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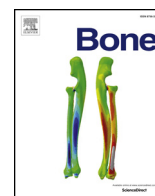
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Full Length Article

Selective inhibition of progesterone receptor in osteochondral progenitor cells, but not in mature chondrocytes, modulated subchondral bone structures



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ABSTRACT

Objective: The presence or relative proportion of progesterone nuclear receptors (PR) in different tissues may contribute to sexual dimorphism in these tissues. PR is expressed in chondrocytes, but its function is mostly unknown. We hypothesized that the PR may regulate chondrocyte metabolism and affect subchondral bone structure.

Methods: We utilized genetic fate mapping and immunohistochemistry to elucidate PR expression in and effect on cartilage. To define sex-dependent and chondrocyte-specific effects of the PR on subchondral bone, we selectively deleted PR in osteochondrogenic progenitor cells marked by Prx1 (Prx1; PRcKO) and Collagen 2 (Col2; PRcKO), or in matured chondrocytes marked by aggrecan (Acan; PRcKO) and evaluated subchondral bone structure at 4 months of age. Chondrocyte aging was monitored by anti-senescence marker p16INK4a, and MMP13, one of the Senescence-Associated Secretory Phenotype (SASP) components.

Results: Compared to wild-type (WT) mice, the female Prx1; PRcKO and the Col2; PRcKO mice had greater total subchondral bone volume and greater subchondral cortical bone thickness, with increased estimated subchondral bone stiffness and failure load in both female and male Col2; PRcKO mice. Moreover, Col2; PRcKO mice from both sexes had greater bone formation and bone strength at the femurs. In contrast, we did not observe any subchondral bone changes in Acan; PRcKO mice other than higher work-to-failure observed in the male Acan; PRcKO mice. Despite no detected difference in articular cartilage between the WT and the PR; chondrocyte conditional deletion mice, there were greater numbers of senescent chondrocytes and increased MMP13 expression, especially in the male mutant mice.

Conclusion: These findings suggest that selective inhibition of PR in osteoprogenitor cells, but not in terminally differentiated chondrocytes, induced an increased subchondral bone phenotype and high estimated subchondral bone strength, which might be associated with the development of osteoarthritis in older age.

1. Introduction

The presence or relative proportion of progesterone nuclear receptors (PR) in different tissues may contribute to sexual dimorphism in these tissues. The function of the PR in chondrocytes is mostly unknown. Over the past ten years, our research group and others have studied PR action on the skeleton. PR is expressed by cultured osteoblasts, osteoclasts [1–3], and chondrocytes [4] and is present *in vivo* in mouse bone [3,5]. Both genetic fate mapping and immunohistochemistry have identified PR (esp. PR-B) expression in

subchondral cartilage and the chondrocytes in the growth plate [5]. Additionally, conditional PR deletion in osteochondrogenic progenitor cells significantly suppressed immunomodulation pathways that might affect disease pathways involved in the development of osteoarthritis (OA) [6].

OA is characterized by degenerative changes in the whole joint leading to pain and ultimately physical disability. The risk of developing OA increases in post-menopausal women [7,8], suggesting a link between OA and hormonal status, which has traditionally been attributed to changes in estrogen levels. Women with low circulating

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estradiol levels were nearly twice as likely to develop OA or experience joint pain as those with higher hormone levels, after adjusting for age, injury history, and body-mass index [9]. Long-term estrogen replacement therapy might provide a moderate level of protection against knee OA [8,10–12] which might be due to attenuation of pro-inflammatory cytokines resulting from estrogen deficiency [8,13]. Gene polymorphisms in the estrogen receptors (ERs) α & β have been found to be associated with knee and hand OA [14–20]. The association of ER γ with OA has been confirmed in animal studies using an ER gain-of-function approach [21]. PR is recognized to be more important than the ER in immunomodulation of female prevalence dominant diseases such as systemic lupus erythematosus, rheumatoid arthritis, and OA [6,22,23]. Progressive OA has been reported to sometimes develop during pregnancy and may result from increased hormone levels, including progesterone [24]. Samples of synovial fluid from OA patients demonstrates high levels of cytokines as well as MMPs [25–27]. Progesterone has consistently been found to have inhibitory effects on MMPs in the endometrium and synovial joints [25,27,28], suggesting a connection between the PR and OA. In this study, we aimed to evaluate the role of the PR in subchondral bone. Since both global and bone-cell specific PR knockout mice have higher bone mineral density (BMD) than their wild type (WT) controls and there are observed sex differences in this relationship [5,6,29], we hypothesized that the PR may regulate osteochondrocyte metabolism and might have a role in regulating subchondral bone structures, which is related to OA incidence and progression [30–36]. For this reason, we set out to use a Cre recombinase driven approach to examine the effects of tissue-specific inactivation of PR in osteochondral progenitor cells or terminally differentiated chondrocytes on subchondral bone architecture.

2. Materials and methods

2.1. Mice

PR-flox mice were obtained from Baylor College of Medicine (Houston, TX, USA). A targeting vector designed to replace part of exon 2 of the PR gene with a selectable marker was employed to create a strain of mice carrying a conditional null PR allele [37]. Prx1-Cre, Col2a1-Cre^{ERT} (Col2-Cre), Aggrecan-Cre^{ERT} (Acan-Cre), PR-Cre, mT/mG, and Ai14D transgenic mice were obtained from the Jackson Laboratory (Farmington, CT, USA). The PR-Cre mice (B6.129S(Cg)-Pgtrm1.1(cre) Shah/AndJ) harbor an internal ribosome entry site and Cre recombinase gene downstream of the progesterone receptor transcriptional stop codon. As such, Cre expression was driven by the endogenous PR promoter/enhancer elements [38]. Ai14D mouse was bred with the PR-Cre mouse to track PR expression. Each Cre line was crossed with PR-flox mice to generate conditional PR knockout mice. Tamoxifen (Sigma-Aldrich, St Louis, MO, USA) was injected intraperitoneally at 5 mg/kg for three consecutive days to both the WT (Cre-) and the Cre+ mice when they were one month of age to activate the Cre and selectively remove PR from the targeted cells. We have previously determined that this dose did not significantly affect bone turnover [39]. Mice were euthanized at four months of age. Two fluorochrome labels were used, alizarin red (20 mg/kg) or calcein (10 mg/kg), which were given to the female WT mice at days -7 and -1 before euthanization. Since we found relatively weak alizarin red staining in the samples, we switched to double calcein labeling for female cKO mice and all the male mice.

PCR-based strategies were used for genotyping mouse genomic DNA. All animal work was done in compliance with the guiding principles of UC Davis' "Care and Use of Animals." Mice were housed in the animal facility under strictly controlled environmental conditions (12-hour light/dark cycle, room temperature 22 °C), and fed *ad libitum* (food and water). The Institutional Animal Care and Use Committee of the University of California, Davis approved the animal protocol.

2.2. Chondrogenic differentiation of bone marrow stromal cells

Mouse bone marrow stromal cells were extracted from PR-tdTomato mice and allowed to expand for 7–10 days. Cells were then placed at 1.25×10^5 density in 5 mL of the pre-warmed completed StemXVivo Chondrogenic Base Media with 0.5 mL of pre-warmed completed StemXVivo Chondrogenic Differentiation Media (R&D Systems, Minneapolis, MN, USA). Media was replaced every 2–3 days. Cell culture images were captured, and RNA was extracted from the cells at culture days 0, 8 and 14 to monitor the levels of PR expression.

2.3. MicroCT measurements

Right femurs were scanned and analyzed using VivaCT 40 (Scanco Medical, Bassersdorf, Switzerland) with a voxel resolution of 10 μ m in all three spatial dimensions and a mono-energetic (70 Kev) X-ray source. We evaluated the entire epiphyseal area. A total metaphysis tissue volume of 3–4 mm³ for each scan was used to obtain the subchondral bone volume/total volume (BV/TV) ratio, subchondral trabecular and cortical bone thickness [6,40,41].

We used a microCT-based finite-element model (FEA) to estimate the biomechanical properties of the subchondral bone. The same microCT images that we used for subchondral bone architectural evaluation were incorporated into the FEA model as previously described [42]. The 3D image voxels were converted to elements; each finite element model mesh had approximately 9–18 million elements. Each element segmented was assigned as bone using Young's modulus of 18 GPa and a Poisson ratio of 0.3 [43]. Details of the numerical method were published elsewhere [44,45]. The boundary conditions that defined the load platen-specimen interface were assumed to be frictionless. Subchondral bone stiffness and changes in the load-carrying capacity of the subchondral bone (estimated failure load) and apparent modulus were calculated from FEA analyses [46].

2.4. Bone and knee histology

The right distal femurs were collected, fixed in 4% formaldehyde with 10% sucrose (W/V) at 4 °C for 2 days, further dehydrated in 30% sucrose overnight and embedded in Optimal Cutting Temperature (OCT) medium (Thermo Fisher, Waltham, MA, USA). Cryosections were prepared and imaged with a Keyence BZ-X9000 all-in-one Fluorescence Microscope (Itasca, IL, USA). Surface-based bone formation was quantitated using Bioquant (Bioquant Image Analysis Corporation, Nashville, TN, USA) [6]. Primary antibodies against p16NIK4a and MMP13 (Abcam, Cambridge, MA, USA) and Alexa-Fluor 594 or 488-conjugated secondary antibody were counterstained with DAPI. Positive stained cells were counted using the cell count software in Keyence BZ-X9000 all-in-one imaging system (Itasca, IL, USA).

The left knee joints were fixed in 10% phosphate-buffered saline formalin for 2 days, decalcified in 10% EDTA for 3 weeks, and embedded in paraffin. Sections were stained with Safranin-O – Fast green for measurement of articular cartilage thickness, subchondral bone plate thickness, subchondral trabecular bone number and diameter, and cartilage content using Bioquant Imaging software (Bioquant Imaging System, Nashville, TN, USA) [40,41].

2.5. Whole bone strength measurements

Each right femur was loaded to failure along its long axis using an MTS 831 electro-servo-hydraulic testing system (MTS Systems Corp., Eden Prairie, MN, USA) at a displacement rate of 0.01 mm/s with a 90 N load cell. Sample loads and displacements were continuously recorded throughout each test. The maximum load was determined from the load-displacement curve, and the work-to-failure was calculated from the area under the load-displacement curve [3,47–49].

2.6. Statistical analysis

The results are expressed as mean \pm standard deviation for bone structure, bone turnover, and bone strength variables. Two-way ANOVA was used to account for genotype and sex. If significant differences were observed, then a Tukey's multiple comparisons test was used to assess pairwise comparisons. A value of $p < 0.05$ was considered statistically significant. Data were analyzed using the GraphPad Prism 7 software package (La Jolla, CA, USA).

3. Results

3.1. PR and Col2 expression in bone and joint

To characterize the pattern of PR expression in cartilage, we generated a reporter mouse strain PR-Cre; tdTomato, in which the Cre was driven by the endogenous PR promoter, and this activated the tdTomato expression. We collected the right distal femurs from PR-Cre; tdTomato mice at three weeks of age and created cryosections. Red fluorescence, corresponding to PR expression, was observed within some areas of the growth-plate cartilage as well as the subchondral cartilage area (Fig. 1A, B). Western Blot confirmed PR expression on protein extracted from distal femurs, including the growth plate (Fig. 1B). When we collected the bone marrow stromal cells (BMSCs) from 4-week old PR-Cre; tdTomato mice and cultured them in chondrogenic media for 14 days, we found decreased PR-RNA level when the BMSCs differentiated into chondrocytes, especially in the females (Fig. 1D, E). Lineage tracing studies *in vivo* suggested Col2-tdTomato+ cells were observed in the articular chondrocytes and growth plate chondrocytes (Fig. 1F, white arrowheads), while the Col1+ green cells were observed in the endosteal and periosteal bone surfaces (Fig. 1F).

3.2. Subchondral bone microarchitectural changes following PR conditional knockouts in osteochondrogenic progenitor cells with noted sex differences

We have previously reported that global PRKO or PR conditional knockouts (PRcKO) in osteoprogenitor cells using Prx1-Cre resulted in high bone mass in long bones, especially in females [5,6,29]. Similarly, in the current analysis, we found that subchondral bone volume was significantly higher in the female Prx1;PRcKO mice, with significantly thicker subchondral cortical bone and thinner articular cartilage (Fig. 2A). The subchondral cortical bone plate was thicker (blue arrowheads) than the WT mice with thinner articular cartilage (black arrowhead) at eight months of age in the female Prx1; PRcKO mice (Fig. 2B).

We next crossed Col2-Cre^{ERT} or Acan-Cre^{ERT} mice with PR-flox/flox mice to conditionally inactivate the PR gene in chondrocytes starting from one month of age, when tamoxifen was given to both wild-type (WT) and these PRcKO mice. Knee joints were collected from homozygous Col2; PRcKO and their WT littermates at four months of age. There were no differences in either body weights or femoral length between the WT and the Col2; PRcKO mice (data-on-file). Measurements of subchondral bone structures by microCT and histology yielded similar results. We chose to present results from microCT measurements in this report. Compared to the wild-type (WT) mice, only the male Col2; PRcKO mice had higher subchondral trabecular bone volume/tissue volume (Fig. 3A, B) but had similar subchondral trabecular bone and cortical bone thickness (Fig. 3A, B). The estimated stiffness was higher in both female and male Col2; PRcKO mice and failure load carried by the subchondral bone was significantly higher in the male Col2; PRcKO mice compared to the WT (Fig. 3C, D). Articular cartilage thickness measured by Safranin-O staining at the femoral chondral and was not different between WT and the Col2; PRcKO mice in either sex (Fig. 3E).

We failed to detect subchondral bone structures using Acan-Cre [50] to selectively removed PR from terminally differentiated chondrocytes.

Although the subchondral bone structure and estimated load carried by subchondral bone area were not different between Acan; PRcKO mice and their WT littermates in both sexes (Fig. 4), there was a 2-fold higher work-to-failure in the male Acan; PRcKO mice compared to their WT (Fig. 4).

These data suggested that PR inactivation in the osteochondral progenitor cells, but not in the terminally differentiated chondrocytes, altered subchondral bone architectures with noted sex differences. Articular cartilage plate thickness did not differ between the WT and mutant mice at four months of age.

3.3. PRcKO in chondrocytes marked by Col2 increased bone formation and bone strength

Consistent with the high subchondral bone mass phenotype in the epiphysis in the Col2; PRcKO mice, we also found the trabecular bone area to be higher at the distal femoral metaphysis (DFM) in Col2; PRcKO mice in both sexes (Fig. 5A). Dynamic histomorphometric analysis of bone formation measured at the distal femoral metaphysis in 4-month-old Col2; PRcKO mice revealed that the female and male Col2; PRcKO mice had 60–90% higher surface-based bone formation rate at both the endosteal and the periosteal bone surfaces compared to their WT littermates (Fig. 5B–E). Selective removal of PR from Col2+ chondrocytes increased bone strength (maximum load and Work-to-Failure) by 15–25% in the male Col2; PRcKO mice (Fig. 5F–G) as compared to their WT littermates. These data suggested that PR inactivation in the Col2+ chondrocytes increased bone formation and whole bone strength, especially in the male Col2; PR conditional KO mice.

3.4. Lack of PR in the Col2+ chondrocytes accelerated chondrocyte aging

To explore the cellular mechanisms of PR on chondrocytes that might affect the age-related cartilage degradation and sex differences, we examined the subchondral cartilage of 4-month-old Col2; PRcKO mice from both sexes. p16^{INK4a} was a marker for senescent cells [51,52]. Using anti-p16^{INK4a} staining as a marker for cell senescence, we observed approximately 18% subchondral cartilage and subchondral bone marrow cells were positive for p16^{INK4a} in all the mice (Fig. 6, white arrowheads). p16^{INK4a} expression was higher particularly in subchondral cartilage as well as in subchondral bone marrow in the male Col2; PRcKO mice. The number of cells positive for a senescence-associated secretory component, MMP13, was expressed at relatively low levels in the cartilage in the WT mice, but its expression was higher at subchondral cartilage/bone marrow in male Col2; PRcKO mice as compared to the WT mice (Fig. 6, red stained cells). Some p16^{INK4a} + senescent cells co-expressed MMP13 in both the female and male Col2; PRcKO mice, (Fig. 6, yellow heads). Taken together, these exploratory findings suggested that selective removal of PR from Col2+ chondrocytes was associated with higher levels of chondrocyte senescence and accompanied by higher MMP13 expressions in the male mutant mice, which might have accelerated cartilage degradation as they aged.

4. Discussion

Mice lacking PR in osteochondral progenitor cells displayed high subchondral trabecular bone volume and subchondral cortical bone plate thickness, which were associated with higher estimated stiffness and failure load carried by the subchondral bone. The femoral bone formation rate and bone strength were both significantly higher when PR was selectively removed from chondrocytes marked by Col2. Additionally, using Acan-cre [50,53], which was more specific to mature articular chondrocytes, did not cause development of a significant bone or joint phenotype except for higher Work-to-Failure in the male mutant mice. Moreover, cellular senescence, marked by p16^{INK4a}, and

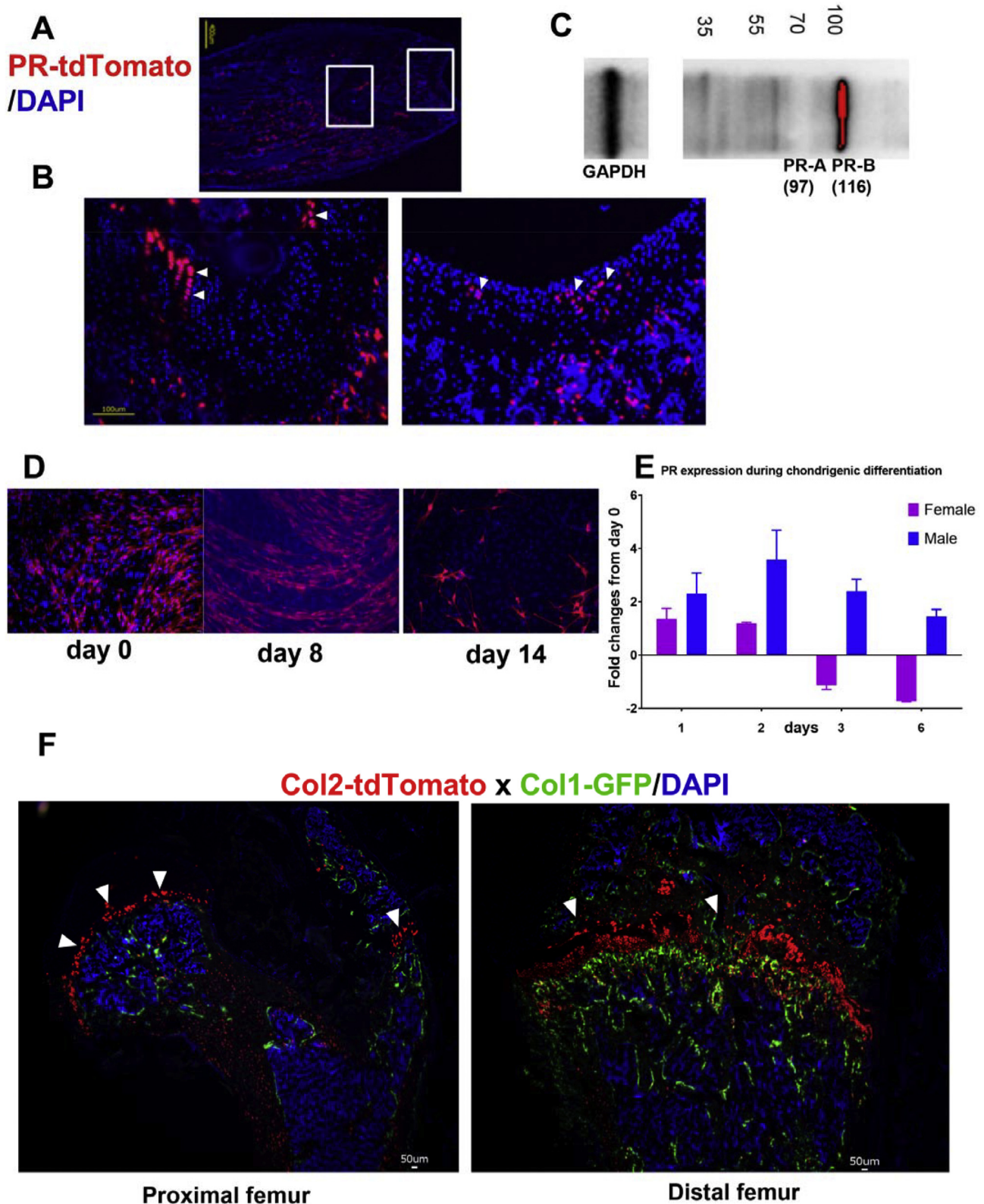


Fig. 1. Characterization of PR expression in joint and chondrocytes.

(A) PR-Cre mice were crossed with tdTomato, a red fluorescent protein. Fields on left and right showed PR expression in the growth plate subchondral bone and cartilage (white arrowheads). (B) Western blot performed on proteins extraction from the subchondral bone and growth plates regions from WT female mice, 1 month of age. (C) Enlarged area indicated in the rectangular boxes in (B) showing PR expressions in the growth plate and the cartilage. (D) Bone marrow stromal cells were collected from one-month-old PR-tdTomato female and male mice and were differentiated into chondrocytes in chondrogenic media for 14 days. Cell nuclei were stained blue with DAPI. (E) Quantitative RT-PCR was performed to detect PR expression in BMSC differentiating into chondrocytes at 1, 2, 3, and 6 days. The result was presented as fold-changes from day 0. (F) Col2-Cre^{ERT} mice were first crossed to tdTomato mice and then to Col1-GFP mice resulting in Col2 + cells expressing red (white arrowheads) and Col1 expressing green fluorescence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

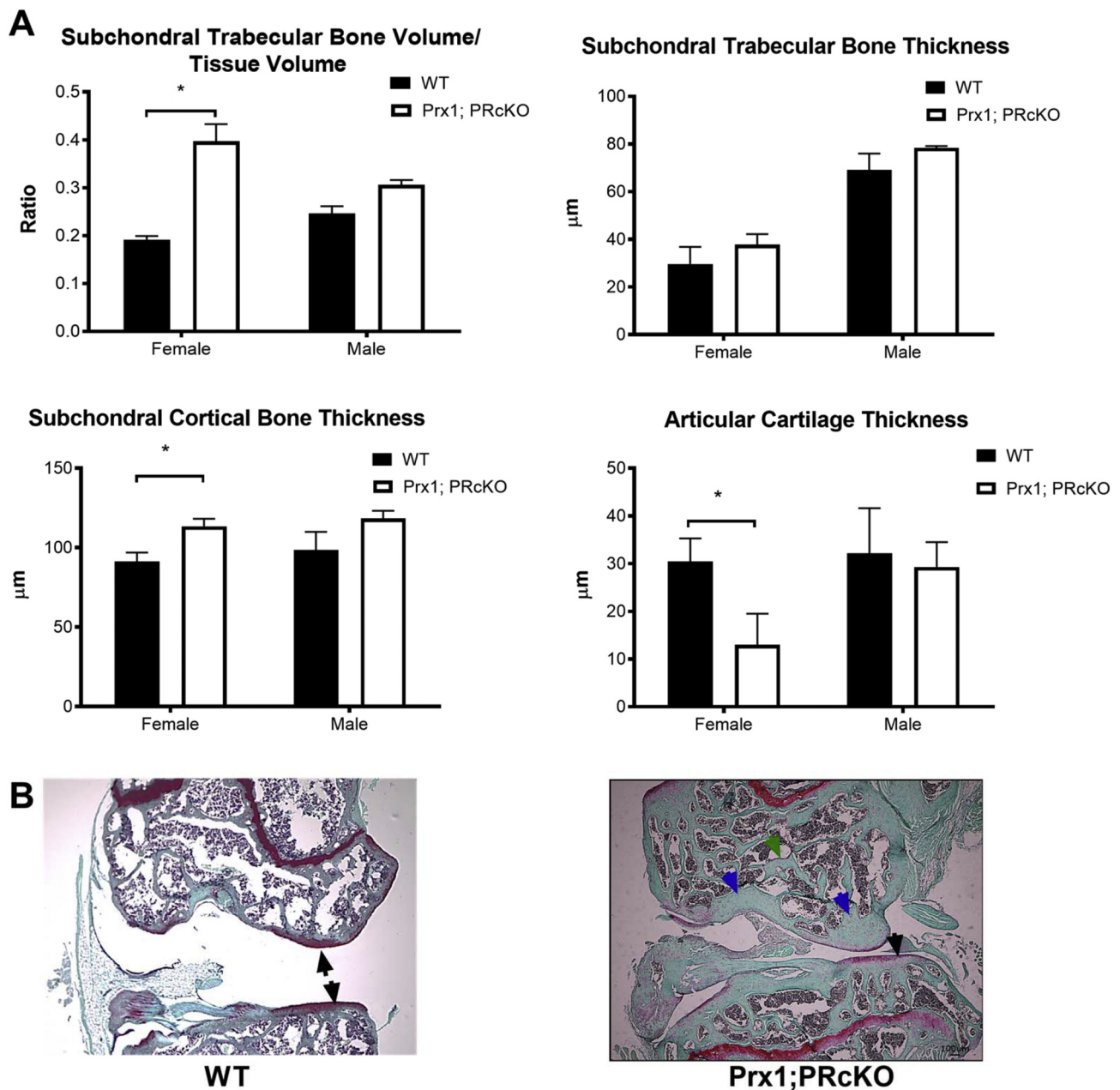


Fig. 2. Subchondral bone microarchitecture in Prx1; PRcKO mice.

(A) Subchondral bone volume and microarchitectures were measured by microCT. (B) Representative histology images from WT or Prx1; PRcKO female mice at eight months of age. Arrows illustrated articular cartilage. $N = 5-8$ /group.

MMP13, a component for the senescence-associated secretory phenotype, were significantly higher in the subchondral cartilage and subchondral bone marrow regions in the 4-month-old male Col2; PRcKO mice. Based on our observations, we propose that PR inhibition in the osteochondral progenitor cells could result in subchondral bone sclerosis and increased OA incidence or progression.

4.1. Contribution of subchondral and whole bone architectural changes to OA

OA affects both bone and joint. Hip and knee OA, in particular, are characterized by loss of articular cartilage, sclerosis, and recurrent inflammation of joint tissues [30–36]. OA is more common in women and the risk of developing OA is increased in post-menopausal women [7,8], suggesting a link between OA and hormonal status. Subchondral bone

might be essential for maintaining knee joint stability by supporting the articular cartilage. Changes in the subchondral bone plate and subchondral trabecular bone have been reported to be associated with incident knee OA [35]. Loss of subchondral trabecular rods with thickening of the remaining trabecular plates have been identified in advanced knee OA surgical specimens [32,54]. Subchondral bone changes are reported present even in early stages of OA, preceding cartilage lesions [30,35]. Subchondral bone and cartilage might play complementary roles in load-bearing joints. Subchondral bone supports articular cartilage and distributes mechanical loads across joint surfaces. Architectural changes in the subchondral bone could alter the load on the overlying cartilage, leading to inconsistent load distribution, and creating localized structural load excess allowing for incident cartilage wear and degeneration. Alternatively, loss of cartilage could also transmit increased and uneven loads to the underlying subchondral

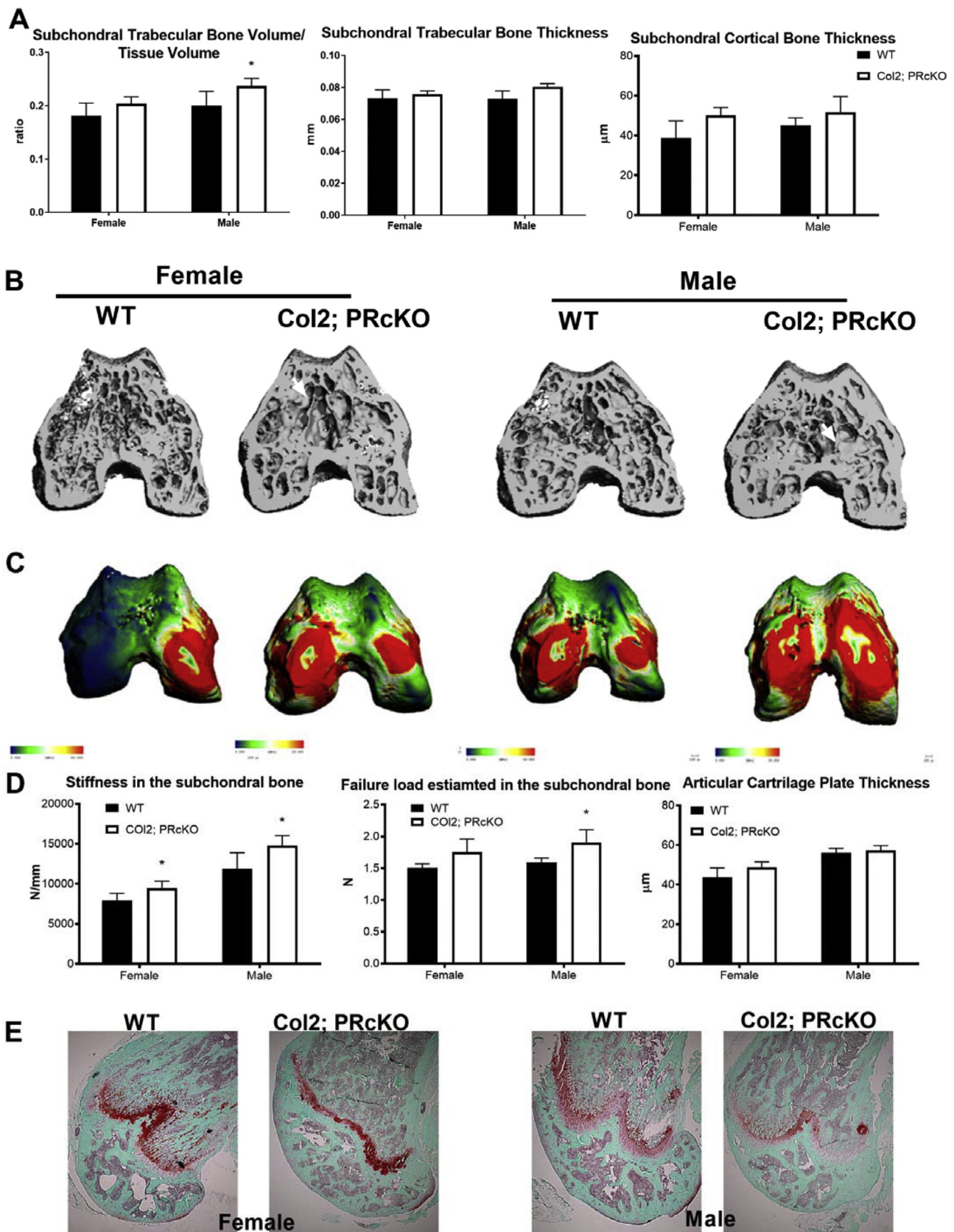


Fig. 3. Subchondral bone microarchitecture and estimated bone strength in Col2; PRcKO mice. (A) Subchondral bone volume, trabecular bone thickness, and cortical bone thickness measured by microCT. (B) Representative microCT images of the subchondral bone from WT or Col2; PRcKO female and male mice. (C) Representative FEA maps of the distal femur subchondral bone from female and male WT and Col2; PRcKO mice. Blue to red indicated low to high stiffness in a continuous manner. (D) Stiffness and failure load were estimated by FEA analyses at the subchondral bone in 4-month-old WT and Col2; PRcKO mice. $N = 8-14/\text{group}$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

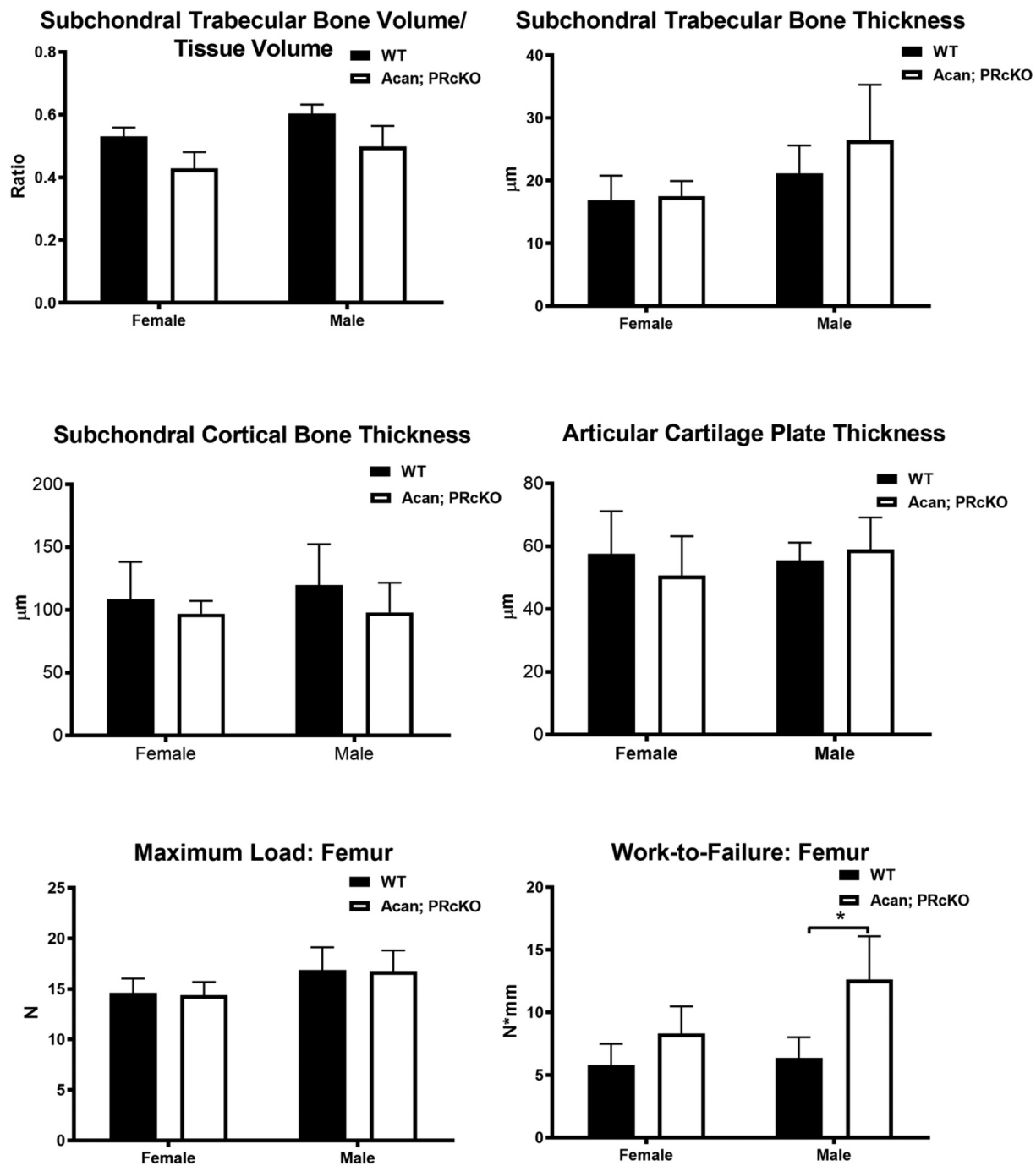


Fig. 4. Subchondral bone microarchitecture and bone strength in Acan; PRcKO mice. Subchondral bone volume, trabecular bone thickness, and cortical bone thickness were measured by microCT. Whole bone strength was measured at the femurs in 4-month-old WT and Acan; PRcKO mice. $N = 5-10/\text{group}$.

bone and thereby induce sclerosis of the subchondral bone [36]. Given these types of potential interaction between bone and cartilage, the current study findings support the concept of the PR as a potentially important contributor to OA: PR had significant demonstrable effects on subchondral bone structures that preceded other changes in the joint and differed by sex in ways reminiscent of sex differences noted in OA. Decreased activity of inflammatory arthritis has been reported during pregnancy [55–59] while multiple pregnancies appear to increase the risk of developing OA [60]. Based on our findings together with these prior observations, we propose that a therapeutic intervention using PR signaling in the osteochondral progenitor cells could be beneficial for maintaining cartilage integrity and could potentially decrease OA

incidence or progression. However, to date, PR inhibition has not been used in the prevention or the treatment of OA.

More carefully designed translational and human clinical trials are needed to evaluate the effects of the progesterone/PR signaling axis in the pathogenesis of OA, considering other factors of the genetic, cellular and physical environments during aging.

4.2. Aging, chondrocyte senescence, and PR

Increased levels of senescence markers, such as senescence-associated beta galactosidase (SA- β Gal) activity, telomere shortening, and accumulation of cytokines and MMPs have been linked to cell

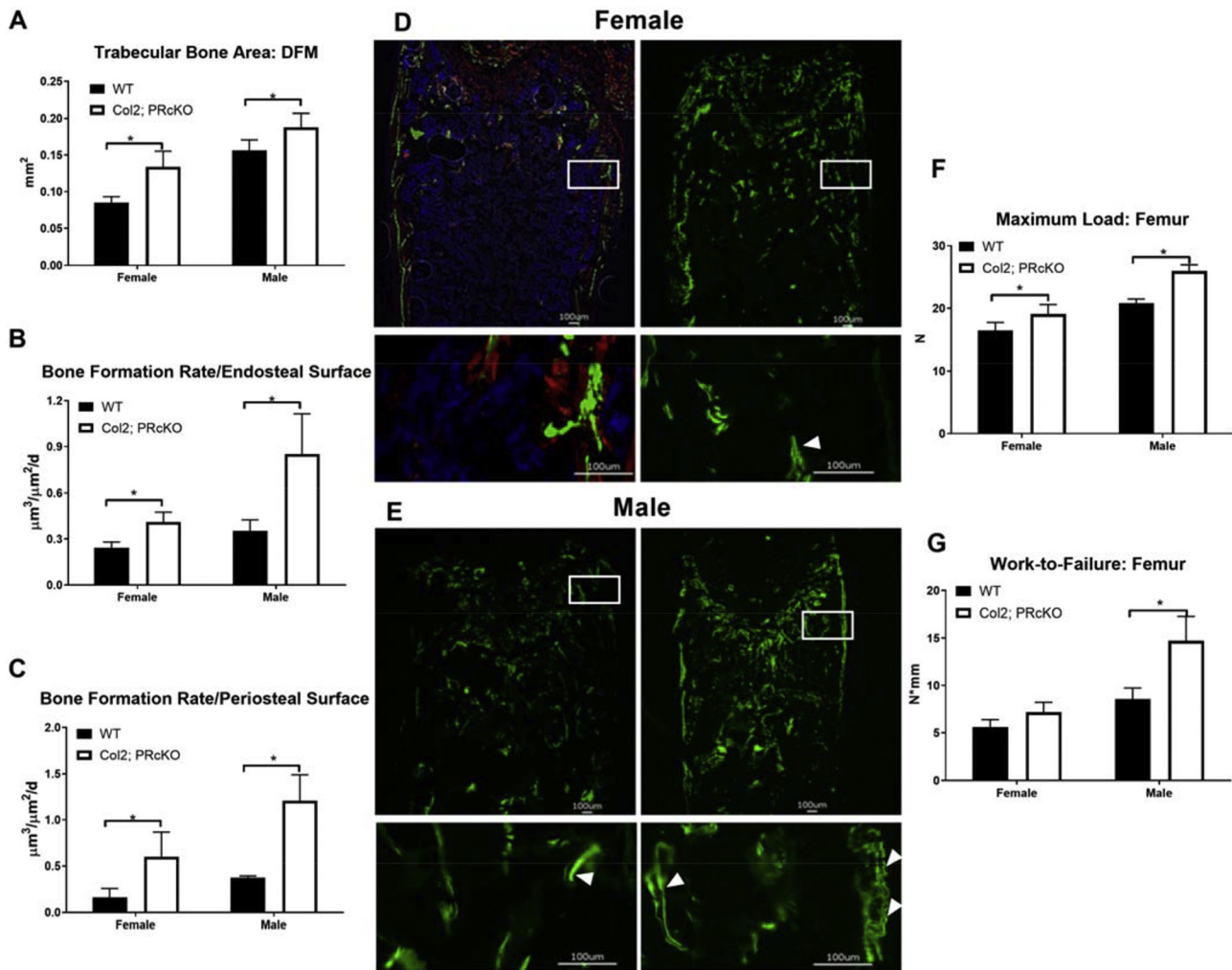


Fig. 5. Col2; PRcKO mice had higher bone formation rate and whole bone strength.

(A) Bone histomorphometry was performed at the distal femur metaphysis from wild type and Col2; PRcKO mice of both sexes at four months of age. Mice were given two fluorescent labels at -7 and -1 -day(s) before sacrifice to calculate bone formation rate at the (B) endosteal and (C) periosteal bone surface. (D) Representative images of double fluorescence labeled distal femurs in the 4-month-old female or (E) male WT or Col2; PRcKO mice. Boxes represent the enlarged periosteal and endosteal surfaces. (F) Maximum load and (G) Work-to-Failure were obtained at the femurs by three-point bending. $N = 8-10$ /group.

senescence within joint tissues and might play a pathologic role in the etiogenesis of primary OA [61–63]. Using histological staining, SA- β Gal has been observed in chondrocytes from elderly persons [61] and in chondrocytes from OA patients [64,65]. Selective removal of p16^{INK4a} positive cells, a marker for senescent cells, delayed the onset of several aging-related phenotypes in mice [66]. Since articular chondrocytes are terminally differentiated cells, it was unlikely that they would experience telomere shortening resulting from classical replicative senescence. Diekmann et al. showed that the expression of p16^{INK4a} itself might be associated with dysfunctional chondrocytes. OA phenotypes might arise in part due to the production of Senescence-Associated Secretory Phenotype (SASP) produced by the senescent chondrocytes [67]. Extrinsic factors, such as repetitive loading, stress, or local hormonal regulation, might also contribute to chondrocyte aging/senescence. Also, the lack of progenitor cells in joints limits the regenerative ability of the cartilage to respond to aging or injury/microfractures. Since chondrocytes under normal physiology do not readily enter cell division, the increased population of senescent chondrocytes would result in an altered ratio of active to senescent chondrocytes over time, as well as indirectly inducing the chronic inflammation associated with primary OA.

Studies on the effects of PR on cell metabolism and viability have often focused on its impact in the reproductive system; for example, the

PR controls cell fate decision for healthy mammary gland development [68]. PR expression levels in bone and joint were approximately 100-fold lower than in other PR-dominant tissues such as the ovary and uterus, but there is evidence of the importance of these signaling pathways in these structural tissues nonetheless [5]. Sex differences in the regulation of PR have been reported in bone [3,37]. Earlier studies using mouse genetic fate-mapping approaches demonstrated that some PRs were expressed by cells at the articular and growth plate regions at 1–2 months-old [5]. PR expression was detected in early osteochondral progenitor cells, and its expression decreased as the osteochondral progenitor cells differentiated into either osteoblasts or chondrocytes. PR-B, the same isotype that we detected in the epiphyseal and metaphyseal regions [5], induced senescence in ovarian cancer cells [69] via a FOXO1 dependent mechanism and was ligand-dependent [70]. FOXO1 is a critical transcriptional factor for chondrocyte senescence. Downregulation of FOXO1 increases the susceptibility of human-derived chondrocytes to oxidative stress [71]. Selectively knocking out FOXO1/2/4 completely or FOXO1 from the chondrocytes marked by Col2 and Acan decreased gene expression associated with cell viability and antioxidant defense and made mice more susceptible to developing post-traumatic OA [72]. The current study confirmed chondrocyte senescence was detected as early as four months with the lack of PR, and that PR expression in osteochondral cells might be protective against

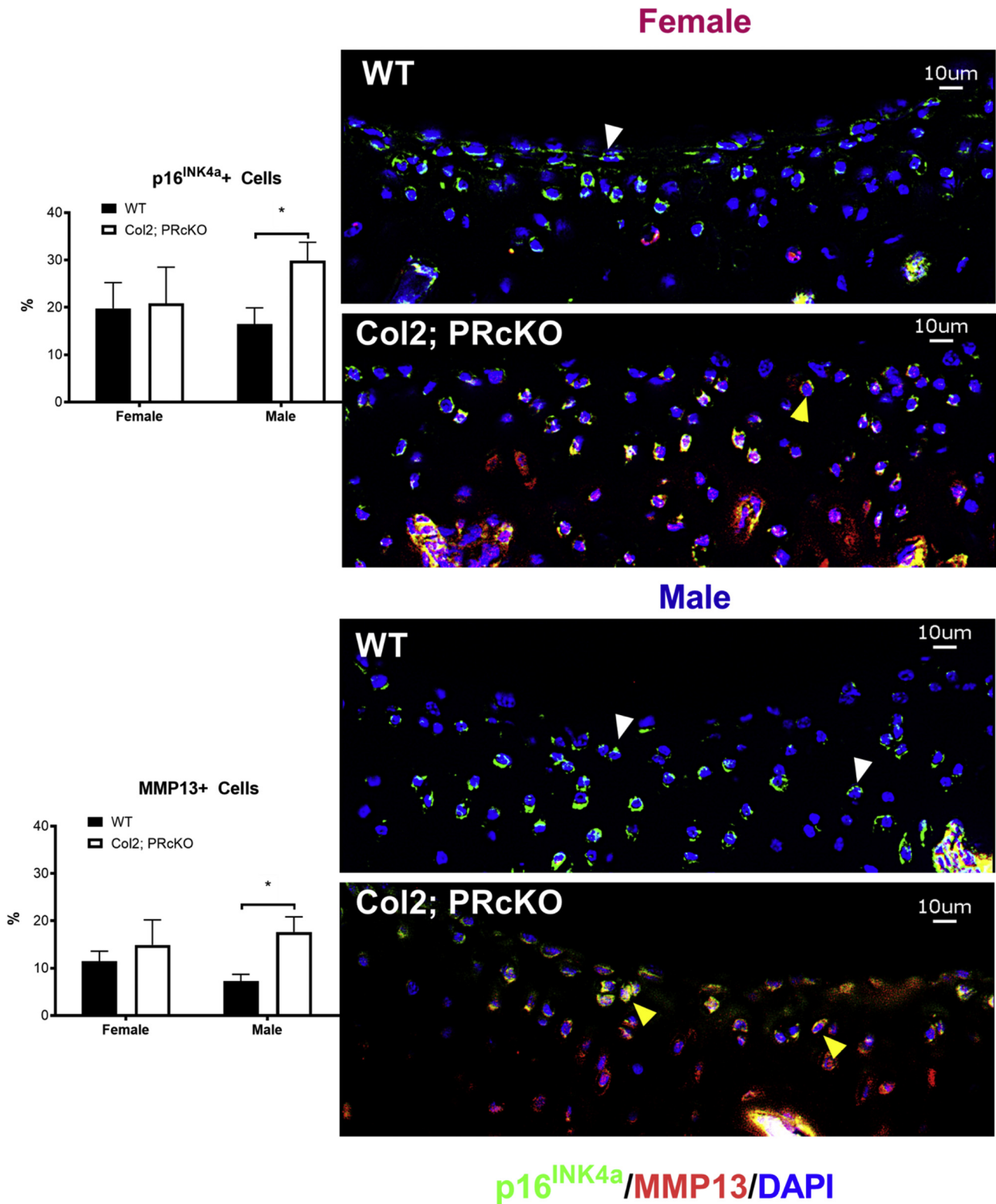


Fig. 6. Col2; PRcKO male mice had higher cellular senescence at the subchondral bone regions. Distal femurs from both female and male 4-month-old WT and Col2; PRcKO mice were stained with anti-p16^{INK4a} (stained in green, white arrowheads) and MMP13 (stained in red). Some of the chondrocytes expressed both p16^{INK4a} and MMP13 (yellow arrowheads). Scale bar 10 μm. *N* = 5–8/group.

chondrocyte aging and prevent OA-like changes as the mice aged. Combining these various pieces of information creates a powerful argument that PRs are important in bone and in the development of OA, and that this influence may start early in the lifespan but may act in part through processes related to senescence throughout the lifespan.

4.3. Limitations and strengths

A primary limitation of the study is that we did not follow the WT or the mutant mice to more advanced ages when OA-like phenotypes may be more prevalent and more directly comparable to typical age-related

primary OA. Further studies to evaluate age-related OA effects, as well as post-traumatic injuries would provide valuable information to determine if the presence of PR inhibition in osteochondral cells could serve as a therapeutic target to prevent the progression of OA or explain the sex difference in OA incidence.

We used a lineage tracing method in combination with immunohistochemistry staining to monitor PR expression in the chondrocytes [5], but we did not use molecular markers such as Col10a, sox9 or ihh to validate expression of the Col2-reporter; nor did we use histological sections to determine subpopulations or stages of chondrocyte maturation to identify chondrocytes in bone. These could be considered for future studies.

Aggrecan is the most abundant protein in cartilage and has been reported to have numerous functional roles from the articular cartilage to the growth plate zones as well as contributing to biomechanical properties [50,73,74]. We used two low dose tamoxifen injections at 5 mg/kg to activate Cre expression, due to a concern for tamoxifen doses affecting bone turnover and bone mass [39]. Prx1 and Col2-Cre targeted greater cell populations than Acan-Cre [50,75,76], which might explain the greater changes that we observed in Prx1; PRcKO and Col2; PRcKO mice compared to the Acan; PRcKO mice. The expression of PR in chondrocytes quickly decreased as the osteochondral progenitors differentiated into the chondrocytes, especially in the females, which could explain the sex difference we observed in the study. Selective deletion of PR from the osteoprogenitors using Prx1-cre or Col2-Cre resulted in high bone mass and bone strength, suggesting inhibitory effects of PR on bone mass acquisition [5]. Although PR selective deletion from Acan + cells did not affect the subchondral bone structures, it did induce higher Work-to-Failure measured at the femur, suggesting that lack of PR signaling in the chondrocytes might impact the material bone strength.

The effects of estrogen (E2) or estrogen receptors (ER) might counter-balance some of our findings in the female mice [77]. We did not monitor the levels of E2 and ER expressions in the current studies. It has been reported that double ER α and β estrogen receptor knockout mice developed OA features such as osteophytosis formation as early as at six months of age, while no cartilage damage was observed [78]. Female Col2; ER α cKO mice had less bone erosion and cartilage degradation in a model of inflammatory arthritis, suggesting that ER α is important for maintaining joint integrity under inflammatory conditions. There is a lack of studies on how hormonal receptors regulate chondrocyte aging and in the pathogenesis of primary OA.

4.4. Conclusion

Loss of function of PR in osteochondral progenitors resulted in a high subchondral bone phenotype and high estimated load carried by the subchondral bone that might precede cartilage degradation, an important component of late stage OA. The lack of PR on osteoprogenitor cells might cause an increased risk of OA as mice age. An aging study to define the role of PR in primary osteoarthritis is warranted.

Declaration of competing interest

None.

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Author contributions

CLD, WY: study conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, and final approval of the manuscript. JJJ: collection and assembly of data, data analysis and interpretation, manuscript writing, and final approval of the manuscript. XPL, AK, MJ, LL: collection and assembly of data, data analysis, interpretation, and final approval of the manuscript. BLW: data analysis and interpretation, editing of drafts, and final approval of the manuscript. NEL: data analysis and interpretation, and final approval of the manuscript.

Data and materials availability

Data is archived at the University of California, Davis.

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