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Deletion of the Transforming Growth Factor β Receptor Type II Gene in Articular Chondrocytes Leads to a Progressive Osteoarthritis-like Phenotype in Mice

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Abstract

Objective—While transforming growth factor β (TGFβ) signaling plays a critical role in chondrocyte metabolism, the TGFβ signaling pathways and target genes involved in cartilage homeostasis and the development of osteoarthritis (OA) remain unclear. Using an in vitro cell culture method and an in vivo mouse genetic approach, we undertook this study to investigate TGFβ signaling in chondrocytes and to determine whether *Mmp13* and *Adamts5* are critical downstream target genes of TGFβ signaling.

Methods—TGFβ receptor type II (TGFβRII)–conditional knockout (KO) (TGFβRII^{Col2ER}) mice were generated by breeding TGFβRII$^{flox/flox}$ mice with *Col2-CreER*–transgenic mice. Histologic, histomorphometric, and gene expression analyses were performed. In vitro TGFβ signaling studies were performed using chondro-genic rat chondrosarcoma cells. To determine whether *Mmp13* and *Adamts5* are critical downstream target genes of TGFβ signaling, TGFβRII/matrix metalloproteinase 13 (MMP-13)– and TGFβRII/ADAMTS-5–double-KO mice were generated and analyzed.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Chen had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Im, O'Keefe, Chen.

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Results—Inhibition of TGFβ signaling (deletion of the *Tgfbr2* gene in chondrocytes) resulted in up-regulation of *Runx2*, *Mmp13*, and *Adamts5* expression in articular cartilage tissue and progressive OA development in TGFβRII^{Col2ER} mice. Deletion of the *Mmp13* or *Adamts5* gene significantly ameliorated the OA-like phenotype induced by the loss of TGFβ signaling. Treatment of TGFβRII^{Col2ER} mice with an MMP-13 inhibitor also slowed OA progression.

Conclusion—*Mmp13* and *Adamts5* are critical downstream target genes involved in the TGFβ signaling pathway during the development of OA.

Osteoarthritis (OA) is the most common degenerative joint disease, affecting >25% of the US population age >18 years. The major pathologic changes of OA include abnormal articular chondrocyte maturation, progressive loss and destruction of articular cartilage, osteophyte formation, and subchondral sclerosis. The etiology of OA is multifactorial, including joint injury, obesity, aging, and heredity (1–3). There are currently no interventions to restore degraded cartilage or decelerate the progression of OA, as the precise signaling pathways involved in initiation and progression of OA are still poorly understood.

Ex vivo studies with tissues obtained from OA patients and in vivo studies with mutant mouse models suggest that the factors involved in development of OA include growth factors, such as transforming growth factor β (TGFβ) and Indian hedgehog (IHH), and signaling molecules, such as Smads, β-catenin, and hypoxia-inducible factor 2α (HIF-2α) (4–8). TGFβ signaling strongly inhibits chondrocyte hypertrophy and maturation. In canonical TGFβ signaling, the TGFβ ligand binds to TGFβ receptor type II (TGFβRII), which then phosphorylates the type I transmembrane serine/threonine kinase receptor. The activated kinase subsequently phosphorylates Smad2 or Smad3 (receptor-activated Smad), which then forms a heteromeric complex with Smad4 (common-mediator Smad), translocates into the nucleus, and interacts with other DNA binding proteins to regulate target gene transcription (9).

Recent genetic manipulations suggest that dys-regulation of TGFβ signaling induces development of OA via the TGFβ/Smad3 signaling pathway in chondrocytes (4,10). Transgenic mice overexpressing a dominant-negative form of TGFβRII (dn-TGFβRII) in skeletal tissue were found to exhibit cartilage disorganization and progressive cartilage degradation resembling the features of OA in humans (10). Similar to the dn-TGFβRII–transgenic mice, *Smad3*-knockout (KO) mice were also found to display progressive articular cartilage degradation and osteophyte formation (4). A recent study showed that a single-nucleotide polymorphism in the *Smad3* gene was correlated with the incidence of hip and knee OA in a cohort of 527 patients (11). More recently, different types of *Smad3* mutations were identified in patients with a syndromic form of aortic aneurysms and early-onset OA (12,13). These observations strongly support the notion that the TGFβ/Smad3 signaling pathway in chondrocytes plays an essential role in the development of OA. However, the critical downstream target genes of TGFβ signaling involved in the development of OA remain unknown.

The progressive loss of articular cartilage is a fundamental feature of OA. Articular cartilage consists of a dense meshwork of interconnected collagen fibrils within which is embedded a rich matrix of negatively charged proteoglycans. The negative charge attracts ions and provides the tissue with a high osmotic pressure that resists compressive force and provides boundary lubrication (14). Human clinical and animal studies have shown that matrix metalloproteinase 13 (MMP-13) plays a pivotal role during cartilage degradation. MMP-13 is a primary collagenase that preferentially cleaves type II collagen in articular cartilage (15). Clinical investigations have revealed that MMP-13 expression is elevated in articular cartilage of OA patients (16). Expression of the constitutively active *Mmp13* gene leads to

an OA-like phenotype in mice (17). In addition to MMP-13, ADAMTS-5, the principal enzyme responsible for degradation of aggrecan in articular cartilage, plays a critical role during OA development. Studies have shown that expression levels of *Adamts5* are significantly increased during OA development (18). Deletion of the *Adamts5* gene prevented articular cartilage degradation in mouse models of surgically or chemically induced OA (19–21).

To determine the mechanism of inhibition of TGFβ signaling in the development of OA, we generated chondrocyte-specific $Tgfbr2^{Col2ER}$ mice by breeding *Col2-CreER*–transgenic mice (22,23) with $Tgfbr2^{flox/flox}$ mice. Gene deletion was induced by injection of tamox-ifen into 2-week-old mice, and alterations in the articular cartilage were analyzed at ages 3 and 6 months. Our studies demonstrated that deletion of the *Tgfbr2* gene at the postnatal/adult stage led to a severe OA-like phenotype. Our in vitro studies demonstrated that inhibition of TGFβ signaling up-regulated *Mmp13* and *Adamts5* expression in articular cartilage tissue. MMP-13 is a collagenase that mainly degrades type II collagen. ADAMTS-5 is an aggrecanase that degrades aggrecan. Type II collagen and aggrecan are the principal matrix components present in articular cartilage. Because both MMP-13 and ADAMTS-5 play critical roles in the development of OA (12,21,24), we reasoned that *Mmp13* and *Adamts5* might be the key downstream target genes of TGFβ signaling in articular chondrocytes during OA development. In this study, we demonstrated that deletion of the *Mmp13* or *Adamts5* gene in mice of the $Tgfbr2^{Col2ER}$ background significantly prevented the OA-like phenotype observed in $Tgfbr2^{Col2ER}$ mice, which suggests that MMP-13 and ADAMTS-5 are critical downstream targets of TGFβ signaling during OA development.

MATERIALS AND METHODS

Generation of $Tgfbr2^{Col2ER}$–conditional KO mice and *Tgfbr2/Mmp13*– and *Tgfbr2/Adamts5*–double-KO mice

Col2-CreER mice were generated in our laboratory (22,23). $Tgfbr2^{flox/flox}$ mice were obtained from the National Cancer Institute (25). To generate *Tgfbr2*–conditional KO mice, $Tgfbr2^{flox/flox}$ mice were crossed with *Col2-CreER*–transgenic mice. The resulting *Col2-CreER*/$Tgfbr2^{flox/flox}$ ($Tgfbr2^{Col2ER}$) mice were injected intraperitoneally (IP) with tamoxifen (1 mg/10 gm body weight/day, for 5 days) at age 2 weeks and were killed at ages 3 and 6 months for histologic analysis. The Cre-negative littermates were used as controls. The primer names and sequences used for mouse genotyping are available online at http://www.rushu.rush.edu/biochem. To generate double-KO mice, $Tgfbr2^{Col2ER}$ mice were crossed with $Mmp13^{flox/flox}$ or $Adamts5^{-/-}$ (26) mice (obtained from The Jackson Laboratory).

Cre recombination efficiency

To determine whether the *Col2-CreER* transgene targets floxed genes specifically in articular cartilage, *Col2-CreER*–transgenic mice were bred with *Rosa-tomato* (*mT/mG*) and *Rosa-lacZ* (*R26R*) reporter mice. Tamoxifen (1 mg/10 gm body weight/day, for 5 days) was injected IP into 2-week-old mice. The mice were killed at age 1 month. Histologic sections were analyzed using a fluorescence microscope and X-Gal staining.

Treatment with MMP-13 inhibitor

An MMP-13 inhibitor, CL82198 (Tocris), was used for the in vivo experiment. The inhibitory effect of CL82198 on MMP-13 enzymatic activity was confirmed using an MMP-13 fluorometric drug discovery kit (Enzo Life Sciences). Both TGFβRII^{Col2ER} mice and Cre-negative control mice were injected IP with tamoxifen (1 mg/10 gm body weight/day for 5 days) at age 2 weeks and then treated with the MMP-13 inhibitor (10 mg/kg body

weight, IP, every other day until the mice were killed) or vehicle (phosphate buffered saline). At age 3 months, the mice were killed and their knee joint tissues were harvested for histologic and histomorphometric analyses.

Histology and immunohistochemistry

Knee joint tissues from 3- and 6-month-old mice were fixed in 4% parafor-maldehyde, decalcified, dehydrated, and embedded in paraffin. Serial midsagittal sections (3-μm thick) were cut and stained with Alcian blue/hematoxylin and eosin for morphologic analysis. Immunohistochemistry was performed on the 3-μm–thick tissue sections using antibodies to TGFβRII (Santa Cruz Biotechnology) and type X collagen (Quartett).

Real-time quantitative polymerase chain reaction (PCR) analysis

Total RNA was extracted from rat chondro-sarcoma cells or mouse articular cartilage tissue using TRIzol according to the protocol of the manufacturer (Invitrogen). DNase I–treated total RNA was reverse-transcribed using oligo(dT), and complementary DNA (cDNA) was amplified by PCR in a total volume of 20 μl. The solution contained 10 μl SYBR Green Master Mix (Thermo Scientific), 1 μl of the diluted (1:5) cDNA, and 10 pmoles of forward and reverse primers (a list of genes specified by these primers is available at http://www.rushu.rush.edu/biochem).

Cell culture and transfection

Rat chondrosarcoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 atmosphere. RUNX-2 and cyclin-dependent kinase 4 (CDK-4) plasmids or *Tgfbr2*, *Runx2*, and *CDK4* small interfering RNAs (siRNAs) were transiently transfected into rat chondrosarcoma cells in 6-cm culture dishes using Lipofectamine 2000 (Invitrogen). Empty vector or scrambled siRNA was used as control. Western blotting and immunoprecipitation assays were performed 24 hours after transfection. The siRNAs *siTgfbr2* (catalog no. L-091837), *siRunx2* (catalog no. L-082676), and *siCDK4* (catalog no. L-082676) (on-target plus rat smart pool) were purchased from Thermo Fisher Scientific.

Luciferase and chromatin immunoprecipitation (ChIP) assays

Plasmid containing the 3.4-kb human *Mmp13* promoter was transfected into rat chondrosarcoma cells, and luciferase assay was performed to check the effect of TGFβ (2 ng/ml) on *Mmp13* transcriptional activity. ChIP assay was performed as previously described (27) to determine whether RUNX-2 specifically binds to the proximal promoter region of the *Mmp13* gene in rat chondrosarcoma cells. Three sets of primers were used in the ChIP assay (further information is available at http://www.rushu.rush.edu/biochem).

Western blot analysis and ubiquitination assay

Western blot analysis and ubiquitination assay were performed as described previously (28,29). For the RUNX-2 ubiquitination assay, the proteasome inhibitor MG132 (10 μM) was added to the cell culture 4 hours before cells were harvested. The rat anti–RUNX-2 monoclonal antibody was purchased from MBL. The rabbit anti–CDK-4 polyclonal antibody (C-22) was purchased from Santa Cruz Biotechnology. The UbiQapture kit was purchased from Enzo Life Sciences.

Statistical analysis

Results are presented as the mean ± SD. Statistical tests included Student's unpaired *t*-test and two-way analysis of variance followed by Tukey's test. *P* values less than 0.05 were considered significant.

RESULTS

Col2-CreER–targeted Cre recombination in articular chondrocytes

To specifically target articular chondrocytes at postnatal/adult stages, we generated *Col2-CreER*–transgenic mice (22,23). To evaluate the targeting efficiency and specificity of these mice, we bred them with *Rosa-tomato* (*mT*/*mG*) and *Rosa-lacZ* (*R26R*) reporter mice.

Analysis of histologic sections from 1-month-old mice using fluorescence microscopy or X-Gal staining showed efficient targeting (>85%) of growth plate and articular chondrocytes in transgenic mice following tamoxifen administration at age 2 weeks (Figures 1A–D). We then used these mice to generate TGFβRII^{Col2ER} mice by breeding *Col2-CreER* mice with TGFβRII$^{flox/flox}$ mice. Immunohistochemical analysis demonstrated that TGFβRII expression was undetectable in the majority of articular chondrocytes from 3-month-old TGFβRII^{Col2ER} mice (Figure 1E).

Postnatal deletion of the *Tgfbr2* gene induces an OA-like phenotype

To determine the role of TGFβ signaling in OA development in postnatal and adult mice, we generated TGFβRII^{Col2ER} mice. Mice were injected with tamoxifen at age 2 weeks. A severe and progressive OA-like phenotype was observed in 3- and 6-month-old TGFβRII^{Col2ER} mice. Histologic examination revealed tears and clefts in the articular cartilage surface, localized damage of articular cartilage tissues, increased numbers of hypertrophic chondrocytes localized at the surface of articular cartilage, early osteophyte formation, and increases in subchondral bone mass (especially at the upper ends of tibiae) in 3-month-old TGFβRII^{Col2ER} mice (Figures 2A and B). Histomorphometric analysis showed a significant reduction in articular cartilage area in 3-month-old TGFβRII^{Col2ER} mice (Figure 2C). We also analyzed the data using the histologic scoring system recommended by the Osteoarthritis Research Society International (OARSI) (30), and we found that TGFβRII^{Col2ER} mice had significantly higher scores for OA damage compared to Cre-negative control mice (Figure 2D). Loss of the entire articular cartilage, extensive formation of osteophytes, and substantially increased subchondral bone mass were observed in 6-month-old TGFβRII^{Col2ER} mice (Figure 2E).

To determine changes in gene expression in articular cartilage tissue during OA development, we extracted RNA from articular cartilage tissue of 1-month-old TGFβRII^{Col2ER} mice and found that expression of *Mmp13*, *Adamts5*, *Adamts4*, *Runx2*, *Alp*, *Oc*, and *Col10* increased significantly (Figure 2F), which suggested that the chondrocyte differentiation process was accelerated in TGFβRII^{Col2ER} mice. Consistent with these findings, increased type X collagen protein levels were also detected in the superficial zone of articular cartilage in 3-month-old TGFβRII^{Col2ER} mice (Figure 2G). In contrast, no significant changes were found in the growth plate cartilage of 3- and 6-month-old TGFβRII^{Col2ER} mice (Figure 2H). These results indicate that postnatal TGFβ signaling in articular chondrocytes plays a critical role in maintaining normal articular cartilage function. Endogenous TGFβ signaling in articular chondrocytes prevents chondrocyte hypertrophy. This unique feature distinguishes articular chondrocytes from growth plate chondrocytes.

Mmp13 and *Adamts5* up-regulation is mediated by RUNX-2 in TGFβRII^{Col2ER} mice

The mechanism of TGFβ regulation of *Mmp13* expression was investigated using an in vitro cell culture system. Treatment with TGFβ significantly inhibited *Mmp13* and *Col10* expression in rat chondrosarcoma cells at the 24- and 48-hour time points (Figures 3A and B). In contrast, transfection of *Tgfbr2* siRNA up-regulated *Mmp13* and *Col10* expression in rat chondrosarcoma cells (Figure 3C). Cotransfection of *Runx2* siRNA significantly inhibited the stimulatory effects of *Tgfbr2* siRNA on *Mmp13* and *Adamts5* expression (Figures 3D and E). We then cloned the 3.4-kb human *Mmp13* promoter and identified a putative RUNX-2 binding site (osteoblast-specific element 2 [OSE-2]) located in the proximal region of the *Mmp13* promoter; this binding site is conserved among humans, mice, and rats (Figure 3F). We found that treatment with TGFβ (2 ng/ml) significantly inhibited *Mmp13* promoter activity. However, this inhibitory effect was largely prevented when the RUNX-2 binding site was mutated (Figure 3G). Using a ChIP assay, we further showed that RUNX-2 directly binds to its binding site (5′-PyGPyGGTPy-3′) at the rat *Mmp13* promoter in rat chondrosarcoma cells, and addition of TGFβ suppressed RUNX-2 binding to this OSE-2 site (Figure 3H). These findings suggest that RUNX-2 may mediate the increase in *Mmp13* expression that occurs with impaired TGFβ signaling.

TGFβ regulates RUNX-2 degradation via up-regulation of CDK-4

In chondrocytes of TGFβRII^{Col2ER} mice, *Runx2* messenger RNA (mRNA) levels were increased 2–3-fold (Figure 2F). However, RUNX-2 protein levels were increased >8-fold in articular chondrocytes of TGFβRII^{Col2ER} mice (Figure 4A). These findings suggest that RUNX-2 protein stability was regulated by TGFβ signaling in articular chondrocytes. In previous studies we showed that TGFβ stimulated cyclin D1 expression in chondrocytes (31), and cyclin D1 and CDK-4 induce RUNX-2 phosphorylation and subsequent ubiquitination and proteasome degradation (28). In the present study, we found that TGFβ up-regulated CDK-4 expression while it down-regulated RUNX-2 protein levels (Figure 4B). TGFβ-mediated down-regulation of RUNX-2 protein levels could be prevented by transfection of *CDK4* siRNA in rat chondrosarcoma cells (Figure 4C). Consistently, treatment with TGFβ enhanced RUNX-2 ubiquitination in rat chondrosarcoma cells, and this effect was significantly prevented by the transfection of *CDK4* siRNA (Figure 4D). Based on these findings, we hypothesize that up-regulation of *Mmp13* expression in TGFβRII^{Col2ER} mice was mediated by increased RUNX-2 protein stability through a CDK-4–dependent mechanism. These findings suggest that TGFβ signaling may regulate downstream target genes such as *Mmp13* and *Adamts5* through activation of RUNX-2 in chondrocytes.

Inhibition of MMP-13 prevents the OA-like phenotype observed in TGFβRII^{Col2ER} mice

To determine the role of *Mmp13* and *Adamts5* in OA development in TGFβ βRII^{Col2ER} mice, we bred TGFβRII^{Col2ER} mice with MMP-13$^{flox/flox}$ mice (32) or ADAMTS-5$^{-/-}$ mice (26) and produced *Col2-CreER*/TGFβRII$^{flox/flox}$/MMP-13$^{flox/flox}$– or *Col2-CreER*/TGFβRII$^{flox/flox}$/ADAMTS-5–double-KO mice. Histologic examination revealed that deletion of the *Mmp13* gene in TGFβRII^{Col2ER} mice significantly ameliorated articular cartilage defects observed in 3- and 6-month-old TGFβRII^{Col2ER} mice. The findings included reduced loss of articular cartilage and a decrease in subchondral sclerosis and osteophyte formation (Figures 5A and E). Histomorphometric analysis further demonstrated a significant increase in pro-teoglycan levels and restoration of articular cartilage morphology when the *Mmp13* gene was deleted in 3- and 6-month-old double-KO mice (Figures 5B and F). Deletion of the *Mmp13* gene had no significant effect on type X collagen expression in 3-month-old TGFβRII/MMP-13–double-KO mice (Figure 5D). These

results suggest that different features of the OA-like phenotype may be regulated by distinct molecular mechanisms.

The articular cartilage areas in MMP-13^{Col2ER} mice (Figures 5B and F) and double-KO mice (Figures 5B and F) were significantly increased compared to those in Cre-negative control mice, which suggests that under normal conditions there is cartilage tissue turnover induced by MMP-13, so that deletion of the *Mmp13* gene could lead to higher articular cartilage volume. We also analyzed the data using the histologic scoring system recommended by the OARSI, and we found that deletion of the *Mmp13* gene was significantly chondro-protective in 3- and 6-month-old TGFβRII^{Col2ER} mice (Figures 5C and G).

To further determine the role of MMP-13 in OA development in TGFβRII^{Col2ER} mice, we investigated the efficacy of the MMP-13 inhibitor CL82198 (33) in reversing the OA-like phenotype of these mice. Immediately following injection of tamoxifen, CL82198 (10 mg/kg) was injected IP every other day into TGFβRII^{Col2ER} mice. Knee joint tissues were harvested at age 3 months, and the effect of CL82198 on the articular cartilage phenotype was examined histologically. The loss of articular cartilage tissues seen in TGFβRII^{Col2ER} mice was significantly ameliorated by treatment with the MMP-13 inhibitor (Figure 5H). Consistent with the histologic findings, histomorphometric analysis confirmed that articular cartilage areas at both the tibial plateau and femoral condyle were significantly increased by treatment with the MMP-13 inhibitor compared to the vehicle control (Figures 5I and J). Analysis using the histologic scoring system recommended by the OARSI showed that the MMP-13 inhibitor effectively slowed histologic damage observed in TGFβRII^{Col2ER} mice (Figure 5K). These results suggest that MMP-13 could be a potential target of drugs for the treatment of OA.

Deletion of the *Adamts5* gene prevents early-stage OA development in TGFβRII^{Col2ER} mice

The articular cartilage defects observed in TGFβRII^{Col2ER} mice could also be prevented in 3-month-old TGFβRII/ADAMTS-5–double-KO mice following deletion of the *Adamts5* gene (Figures 6A and B). Analysis using the histologic scoring system recommended by the OARSI yielded a similar result. Deletion of the *Adamts5* gene was significantly chondroprotective in 3-month-old TGFβRII^{Col2ER} mice (Figure 6C). However, deletion of the *Adamts5* gene had no significant effect on articular cartilage defects observed in 6-month-old TGFβRII^{Col2ER} mice (Figure 6D). These results suggest that although both *Mmp13* and *Adamts5* are downstream target genes of TGFβ signaling in articular chondrocytes, *Mmp13* may have a more significant and prolonged effect on OA development in TGFβRII^{Col2ER} mice. In this study, we found that deletion of the *Mmp13* or *Adamts5* gene not only inhibited articular cartilage degradation but also prevented defects of subchondral sclerosis and osteophyte formation observed in TGFβRII^{Col2ER} mice.

DISCUSSION

Although dn-TGFβRII–transgenic mice and *Smad3*-KO mice have already been shown to have an OA-like phenotype (10,29,34), it remains unclear whether TGFβ signaling plays a critical role in postnatal OA development, since in dn-TGFβRII–transgenic mice and *Smad3*-KO mice, TGF β signaling was inhibited at an early embryonic stage. The *Col2-CreER*–transgenic mouse model is a valuable tool, allowing chondrocyte-specific gene targeting in an inducible manner (22,23,35). In the present study, the *Tgfbr2* gene was deleted specifically in chondrocytes at the postnatal stage. TGFβRII^{Col2ER} mice exhibit a phenotype similar to that of human OA, including increased chondrocyte hypertrophy observed in the superficial zone of articular cartilage, tears and clefts in the articular surface, severe loss of articular cartilage tissue, osteophyte formation at the margins of cartilage

tissue, especially at the late stage of disease (in 6-month-old mice), and subchondral sclerosis.

It is interesting to note that inhibition of TGFβ signaling at the postnatal stage leads to articular cartilage damage and a severe OA–like phenotype, while the morphology of growth plate cartilage is relatively normal. In the present study, we induced *Tgfbr2* gene deletion at age 2 weeks to compare roles of TGFβ signaling in both articular and growth plate chondrocytes. We found that deletion of the *Tgfbr2* gene in chondrocytes at the postnatal stage (in 2-week-old mice) led to a severe OA–like phenotype in articular cartilage but had no significant effect on growth plate cartilage, which suggests that TGFβ signaling plays a specific role in maintaining integrity of articular cartilage at the postnatal stage. To further determine changes in growth plate cartilage development, we also performed gene expression analysis using primary sternal chondrocytes isolated from Cre-negative and TGFβRII^{Col2ER} mice. We found that expression of chondrocyte marker genes such as *Runx2*, *Alp*, *Oc*, *Mmp13*, and *Col10* was not significantly changed (data not shown). We also found that there was no significant change in type X collagen protein expression in growth plate chondrocytes from TGFβRII^{Col2ER} mice. This finding suggests that TGFβ signaling may play a specific role in maintaining integrity of articular cartilage at the postnatal and adult stages and in preventing abnormal differentiation of articular chondrocytes. This unique feature distinguishes articular chondrocytes from growth plate chondrocytes.

In the present study, we demonstrated that *Mmp13* mRNA levels were significantly up-regulated in TGFβRII^{Col2ER} mice. Our in vitro studies demonstrated that inactivation of TGFβ signaling stimulates *Mmp13* gene transcription in a RUNX-2–dependent manner. Deletion of the *Mmp13* gene significantly prevented the articular cartilage destruction observed in TGFβRII^{Col2ER} mice. Treatment of TGFβRII^{Col2ER} mice with an MMP-13 inhibitor also significantly inhibited articular cartilage degradation. While the MMP-13 inhibitor used in this study has nonspecific inhibitory effects on other MMPs, our studies suggest that MMP-13 inhibition is a possible therapeutic strategy for the treatment of OA. Although deletion of the *Adamts5* gene did not protect against articular cartilage degradation and other features of OA observed in 6-month-old TGFβRII^{Col2ER} mice, deletion of the *Adamts5* gene fully prevented the articular cartilage defects observed in 3-month-old TGFβRII^{Col2ER} mice. These results further suggest that up-regulation of *Adamts5* may play an important role in the development of early-stage OA in TGFβRII^{Col2ER} mice.

While deletion of the *Mmp13* gene significantly prevented the degradation of articular cartilage observed in TGFβRII^{Col2ER} mice, the rescue was incomplete. These findings are consistent with those in a study in which MMP-13^{Col2ER} mice were subjected to meniscal injury (36). There are 2 possible reasons for these findings. First, the Cre recombination efficiency mediated by *Col2-CreER* is ~85% in articular chondrocytes; thus, MMP-13 activity in the other 15% of chondrocytes remains normal in TGFβRII/MMP-13–double-KO mice. Second, it is known that deletion of *Adamts4* and *Adamts5* genes protects against OA development (19–21), and deletion of the *Adamts5* gene may be compensated for by ADAMTS-4, a member of the same family. In exploring the roles of both collagenase and aggrecanase in OA development in TGFβRII^{Col2ER} mice, further investigation is required to determine if double deletion of the *Mmp13* and *Adamts5* genes could fully protect against the OA-like phenotype observed in TGFβRII^{Col2ER} mice.

Deletion of the *Mmp13* gene significantly prevented several features of OA observed in TGFβRII^{Col2ER} mice, such as articular cartilage degradation, osteophyte formation, and subchondral sclerosis. However, *Mmp13* deletion failed to inhibit chondrocyte hypertrophy, since type X collagen was still highly expressed in the superficial zone of articular cartilage in the TGFβRII/MMP-13–double-KO mice. This is probably because the *Mmp13* gene is

downstream of RUNX-2. Most hypertrophic chondrocyte marker genes are induced by the *Runx2* gene, which is independent of *Mmp13* gene expression. It would be interesting to determine whether deletion of the *Runx2* gene completely prevents the OA-like phenotype observed in TGFβRII^{Col2ER} mice.

The current study shows that TGFβ signaling regulates the expression of genes that are critical in the maintenance of the articular cartilage matrix. In the absence of TGFβ signaling, *Mmp13* and *Adamts5* are induced and lead to articular cartilage tissue degeneration and the development of OA. However, it is still not known how TGFβ signaling is reduced or inactivated during the development of OA in patients. Potential causes of TGFβ signaling inactivation in articular chondrocytes include a loss-of-function mutation in the *Tgfbr2* gene or mutation of other genes responsible for mediating TGFβ signaling, such as *Smad3*. There are several lines of evidence that mechanical loading leads to inactivation of TGFβ signaling in bone cells (37). Using immunohistochemistry, we recently demonstrated that levels of TGFβRII and phosphorylated Smad3 were significantly down-regulated in a mouse model of OA induced by meniscus injury (results not shown), which suggests that 1 potential mechanism of meniscus injury–induced OA may be mediated by down-regulation of TGFβ signaling in articular chondrocytes. Finally, tumor necrosis factor α and interleukin-1β are 2 important proinflammatory cytokines involved in the development of OA. Recent studies demonstrate that these cytokines inhibit TGFβ/Smad signaling in other cell types (37–39). Since many other signaling pathways and transcription factors are involved in OA development, it would be interesting to investigate the interaction of TGFβ signaling with other signaling pathways during OA development, such as the Wnt/β-catenin, IHH, and HIF-2α pathways.

Previous studies demonstrate that TGFβ signaling has different effects on cells at various stages of differentiation. In mesenchymal progenitor cells, TGFβ promotes chondrogenesis (40,41). However, in chondrocytes, TGFβ inhibits differentiation and hypertrophy (42,43). It would be interesting to know whether inhibition of TGFβ signaling in mesenchymal progenitor cells leads to a phenotype similar to the one we observed in the current study. This question could be addressed by generating and analyzing *Prx1-CreER*/TGFβRII$^{flox/flox}$ mice or *Nestin-CreER*/TGFβRII$^{flox/flox}$ mice. It has been reported that exogenous application of TGFβ may cause fibrosis and osteophyte formation (36,44). In the present study, we found that deletion of the *Tgfbr2* gene does not cause synovial fibrosis and does lead to slight osteophyte formation. A possible explanation for this discrepancy is that we have selectively deleted the *Tgfbr2* gene in articular chondrocytes. However, exogenous application of TGFβ could nonspecifically target other tissues in the joint.

In the present study, we induced *Tgfbr2* gene deletion in 2-week-old mice. Since mice are still growing at this stage, which may affect articular cartilage structure, further investigation is still required using deletion of the *Tgfbr2* gene in mice 2 months of age or even older. Using *Col2-CreER*– and *Aggrecan-CreER*–transgenic mice, we are currently testing whether deletion of the *Tgfbr2* gene at different ages of adult mice (2, 4, and 6 months) will also produce an OA-like phenotype. These studies will help us determine the effect of *Tgfbr2* gene deletion on OA development and progression in adult mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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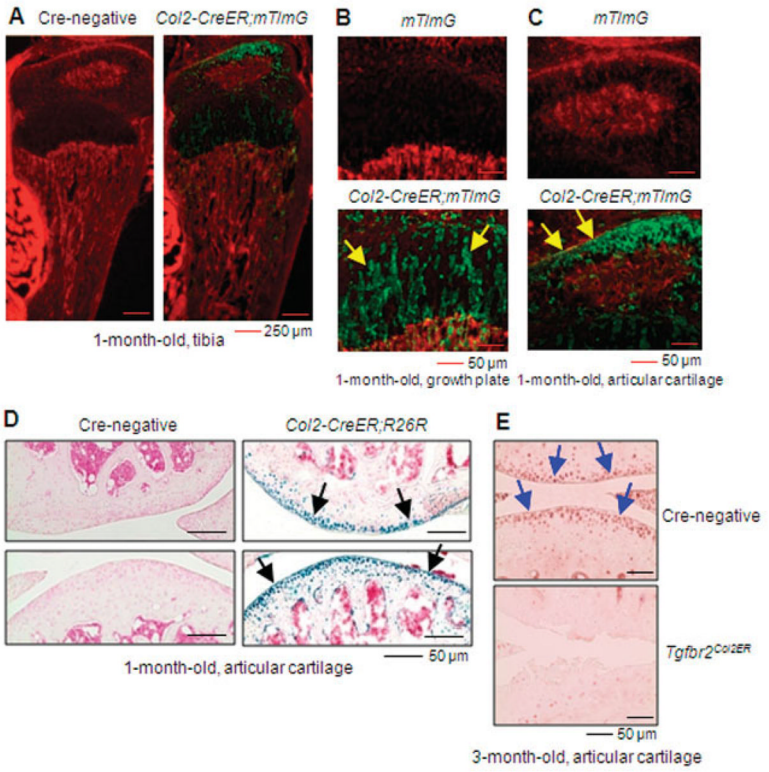

Figure 1.

Directed Cre recombination in articular chondrocytes from *Col2-CreER* mice. *Col2-CreER;mT/mG* mice and *Col2-CreER;R26R* mice were generated by breeding *Col2-CreER*–transgenic mice with *Rosa-Tomato* (*mT/mG*) mice and *Rosa-lacZ* (*R26R*) mice, respectively. Tibia samples were harvested from 1-month-old mice after they were injected with tamoxifen at age 2 weeks. **A–C,** Analysis of histologic sections from *mT/mG* mice and *Col2-CreER;mT/mG* mice using fluorescence microscopy demonstrated the efficiency of Cre recombination in the growth plate and articular chondrocytes. **Arrows** in **B** indicate *Col2-CreER*–targeted growth plate chondrocytes; **arrows** in **C** indicate *Col2-CreER*–targeted articular chondrocytes. **D,** X-Gal staining showed that the efficiency of *Col2-CreER*–mediated Cre recombination was >85% in articular chondrocytes. **Arrows** indicate Col2-CreER–targeted articular chondrocytes. **E,** Immunohistochemical analysis showed that transforming growth factor β receptor type II (TGFβRII) expression was dramatically reduced in articular chondrocytes from 3-month-old TGFβRII^{Col2ER} mice. **Arrows** indicate TGFβRII-positive articular chondrocytes.

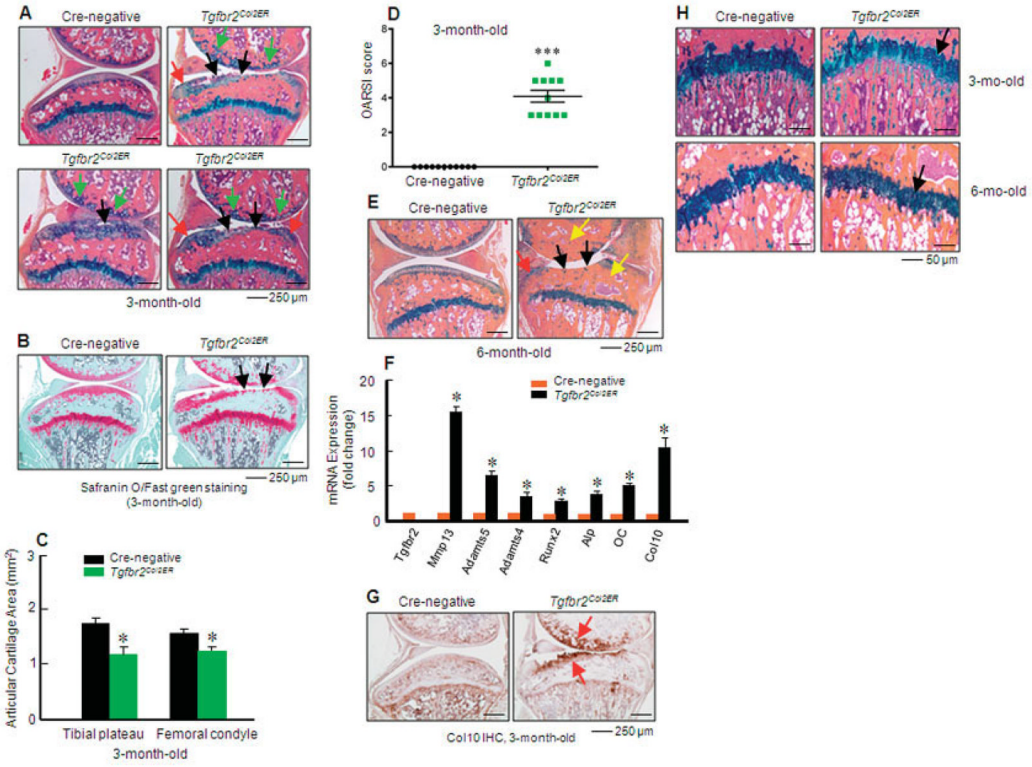

Figure 2.

Transforming growth factor β receptor type II (TGFβRII)–conditional knockout (TGFβRII^{Col2ER}) mice show a progressive osteoarthritis (OA)–like phenotype. **A** and **B,** Knee joint samples were dissected from 3-month-old mice, and Alcian blue/hematoxylin and eosin and Safranin O–fast green staining were performed. TGFβRII^{Col2ER} mice displayed early signs of an OA-like phenotype, including enhanced chondrocyte hypertrophy in the superficial zone (**green arrows**), tears and clefts in the articular surface (**black arrows**), and osteophyte formation (**red arrows**). **C,** Histomorphometric analysis showed that articular cartilage area in both the tibia and femur was significantly reduced in 3-month-old TGFβRII^{Col2ER} mice (* = $P < 0.05$ versus Cre-negative mice, by Student's unpaired *t*-test; n = 11 mice per group). **D,** Analysis using the histologic scoring system recommended by the Osteoarthritis Research Society International (OARSI) revealed articular cartilage destruction in 3-month-old TGFβRII^{Col2ER} mice (** = $P < 0.01$ versus Cre-negative mice, by Student's unpaired *t*-test). **E,** Severe loss of articular cartilage tissue (**black arrows**), osteophyte formation (**red arrow**), and subchondral sclerosis (**yellow arrows**) were observed in 6-month-old TGFβRII^{Col2ER} mice. **F,** Total RNA was isolated from articular cartilage of 1-month-old TGFβRII^{Col2ER} mice and their littermates, and real-time polymerase chain reaction was performed. Loss of TGFβ signaling resulted in increased expression of hypertrophic chondrocyte marker genes (* = $P < 0.05$ versus Cre-negative mice, by Student's unpaired *t*-test; n = 4 mice per group). **G,** Immunohistochemical (IHC) analysis showed that type X collagen protein levels were increased in TGFβRII^{Col2ER} mice, especially in the superficial zone (**red arrows**). **H,** Structure and morphology of growth plate cartilage (**black arrows**) were not significantly changed. Values in **C, D,** and **F** are the mean ± SD. In **D,** squares represent individual mice.

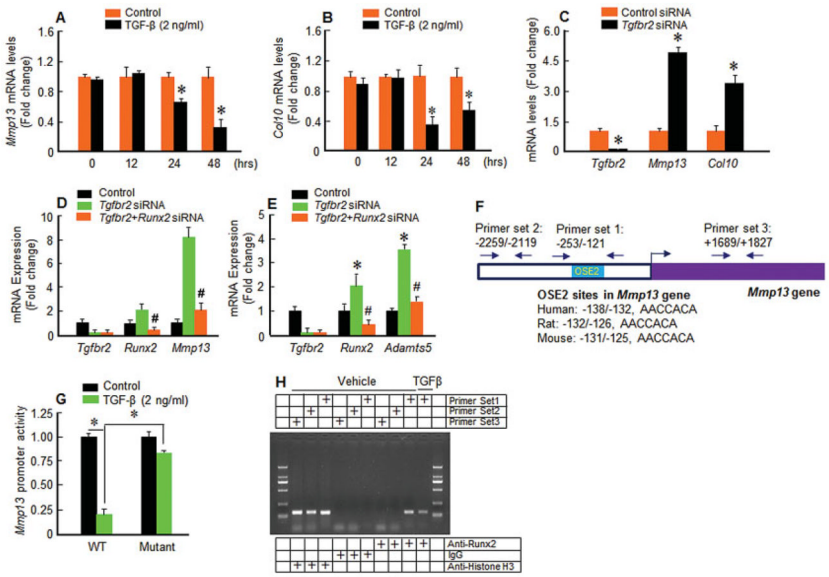

Figure 3.

Mmp13 up-regulation is mediated by a RUNX-2–dependent mechanism in transforming growth factor *β* receptor type II (TGF*β*RII)–conditional knockout (TGF*β*RII^{Col2ER}) mice. **A** and **B,** Treatment with TGF*β* (2 ng/ml) inhibited *Mmp13* (**A**) and *Col10* (**B**) expression at 24 and 48 hours in rat chondrosarcoma cells (* = $P < 0.05$ versus control, by Student's unpaired *t*-test; n = 3 wells per group). **C,** Transfection of *Tgfbr2* small interfering RNA (siRNA) up-regulated *Mmp13* and *Col10* expression in rat chondrosarcoma cells (* = $P < 0.05$ versus control siRNA, by Student's unpaired *t*-test; n = 3 wells per group). **D** and **E,** Transfection of *Runx2* siRNA inhibited *Tgfbr2* siRNA–induced *Mmp13* (**D**) and *Adamts5* (**E**) expression in rat chondrosarcoma cells (# = $P < 0.05$ versus *Tgfbr2* siRNA alone; * = $P < 0.05$ versus control, by Student's unpaired *t*-test). **F,** Shown are locations and sequences of osteoblast-specific element 2 (OSE-2) sites in the *Mmp13* promoter. **G,** Rat chondrosarcoma cells were transfected with 3.4-kb wild-type (WT) or mutant (OSE-2 site mutation) human *Mmp13* promoter and treated with TGF*β*. TGF*β* significantly inhibited *Mmp13* promoter activity, and the inhibitory effect of TGF*β* on the *Mmp13* promoter was prevented when rat chondrosarcoma cells were transfected with the mutant *Mmp13* promoter (* = $P < 0.05$ by Student's unpaired *t*-test; n = 3 wells per group). **H,** Chromatin immunoprecipitation assay was performed using digested chromatin from rat chondrosarcoma cells that were either left untreated or treated with TGF*β* (2 ng/ml). Purified DNA was subjected to polymerase chain reaction using *Mmp13*-specific primer set 1 (−253/−121), which amplifies the fragment spanning the RUNX-2 binding sequence, or the negative control primer set 2 (−2259/−2119) or the positive control primer set 3 (+1689/+1827). Values in **A–E** and **G** are the mean ± SD.

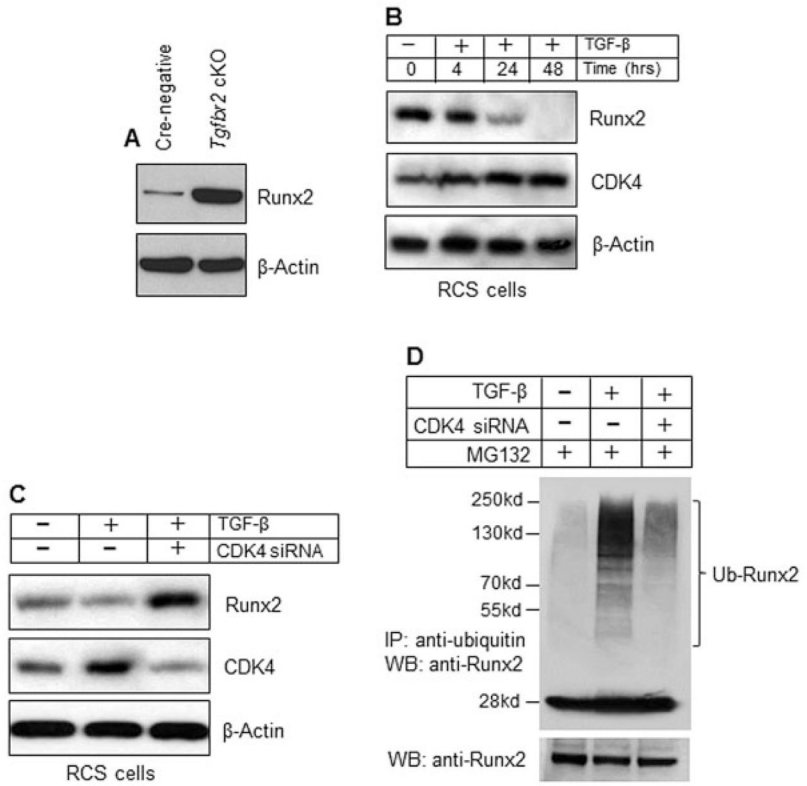

Figure 4.

Transforming growth factor β (TGFβ) enhances RUNX-2 degradation through cyclin-dependent kinase 4 (CDK-4)–mediated ubiquitination. **A,** Cell lysates were extracted from primary articular chondrocytes isolated from 1-month-old TGFβ receptor type II (TGFβRII)–conditional knockout (cKO) (TGFβRII^{Col2ER}) mice and their Cre-negative littermates. Western blot (WB) analysis showed that RUNX-2 protein levels were significantly increased (>8-fold) in articular chondrocytes from TGFβRII^{Col2ER} mice. **B,** Rat chondrosarcoma (RCS) cell cultures were treated with TGFβ (2 ng/ml) for 4, 24, and 48 hours, and Western blot analysis was performed. Treatment with TGFβ reduced RUNX-2 protein levels and increased CDK-4 expression in rat chondrosarcoma cells. **C,** Rat chondrosarcoma cells were transfected with *CDK4* small interfering RNA (siRNA) and treated with TGFβ (2 ng/ml). Western blot analysis showed that inhibition of CDK-4 expression enhanced RUNX-2 protein levels in rat chondrosarcoma cells. **D,** RUNX-2 ubiquitination (Ub) assay was performed using rat chondrosarcoma cells transfected with *CDK4* siRNA and treated with TGFβ. The proteasome inhibitor MG132 (10 μM) was added to the cell culture 4 hours before cell lysates were collected. Ubiquitinated proteins were immunoprecipitated (IP) using a UbiQapture kit, and polyubiquitinated RUNX-2 was detected using an anti–RUNX-2 antibody. Treatment with TGFβ significantly enhanced RUNX-2 ubiquitination, and knocking down *CDK4* expression significantly prevented RUNX-2 ubiquitination induced by TGFβ.

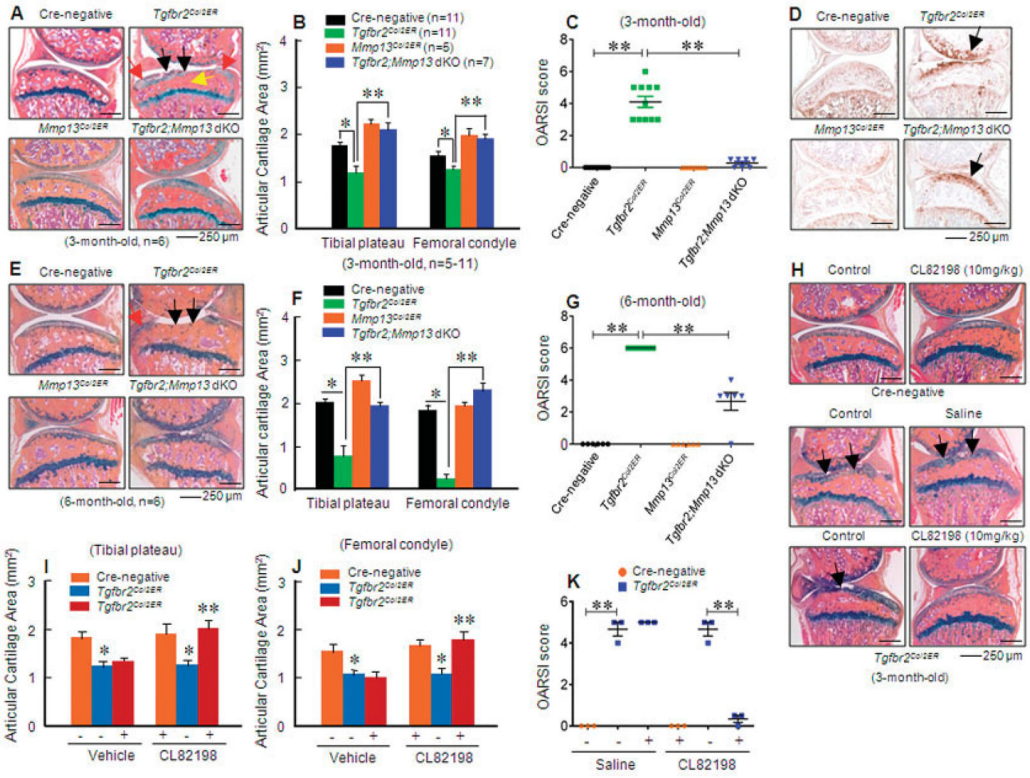

Figure 5.

Mmp13 deletion prevents the osteoarthritis (OA)–like phenotype observed in transforming growth factor *β* receptor type II (TGF*β*RII)–conditional knockout (TGF*β*RII^{Col2ER}) mice. **A** and **E,** Alcian blue/hematoxylin and eosin staining was performed in 3-month-old (**A**) and 6-month-old (**E**) mice. TGF*β*RII^{Col2ER} mice displayed a phenotype like that of progressive OA. Deletion of the *Mmp13* gene in TGF*β*RII^{Col2ER} mice prevented their OA-like phenotype, including articular cartilage degradation (**black arrows**), osteophyte formation (**red arrows**), and subchondral sclerosis (**yellow arrow**). **B, C, F,** and **G,** Analysis using histomorphometry (**B** and **F**) and the histologic scoring system recommended by the Osteoarthritis Research Society International (OARSI) (**C** and **G**) showed that *Mmp13* deletion in 3- and 6-month-old TGF*β*RII^{Col2ER} mice protected against articular cartilage degradation induced by the loss of TGF*β* signaling (* = $P < 0.05$; ** = $P < 0.01$ by two-way analysis of variance [ANOVA] followed by Tukey's test). **D,** There were no significant changes in type X collagen protein levels (**arrows**) between TGF*β*RII^{Col2ER} mice and TGF*β*RII/matrix metalloproteinase 13 (MMP-13)–double-knockout (dKO) mice. **H–J,** The MMP-13 inhibitor CL82198 (10 mg/kg) was injected into TGF*β*RII^{Col2ER} mice. Histologic examination (**H**) and histomorphometric analyses (**I** and **J**) showed that the MMP-13 inhibitor protected against articular cartilage degradation observed in TGF*β*RII^{Col2ER} mice (* = $P < 0.05$ versus Cre-negative control; ** = $P < 0.05$ versus TGF*β*RII^{Col2ER} mice receiving vehicle, by two-way ANOVA followed by Tukey's test). **Arrows** in **H** indicate loss of cartilage tissue. **K,** Analysis using the OARSI scoring system showed that the MMP-13 inhibitor slowed articular cartilage degeneration in 3-month-old TGF*β*RII^{Col2ER} mice (** = $P < 0.01$ by two-way ANOVA followed by Tukey's test). Values in **B, C, F, G,** and **I–K** are the mean ± SD. In **C, G,** and **K,** symbols represent individual mice. MMP-13^{Col2ER} = MMP-13 knockout.

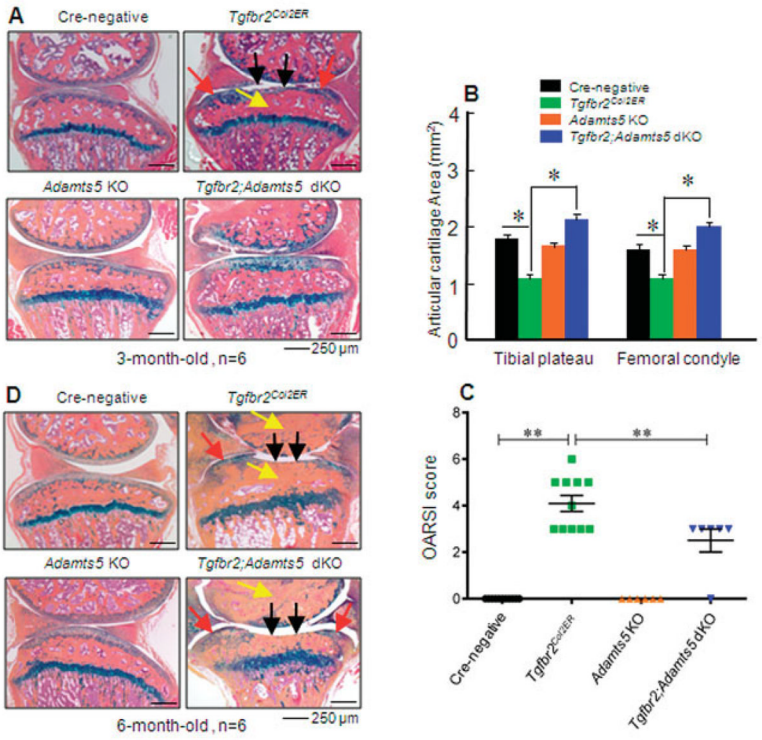

Figure 6.

Deletion of the *Adamts5* gene partially prevents the osteoarthritis (OA)–like phenotype observed in transforming growth factor *β* receptor type II (TGF*β*RII)–conditional knockout (KO) (TGF*β*RII^{Col2ER}) mice. **A** and **D,** Histologic examination of knee joint tissues dissected from 3-month-old (**A**) and 6-month-old (**D**) mice was performed. Alcian blue/hematoxylin and eosin staining demonstrated that 3- and 6-month-old TGF*β*RII^{Col2ER} mice displayed progressive development of OA. The OA-like phenotype induced by the loss of TGF*β* signaling was completely prevented in 3-month-old TGF*β*RII^{Col2ER} mice with deletion of the *Adamts5* gene (TGF*β*RII/ADAMTS-5–double-KO [dKO] mice), but there was no effect in 6-month-old TGF*β*RII/ADAMTS-5–double-KO mice. **Black arrows** indicate loss of articular cartilage; **red arrows** indicate osteophyte formation; **yellow arrows** indicate subchondral sclerosis. **B,** Histomorphometric analysis showed that articular cartilage areas in the tibial plateau and femoral condyle were decreased in TGF*β*RII^{Col2ER} mice compared to Cre-negative control mice (* = $P < 0.05$ by two-way analysis of variance [ANOVA] followed by Tukey's test). In contrast, deletion of the *Adamts5* gene in 3-month-old TGF*β*RII^{Col2ER} mice completely protected against this articular cartilage degradation (* = $P < 0.05$ by two-way ANOVA followed by Tukey's test). **C,** Analysis using the histologic scoring system recommended by the Osteoarthritis Research Society International (OARSI) showed that deletion of the *Adamts5* gene in 3-month-old TGF*β*RII^{Col2ER} mice protected against articular cartilage loss induced by the loss of TGF*β* signaling (** = $P < 0.01$ by two-way ANOVA followed by Tukey's test). Values in **B** and **C** are the mean ± SD. In **C,** symbols represent individual mice.