



Attenuation of the Progression of Articular Cartilage Degeneration by Inhibition of Tgf- β 1 Signaling in a Mouse Model of Osteoarthritis

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**Attenuation of the progression of articular cartilage degeneration by
inhibition of Tgf- β 1 signaling in a mouse model of osteoarthritis**

(Delay in the development of OA by inhibition of Tgf- β 1)

A Thesis Presented by

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to

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Abstract

Background

The goal of this study is to understand role of transforming growth factor beta 1 (TGF- β 1) in development of osteoarthritis (OA). Results from studies indicate that the genetic inactivation of Smad-3, or the disruption of the interaction of Tgf- β 1 with its receptor Tgf- β type II receptor (Tgfbr2), in germline cells results in OA-like knee joints in mice at one month of age. However, other studies suggest that the increased expression of Tgf- β 1 in mature knee joints causes OA in animal models. A human genetic study reports that a two-nucleotide deletion, 741-742del AT, and/or a nucleotide change, 859C>T or 782C>T in SMAD-3 are associated with early-onset OA. This observation is consistent with the finding that the lack of Tgf- β 1 signaling in the germline cell results in OA in developing joints and that increased Tgf- β 1 signaling causes OA in mature joints. The plausible explanation for this “conflicting” role of TGF- β 1 in the pathogenesis of OA is that the effective TGF- β 1 signaling acts in either a dose-dependent or a developmental stage-dependent manner. The present study addresses the question as to whether inhibition of Tgf- β 1 signaling prevents mature knee joints from being degenerated in mouse models of OA.

Methods

- 1) Using conditional knock out techniques with aggrecan-CreERt2 mice and floxed *Tgfbr2* mice, *Tgfbr2* was removed from articular cartilage of knee joints in 2-month-old mice. Mice without *Tgfbr2* were kept for another 6 months or longer. Knee joints from the mice (n=8) and their corresponding control (n=4) were collected for morphological analysis.
- 2) Mice without *Tgfbr2* at two months old were subjected to DMM to induce articular cartilage degeneration. Knee joints from the mice at 4 and 8 weeks post surgery (n=8 in each group) were collected for morphological analysis.

Results

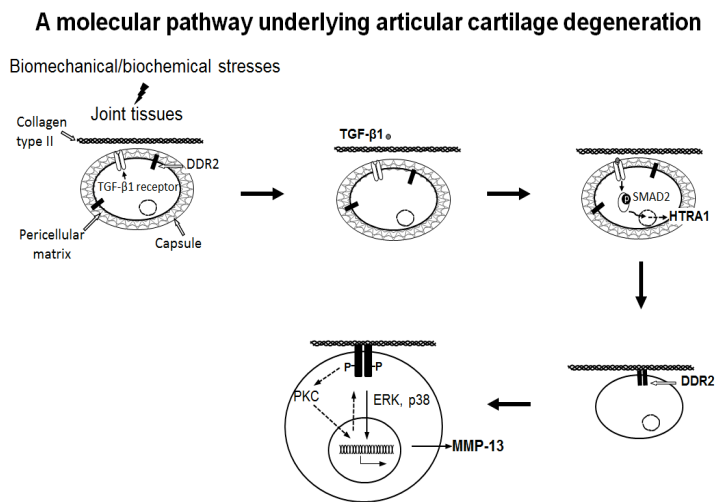
- 1) We did not find the initiation and acceleration of articular cartilage degeneration by the genetic inactivation of *Tgfbr2* in knee joints of mice at the age of 9 months or older. We also did not find hypertrophic chondrocytes in the articular cartilage of the mice.
- 2) We found that removal of *Tgfbr2* in articular cartilage of knee joints delayed articular cartilage degeneration, at least 6 weeks, compared to that in wild-type littermates.

Conclusion

Inhibition of Tgf- β 1 signaling attenuated articular cartilage degeneration in mature knee joints of mouse models of OA. Therefore, inhibition activity of TGF- β 1, not application of TGF- β 1, may be considered in treatment of OA in mature joints.

Introduction

Osteoarthritis (OA) is a progressive synovial joints disease, caused by the imbalance between the anabolic and catabolic activities of the articular chondrocytes and consequently leads to the degradation of the cartilage and failure in repairing of the damaged joint damage. (1) The cytokines and growth factors that involve the initiation are very complex, but the ultimate consequence of the breakdown of the articular cartilage in the development OA follows a consistent pathological pattern, regardless of the nature of the initiating factors for OA. Results from studies using animal and human models of OA tissues indicate that in the process of the articular cartilage degeneration, the earliest indication of change is chondrocyte clustering and a general up-regulation in synthetic activity of chondrocytes, including increased expression of extracellular matrix proteins and cartilage degrading enzymes. At later stages of the degenerative process, fibrocartilage and osteophytes are formed. The complexity of the initiating factors for OA suggests that there may be multiple initiating pathways towards a common target(s) underlying the pathogenesis of OA. Based on the discoveries from the recent investigations in our lab and other research groups, we are proposing a causal sequence of the molecular events that contributes the articular cartilage degeneration (3-6).



Role of MMP13 in the pathogenesis of OA: Expression of MMP13 is hardly detected in normal mature articular cartilages, but the activity and expression of the enzyme are increased in human OA cartilages and in mouse models of OA. MMP-13 is believed to play an important role in OA because of its ability of degrading both aggrecans and collagen type II (7, 8). The constitutive expression of Mmp-13 in mouse cartilage results in OA-like changes to the knee joints (9) and removal of this enzyme prevents articular cartilage erosion in a joint instability mouse model of OA (10). Results from another study demonstrated that articular cartilage degradation is completely irreversible after induction of MMP-mediated degradations of aggrecan and collagen type II (11). If the degradation of collagen type II is irreversible, then, in order to intervene in the progression of OA, the inhibition of the MMP-13 activity and expression becomes crucial. Results from our recent studies suggest that DDR2 may be a “stimulant” in chondrocytes.

Role of DDR2 in the induction of MMP13 in chondrocytes: Synthesis of chondrocytes and releasing of matrix-degrading enzymes can be incited by altered mechanical forces, and consequently lead to the degradation of the pericellular matrix of chondrocytes, and enhances the exposure of the chondrocytes to native collagen type II. Interaction of the chondrocytes with native collagen type II fibrils results in up-regulated activity and expression of DDR2 and induction of MMP-13. MMP-13 functions as cleaving collagen type II and aggrecans (7, 8). The resulting fragments of collagen type II and aggrecans may further increase the synthesis of MMP-13 through interaction with other cell-surface receptors (12). This result is a positive feedback amplification loop that leads to the irreversible destruction of the articular cartilage. Experiments found that 1) reduced expression of DDR2 attenuated articular cartilage degeneration in the mouse models of OA; 2) the levels of MMP-13 and DDR2 mRNAs were elevated in human chondrocytes and mouse primary chondrocytes cultured on native collagen type II, indicating that native collagen type II induces expression of DDR2 and MMP-13 mRNAs; and 3) When human chondrocytes were cultured on denatured collagen type II (gelatin), the levels of MMP-13 and DDR2 mRNAs were not elevated, suggesting that the chondrocytes respond in a specific manner to triple-helical collagen type II. Based

on these results, we concluded that the activation of DDR2, through the interaction of the receptor with collagen type II, increases the expression of MMP-13 in chondrocytes, resulting in the destruction of articular cartilage(13-18).

Role of the high temperature requirement A1 (HTRA1) the pathogenesis of OA:

Chondrons are composed of chondrocytes, a pericellular matrix, and a capsule surrounding the pericellular matrix. The pericellular matrix separates chondrocytes from the adjacent interterritorial or territorial matrices containing collagen type II, and also prevents newly synthesized collagen type II from binding to DDR2 during the developmental stage or in mature articular cartilage. Results from human and mouse genetic studies indicate that maintaining the integrity of the pericellular matrix is one of the key elements in protecting against OA. The pericellular matrix of chondrocytes is disrupted in human OA cartilages. It is obvious that enzymes that are capable of degrading the pericellular molecules will play an important role in the pathogenesis of OA, and HTRA1 may be one such enzyme (19, 20). The expression of HTRA1 is predominantly increased in human OA cartilages and in the articular cartilages of joints from animal models of OA. More importantly, HTRA1 can degrade the pericellular components such as decorin, biglycan, COMP, fibronectin, and aggrecan. It is also found that pericellular collagen type VI was not present in chondrocytes expressing HTRA1 in mouse OA joints, which is indicative of the disruption of the pericellular matrix of chondrocytes. Also, those cells without surrounding collagen type VI were associated with an increased expression of Ddr2, which suggests that HTRA1 may contribute to the development of OA through the degradation of the pericellular network, resulting in activation of DDR2 (21, 22).

Role of transforming growth factor beta 1 (TGF- β 1) in the pathogenesis of OA:

TGF- β 1 has been considered an anabolic factor to articular chondrocytes, based largely on results from *in vitro* and *ex vivo* experiments in which TGF- β 1 can stimulate chondrocytes to synthesize and release extracellular matrix molecules, including proteoglycans and type II collagens (23, 24). In addition, results from two studies indicate that the genetic inactivation of *Smad-3* or disruption of the interaction of Tgf- β 1 with its receptor, Tgf- β type II receptor (Tgfbr2), in germline cells causes OA-like knee joints in mice (25, 26).

Moreover, a human genetic study reports that a two-nucleotide deletion, 741-742del AT (nonsense mutation), in SMAD-3 causes early-onset OA in a human family (27). This is consistent with the results from the animal models, indicating that the lack of Tgf- β 1 signaling in the germline cell results in OA. However, observations from other studies also suggest that the increased TGF- β 1 signaling may initiate and accelerate articular cartilage degeneration in mature joints.

First, the enhanced production of extracellular matrix molecules, due to an increase in the synthetic activity of chondrocytes, is not necessarily beneficial or physiological in maintenance of the homeostasis of mature articular cartilage. For instance, one of the earliest pathological signs in articular cartilage degeneration is the overproduction of proteoglycans in mouse models of OA (5,6). A study by Van den Berg et. al. reports that the constitutive overexpression of active TGF- β 1 in adult mouse knee joints results in OA associated with increase in the production of proteoglycans in articular cartilage, hyperplasia of synovium and chondro-osteophyte formation (28). Thus, the overproduction of proteoglycans in articular cartilage could be a pathologic response of the chondrocyte in the early stages of articular cartilage degeneration. This raises an interesting question: does TGF- β 1 disrupt homeostasis of articular cartilage instead of repairing the damaged cartilage in mature joints? Second, the human genetic study reports that a nucleotide change, 859C>T or 782C>T in SMAD-3, increases the level of TGF- β 1 and activity of the TGF- β 1 signaling pathway in two human families associated with early-onset OA (27). This is in agreement with the observation from two other studies indicating that the level of TGF- β 1 is significantly higher in human osteoarthritic tissues than in healthy articular cartilages (29, 30). Studies with animal models, by Itayem et al., suggest that intra-articular injections of TGF- β 1 into adult rat knee joints may cause early onset of OA (31, 32). Third, to confirm the *in vitro* observation that TGF- β 1 induced HTRA1 in human chondrocytes, Dr. Li investigated whether Tgf- β 1 induced HtrA1 in articular chondrocytes of two mouse models of OA (33). He found increases in the expression of Tgf- β 1, p-Smad2/3 and HtrA1 in articular chondrocytes of knee joints in the mouse models and increased expressions of p-Smad2/3 and HtrA1 were co-localized in the chondrocyte. In addition, TGF- β 1-induced expression of HTRA1 was

inhibited by an ALK-5 inhibitor, SB431542, in human and mouse chondrocytes. This suggests that Tgf- β 1 canonical signaling may be activated to induce Htra1 in articular chondrocytes of the mouse models of OA. More importantly, results from another independent research group demonstrate that TGF- β 1 induces HTRA1 in human primary chondrocytes (34).

Data from aforementioned investigations raise the question as to what the exact role of TGF- β 1 is in the development of OA. Our explanations for this “conflicting” role of TGF- β 1 in the pathogenesis of OA are: 1) Effective TGF- β 1 signaling acts in a dose-dependent manner. In this scenario, an appropriate level of TGF- β 1 is required for the development and maintenance of articular cartilages. Therefore, TGF- β 1 below or above this level results in articular cartilage degeneration. 2) Effective TGF- β 1 signaling acts in a developmental stage-dependent manner. In this scenario, TGF- β 1 is required for the development of articular cartilage; however, once a joint is formed, TGF- β 1 is no longer needed. Therefore, induction of TGF- β 1 in an adult joint causes articular cartilage degeneration.

Hypothesis

The deficiency of TGF- β 1 may have chondroprotective effect on articular cartilage degeneration in mature joints.

Specific Aim

- 1) To investigate **biological effect** and **biochemical** changes of Tgfr2 deficiency on articular cartilage of mature joints;
- 2) To investigate **chondron-protective** effect and **biochemical** changes of Tgfr2 deficiency on mature knee joints against OA.

Materials and Methods

The efficiency of *Tgfb2* removal in *AgcCreER^{+/-};Tgfb2^{f/f}* mice. Three of *AgcCreER^{+/-};Tgfb2^{f/f}* and 3 of *AgcCreER^{+/-};Tgfb2^{+/+}* mice at the age of 2 months old were injected intraperitoneally with 4OH-Tamoxifen at 1mg/10g body weight/daily for 5 consecutive days. The mice were then sacrificed and articular cartilages were collected for isolation of the genomic DNAs. SYBR Green real-time PCR was performed on iCycler (Bio-Rad). For an internal positive control, primers that target cartilage oligomeric matrix protein were used: forward 5'-ACCCACAACAGGCACATT -3" and reverse 5'- TCAGTCATAGGAAGCAGG-3' to generate a 142 bp PCR product. For the detection of *Tgfb2* gene, the primers for the exon 2 of *Tgfb2* were used: forward 5'- AACAGTGATGTCATGGCCAG -3" and reverse 5'- CAGACTTCATGCGGCTTCTC -3' to generate a 155 bp PCR product. PCR was performed using 25 µl of 1X PCR buffer containing each primer at 200 nM and 0.5 µl of genomic DNA. Real-time PCR reaction was carried out at 95°C for 3 minutes followed by 50 cycles of 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. Each sample was tested in triplicate.

Histological examination of mouse knee and temporomandibular (TM) joints by Safranin O/Fast green. At age of 2 months old, eight mice from *AgcCreERT2^{+/-};Tgfb2^{f/f}* mice were injected intraperitoneally with 4OH-tamoxifen at 1mg/10g body weight/daily for 5 consecutive days and other eight *AgcCreERT2^{+/-};Tgfb2^{f/f}* mice were injected intraperitoneally with sunflower seed oil for 5 consecutive days. The mice were maintained under a 12-hour lighting schedule. At the age of 6 months old, four mice from each group were sacrificed for the collection of knee and temporomandibular (TM) joints. The rest of the mice were kept alive to the age of 12 months old and sacrificed for the collection of knee and TM joints. Four wild-type littermates at the age of 6 and 12 months were also sacrificed for the collection of knee and TM joints. For knee joints, the samples were fixed in 4% paraformaldehyde for 6 hours at room temperature and processed for paraffin embedding. For TM joints, mouse heads were cut along the midsagittal plane. The right half of the mouse heads were fixed and embedded. The samples were then sectioned by serial sectioning at a 6 µm-thickness from lateral to medial direction for the knee joints and in an anterior-posterior direction for the TM joints. Every tenth section was collected for Safranin O/Fast green staining.

Immunohistostaining of type X collagen. Eight paraffin sections, distributed throughout each joint, from mice at the age of 12 months (n=4) were selected for immunohistostaining. Paraffin

sections from the growth plate of tibia of C57/BL mice at the age of one month old were used as positive control. The sections were incubated with a rabbit polyclonal antibody (1:200) against type X collagen (Cat. No. ab58632, Abcam, www.abcam.com). After overnight incubation at 4°C, the sections were washed and subsequently treated with a biotinylated secondary antibody. Color development was performed using a peroxidase substrate (VECTOR NovaRED Substrate, Cat. No. SK-4800, Laboratories, Burlingame, CA) following treatment of the sections with a mixture of avidin and biotinylated horseradish peroxidase (VECTASTAIN ABC Kit, Cat. No. PK-4000, Vector Laboratories, Burlingame, CA). Sections were counterstained with 0.2% Fast Green solution. Staining without primary antibody were performed as negative controls.

Inducible expression of CreERT2 in the articular cartilage of condyles of mandibles in adult mouse TM joints. *Agc1tm(IRES-CreERT2)* mice were bred with *Rosa26* Cre reporter (*R26R*) mice. Mice containing both *Agc1tm(IRES-CreERT2)* and *R26R* reporter were identified by PCR. A pair of PCR primers for *Agc1tm(IRES-CreERT2)* was: forward 5'- -3' and reverse 5'- -3'. A pair of PCR primers for *R26R* reporter was: forward 5'- -3' and reverse 5'- -3'. The mice and their corresponding controls were maintained under a 12-hour lighting schedule. At the age of 6 months old, the mice were injected intraperitoneally with 4OH-tamoxifen at 1mg/10g body weight/daily for 5 consecutive days. The mice were sacrificed for the collection of mandibles of TM joints. The mandibles were then stained with X-gal.

Destabilization of the medial meniscus (DMM). Two groups of mice (n=8), *AgcCreERT2^{+/-};Tgfb2^{f/f}*, *AgcCreERT2^{+/-};Tgfb2^{+/+}*, at the age of 12 weeks were generated, see the histological examination section above. The mice were subjected either to DMM or Sham surgery. Briefly, after the mice 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. The medial meniscotibial ligament was sectioned. The joint capsule was then, closed with a continuous 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 12-hour lighting schedule for further experiments. Sham surgery in which the ligament was visualized but not transected in mice was performed as a negative control.

Evaluation of articular cartilage conditions by a scoring system. The pathologic condition of the joints was evaluated by a scoring system designed to assess the histology of OA in mouse joints; the system is recommended by the OARSI histopathology initiative (11). The score 0 is

for normal mouse articular cartilage; 0.5 for Loss of Safranin-O without structural changes; 1 for small fibrillations without loss of cartilage; 2 for vertical clefts down to the layer immediately below the superficial layer and some loss of surface lamina; 3 for vertical clefts/erosion to the calcified cartilage extending to <25% of the articular surface; 4 for vertical clefts/erosion to the calcified cartilage extending to 25 to 50% of the articular surface; 5 for vertical clefts/erosion to the calcified cartilage extending to 50 to 75% of the articular surface; and 6, the maximal score, for vertical clefts/erosion to the calcified cartilage extending >75% of the articular surface.

Statistic analysis. There were 9 to 10 paraffin sections from each knee joint. The score from the section of the worst condition was selected to represent the joint. Thus, six to eight scores were obtained for each experimental group. An average score for each experimental group was then calculated from the scores.

The t-test with a significance level of 0.05 was used to determine whether a significant difference between any two average scores was present. With regard to the determination of sample size in this study, a pilot study and power analysis on the effect of the Tgfbr2-deficiency was performed. From the results, it was concluded that a sample size 6 is required to achieve the specified confidence interval with at least 50% reduction of the score in the treatment group and with a 95% confidence level.

Immunohistostaining for p-Smad2/3. Paraffin sections of randomly selected 4 animals from each experimental group were used for immunohistostaining for protein expression of p-Smad2/3. Sections were de-paraffinized and quenched for endogenous peroxidase activity. The sections were incubated with a rabbit polyclonal antibody (1:500) against p-Smad2/3 (Cat. No. 3101, Cell Signaling Technology, www.cellsignal.com). After overnight incubation at 4 °C, sections were washed and subsequently treated with a biotinylated secondary antibody (goat anti-rabbit IgG-B). Coloring was developed with the use of a peroxidase substrate (VECTOR NovaRED Substrate, Cat. No. SK-4800, Laboratories, Burlingame, CA) following treatment of the sections with a mixture of avidin and biotinylated horseradish peroxidase (VECTASTAIN ABC Kit, Cat.No. PK-4000, Vector Laboratories, Burlingame, CA). Sections were counterstained with 0.2% Fast Green solution. Staining without the primary antibody was also performed as negative controls.

Results

No morphological effect of a genetic inactivation of *Tgfb2* in the articular cartilage of adult mouse joints.

First, we used a specific mouse strain, Cre-recombinase and the modified estrogen receptor (CreERT2) driven by the aggrecan promoter, *Agc1tm(IRES-CreERT2)*. The result from a previous study demonstrates that CreERT2 is highly inducible in the articular chondrocytes of adult knee joints in *Agc1tm(IRES-CreERT2)* mice (16). In our experiment, we examined the efficiency of the ablation of *Tgfb2* by *Agc1tm(IRES-CreERT2)* in the articular chondrocyte of adult mouse knee joints. After several rounds of crossing *Agc1tm(IRES-CreERT2)* mice with floxed *Tgfb2* mice, we obtained compound mutant mice, heterozygous CreERT2 driven by the aggrecans promoter and homozygous floxed *Tgfb2* (*AgcCreERT2^{+/-};Tgfb2^{f/f}*). We found that the exon 2 genomic DNA of *Tgfb2* was not detected in 86% articular chondrocytes of adult mouse knee joints, figure 1. The loss of the exon 2 resulted in a pre-mature stop codon immediately right after the exon 1 of *Tgfb2*. This result indicated that *Tgfb2* was deleted in the majority of articular chondrocytes of adult knee joints in *AgcCreERT2^{+/-};Tgfb2^{f/f}* mice.

Second, we examined morphologic conditions of the articular cartilage of knee joints from *AgcCreERT2^{+/-};Tgfb2^{+/+}*, *AgcCreERT2^{+/-};Tgfb2^{f/f}* mice and their wild-type littermates at the ages of 6 and 12 months old. Knee joints from four mice in each group were used for histology analysis. We found that there was no morphological differences between *AgcCreERT2^{+/-};Tgfb2^{+/+}* and the wild-type littermates, indicating no morphological effect by the insertion of the *CreERT2* in the mouse genome. This is consistent with the observation by another independent investigation (16). We also did not find the morphological differences between *AgcCreERT2^{+/-};Tgfb2^{f/f}* and *AgcCreERT2^{+/-};Tgfb2^{+/+}* mice, figure 2. This indicated that the removal of *Tgfb2* from the articular cartilage did not cause any morphological changes in knee joints of mice after mature (adult mice).

Third, data from in vivo experiments indicate that inhibition activity of TGF- β 1 signaling results in the pre-mature hypertrophy of articular chondrocytes in mouse joints during early development (before mouse mature at the age of 6 to 8 weeks). Type X collagen is one of the markers for chondrocyte hypertrophy. We examined the protein expression of type X collagen in the articular cartilage of knee joints of mice at 12 months old. In these mice, *Tgfb2* had been removed from the articular cartilages at the age of 2 months. The mice continued to live for 10

more months. However, we did not detect the protein expression of type X collagen in the articular cartilage of knee joints in these mice, figure 3.

Forth, although a previous study reports that the expression of CreERT2 is inducible in the articular chondrocyte of adult knee joints in *Agc1tm(IRES-CreERT2)* mice, it is unknown whether CreERT2 is also inducible in the articular cartilage of adult temporomandibular (TM) joints. By crossing *Agc1tm(IRES-CreERT2)* mice with Rosa-26 reporter mice, we found that CreERT2 was highly expressed in the articular cartilage of TM joints in mice at the age of 6 months old, figure 4. This result indicated that *Agc1tm(IRES-CreERT2)* mouse strain could also be used to remove a gene of interest in the articular cartilage of adult TM joints.

Fifth, we examined morphology of TM joints from *AgcCreERT2^{+/-};Tgfr2^{+/+}*, *AgcCreERT2^{+/-};Tgfr2^{f/f}* mice and their wild-type littermates at the ages of 6 and 12 months old. We found that there were no morphological differences in TM joints among the mice, figure 5. This result was consistent with our observation in adult knee joints, in which the removal of *Tgfr2* from the adult articular cartilage did not cause any overt morphological changes in the joints.

Chondro-protective effect of the genetic inactivation of *Tgfr2* on the articular cartilage of adult mouse knee joints against the development of OA.

First, we determined whether the removal of *Tgfr2* from the articular cartilage of adult knee joints could attenuate the progression of the cartilage degeneration. We performed DMM surgery on the knee joints of adult *AgcCreERT2^{+/-};Tgfr2^{f/f}* mice and their corresponding controls. We found significant disparities in the progressive process of the articular cartilage degeneration in knee joints between *AgcCreERT2^{+/-};Tgfr2^{f/f}* and *AgcCreERT2^{+/-};Tgfr2^{+/+}* mice at 8 and 16 weeks following DMM surgery, figure 6. The progression towards OA was dramatically delayed in the *AgcCreERT2^{+/-};Tgfr2^{f/f}* mice after the surgery. There were no abnormal morphological changes in the sham surgery groups.

The condition of the articular cartilage was also evaluated with a modified Mankin scoring system, table 1. Mice at 8 weeks following sham surgery were used as a normal control (score=0). At 8 weeks following DMM surgery, the average score for *AgcCreERT2^{+/-};Tgfr2^{f/f}* mice and *AgcCreERT2^{+/-};Tgfr2^{+/+}* littermates were 0.9 and 2.2, respectively. At 16 weeks following surgery, the average scores were 2.3 for *AgcCreERT2^{+/-};Tgfr2^{f/f}* mice and 4.7 for

AgcCreERT2^{+/-};Tgfbr2^{+/+} littermates. The scores were significantly differences between two groups at both time points.

Second, we examined the protein expression of p-Smad2/3 in the articular cartilage of knee joints from mice at 8 weeks after DMM. The protein of p-Smad2/3 was hardly detected in the articular cartilage of knee joints from all of the articular cartilage *Tgfbr2*-deficient mice. However, the protein of p-Smad2/3 was present in the articular cartilage of knee joints from the all of the wild-type littermates. The location of the p-Smad2/3 was localized and randomly scatted, figure 7.

Discussion

TGF- β 1 in the development of articular cartilage

Results from three independent research groups indicate that Tgf- β 1 is required for the development of articular cartilage of knee joints in mice. They find that Tgf- β 1/Smad2/3 may play a significant role in the control of chondrocyte hypertrophic differentiation in articular cartilage. The complete removal of Smad3 in mouse germline cells results in a high number of hypertrophic chondrocytes in the basal layer of articular cartilage of knee joints in mice at 1 month old. The deficiency of Smad3 may enhance bone morphogenetic protein (BMP) signaling and deregulate p38, which leads to chondrocyte hypertrophy. In addition, the lack of the Tgf- β 1/Smad2/3 signaling may activate Runx2-inducible expression of Mmp-13, which leads to the degeneration of articular cartilage. Clearly, TGF- β 1 is one of the critical factors in the development of articular cartilage. However, there is no information suggesting that this also is the case in adult (mature) articular cartilage.

TGF- β 1 in the maintenance of articular cartilage

Is TGF- β 1 one of the key molecules in the maintenance of articular cartilage in adult joints? As of today, one study reports that the protein of Tgf- β 3 is present in the adult articular cartilage of mouse knee joints. Results from another study show the presence of Alk1 and Alk5 in the adult articular cartilage of mouse knee joints. As mice age, the ratio of Alk1/Alk5 is increased. This suggests that the presence of Alk1 with a dramatic decrease in the protein expression of Alk5 in adult articular cartilage causes the induction of cartilage-degrading enzymes, such as MMP-13, in the aged cartilage. However, a question remains as to which comes first, articular cartilage degeneration along with aging or the increased ratio of Alk1/Alk5 in mouse adult articular cartilage?

To understand whether or not Tgf- β 1 plays any role in the maintenance of mature articular cartilage, we utilized conditional knockout approach to remove *Tgfbr2* in the articular cartilage of mice at the age of 8 weeks old. Usually it takes about 6 to 8 weeks for laboratory mice to be completely mature. The mice were kept up to 12 months old. There were no any overt morphological changes observed in the articular cartilage of knee and TM joints of the

Tgfb2-deficient mice. The protein expression of type X collagen, one of the molecular markers for chondrocyte hypertrophy, in the articular cartilage of knee joints of the *Tgfb2*-deficient mice were also examined. There was no presence of type X collagen in the cartilage detected. This suggests that the removal of *Tgfb2* from the mature articular cartilage may not have any effect on chondrocyte hypertrophic differentiation. In addition, there was no presence of Tgf- β 1 detected in mature articular cartilage of mouse knee joints. The aforementioned results from this investigation suggest that Tgf- β 1 may have, if any, an insignificant role in the maintenance of mature articular cartilages.

TGF- β 1 in the degenerative process of adult articular cartilage

Results from numerous independent studies and data from our previous investigation demonstrate that the protein expression of TGF- β 1 is dramatically increased in human osteoarthritic cartilages and in the articular cartilage of mouse models of OA. Since data from in vitro experiments indicate that TGF- β 1 can stimulate chondrocytes to synthesize extracellular matrix molecules, such as type II collagen and proteoglycans, it has been suggested that the increased expression of TGF- β 1 may be partly a counter to articular cartilage degeneration. However, other investigations find that the up-regulated expression of TGF- β 1 causes articular cartilage degeneration. First, mature articular cartilage is a relatively quiescent tissue. A study by Verzijl, et al indicates that the half-life of type II collagen in humans is 117 years (35). The long half-life of type II collagen indicates a slow turnover of the collagen in mature articular cartilages. This also suggests that it may not be needed for chondrocytes to continually produce type II collagen in mature articular cartilage. Thus, the TGF- β 1-stimulated overproduction of type II collagen or/and proteoglycans in mature articular cartilage, in fact, may disrupt the homeostasis of the cartilage, which eventually leads to cartilage degeneration. Second, an independent research group finds that TGF- β 1 induces a serine protease, high temperature requirement A1 (HTRA1) in human chondrocytes (34). Results from our in vitro experiments confirmed this observation (33). Furthermore, we found increases in the expression of Tgf- β 1, p-Smad2/3 and HtrA1 in articular chondrocytes of knee joints in mouse models of OA and increased expressions of p-Smad2/3 and HtrA1 were co-localized in the chondrocyte. In addition, TGF- β 1-induced expression of HTRA1 was inhibited by an ALK-5 inhibitor, SB431542,

in human and mouse chondrocytes. This suggests that Tgf- β 1 canonical signaling is activated to induce HtrA1 in articular chondrocytes of the mouse models of OA.

A consensus of opinions in the OA research field is that mechanical stress is the primary initial insult to articular cartilage during the development of OA. Induction of TGF- β 1 is one of the responses to mechanical stress in chondrocytes. Our studies demonstrated that normal mechanical loading of defective joints or an overloading of normal joints could stimulate chondrocytes to synthesize and release Tgf- β 1 in mouse models of OA (15). Based upon the data from our investigations and others' studies, we propose a molecular pathway (see the following flow chart) underlying articular cartilage degeneration as follows: Excessive mechanical stresses can stimulate chondrocytes and other joint tissues to synthesize and release TGF- β 1. The active TGF- β 1 binds to its cognate receptor, TGFBR2, which induces expression of HTRA1 in chondrocytes. Consequences of induction of HTRA1 are degradation of the pericellular matrix and enhanced exposure of chondrocytes to type II collagen. Interaction of chondrocytes with type II collagen results in enhanced signaling through a cell surface receptor tyrosine kinase, discoidin domain receptor 2 (DDR2), for native type II collagen. This induces the expression of MMP-13 as well as expression of DDR2 itself. MMP-13 degradation of type II collagen and aggrecan results in type II collagen and aggrecan fragments, which in turn may activate signals that further increase the synthesis of MMP-13. The end result is a feedback amplification loop that causes irreversible articular cartilage degeneration.

Conclusions

In summary, we believe that TGF- β 1 is a pathogenic factor in the development of OA in adult joints. Therefore, inhibition of activity of TGF- β 1, not application of TGF- β 1, should be considered in the prevention and treatment of OA in the joints.

Table.

(Table 1.) The recommended semi-quantitative scoring system: Grade Osteoarthritic damage

- 0: Normal
- 0.5: Loss of Safranin-O without structural changes
- 1: Small fibrillations without loss of cartilage
- 2: Vertical clefts down to the layer immediately below the superficial layer and some loss of surface lamina
- 3: Vertical clefts/erosion to the calcified cartilage extending to <25% of the articular surface
- 4: Vertical clefts/erosion to the calcified cartilage extending to 25-50% of the articular surface
- 5: Vertical clefts/erosion to the calcified cartilage extending to 50-75% of the articular surface
- 6: Vertical clefts/erosion to the calcified cartilage extending >75% of the articular surface

Figure legends.

Figure 1. The percentage of the Tgfb2-chondrocytes in knee joints of mice

Chondrocytes in wild-type littermates contains Tgfb2, 100%. However, there were about less than 14% of chondrocytes with Tgfb2 in the conditionally Tgfb2 knockout mice. There were significant differences in the number of the Tgfb2-cells between two groups, $p < 0.01$ (t-test).

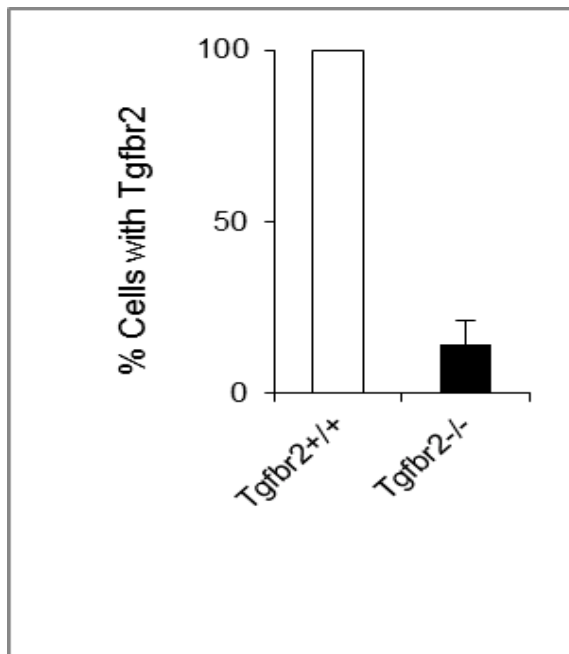


Figure 2. Morphology of articular cartilages in mouse knee joints

There were no overt morphological changes seen in the articular cartilages of knee joints in the *Tgfr2*-deficient mice at the ages of 6 and 12 months.

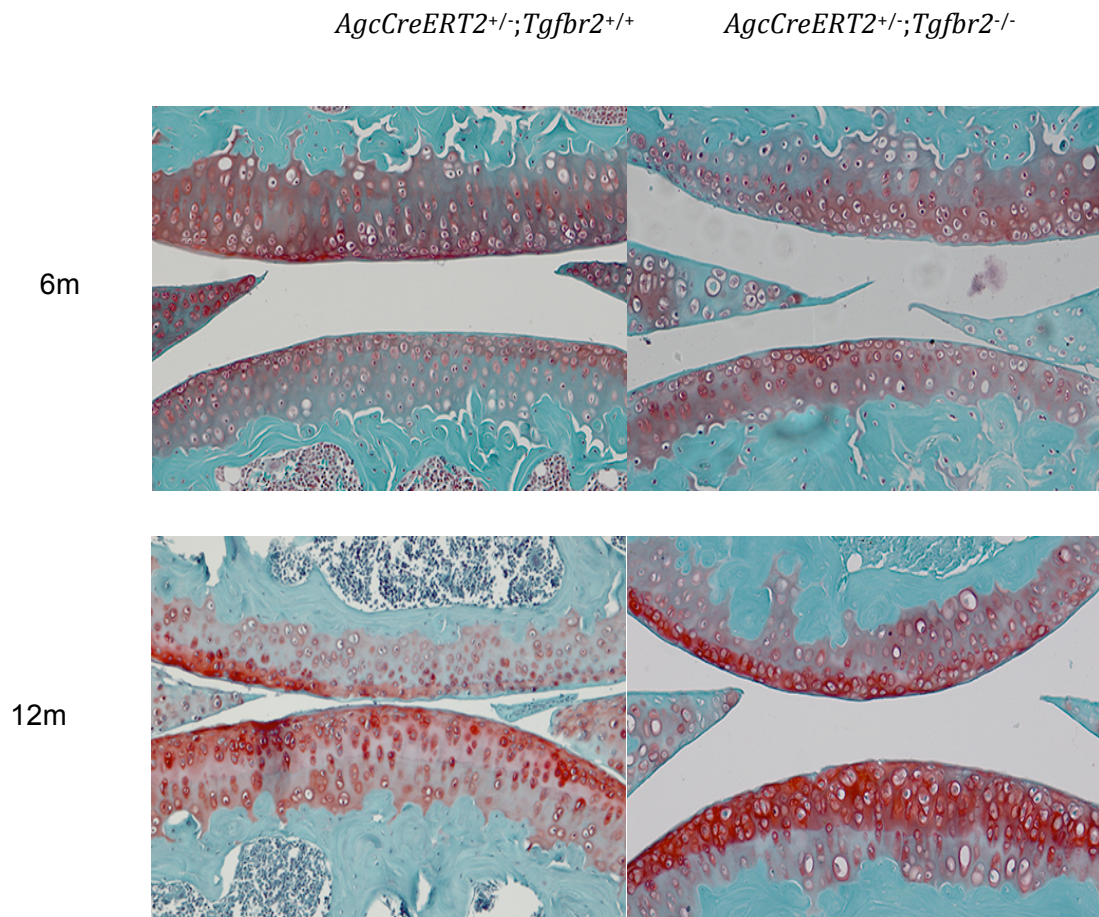


Figure 3. Immunohistostaining for collagen type X

Collagen type X was present in the hypertrophic chondrocytes of growth plates in mice at the age of one month old. There were no positive staining of collagen type X in the articular cartilage of both *tgfr2*-deficient mice and their wild-type littermates at the age of two months old.

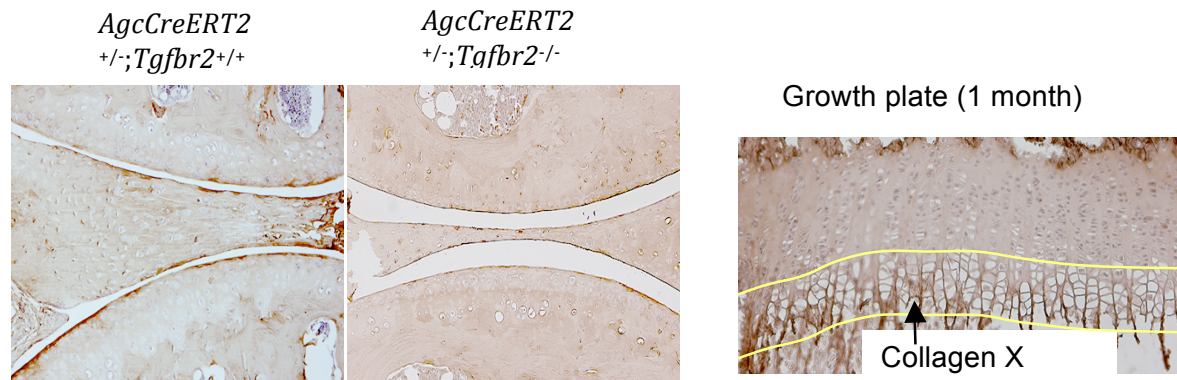


Figure 4. Lac-Z staining in the articular cartilage of temporomandibular (TM) joints in mice

There was an intensive Lac-Z staining in the articular cartilage of TM joints of *AgcCreERT2/+; Rosa26^{floxlacZ/+}* mice at the age of 1 and 6 months.

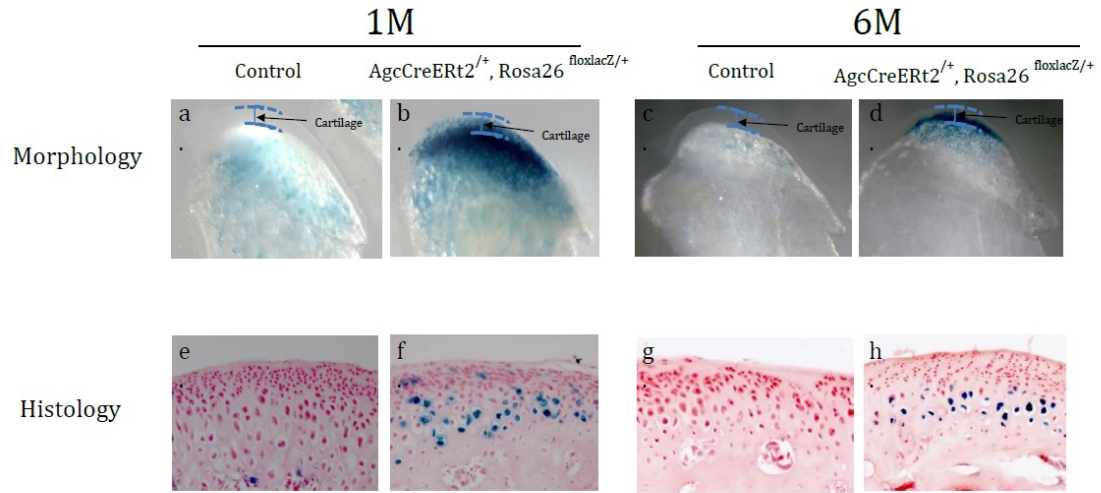


Figure 5. Morphology of articular cartilages in mouse TM joints

There were no overt morphological changes seen in the articular cartilages of TM joints in the *Tgfr2*-deficient mice at the ages of 6 and 12 months.

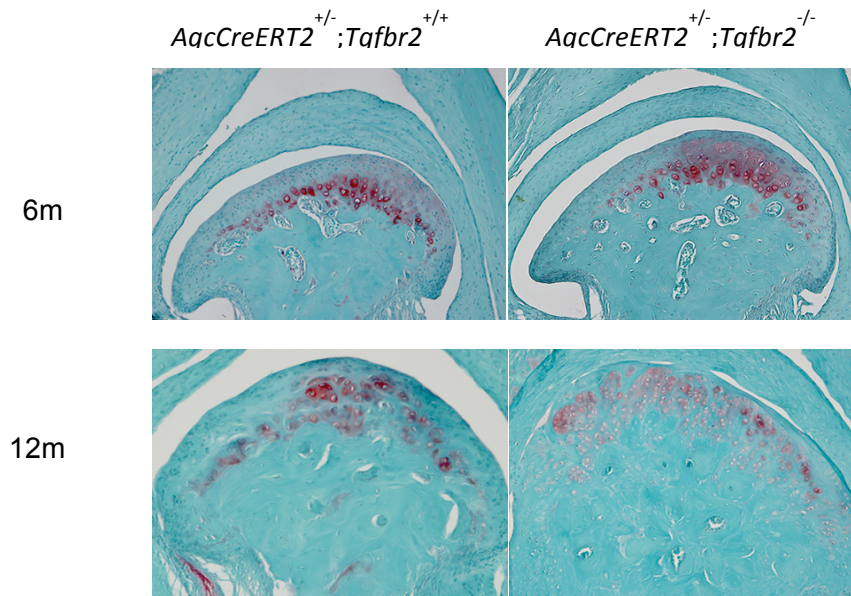


Figure 6. Morphology of the articular cartilage of knee joints from the *Tgfr2*-deficient mice following DMM surgery

Fibrillation was seen (the arrow) in the articular cartilage of the wild-type littermates at 8 weeks following the surgery. At 16 weeks following the surgery, a complete loss of articular cartilage was evident in the cartilage. A delay of the degenerative process was seen in the *Tgfr2*-deficient mice following surgery, which was no fibrillation observed in the *Tgfr2*-deficient mice at the 8 weeks. But the fibrillation appeared in the *Tgfr2*-deficient mice at 16 weeks (Bar=100 μ m).

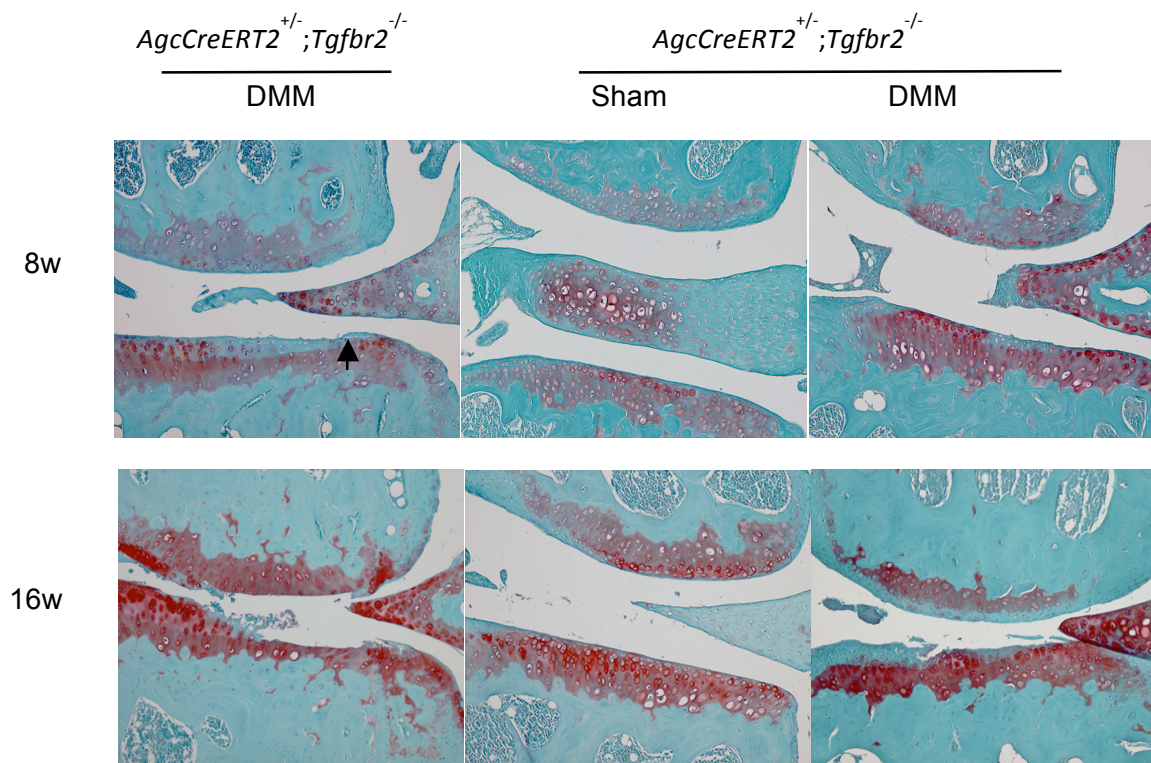
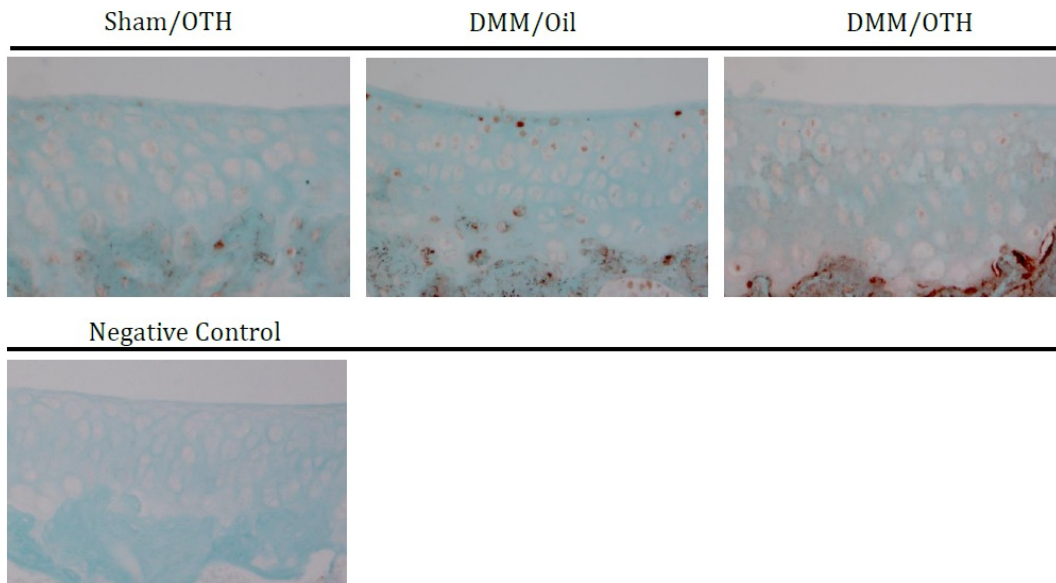


Figure 7. Immunohistostaining of p-Smad2/3 in the articular cartilage of the Tgfr2-deficient mice

There were Tgfr2-positive staining cells in the articular cartilage of mice after DMM surgery. However, the Tgfr2-positive staining cells were hardly detected in the Tgfr2-deficient mice and in sham control mice. The similar positive staining background appeared in the bone marrow of all mice indicated that the paraffin sections were treated under a consistent immunostaining condition.



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