The Impact of Proteoglycan-4 and Parathyroid Hormone on Articular Cartilage

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Abstract

Proteoglycan-4 (*Prg4*) protects synovial joints from arthropathic changes by mechanisms that are incompletely understood. Parathyroid hormone (PTH), known for its anabolic actions in bone, increases *Prg4* expression and has been reported to inhibit articular cartilage degeneration in arthropathic joints. To investigate the effect of Prg4 and PTH on articular cartilage, 16-week-old *Prg4* mutant and wildtype mice were treated with intermittent PTH (1–34) or vehicle control daily for six weeks. Analyses included histology of the knee joint, micro-CT of the distal femur, and serum biochemical analysis of type II collagen fragments (CTX-II). Compared to wildtype littermates, *Prg4* mutant mice had an acellular layer of material lining the surfaces of the articular cartilage and menisci, increased articular cartilage degradation, increased serum CTX-II concentrations, decreased articular chondrocyte apoptosis, increased synovium SDF-1 expression, and irregularly contoured subchondral bone. PTH-treated *Prg4* mutant mice developed a secondary deposit overlaying the acellular layer of material lining the joint surfaces, but PTH-treatment did not alter signs of articular cartilage degeneration in *Prg4* mutant mice. The increased joint SDF-1 levels and irregular subchondral bone found in *Prg4* mutant mice introduce novel candidate mechanisms by which Prg4 protects articular cartilage.

Keywords

Proteoglycan-4; PTH; cartilage; degeneration; SDF-1

INTRODUCTION

The proteoglycan-4 (*Prg4*) protein products, lubricin and superficial zone protein, are secreted glycoproteins expressed in synovial joints by synoviocytes and superficial zone articular chondrocytes[1]. Prg4 has well known boundary lubricating properties in joints, and has actions inhibiting cell adhesion and suppressing synoviocyte proliferation[1,2,3]. Characterization of the joints in *Prg4* mutant (*Prg4*$^{-/-}$) mice demonstrated that Prg4 protects synovial joints from early arthropathic changes, which are non-inflammatory in nature[1].

Prg4^{-/-} mice have hyperplasia of synovial intimal cells, disappearance of superficial zone chondrocytes, abnormal protein deposition on cartilage surfaces, articular cartilage degradation, and precocious joint failure[1,3].

Studies demonstrating an increased coefficient of friction in loaded joints from *Prg4*^{-/-} mice indicate that the boundary lubricating properties of Prg4 play an important role protecting cartilage from load induced wear[4,5]. In addition to validating the role of boundary lubrication, investigations of the *Prg4*^{-/-} mice have introduced novel candidate mechanisms by which Prg4 protects articular cartilage. Coles et al. postulated that lubricin's ability to form a surface covering maintains articular cartilage surface integrity by actions beyond boundary lubrication[3]. Based on findings that Prg4 restricts synovial intimal cell proliferation via an adhesion dependent mechanism, Rhee et al. speculated that Prg4 actions limiting synovial cell proliferation protect articular cartilage from physical invasion by hyperplastic synovium[1].

PRG4 loss-of-function mutations in humans result in an autosomal recessive disorder, camptodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome, which is characterized by precocious joint failure recapitulated in the *Prg4*^{-/-} mice[6]. In addition to CACP syndrome, reduced PRG4 levels have been associated with osteoarthritis (OA) and rheumatoid arthritis (RA), the two most common degenerative joint conditions[7,8]. These findings of decreased PRG4 levels in arthropathic joints, coupled with studies demonstrating that exogenous lubricin administration inhibits the onset and progression of OA in rats suggest that PRG4 plays a regulatory role in the pathogenesis of degenerative joint disease[9,10].

Intermittent parathyroid hormone (PTH) (1–34) injection, currently the only FDA approved anabolic therapy for osteoporosis, has been reported to inhibit the progression of articular cartilage degeneration in rodent OA models[11,12]. Subcutaneous intermittent PTH injection has recently been shown to increase *Prg4* and proteoglycan expression in articular cartilage of degenerating joints responsive to PTH treatment[12]. While it has been postulated that PTH protects articular cartilage from arthropathic changes by restricting chondrocyte maturation, PTH actions increasing *Prg4* and proteoglycan expression in articular chondrocytes are strong candidate mechanisms mediating PTH chondroprotective properties[11,12].

Prg4^{-/-} mice were studied to further elucidate Prg4 functions protecting articular cartilage *in vivo*. In light of recent *in vivo* findings that *Prg4* is a PTH-responsive gene in degenerating articular cartilage protected by intermittent PTH-treatment[12], we investigated the impact of PTH on articular cartilage degeneration in the arthropathic joints of adult *Prg4*^{-/-} mice.

METHODS

Breeding of *Prg4* mutant mice, and administration of PTH

Prg4 mutant (*Prg4*^{-/-}) mice, generated by homologous recombination in 129Sv/Ev-derived embryonic stem cells[1], were backcrossed onto a C57BL6 genetic background. 16-week-old *Prg4*^{-/-} and *Prg4* wildtype (*Prg4*^{+/+}) littermate mice were given daily subcutaneous injections of either recombinant human PTH (1–34) (50 μg/kg) (Bachem, Torrence, CA) or vehicle (0.9% NaCl) control for 6 weeks. The daily 50μg/kg PTH (1–34) dose, a moderate murine anabolic dose, was carried out for 6 weeks duration to thoroughly assess PTH actions on skeletal and joint remodeling. 24 hours following final injection, mice were sacrificed and tissues harvested.

Histology

Hindlimbs were fixed in 10% phosphate-buffered formalin (PBF) for 48 hours at 4°C, and then decalcified in 14% EDTA pH 7.2 for 14 days at room temperature. Knee joints were embedded in paraffin. 5 μm serial sagittal knee sections were cut, laterally to medially across the knee joint. H&E staining was performed to assess histopathology. Safranin O-fast green staining was carried out to evaluate proteoglycan content and articular cartilage degradation. The Osteoarthritis Research Society International (OARSI) osteoarthritis cartilage histopathology assessment system was adopted to score the severity and extent of articular cartilage degradation in the tibial plateau[13,14]. All histological scoring was performed in a blinded manner.

Immunohistochemistry (IHC)

IHC was carried out in sagittal knee sections to analyze the expression of type 1 collagen (col1), type 2 collagen (col2), and stromal cell-derived factor-1 (SDF-1). Staining was performed with antibodies to col1, col2, SDF-1, or an IgG matched isotype control (ABCAM, Cambridge, MA), in conjunction with a HRP Cell & Tissue Staining Kit (R&D Systems, Minneapolis, MN). Col1 and col2 labeling were evaluated to elucidate the cartilaginous make-up of the ectopic deposits lining the joint surfaces of $Prg4^{-/-}$ mice. SDF-1 labeling was assessed to characterize the localization and intensity of SDF-1 expression in synovial cells. Representative images (20X) of the synovium were acquired in the anterior aspect of the knee joint with Olympus FV-500 confocal microscope, and SDF-1 intensity was graded as (1) low, (2) moderate, (3) high.

Serum biochemical analyses

Via cardiac puncture at euthanasia, serum was isolated, and stored at −80°C. Serum Pre-Clinical CartiLaps ELISA (Immunodiagnostic Systems, Fountain Hills, AZ) was carried out in duplicate to assess degradation products of C-terminal telopeptides of type II collagen (CTX-II).

TUNEL staining

TUNEL staining was carried out in sagittal knee sections to assess chondrocyte apoptosis in articular cartilage. The region of interest was the full thickness cartilage of the tibial plateau, between the anterior and posterior menisci. Exposed ends of DNA fragments, induced by apoptotic signals, were bound by Klenow enzyme and subsequently detected following the protocol of the FragEL™ DNA Fragmentation Detection Kit (EMD Chemicals, Gibbstown, NJ).

Immunofluorescence (IF)

SDF-1 IF was carried out in sagittal knee sections to quantitatively assess SDF-1 expression in the synovium. SDF-1 IF was performed using Zenon Alexa Fluor 488 Rabbit Labeling Kit (Invitrogen, Carlsbad, CO) and SDF-1 alpha rabbit polyclonal AB (ABCAM). Sections were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Representative images (20X) of the synovium were acquired in the anterior aspect of the knee joint with Olympus FV-500 confocal microscope, and analysis was performed using Image Pro Plus 5.1 Software.

Micro-computed tomography (micro-CT)

Femurs were fixed in 10% PBF for 48 hours at 4°C, stored in 70% EtOH, and scanned in H_2O at an 18-μm isotropic voxel size via eXplore Locus SP (GE Healthcare Pre-Clinical Imaging, London, ON, Canada). Calibrated three-dimensional images were reconstructed,

and distal femur subchondral bone was assessed via GE Medical Systems MicroView v2.2 software.

Statistical analyses

Unpaired *t* tests and the Mann-Whitney U test (semi-quantitative histological scoring) were performed using GraphPad Instat Software (GraphPad Inc., San Diego, CA). Significance was noted at p<0.05. Data are presented as mean ± standard error of the mean.

RESULTS

Histological abnormalities in the cartilage and meniscus of *Prg4*$^{-/-}$ mice

Vehicle- and PTH-treated *Prg4*$^{-/-}$ mice had an acellular layer of material lining the surfaces of the articular cartilage and menisci (Fig. 1). Rhee et al. speculated that the acellular layer of material lining the *Prg4*$^{-/-}$ joint surfaces is composed of adsorbed proteins from the synovial fluid[1]. Interestingly, the formation of a secondary deposit overlaying the acellular layer of material lining the surfaces of the articular cartilage and posterior meniscus was detected in 5 of 10 PTH-treated *Prg4*$^{-/-}$ mice, whereas 0 of 10 vehicle-treated *Prg4*$^{-/-}$ mice demonstrated these deposits. PTH-treatment had no observed histologic effects on the joints of *Prg4*$^{+/+}$ mice (Fig. 1).

Nature of the deposits overlying the joint surfaces of *Prg4*$^{-/-}$ mice

The acellular layer lining the surfaces of articular cartilage and menisci in *Prg4*$^{-/-}$ mice, and the overlaying secondary deposit noted in PTH-treated *Prg4*$^{-/-}$ mice were weakly labeled by Safranin O staining, suggesting the deposits were not primarily proteoglycan in nature (Fig. 2). Col2 labeled the most outward layer of the joint surfaces, but failed to stain underlying layers (Fig. 2). Similar to a narrow intense band of col2 lining physiologic joint surfaces in *Prg4*$^{+/+}$ mice, a thin intense band of col2 labeled the periphery of the acellular layer lining the joint surfaces in vehicle-treated *Prg4*$^{-/-}$ mice. Col2 labeling of the secondary deposit in PTH-treated *Prg4*$^{-/-}$ mice was not restricted superficially to the periphery but instead permeated through the deposit. Col1 staining was not localized to the ectopic deposits in vehicle- or PTH-treated *Prg4*$^{-/-}$ mice, demonstrating that the deposits do not have a fibrocartilaginous composition (Fig. 2).

Articular cartilage degradation in *Prg4*$^{-/-}$ mice

Prg4$^{-/-}$ mice had articular cartilage surfaces characterized by fibrillations, fissures, and mid-zone excavations (Fig. 3A). Histologic scoring indicated that the severity and extent of articular cartilage degradation was greater in *Prg4*$^{-/-}$ mice, and that degradation was not altered by PTH-treatment (Fig. 3B). Serum C-terminal telopeptides of type II collagen (CTX-II), were assayed as a systemic marker for articular cartilage degradation. Serum CTX-II levels were increased in *Prg4*$^{-/-}$ vs. *Prg4*$^{+/+}$ mice, and were not affected by PTH-treatment (Fig. 3C).

Decreased articular chondrocyte apoptosis in *Prg4*$^{-/-}$ mice

TUNEL staining of sagittal knee sections was performed to evaluate the effect of Prg4 and the impact of PTH-treatment on articular chondrocyte apoptosis. The frequency of TUNEL+ chondrocytes was significantly lower in *Prg4*$^{-/-}$ mice. There was no effect of PTH on the frequency of TUNEL+ chondrocytes in the articular cartilage of either *Prg4*$^{-/-}$ or *Prg4*$^{+/+}$ mice (Figs. 4A–C). Total number of chondrocytes per cartilage area were similar in *Prg4*$^{-/-}$ vs. *Prg4*$^{+/+}$ mice, and PTH did not alter total number of chondrocytes per cartilage area (Fig. 4D).

Increased SDF-1 expression in the synovium of *Prg4*^{−/−} mice

Expression of SDF-1, a chemokine best known for physiologic roles regulating the homing and mobilization of hematopoietic cells in the bone marrow[15], was assessed in the knee joint since increased SDF-1 levels have been reported to play a multifaceted role in degenerative joint disease[16,17,18]. Additional rationale for evaluating SDF-1 expression in the joints of *Prg4*^{−/−} mice was based on findings that *Prg4*^{−/−} mice have altered SDF-1 expression in bone marrow[19].

SDF-1 IF labeling of sagittal knee sections revealed increased SDF-1 in the synovium of *Prg4*^{−/−} mice (Figs. 5A, C). While the *Prg4*^{+/+} synovium had low to moderate SDF-1 expression limited to the surface synovium approximating lining synovial intimal cells, the *Prg4*^{−/−} synovium had intense SDF-1 expression throughout the hyperplastic tissue matrix. SDF-1 IHC labeling demonstrated the increased SDF-1 expression in the *Prg4*^{−/−} synovium was localized to synoviocytes within the hyperplastic tissue matrix (Figs. 5B, D). There was no difference in SDF-1 expression in the synovium of vehicle- vs. PTH-treated mice (Figs. 5A–D).

Irregular subchondral bone in *Prg4*^{−/−} mice

Based on reports that *Prg4* is expressed in bone[20] and radiographic findings that *Prg4*^{−/−} mice and humans afflicted by CACP have periarticular osteopenia[1,6], micro-CT analysis was employed to assess subchondral bone morphology in the distal femur of *Prg4*^{−/−} mice (Fig. 6). Sagittal section images of the distal femur confirmed the expected bone forming anabolic effects of PTH in the metaphyseal trabecular and cortical bone of *Prg4*^{−/−} and *Prg4*^{+/+} mice (Fig. 6A). While a side view (Fig. 6B) and posterior view (Fig. 6C) demonstrated no obvious morphological differences, the anterior view of the distal femur (Fig. 6D) revealed abnormalities in the surface contour of the subchondral bone in *Prg4*^{−/−} mice. Specifically, the subchondral bone of the patellar groove had irregular contours characterized by a medial clefting with elevated lateral ridges (Fig. 6D). Perpendicular to a line drawn through the height of the lateral ridges, the depth of the patellar groove was greater in *Prg4*^{−/−} vs. *Prg4*^{+/+} mice (Figs. 6D, E).

DISCUSSION

The present study investigated Prg4 actions regulating articular cartilage protection, and tested whether intermittent PTH administration could inhibit articular cartilage degeneration in *Prg4* mutant mice. Treatment began at 16 weeks, an age at which *Prg4* mutant mice have an intermediate degree of joint failure, as indicated by a significant loss of cartilage structure, stiffness, and frictional properties[1,3]. The intermediate stage was evaluated since this is the period when the majority of arthropathic patients become symptomatic and present for clinical care. While intra-articular injection delivers a controlled local dose to a targeted joint, PTH was administered via subcutaneous injection to avoid the risk of iatrogenic trauma to the joints.

In bone PTH exerts its anabolic actions by signaling at stromal/osteoblastic cells, the predominant PTH/PTH-related protein (PTHrP) receptor (PPR) expressing cells[21]. During skeletal development the PPR is expressed in growth plate prehypertrophic zone chondrocytes, contributing to a signaling feedback loop involving indian hedgehog which regulates endochondral bone formation[22]. Basal PTHrP appears to regulate articular chondrocyte maintenance[23] and PPR expression is upregulated in articular chondrocytes within OA joints[12], yet the role of PPR signaling in articular cartilage is largely unknown. *In vitro* studies demonstrating that PTH administration inhibits terminal differentiation of articular chondrocytes, and *in vivo* OA studies reporting that PTH treatment significantly

suppresses chondrocyte maturation and apoptosis imply that PTH protects articular cartilage from arthropathic changes by restricting chondrocyte maturation[11,12]. The recent report that PTH upregulates *Prg4* and proteoglycan expression in articular cartilage within degenerating joints responsive to PTH, highlights *Prg4* as a strong candidate regulator of PTH actions inhibiting articular cartilage degeneration in arthropathic joints[12].

Surprisingly, the only observed PTH effect in the joints of *Prg4* mutant mice was that PTH induced the formation of a secondary deposit overlaying the acellular layer of material lining the surfaces of the articular cartilage and menisci in half of the PTH-treated mutant animals. The col2 IHC labeling through the full thickness of the secondary deposit in PTH-treated *Prg4* mutant mice vs. the restricted col2 labeling at the outward periphery of the acellular layer lining the joint surfaces in vehicle-treated *Prg4* mutant mice substantiates that the secondary deposit was due to PTH-treatment. Taking into consideration that subcutaneous PTH administration increased *Prg4* mRNA and proteoglycan expression in the articular cartilage of degenerating OA joints within wildtype mice[12], the finding that the secondary deposit lining the joint surfaces of PTH-treated *Prg4* mutant mice was weakly proteoglycan in nature suggests that *Prg4* supports PTH actions increasing proteoglycan expression in arthropathic joints. Although the composition of this secondary deposit is unclear at present, its formation indicates that PTH signaling was functional within the joints of the *Prg4* mutant mice. Similar to reports that intermittent PTH did not affect articular chondrocytes in the knee joints of healthy rats or sham operated mice[11,12], intermittent PTH had no impact on the joints of *Prg4* wildtype mice.

While the minimal impact of PTH in the arthropathic joints of *Prg4* mutant mice implies that Prg4 is required for PTH to exert chondroprotective effects, it is unclear whether Prg4 supports PTH actions inhibiting articular cartilage degeneration. Keeping in mind the lack of Prg4 throughout development in *Prg4* mutant mice is different than acutely reduced Prg4 levels in degenerating joints, it is possible that the damage caused by the complete absence of Prg4 cannot be modified by the actions of PTH.

The unexpected finding that TUNNEL staining was significantly decreased in articular chondrocytes in *Prg4* mutant mice suggests that *Prg4* mutant articular chondrocytes may have cell autonomous alterations protecting against apoptosis. Taking into consideration a proposed hypothesis that PTH protects articular cartilage from arthropathic changes by restricting chondrocyte maturation[11,12], PTH may not have altered articular cartilage degeneration in *Prg4* mutant mice due to the anti-apoptotic nature of the *Prg4* mutant chondrocytes.

SDF-1 is expressed and secreted by synoviocytes, and the SDF-1 receptor, CXCR4, is expressed by articular chondrocytes[24]. While there is no known physiologic role for SDF-1/CXCR4 paracrine signaling in synovial joints, increased SDF-1 levels in the joints of OA and RA patients has been implicated in the degeneration of articular cartilage[16,17,18]. In light of *in vitro* articular chondrocyte studies demonstrating that supra-physiologic SDF-1 levels stimulate the secretion of matrix metalloproteinase-3 (MMP-3), MMP-9, and MMP-13[16,17], we speculate that increased SDF-1 levels in the joints of *Prg4* mutant mice stimulate articular chondrocytes to release MMPs which mediate the accelerated degradation of articular cartilage. Since MMP mediated degradation of articular cartilage is required for the release of type 2 collagen fragments into the circulation, the increased serum CTX-II levels observed in this study provides evidence that MMP activity is upregulated in the joints of *Prg4* mutant mice.

The irregularly contoured subchondral bone surface of the patellar groove in *Prg4* mutant mice illustrates that *Prg4* loss-of-function mutation-induced changes in the joints are not

limited to the synovium and cartilage. Of interest, prior work has suggested that changes in the shape and contour of the subchondral bone adversely impact the mechanical loading properties of the adjacent articular cartilage, which has been speculated to induce pathologic changes in cartilage structure and integrity[25]. Regardless of whether the irregularly contoured subchondral bone in *Prg4* mutant mice is due to Prg4 actions supporting skeletogenesis or protecting articular cartilage, the altered subchondral bone morphology likely contributes to the degradation of articular cartilage in *Prg4* mutant mice.

This investigation, which did not demonstrate a protective effect of PTH in the arthropathic joints of mature *Prg4* mutant mice, uncovered novel candidate mechanisms by which Prg4 protects articular cartilage. Increased SDF-1 levels in the joints of *Prg4* mutant mice suggest that Prg4 functions as an as an upstream regulator of catabolic cytokine expression in degenerating joints. Although it is unknown whether changes in subchondral bone precede or are secondary to alterations in articular cartilage, the irregularly contoured subchondral bone likely contributes to the disruption of the articular cartilage in *Prg4* mutant mice. In summary, Prg4 is a dynamic factor that protects articular cartilage by diverse mechanisms.

Acknowledgments

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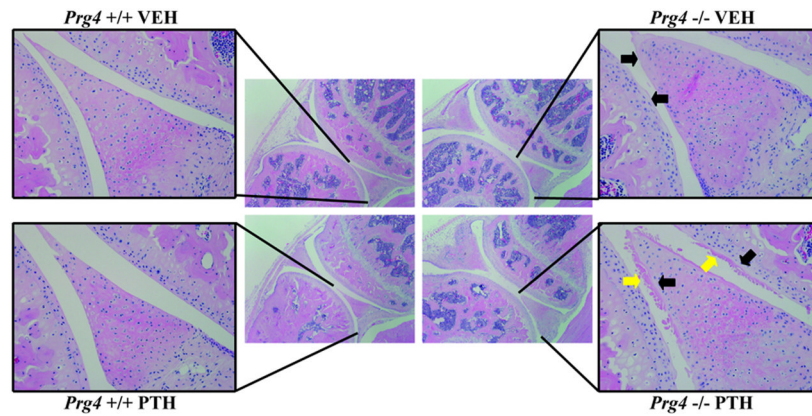
Figure 1.
Knee joint histopathology. 16-week-old *Prg4* mutant (*Prg4*$^{-/-}$) and wildtype (*Prg4*$^{+/+}$) mice were administered intermittent PTH (1–34) (50µg/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injection daily for 6 weeks. Knee joints were isolated from 22-week-old *Prg4* mice for histological evaluation. Representative images of H&E stained sagittal knee sections (4X), with (**inset**) 20X image of the articular cartilage and posterior meniscus (**black arrows** indicate the acellular layer lining joint surfaces; **yellow arrows** indicate the secondary deposit associated with PTH-treatment).
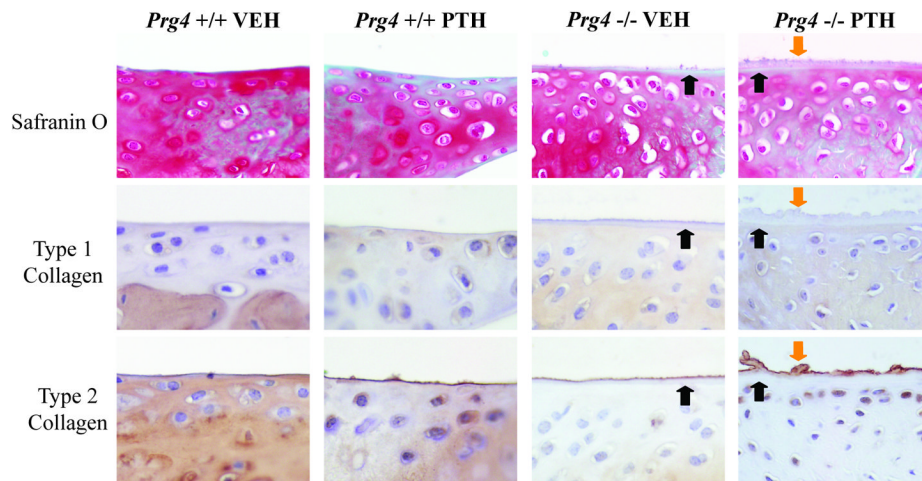
Figure 2.
Nature of the deposits overlaying the joint surfaces of *Prg4* mutant mice. Representative images of meniscal surfaces (40X) in sagittal knee sections labeled with Safranin O-fast green stain, type 1 collagen IHC stain, type 2 collagen IHC stain (**black arrows** indicate labeling of the acellular layer lining joint surfaces; **orange arrows** indicate labeling of the secondary deposit associated with PTH-treatment).
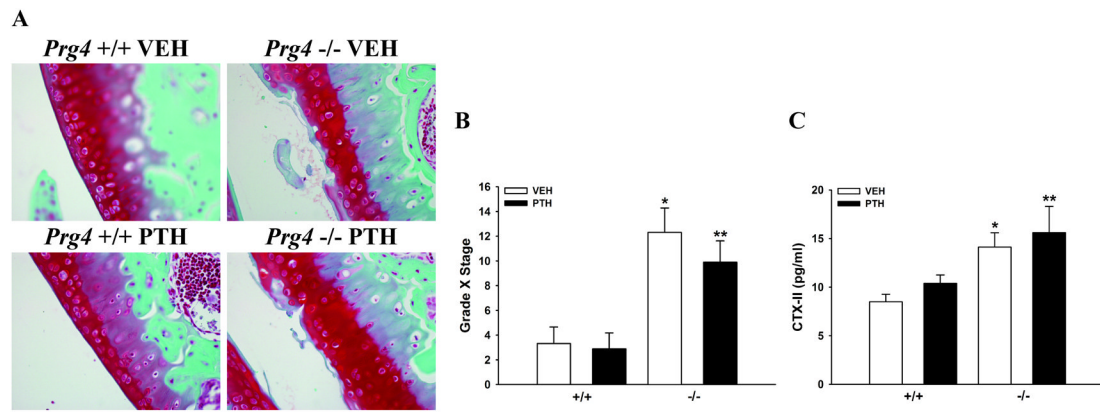
Figure 3.
Articular cartilage degradation. **A**: Representative images of Safranin O-fast green stained articular cartilage (40X) in sagittal knee sections. **B**: Bar graph represents the OARSI score (Grade X Stage) for the articular cartilage in the tibial plateau (n=8–10/gp). *p<0.01 vs. +/+ VEH; **p<0.01 vs. +/+ PTH. **C:** Bar graph represents serum C-terminal telopeptides of type II collagen (CTX-II) levels (n=8–10/gp). *p<0.01 vs. +/+ VEH; **p=0.08 vs. +/+ PTH.
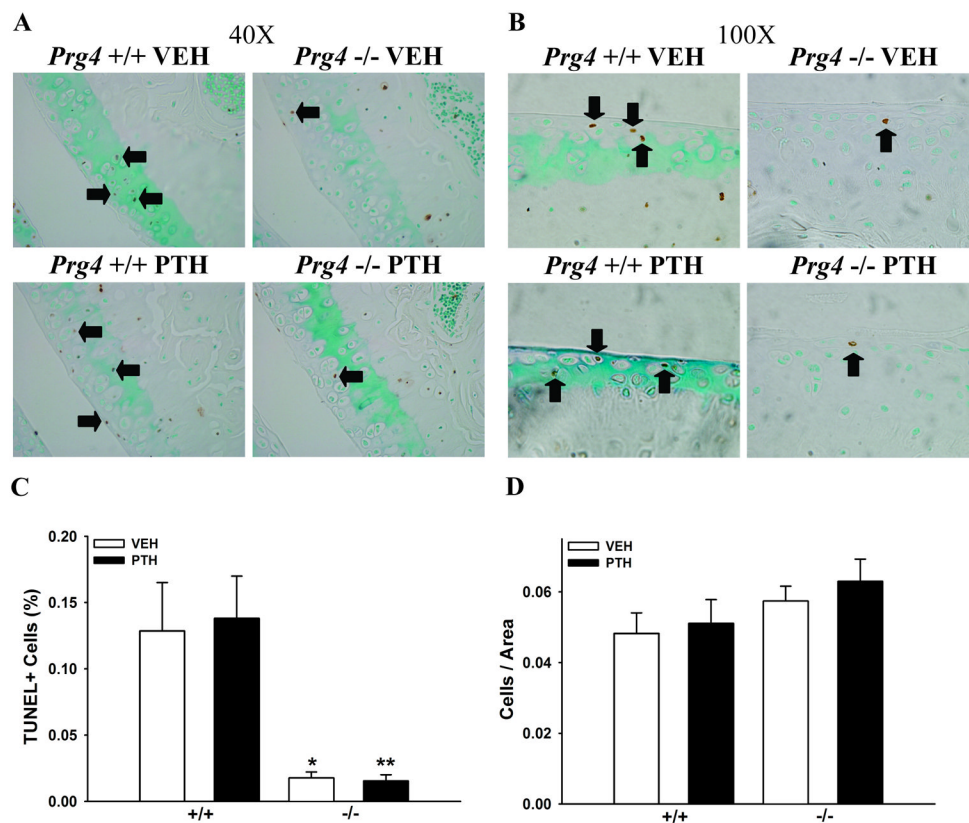
Figure 4.
Articular chondrocyte apoptosis. Representative images of TUNEL stained articular chondrocytes in sagittal knee sections: (**A**) 40X, (**B**) 100X (**black arrows** point to selected TUNEL+ chondrocytes). **C**: Bar graph represents the number of TUNEL+ chondrocytes per total chondrocytes (n 5/gp). *p<0.01 vs. +/+ VEH; **p<0.001 vs. +/+ PTH. **D**: Bar graph represents the total number of chondrocytes per cartilage area (n 5/gp).
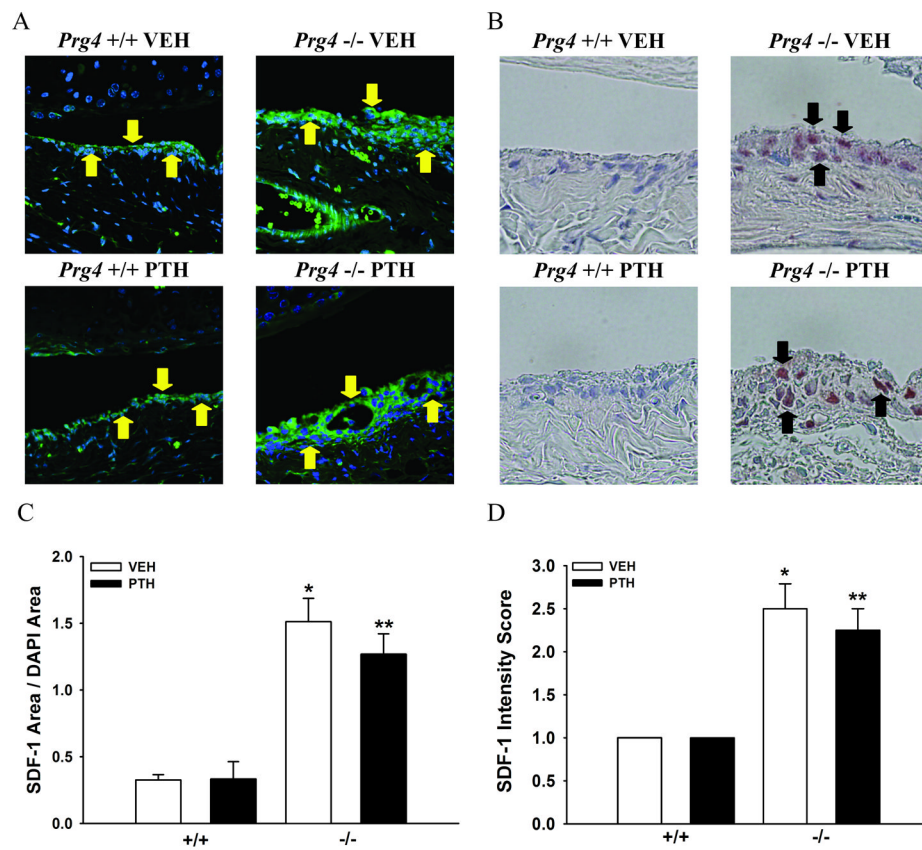
Figure 5.
Synovium SDF-1 expression. **A**: Representative images of stromal cell-derived factor-1 (SDF-1) immunofluorescence labeled synovium (60X) (SDF-1/Green and DAPI/Blue) (**yellow arrows** indicate SDF-1+ synovium) in sagittal knee sections. **B**: Representative images of SDF-1 immunohistochemistry labeled synovium (60X) (SDF-1/Brown) (**black arrows** indicate SDF-1+ synovial cells) in sagittal knee sections. **C**: Bar graph represents SDF-1 area/DAPI area in the synovium (n 4/gp). $^{*}p<0.001$ vs. +/+ VEH; $^{**}p<0.01$ vs. +/+ PTH. **D**: Bar graph represents synovial cell SDF-1 intensity score (n=4/gp). $^{*}p<0.05$ vs. +/+ VEH; $^{**}p<0.05$ vs. +/+ PTH.
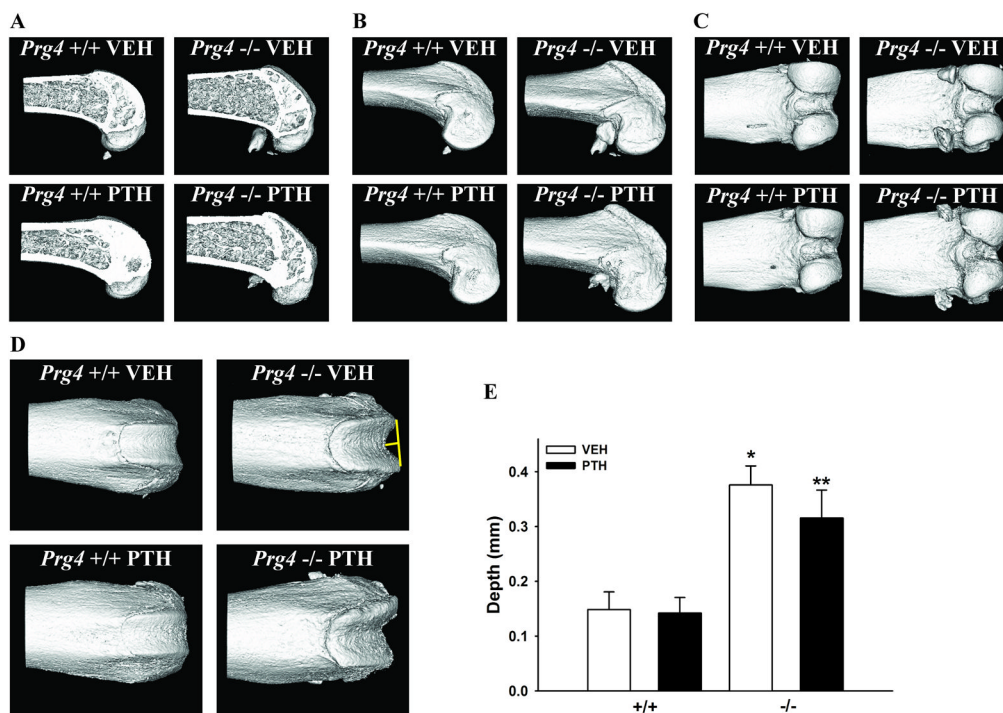
Figure 6.

Subchondral bone morphology. Representative micro-CT images of the distal femur subchondral bone: (**A**) sagittal section view, (**B**) side view, (**C**) posterior view, (**D**) anterior view (**yellow line** indicates depth of patellar groove). **E**: Bar graph represents patellar groove depth (n=7–8/gp). [*]p<0.001 vs. +/+ VEH; [**]p<0.01 vs. +/+ PTH.