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Original Article

Comparison of Kindlin-2 deficiency-stimulated osteoarthritis-like lesions induced by *Prg4*^{CreERT2} versus *Aggrecan*^{CreERT2} transgene in mice

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A R T I C L E I N F O

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A B S T R A C T

Background: Genetically modified mice are the most useful tools for investigating the gene functions in articular cartilage biology and the pathogenesis of osteoarthritis. The *Aggrecan*^{CreERT2} mice are one of the most reported mouse lines used for this purpose. The *Prg4* (proteoglycan 4) gene encodes the lubricin protein and is expressed selectively in chondrocytes located at the superficial layer of the articular cartilage. While the *Prg4*^{GFPCreERT2} knock-in inducible-Cre transgenic mice were generated a while ago, so far, few studies have used this mouse line to perform gene functional studies in cartilage biology.

Methods: We have recently reported that deleting the *Fermt2* gene, which encodes the key focal adhesion protein Kindlin-2, in articular chondrocytes by using the *Aggrecan*^{CreERT2} transgenic mice, results in spontaneous osteoarthritis (OA) lesions, which highly mimics the human OA pathologies. In this study, we have compared the Kindlin-2 deficiency-caused OA phenotypes induced by *Prg4*^{GFPCreERT2} with those caused by *Aggrecan*^{CreERT2} using imaging and histological analyses.

Results: We find that Kindlin-2 protein is deleted in about 75% of the superficial articular chondrocytes in the tamoxifen (TAM)-treated *Prg4*$^{GFPCreERt2/+}$; *Fermt2*$^{fl/fl}$ mice compared to controls. At 6 months after TAM injections, the OARSI scores of *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ and *Prg4*$^{GFPCreERt2/+}$; *Fermt2*$^{fl/fl}$ mice were 5 and 3, respectively. The knee joints histological osteophyte and synovitis scores were also significantly decreased in *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice compared to those in *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice. Furthermore, magnitudes of upregulation of the extracellular matrix-degrading enzymes Mmp13 and hypertrophic chondrocyte markers Col10a1 and Runx2 were decreased in *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ versus *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice. We finally examined the susceptibility of *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mouse model to surgically induce OA lesions. The pathological features of OA in the TAM-DMM model exhibited significant enhancement in cartilage erosion, proteoglycan loss, osteophyte, and synovitis and an increase in OARSI score in articular cartilage compared with those in corn-oil DMM mice.

Conclusion: Kindlin-2 loss causes milder OA-like lesions in *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ than in *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice. In contrast, Kindlin-2 loss similarly accelerates the destabilization of the medial meniscus-induced OA lesions in both mice.

Translational Potential of this Article: Our study demonstrates that *Prg4*^{GFPCreERT2} is a useful tool for gene functional study in OA research. This study provides useful information for investigators to choose appropriate Cre mouse lines for their research in cartilage biology.

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1. Introduction

Osteoarthritis (OA) is a complex synovial joint disorder and a leading cause of disability that results in tremendous economic and health burdens to societies and patients. More than 500 million people are affected by OA worldwide [1]. However, so far, there is no effective treatment that can halt or reverse the disease, in part due to our lack of knowledge of the fundamental molecular mechanism of the initiation and progression of OA. Transgenic mouse models play important roles in OA research [2]. Genetically modified mice using an inducible Cre-loxP strategy are the most useful tools for investigating the gene functions in the articular cartilage biology and the pathogenesis of OA. Inducible *Aggrecan*^{CreERT2} mice are one of the most reported mouse lines used to conditionally knockout genes in the articular chondrocytes of adult mice to investigate the OA mechanism. By using *Aggrecan*^{CreERT2} mice, the EGFR pathway, focal adhesion pathway, HIFs pathway, β-catenin signaling, BMP pathway, and other pathways are identified to play important roles in OA onset and development [3–8]. Notably, the *Aggrecan*^{CreERT2} is active not only in the articular chondrocytes but also in other cell types, including the growth plate chondrocytes and the nucleus pulposus cells [9]. This may complicate the interpretation of data produced by using this mouse line.

Healthy articular cartilage is coated on the surface by lubricin molecules, which provide lubrication boundary and low friction of the cartilage surface. Lubricin encoded by the *Prg4* (proteoglycan 4) gene is a secreted proteoglycan protein that is predominantly expressed by chondrocytes in the superficial zone of articular cartilage and synovial fibroblasts [10,11]. *Prg4*^{GFPCreERT2} knock-in mice that express tamoxifen-inducible Cre recombinase from the internal *Prg4* locus were generated and reported [12]. *Prg4*^{GFPCreERT2} is expressed by chondrocytes primarily in the superficial layer of articular cartilage in adult mice [12,13]. So far, few reported studies had used this mouse line to induce gene conditional deletion in adult mice for OA study.

Kindlin-2 is an essential focal adhesion protein encoded by the *Fermt2* gene. It is crucial for early embryonic development, and loss of Kindlin-2 leads to embryonic lethality at E7.5 in mice [14,15]. We have recently reported that the expression of the Kindlin-2 protein in the articular chondrocytes is reduced by more than 90% in TAM-inducible *Aggrecan*^{CreERT2} mice and discovered that deleting the key focal adhesion protein Kindlin-2 in the chondrocytes by using *Aggrecan*^{CreERT2} mice leads to the quick development of spontaneous OA-like phenotypes [7]. In this study, we determine whether the *Prg4*^{GFPCreERT2} mouse line is a useful tool to perform gene conditional knockout in OA study by comparing the Kindlin-2 deficiency-stimulated OA-like lesions induced by *Aggrecan*^{CreERT2} and *Prg4*^{GFPCreERT2} mouse lines.

2. Materials and methods

2.1. Mouse lines and sample collections

Prg4^{GFPCreERT2} mice were purchased from Jackson Laboratory with C57BL/6 genetic background. The *Aggrecan*^{CreERT2} and *Fermt2*$^{fl/fl}$ mice and the preparation of *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice were previously described [7,9,14,16]. All transgenic mice used in this study have been crossed at least 10 generations with the wild-type C57BL/6 mice. We mated *Fermt2*$^{fl/fl}$ female mice with male *Prg4*$^{GFPCreERT2/GFPCreERT2}$; *Fermt2*$^{fl/fl}$ mice to obtain *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice for experiments. To induce the Cre expression, three-month-old male mice were given tamoxifen (TAM) (Sigma, t5648) via intraperitoneal injection for 10 consecutive days at a dose of 100 mg/kg/dose diluted in corn oil. Three-month-old male mice injected with the same dose of corn oil were used as controls in this study. Six months after the TAM injection, knee samples of hindlimbs were collected. For OA-induced models, DMM surgeries were performed on the right knee joint of mice one month after TAM(or corn oil) injection, and SHAM surgeries were performed at the same time on the left knee joint. Knee joint samples were collected two months after DMM and SHAM surgeries. Mice were randomly divided into experimental and control groups. Animals were maintained 5 mice/cage at a temperature of 20–24 °C, exposed to a 12-h/12-h light and dark period, and given water and rodent food.

2.2. Micro-computed tomography (μCT) analysis

According to our protocol described previously [7], the knee joints were subjected to μCT analysis. We used a high-resolution μCT scanner (Bruker MicroCT, SkyScan1172) with a source voltage of 60 kV with a current of 100 μA to scan the knee joint of mice resulting in a 13 μm image pixel size. The same protocol was used to scan formalin-fixed mouse knee joints resulting in 10 μm image pixel size. Each set of scanned images was evaluated under the same thresholds to allow three-dimensional structure rendering for each sample.

2.3. Histology and immunostaining assays

Mouse knee joints were fixed in 4% PFA for 24 h, decalcified with 10% ethylenediaminetetraacetic acid (15% EDTA; pH7.2) for 14 days, dehydrated, paraffin-embedded, and 5-μM sliced. The paraffin sections were stained with SO&FG (Solarbio, cat. no. G1371)/H&E (Thermo Fisher, cat. no. 7211&7111), respectively, for morphological analysis according to the manufacturer's instructions. Paraffin sections were used for immunofluorescence (IF) staining and immunohistochemistry (IHC) staining. For IF staining, after blocking with QuickBlock™ Blocking Buffer (Beyotime) with Triton 100 (Sigma–Aldrich), sections were incubated with primary antibodies (4 °C, overnight). Then, sections were incubated with anti-mouse/rabbit Alexa Fluor 568 secondary antibodies. Finally, sections were examined by confocal microscope (LSM 980, ZEISS) and evaluated using ImageJ software. For IHC staining, sections were deparaffinized by xylene, and then they were rehydrated using ethanol. Citrate buffer (0.1 mol L^{-1}, pH 6.0) was used to perform antigen retrieval. After blocking using a peroxidase-blocking solution and normal horse serum, sections were incubated with primary antibodies (4 °C, overnight). Then, sections were incubated with biotinylated IgG and streptavidin-horseradish peroxidase. DAB Peroxidase Substrate Kit was used to visualize the immunoreactivity. Finally, sections were counterstained by hematoxylin and then mounted. Histological scoring and quantitative IF and IHC staining analyses were performed in a double-blinded manner.

2.4. Statistics

The sample size of each experiment is determined according to the experience of previous animal experiments in our laboratory. Statistical analyses were completed using the Prism GraphPad. Results are expressed as mean ± s.d. The two-sided unpaired Student's *t*-test was used between the comparable groups, followed by Tukey's post hoc test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Kindlin-2 protein was significantly reduced in articular chondrocytes of the superficial zone in *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice

To delete the *Fermt2* gene, which encodes the Kindlin-2 protein, in the articular chondrocytes of the superficial zone, 3-month-old *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice were genotyped, bred, and given TAM diluted in corn oil for 10 consecutive days (Suppl. Fig. 1A–C). *Fermt2*$^{fl/fl}$ mice and *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice injected with corn oil alone served as control. *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice were sacrificed after 6 months of TAM or corn oil injections. The knee joints of the hindlimbs were collected, and tissues were processed to obtain paraffin sections. Immunostaining was performed on paraffin sections, and results showed that GFP protein was detected by anti-GFP antibodies in the superficial

layer and middle layer of articular cartilage of both *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice injected with corn oil alone and TAM (Fig. 1A and B). Of note, GFP proteins were observed under the microscope without antibody staining. While Cre proteins were detected in the cytoplasm of articular chondrocytes in *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ control mice, they were observed to locate within nuclear area stained with DAPI in around 80% of articular chondrocytes of *Prg4*^{GFPCreERT2} *Fermt2*$^{fl/fl}$ mice injected with TAM (Fig. 1A, C). This indicates that in the TAM-injected mice, the Cre is translocated to the nucleus, functioning as the recombinase to delete the floxed gene. As a result, the percentage of Kindlin-2 positive articular chondrocytes was decreased by around 75% in TAM-injected mice compared to that of controls (Fig. 1A, D), suggesting that expression of Kindlin-2 protein was dramatically decreased in articular chondrocytes of the superficial zone in the TAM-induced *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice. However, the intracellular Prg4, as an important factor for joint function, was not markedly altered in the articular chondrocytes in either corn oil or TAM-injected *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice compared to that in control mice (Fig. 1A, E), suggesting that deleting one allele of the *Prg4*^{GFPCreERT2} transgene does not impact the expression level of Prg4 protein in the articular cartilage.

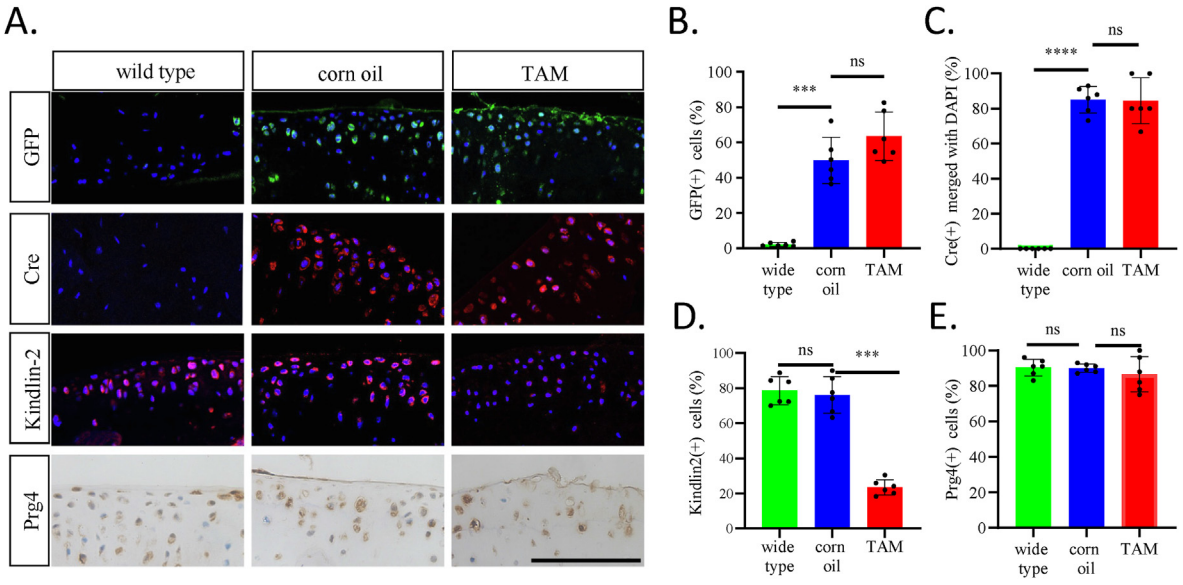

Figure 1. Deletion of Kindlin-2 in knee articular chondrocytes of *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice (A) IF staining with GFP, Cre and Kindlin-2 and IHC staining with Prg4 on knee joint articular cartilage paraffin sections for control group (*Fermt2*$^{fl/fl}$), corn oil group (corn oil injected *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$) and TAM group (TAM-injected *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$). Scale bar, 100 μm (B–E) Quantitative data of panel A. Quantitative data are shown as mean ± s.d. NS, no statistical significance, ***$P < 0.001$, ****$P < 0.0001$.

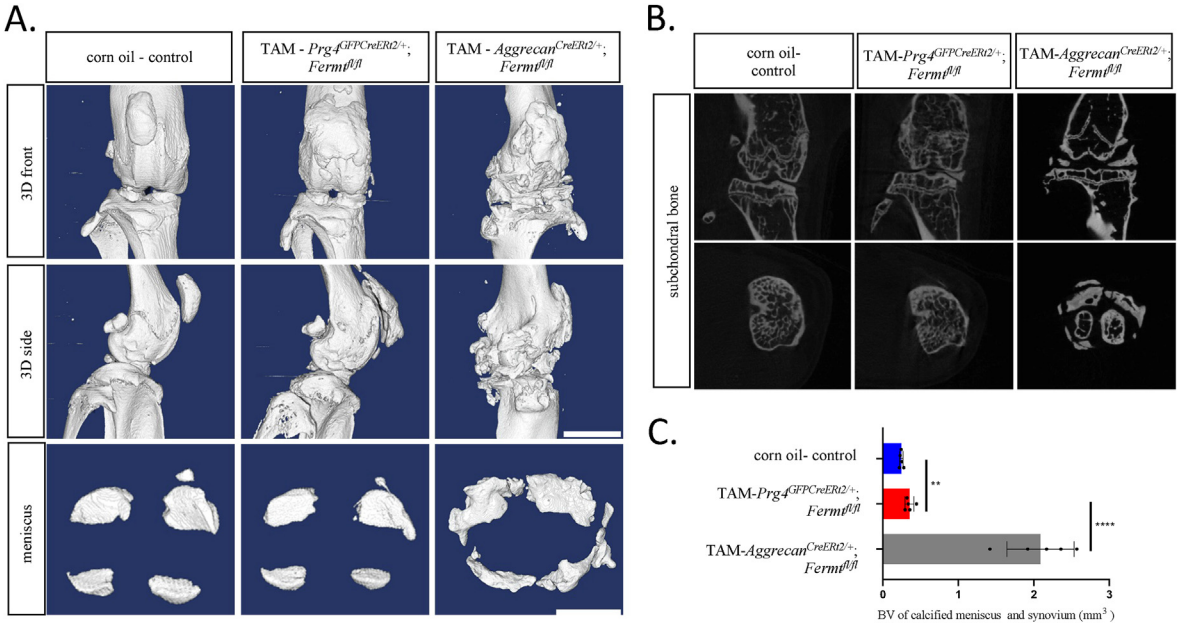

Figure 2. μCT analysis of knee joints from *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ and *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice (A). μCT scans of knee joints from corn oil and tamoxifen injected *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ and tamoxifen injected *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice at 6 months after TAM or corn oil injection. Scale bar, 1 mm (B–C). BV of calcified meniscus and synovium (mm^3). Quantitative data are shown as mean ± s.d. NS, no statistical significance, **$P < 0.01$, ****$P < 0.0001$. n = 6 mice per group and results from one representative replicate are shown.

3.2. Kindlin-2 deficiency causes milder OA-like phenotypes in *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ than in *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice

We next compared the structural differences of the knee joints between corn oil- and TAM-treated *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice. At 6 months after corn oil and TAM injections, hindlimbs were collected for further analyses. Consistent with our previous observations [7], knee joints of TAM-treated *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice displayed macroscopic OA-like abnormalities, including joint enlargement, visible cartilage damage, and incorrect joint alignment. However, the appearance of the knee joints in TAM-induced *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice was indistinguishable from the controls. Then, micro-computerized tomography (μCT) analyses were performed on the knee joints of the hindlimbs. In further consistency of our previous results, at six months after TAM injections, the *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice displayed severe OA-like phenotypes, including whole joint deformation, meniscus, and synovium calcification, subchondral bone sclerosis, and massive osteophyte formation in the knee joints (Fig. 2A and B). In contrast, deleting Kindlin-2 in Prg4-positive chondrocytes only induced mild osteophyte formation without causing marked abnormalities in the menisci and subchondral bone when compared to that in the control group (Fig. 2A and B). Results of quantification analysis on the calcified meniscus and synovium of hindlimb knee joints showed that the calcification of meniscus and synovium was slightly increased in the TAM-induced *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice compared to that in control, which was much more severe in TAM-treated *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice (Fig. 2C).

3.3. The OA-like lesions measured by histological analyses are less severe in *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ than in *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice

We next performed safranin O and fast green staining of the knee joint sections from each group for histological analyses. The histological OA severity was scored using the Osteoarthritis Research Society International (OARSI) score system. Histological evaluations revealed that Kindlin-2 deletion in Prg4-positive chondrocytes resulted in a significantly increased OARSI score (Fig. 3A and B). Consistent with the μCT analysis, the OARSI score was significantly lower in TAM-treated *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice than that in *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice. TAM-treated *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ knee joints displayed several early-stage OA-like phenotypes, including more chondrocyte hypertrophy in the superficial layer of articular cartilage, clustering and rough articular cartilage surface and spontaneous fissures, and loss of safranin O-stained proteoglycans (Fig. 3A, C). Synovitis score was evaluated by using Krenn's synovitis scoring system. While we observed marked enlargement of the synovial lining cells in the knee joint sections in both TAM-injected *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ and *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice, the synovial stroma and inflammatory infiltration was much less severe in the former than that in the latter (Fig. 3A, D-E). In addition, histological staining showed that the structure and morphology

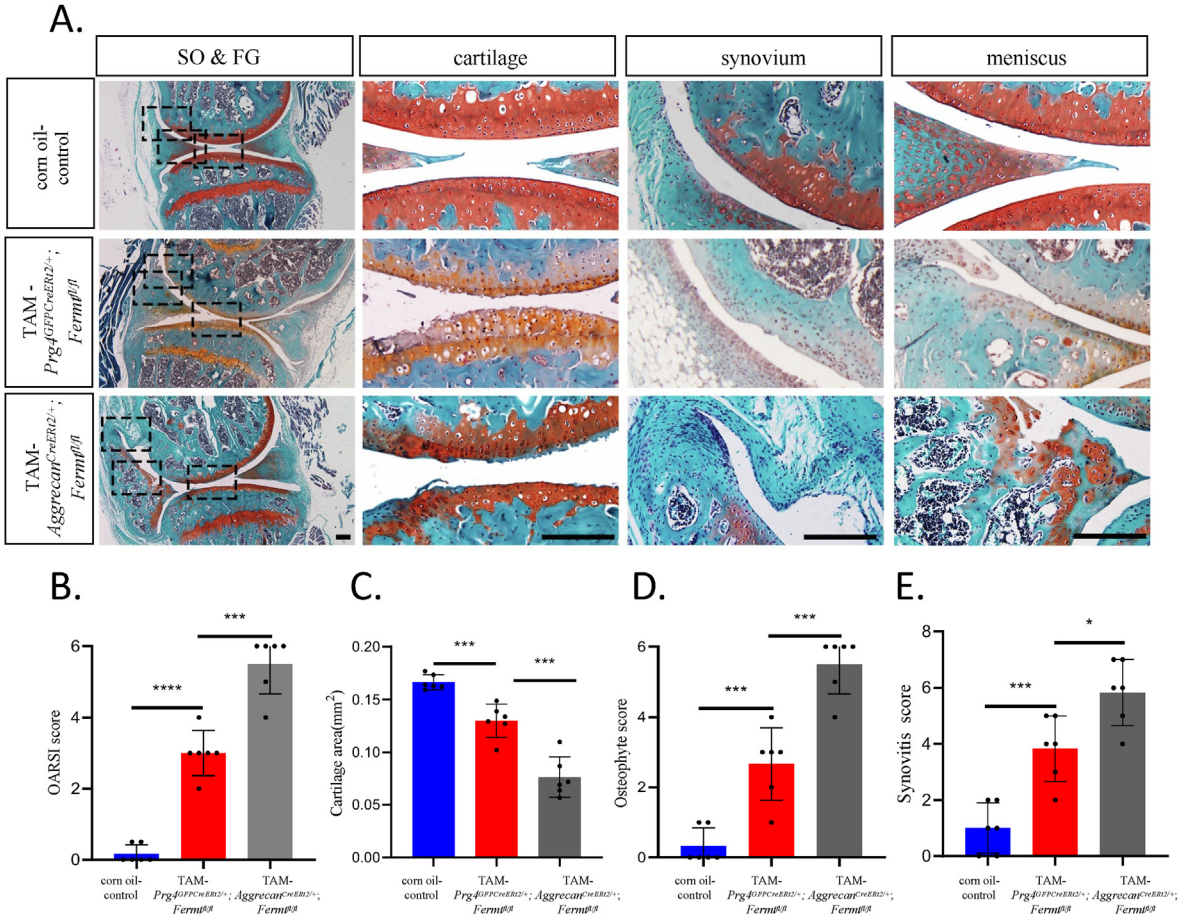

Figure 3. Histological analysis of knee joints from *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ and *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice (A) SO&FG staining on paraffin sections of the corn oil and tamoxifen injected *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ and tamoxifen injected *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice (B) Quantification of OARSI score (C) Quantitative analysis of the cartilage area (D) Osteophyte score evaluated by using Krenn's synovitis scoring system (E) Synovitis score was performed using histological sections. Quantitative data are shown as mean ± s.d. NS, no statistical significance, $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$. n = 6 mice per group and results from one representative replicate are shown.

of the growth plates in TAM-injected *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice were not markedly altered compared to those in controls with the similar expression level of Kindlin-2 protein in both phenotypes, while growth plates showed obviously expanded in TAM-injected *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice (Supp. Fig. 2A–C). Collectively, the OA-like defects were milder in *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice than in age- and sex-matched *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice.

3.4. The magnitudes of OA-related gene upregulation are decreased in *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ relative to *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice

In our previous studies, we discovered that Kindlin-2 deletion in articular cartilage largely up-regulated expression of Runx2 and accelerated chondrocyte differentiation and hypertrophy. The IF staining analyses were performed to determine the expression of the extracellular matrix protein aggrecan, matrix-degrading enzymes Mmp13, and hypertrophic chondrocyte markers Col10 and Runx2. Results showed that the expression of aggrecan protein was not markedly altered in articular chondrocytes from TAM-treated *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice compared to that in controls, while it is slightly down-regulated in articular chondrocytes from TAM-treated *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice (Fig. 4A and B). Mmp13 protein is accumulated both in the middle and deep layer of articular cartilage of both TAM-injected *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ and *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice (Fig. 4A, C). Likewise, the expression levels of catabolic ECM enzymes Mmp3 were significantly increased in both TAM-injected *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ and *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice (Suppl. Fig. 3A and B). In agreement with our previous findings, articular chondrocytes in the superficial layer and middle layer of articular cartilage displayed hypertrophic differentiation with increased expression of Col10 and Runx2 proteins in TAM-induced *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ and *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ (Fig. 4A, D-E).

3.5. Kindlin-2 loss similarly accelerates the DMM-induced OA lesions in *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ and *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice

We finally examined the susceptibility of *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mouse model to surgically induced OA lesions. Mice were injected with TAM or corn oil at three-month-old. After six months of injection, DMM and sham surgeries were performed on the right knee and left knee of the hindlimbs, respectively. Knee joint samples were collected at 3 months after surgery for further analyses. Results from μCT analyses showed that the surface of the knee joint was smooth without obvious osteophyte hyperplasia for the corn oil sham group. As expected, DMM increased the mineralized volume of the meniscus, promoted synovial osteophyte formation at the joint edge, and induced subchondral bone sclerosis (Fig. 5A and B). Mice with DMM surgeries showed marked pathological features of OA, indicating that the DMM OA model was successfully established. In the DMM model, the TAM-injected group markedly exacerbated the progression of OA, with meniscus mineralization, marginal synovial osteophyte formation, and subchondral bone sclerosis being more drastic than those in the control DMM model, indicating that the lacking Kindlin-2 in Prg4-expressing chondrocytes accelerates the progression of DMM-induced OA-like phenotype (Fig. 5A and B). To

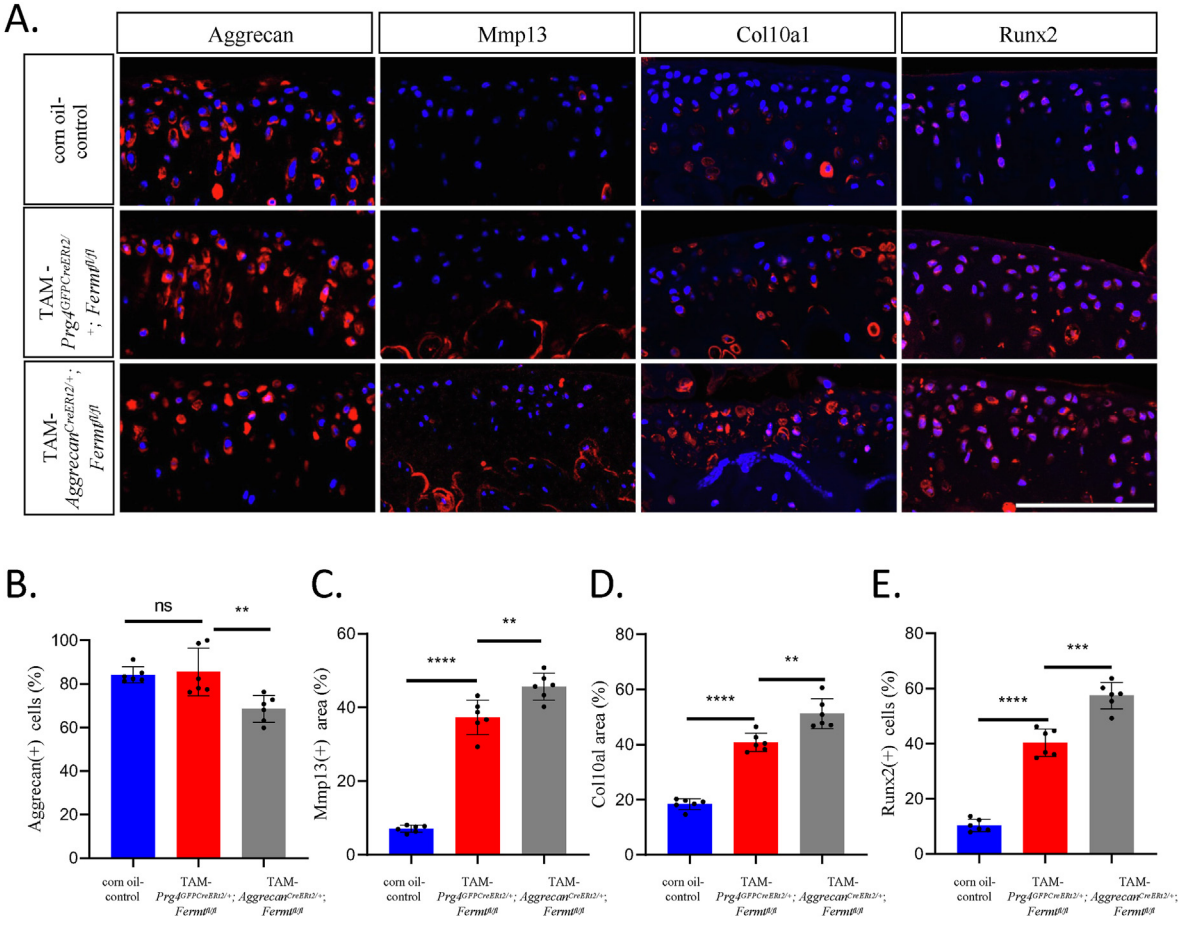

Figure 4. Immunofluorescence staining of cartilage degeneration-associated factors (A) IF staining of Aggrecan, Mmp13, Col10a1 and Runx2 on knee joint sections. Scale bar, 100 μm (B–E). Quantitative analysis of the fluorescent signal intensities of Aggrecan (B), Mmp13 (C), Col10a1 (D) and Runx2 (E). Quantitative data are shown as mean ± s.d. NS, no statistical significance, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

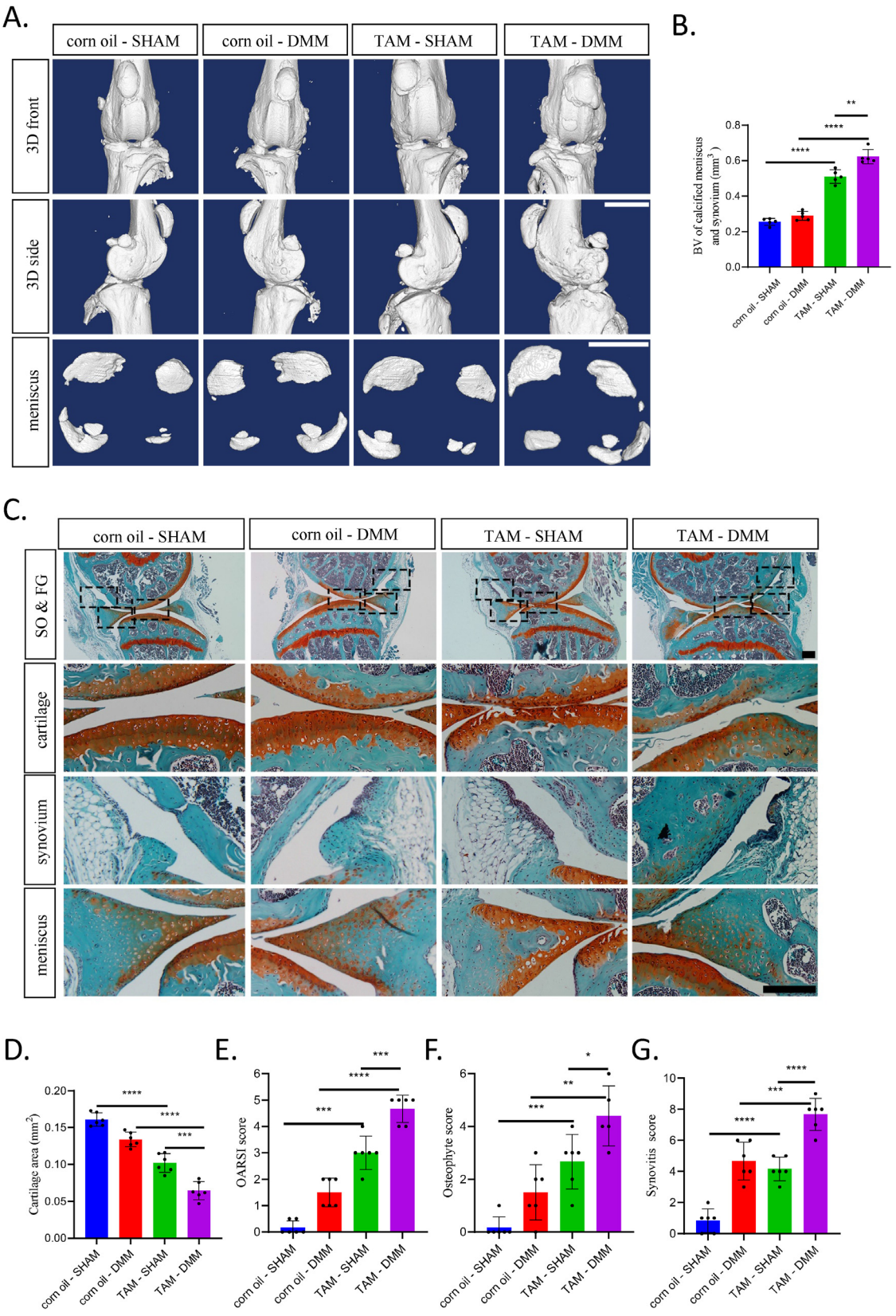

Figure 5. μCT and histological analyses of the knee joint sections from DMM-treated *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice (A) μCT scans 3D pictures of knee joints from corn oil and TAM-injected *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice at 8 weeks after sham or DMM surgery. Scale bar, 1 mm. n = 5 mice per group and results from one representative replicate are shown (B) The quantification BV of calcified meniscus and synovium (C) SO&FG-stained knee joint sections of corn oil and TAM injected *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice at 8 weeks after sham or DMM surgery (D–G) Quantification of OARSI score (B), cartilage area (C), osteophyte score (D) and synovitis score (E) was performed using histological sections. Quantitative data are shown as mean ± s.d. NS, no statistical significance, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. n = 6 mice per group and results from one representative replicate are shown.

confirm that with histological analysis, SO/FG staining was down, and results showed that the articular cartilage of the corn-oil sham model was thicker, with more proteoglycan content, less synovial tissue, and no pathological features of OA (Fig. 5C). In the corn-oil DMM group, there were pathological features of OA, such as knee cartilage erosion, proteoglycan loss, osteophyte and synovial hyperplasia at the joint edge, and the corresponding cartilage injury score, osteophyte score, and synovitis score increased (Fig. 5C–G), indicating that OA modeling was successfully established. Compared with the corn oil-sham group, TAM-sham showed obvious pathological features of spontaneous OA, such as cartilage injury, joint edge osteophyte formation, and synovial inflammatory infiltration, and the corresponding cartilage injury score, osteophyte score, and synovitis score also increased (Fig. 5C–G). Consistent with previous μCT data, the pathological features of OA in the TAM-DMM model exhibited significant enhancement in cartilage erosion, proteoglycan loss, osteophyte, and synovitis and an increase in OARSI score in articular cartilage compared with those in corn-oil DMM mice (Fig. 5C–G), indicating that loss of Kindlin-2 in Prg4 positive chondrocytes could not only lead to spontaneous OA, but also promote DMM-induced OA lesions in adult mice.

4. Discussions

In this study, we establish that the *Prg4*^{GFPCreERT2} mouse line can be used to efficiently delete the expression of the key focal adhesion protein Kindin-2 in articular chondrocytes of the superficial middle layer of adult articular cartilage. Mice lacking Kindlin-2 in Prg4-expressing articular chondrocytes display OA-like lesions, suggesting that the *Prg4*^{GFPCreERT2} mouse line could be a useful tool for performing gene functional studies for OA research. Importantly, in this study, we have compared the OA-like phenotypes caused by Kindlin-2 deficiency mediated by *Prg4*^{GFPCreERT2} and *Aggrecan*^{CreERT2} transgenes. We find that the Kindlin-2 loss-induced OA-like lesions are significantly lighter in *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ than in *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice. All OA parameters, including the OARSI, synovitis, and osteophyte scores, are much less severe in *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ than in *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice.

It should be noted that the TAM-treated *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice have a 100% penetration to cause severe spontaneous OA in the knee joints and show joint defects in the temporomandibular joint (TMJ) [7,16]. In the TAM-injected *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mouse model, we generated 11 samples at different time points, and the penetration of OA-like phenotypes is also 100%, although the OA lesions are much less severe than those in *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice. The differences could be due to the variations between the deletion efficiency of the two Cre mouse lines. The loss of Kindlin-2 in adult chondrocytes may affect the morphology of the growth plate in the *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ mice, which might lead to an extra contribution to OA phenotypes. Our study provides an important reference for choosing tools for the mouse genetic study of OA.

Lubricin, encoded by the *Prg4* (proteoglycan 4) gene, is a secreted proteoglycan protein that is predominantly expressed by chondrocytes in the superficial zone of articular cartilage and synovial fibroblasts [10,11]. In this study, we used adult heterozygous *Prg4*$^{GFPCreERT2/+}$ to drive Cre recombinase expression because 9-month-homozygous mice *Prg4*$^{GFPCreERT2/GFPCreERT2}$ exhibit loss of articular chondrocytes in the upper superficial layer and proteinaceous deposit in articular cartilage, and displayed synovial hyperplasia [12], which could affect our observation of target gene functional study. In our study, using 3-month-old heterozygous *Prg4*$^{GFPCreERT2/+}$ mice to drive Cre recombinase expression by injecting TAM for 10 consecutive days could efficiently delete our target gene in the chondrocytes of the superficial and middle layer of articular cartilage without affecting the expression level of Prg4 protein or leading to any OA phenotypes. Our target gene was efficiently deleted at 2 months after TAM injections when we did not observe any notable changes in articular cartilage or other tissues. We started to observe cartilage lesions at 6 months after TAM injection, while we could observe obvious cartilage degradation in *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ as early as 3 months after TAM injection [7], suggesting Kindlin-2 efficiency induced by *Prg4*$^{GFPCreERT2/+}$ mice develops OA-lesions much slower than those induced by *Aggrecan*^{CreERT2}. Interestingly, we find that the levels of the acceleration of the DMM-induced OA lesions stimulated by *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ and *Aggrecan*$^{CreERT2/+}$; *Fermt2*$^{fl/fl}$ are similar. This may be because the superficial layer of the articular cartilage is the major initiation and contribution to DMM-induced OA lesions, in which both mice show a similar level of Kindlin-2 deletion.

The pathogenesis of OA is extremely complex, with feedback and interactions of synovial joint tissues, including articular cartilage, synovium, subchondral bone, and others [1]. How the molecular signaling crosstalk among all kinds of cell types leads to the initiation and progression of OA is still unclear. The limitation of using the *Prg4*^{GFPCreERT2} mouse line to study gene functions in articular chondrocytes is that the Cre expression driven by the internal *Prg4* promoter is not only limited in those articular chondrocytes but also in some synovial cells, as reported before [12], which might complicate the data interpretation in terms of facing the difficulty to identify the original initiated tissue leading to the phenotype. In this study, we did detect Cre expression in the synovium region in *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice by IF staining; however, Kindlin-2 protein can be detected in a very limited number of synovial cells. Therefore, we did not observe any decrease of Kindlin-2 expression in the synovial cells in TAM-induced *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice without knowing the reason (Data not shown). On the other hand, the advantage of using the *Prg4*^{GFPCreERT2} mouse line in the OA study compared to the most used *Aggrecan*^{CreERT2} and *Col2a1*^{CreERT2} lines is that the Cre is expressed in the growth plate chondrocytes in *Prg4*^{GFPCreERT2} mice, which should have less direct effects on the subchondral bone which is another tissue that might affect the onset and development of OA. Interestingly, we found that bone volume fraction was significantly decreased with a lower number of osterix-positive osteoblasts and reduced TRAP staining in the subchondral bone in the primary ossification center in TAM-induced *Prg4*$^{GFPCreERT2/+}$; *Fermt2*$^{fl/fl}$ mice compared to controls, suggesting that articular cartilage lesions initiated from loss of Kindlin-2 might trigger biological changes of subchondral bone during the development of OA.

One major limitation of the *Aggrecan*^{CreERT2} mouse line as a tool for OA research is related to the fact that the TAM-induced Cre recombinase is extensively expressed in all chondrocytes, including the growth plate cartilage, which is an important tissue cross-talking with the subchondral bone and affects the biology of articular cartilage in adult mice. *Prg4*^{GFPCreERT2} mouse line, which expresses Cre recombinase exclusively in articular chondrocytes and synovial cells, is demonstrated to be a useful tool for OA research in this study. This study provides useful information for investigators to choose appropriate Cre mouse lines for their research in articular cartilage biology.

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Ethical statement

All research protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Southern University of Science and

Technology. All relevant guidelines for the work with animals were adhered in this study.

Declaration of competing interest

The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2023.05.005.

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