Delay in articular cartilage degeneration of the knee joint by the conditional removal of discoidin domain receptor 2 in a spontaneous mouse model of osteoarthritis

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Background: Discoidin domain receptor 2 (*Ddr2*) is a rate-limiting factor in articular cartilage degeneration, a condition which normally leads to joint destruction. In human osteoarthritic tissues and mouse models of osteoarthritis (OA), the expression of *Ddr2* increases and interacts with collagen type II, inducing the expression of matrix metalloproteinase 13 (MMP-13) and the receptor itself in chondrocytes. Moreover, conditional deletion of Ddr2 can significantly delay the progression of articular cartilage degeneration in post-traumatic OA mouse models. However, the biological effect of the conditional removal of *Ddr2* in aging-related OA is still unknown. Therefore, this investigation was to determine whether the conditional removal of Ddr2 in articular cartilage could delay the cartilage degeneration in an aging-related mouse model (*Col11a1*$^{+/-}$) of OA.

Methods: Mice *Acan*$^{+/CreERT2}$ were bred with *Ddr2*$^{flox/flox}$ mice to generate *Acan*$^{+/CreERT2}$;*Ddr2*$^{+/flox}$ mice. *Acan*$^{+/CreERT2}$;*Ddr2*$^{+/flox}$ mice were crossed with *Ddr2*$^{flox/flox}$ mice to produce *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$ mice. A similar breeding procedure was used to generate *Col11a1*$^{+/-}$;*Ddr2*$^{flox/flox}$ mice, in which *Acan*$^{+/CreERT2}$ mice were replaced by *Col11a1*$^{+/-}$ mice. *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$ mice were bred with *Col11a1*$^{+/-}$;*Ddr2*$^{flox/flox}$ mice to produce *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice that were then treated with tamoxifen or oil at the age of 10 weeks. Knee joints from oil- and tamoxifen-treated *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice, and *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$ mice at the ages of 3, 9 and 15 months were collected for histology and immunohistochemistry analyses. The protein expressions of Ddr2 and Mmp-13 and the degraded collagen type II were examined.

Results: The cartilage degeneration was significantly delayed in tamoxifen-treated *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice. The scores, representing the severity of the cartilage damage, between oil- and tamoxifen-treated mice were: (mean ± SD) 1.33±0.47 *vs.* 1.29±0.45 (P>0.05) at the age of 3 months, 3.50±0.50 *vs.* 2.14±0.35 (P<0.001) at the age of 9 months, and 5.33±0.47 *vs.* 2.71±0.55 (P<0.001) at the age of 15 months. The protein expressions of Ddr2, Mmp-13 and the degraded collagen type II were significantly decreased in tamoxifen-treated mice.

Conclusions: The removal of Ddr2 could significantly attenuate the cartilage degeneration in *Col11a1*$^{+/-}$ mice.

Keywords: Discoidin domain receptor 2 (Ddr2); matrix metalloproteinase 13 (MMP-13); articular cartilage; osteoarthritis (OA); mouse model

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Introduction

The preservation of articular cartilage is one of the effective ways to prevent joints from being destroyed. Understanding the molecular basis underlying the progressive process of articular cartilage degeneration can provide valuable insights into the potential therapeutic targets for the preservation of articular cartilage. On the basis of results from previous investigations, it was concluded that a cell surface receptor tyrosine kinase, discoidin domain receptor 2 (*Ddr2*) for native collagen type II, may be a rate-limiting factor in articular cartilage degeneration (1-4). In related studies, the expression of *Ddr2* was found to be increased in human osteoarthritic tissues and degenerative articular cartilages of osteoarthritis (OA) mouse models (5-8), and co-localized with the elevated expression of matrix metalloproteinase 13 (MMP-13) (9). At an early stage, activation of the collagen receptor DDR2 leads to upregulated expression of MMP13. This results in generation of fragments of collagen II and fibronectin in the territorial matrix. These fragments, as well as cytokines such as IL-1, further stimulate signaling pathways that induce MMP-13 expression. The result is a time-dependent increase in the degradation of the cartilage extracellular matrix. Based on previous studies of other independent research groups and our group, MMP-13 can degrade both proteoglycans and type II collagen effectively, and the expression of MMP13 is hardly detected in normal mature articular cartilage, while the activity and expression of this enzyme are increased in human osteoarthritic cartilage and in joints of OA mouse models (1,5,6,8). In addition, *in vitro* experiments demonstrated that the interaction of *Ddr2* with collagen type II can induce the expression of MMP-13 in chondrocytes through the activation of Ras/Raf/MEK/ERK signaling pathways and p38 signaling pathways: the activation of DDR2 by type II collagen will lead to the phosphorylation of MEK, which subsequently activate the downstream molecule ERK via the Ras/Raf/MEK/ERK pathway, and p38 via p38 signaling pathways in chondrocytes. Then the phosphorylated ERK and p38 translocate into nucleus, result in elevated levels of MMP-13 mRNA, and therefore increase the expression of MMP-13 (5). More importantly, the conditional deletion of *Ddr2* was revealed to significantly delay the progression of articular cartilage degeneration in a post-traumatic OA mouse model, with destabilization of the medial meniscus (DMM) (1). However, a question remains: what is the biological effect of the conditional removal of *Ddr2* in other types of OA, such as in the case of aging-related OA? Collagen type XI-haploinsufficiency (*Col11a1*$^{+/-}$) mouse strain provides us with an excellent opportunity to determine whether the conditional deletion of *Ddr2* can significantly attenuate the pathogenesis of OA in an aging-related mouse model.

Chondrodysplasia (*cho*) in mice is a spontaneous mutant mouse strain (10). The genetic defect of *cho* is identified with a single nucleotide deletion in *Col11a1*, which leads to the absence of the normal collagen type XI in homozygous mutant mice (*cho/cho*) (11). Thus, the condition in *cho/cho* mice is equal to that in functional knockout collagen type XI in mice (*Col11a1*$^{-/-}$). Despite having normal life span (about 30 months on average) without any overt abnormalities, heterozygous *cho*/+ (*Col11a1*$^{+/-}$) mice may develop OA-like changes in knee joints starting at the age of 3 months old and form a typical osteoarthritic joint at the age of 15 months old (12). They may further present the pathological appearance of chondrocyte clusters and undergo an increase in the production of proteoglycans in the extracellular matrix, particularly in the pericellular matrix of chondrocytes. The degradation of proteoglycans and collagen type II is seen in *Col11a1*$^{+/-}$ mice with aging. Finally, fibrillation, missing cartilage, synovial inflammation, and osteophyte formation are evident in the mutant mice. This pathological progression is consistent with what is seen in the majority of human aging-related OA cases (13,14). Thus, *Col11a1*$^{+/-}$ mouse strain is a good aging-related OA mouse model.

In this study, for the first time, we explored the biological effect of the conditional removal of Ddr2 in aging-related OA, and used a mouse genetic approach to specifically remove *Ddr2* in the articular cartilage of knee joints in *Col11a1*$^{+/-}$ mice at the age of the 10 weeks, an age at which *Col11a1*$^{+/-}$ mice do not demonstrate articular cartilage degeneration. Mice were then euthanized at the ages of 3, 9, and 15 months so that knee joints could be collected, and the joints were then examined for evidence of articular

cartilage degeneration.

We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/atm-20-5786).

Methods

The conditional deletion of Ddr2 in the articular cartilage of knee joints of Col11a1$^{+/-}$ mice

All animal work described in this study was approved by and was conducted in accordance with the Institutional Animal Care and Use Committee of Harvard Medical School. Three mouse strains were used in this experiment. The first mouse strain, *AcanCreERT2*, was made available by the Jackson Laboratory (B6.Cg-*Acan*$^{tm1(cre/ERT2)Crm}$/J, stock# 019148), and expresses a recombinant protein consisting of Cre-recombinase and a modified estrogen receptor (*CreERT2*), driven by the endogenous aggrecan promoter (14). The second strain was the floxed *Ddr2* (*Ddr2*$^{flox/flox}$) mouse strain (1), with the exon 9 of *Ddr2* flanked by loxP sites. The third strain was the *Col11a1*$^{+/-}$ mice. For the generation of *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice, *Acan*$^{+/CreERT2}$ mice were bred with *Ddr2*$^{flox/flox}$ mice to produce *Acan*$^{+/CreERT2}$;*Ddr2*$^{+/flox}$ mice. *Acan*$^{+/CreERT2}$;*Ddr2*$^{+/flox}$ mice were then crossed with *Ddr2*$^{flox/flox}$ mice to produce *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$ mice. A similar breeding procedure was used to generate *Col11a1*$^{+/-}$;*Ddr2*$^{flox/flox}$ mice. Finally, *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$ mice were bred with *Col11a1*$^{+/-}$;*Ddr2*$^{flox/flox}$ mice to produce *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice (*Figure 1A*). The *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice were identified by polymerase chain reaction (PCR). The detailed experimental procedures of PCR for the identification of *Acan*$^{+/CreERT2}$, *Ddr2*$^{flox/flox}$, and *Col11a1*$^{+/-}$ are described in our previous publications (1,12).

To specifically delete *Ddr2* from the articular cartilage of knee joint in *Col11a1*$^{+/-}$ mice, 10-week-old *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice were intraperitoneally injected with tamoxifen at 2 mg/10 g body weight daily with the concentration of 1mg/100ul for 10 consecutive days. *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$; *Col11a1*$^{+/-}$ mice were treated with tamoxifen. Meanwhile, *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice treated with sunflower seed oil and their littermates, *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$ mice (as wild-type control), were used as the two control groups. They were maintained in a mouse facility under a 12-hour light and dark cycle, and euthanized at the ages of 3, 9, and 15 months for knee joint collection.

Measurement of the removal efficiency of Ddr2 from the articular cartilage of knee joints

First, three 10-week-old *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice were intraperitoneally injected with tamoxifen at 2 mg/10 g body weight each day for 10 consecutive days. The other three *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice were injected with oil as control.

Second, the mice were euthanized at the age of 3 months old, and articular cartilages of knee joints were carefully sliced off from the tibial and femoral condyles, and collected individually from each mouse. For genomic DNA isolation, the articular cartilages were digested in a 1.7 mL Eppendorf tube containing 200 mL of the tail buffer and 2 mL of protease K (10 mg/mL) (Sigma, Cat#P2308). The tubes were shaken with an Eppendorf ThermoMixer at 300 rpm at 56 ℃ overnight. Then, 200 mL of isopropanol were added to precipitate DNA after digestion. The samples were stored at –20 ℃ for 3 to 4 hours. The DNA was pelleted at 12,000 ×g at 4 ℃ for 15 min.

Third, for the detection of the exon 9 of *Ddr2* in mice after injection, PCR was performed with the genomic DNA isolated from the articular cartilage; the forward primer was 5'-AGTAGGTGCTAGCTACCTCCCACC-3', and the reverse primer was 5'-GATGGGAGTCCATTGTTGTC-3'. For the quantitative measurement of the exon 9 of *Ddr2*, duplex real-time PCR was performed using TaqMan Copy Number Assay. For the target gene, *Ddr2* exon 9 (Transcript: ENSMUST00000027985), TaqMan probe (Cat# 4400291), was labeled with FAM; for the reference gene, Tfrc, TaqMan probe (Cat# 4458366) was labeled with VIC. PCR was performed in 20 ml of reaction buffer containing 20 ng of genomic DNA according to the company's protocol. Real-time PCR reaction was carried out at 95 ℃ for 10 min followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 60 s, using StepOnePlus real-time PCR System (ThermoFisher Scientific). Each sample was tested in triplicate.

Histology

Acan$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice were injected peritoneally with tamoxifen or oil at 10 weeks of age, and knee joints were collected at 3, 9, and 15 months of age. There were eight joints collected from each experimental group, with one joint being collected from each chosen mouse. Knee joints were then fixed and processed for paraffin embedding, and 6-μm thick serial sections were cut from the anterior to posterior side of each joint. Every

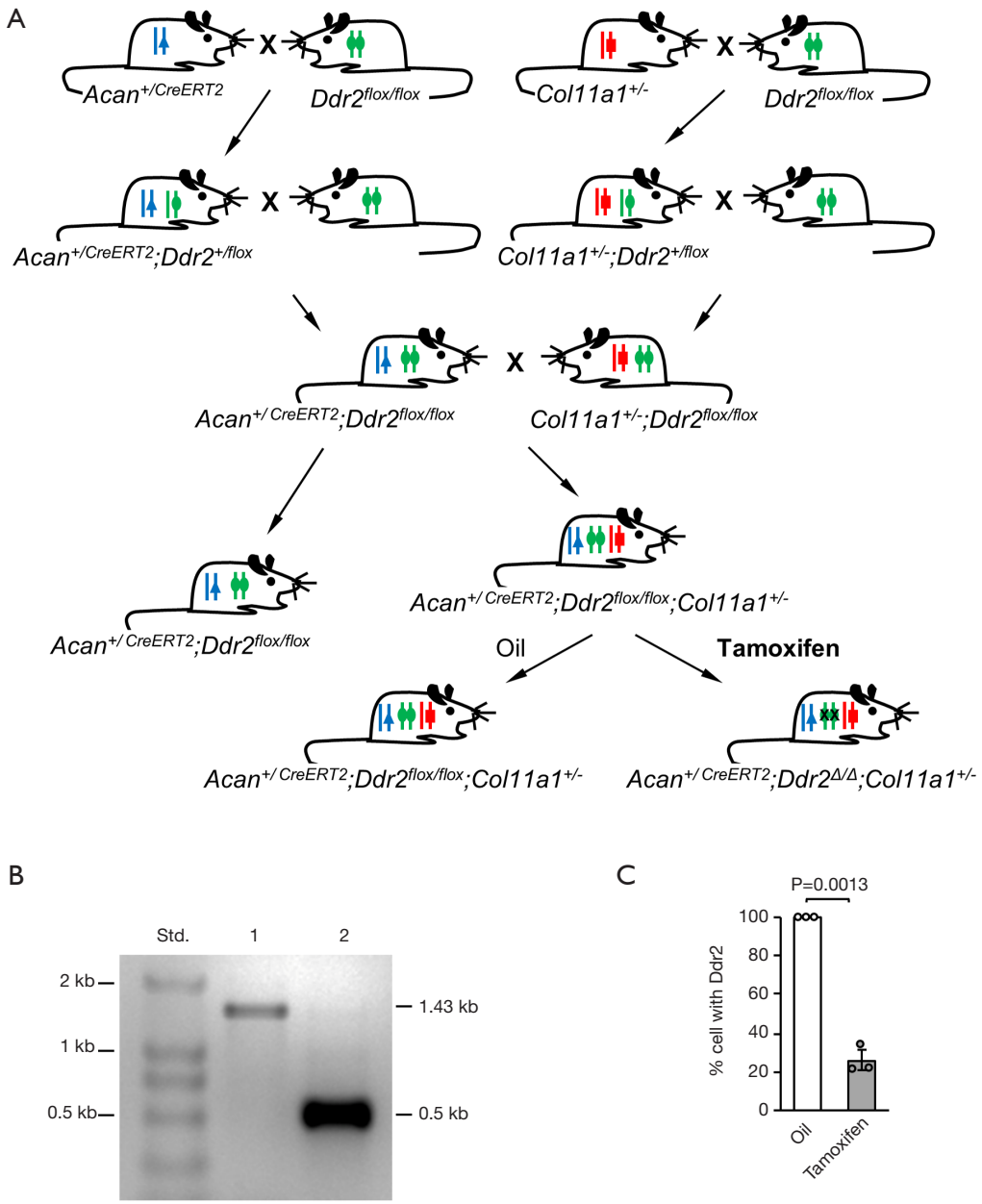


Figure 1 The generation of *Acan*$^{+/CreERT2}$;*Ddr2*$^{\Delta/\Delta}$;*Col11a1*$^{+/-}$ mice, detection for the deletion of the exon 9 of Ddr2 in articular chondrocytes, and the percentage of articular chondrocytes containing the exon 9 of Ddr2. (A) Generation of *Acan*$^{+/CreERT2}$;*Ddr2*$^{\Delta/\Delta}$;*Col11a1*$^{+/-}$ mice. Three specific genes (*Acan*$^{+/CreERT2}$, *Ddr2*$^{flox/flox}$, and *Col11a1*$^{+/-}$) were established in mice after the several rounds of mice breeding. The *Ddr2* could be removed from adult articular chondrocytes by the injection of tamoxifen into the *Acan*$^{+/CreERT2}$; *Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice. Mice with the *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$ background were used as littermate controls. (B) Detection for the deletion of the exon 9 of *Ddr2* in articular chondrocytes. A 1.43-kb band of PCR product indicates articular chondrocytes containing the exon 9 of *Ddr2* (Lane 1) from *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice treated with sunflower seed oil. A 0.5-kb band indicates articular chondrocytes without the exon 9 of *Ddr2* (Lane 2) from *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice treated with tamoxifen (*Acan*$^{+/CreERT2}$;*Ddr2*$^{\Delta/\Delta}$;*Col11a1*$^{+/-}$ mice). (C) The percentage of articular chondrocytes containing the exon 9 of *Ddr2*. In tamoxifen-treated *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice (*Acan*$^{+/CreERT2}$;*Ddr2*$^{\Delta/\Delta}$;*Col11a1*$^{+/-}$) about 25% of articular chondrocytes contained the exon 9 of *Ddr2*, indicating that the exon 9 of *Ddr2* was deleted from 75% of chondrocytes in those mice. Oil-treated *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice were used as the control. P=0.0013.

tenth section was stained with Safranin O/Fast green.

Knee joint morphology was evaluated by a modified Mankin score system as recommended by the Osteoarthritis Research Society International (15). We made minor modifications to this scoring system. We added a chondrocyte cluster and/or considered an increase in Safranin O staining as score 1 and a loss of Safranin O staining as score 2 since these morphological appearances are the earliest pathological signs in articular cartilage degeneration. We then ranked fibrillation above the tidemark as 3, fibrillation reaching to the tidemark as 4, cartilage missing less than 25% of the cartilage surface as 5, cartilage missing less than 50% of the cartilage surface as 6, cartilage missing less than 75% of the cartilage surface as 7, and cartilage missing more than 75% of the cartilage surface as 8. The morphological conditions of meniscus, subchondral bone, and synovial membrane were also evaluated to assess the overall condition of the joint.

Immunohistostaining

Unstained paraffin sections were used for immunohistostaining. Four animals were randomly selected from three groups at the age of 9 months old. Eight to ten paraffin sections from each joint were used for immunohistostaining with a polyclonal antibody. The sections were incubated with the antibody against *Ddr2* (1:300 dilution, Abcam, cat. no. ab203219), the antibody against MMP-13 (1:300 dilution, Abcam, cat. no. ab84594), or the antibody against degraded collagen type II (1:400 dilution, IBEX Pharmaceuticals Inc, cat. no. 50–1053) at 4 ℃, overnight. The samples were then incubated with a biotinylated secondary antibody (goat anti-rabbit IgG). After that, the samples were treated with a mixture of avidin and biotinylated horseradish peroxidase (VECTASTAIN ABC Kit, Vector Laboratories, cat. no. PK-400). NovaRED peroxidase substrate (Vector Laboratories, cat. no. SK-4800) was used for color detection. Sections were counterstained with 0.2% Fast Green solution. For negative controls, staining with isotype-matched normal IgG (Vector Laboratories) and staining without primary antibodies were also performed.

Statistics

For histology, all of the sections stained by Safranin O/Fast green were examined. From each knee joint, ten to twelve paraffin sections were scored, and the worst score was selected to represent that joint. Six to eight scores were obtained, and an average score was calculated from each group. Normally distributed data were analyzed by the Student's *t*-test, and are presented as mean ± 95% confidence intervals (CI). A P value <0.05 was used to determine the presence of statistically significant differences between any two scores.

Results

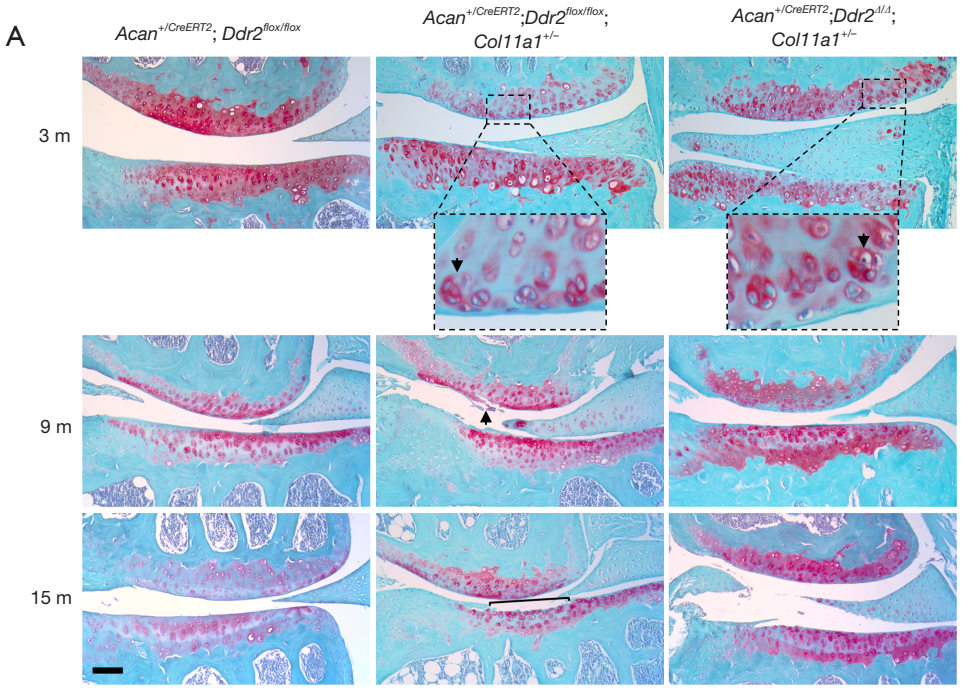
The genetic removal of Ddr2 from the articular cartilage of knee joints in Col11a1$^{+/-}$ mice

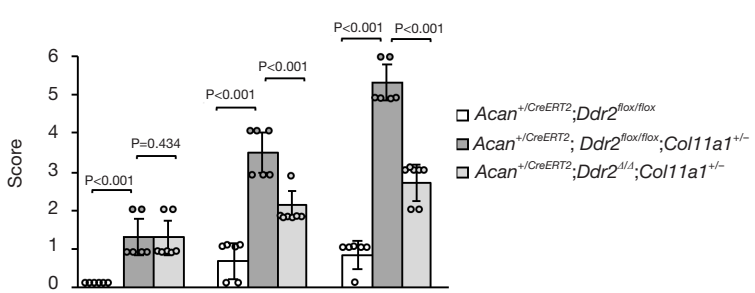
The average size of cartilage pieces was about 1.5×1.5×0.15 mm^3. From each knee joint, 1.5 to 2.0 mg of the cartilages were collected. For detection of the exon 9 of *Ddr2*, PCR was carried out with the genomic DNA. PCR product, 1.43-kb in size, was detected in the DNA of *Acan$^{+/CreERT2}$;Ddr2$^{flox/flox}$;Col11a1$^{+/-}$* mice treated with oil, whereas a size of 0.5-kb was detected in the DNA of *Acan$^{+/CreERT2}$;Ddr2$^{flox/flox}$;Col11a1$^{+/-}$* mice treated with tamoxifen; this indicated that the exon 9 of *Ddr2* was deleted in tamoxifen-treated *Acan$^{+/CreERT2}$;Ddr2$^{flox/flox}$;Col11a1$^{+/-}$* mice (*Figure 1B*).

For the quantitative measurement of the exon 9 of *Ddr2* in chondrocytes, genomic DNA was also used. About 75% of chondrocytes in *Acan$^{+/CreERT2}$;Ddr2$^{\Delta/\Delta}$;Col11a1$^{+/-}$* mice did not have the exon 9 of *Ddr2* (*Figure 1C*). The number of cells with the exon 9 of *Ddr2* was significantly lower in *Acan$^{+/CreERT2}$;Ddr2$^{\Delta/\Delta}$; Col11a1$^{+/-}$* mice than in *Acan$^{+/CreERT2}$;Ddr2$^{flox/flox}$;Col11a1$^{+/-}$* mice.

Delay of the articular cartilage degeneration of knee joints in Acan$^{+/CreERT2}$;Ddr2$^{\Delta/\Delta}$;Col11a1$^{+/-}$ mice

The progressive process of articular cartilage degeneration was significantly delayed in *Col11a1$^{+/-}$* mice without *Ddr2*. At the age of 3 months old, the major pathological indicator in the articular cartilage of *Col11a1$^{+/-}$* mice with or without *Ddr2* was chondrocyte clusters (*Figure 2A*). The scores for the evaluation of cartilage condition indicated no significant difference between these two groups (*Figure 2B*). At the age of 9 months old, fibrillation was only seen in the articular cartilage of knee joints in *Col11a1$^{+/-}$* mice with *Ddr2*. The score was significantly lower in *Col11a1$^{+/-}$* mice without *Ddr2* than in *Col11a1$^{+/-}$* mice with *Ddr2*. At the age of 15 months, the missing cartilage was observed only in *Col11a1$^{+/-}$* mice with *Ddr2*. Again, the score was significantly lower in *Col11a1$^{+/-}$* mice without *Ddr2* than





Age (months)	*Acan*$^{+/CreERT2}$; *Ddr2*$^{flox/flox}$		*Acan*$^{+/CreERT2}$; *Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$		*Acan*$^{+/CreERT2}$; *Ddr2*$^{\Delta/\Delta}$;*Col11a1*$^{+/-}$	
	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD
3	6	0.00±0.00	6	1.33±0.47	7	1.29±0.45
9	6	0.67±0.47	6	3.50±0.50	7	2.14±0.35
15	6	0.83±0.37	6	5.33±0.47	7	2.71±0.45

Figure 2 Morphology of articular cartilages of mouse knee joints. (A) Chondrocyte clusters appeared in both oil-treated mice (*Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$) and tamoxifen-treated mice (*Acan*$^{+/CreERT2}$;*Ddr2*$^{\Delta/\Delta}$;*Col11a1*$^{+/-}$) at the age of 3 months (arrows in the insets of the top panel). Fibrillation was seen in *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice (arrow in the middle panel), whereas proteoglycan depletion was observed in *Acan*$^{+/CreERT2}$;*Ddr2*$^{\Delta/\Delta}$;*Col11a1*$^{+/-}$ mice at the age of 9 months. The loss of articular cartilage was seen *Acan*$^{+/CreERT2}$; *Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice at the age of 15 months (as indicated in the bracket area of cartilage in the bottom panel). However, there was no loss of the cartilage observed in tamoxifen-treated *Acan*$^{+/CreERT2}$;*Ddr2*$^{\Delta/\Delta}$;*Col11a1*$^{+/-}$ mice. Bar =100 μm. (Staining: Safranin O/Fast green). (B) We used the morphology of the articular cartilage in wild-type mice at the age of 3 months as normal articular cartilage (score 0). There were no significant differences in the scores between oil- or tamoxifen-treated mice at the age of 3 months. However, the scores were significantly lower in tamoxifen-treated mice (*Acan*$^{+/CreERT2}$;*Ddr2*$^{\Delta/\Delta}$;*Col11a1*$^{+/-}$) at the ages of 9 and 15 months, compared with those in oil-treated mice (*Acan*$^{+/CreERT2}$; *Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$). P<0.001 in the 9- and 15-month groups.

in *Col11a1*$^{+/-}$ mice with *Ddr2*. The morphological analyses demonstrated that the deletion of *Ddr2* could significantly delay the progression of *Col11a1*$^{+/-}$ mouse knee joints into OA-like joints. There were no abnormal morphological changes in the wild-type groups with the exception of a slight degradation of proteoglycans in the cartilage at the age of 15 months old.

With regard to other joint tissues, synovial tissue inflammation and osteophyte formation were seen in *Col11a1*$^{+/-}$ mice at the age of 15 months (12). However, we did not observe these changes in *Col11a1*$^{+/-}$ mice without *Ddr2*. We did not detect any pathological change in the subchondral bone of both *Col11a1*$^{+/-}$ mice with and without *Ddr2*.

In one of our previous investigations, we found that the process of articular cartilage degeneration was significantly attenuated in *Ddr2*$^{+/-}$;*Col11a1*$^{+/-}$ mice (conventional heterozygous knockout *Ddr2* in *Col11a1*$^{+/-}$ mice) in which 50% of the *Ddr2* was removed in *Col11a1*$^{+/-}$ mice (15). In this present study, we re-evaluated the condition of the articular cartilages of knee joints in *Col11a1*$^{+/-}$ and *Ddr2*$^{+/-}$;*Col11a1*$^{+/-}$ mice. Results showed that there were no significant differences in the scores between *Ddr2*$^{+/-}$;*Col11a1*$^{+/-}$ and *Acan*$^{+/CreERT2}$;*Ddr2*$^{\Delta/\Delta}$;*Col11a1*$^{+/-}$ mice at the ages of 3, 9, and 15 months (*Figure 3*).

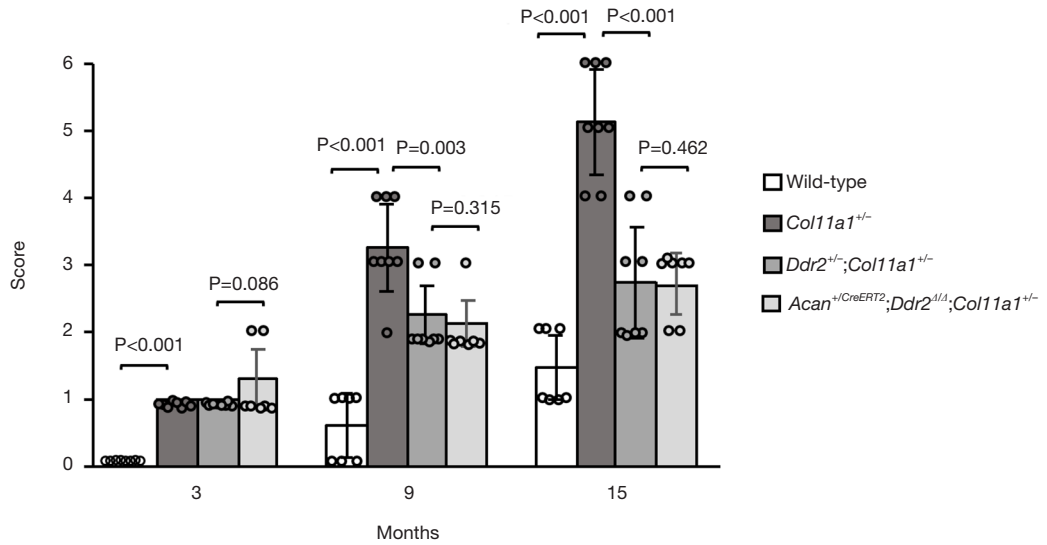
Decrease in Ddr2 and MMP-13 expression and the amount of the degraded collagen type II in Acan$^{+/CreERT2}$*;Ddr2*$^{\Delta/\Delta}$*; Col11a1*$^{+/-}$ *mice*

The protein expression of *Ddr2* could barely be detected in the articular cartilage of knee joints in *Acan*$^{+/CreERT2}$;*Ddr2*$^{\Delta/\Delta}$;*Col11a1*$^{+/-}$ mice (*Figure 4*), which is consistent with the result of the removal efficiency, indicating that *Ddr2* was deleted in majority of articular chondrocytes. We also found that the protein expression of MMP-13 the degraded collagen type II could also barely be detected in the articular cartilages in *Acan*$^{+/CreERT2}$;*Ddr2*$^{\Delta/\Delta}$;*Col11a1*$^{+/-}$ mice. The protein expression of Ddr2 and MMP-13 and the amount of the degraded collagen type II, were dramatically increased in *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Cl11a1*$^{+/-}$ mice (*Figure 4*). This is in line with our previous observation that the expression of Ddr2 and MMP-13 and the amount of the degraded collagen type II were increased in *Col11a1*$^{+/-}$ mice (5).

Discussion

Results from investigations by numerous research groups have confirmed that the increase in the expression of *Ddr2* is associated with the acceleration of articular cartilage degeneration, which eventually leads to OA (2,5,8,16). In one of our previous studies, we conventionally removed one copy of *Ddr2* in *Col11a1*$^{+/-}$ mice (*Ddr2*$^{+/-}$;*Col11a1*$^{+/-}$ mice) by crossing heterozygous *Ddr2*-knockout (*Ddr2*$^{+/-}$) mice with *Col11a1*$^{+/-}$ mice. The result indicated that the removal of one copy of *Ddr2* in *Col11a1*$^{+/-}$ mice could delay the progression of articular cartilage degeneration in knee joints. In addition, the result from one of our other investigations demonstrated that the conditional deletion of *Ddr2* in the articular cartilage of adult mouse knee joints significantly attenuated the cartilage degeneration induced by post-traumatic injury (the destabilization of the medial meniscus, DMM). One question that remains is the exact biological effect conferred by the conditional removal of *Ddr2* in the articular cartilage of adult knee joints in *Col11a1*$^{+/-}$ mice. In the present study, we conditionally deleted *Ddr2* in the articular cartilage of knee joints in adult *Col11a1*$^{+/-}$ mice. We found that the deletion of *Ddr2* significantly delayed the progressive process of the cartilage degeneration in *Col11a1*$^{+/-}$ mice, suggesting that the inhibitory activity of *Ddr2* can protect knee joints against the development of OA initiated by post-traumatic injury or non-traumatic factors. We noticed that there was no significant difference in the deceleration of articular cartilage degeneration between *Ddr2*$^{+/-}$;*Col11a1*$^{+/-}$ mice and *Acan*$^{+/CreERT2}$;*Ddr2*$^{\Delta/\Delta}$;*Col11a1*$^{+/-}$ mice. We speculate that one reason for this was that *Ddr2* was only deleted in 75% of chondrocytes in *Acan*$^{+/CreERT2}$;*Ddr2*$^{\Delta/\Delta}$;*Col11a1*$^{+/-}$ mice. Another possibility is that more time is required, for example 24 months old or longer, for the mice to display a marked difference.

Results from immunohistostaining demonstrated that the removal of *Ddr2* in the knee joints of *Col11a1*$^{+/-}$ mice could dramatically downregulate the expression of MMP-13. The reduction in the amount of the degraded collagen type II in *Col11a1*$^{+/-}$ mice without *Ddr2* also indicated a decreased activity of MMP-13 in the cartilage. This suggests that the chondro-protective effect in the articular cartilage of knee joints in *Col11a1*$^{+/-}$ mice without *Ddr2* may be due to inhibiting the expression of MMP-13 by the deletion of *Ddr2*. With regard to the mechanism by which the cartilage is protected by the removal of *Ddr2*, based on our previous investigations and other studies, we find that TGF-β1 and HTRA1 also play important roles in the pathological development of OA. Therefore, we hypothesize the possible molecular pathway underlying articular cartilage degeneration as follows: excessive mechanical stresses



Age (months)	Wild-type		*Col11a1*$^{+/-}$		*Ddr2*$^{+/-}$;*Col11a1*$^{+/-}$		*Acan*$^{+/CreERT2}$; *Ddr2*$^{\Delta/\Delta}$;*Col11a1*$^{+/-}$	
	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD
3	8	0.00±0.00	8	1.00±0.00	8	1.00±0.00	7	1.29±0.45
9	7	0.59±0.49	8	3.25±0.66	8	2.25±0.43	7	2.14±0.35
15	7	1.46±0.49	8	5.13±0.78	8	2.75±0.83	7	2.71±0.45

Figure 3 Evaluation of the condition of articular cartilage in mouse knee joints. There were no significant differences in the scores between Ddr2$^{+/-}$;Col11a1$^{+/-}$ mice and tamoxifen-treated Acan$^{+/CreERT2}$;Ddr2$^{\Delta/\Delta}$;Col11a1$^{+/-}$ mice, at all the ages, although the scores showed significant differences between wild-type controls and both the genetic mutant mouse groups.

induced by aging-related overloading of normal joints can stimulate chondrocytes and other joint tissues to synthesize and release latent TGF-β1 into synovial fluid. The latent TGF-β1 is then activated and binds to TGFβ receptor II which induces expression of HTRA1 in chondrocytes, leading to degradation of the pericellular matrix and enhanced exposure of chondrocytes to type II collagen. As the pericellular matrix of chondrocytes plays significant roles in keeping *Ddr2* inactive. The degradation of the pericellular matrix can expose chondrocytes to collagen type II. The interaction of chondrocytes with collagen type II activates Ddr2, resulting in induction of MMP-13 as well as expression of DDR2 itself. The result is the degradation of collagen type II and proteoglycans, leading to type II collagen and aggrecan fragments, which in turn activates signals that further increase the synthesis of MMP-13. This feedback amplification loop eventually results in irreversible articular cartilage degeneration, which eventually leads to OA (1,5-9,12,16). We observed that *Ddr2* was not completely deleted from adult articular chondrocytes in *Acan*$^{+/CreERT2}$;*Ddr2*$^{\Delta/\Delta}$;*Col11a1*$^{+/-}$ mice. This might be due to the difficulty in removing *Ddr2* from every aggrecan-producing chondrocyte via tamoxifen injection. In addition, our previous experiment showed that articular chondrocytes below the tidemark in adult knee articular cartilages do not express aggrecan (1). In the examination of the efficiency of *Ddr2* removal experiment, the collection of articular cartilage included the cartilage below the tidemark. This might partly account for the non-aggrecan–expressing cells (about 25%) that contained *Ddr2*. We also noticed that the articular cartilage was continually damaged in *Acan*$^{+/CreERT2}$;*Ddr2*$^{\Delta/\Delta}$;*Col11a1*$^{+/-}$ mice as the mice aged.

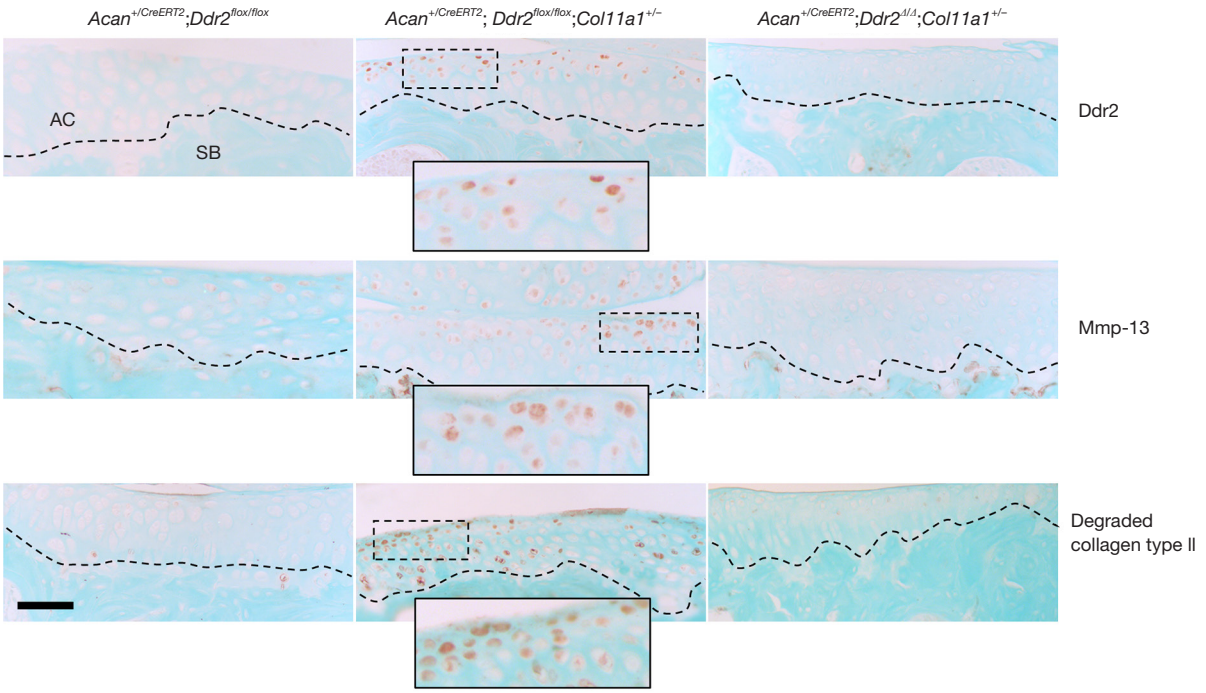


Figure 4 Immunohistostaining of Ddr2, MMP-13, and degraded collagen type II in the articular cartilage of mouse knee joints at 9 months of age. The protein expression of *Ddr2*, MMP-13, and degraded collagen type II were increased in oil-treated *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice (as indicated by the brown-color–stained cells in the middle column of the figure). However, the protein expression of Ddr2, MMP-13, and degraded collagen type II were not detected in the articular cartilage of tamoxifen-treated *Acan*$^{+/CreERT2}$;*Ddr2*$^{flox/flox}$;*Col11a1*$^{+/-}$ mice (*Acan*$^{+/CreERT2}$;*Ddr2*$^{\Delta/\Delta}$;*Col11a1*$^{+/-}$; right column) and wild-type mice (left column). The dashed line represents the boundary between articular cartilage (AC) and subchondral bone (SB). Bar =100 μm.

This suggests that there may be other extracellular matrix–degrading enzymes, apart from MMP-13, that cause the degradation of the cartilage in *Acan*$^{+/CreERT2}$;*Ddr2*$^{\Delta/\Delta}$;*Col11a1*$^{+/-}$ mice.

We are aware that in the last 15 years, results from other research groups have demonstrated that the genetic inactivation or inhibition of genes can protect articular cartilage from being degraded. These genes include hypoxia-inducible factor-2α (HIF-2α), Indian hedgehog (IHH), the Zn^{2+} importer ZIP8, toll-like receptor, Wnt/b-Catenin signaling, and others. The genetic removal of Hif-2α or Ihh can inhibit chondrocyte hypertrophy, resulting in the inhibition expression of MMP-13 (17,18). The delay of the OA progression by the deletion of Zip8 may be due to a downregulation of the expression of extracellular matrix-degrading enzymes, MMP3 and MMP-13 (19). Toll-like receptor and Wnt/β-Catenin signaling may also be involved in the initiation and progression of articular cartilage degeneration (20). Taken together, these studies suggest that numerous molecular pathways interact with each other, eventually leading to MMP-13 induction in chondrocytes. Thus, the removal of any one of these pathways can significantly inhibit induction of MMP-13 in chondrocytes. Obviously, as this study is a preliminary investigation of aging-related OA mouse model, more indepth experiments are needed to understand the exact mechanisms by which these genes contribute to the development of OA.

In conclusion, the study presented here provides additional data to support our observation that the increase of the activity and expression of *Ddr2* may accelerate the progressive process of articular cartilage degeneration of joints, which eventually results in OA. Thus, biological reagents, such as large- or small-molecule inhibitors of *Ddr2*, may be used as disease-modifying OA drugs to treat the disease.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animal work described in this study was approved by and was conducted in accordance with the Institutional Animal Care and Use Committee of Harvard Medical School.

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