MUSCULOSKELETAL PATHOLOGY

High-Temperature Requirement A1 Protease as a Rate-Limiting Factor in the Development of Osteoarthritis

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Preserving the mature articular cartilage of joints is a critical focus in the prevention and treatment of osteoarthritis. We determined whether the genetic inactivation of high-temperature requirement A1 (HtrA1) can significantly attenuate the degradation of articular or condylar cartilage. Two types of mouse models of osteoarthritis were used, a spontaneous mutant mouse model [type XI collagen—haploinsufficient (*Col11a1*^{+/-}) mice] and two post-traumatic mouse models [destabilization of the medial meniscus (DMM) on the knee and a partial discectomy (PDE) on the temporomandibular joint]. Three different groups of mice were generated: i) *HtrA1* was genetically deleted from *Col11a1*^{+/-} mice (*HtrA1*^{-/-};*Col11a1*^{+/-}), ii) *HtrA1*-deficient mice (*HtrA1*^{-/-}) were subjected to DMM, and iii) *HtrA1*^{-/-} mice were subjected to PDE. Knee and temporomandibular joints from the mice were characterized for evidence of cartilage degeneration. The degradation of articular or condylar cartilage was significantly delayed in *HtrA1*^{-/-};*Col11a1*^{+/-} mice and *HtrA1*^{-/-} mice after DMM or PDE. The amount of collagen type VI was significantly higher in the articular cartilage in *HtrA1*^{-/-};*Col11a1*^{+/-} mice, compared with that in *Col11a1*^{+/-} mice. The genetic removal of *HtrA1* may delay the degradation of articular or condylar cartilage in mice. (*Am J Pathol* 2019, 189: 1423–1434; <https://doi.org/10.1016/j.ajpath.2019.03.013>)

More than 30 million individuals in the United States have osteoarthritis (OA).¹ However, there are no disease-modifying OA drugs (DMOADs) available. Existing drugs for the treatment of OA primarily treat the pain associated with a rapidly deteriorating joint. Unfortunately, no amount of analgesics or joint lubricating agents can prevent the cartilage from being destroyed in the development of OA. However, one of the unique characteristics in the development of OA is the extremely long delay (spanning decades) between an initiating event and the onset of the pain, lack of mobility, and reduced quality of life associated with advanced OA. Understanding of the molecular basis underlying this long delay period can provide invaluable information in the effort to develop novel therapeutic targets

for the development of DMOAD to prevent joint destruction.

Articular cartilage degeneration is one of the pivotal pathologic processes in the development of OA. Regardless of the complexity of the OA etiology, the pathologic progress of the articular cartilage degeneration is consistently characterized by the increased activity of chondrocytes at the early stages of the cartilage degeneration, followed by the degradation of proteoglycans and type II collagen, and the final appearance of fibrillation and missing cartilage.^{2–5} This consistent pathologic change suggests that there may be

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multiple initiating pathways toward the common targets that contribute to articular cartilage degeneration. Therefore, the identification of molecular pathways underlying the cartilage degeneration may not only help us understand the disease better but may also provide us with therapeutic targets for the design of novel clinical protocols in the prevention and treatment of OA.

High-temperature requirement A1 (HTRA1) is a serine protease.^{6,7} Substrates of HTRA1 have been identified, including decorin, biglycan, fibromodulin, aggrecans, and fibronectin. These molecules are pericellular components of chondrocytes in articular cartilage. The level of HTRA1 is increased in synovial fluids obtained from OA and rheumatoid arthritis patients.^{7,8} HTRA1 is the most abundant protease in human OA cartilage.⁹ The increased expression of HtrA1 is associated with osteoarthritic joints in the mice.¹⁰ The elevated expression of the enzyme is associated with the disruption of the pericellular matrix of chondrocytes.¹¹ Together, the aforementioned findings indicate that the increase in the expression and activity of HTRA1 may be involved in the pathogenesis of OA. More important, however, more *in vivo* data are needed to demonstrate a causal relationship between HTRA1 and the development of OA.

Herein, we present evidence to indicate that HTRA1 may be a rate-limiting factor in the progressive process of articular or condylar cartilage degeneration. Genetic approaches were used to remove *HtrA1* in a spontaneous mutant mouse model of OA. Surgical destabilization of the medial meniscus (DMM) in *HtrA1*-deficient (*HtrA1*^{-/-}) mice and partial discectomy (PDE) on temporomandibular joints (TMJs) of *HtrA1*^{-/-} mice was performed. Knee joints and TMJs were collected at different ages of mice and analyzed for the pathologic changes of articular or condylar cartilage degeneration.

Materials and Methods

Generation and Characterization of *HtrA1*^{-/-} Mice

The more detailed information of the generation of *HtrA1*^{-/-} mice has been described in a previous publication.¹² In this investigation, RT-PCRs were performed to verify whether *HtrA1* was completely knocked out. Total RNAs were isolated from brain, heart, and muscles for the synthesis of cDNAs. The cDNAs were synthesized with oligo(dT). The PCR primers for the detection of the first exon of *HtrA1* were used: forward primer, 5'-AGGTTGAGCTGAAGAATGGAGC-3'; and reverse primer, 5'-AATTCTCCAGGTCTCAGCTCTG-3'.

Morphologic Characterization of Articular and Condylar Cartilages in *HtrA1*^{-/-} Mice

Knee joints (*n* = 4) and TMJs (*n* = 4) were collected from *HtrA1*^{-/-} and their wild-type littermates at the age of 15

months. All joints were fixed and processed for paraffin embedding. Serial sections (6 μm thick) were cut from the anterior to the posterior side of each joint. Every tenth section was collected for Safranin O/Fast Green staining.

Examination of Bone Density of *HtrA1*^{-/-} Mice

A radiographic analysis was performed on two *HtrA1*^{-/-} mice and two wild-type littermates at the age of 15 months. The mice were exposed to the imaging system (Bruker In-Vivo FX PRO, Billerica, MA) at 35 peak kV X-rays for 30 seconds. The radiographic images were taken to indicate the density of the mice skeletons.

A high-resolution desktop microtomographic imaging system (μCT40; Scanco Medical AG, Brüttisellen, Switzerland) was also used to assess the bone density of the tibial epiphysis. Protocols suggested by the *Journal of Bone Mineral Research* guidelines were used for image acquisition and analysis.¹³ Morphometric variables were computed from the binarized images using direct, three-dimensional techniques that did not rely on any prior assumptions about the underlying structure.¹⁴⁻¹⁶ Measurements of the whole cross-section of the tibial epiphysis included total volume (mm³), bone volume (mm³), bone volume fraction (bone volume/total volume; %), bone mineral density (mgHA/cm³), and tissue mineral density (mgHA/cm³).

DMM of Mouse Knee Joint

The experimental procedure was performed after approval from the Harvard Medical School (Boston, MA) Institutional Animal Care and Use Committee. *HtrA1*^{-/-} mice and their wild-type littermates, at the age of 10 weeks, were anesthetized with ketamine (90 μg/g body weight) and xylazine (25 μg/g body weight) intraperitoneally. Right knees were prepared for DMM surgery. Buprenorphine was provided perioperatively at 0.09 μg/kg. The intercondylar region was exposed, and the medial meniscotibial ligament was divided into sections. Buprenorphine hydrochloride (Par Pharmaceutical, Chestnut Ridge, NY) was also provided subcutaneously at 15 ng/g body weight twice per day for 2 days post-surgically. Knee joints were harvested from *HtrA1*^{-/-} mice (*n* = 8) and wild-type littermates (*n* = 8) at 8 and 16 weeks after the surgery.

A PDE of Disk in Mouse TMJ

The experimental procedure was performed after approval from the Harvard Medical School Institutional Animal Care and Use Committee. The detailed surgical procedure has been described previously.⁴ Briefly, *HtrA1*^{-/-} mice and their wild-type littermates, at the age of 10 weeks, were used for the surgery. Each mouse was anesthetized with ketamine and xylazine intraperitoneally. The left TMJ was open, and the lateral part of the disk was removed. Buprenorphine hydrochloride (analgesic) was provided for 2 days post-surgically.

Immediately after the surgery, powder food was supplied to the mice for a week and then replaced with regular food. The joints were harvested from *HtrA1*^{-/-} mice (*n* = 8) and wild-type littermates (*n* = 8) at 4 and 12 weeks after the operation.

Generation of the Compound Mutant, *HtrA1*^{-/-}; *Col11a1*^{+/-}, Mice

Collagen type XI-haploinsufficient (*Col11a1*^{+/-}) mice are a spontaneous mouse model of OA. It takes approximately 12 to 15 months for *Col11a1*^{+/-} mice to develop a typical OA knee joint.³ In this experiment, *HtrA1*^{-/-} mice were crossed with *Col11a1*^{+/-} mice. The double-heterozygous knockout, *HtrA1*^{+/-}; *Col11a1*^{+/-}, mice were identified by PCR. The PCR procedure for the identification of *Col11a1*^{+/-} mice has been described previously.³ The PCR procedure for the identification of *HtrA1*^{+/-} mice was described above in the section of the generation and characterization of *HtrA1*^{-/-} mice. The double-heterozygous knockout male and female mice were crossed to generate the compound mutant, *HtrA1*^{-/-}; *Col11a1*^{+/-}, mice. Littermates, *Col11a1*^{+/-} mice, and wild-type mice were used as controls. The mice were euthanized at the ages of 3, 9, and 15 months for the collection of knee joints and TMJs.

Histologic and Immunohistostaining Analysis of the Articular and Condylar Cartilages of Mouse Joints

For histology, the same experimental procedure described above in the section of the morphologic characterization of articular and condylar cartilages in *HtrA1*^{-/-} mice was used. Eight joints were collected from each experimental group. For the evaluation of the morphology of mouse articular and condylar cartilages, a scoring system, recommended by the Osteoarthritis Research Society International,¹⁷ was modified. On the basis of the morphologic appearance during the progressive process of the cartilage degeneration, the following ranking was used: chondrocyte cluster and/or Safranin O staining increase, 1; loss of Safranin O staining, 2; fibrillation above the tidemark, 3; fibrillation reaching to the tidemark, 4; cartilage missing <25% of the cartilage surface, 5; cartilage missing <50% of the cartilage surface, 6; cartilage missing <75% of the cartilage surface, 7; and cartilage missing >75% of the cartilage surface, 8. The morphologic conditions of meniscus, subchondral bone, and synovial membrane were also analyzed to assess the overall condition of the joint.

For immunohistostaining, four randomly selected knee joints from *Col11a1*^{+/-} mice and their littermates at the age of 9 months were used. Eight sections from each joint were collected for immunohistostaining. The sections were incubated with a polyclonal antibody against HtrA1 (1:200 dilution; Abcam Inc., Cambridge, MA; catalog number ab-38611), a polyclonal antibody against discoidin domain receptor 2 (*Ddr2*; 1:300 dilution; Abcam Inc.; catalog number ab203219), or a polyclonal antibody against matrix

metalloproteinase-13 (*Mmp-13*) (1:300 dilution; Abcam Inc.; catalog number ab84594) at 4°C, overnight. After that, the samples were incubated with a biotinylated secondary antibody (goat anti-rabbit IgG-B). Sections were treated with a mixture of avidin and biotinylated horseradish peroxidase (VECTASTAIN ABC Kit; Vector Laboratories, Burlingame, CA; catalog number PK-4000). Then, NovaRED peroxidase substrate (Vector Laboratories; catalog number SK-4800) was used for color detection. Sections were counterstained with 0.2% Fast Green solution.

Measurement of the Level of *Ddr2* and *Mmp13* mRNA in Articular Cartilages by Real-Time PCR

Knee joints of *HtrA1*^{-/-}; *Col11a1*^{+/-} mice (*n* = 3), *Col11a1*^{+/-} mice (*n* = 3), and wild-type littermates (*n* = 3), at the age of 9 months, were obtained. Articular cartilages were sliced off from the tibia and femoral condyles and then were pooled together for the isolation of total RNAs by using an Rneasy Micro Kit (Qiagen, Germantown, MD; catalog number 74004). The cDNA was synthesized with oligo(dT) primer. PCR primers were as follows: *Ddr2*, 5'-CTGTCGGATGAGCAGGTTAT-3' (forward) and 5'-CTCGGCTCCTTGCTGAAGAA-3' (reverse); *Mmp13*, 5'-GTGTGGAGTTATGATGATGT-3' (forward) and 5'-TGCGATTACTCCAGATACTG-3' (reverse); and *Gapdh*, 5'-ACTGAGGACCAGGTTGTC-3' (forward) and 5'-TGCTGTAGCCGTATTCATTG-3' (reverse). The concentrations of the primers used in PCRs were as follows: *Ddr2*, 600 nmol/L; *Mmp-13*, 600 nmol/L; and *Gapdh*, 200 nmol/L. PCR was performed using the StepOnePlus (Applied Biosystems, Beverly, MA). Each cDNA sample was analyzed in triplicate. The experiment was performed twice.

Western Blot Analysis for Examination of Collagen Type VI in Mouse Articular Cartilages

Knee joints of *Col11a1*^{+/-} mice (*n* = 3) and *HtrA1*^{-/-}; *Col11a1*^{+/-} mice (*n* = 3), at 9 months of age, were collected. Articular cartilages were collected from the surface of tibia and femoral condyles and were then pooled and transferred into a tube containing 90 µL of RIPA Lysis & Extraction Buffer (G-Biosciences, St. Louis, MO; catalog number 786-489) and 1× protease and phosphatase inhibitors (ThermoFisherScientific, Waltham, MA; catalog number 78443). The tissues were homogenized and centrifuged. The supernatant was collected for the analysis. Equal amounts of total proteins were loaded into an acrylamide/bisacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with a rabbit polyclonal antibody against collagen type VI (Abcam Inc.; catalog number ab199720) overnight. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibodies. Western blot analyses were developed by enhanced chemiluminescence (PerkinElmer, Waltham, MA) and detected by X-ray films.

Statistical Analysis

In the histologic examination, 10 to 12 paraffin sections were scored. The section with the worst score was used to represent that joint. An average score was calculated from the 10 to 12 scores for each group. *t*-Tests were used in this study. To determine sample size, a pilot study was performed on the effect of the *HtrA1* deficiency on mice by DMM. The result indicated that a sample size of a minimum of 5 was required to achieve the 95% CI with at least 50%

reduction of the score in the treatment group. For real-time quantitative PCR analysis also, the *t*-test was used.

Results

Characterization of *HtrA1*^{-/-} Mice

A 148-bp DNA PCR product of the first exon of *HtrA1* was generated from cDNA of brain, heart, and muscle of wild-type littermates. However, the PCR product was not

Figure 2 Morphology and evaluation of temporomandibular joints (TMJs) in *HtrA1*^{-/-} mice after partial discectomy (PDE). **A:** Morphology of the condylar cartilage of TMJs. At 4 weeks after PDE, the morphologic changes are similar in both wild-type (WT) mice and *HtrA1*^{-/-} mice, characterized by the overproduction of proteoglycans in the condylar cartilage and in the fossa of temporal bones of TMJs. At 12 weeks after PDE, the fibrillation appears in the wild-type mice (**arrow**) but is not seen in *HtrA1*^{-/-} mice. **B:** Evaluation of the condylar cartilage of TMJs in mice. There are no significant differences in the scores between the wild-type littermates and *HtrA1*^{-/-} mice at 4 weeks after PDE. However, the score is significantly higher in the wild-type mice, compared with that in *HtrA1*^{-/-} mice at 12 weeks after PDE. There is significant difference between the sham and PDE in wild-type mice at 12 weeks after operation. **P < 0.01, ***P < 0.001. Scale bar = 50 μ m (A). KO, knockout.

detected in cDNA of brain, heart, and muscle from *HtrA1*^{-/-} mice (Supplemental Figure S1A), indicating that there was no expression of *HtrA1* mRNA in *HtrA1*^{-/-} mice.

The morphology of the articular cartilage of knee joints and the condylar cartilage of TMJ from *HtrA1*^{-/-} mice, at the age of 15 months, was examined by histology. No abnormal morphologic changes were observed in the cartilages of *HtrA1*^{-/-} mice at 15 months of age (Supplemental Figure S1B), indicating that the deletion of *HtrA1* did not cause any overt morphologic change in the cartilages of knee joints and TMJs in the mice.

The density of bone in *HtrA1*^{-/-} mice, at 15 months of age, was examined by X-ray. No significant differences were observed in the bone density between *HtrA1*^{-/-} mice and their wild-type littermates (Supplemental Figure S1C). The density of the tibial epiphysis was examined in *HtrA1*^{-/-} mice by micro-computed tomography. Again, no significant differences were observed in the total bone volumes and bone tissue mineral densities in the tibial epiphyses between *HtrA1*^{-/-} and wild-type littermates at the age of 15 months (Supplemental Table S1 and Supplemental Figure S1D). This indicated that the removal

Figure 3 Morphology and evaluation of the articular cartilage of knee joints in compound mutant mice. **A:** Morphology of articular cartilages of mouse knee joints in *Col11a1*^{+/-} mice, *HtrA1*^{-/-};*Col11a1*^{+/-} mice, and the wild-type (WT) littermates. At 3 months of age, chondrocyte clusters (arrows, insets in top row) appear in *Col11a1*^{+/-} mice and *HtrA1*^{-/-};*Col11a1*^{+/-} mice and no significant differences in the morphology of knee joints are seen in both groups of the mice. At 9 months of age, the fibrillation is seen in *Col11a1*^{+/-} mice (arrow, inset in middle row), but not in *HtrA1*^{-/-};*Col11a1*^{+/-} mice. At 15 months, loss of articular cartilages is observed in *Col11a1*^{+/-} mice. The morphology of the articular cartilage is similar in *HtrA1*^{-/-};*Col11a1*^{+/-} mice at both 9 and 15 months of age. Boxed areas are shown at higher magnification in the insets. **B:** Evaluation of the articular cartilage of knee joints in *Col11a1*^{+/-} mice, *HtrA1*^{-/-};*Col11a1*^{+/-} mice, and the wild-type littermates. There are no significant differences in the scores between *Col11a1*^{+/-} mice and *HtrA1*^{-/-};*Col11a1*^{+/-} mice at 3 months of age. However, the scores are significantly higher in *Col11a1*^{+/-} mice, compared with those in *HtrA1*^{-/-};*Col11a1*^{+/-} mice at the ages of 9 and 15 months. The scores are slightly increased in the wild-type littermates at the ages of 9 and 15 months, compared with that at the age of 3 months (the morphology of articular cartilage in wild-type littermates was used as normal articular cartilage; score, 0). ****P* < 0.001. Scale bar = 100 μm (A).

of *HtrA1* did not affect the bone density in the epiphysis of adult mice.

Chondroprotective Effect of the Genetic Deletion of *HtrA1* on Cartilages in Injurious Mouse Models of OA

First, significant disparities were observed in the progressive process of articular cartilage degeneration in knee joints between *HtrA1*^{-/-} mice and the wild-type littermates after DMM surgery at the age of 10 weeks (Figure 1A). The progressive process of articular cartilage degeneration was significantly delayed in *HtrA1*^{-/-} mice after DMM surgery. The severity of the cartilage damage was evaluated by the scoring system. At 8 weeks after DMM surgery, the average scores for *HtrA1*^{-/-} mice and wild-type littermates were 1.75 and 3.25, respectively. At 16 weeks after DMM surgery, the average scores were 2.38 for *HtrA1*^{-/-} mice and 5.25 for wild-type littermates. The scores were significantly different between two groups at each time point (Figure 1B).

Second, the progression of OA was significantly delayed in *HtrA1*^{-/-} mice after PDE surgery. At 12 weeks after the surgery, significantly less damaged condylar cartilages were seen in *HtrA1*^{-/-} mice (Figure 2). However, no difference was observed in the morphology of condylar cartilages between *HtrA1*^{-/-} mice and their wild-type littermates at 4 weeks after the surgery.

Chondroprotective Effect of the Genetic Removal of *HtrA1* on Cartilages in an Aging-Related Mouse Model of OA

Col11a1^{+/-} mice developed normally, with the exception of early-onset OA. At 3 months of age, chondrocyte clusters appeared in the articular cartilage of *Col11a1*^{+/-} mice (Figure 3A). At 9 months of age, fibrillation and localized proteoglycan degradation were seen in the articular cartilage of *Col11a1*^{+/-} mice. At 15 months, *Col11a1*^{+/-} mice revealed several features of a typical OA knee joint, including loss of articular cartilage and misshapen menisci. However, although chondrocyte clusters appeared in the articular cartilage of knee joints in *HtrA1*^{-/-};*Col11a1*^{+/-} mice at 3 months of age, there was no fibrillation or loss of articular cartilage observed in *HtrA1*^{-/-};*Col11a1*^{+/-} mice at 9 and 15 months of age (Figure 3A). The condition of the articular cartilage was also evaluated by the scoring system. Articular cartilage of knee joints in wild-type littermates at 3 months of age was used as a reference point for normal articular cartilage, with a score of 0. The scores in each group are indicated in Figure 3B. The scores increased slightly with age in *HtrA1*^{-/-};*Col11a1*^{+/-} mice, but were not as dramatically increased as they were in *Col11a1*^{+/-} mice. Also, there was no significant increase in scores as *HtrA1*^{-/-};*Col11a1*^{+/-} mice aged from 9 to 15 months of age.

TMJ was also characterized in *HtrA1*^{-/-};*Col11a1*^{+/-} mice (Figure 4A). A slight increase in the production of proteoglycans was seen in both *Col11a1*^{+/-} and *HtrA1*^{-/-};

Col11a1^{+/-} mice at the age of 3 months. There was no significant difference between these two groups of mice at the age of 3 months. However, fibrillation and loss of condylar cartilage were seen in *Col11a1*^{+/-} mice, but not in *HtrA1*^{-/-};*Col11a1*^{+/-} mice at the ages of 9 and 15 months. Scores of the condylar cartilages in *HtrA1*^{-/-};*Col11a1*^{+/-} mice were significantly lower, compared with that in *Col11a1*^{+/-} mice, at the ages of 9 and 15 months (Figure 4B).

Inhibition of Expression of Ddr2 and Mmp-13 and the Protection of Collagen Type VI Degradation in the Articular Cartilage of Knee Joints in Mice by the Deletion of *HtrA1*

First, immunohistostaining of HtrA1, Ddr2, and Mmp-13 was performed on the articular cartilage of *HtrA1*^{-/-};*Col11a1*^{+/-} mice and their corresponding controls at 9 months of age. The protein expression of these three genes was not detected in the articular cartilage of knee joints in wild-type littermates (Figure 5A). However, the protein expression of these genes was increased in the articular cartilage of *Col11a1*^{+/-} mice. This is consistent with the previous observations.^{11,18} In the articular cartilage of *HtrA1*^{-/-};*Col11a1*^{+/-} mice, there was no protein expression of HtrA1. This is also consistent with the result, which indicates that *HtrA1* is knocked out in *HtrA1*^{-/-} mice described above. The protein expressions of Ddr2 and Mmp-13 were also hardly detected in the articular cartilage of *HtrA1*^{-/-};*Col11a1*^{+/-} mice.

Second, real-time quantitative PCR was performed to measure the levels of *Ddr2* and *Mmp13* mRNA. There were no increases in the levels of *Ddr2* and *Mmp13* mRNA in the articular cartilages of knee joints in *HtrA1*^{-/-};*Col11a1*^{+/-} mice, compared with that in wild-type littermates, at the age of 9 months (Figure 5B). This is in line with the result from the immunohistostaining above.

Third, the protein level of collagen type VI was examined in the articular cartilage of knee joints by Western blot analysis. Collagen type VI is present exclusively in the pericellular matrix of chondrocytes in cartilage.¹⁹ Thus, degradation/disappearance of collagen type VI is an indication of the pericellular matrix destruction. In our experiment, the protein level of collagen type VI was significantly higher in the articular cartilage of *HtrA1*^{-/-};*Col11a1*^{+/-} mice, compared with that in *Col11a1*^{+/-} mice at the age of 9 months (Figure 5C).

Discussion

Protective Effect of the Genetic Deletion of *HtrA1* on Articular and Condylar Cartilage Degeneration during the Development of OA

An independent research group reported that the genetic inactivation of *HtrA1* enhanced trabecular bone mass in mice.²⁰ Thus, experiments were performed to examine whether the genetic removal of *HtrA1* in our investigation resulted in increases in bone formation. The results showed

B

Score

□ WT
■ *Col11a1*^{+/-}
▒ *HtrA1*^{-/-}; *Col11a1*^{+/-}

3 months 9 months 15 months

*** **

Time Point	WT	<i>Col11a1</i> ^{+/-}	<i>HtrA1</i> ^{-/-} ; <i>Col11a1</i> ^{+/-}
3 months	~0.8	~0.9	~0.9
9 months	~1.4	~2.9	~1.6
15 months	~1.5	~4.7	~1.9

age of 15 months. Because there are several other HtrA family members in mice, they may be able to compensate for the loss of *HtrA1* during development. On the basis of the result from the characterization of the *HtrA1*^{-/-} mice, this mouse strain may be an excellent animal model to investigate whether the deletion of *HtrA1* has chondroprotective effects.

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Figure 5 Immunohistostaining and relative expression levels of discoidin domain receptor 2 (*Ddr2*) and matrix metalloproteinase-13 (*Mmp-13*) and Western blot analysis of collagen type VI in the articular cartilage of mice at 9 months of age. **A:** Immunohistostaining of HtrA1, *Ddr2*, and *Mmp-13* in the articular cartilage of mice at 9 months of age. The protein expression levels of HtrA1, *Ddr2*, and *Mmp-13* were increased in *Col11a1*^{+/-} mice (brown-staining cells). But the protein expression of all three genes is not detected in the articular cartilage in *HtrA1*^{-/-};*Col11a1*^{+/-} mice and their wild-type (WT) littermates [dashed lines indicate the borders between articular cartilage (AC) and subchondral bone (SB)]. Boxed areas are shown at higher magnification in the insets. **B:** Relative expression levels of *Ddr2* and *Mmp-13* in the articular cartilage of mice at 9 months of age. The levels of *Ddr2* and *Mmp-13* mRNAs are significantly increased in the articular cartilage of *Col11a1*^{+/-} mice, in comparison with *HtrA1*^{-/-};*Col11a1*^{+/-} mice. There are no increases in the levels of *Ddr2* and *Mmp-13* in *HtrA1*^{-/-};*Col11a1*^{+/-} mice, compared with that in their wild-type littermates. **C:** Western blot analysis of collagen type VI in the articular cartilage of *Col11a1*^{+/-} and *HtrA1*^{-/-};*Col11a1*^{+/-} mice. The intensity of collagen type VI band is much higher in *HtrA1*^{-/-};*Col11a1*^{+/-} mice. The level of collagen type VI in *Col11a1*^{+/-} mice is approximately 60% of the collagen in *HtrA1*^{-/-};*Col11a1*^{+/-} mice. ****P* < 0.001. Scale bar = 100 μ m (**A**).

models (DMM and PDE mice). Knee joints and TMJs were examined in the models. The progressive process of the articular or condylar cartilage degeneration was significantly delayed in mice without HtrA1. This ruled out the possibility that the chondroprotective effect seen with the genetic removal of *HtrA1* in mice was either a joint- or a model-specific event. The modified Mankin scores of knee joints were slightly increased from 2.00 to 2.38 and the scores of

TMJ were up from 1.50 to 1.86 from the age of 9 to 15 months in *HtrA1*^{-/-};*Col11a1*^{+/-} mice. In the post-traumatic models, the scores of knee joints were increased from 1.75 to 2.38 in *HtrA1*^{-/-} mice at 8 and 16 weeks, respectively, after DMM. For TMJ, the scores were increased from 0.83 to 1.33 in *HtrA1*^{-/-} mice at 4 and 12 weeks, respectively, after PDE. The slight increases in the scores indicate that HtrA1 may be one of the rate-limiting factors in the

Figure 6 A schematic of a molecular signaling cascade leading to articular cartilage degeneration in the development of osteoarthritis. **1:** Excessive mechanical stress induces transforming growth factor (TGF)- β 1 in articular chondrocytes. **2:** TGF- β 1/phosphorylated Smad2/3 signaling induces HTRA1. **3:** The pericellular matrix of chondrocytes is destroyed by HTRA1 to expose discoidin domain receptor 2 (DDR2) to native collagen type II. **4:** The interaction of DDR2 with the collagen type II activates DDR2 to induce matrix metalloproteinase-13 (MMP-13) via extracellular signal-regulated kinase (ERK) and p38 signaling pathways. In the end, the collagen type II and proteoglycan are degraded by MMP-13. TBR, TGF- β receptor.

acceleration of articular or condylar cartilage degeneration during the development of OA. The genetic deletion of *HtrA1* did not prevent the formation of chondrocyte clusters or the increase in the production of proteoglycans in articular and condylar cartilages at early stages of cartilage degeneration. This suggests that increases in chondrocyte activities, such as cluster formation and overproduction of proteoglycans, are caused by other factors.

A Possible Role of the Increased *HTRA1* in the Acceleration of the Progressive Process of Articular/Condylar Cartilage Degeneration

We propose a causal sequence of molecular events that is responsible for articular cartilage degeneration. HTRA1 is one of the key molecules in this molecular sequence (Figure 6).

Excessive mechanical stresses are initial insults to stimulate chondrocytes in cartilages to synthesize and release transforming growth factor- β 1 (TGF- β 1).^{21,22} The protein expression of Tgf- β 1 was increased in the articular cartilage of knee joints in *Col11a1*^{+/-} and DMM mice at early stages of articular cartilage degeneration.²³ It is likely that chondrocytes are not protected properly and can sense an excessive stress under both conditions. In *Col11a1*^{+/-} mice, chondrocytes reside in the defective extracellular matrix. Thus, chondrocytes under this condition can feel insulted by normal mechanical stimulation. In DMM mice, the altered geometry of the knee joint can exert excessive stress on the chondrocytes, even those residing in the normal extracellular matrix. Moreover, the conditionally genetic removal of Tgf- β receptor II (*Tgfb2*) in mature articular cartilage protects knee joints against the development of OA in mice.²⁴ More important, the results from other independent research groups

also indicate that TGF- β 1 may be a detrimental factor in the pathogenesis of OA in adult joints.²⁵⁻³⁰

TGF- β 1 induces HTRA1 in chondrocytes, which leads to the degradation of the pericellular matrix of chondrocytes. The elevated protein expression of phosphorylated Smad2/3 colocalizes with the increased expression of HtrA1 in the articular cartilage of knee joints in *Col11a1*^{+/-} and DMM mice.²³ TGF- β 1 induces the expression of HTRA1 in human chondrocytes.³¹ As for the destruction of the pericellular matrix by HtrA1, collagen type VI disappears in chondrocytes expressing HtrA1 in *Col11a1*^{+/-} mice.¹⁸ In articular cartilage, collagen type VI exclusively surrounds chondrocytes to make up the pericellular capsule of chondrocytes. The degradation (disappearance) of collagen type VI is an indication of the integrity of the pericellular matrix. In this study, the removal of *HtrA1* could protect collagen type VI from being degraded in *Col11a1*^{+/-} mice. This indicated that more chondrocytes had an intact pericellular matrix in the articular cartilage of *HtrA1*^{-/-};*Col11a1*^{+/-} mice than that in the articular cartilage of *Col11a1*^{+/-} mice. Western blot analysis was performed to compare collagen type VI in *HtrA1*^{-/-};*Col11a1*^{+/-} mice with *Col11a1*^{+/-} mice. Wild-type mice were not used as one of the controls because the extraction of extracellular matrix components was much different (easier in *Col11a1*^{+/-} mice than in wild-type littermates). Thus, it is difficult to quantitatively compare the amount of collagen type VI in the extracellular matrix of *Col11a1*^{+/-} mice and their wild-type littermates by Western blot analysis.

The disappearance of the pericellular matrix elicits interaction of the collagen type II with *DDR2*. The pericellular matrix separates chondrocytes from the adjacent interterritorial or territorial matrices containing collagen type II.^{32,33} It is conceivable that one of the consequences of the degradation of the pericellular matrix is to expose chondrocytes to

collagen type II. This, in turn, results in the interaction of collagen type II with DDR2. The removal of HTRA1 or inhibiting activity of this enzyme could protect the pericellular matrix and prevent DDR2 from being activated. Results from the immunohistostaining experiments showed that the expression of Ddr2 was not increased by the genetic deletion of *HtrA1* in the articular cartilage of *Col11a1*^{+/-} mice. Non-up-regulated expression of Ddr2 reflected the inactive status of Ddr2 because the activation of Ddr2 increases the expression of the receptor itself in chondrocytes. In addition, results from the experiments with the conditionally induced *Ddr2* in transgenic mice support the pericellular matrix-degrading role of HtrA1 in the development of OA.¹⁸ Thus, maintaining the integrity of the pericellular matrix may protect joints against the development of OA.^{34,35}

Activation of DDR2 induces expression of MMP-13. The activation of DDR2 by the interaction of chondrocytes with native collagen type II *in vitro* induces MMP-13. The blockage of extracellular signal-regulated kinase and p38 signaling pathways inhibits the induction of MMP-13 in chondrocytes.^{36–38} The genetically conditional removal of *Ddr2* in articular cartilage significantly attenuates the progressive process of the cartilage degeneration in mouse models of OA.^{39,40} Also, the activation of DDR2 induces the expression of MMP-13 in chondrocytes.^{41–44}

In summary, the excessive mechanical stress can stimulate chondrocytes to synthesize and release TGF- β 1 in adult joints. This factor then binds to its receptor, inducing signal transduction via phosphorylated SMAD2/3, which results in the induction of HTRA1. HTRA1 degrades the pericellular matrix of the chondrocytes. The destruction of the pericellular matrix exposes chondrocytes to collagen type II. This, in turn, elicits interaction of DDR2 with collagen type II and activates DDR2. The activation of DDR2 induces both expressions of MMP-13 and the receptor itself. MMP-13 degrades collagen type II and aggrecans. The end result is OA.

HTRA1 as a Potential Therapeutic Target for Development of DMOADs

A DMOAD should be able to reverse, stop, or significantly attenuate the developmental process of the disease. There are currently no DMOADs as there are no approved therapeutic targets against which a drug can be developed. Our data demonstrate that HTRA1 may be an ideal target for the development of DMOADs. The genetic inactivation of *HtrA1* significantly delays the progressive process of articular and condylar cartilage degeneration in mouse models of OA. Thus, biological reagents that can inhibit the enzymatic activity of HTRA1 may be used as DMOADs to treat OA. Because HTRA1 is a serine protease, one of the challenges to pharmaceutical companies is to develop an effective drug that can specifically inhibit HTRA1 with little or no effect on other proteases. A recent study reports an allosteric mechanism of HTRA1 activation and regulation.⁴⁵ This

provides possibilities for developing highly selective biological reagents, including small or large molecules and cyclic peptides, to target HTRA1.

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Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.03.013>.

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