Histol Histopathol (2014) 29: 609-618
DOI: 10.14670/HH-29.10.609

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Induction of high temperature requirement A1, a serine protease, by TGF-beta1 in articular chondrocytes of mouse models of OA

Lin Xu[1,2], Imanoel Golshirazian[1], Brian J. Asbury[1] and Yefu Li[1, 2]

[1]Department of Developmental Biology, Harvard School of Dental Medicine and [2]Faculty of Medicine, Harvard Medical School, Boston, USA

Summary. The goal of this study is to determine whether transforming growth factor beta 1 (Tgf-ß1) induces the high temperature requirement A1 (HtrA1) in articular chondrocytes of two mouse models of osteoarthritis (OA). Early onset articular cartilage degeneration in the mouse models was characterized by histology. Expression profiles of Tgf-ß1, p-Smad1, p-Smad2/3 and HtrA1 in knee joints of the mouse models were examined by immunofluorescene. By *in vitro* and *ex vivo* experiments, human primary chondrocytes and articular cartilages from femoral condyles of mice were treated with recombinant human TGF-ß1 and an ALK5 chemical inhibitor, SB431542. The level of HTRA1 mRNA in human and mouse articular chondrocytes was examined by real-time PCR. Chondrocyte clusters were present in the articular cartilage of knee joints in the mouse models. Increased expressions of Tgf-ß1, p-Smad2/3 and HtrA1 were detected in the articular chondrocyte of knee joints in the mouse models. The increased expressions of p-Smad2/3 and HtrA1 were co-localized in the articular chondrocyte of the knee joints. The expression of p-Smad1 was hardly detectable in the mouse models and their corresponding wild-type littermates. The level of HTRA1 mRNA was increased in human and mouse articular chondrocytes treated with TGF-ß1, compared with that in chondrocytes without the treatment of TGF-ß1. The TGF-ß1-induced expression of HTRA1 in human and mouse articular chondrocytes was inhibited by SB431542. These results suggest that the Tgf-ß1 canonical signaling was activated to induce HtrA1 in the articular chondrocytes of the mouse models of OA.

Key words: TGF-ß1, HTRA1, Chondrocyte, Articular Cartilage, Osteoarthritis

Offprint requests to: Lin Xu, MD/PhD, REB. Rm514, 188 Longwood Ave., Boston, MA 02115. email: lin_xu@hms.harvard.edu and Yefu Li, MD/PhD, REB. Rm 504, 188 Longwood Ave., Boston, MA 02115, USA. e-mail: yefu_li@hms.harvard.edu

Introduction

High temperature requirement A 1 (HTRA serine peptidase 1) is a serine protease and one of the four HTRA family members in human and mouse genomes (Clausen et al., 2002). Mammalian HTRA1 was originally identified as a gene down-regulated in SV40 transformed fibroblasts. It contains several domains including an insulin-like growth factor binding protein (IGFBP) domain, a Kazal-type serine protease inhibitor (KI) domain and a PDZ domain plus a signal sequence for secretion of HTRA1. The presence of the KI domain suggests that HTRA1 may be a self-regulating enzyme and/or may regulate other serine proteases. Substrates of HTRA1 have been identified, including decorin, biglycan, fibromodulin, aggrecans and fibronectin (Oka et al., 2004; Tsuchiya et al., 2005; Grau et al., 2006). All of these molecules are the pericellular components of chondrocytes in articular cartilages. It is conceivable that up-regulated expression and activity of this enzyme may cause degradation of the pericellular matrix of chondrocytes, which eventually leads to the destruction of a joint. In fact, HTRA1 has been implicated in rheumatoid arthritis (RA) and osteoarthritis (OA). The

level of HTRA1 is higher in synovial fluids obtained from RA and OA patients (Grau et al., 2006). HTRA1 is the most abundant protease in human OA cartilages (Wu et al., 2007). A study with one mouse model of RA, an anti-type II collagen monoclonal antibody induced mouse model, demonstrates that expression of HtrA1 is increased in arthritic joints (Tsuchiya et al., 2005). Results from our previous studies also indicated that the protein level and activity of HtrA1 were increased in knee and temporomandibular joints of genetic and non-genetic mouse models of OA (Polur et al., 2010). More importantly, the elevated expression of the enzyme was associated with disruption of the pericellular matrix of chondrocytes in the articular cartilage of the mouse models of OA. Degradation of the pericellular matrix of chondrocytes has been described as one of the early pathological changes in human OA (Poole et al., 1991). The fact that many of the pericellular matrix components are substrates of this enzyme suggests that the degradation of the pericellular matrix by HTRA1 may contribute to articular cartilage degeneration of joints. In fact, results from human and mouse genetic studies demonstrate that deletion of one or two of the pericellular matrix components of the chondrocyte results in early onset of OA (Hecht et al., 1995; Alexopolulos et al., 2009; Wadhwa et al., 2005; Borochowitz et al., 2004; van der Weyden et al., 2006). Clearly, HTRA1 plays a role in the pathogenesis of OA. A remaining question concerns which factor(s) cause induction of HTRA1 in chondrocytes.

One study reports that transforming growth factor beta 1 (TGF-ß1) induces the expression of HTRA1 in human primary chondrocytes *in vitro* (Urano et al., 2010). Results from our previous *in vitro* experiment confirmed this observation (Li et al., 2011). Moreover, the level of TGF-ß1 is significantly higher in human osteoarthritic tissues than in healthy articular cartilages (Schlaak et al., 1996; Kawamura et al., 2012). This prompted us to investigate whether the expression of Tgf-ß1 and its canonical signaling was increased in articular chondrocytes of the mouse models of OA. We also investigated whether the activated Tgf-ß1 canonical signaling was associated with induction of HtrA1 in the mouse models. Finally, by *in vitro* and *ex vivo* experiments, we treated human primary chondrocytes and femoral articular cartilages of C57BL wild-type mice with recombinant human TGF-ß1 and an ALK5 inhibitor, SB431542 for evidence of induction of HTRA1 in chondrocytes.

Materials and methods

A genetic mouse model of OA

We have characterized genetic form of a mouse model of OA, *Col11a1*$^{+/-}$ mice or heterozygous chondrodysplasia *(cho/*$^{+}$*)* mice. We identified a mutation in *Col11a1* as the genetic cause of cho in mice (Li et al., 1995). The mutation, a single nucleotide deletion, resulted in premature termination of the α1 chain of type XI collagen. The protein product of Col11a1 was not detectable in *cho/cho* mice, which exhibited prenatal lethality. Heterozygous cho (or *Col11a1*$^{+/-}$) mouse genotyping was performed by direct DNA sequence. Mouse genomic DNA was isolated from a 3 mm mouse tail segment. A 528 base-pair DNA fragment was generated by PCR. The forward primer was: 5'-GGTGTTGTCCTGGGTAAACAG-3' and the reverse primer was 5'- GCAGTCACCATTGTCTAATCATC-3'. A primer, 5'- AGCTAAGCCACTGAGGCACAA-3', next to the forward primer was used for direct DNA sequencing of the PCR product. *Col11a1*$^{+/-}$ mice and their wild-type littermates identified by the DNA sequence were separated and maintained under a daily schedule of 12 hours with light and 12 hours without light.

A non-genetic mouse model of OA

The experimental surgical procedure was performed following approval from the Harvard Medical School Institutional Animal Care and Use Committee. Male and female C57BL mice at the age of 10 to 12 weeks were anesthetized intra-peritoneally with Ketamine (90 mg) and Xylazine (10 mg)/kg mouse body weight. The right knees were prepared for aseptic surgery. The joint capsule immediately medial to the patellar tendon was opened to provide visualization of the medial meniscotibial ligament. The ligament was sectioned and the medial meniscus displaced medially, resulting in the destabilization of the medial meniscus (DMM). The joint capsule was closed with 8-0 tapered Vicryl suture and the subcutaneous layer was closed with 7-0 cutting Vicryl. The skin was closed by the application of tissue adhesive. The mice were maintained under a daily schedule of 12 hours with light and 12 hours without light for further experiments. Sham surgery in which the ligament was visualized but not transected in mice was also performed as a control.

Histology

Knee joints from *Col11a1*$^{+/-}$ mice and their wild-type littermates at the age of 3 months (n=4 each) and knee joints from wild-type mice at 4 weeks following DMM and sham surgery (n=4 each) were collected and fixed. Before they were processed for paraffin embedding, the samples were decalcified in Morse's solution. For each knee joint from *Col11a1*$^{+/-}$ mice and their wild-type littermates, 6 μm thick serial sections from medial to lateral sides were cut. For each knee joint from DMM surgery, 6 μm thick serial coronal sections were cut. Every tenth section was collected for Safranin O/Fast green staining. Sections from knee joints were also used for immunofluorescene (see below).

Immunofluorescence

For analysis of the protein expression of Tgf-ß1, p-Smad1, p-Smad2/3 and HtrA1 in *Col11a1*$^{+/-}$ mice and

DMM mice, eight to ten paraffin sections, evenly distributed throughout each joint, were selected for immunohistostaining. Sections were de-paraffinized and quenched for endogenous peroxidase activity. The sections were incubated with a rabbit polyclonal antibody (1:200) against Tgf-ß1 protein (Cat. No. 5559-100, BioVsion, www.biovision.com), or a rabbit polyclonal antibody (1:400) against p-Smad1 (Cat. No. 06-702, Millipore. Temecula, Ca). After overnight incubation at 4°C, the sections were washed with PBS three times and the sections were treated with a secondary antibody (1:500), Alexa Fluor488 donkey anti-rabbit IgG at room temperature for 30 min. The sections were then washed three times with PBS and examined under a fluorescence microscope (ZEISS, AXIO Imager. M1). Staining with the isotype-matched normal IgGs (Vector Laboratories) and staining without primary antibodies were also performed as negative controls.

For double immunohistostaining, the sections were incubated with a rabbit polyclonal antibody (1:500) against p-Smad2/3 (Cat. No. 3101, Cell Signaling Technology, www.cellsignal.com) and a goat polyclonal antibody (1:200) against HtrA1 (Cat. No. sc-15465, Santa Cruz Biotechnology, CA) at 4°C overnight. After washing with PBS three times, the sections were treated with mixed secondary antibodies (1:500), Alexa Fluor488 donkey anti-rabbit IgG and Alexa Fluor594 donkey anti-goat IgG at room temperature for 30 min. The sections were then washed three times with PBS and examined under a fluorescence microscope (ZEISS, AXIO Imager. M1).

Human primary chondrocytes culture

Human primary chondrocytes were purchased from Cell Applications, Inc. (www.cellapplications.com). The chondrocytes are derived from normal human articular cartilage and can be cultured at least 10 population doublings. The human chondrocytes that we used in our experiment were at third passage. The chondrocytes were grown to 70% confluence in human chondrocyte growth medium (Cell Applications, Inc.). Cells were serum starved in human chondrocyte basal medium (Cell Applications, Inc.) for 24 hours prior to treatment. Cells were cultured in a 6-well plate without TGF-ß1 (as a control) or with TGF-ß1 (Cat. No. 100-B, R&D systems, Inc., Minneapolis, MN) at a concentration of 10ng/ml or TGF-ß1 plus SB431542 (10 μM) for 18 hrs at 37°C (SB431542 was added 30 min. prior to the addition of TGF-ß1). At the end of the culture, cells were briefly rinsed with PBS then lysed with Denaturation solution (Ambion), followed by RNA extraction. This experiment was performed twice.

Ex vivo articular cartilage culture of mouse femoral heads

Fifteen C57BL wild-type mice (the Jackson Laboratory, www.jax.org) at the age of 8 weeks were sacrificed for collection of femoral articular cartilage. Articular cartilage covering the femoral head was removed and stored in Dulbecco's Modified Eagle Medium (DMEM). Articular cartilages were pooled in 5 ml of DMEM and homogenized with PT-DA 2112/EC polytron (www.kinematica-inc.com) at the lowest speed for 30 seconds. The sample was then filtered through a 100 μm Nylon Cell Strainer (BD Biosciences, www.bdbiosciences.com). The filtered sample was centrifuged. The pellet was re-dissolved in 2 ml of DMEM and was then divided into 6 wells in a 12-well culture plate. The samples in 2 wells were cultured without TGF-ß (as a control) and 2 wells with TGF-ß1 (10 ng/ml) and 2 wells with TGF-ß1 (10 ng/ml) plus SB431542 (10 μM) for 18 hrs at 37°C (SB431542 was added 30 min. prior to the addition of TGF-ß1). At the end of the culture, the cartilages were collected for isolation of total RNAs. This experiment was performed twice.

Analysis of HTRA1 mRNA in articular chondrocytes by real-time PCR

Total RNAs were isolated from human primary chondrocytes and the mouse femoral articular cartilages using the ToTALLY RNA Kit (Ambion). The cDNAs were synthesized with 200 ng of total RNAs and oligo(dT) primer using Super-Script First-strand Synthesis System (Lifetechnologies). Real-time PCR conditions were optimized for maximal PCR efficiency by adjusting the concentrations of PCR primers: human HTRA1, forward: 5'-CGAATGATGTCACTCACGTC-3' and reverse: 5'-TTTTCCTTGAGACCACCAGC-3', human GAPDH, forward: 5'-CAACGGATTTGGTC GTATTGG-3' and reverse: 5'-ATGTAAACCATGTA GTTGAGGTC-3', mouse HtrA1, forward 5'-GAT CCGAATGATGTCGCTCAC-3' and reverse 5'-ATTTTCCTTGAGCCCTCCGGC-3' and mouse Gapdh, forward 5'-ACTGAGGACCAGGTTGTC-3' and reverse 5'-TGCTGTAGCCGTATTCATTG-3'. We used GAPDH as the internal control in our real-time PCR experiments. The optimum concentrations of primers for each gene were: forward 250 nM/reverse 250 nM. The t-test analysis, with 5% significance level, was used to detect differences in mRNA levels between the control and experimental groups. PCR was performed using 25 μl of 1X PCR buffer containing 3 μM $MgCl_2$, 200 nM dNTPs, PCR primers, 1X SYBR Green, 0.5 unit Taq DNA polymerase (Denville) and 0.5 μl cDNA. Real-time PCR was performed using the Icycler iQ detection system (BioRad, CA) and the PCR reaction was carried out at 95°C for 3 minutes followed by 50 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 4 min. At the end of the PCR cycles, a melting curve, using a temperature range from 55°C to 95°C with +0.5°C intervals, was generated to test the specificity of the PCR product.

Statistical analysis for qPCR analysis

In each qPCR experiment, a cDNA sample was tested in triplicate. Thus we obtained three individual numbers for each cDNA sample. We then calculated an average number of each cDNA sample. The experiment was performed twice. The t-test was applied to see if the difference between the means of the treatment group and control group was statistically significant (the P value was less than 0.05).

Results

Early onset articular cartilage degeneration in knee joints of two mouse models of OA

The detailed information of the OA-like phenotype in *Col11a1*$^{+/-}$ and DMM mice has been published (Xu et al., 2003, 2010). Briefly, *Col11a1*$^{+/-}$ mice developed without overt gross abnormality. However, knee joints revealed chondrocyte clusters, one of the early signs of articular cartilage degeneration, in *Col11a1*$^{+/-}$ mice starting at the age of 3 months (Fig. 1.). In DMM mice, examination of knee joints also revealed the chondrocyte cluster starting at 4 weeks following surgery (Fig. 1). Chondrocyte clusters were seen in the un-calcified layer of the articular cartilage and the location of the clusters was variable in the layer of the articular cartilage. All of the knee joints from *Col11a1*$^{+/-}$ and DMM mice showed the presence of the chondrocyte clusters.

Increased expression of Tgf-ß1 in the articular cartilage of knee joints in Col11a1$^{+/-}$ *and DMM mice*

We found that protein expression of Tgf-ß1 was increased in *Col11a1*$^{+/-}$ and DMM mice (green-color stained cells in Fig. 2). The increased expression of Tgf-ß1 was predominantly within the un-calcified layer of the articular cartilage. Our immunohistostaining did not detect the protein expression of Tgf-ß1 in either the wild-type control mice or the sham surgery control mice.

We noticed that there was weak (dim) fluorescent staining in the wild-type and sham mice. However, the staining in those mice was similar to that in our two

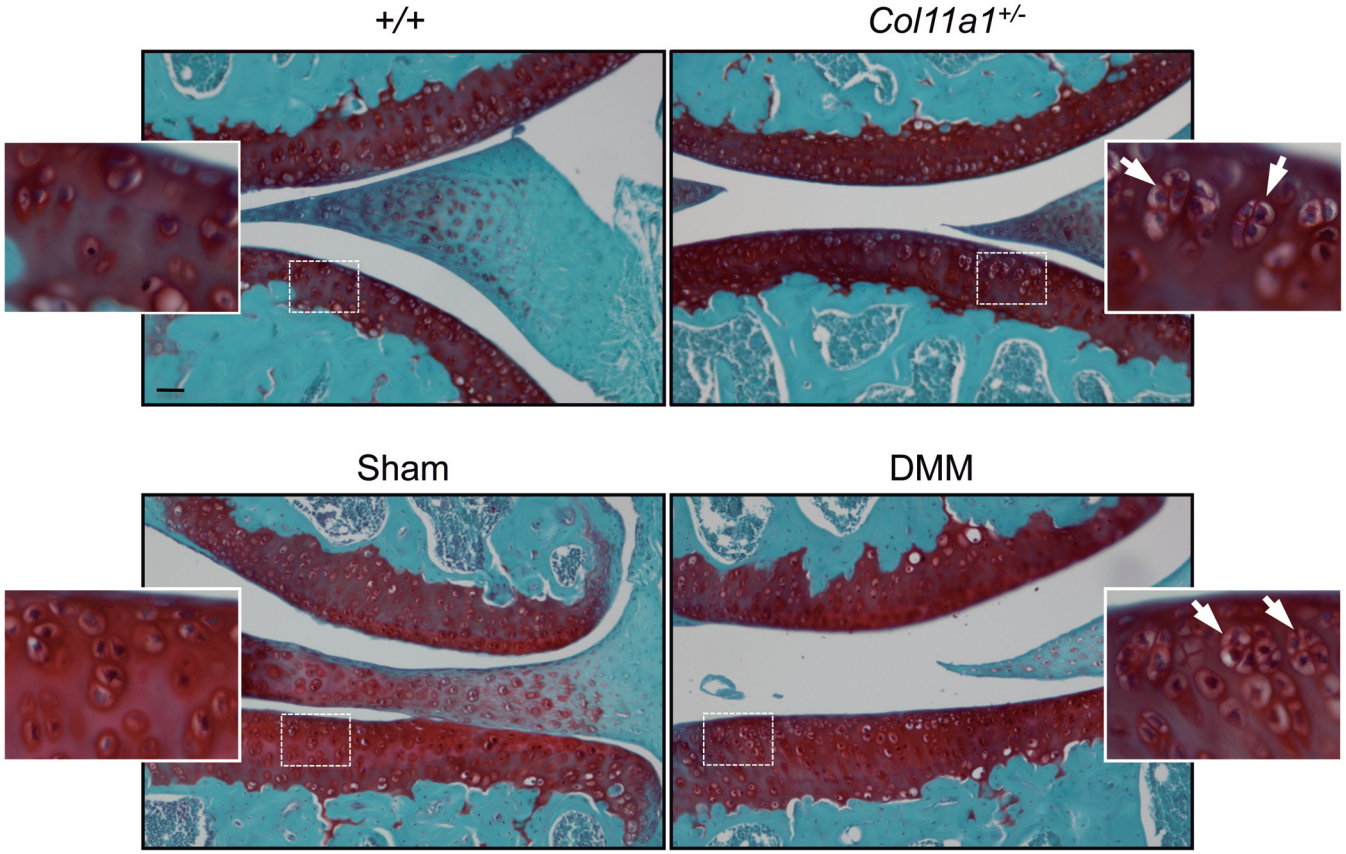

Fig. 1. Morphology of articular cartilage of mouse knee joints. Each image represents knee joints from four mice. Compared with wild-type mice at the age of 3 months old, chondrocyte clusters were seen in the superficial layer of articular cartilages in *Col11a1*$^{+/-}$ mice, see the inset on top panel. The chondrocyte clusters were also observed in knee joints of mice at 4 weeks following DMM surgery, see the inset on bottom panel. The chondrocytes cluster appeared in all of *Col11a1*$^{+/-}$ mice and mice with DMM surgery. Bar: 50 μm.

negative control groups, including the isotype-matched normal IgGs and without primary antibodies. We believe that the appearance of the weak fluorescent signal in the wild-type and sham mice was, in fact, the background effect of the staining.

Co-localization expression of p-Smad2/3 with HtrA1 in knee joints of Col11a1$^{+/-}$ and DMM mice

Receptor-regulated SMADs 2 and 3 (R-SMAD2/3) are down-stream factors of the TGF-ß1 canonical signaling pathway. Additionally, it has been reported that R-SMAD1 is also a down-stream factor in TGF-ß1 signaling. Thus, we used those molecules to indicate whether TGF-ß1 was activated in mouse articular chondrocytes. We found that protein expression of p-Smad2/3 was increased in *Col11a1*$^{+/-}$ and DMM mice (green-color stained cells in Fig. 3). The expression pattern of p-Smad2/3 was similar to that of Tgf-ß1, which was predominantly within the un-calcified layer of the articular cartilage. We did not detect the protein expression of p-Smad2/3 in the control mice.

We also observed that the expression of HtrA1 was increased in the knee joint of *Col11a1*$^{+/-}$ and DMM mice (red-color stained cells in Fig. 3). This is consistent with the result from our previous experiment (Polur et al.,

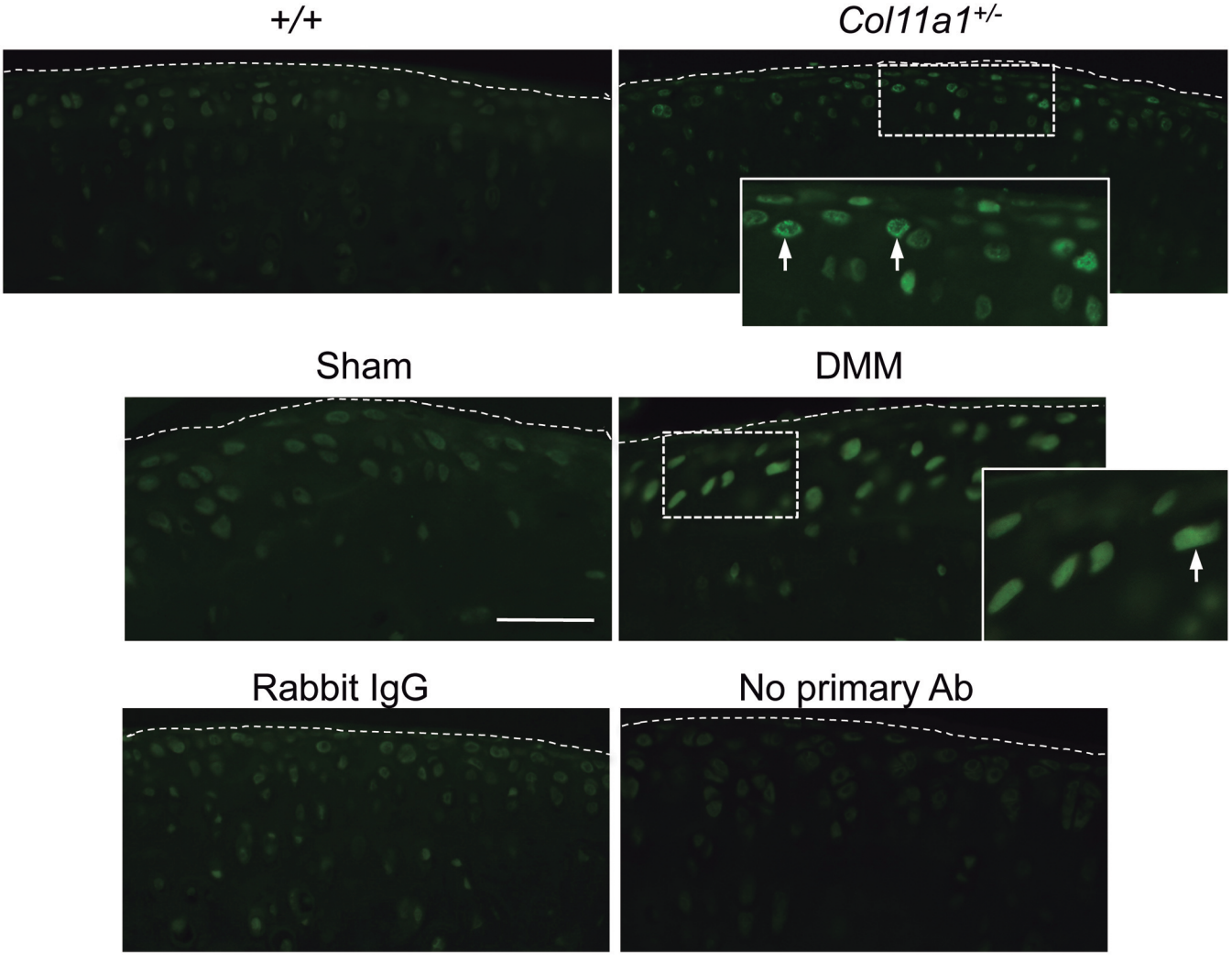

Fig. 2. Protein expression of Tgf-ß1 in articular cartilages of mouse knee joints. Knee joints from *Col11a1*$^{+/-}$ mice and DMM mice were used for examination of the protein expression of Tgf-ß1. The green-color cells (see the cell in the inset) in *Col11a1*$^{+/-}$ mice and DMM mice indicate positive staining for Tgf-ß1. The positive staining of the cells was predominantly distributed in the superficial layer of the articular cartilage. The positive staining cells were seen in all of *Col11a1*$^{+/-}$ mice and DMM mice. The location of the increased expression of Tgf-ß1 was randomly throughout the superficial layer of the entire articular cartilages. In contrast, there were hardly the positive staining of cells for Tgf-ß1 in wild-type mice (as control for *Col11a1*$^{+/-}$ mice) and sham surgery mice (as control for DMM surgery mice). We noticed the weak immunofluorescent signal in the wild-type and sham mice. However, based on the immunostaining in two negative controls, the weak signals resulted from the background effect of the immunostaining. Bar: 100 μm.

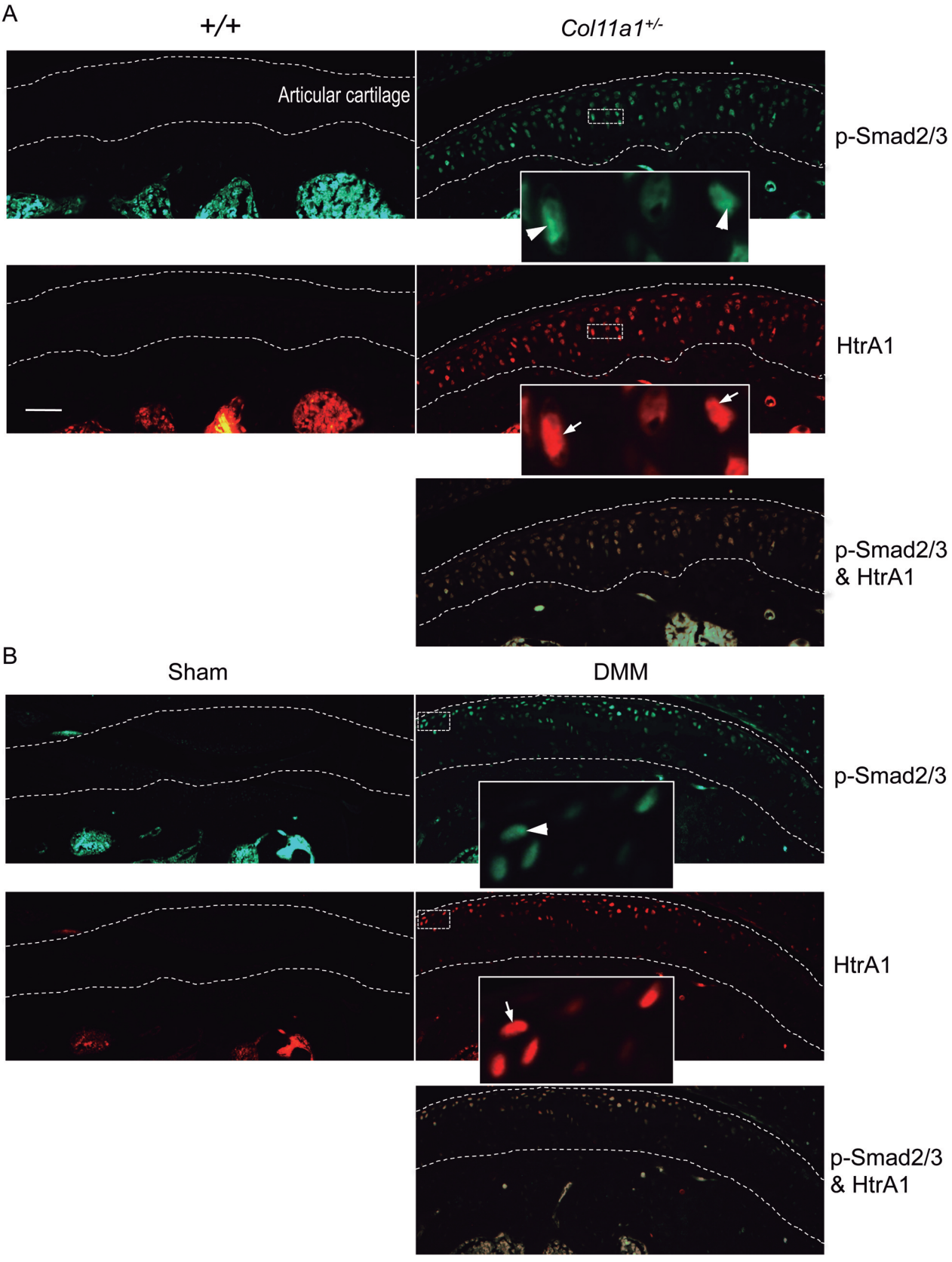

Fig. 3. Co-localization expression of p-Smad2/3 and HtrA1 in the articular cartilage of mouse knee joints. The green-stained cells indicate positive staining for p-Smad2/3 and the red-stained cells indicate positive staining for HtrA1. A comparison of the top and bottom pictures in the panels A or B shows that the same cells stain positively for both p-Smad2/3 and HtrA1, in the articular cartilage of knee joints of *Col11a1*$^{+/-}$ mice (**A**) and DMM mice (**B**). Bar: 100 μm.

2010). Interestingly, we found that the increased expression of p-Smad2/3 was co-localized in the same cells with the elevated expression of HtrA1 in the knee joints of the mouse models of OA (Fig. 3). It is worth mentioning that in our previous study, we found that the increased expression of HtrA1 was associated with the disappearance of the type VI collagen (Polur et al., 2010). Since type VI collagen is exclusively present in the pericellular matrix of articular chondrocytes in knee joints, the lack of type VI collagen is an indicator for the disruption of the pericellular matrix.

Regarding the protein expression of p-Smad1, we could hardly detect p-Smad1 in *Col11a1*$^{+/-}$ and DMM mice and their corresponding control mice (data not shown).

Inhibition TGF-ß1-induced expression of HTRA1 by an ALK5 inhibitor, SB431542, in human and mouse articular chondrocytes

In human chondrocyte culture, mRNAs were isolated from cells treated without TGF-ß1, with TGF-ß1 alone and with TGF-ß1 plus SB431542 for 18 hrs. The level of HTRA1 mRNA was increased (about 2.8-fold) in human chondrocytes treated with TGF-ß1, compared with that in human chondrocytes without TGF-ß1 (Fig. 4a). We also found that the level of HTRA1 mRNA was significantly reduced (about 85%) in cells treated with TGF-ß1 and SB431542, compared with that in cells treated with TGF-ß1 alone (Fig. 4a).

In *ex vivo* experiments, pieces of the mouse femoral articular cartilages were collected. There were several chondrons in each piece of the cartilage. After 18-hr treatment without TGF-ß1, with TGF-ß1 alone and with TGF-ß1 plus SB431542, mRNAs from the chondrocytes were isolated and measured by qPCR. We found that the level of HtrA1 mRNA was significantly increased (about 3.4-fold), in mouse chondrocytes treated with TGF-ß1 (Fig. 4b). We also found that the level of HtrA1 mRNA was significantly reduced (about 90%) in cells treated with TGF-ß1 and SB431542 (Fig. 4b). These observations were consistent with our results from the human culture experiments.

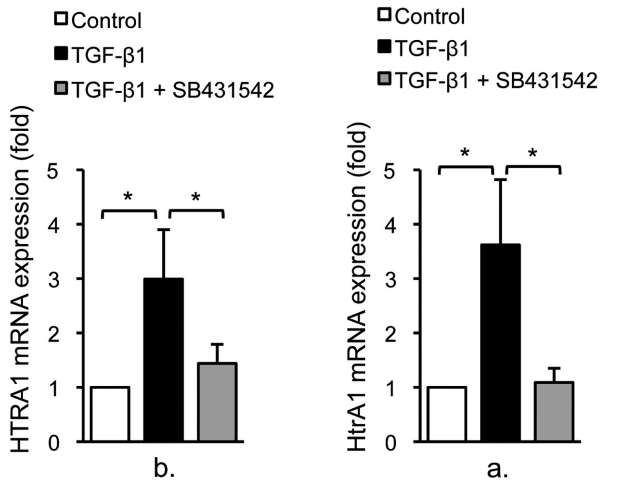

Fig. 4. The level of HTRA1 mRNA in articular chondrocytes treated with TGF-ß1 or/and SB431542. The level of HTRA1 mRNAs in articular chondrocytes was examined by real-time PCR. The level of HTRA1 mRNA was significantly increased about 2.8 times in human primary chondrocytes **(a)** and about 3.4 times in mouse articular chondrocytes **(b)** treated with TGF-ß1, compared with that in chondrocytes without the treatment of TGF-ß1. The level of HTRA1 mRNA was significantly decreased about 85% in human primary chondrocytes **(a)** and about 90% in mouse articular chondrocytes **(b)** treated with TGF-ß1 and SB431542, compared with that in chondrocytes with the treatment of TGF-ß1 alone.

Discussion

Roles of TGF-ß1 in the pathogenesis of OA

Although results from numerous studies suggest that TGF-ß1 may have a beneficial effect on the joint (protecting against OA), other studies suggest that TGF-ß1 may have a deleterious effect on joints (resulting in OA). On one hand, the increased expression of TGF-ß1 in adult knee joints causes early onset OA-like changes in animal models. Studies by Itayem et al. show that after injection of TGF-ß1 into the knee of the adult rats for three consecutive days, the number of chondrocytes is reduced and the morphology of chondrocytes becomes a star-like phenotype. They suggest that intra-articular injections of TGF-ß1 into knee joints may cause early onset of OA (Itayem et al., 1997, 1999). Another study by Bakker et al. demonstrates that constitutive expression of TGF-ß1 in synovium in adult mouse knee joints may cause hyperplasia in multiple joint tissues, including enhanced glycosaminoglycan content of articular cartilage, chondro-osteophyte formation and hyperplasia of synovium, which eventually leads to OA (Bakker et al., 2001). On the other hand, results from two studies (Serra et al., 1997; Yang et al., 2001) indicate that the genetic inactivation of *Smad-3* or the disruption of the interaction of TGF-ß1 with its receptor, Tgf-ß type II receptor (Tgfbr2), in germline cells results in juvenile OA-like knee joints in mice at one month old. In general, it takes about 6 to 8 weeks for mice to reach maturity. Interestingly, a recent human genetic study (van de Laar et al., 2011) reports that a nucleotide change, 859C>T or 782C>T, in *SMAD3* increases the level of TGF-ß1 and the activity of the TGF-ß1 signaling pathway in two human families associated with early-onset OA. They also report that a two-nucleotide deletion, 741-742del AT (nonsense mutation), in *SMAD3* causes early-onset OA in a human family. The observation in the human families is consistent with the result from the animal models, indicating that the increased Tgf-ß1 signaling causes OA in knee joints and that the lack of Tgf-ß1 signaling in the germline cell results in OA. Two plausible explanations for this "conflicting" role of TGF-ß1 in the pathogenesis of OA

are: 1) Effective TGF-ß1 signaling acts in a dose-dependent manner. In this scenario, an appropriate level of TGF-ß1 is required for the development and maintenance of articular cartilages. Therefore, TGF-ß1 below or above this level results in articular cartilage degeneration. The end result is OA. 2) Effective TGF-ß1 signaling acts in a developmental stage-dependent manner. In this scenario, TGF-ß1 is required for the development of articular cartilage. However, once a joint is formed, TGF-ß1 is not needed anymore. Therefore, induction of TGF-ß1 in an adult joint causes articular cartilage degeneration.

Increased Tgf-ß1 canonical signaling pathway associated with early articular cartilage degeneration in knee joints of adult mice

In this present study, we found that the protein presence of Tgf-ß1 was hardly detected in the articular cartilage of knee joints in adult wild-type mice. Clearly, the level of Tgf-ß1 protein, if any, is quite low in the articular cartilage of knee joints in adult mice. However, the protein expression of Tgf-ß1 and p-Smad2/3 were dramatically increased in the articular cartilage of knee joints associated with early articular cartilage degeneration in our mouse models of OA. We believe that this increased expression is a result of the chondrocytes in articular cartilage detecting excessive mechanical stimulation. In the case of *Col11a1*$^{+/-}$ mice, chondrocytes residing in the defective extracellular matrix are not protected properly. Thus they detect excessive mechanical stimulation even under normal mechanical joint loading. In the case of DMM mice, although chondrocytes reside in a normal extracellular matrix, the lateral movement of the medial meniscus by the surgery creates the incongruence between joint surfaces. Under this condition, the joint surfaces distribute normal mechanical loading unevenly on the joints. Thus, chondrocytes in a small area can detect excessive mechanical stimulation. The excessive mechanical stimulation causes induction of TGF-ß1 in chondrocytes (Lee et al., 2005) and cells of other joint tissues (Maeda et al., 2011).

Induction of HTRA1 by TGF-ß1 canonical signaling pathway in chondrocytes

One of the findings in this study was that the protein expression of TGF-ß1 and its signaling activity were not only elevated, but also appeared at an early stage of articular cartilage degeneration in adult mouse knee joints. We also found that the increased expression of p-Smad2/3 was co-localized in the same cells expressing HtrA1. This suggests that the activation of Tgf-ß1 signaling may result in induction of HtrA1 in articular cartilage of the knee joints. Results from our *in vitro* and *ex vivo* experiments provide direct evidence that TGF-ß1 could indeed induce HTRA1 in chondrocytes via p-SMAD2/3.

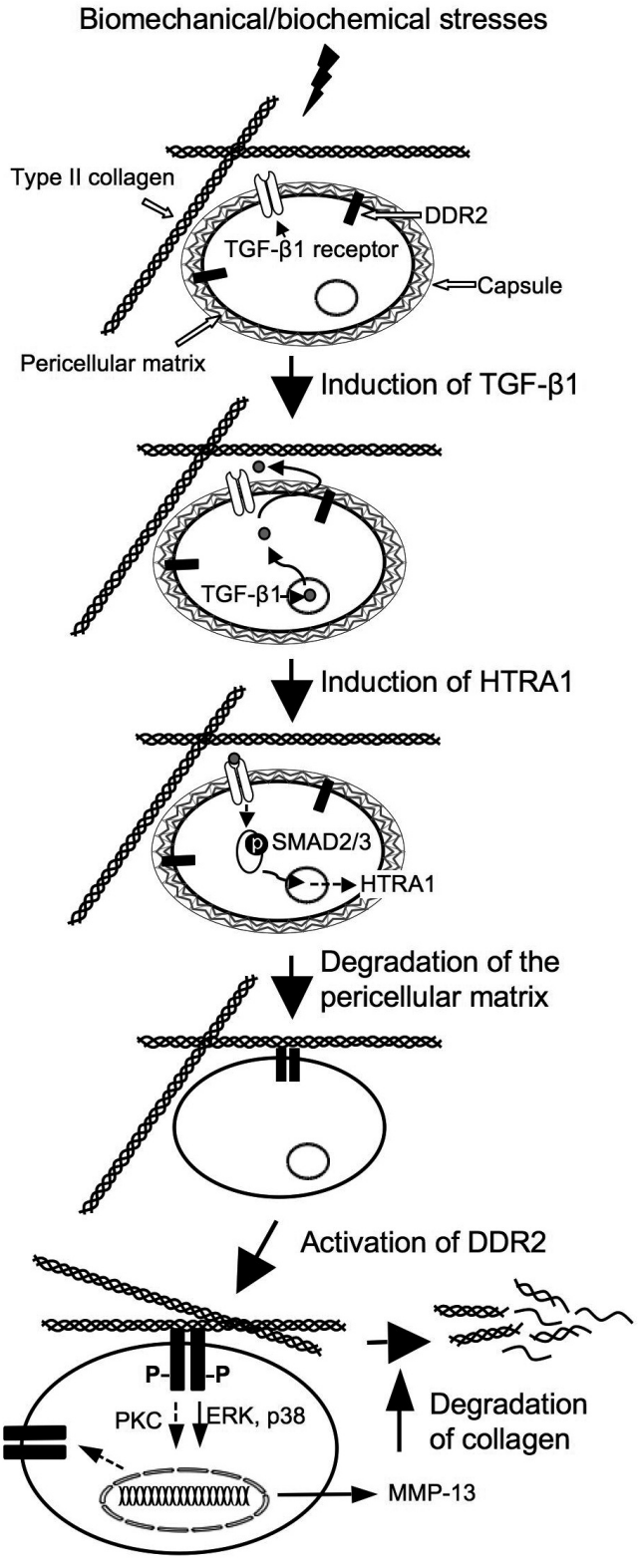

Fig. 5. A schematic illustration of a molecular pathway underlying articular cartilage degeneration.

We also noticed that HTRA1 may be able to inhibit signaling of various TGF-ß proteins, such as bone morphogenetic protein 4 (BMP4), TGF-ß1 and TGF-ß2, and growth differentiation factor 5 (GDF5) based on the structural and functional similarity of the IGFBP domain of HTRA1 with follistatin, a potent antagonist of activin (a TGF-ß family protein) (Zumbrunn and Trueb, 1996). This suggests that there may be a negative feedback loop between TGF-ß1 and HTRA1, which regulates the activity and expression of these two molecules in chondrocytes.

A plausible mechanistic explanation of the high level of TGF-ß1 resulting in OA

Based on the results from our studies and others' investigations (Poole et al., 1988; Hunziker et al., 1997; Poole, 1997; Xu et al., 2005, 2007, 2011; Klatt et al., 2009; Vonk et al., 2011), we propose a molecular chain of events underlying articular cartilage degeneration (Fig. 5).

Excessive mechanical stresses, either by normal mechanical loading of defective joints such as in the case of *Col11a1*$^{+/-}$ mice or overloading of normal joints in the case of the DMM mouse model of OA, can stimulate chondrocytes or cells in other joint tissues to synthesize TGF-ß1. TGF-ß1 is then activated and binds to its cognate receptor, TGFßRII. This, in turn, activates TGF-ß1 signaling via SMAD2/3 and induces expression of HTRA1. Since the pericellular components such as laminin, fibronectin, biglycan, decrin, fibromodulin, matrilin 3, and cartilage oligo matrix protein are the substrates of HTRA1, this enzyme can degrade the pericellular matrix. In normal articular cartilage, the pericellular matrix separates chondrocytes from adjacent interterritorial or territorial matrix containing type II collagen fibers. Thus, the disappearance of the pericellular matrix exposes chondrocytes to type II collagen. The enhanced exposure of chondrocytes to type II collagen activates a cell membrane receptor tyrosine kinase for native type II collagen, discoidin domain receptor 2 (DDR2). The activation of DDR2 then induces MMP-13, which leads to destruction of the type II collagen-proteoglycans network. The end result is OA. One legitimate question, from an evolutionary standpoint, is: if this molecular pathway leads to irreversible articular cartilage degradation, why do we still retain it? One explanation may be that this molecular pathway is needed for proper tissue turnover during development.

It is worth mentioning that the result from a recent study by an independent research group support our hypothesis of the HTRA1-DDR2-MMP-13 as a degradative pathway underlying articular cartilage degeneration (Holt et al., 2012).

In summary, the result from this present study suggests that TGF-ß1 may be able to induce HTRA1 in articular chondrocytes in mature joints and that increased protein expression and activity of TGF-ß1 may be detrimental to an adult joint. Therefore, inhibition activity of TGF-ß1, not application of TGF-ß1, should be considered in the prevention and treatment of OA in adult joints.

Acknowledgements. This study was supported by grants from the National Institutes of Health, NIAMS, in the United States, R01 AR051989 and RC4 AR060546 (LX and LY).

References

Alexopoulos L.G., Youn I., Bonaldo P. and Guilak F. (2009). Developmental and osteoarthritic changes in Col6a1-knockout mice. Arthritis Rheum. 60, 771-779.

Bakker A.C., van de Loo F.A., van Beuningen H.M., Sime P., van Lent P.L., van der Kraan P.M., Richards C.D. and van den Berg W.B. (2001). Overexpression of active TGF-beta-1 in the murine knee joint: evidence for synovial-layer-dependent chondro-osteophyte formation. Osteoarth. Cartil. 9, 128-136.

Borochowitz Z.U., Scheffer D., Adir V., Dagoneau N., Munnich A. and Cormier-Daire V. (2004). Spondylo-epi-metaphyseal dysplasia (SEMD) matrilin 3 type: homozygote matrilin 3 mutation in a novel form of SEMD. J. Med. Genet. 41, 366-372.

Clausen T., Southan C. and Ehrmann M. (2002). The HtrA family of proteases: implications for protein composition and cell fate. Molecular Cell. 10, 443-455.

Grau S., Richards P.J., Kerr B., Hughes C., Caterson B., Williams A.S., Junker U., Jones S.A., Clausen T. and Ehrmann M. (2006). The role of human HtrA1 in arthritic disease. J. Biol. Chem. 281, 6124-6129.

Hecht J.T., Nelson L.D., Crowder E., Wang Y., Elder F.F., Harrison W.R., Francomano C.A., Prange C.K., Lennon G.G. and Deere M. (1995). Mutations in exon 17B of cartilage oligomeric matrix protein (COMP) cause pseudoachondroplasia. Nat. Genet. 10, 325-329.

Holt D.W., Henderson M.L., Stockdale C.E., Farrell J.T., Kooyman D.L., Bridgewater L.C. and Seegmiller R.E. (2012). Osteoarthritis-like changes in the heterozygous sedc mouse associated with the HtrA1-Ddr2-Mmp-13 degradative pathway: a new model of osteoarthritis. Osteoarth. Cartil. 20, 430-439.

Hunziker E.B., Michel M. and Studer D. (1997). Ultrastructure of adult human articular cartilage matrix after cryotechnical processing. Microsc. Res. Tech. 37, 271-284.

Itayem R., Mengarelli-Widholml S. and Reinholt F. (1999). The lone-term effect of a short course of transforming growth factor,1 on rat articular cartilage. APMIS 107, 183-192.

Itayem R., Mengarelli-Widholm S., Hulth A. and Reinholt F. (1997). Ultrastructural studies on the effect of transforming growth factor-,1 on rat articular cartilage. APMIS 105, 221-228.

Kawamura I., Maeda S., Imamura K., Setoguchi T., Yokouchi M., Ishidou Y. and Komiya S. (2012). SnoN suppresses maturation of chondrocytes by mediating signal cross-talk between transforming growth factor-, and bone morphogenetic protein pathways. J. Biol. Chem. 287, 29101-29113.

Klatt A.R., Zech D., Kühn G., Paul-Klausch B., Klinger G., Renno J.H., Schmidt J., Malchau G. and Wielckens K. (2009). Discoidin domain receptor 2 mediates the collagen II-dependent release of interleukin-6 in primary human chondrocytes. J. Pathol. 218, 241-247.

Lee J.H., Fitzgerald J.B., Dimicco M.A. and Grodzinsky A.J. (2005). Mechanical injury of cartilage explants causes specific time-

dependent changes in chondrocyte gene expression. Arthritis Rheum. 52, 2386-2395.

Li Y., Lacerda D.L., Warman M.L., Beier D.R., Yoshioka H., Ninomiya Y., Oxford JT., Morris N.P., Andrikopoulos K., Ramirez F., Wardell B.B., Lifferth G.D., Teuscher C., Woodward S.R., Taylor B.A., Seegmiller R.E. and Olsen B.R. (1995). A fibrillar collagen gene, Col11a1, is essential for skeletal morphogenesis. Cell 80, 423-430.

Li Y., Li Y., Xu L., Servais J., Frank E., Lazarev A., Olsen B.R., Grodzinsky A.J. and Medici D. (2011). Mechanical loading-induced TGF-ß1 mediates cartilage degradation caused by upregulation of HTRA1/DDR2. Trans. Orthop. Research Soc. Long Beach, CA.

Maeda T., Sakabe T., Sunaga A., Sakai K., Rivera A.L., Keene D.R., Sasaki T., Stavnezer E., Lannotti J., Schweitzer R., Ilic D., Baskaran H. and Sakai T. (2011). Conversion of mechanical force into TGF-ß-mediated biochemical signals. Curr. Biol. 21, 933-941.

Oka C., Tsujimoto R., Kajikawa M., Koshiba-Takeuchi K., Ina J., Yano M., Tsuchiya A., Ueta Y., Soma A., Kanda H., Matsumoto M. and Kawaichi M. (2004). HtrA1 serine protease inhibits signaling mediated by Tgfbeta family proteins. Development 131, 1041-1053.

Polur I., Lee P.L., Servais J.M., Xu L. and Li Y. (2010). Role of HTRA1, a serine protease, in the progression of articular cartilage degeneration. Histol. Histopathol. 25, 599-608.

Poole C.A. (1997). Articular cartilage chondrons: form, function and failure. J. Anat. 191, 1-13.

Poole C.A., Flint M.H. and Beaumont B.W. (1988). Chondrons extracted from canine tibial cartilage: preliminary report on their isolation and structure. J. Orthop. Res. 6, 408-419.

Poole C.A., Matsuoka A. and Schofield J.R. (1991). Chondrons from articular cartilage. III. Morphologic changes in the cellular microenvironment of chondrons isolated from osteoarthritic cartilage. Arthritis Rheum. 34, 22-35.

Schlaak J.F., Pfers I., Meyer Zum Büschenfelde K.H. and Märker-Hermann E. (1996). Different cytokine profiles in the synovial fluid of patients with osteoarthritis, rheumatoid arthritis and seronegative spondylarthropathies. Clin. Exp. Rheumatol. 14, 155-162.

Serra R., Johnson M., Filvaroff E.H., LaBorde J., Sheehan D.M., Derynck R. and Moses H.L. (1997). Expression of a truncated, kinase-defective TGF-beta type II receptor in mouse skeletal tissue promotes terminal chondrocyte differentiation and osteoarthritis. J. Cell Biol. 139, 541-552.

Tsuchiya A., Yano M., Tocharus J., Kojima H., Fukumoto M., Kawaichi M. and Oka C. (2005). Expression of mouse HtrA1 serine protease in normal bone and cartilage and its upregulation in joint cartilage damaged by experimental arthritis. Bone 37, 323-336.

Urano T., Narusawa K., Kobayashi S., Shiraki M., Horie-Inoue K., Sasaki N., Hosoi T., Ouchi Y., Nakamura T. and Inoue S. (2010). Association of HTRA1 promoter polymorphism with spinal disc degeneration in Japanese women. J. Bone Miner. Metab. 28, 220-326.

van der Weyden L., Wei L., Luo J., Yang X., Birk D.E., Adams D.J., Bradley A. and Chen Q. (2006). Functional knockout of the matrilin-3 gene causes premature chondrocyte maturation to hypertrophy and increases bone mineral density and osteoarthritis. Am. J. Pathol. 169, 515-527.

van de Laar I.M., Oldenburg R.A., Pals G., Roos-Hesselink J.W., de Graaf B.M., Verhagen J.M., Hoedemaekers Y.M., Willemsen R., Severijnen L.A., Venselaar H., Vriend G., Pattynama P.M., Collée M., Majoor-Krakauer D., Poldermans D., Frohn-Mulder I.M., Micha D., Timmermans J., Hilhorst-Hofstee Y., Bierma-Zeinstra S.M., Willems P.J., Kros J.M., Oei E.H., Oostra B.A., Wessels M.W. and Bertoli-Avella A.M. (2011). Mutations in SMAD3 cause a syndromic form of aortic aneurysms and dissections with early-onset osteoarthritis. Nat. Genet. 43, 121-126.

Vonk L.A., Doulabi B.Z., Huang C., Helder M.N., Everts V. and Bank R.A. (2011). Collagen-induced expression of collagenase-3 by primary chondrocytes is mediated by integrin α1 and discoidin domain receptor 2: a protein kinase C-dependent pathway. Rheumatology. (Oxford). 50, 463-472.

Wadhwa S., Embree M., Ameye L. and Young M.F. (2005). Mice deficient in biglycan and fibromodulin as a model for temporomandibular joint osteoarthritis. Cells Tissues Organs. 181, 136-143.

Wu J., Liu W., Bemis A., Wang E., Qiu Y., Morris E.A., Flannery C.R. and Yang Z. (2007). Comparative proteomic characterization of articular cartilage tissue from normal donors and patients with osteoarthritis. Arthritis Rheum. 56, 3675-3684.

Xu L., Flahiff C.M., Waldman B.A., Wu D., Olsen B.R., Setton L.A. and Li Y. (2003). Osteoarthritis-like changes and decreased mechanical function of articular cartilage in the joints of chondrodysplasia (cho) mice. Arthritis Rheum. 48, 2509-2518.

Xu L., Peng H., Wu D., Hu K., Goldring M.B., Olsen B.R. and Li Y. (2005). Activation of the discoidin domain receptor 2 induces expression of matrix metalloproteinase 13 associated with osteoarthritis in cho/+ mice. J. Biol.Chem. 280, 548-555.

Xu L., Peng H., Glosson S., Lee P.L., Hu K., Ijiri K., Olsen B.R., Goldring M.B. and Li Y. (2007). Increased expression of a collagen receptor discoidin domain receptor 2 in articular cartilage as a key event in the pathogenesis of osteoarthritis. Arthritis Rheum. 56, 2663-2673.

Xu L., Servais J., Polur I., Kim D., Lee PL., Chung K. and Li Y. (2010). Attenuation of osteoarthritis progression by reduction of the discoidin domain receptor 2 in mice. Arthritis Rheum. 62, 2736-2744.

Xu L., Polur I., Servais J.M., Hsieh S., Lee P.L., Goldring M.B. and Li Y. (2011). Intact pericellular matrix of articular cartilage is required for un-activated discoidin domain receptor 2 in the mouse model. Am. J. Pathol. 179, 1338-1346.

Yang X., Chen L., Xu X., Li C., Huang C. and Deng C.X. (2001). TGF-beta/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. J. Cell Biol. 153, 35-46.

Zumbrunn J. and Trueb B. (1996). Primary structure of a putative serine protease specific for IGF-binding proteins. FEBS Lett. 398, 187-192.

Accepted October 17, 2013