experiments comparing two groups of data, unpaired Student t-test was performed. For data that multiple groups are involved, one-way analysis of variance (ANOVA) was performed followed by Turkey’s post-hoc test. P values less than 0.05 were considered significant.

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Table 1. The names of sequences of primers used in this project.

| Genes  | Primer sequence (forward primers) | Primer sequence (reverse primers) |
|--------|----------------------------------|----------------------------------|
| Runx2  | GACGTGTGCTTACGTCATGGC            | ACTTGGTTTTCCATACAGCGCGGA        |
| Mmp9   | GCACAGGCCAATTCTAGCCG             | TGTAGTTAGTGGTCCTCCACCTGG        |
| Mmp13  | CTTCTCCTCTGTTGACGCAGCTC          | CTGTGGAGTCATCGTGTAGACT          |
| Adamts4| ATGGCCTCAACCTCATTCCAG            | GCAAGAGGTTGGTGAATCTTG           |
| Adamts5| GGAGGGAGGCCATTTACAC              | CGTAGAAGAAGTGGCCACCTTT          |
| Adamts7| GCAGAGCTTGGCTTGCTTGCTCTCA       | GCCACAGATAAGGTTGGTG             |
| Adamts12| GACGCCAGGGCAAGAAGATTCTT        | CCCAGTGFAGCCGTCGAGATTA          |
| Coll1A1| TTCTGCTGTCAATGGTTGCTTGGAC       | GGAGTAGAATTGGTGTCCTG            |
| Actin  | GCGTGATATCTCCTCCTCATG           | CCAGTTGTAACAAATGGCAGT          |
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**Author Contributions**

Di Chen contributed to the experimental design and data interpretation. Lifan Liao, Shanxing Zhang, Jianhong Gu, Jian Huang, Lan Zhao, Chundo Oh, Jun Li and Baoli Wang carried out all experiments. Takeshi Takarada and Yukio Yoneda contributed to this work by providing Runx2\textsuperscript{flox/flox} mice and advising on how to breed and genotype the mice. Lifan Liao, Meiqing Wang and Di Chen contributed to the manuscript preparation and revision.

**Additional Information**

**Competing Interests:** The authors declare that they have no competing interests.

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