Mice Lacking Wnt9a or Wnt4 Are Prone to Develop Spontaneous Osteoarthritis With Age and Display Alteration in Either the Trabecular or Cortical Bone Compartment

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ABSTRACT
Osteoarthritis (OA) is a common degenerative disease of the joint, with a complex multifactorial not yet fully understood etiology. Over the past years, the Wnt signaling pathway has been implicated in osteoarthritis. In a recent genomewide association study (GWAS), the chromosomal location on chromosome 1, linked to the Wnt3a-Wnt9a gene locus, was identified as the most significant locus associated with a thumb osteoarthritis endophenotype. Previously, it was shown that WNT9a is involved in maintaining synovial cell identity in the elbow joint during embryogenesis. Here, we report that the conditional loss of Wnt9a in the Prx1-Cre expressing limb mesenchyme or Prg4-CreER expressing cells predispositions the mice to develop spontaneous OA-like changes with age. In addition, the trabecular bone volume is altered in these mice. Similarly, mice with a conditional loss of Wnt4 in the limb mesenchyme are also more prone to develop spontaneously OA-like joint alterations with age. These mice display additional alterations in their cortical bone. The combined loss of Wnt9a and Wnt4 increased the likelihood of the mice developing osteoarthritis-like changes and enhanced disease severity in the affected mice. © 2022 The Authors. Journal of Bone and Mineral Research published by Wiley Periodicals LLC on behalf of American Society for Bone and Mineral Research (ASBMR).

KEY WORDS: Wnt9a; Wnt4; RISK FACTOR; SPONTANEOUS OSTEOARTHRITIS; AGING; MCT

Introduction
Osteoarthritis (OA) is a degenerative disease of synovial joints occurring primarily in older individuals. Hallmarks of this disease are the progressive loss of articular cartilage, osteophyte formation, subchondral bone changes, and bone cysts arising at more advanced stages leading to chronic pain and disability. A signaling pathway that receives growing attention concerning OA is the Wnt pathway. In the developing and mature skeleton, this pathway participates in the regulation of chondrocyte differentiation, joint development and homeostasis, osteoblastogenesis, and bone homeostasis. The Wnt glycoprotein family consists of 19 ligands, which—dependent on the receptor complexes available at the cell surface—can activate either the canonical β-catenin dependent or a noncanonical Wnt signaling pathway. Maintenance of homeostatic Wnt activity in the articular cartilage is crucial for sustaining a healthy joint, because excessive or insufficient Wnt activity leads both to articular cartilage defects. Recent data suggest that Wnt/β-catenin signaling in the articular cartilage superficial zone is of particular importance. As for the relevant Wnt ligands, data from humans and mice have implicated a role for WNT16 in OA. Wnt16 is expressed already during development in the murine joints. It is a weak activator of the β-catenin-dependent pathway and presumably antagonizes excessive Wnt/β-catenin signaling. Another ligand implicated is WNT9a, which plays a role in early joint development. Wnt9a overexpression induces signs of ectopic joint formation by altering the fate of prechondrogenic...
mesenchymal cells. Conversely, loss of WNT9a activity leads to alterations of distinct joints, compromising cell fate maintenance of synovial cells, resulting in their aberrant differentiation into chondrocytes. In an experimental model of chronic rheumatoid arthritis, loss of Wnt9a leads to a more severe disease phenotype. In both contexts, WNT9a signals presumably through the Wnt/β-catenin pathway. Concomitantly, canonical Wnt/β-catenin signaling also plays a role in chondrocytes, where it transiently regulates the expression of Indian hedgehog, thereby influencing the onset of hypertrophy. Concomitantly, canonical Wnt/β-catenin signaling positively regulates hypertrophic differentiation during endochondral bone formation. Furthermore, β-catenin stabilization promotes hypertrophic chondrocyte-to-osteoblast differentiation and it is the co-transcriptional activity of β-catenin which is required in hypertrophic chondrocytes for the differentiation of chondrocyte-derived osteoblasts. The Wnt9a locus is located on mouse chromosome 11 and clustered head-to-head with Wnt3a. This organization is conserved in the human genome, where the cluster is located in the 1q42 chromosomal region. In a recent genomewide association study (GWAS) on osteoarthritis endophenotypes, this region was identified as a novel locus associated with thumb OA endophenotypes, joint space narrowing in the knee, and hip cartilage thickness alterations. Previously it has been shown that Wnt4, also being expressed in the joint during development, acts together with Wnt9a, maintaining joint cell identity and regulating chondrocyte maturation during embryonic development. Wnt4 mutants, which die at birth, do not display any apparent skeletal phenotype. Ectopic WNT4 signaling in chondrocytes, on the one hand, accelerates hypertrophic chondrocyte differentiation and, on the other hand, delays the appearance of synovial cells, resulting in their aberrant differentiation and interferes with nuclear factorκB (NfκB) activity and regulating chondrocyte maturation during embryonic development, acts together with Wnt9a, maintaining joint cell identity and regulating chondrocyte maturation during embryonic development.

Materials and Methods

Animal models

The following mouse strains were used: Wnt9a floxed and Wnt9a germline deleted (Wnt9a"fl/fl"), Wnt4 floxed, Wnt4 knockout, Ppx1-Cre, Prg4-GFPCreER2 (Prg4-CreER), Tet-off Osx-GFP-Cre (Osx-Cre). Mouse strains were maintained on a C57Bl/6J (Charles River Laboratories, Worcester, MA, USA) background. The Cre-activity was in all cases provided via the male. For the Osx-Cre line, Cre-activity was suppressed during pregnancy by doxycycline (200 μg/mL; Sigma-Aldrich, St. Louis, MO, USA; D9891) in the drinking water. Experimental mice were generated by crossing Wnt9a"fl/fl" or Wnt4"fl/fl" male mice heterozygous for the respective Cre-allele with either Wnt9a"fl/+" or Wnt4"fl/+" female mice. To induce Cre-activity in the Prg4-CreER mice, 6-week-old Wnt9aΔfl;Prg4-CreER (Wnt9aΔfl/Prg4CreER−/−) and Wnt4Δfl;Prg4-CreER (Wnt4Δfl/Prg4CreER−/−) mice, as well as their respective littermate controls (Wnt9aΔfl/Prg4CreER+/+; Wnt4Δfl/Prg4CreER+/+), were intraperitoneally (i.p.) injected on 3 consecutive days with 75 μL (20 mg/mL) Tamoxifen (Sigma-Aldrich; T5648), which was dissolved in corn oil (Sigma-Aldrich; CB267) at 60 °C. Tamoxifen-treated mice were analyzed at 6 and 12 months of age. Double-mutant mice were generated by crossing male Wnt9aΔfl;Wnt4Δfl−/−; Ppx1-Cre mice with Wnt9aΔfl;Wnt4Δfl−/− females. Following 3R criteria (Replacement, Reduction, Refinement), with the exception of the experiments involving the Osx-Cre line, mice of both sexes were included (for details on sex distribution and animal numbers see Tables S1–S3). Whenever possible, littermates of the same sex were housed together. Mice were housed in individually ventilated cage (IVCs) type II long (Tecniplast USA, Inc., West Chester, PA, USA) with appropriate bedding and nesting material, in a temperature controlled (22 °C–24 °C) environment under a 12-hour light/dark cycle, and fed with a rodent chow diet (1314 [breeding] and 1324 [maintenance]; Altromin, Lage, Germany). Food and water were available ad libitum. Scoring of the samples was performed in a semi-blinded fashion. Adult mice were euthanized by cervical dislocation and juveniles by decapitation. Genotyping was performed using previously published polymerase chain reaction (PCR) protocols. Animal experiments were performed in accordance with local, institutional, and national regulations under the licenses 84-02.04.2014.A056 and 84-02.04.2017.A295.

Micro-computed tomography analysis

Mouse limbs were fixed for 48 hours in 4% paraformaldehyde at room temperature (RT), washed in 70% ethanol, and scanned using the SkyScan 1176 micro-computed tomography (μCT) (Bruker, Kontich, Belgium) with an 0.5-mm aluminum filter at 50 kV, 500 μA, 8.52 μm image pixel size, and 955 ms exposure. Sections were reconstructed using the NRecon v1.7.4.6 software (SkyScan; Bruker) with beam hardening correction set to 40%. The CT Analyzer v1.18.9.0 software (Bruker) was used for the analysis of the medial and lateral subchondral cortical bone plates of the proximal tibia (639 μm wide, 852 μm deep) (sagittal sections) and the subchondral trabecular bone (Aβ) in the medial and lateral region of the epiphysis between the subchondral bone plate and the proximal growth plate (639 μm wide, 852 μm deep) (sagittal sections) (see also exemplary images in Fig. S4A and S5A). The subchondral trabecular bone was analyzed in the 426-μm to 2130-μm metaphyseal region below the growth plate of the distal femur (transaxial sections). The cortical bone in the femur was analyzed in the 2556-μm to 2982-μm region below the distal growth plate (transaxial sections) (see also exemplary images in Fig. S7A-D). In the mid-diaphysis region, the bone parameters examined were: bone volume to tissue volume (BV/TV), trabecular thickness (TB.Th), trabecular number (TB.N), and trabecular separation (TB.Sp).

Histology and immunostainings

For histological analysis and tartrate-resistant acid phosphatase (TRAP) staining on 5-μm sections, mouse knees were decalcified in 10% ethylenediamine tetraacetic acid (EDTA)/Tris-buffered saline, dehydrated, embedded in paraffin, and sectioned. For all subsequent procedures, sections were deparaffinized...
and rehydrated into distilled water. To assess cartilage defects, sections were stained for 90 seconds in 0.02% Fast Green FCF (J.T.Baker, Phillipsburg, NJ, USA; M377-03) followed by 30 minutes staining with 0.1% Safranin O (Sigma-Aldrich; S-8884).

For immunohistochemistry, sections were pretreated for antigen-retrieval followed by 3% hydrogen peroxide for 30 minutes prior to blocking with 10% serum for 30 minutes. Alternatively, sections for fluorescent staining were treated with 2.5 mg/mL ammonium chloride twice for 5 minutes each, treated for antigen-retrieval, and blocked with 20% fetal calf serum (FCS) for 30 minutes. For the list of primary antibodies, dilutions, and antigen-retrieval conditions, see Table S4. Immunohistochemical staining was developed using the appropriate species-specific biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) at a dilution of 1:250 in combination with the Vectastain Elite ABC Kit (Vector Laboratories; PK-6100) and Diaminobenzidine (Sigma-Aldrich; D4293 or D5905). Sections were counterstained with 0.005% Methyl Green solution (Sigma-Aldrich; 67060).

Osteoarthritis Research Society International scoring, scoring of osteophytes, and ectopic mineralization

A semiquantitative 0–6 subjective scoring system based on the guidelines published by Glasson and colleagues(38) was applied to all four quadrants of the articular knee joint (medial femoral condyle [MFC], medial tibial plateau [MTP], lateral femoral condyle [LFC], and lateral tibial plateau [LTP]) individually and combined as the mean of all quadrants. Number of osteophytes were scored over multiple Safranin O–stained knee sections (femoral and tibial head) focusing on locations defined in Blaney Davidson and colleagues(31). The ectopic mineralization was scored using μCT images rotated along the longitudinal axis and a semiquantitative 0–2 scoring system based on the publication by Rai and colleagues(52) was applied, with 0 = no nodules, 1 = <5 nodules, and 2 = ≥5 nodules.

Isolation and cultivation of primary cells

Wild-type (WT) osteoblasts, deep-layer chondrocytes (DLCs), superficial zone cells (SFZs), and synovial fibroblasts (SFBS) were isolated from the calvaria, femoral, and tibial heads of the knee, and paws of 7-day-old WT pups, respectively. Wnt9a+/fl and Wnt4+/fl SFZs and DLCs were isolated from the femoral and tibial heads of knees of 7-day-old pups with the respective genotype. For cell isolation, paws were skinned and the required tissues cleared of soft tissue. Calvaria were predigested with 2 mg/mL Collagenase/Dispase (Roche Diagnostics, Mannheim, Germany; 10269638001) and 2 mg/mL Collagenase II (Worthington Biochemical Corporation, Lakewood, NJ, USA; LS004176) for 20 and 30 minutes, respectively, at 37°C while shaking. To harvest osteoblasts, calvariae were digested twice with 2 mg/mL Collagenase II for 30 minutes at 37°C while shaking. Calvarial cells were cultured in α minimal essential medium (αMEM) (Sigma-Aldrich; M4526) with 10% FCS and used at passage 1 for RNA isolation. For isolation of chondrocyte populations, femoral and tibial heads of knees were predigested with 0.3% trypsin for 1 hour. SFZs were harvested by digestion with 3 mg/mL Collagenase II for 1.5 hours at 37°C while shaking. Cells in suspension were then incubated for 20 minutes on a fibronectin-coated (Thermo Fisher Scientific, Waltham, MA, USA; 33010-018) plate (10 μg/mL) before rinsing with fresh medium and further incubation. To isolate DLCs, the remaining tissue was then treated with 0.5 mg/mL Collagenase II for 18 hours at 37°C while shaking. Both cell populations, SFZs and DLCs, were incubated with Dulbecco’s modified Eagle medium (DMEM)/F-12 with 10% FCS (Sigma-Aldrich; D8437). For the isolation of SFBS, tarsal and metatarsal elements of the hind paws were isolated and digested with 1 mg/mL collagenase IV (310 units/mg dry weight [dw]; Worthington Biochemical Corporation; LS004188) in high-glucose DMEM (Sigma-Aldrich; D6429) for approximately 20 minutes under heavy stirring. Isolated cells were cultured in high-glucose DMEM with 10% FCS up to passage 4 or 5. All cell populations were cultivated at 37°C, 5% CO2, and evaluated for the expression of marker genes by quantitative PCR (qPCR) (Fig. S1A).

Lentiviral preparation and application to primary cells and treatment of primary cells with recombinant protein

For preparation of the lentiviruses used for in vitro deletion, calcium-phosphate transfection was performed in HEK-293T cells with the following plasmid mixture: 3.5 μg pMD2- VSVG (Addgene, Cambridge, MA, USA; #12259), 6.5 μg pSRE2 (Addgene; #12620), and either 10 μg p156RRl-nlsCre (Addgene; #12106) or p156RRl-nlsCre- (for control). After 6 to 8 hours, the medium was exchanged with medium of the respective target cell type. Two days later, supernatant was collected and filtered (0.45 μm). For application on primary cells, the filtrated supernatant was mixed 1:1 with the respective medium of the targeted primary cell type, supplemented with 10 μg/mL polybrene, and applied to the primary cells, which had been cultured to a density of ~70%. Cells were lysed for RNA isolation 24 hours later. Deletion efficiency was detected by qPCR.

For expression analysis after treatment with recombinant WNT-ligands, SFZs and DLCs from WT mice were cultured to a density of ~70% before stimulation with 300 ng/mL murine rWNT9a (R&D Systems, Minneapolis, MN, USA; 8148-WN) or 100 ng/mL murine rWNT3a (R&D Systems; 1324-WN) for 48 hours. Control cells were treated with 0.1% bovine serum albumin (BSA).

RNA isolation, complementary DNA synthesis, and qPCR analysis

Cultivated osteoblasts, SFZs, DLCs, or SFBS were lysed and total RNA was isolated using the RNaqueous Kit (Invitrogen, Carlsbad, CA, USA; AM1912). A total of 500 ng total RNA was used for first-strand complementary DNA (cDNA) synthesis with the PrimeScript RT Reagent Kit (TaKaRa Bio, Otsu, Japan; RR037). For PCR, 3 μL cDNA and PerfeCTa SYBR Green FastMix (QuantaBio, Beverly, MA, USA; 95072) was mixed with the respective primers (see Table S5) in a total reaction volume of 20 μL. Gene expression was monitored using a BioRad CFX96 cycler (Bio-Rad Laboratories, Hercules, CA, USA). Reaction conditions were 95°C 30 seconds, 45× (95°C 15 seconds, 60°C 30 seconds, 72°C 20 seconds + plate read), 72°C 5 minutes, melting curve: from 55°C to 99°C, in 0.5°C increments for 5 seconds + plate read. Values were calculated using the comparative threshold cycle (ΔCt) method and normalized to the expression levels of housekeeping genes (Hprt, Sdha).

Image acquisition

Images were acquired on the Zeiss Axiosmager.M2 equipped with the AxioCam MRC 6.45 μm color camera (Zeiss, Jena, Germany) for histological and bright field images and the AxioCam MRm 6.45 μm monochromatic camera (Zeiss) in combination with the Apollo 2.0 (Zeiss) for fluorescent images, or the Zeiss Observer.Z1
equipped with the AxioCam ICc 1 color camera using the Zen 2 and Zen 3 software (Zeiss).

Statistical analysis

Normal distribution of data was tested using the D’Agostino-Pearson omnibus test. Statistical analysis was performed using the GraphPad Prism software 6.0 (GraphPad Software, Inc., La Jolla, CA, USA), applying as a nonparametric test the Kruskal-Wallis test with a post-hoc Dunn’s test and for equal distributed multiple samples the one-way ANOVA with a post-hoc Dunnett’s test. For two-sample comparisons, a two-tailed unpaired Student’s t-test or the Mann-Whitney test were used. The specific test used is specified in the figure legends. Data are displayed as box and whisker plots with the median, minimum to maximum, and the mean symbolized by +. The individual number of biological replicates for the different experiments is noted in the main text or figure legends. For a statistical analysis, the minimum number of independent biological samples was considered n ≥ 3.

Results

Wnt9a and Wnt4 are expressed in cells of the joint and signal through the Wnt/β-catenin pathway

Expression analysis of Wnt9a and Wnt4 revealed their differential expression in osteoblasts and the different cell populations of the joint. Wnt9a was expressed highest in the DLCs, followed by SFBs, and lowest in osteoblasts (OBs) relative to its expression in superficial zone cells of the articular cartilage (SFZs). Wnt4, on the other hand, was expressed highest in OBs, followed by DLCs, and least in SFBs relative to its expression in SFZs (Fig. 1A). In accordance with the literature, we observed that recombinant WNT9a induced Axin2 expression by about 20-fold in DLCs and SFZs (Fig. 1B). On the other hand, Axin2 expression was downregulated in Wnt9a-deficient SFZs and DLCs, but only in the latter, this was statistically significant (Fig. 1C). The Wnt target genes Nkd1 (Naked1) and Wisp1 (Wnt1-induced signaling pathway protein 1) were not significantly altered in SFZs but also downregulated in DLCs (Fig. 1C). The Wnt9a-deletion efficiency was high, leading to a reduction in transcript levels by over 90% (Fig. S1B). Interestingly, Wnt4 expression was tendentially increased in Wnt9a-deficient SFZs but not DLCs (Fig. S1B). Contrary to the results for Wnt9a-deficient cells, Axin2 and Nkd1 were downregulated in Wnt4-deficient SFZs but not significantly changed in DLCs (Fig. 1C). Wisp1 expression was not altered in Wnt4-deficient SFZs or DLCs (Fig. 1C). Yet, in both cell types, the Wnt4-deletion efficiency was only modest, reflected in the Wnt4 transcriptional level being reduced by about 60% in SFZs and 50% in DLCs (Fig. S1C). Nonetheless, Wnt9a expression was increased by about twofold in Wnt4-deficient DLCs but not SFZs (Fig. S1C). Finally, the combined loss of Wnt9a and Wnt4 led to a reduced expression of Axin2 in SFZs and DLCs and of Wisp1 in DLCs, whereas Nkd1 was not significantly altered in

Fig. 1. Relative expression of Wnt9a and Wnt4 in cell populations of the joint and osteoblasts and expression changes of canonical Wnt signaling target genes. (A) Fold change in the expression of Wnt9a and Wnt4 in DLCs, SFBs, and OBs relative to SFZs (n = 3 each). (B) Fold change in the expression of the Wnt target gene Axin2 in response to exogenous recombinant WNT3a or WNT9a relative to the BSA-treated control (n = 4 each). (C) Fold change in the expression of the Wnt target genes Axin2, Nkd1, and Wisp1 in SFZs and DLCs after lentiviral-mediated deletion of either Wnt9a, Wnt4, or the combined deletion of Wnt9a and Wnt4 relative to control cells treated with the respective lentivirus not producing CRE (LV-Ø) (n = 3 each). Data in A–C are presented as dot plots showing the median, minimum to maximum, and the mean symbolized by +. Values of p were determined using a two-tailed unpaired Student’s t-test. DLC = deep-layered chondrocyte; Ob = osteoblast; SFB = synovial fibroblast; SFZ = superficial zone cell.
Spontaneous development of histological OA-like phenotypes in mice with altered Wnt9a and Wnt4 signaling in the limb mesenchyme

Wnt9a homozygous mutant mice die within the first day after birth for still unknown reasons.\(^{(10)}\) Hence, to address the role of Wnt9a in joint maintenance during aging, we generated mice carrying a floxed Wnt9a allele over a null allele (Wnt9a\(^{fl/-}\)) and used the Prx1-Cre line to conditionally delete Wnt9a in the limb mesenchyme. The analysis of the mice was carried out at 6, 9, and 12 months of age. Wnt9a\(^{+/+}\) mice had no apparent health problems. However, radiographic examination of aged mice revealed alterations in their knee joints and other joints, including ectopic calcifications and roughness of the bone surface (Figs. 2A and S1E,F). These were most apparent at the age of 12 months and older (Figs. 2A and S1E,F). Wnt4\(^{-/-}\) mice also die within the first day after birth.\(^{(19)}\) As mentioned, Wnt4 can partially compensate for the loss of Wnt9a in embryonic skeletal development\(^{(10)}\) and in vitro a cell-type specific cross-regulation was observed (Fig. S1B,C). Therefore, we also addressed the consequences of loss of Wnt4 for joint maintenance during aging. For this, mice carrying a floxed Wnt4 allele over a null allele (Wnt4\(^{fl/-}\)) were generated, and Wnt4 was subsequently deleted in the limb mesenchyme using the Prx1-Cre line. The Wnt4\(^{fl/-}\) mice are viable and have no apparent health

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**Fig. 2.** Characterization of morphological phenotypic changes in the joints of aged Prx1-Cre conditional deleted Wnt9a, Wnt4, and Wnt9a;Wnt4 mice. (A) Representative µCT images of the knee joint (posterior view, right leg) of 6-month-old, 9-month-old, and 12-month-old control (representative example from a littermate control of one of the following genotypes Wnt9a\(^{fl+/+}\), Wnt4\(^{fl+/+}\), or Wnt9a\(^{fl+/+}\), Wnt4\(^{fl+/+}\), and Wnt9a\(^{fl+/+}\) specimens. (B) Representative images of Safranin O–stained sections through the knee joint (frontal plane) of 6-month-old, 9-month-old, and 12-month-old control, Wnt9a\(^{fl+/+}\), Wnt4\(^{fl+/+}\), and Wnt9a\(^{fl+/+}\) specimens. (A,B) F: female, M: male. (C) Graphical representation of the OARSI score in the different mutants (data combined from males and females) at the 6-month, 9-month, and 12-month time point. The number of individual specimens analyzed in A–C are: controls (6 months: n = 27; 9 months: n = 28, 12 months: n = 27), Wnt9a\(^{fl+/+}\) (6 and 9 months: n = 10; 12 months: n = 11), Wnt4\(^{fl+/+}\) (6, 9, and 12 months: n = 8 each), and Wnt9a\(^{fl+/+}\) (6 months: n = 9; 9 and 12 months: n = 8 each). (D) Quantitative assessment of the subch. C.Th in the lateral and medial tibial plateau of 12-month-old mutant and corresponding littermate control male specimens. (E) Quantitative analysis of the subch. BV/TV in the lateral and medial tibial plateau 12-month-old mutant and corresponding littermate control male specimens. The number of individual male specimens analyzed in D,E are as follows: control (Wnt9a\(^{fl+/+}\)): n = 5, Wnt9a\(^{fl+/+}\): n = 6, Wnt4\(^{fl+/+}\): n = 5, Wnt4\(^{fl+/+}\): n = 5, and control (Wnt9a\(^{fl+/+}\)): n = 5, Wnt9a\(^{fl+/+}\): n = 6, Wnt4\(^{fl+/+}\): n = 5, and control (Wnt9a\(^{fl+/+}\)): n = 5, Wnt9a\(^{fl+/+}\): n = 4. Data in C–E are presented as box and whisker plots showing the median, minimum to maximum, and the mean symbolized by +. Values of p in C were determined using a Kruskal-Wallis test with Dunn’s post hoc testing, and in D and E using a two-tailed unpaired Student’s t test. C.Th = cortical thickness; subch = subchondral; BV/TV = ratio of trabecular bone volume to the total tissue volume.
problems. Radiographic examination of their knees revealed no alterations at the age of 6, 9, or 12 months (Fig. 2A). Nonetheless, when Wnt4 was conditionally deleted in the Wnt9aΔPrx1−/− mutant background an aggravation of the radiologically visible destructive joint phenotype was noted in the Wnt9aΔPrx1−/−; Wnt4ΔPrx1−/− (Wnt9a/4ΔPrx1−/−) double mutants compared to the Wnt9aΔPrx1−/− single mutants, with alterations being already visible at 6-month (Fig. 2A). The respective male and female mutant

Fig. 3. Characterization of the histological and immunohistological changes in the articular joint cartilage of 6-month-old Prx1-Cre conditional deleted Wnt9a, Wnt4, and Wnt9a;Wnt4 mice. (A–G) Representative images of the affected articular cartilage regions of the lateral tibial head of 6-month-old control (Wnt9aΔPrx1+/+), Wnt9aΔPrx1−/−, Wnt4ΔPrx1−/−, and Wnt9a/4ΔPrx1−/− specimens. F: female, M: male. (A) Safranin O–stained sections; the thickness of the proteoglycan-rich region is indicated by the double arrow. Immunohistochemistry for type 2 Collagen (B), type 10 Collagen (C), for MMP13 (D), for ADAMTS-5 (E), and for cleaved CASPASE-3 (F). (G) Immunofluorescent staining for type 2 Collagen (red) and Lubricin (PRG4, green). (G′) Magnification of the Lubricin-positive region corresponding to the images in G. Arrows point at alterations and cartilage defects are marked by asterisks. Scale bars = 50 μm.
Table 1. Summary of the Mean Values ± SD of the OARSI Scores and the Number of Affected and Unaffected Female and Male Animals Combined for the Different Time Points (Controls) and Prx1-Cre Mediated Conditional Deletion of Wnt9a, Wnt4, and Wnt9a in Combination with Wnt4

| Age (months) | Controls | Wnt9aΔPrx1/Δ | Wnt4ΔPrx1/Δ | Wnt9a/4ΔPrx1/Δ |
|--------------|----------|---------------|--------------|-----------------|
|              | Mean ± SD | Aff/Unaff (n) | Mean ± SD | Aff/Unaff (n) | Mean ± SD | Aff/Unaff (n) |
| 6            | 0.33 ± 0.52 | 10/17 | 1.38 ± 0.77 | 10/0 | 0.97 ± 1.04 | 6/2 | 1.53 ± 1.45 | 6/3 |
| 9            | 0.24 ± 0.43 | 8/20 | 1.23 ± 1.25 | 6/4 | 1.06 ± 0.68 | 7/1 | 1.52 ± 1.85 | 7/1 |
| 12           | 0.65 ± 0.93 | 16/11 | 1.26 ± 1.39 | 8/3 | 2.22 ± 2.00 | 7/1 | 2.47 ± 2.01 | 6/2 |

Aff = affected (OARSI >0); Unaff = unaffected (OARSI = 0).

Table 2. Summary of the Average Number of Osteophytes Detected at the Knee Joint and the Corresponding Number of Affected and Unaffected Animals at the Age of 6 Months

| Sex | Controls | Wnt9aΔPrx1/Δ | Wnt4ΔPrx1/Δ | Wnt9a/4ΔPrx1/Δ |
|-----|----------|---------------|--------------|-----------------|
|     | Average number of osteophytes | Aff/Unaff (n) | Average number of osteophytes | Aff/Unaff (n) | Average number of osteophytes | Aff/Unaff (n) |
| F & M | 0.43 | 6/22 | 1.96 | 11/0 | 1.31 | 6/1 | 3.33 | 8/1 |
| F | 0.43 | 3/9 | 1.92 | 6/0 | 1.50 | 3/0 | 4.50 | 1/0 |
| M | 0.22 | 3/13 | 2.00 | 5/0 | 1.13 | 3/1 | 3.19 | 7/1 |

Upper row shows the combined average values for females (F) and males (M). Lower row shows the separate values for females and males. Aff = affected (at least one osteophyte); Unaff = unaffected (no osteophytes detected).

Fig. 4. Characterization of morphological phenotypic changes in the joints of aged Prg4-CreER conditional deleted Wnt9a and Wnt4 mice. (A) Representative μCT image of the knee joint (posterior view, right leg) of 6-month-old and 12-month-old control (representative example from a littermate control male specimens). (B) Representative images of Safranin O–stained sections through the knee joint (frontal plane) of 6-month-old and 12-month-old control, Wnt9aΔPrx1/Δ, and Wnt4ΔPrx1/Δ specimens. (C) Representation of the OARSI score in the different mutants (data combined from males and females) at the 6-month and 12-month time point. The number of individual specimens analyzed in A–C are as follows: controls (Wnt9aΔPrx1/Δ and Wnt4ΔPrx1/Δ): 6 months: n = 8, 12 months: n = 8 each, Wnt9aΔPrx1/Δ: 6 months: n = 8, 12 months: n = 10, Wnt4ΔPrx1/Δ: 12 months: n = 8. (D) Quantitative assessment of the subch. Th in the lateral and medial tibial plateau of 12-month-old mutant and corresponding littermate control male specimens. (E) Quantitative analysis of the subch. Th in the lateral and medial tibial plateau 12-month-old mutant and corresponding littermate control male specimens. The number of individual male specimens analyzed in D and E are as follows: control (Wnt9aΔPrx1/Δ): n = 3, Wnt9aΔPrx1/Δ: n = 5, control (Wnt4ΔPrx1/Δ): n = 4, Wnt4ΔPrx1/Δ: n = 4. (C–E) Data are presented as box and whisker plots showing the median, minimum to maximum, and the mean symbolized by . Values of p in C were determined using the Mann-Whitney test for the 6-month and the Kruskal-Wallis test with Dunn’s post-hoc testing for the 12-month time point. In D and E a two-tailed unpaired Student’s t test was used. Ct.Th = cortical thickness; subch = subchondral; TBI/TIV = ratio of trabecular bone volume to the total tissue volume.
mice were on average slightly lighter than their littermate controls, yet, the weight differences were not in all cases statistically significant (Fig. S2A).

Histologically, knee joint cartilage defects were already visible at the age of 6 months in all Wnt9aΔPrx1/C0, and some Wnt4ΔPrx1/C0 and Wnt9a/4ΔPrx1/C0 mice, compared to their littermate controls (Wnt9aΔPrx1/C0, Wnt4ΔPrx1/C0, Wnt9aΔPrx1/C0, Wnt4ΔPrx1/C0; Wnt9aΔPrx1/C0, Wnt4ΔPrx1/C0) (Figs. 2B and 3A, Table 1). The knees of the mutant and control animals were quantitatively assessed according to the Osteoarthritis Research Society International (OARSI) scoring system at the three different time points (30) (Fig. 2C; Table 1). Based on the average OARSI scores, the articular cartilage defects did not
worsen for the Wnt9aΔPrx1−/− mutants with age (Table 1). Differentiation of the OARSI score according to the sex of the mice revealed a trend for the male mice being more susceptible in all groups (Fig. S3A). Yet the differences were only statistically significant for the controls and for 6-month-old and 12-month-old Wnt4ΔPrx1−/− specimens. In general, the articular cartilage on the lateral side within the knee joint was more affected in the mutants (Fig. S3B). Numerous osteocytes were observed in the knees of 6-month-old Wnt9a/ΔPrx1−/− double mutants primarily in the femoral head and occasionally in the tibial head (Fig. S4A). Fewer osteocytes were detected in the knee joints of 6-month-old Wnt9aΔPrx1−/− and Wnt4ΔPrx1−/− mutants (Table 2), which were also smaller. The osteocytes were found primarily in the femoral head and located predominantly on the medial side. A gender bias was not observed (Table 2). Analysis of the subchondral bone thickness of the different mutant tibias revealed a significant decrease of the cortical bone on the medial side in 12-month-old male Wnt9aΔPrx1−/− and Wnt9aΔPrx1−/− double mutants (Fig. 2D, for the area examined see Fig. S4A)

For the younger stages, significant changes were observed in 9-month-old male Wnt9aΔPrx1−/−, where the subchondral cortical bone was thickened on the lateral side, and in female Wnt9aΔPrx1−/− mutants a thinning on the medial side was observed at the age of 6 months (Fig. S4B). Cortical bone thinning on both the lateral and medial side was also observed in 9-month-old female Wnt9a/ΔPrx1−/− double mutants (Fig. S4D).

For the male Wnt4ΔPrx1−/− mutants, no significant differences were observed with respect to their littermate controls at any of the three time points analyzed (Figs. 2D and S4C). In female Wnt4ΔPrx1−/− mutants, significant differences in subchondral bone thickness were only observed on the lateral side in 9-month-old specimens, which displayed thinning (Fig. S4C). The trabecular bone volume fraction (tBV/TV) of the subchondral bone in the tibial epiphyses was only assessed at 12 months (for area analyzed see imageFig. S5A). In male and female Wnt9aΔPrx1−/− mice, the epiphysial trabecular bone volume fraction was significantly decreased on the medial side of the tibial head. Although it was not significantly altered in the Wnt4ΔPrx1−/− male or female

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**Fig. 6.** Alterations in trabecular and cortical bone in aged Prx1-Cre and Prg4-CreER conditional Wnt4 mutants. (A) Representative μCT images of the trabecular bone structure in the distal femoral head of 6-month-old and 9-month-old male control (Wnt4ΔPrx1+/+) and Wnt4ΔPrx1−/− mice, with the quantification of the different bone parameters in 6-, 9-, and 12-month-old male mice on the right side. (B) Representative μCT images of the trabecular bone structure in 6- and 12-month-old male control (Wnt4ΔPrx1+/+) and Wnt4ΔPrx1−/− mice, with the quantification of the different bone parameters in 6-month-old male or female mice. (C) Representative μCT images of the cortical bone structure in tibia of 6-month-old, 9-month-old, and 12-month-old male control (Wnt4ΔPrx1+/+) and Wnt4ΔPrx1−/− mice, with the quantification of the cortical bone thickness on the right. (D) Representative μCT images of the cortical bone structure in tibia of 6-month-old and 12-month-old male control (Wnt4ΔPrx1+/+) and Wnt4ΔPrx1−/− mice, with the quantification of the cortical bone thickness on the right. The following number of mice were analyzed: (A,C) controls: 6 months: n = 6; 9 months: n = 4; 12 months: n = 5, and Wnt4ΔPrx1−/−: 6 months: n = 4; 9 months: n = 4, 12 months: n = 5; (B,D) controls: 6 months: n = 6; 12 months: n = 4, and Wnt4ΔPrx1−/−: 6 months: n = 3; 12 months: n = 4. Data in A–D are presented as box and whisker plots showing the median, minimum to maximum, and the mean symbolized by +. Values of p were calculated using a two-tailed unpaired Student’s t-test.
In the articular cartilage of Wnt9a/Wnt4 double-mutants (Figs. 2E and S5A,B), the tBV/TV values were significantly reduced on the lateral and medial tibial sides, whereas again in females only the medial side was significantly affected (Figs. 2E and S5A,B). Immunohistochemical examination of the knee joints of 6-month-old mice revealed no obvious loss of type 2 Collagen in the articular cartilage of Wnt9aΔPrx1/− , Wnt4ΔPrx1/− , or Wnt9a/Wnt4ΔPrx1/− mice (Fig. 3B). Some cells, close to defects in the articular cartilage in Wnt9aΔPrx1/− and Wnt4ΔPrx1/− mutants, stained positive for type 10 Collagen, present normally in the matrix of hypertrophic chondrocytes (indicated by arrows in Fig. 3C). Matrix metalloproteinase 13 (MMP13) was present in the superficial layer of the articular cartilage in Wnt9aΔPrx1/− and Wnt4ΔPrx1/− mice, and in addition in chondrocytes beneath the superficial layer in areas close to articular cartilage defects in Wnt9a/Wnt4ΔPrx1/− mice, but not in the control (indicated by arrows in Fig. 3D). A disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5)-producing cells were observed in the superficial layer of the articular cartilage in the control and mutant mice. In the articular cartilage of Wnt9aΔPrx1/− , Wnt4ΔPrx1/− , and Wnt9a/Wnt4ΔPrx1/− mutant mice, ADAMTS-5-positive chondrocytes were also observed in deeper layers close to the ossifying zone (indicated by arrows in Fig. 3D). A signal for cleaved Caspase 3 was detected in the articular cartilage of control and mutant mice (Fig. 3F). Immunofluorescent double-staining for type II Collagen and Lubricin (PRG4) revealed a reduced signal for PRG4 in the articular chondrocytes of Wnt9aΔPrx1/− and Wnt9a/Wnt4ΔPrx1/− mutant mice compared to the control (Fig. 3G, H). Overall, the articular cartilage layer appeared to be thinner in the knee joints of Wnt9aΔPrx1/− and Wnt9a/Wnt4ΔPrx1/− mice.

Postnatal deletion of Wnt9a in Prg4-expressing cells predispositions aged mice for articular cartilage defects

The Prx1-Cre line is active from E10.5 onward in all cells of the limb mesenchyme, initially excluding the migrating myoblasts.
derived from the myotome compartment of the somite. From E13.5 onward, however, it is also active in muscle and tendon cells [27,33]. Hence, Prxl-Cre activity is not restricted to skeletal cells or cells of the synovial joint compartments. To delete Wnt9a or Wnt4 postnatally in a more restricted fashion, we used the Tamoxifen-inducible Prg4-CreER line, which is primarily active in the superficial cell layer, synoviocytes, and ligaments of the joint [23]. The radiographic and histological examination of Wnt9a\textsuperscript{fl}\textsuperscript{–/–}\textsuperscript{Δ} mutant mice revealed no apparent differences at 6 months reflected in the OARSI score, which showed only a minor and non-significant increase (n = 8) (Fig. 4A–C). At the 12-month time point, the \( \mu \)CT images did also not reveal any changes. Yet at this time point, lesions were detected histologically within the articular cartilage of Wnt9a\textsuperscript{fl}\textsuperscript{–/–}\textsuperscript{Δ} mutant but not Wnt4\textsuperscript{fl}\textsuperscript{–/–}\textsuperscript{Δ} mice (Fig. 4B). OARSI scoring of the 12-month samples revealed higher scores in the affected Wnt9a\textsuperscript{fl}\textsuperscript{–/–}\textsuperscript{Δ} specimens, yet, averaged these were not statistically significant (\( \rho = 0.076 \)) (Fig. 4C, Table 3). The mean value of the OARSI scores of the defects in the Wnt4\textsuperscript{fl}\textsuperscript{–/–}\textsuperscript{Δ} mutant mice was comparable to that in the littermate control mice (Fig. 4C, Table 3, Fig. S5A). Given that we did not detect a difference at the 12-month time point, we did not analyze 6-month-old Wnt4\textsuperscript{fl}\textsuperscript{–/–}\textsuperscript{Δ} mutant mice in further detail. No obvious bias toward cartilage defects occurring at the lateral or medial side within the knee joint was observed (Fig. S6A). Gender-based differentiation of the OARSI scores revealed a trend for the male Wnt9a\textsuperscript{fl}\textsuperscript{–/–}\textsuperscript{Δ} mice being more susceptible (Fig. S6B). Yet the difference was only statistically significant in the 12-month-old male mice. Analysis of the subchondral bone of the mutant tibial epiphyses based on the \( \mu \)CT data revealed no significant differences in the cortical bone thickness or the BV/TV ratio in 12-month-old male and female Wnt9a\textsuperscript{fl}\textsuperscript{–/–}\textsuperscript{Δ} and Wnt4\textsuperscript{fl}\textsuperscript{–/–}\textsuperscript{Δ} mutant mice, except for female Wnt9a\textsuperscript{fl}\textsuperscript{–/–}\textsuperscript{Δ} mice, which showed a significant reduction in subchondral cortical bone thickness on the medial side (Figs. 4CD and S6C–E). Regarding the individual subchondral trabecular bone parameters, in 12-month-old male Wnt4\textsuperscript{fl}\textsuperscript{–/–}\textsuperscript{Δ} mice, the trabecular number was significantly increased on the lateral side of theibia (Fig. S5D). In essence, the postnatal loss of Wnt9a in Prg4-expressing cells appears to also predispose the mice to defects in their articular cartilage.

Deletion of Wnt9a results in alterations in metaphyseal trabecular bone volume

\( \mu \)CT analysis of Wnt9a\textsuperscript{ΔPrx1/–} and Wnt9a\textsuperscript{ΔPrg4/–} aged mice revealed an alteration in their trabecular bone volume. In both mutants, the tBV/TV was significantly reduced in the femurs of older male mice (Fig. 5A; see Fig. S7A for evaluated area). In the Wnt9a\textsuperscript{ΔPrx1/–} mice, the BV/TV ratio was significantly decreased at all ages analyzed (6 months: \( p = 0.009 \); 9 months: \( p = 0.046 \); 12 months: \( p = 0.044 \)) and associated with a significant decrease in the trabecular number (Tb.N) (Fig. 5A). Yet the thickness of the trabecular structures did not change and surprisingly, nor was the trabecular separation significantly altered (Fig. 5A). In female Wnt9a\textsuperscript{ΔPrx1/–} mice, BV/TV was also reduced at the 6-month and 9-month time points but moderately increased at 12 months; yet these differences were not statistically significant (Fig. S7B). In male Wnt9a\textsuperscript{ΔPrg4/–} mice, a reduction in BV/TV was detected in 6-month-old and 12-month-old specimens, with the difference to the control specimens being only statistically significant at the 12-month time point (\( p = 0.003 \); Fig. S8). Again, the bone phenotype here was associated with a significant decrease in trabecular number, while trabecular thickness and separation did not change significantly (Fig. S8). In the female Wnt9a\textsuperscript{ΔPrg4/–} mice, a moderate but nonsignificant BV/TV...
reduction was observed in 6-month-old specimens (Fig. S7C).

Regarding the cortical bone thickness in the metaphyseal region, no significant differences were observed in the male Wnt9aΔPrx1/− and Wnt9aΔPrg4/− mice (Fig. 5C,D; see Fig. S7D for evaluated area). Yet in 12-month-old female Wnt9aΔPrx1/− and Wnt9aΔPrg4/− mice, a significant decrease in cortical bone thickness was observed. In the females of both genotypes, a slight but not significant decrease could already be observed at 9 months in the Wnt9aΔPrx1/− and at 6 months in the Wnt9aΔPrg4/− mice (in the latter the number of independent samples was only two) (Fig. S7E,F).

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\text{Age (months)} & \begin{array}{c} \text{Controls} \end{array} & \begin{array}{c} \text{Wnt9aΔOsx/−} \end{array} & \begin{array}{c} \text{Wnt4ΔOsx/−} \end{array} \\
\hline
12 & \begin{array}{c} \text{Mean ± SD} \end{array} & \begin{array}{c} \text{Aff/Unaff (n)} \end{array} & \begin{array}{c} \text{Mean ± SD} \end{array} & \begin{array}{c} \text{Aff/Unaff (n)} \end{array} & \begin{array}{c} \text{Mean ± SD} \end{array} & \begin{array}{c} \text{Aff/Unaff (n)} \end{array} \\
\hline
12 & 0.61 ± 0.60 & 8/3 & 1.16 ± 0.50 & 8/0 & 0.68 ± 0.41 & 6/1 \\
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Aff = affected (OARSI >0); Unaff = unaffected (OARSI = 0).

Aff = affected (OARSI >0); Unaff = unaffected (OARSI = 0).

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\text{Age (months)} & \begin{array}{c} \text{Controls} \end{array} & \begin{array}{c} \text{Wnt9aΔOsx/−} \end{array} & \begin{array}{c} \text{Wnt4ΔOsx/−} \end{array} \\
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12 & \begin{array}{c} \text{Mean ± SD} \end{array} & \begin{array}{c} \text{Aff/Unaff (n)} \end{array} & \begin{array}{c} \text{Mean ± SD} \end{array} & \begin{array}{c} \text{Aff/Unaff (n)} \end{array} & \begin{array}{c} \text{Mean ± SD} \end{array} & \begin{array}{c} \text{Aff/Unaff (n)} \end{array} \\
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12 & 0.61 ± 0.60 & 8/3 & 1.16 ± 0.50 & 8/0 & 0.68 ± 0.41 & 6/1 \\
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separation in 9- and 12-month-old male, and 12-month-old female Wnt4ΔPrx1/− mice (Figs. 6A and S8A). The cortical bone thickness was except for the 9-month time point in Wnt4ΔPrx1/− mice significantly decreased in the male Wnt4ΔPrx1/− and Wnt4ΔPrg4/− mice (Fig. 6C,D). It was also significantly decreased in the female Wnt4ΔPrx1/− but not the Wnt4ΔPrg4/− mice (Fig. S8C,D).

To our surprise, in the double conditional Wnt9aΔPrx1/−/− mice, the trabecular bone volume was significantly increased in 9-month-old (p = 0.0002) and 12-month-old (p = 0.0036) female mice. This was associated with a significant increase in Tb.N (p = 0.0002 [9 months] and p = 0.0017 [12 months]) and Tb.Th, at least for the 9-month-old specimens (p = 0.0016). Trabecular separation was not decreased (Fig. 7A). In male mice, no significant differences were detected in the bone volume fraction or the bone morphometric parameters (Fig. 7B). Despite the increase in trabecular bone volume in female Wnt9aΔPrx1/−/− mice, the cortical bone thickness was not increased but decreased in female and male Wnt9aΔPrx1/−/− mice (Fig. 7C,D).

Evaluation of the cortical thickness in the mid-diaphysis region of the femur in the male mice of the different mutant genotypes at 12 months revealed a picture similar as in the metaphyseal region (Fig. 5A–D). Taken together, it appears that the conditional loss of Wnt9a in either Prx1-Cre–expressing or Prg4-CreER–expressing cells primarily has an effect on the trabecular bone volume. Significant alterations in the cortical bone thickness were detected only in old mice at the age of 12 months but not at 9 months (Figs. 5, 7E, and S9A,B and data not shown). Loss of Wnt4 in either Prx1-Cre–expressing or Prg4-CreER–expressing cells, primarily affected the cortical bone thickness and not the trabecular bone (Figs. 5, 8, and S9A,B). In contrast, the combined loss of both Wnts in Prx1-Cre–expressing cells led to an increase of the BV/TV ratio in females and a decrease in cortical bone thickness in female and male mice (Figs. 7 and S9C).

One-year-old mice lacking Wnt9a in Osx-Cre–expressing cells are also more prone to develop OA-like changes in their articular cartilage. Because Wnt9a and Wnt4 are also expressed in osteoblasts (Fig. 1A) and Prx1Cre is also active in osteoblasts, we wanted to rule out a possible contribution of this cellular compartment to the OA-like phenotypic changes in the knee joints of Wnt9aΔPrx1/−/− and Wnt4ΔPrx1/−/− mice. For this, Wnt9a or Wnt4 were deleted postnatally within osteoblast precursors using the Osx-Cre line in male mice. Deletion of neither Wnt9a nor Wnt4 in osteoblasts led to severe histologically visible alterations in their knee joints at the age of 12 months (Fig. 8A). Nevertheless, the OARSI score for the Wnt9aΔOsx/−/− mice was slightly increased, with the articular defects being significantly different in the lateral tibial plateau compartment of the joint (Fig. 8B,C, Table 4). In contrast, the OARSI score for the Wnt4ΔOsx/−/− mice was similar to control animals (Fig. 8B). In the subchondral region, neither the cortical thickness nor the trabecular BV/TV ratio were changed in the Wnt9aΔOsx/−/− or Wnt4ΔOsx/−/− mice (Fig. 8D,E). Surprisingly, the deletion of either Wnt gene in the Osx-Cre–expressing cells did, with one exception, not lead to significant alterations in the trabecular BV/TV values or trabecular bone parameters in 3-month-old and 6-month-old specimens (Fig. 9A,B). A decrease in the bone volume fraction was only detected in 6-month-old Wnt9aΔOsx/−/− mice, which was associated with a significant decrease in trabecular number (Fig. 9A). This decrease was only temporary, as no significant differences were observed in the 12-month-old Wnt9aΔOsx/−/− mice. The cortical bone thickness of the femur was not altered in the metaphyseal or central diaphyseal region of Wnt9aΔOsx/−/− conditional knockout mice (Figs. 9C, S9D). Similar to what had been observed in aged male and female Wnt4ΔPrx1/−/− mice (Figs. 6A and S8), trabecular separation was significantly increased in 6-month-old and 12-month-old Wnt4ΔOsx/−/− mice (Fig. 9B). However, the trabecular BV/TV ratio did not change. Similar to the observation of a decreased cortical thickness in femurs of Wnt4ΔPrx1/−/− and Wnt4ΔPrg4/−/− mice, the deletion of Wnt4 in the Osx-Cre expressing cells also resulted in a statistically significant decreased cortical thickness in the metaphyseal region of 6-month-old and 12-month-old mice and central diaphyseal region of 12-month-old mice (Figs. 9D, S9D).

**Discussion**

In the aging population, OA is one of the most common degenerative joint diseases. OA is a disease affecting the entire joint with cartilage degeneration, subchondral bone changes, formation of osteophytes at late stages, and changes in the synovium.
In recent years, different OA subtypes have been classified based on clustering approaches, transcriptomic variations, and differences in metabolic activities. Yet these subtypes can differ in the prevalence of clinical features such as joint space narrowing or osteocyte frequency.

Our analysis showed that mice with genetically altered Wnt9a expression in distinct cell populations using Cre-lines, which are active either in limb mesenchymal cells (Prx1-Cre) or a more joint-restricted population of cells (Prx4-CreER), are prone to spontaneously develop articular cartilage defects in their knee joints with age. Cartilage thinning was observed primarily in the knee joints of Wnt9a<sup>ΔPrx1/-</sup> mice. Consistent with previously published data that canonical Wnt signaling is involved in the regulation of Prg4 expression, PRG4 levels were reduced in the articular cartilage of Wnt9a<sup>ΔPrx1/-</sup> mice. Mice in which Wnt4 has been inactivated using the Prx1-Cre line were also more prone to articular cartilage degeneration. Yet in contrast to Wnt9a<sup>ΔPrx1/-</sup> mice, in Wnt4<sup>ΔPrx1/-</sup> mice, cartilage degeneration was not associated with thinning of the proteoglycan-rich cartilage layer or down-regulation of PRG4 levels. When Wnt4 was inactivated in a more restricted fashion using the Prx4-CreER line, the degree of cartilage degeneration was similar to the controls. One caveat using the Prg4-CreER line is that, given that we used a regimen of only three tamoxifen injections compared to the original protocol by Kozhemyakina and colleagues using 10 injections, the deletion may have been less efficient. Hence, influencing the severity of the phenotype. Cartilage damage was even more prominent in mice, in which Wnt9a and Wnt4 were inactivated together using the Prx1-Cre line, suggesting that they may either partially compensate for each other’s function or have additive effects. Here, thinning of the proteoglycan-rich cartilage layer and a reduced PRG4 signal were also noted. In all genotypes, the lateral side appeared to be predominantly affected. The phenotypic variability reflected in the scattered OARSI scores is likely due to variations in the deletion efficiency, yet this has not been demonstrated. Interanimal variability is a common feature of spontaneous OA animal models. In surgical models, male mice develop more severe OA. A similar sex-specific trend, with males being more susceptible than females, was observed here in this study as well. As the mutant mice were on average lighter than their control littersmates, we would exclude weight as a contributor to the phenotype.

Cartilage degeneration and thinning are two hallmarks of OA. Others affecting the bone are osteophyte formation and subchondral bone thickening—a characteristic feature of late-stage OA. Osteophytes develop at distinct sites in the joint depending on the OA model used. Compared to induced and experimental models of OA, osteophyte formation occurs later and less frequently in spontaneous models. The osteophytes we noted in the different conditional Prx1-Cre deleted mutants were predominantly located at the femoral head. In addition, we frequently observed ectopic mineralization at ligament and tendon attachment sites or within ligaments. The ectopic mineralization was most prominent in the Wnt9a<sup>ΔPrx1/-</sup> double mutants, where significant differences were already noted at 6 months. These sites have in common that they are under mechanical tension. Contraction-dependent activation of the Wnt/β-catenin signaling pathway is involved in the embryo to suppress chondrogenesis in the developing joint. In newborn Wnt9a mutants, the formation of a single ectopic cartilage nodule has previously been observed only in the elbow joint, where synovial cells differentiated into chondrocytes. In newborn Wnt9a<sup>ΔPrx1/-</sup> mice, the formation of ectopic cartilage nodules was observed at additional sites, including the ankle joint, the lateral side of the knee joint, and occasionally ligaments within the autopod. This likely explains why ectopic mineralization was most prominently observed in the Wnt9a<sup>ΔPrx1/-</sup> double mutants. In synovial cells, as well as cells of the ligaments, Wnt signaling appears to be necessary to suppress chondrogenesis. These ectopic sites of cartilage formation eventually undergo endochondral ossification leading to the appearance of ectopic mineralized structures.

Alterations in the bone, and particular of the subchondral bone, are suspected to affect the forces exerted on articular cartilage and may, therefore, be a crucial contributor to OA development and progression. Regarding OA, in recent years, a biphasic paradigm of bone remodeling and modeling has been proposed, with resorption being predominant in early and bone formation in late OA. In the Wnt9a<sup>ΔPrx1/-</sup> mice, a tendency of thinning on the medial side and thickening on the lateral side of the subchondral cortical bone was noted in both sexes at all stages analyzed (Figs. 2D and S4A, Table 5). Likewise, the subchondral trabecular bone volume was significantly reduced on the medial side in 12-month-old male and female Wnt9a<sup>ΔPrx1/-</sup> mice (Figs. 2E, S5A,B, and Table 5). Except for the thinning of the subchondral bone on the medial side of 12-month-old female Wnt9a<sup>ΔPrx4/-</sup> mice, no similar trends were observed in aged Wnt9a<sup>ΔPrx4/-</sup> mice (Figs. 4DE, S6D–E, and Table 5). Again, a possible limitation here is that deletion in the Wnt9a<sup>ΔPrx4/-</sup> mice may have been incomplete, given that tamoxifen was only injected on 3 consecutive days. No trends regarding alterations in subchondral cortical or trabecular bone were noted in male Wnt9a<sup>ΔPrx1/-</sup> mice (Fig. 2D,E). In female Wnt9a<sup>ΔPrx1/-</sup> mice, trends toward subchondral cortical bone thinning and increased subchondral trabecular bone volume were noted on the lateral side (Figs. 5C4 and S5B). No changes concerning subchondral cortical thickness were observed in aged Wnt9a<sup>ΔPrx4/-</sup> mice, whereas for the subchondral trabecular volume, a tendency for a reduction was observed in Wnt9a<sup>ΔPrx4/-</sup> males and females on the medial side, whereas on the lateral side, females showed the tendency for an increase (Fig. 4DE, S6C–E, and Table 5). In Wnt9a<sup>ΔPrx1/-</sup> mice, subchondral cortical bone thinning on the medial side was observed in male and tendentially also in female mice. In males, this tendency was also observed on the lateral side, yet in females, the opposite trend was noted. The subchondral trabecular volume was significantly reduced in male and female Wnt9a<sup>ΔPrx1/-</sup> mice on the medial side, and in males, also on the lateral side (Figs. 2DE, 5AD, S5, and Table 5). In essence, significant alterations in the subchondral bone structures were always associated with a decrease, resembling the early changes in subchondral bone in patients with OA. In some OA animal models a subsequent thickening of the cortical bone plate was observed with OA progression. However, this was not observed in this study, similar to the situation in a feline OA model, where the cortical bone plate also remained thin.

It is currently controversially discussed whether osteopenia and osteoporosis may also influence the health status of the joint or vice versa. Some studies showed that high BMD is associated with an increased risk of OA. Others suggest that a higher BMD reduces the risk at certain joints, whereas intermediate levels may increase the risk. Whether there is also an effect on OA progression is still debated. On the other hand, increased bone resorption was observed in patients with progressive knee OA. A Korean study showed that decreased BMD in the femur correlated with severe knee OA. Furthermore, in an experimental OA animal model, it has been shown...
that induced osteoporosis increased the severity of cartilage damage.\(^{4,59}\) Bisphosphonates, frequently applied as therapeutic agents for osteoporosis prevention and treatment, can retard the progression of OA, at least in animal models. Mechanistically, they may act by suppressing subchondral bone resorption.\(^{53}\) In clinical studies of patients with knee OA treated with the bisphosphonates alendronate or risedronate, resulting effects have been reported ranging from beneficial significant and nonsignificant effects to no effects.\(^{48,56-58}\) Wnt9a\(^{Δ}\)Prx1\(^{−}\)−, Wnt9a\(^{Δ}\)Prg4\(^{−}\)−, Wnt4\(^{Δ}\)Prx1\(^{−}\)−, and Wnt4\(^{Δ}\)Prg4\(^{−}\)− mutants displayed bone alterations (summarized in Table 4). In male Wnt9a mutants, primarily the trabecular bone volume was reduced, whereas in Wnt4 mutants, only the cortical bone was significantly altered. Although the Ptrx1-Cre line is active in mesenchymal cells including osteoblast precursors, the Prg4-CreER line is reported to recombine in articular chondrocytes, synovium, ligaments, and tendons but not in osteoblasts.\(^{28}\) Wnt9a and Wnt4 are both expressed in osteoblasts. Yet deletion of Wnt9a in a more osteoblast-restricted manner using the Osx-Cre line did not fully recapitulate the trabecular bone phenotype as only a temporary decrease in the BV/TV was observed at the 6-month time point. Still, no alterations were seen in the subchondral bone compartment. Wnt4\(^{Δ}\)Osx\(^{−}\)− mutants, like Wnt4\(^{Δ}\)Prx1\(^{−}\)− and Wnt4\(^{Δ}\)Prg4\(^{−}\)− mutants, displayed a thinning of the cortical bone. Together this suggests that Wnt9a acts primarily in a non-cell-autonomous fashion on the bone, whereas Wnt4 may act in a cell-autonomous and non-cell-autonomous way on cortical bone. Interestingly, the Wnt9a\(^{Δ}\)Osx\(^{−}\)− mutants also showed an increased incidence of low-grade cartilage damage. Yet the OARSI score was not statistically significant (\(p = 0.064\)) compared to the control. As the Osx-Cre line is also active in early hypertrophic chondrocytes in the growth plate, it may likewise be operating in the corresponding region in the articular cartilage, although this has not been reported so far.\(^{59}\) The loss of Wnt9a in this region could then be responsible for the increased low-grade cartilage damage prevalence, or alternatively heterozygous Wnt9a mice may have a higher prevalence by themselves. We performed the conditional deletion of the Wnt genes in a heterozygous background, as this has the advantage that only one floxed allele has to be deleted. Yet on the other hand, a disadvantage of this approach is that in the Wnt9a\(^{Δ}\)Osx\(^{−}\)− mice, we cannot exclude the possibility that the mild phenotype is due the Wnt9a heterozygosity.

Taken together, our data show that the loss of Wnt9a in mesenchymal and joint-associated cells predisposed the mutant mice to develop OA-like joint alterations with age. Likewise, the genomic locus encompassing WNT9A was identified in a GWAS study as a locus associated with an OA endophenotype of the thumb. In addition, these Wnt9a mutants are osteopenic. Conditional Wnt9a deletion in Osx-Cre expressing osteoblastic cells led only to a temporary osteopenic phenotype. Loss of Wnt4 in mesenchymal cells of the limb also predisposed the mutant mice to develop OA-like joint alterations with age. Yet alterations in Wnt4 have so far not been reported in OA patients. In humans, the WNT74 locus is associated with low BMD and increased fracture risk. A common feature in all conditional Wnt4 mutant mice was thinning of the cortical bone, yet, no fractures were observed in the mutant mice.

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**Conflicts of Interest**

All authors declare that they have no conflict of interest and nothing to disclose.

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**Data Availability Statement**

The data that supports the findings of this study are available in the supplementary material of this article.

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