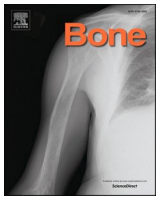
Bone 163 (2022) 116497

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone



Mesenchymal cell-derived Wnt1 signaling regulates subchondral bone remodeling but has no effects on the development of growth plate or articular cartilage in mice

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ARTICLE INFO

Keywords:
Wnt1
Chondrocyte
Growth plate
Articular cartilage
Subchondral bone

ABSTRACT

Chondrocyte differentiation is a principal progress in endochondral ossification and in the formation of secondary ossification center (SOC) during the long bone development. We have previously reported that targeted deletion of *Wnt1* in mesenchymal progenitors ($Wnt1^{-/-}_{Prrx}$) leads to spontaneous fractures and severe osteopenia in mouse long bones, suggesting that Wnt1 is a key regulator of bone metabolism. However, the effect of Wnt1 on the regulation of cartilage development and chondrocyte differentiation remained unknown. In this study, WNT1 protein expression was observed in lateral superficial cartilage and growth plate pre-hypertrophic chondrocytes in mice. *Wnt1* mRNA expression was detected in epiphyseal cartilage from E16.5 to 3 month-old mice. Detailed histological analyses revealed that the average thickness and chondrocyte density of proximal tibial articular cartilage and growth plate were unchanged between $Wnt1^{-/-}_{Prrx}$ and control mice. However, μCT analysis of tibial epiphyses showed that the subchondral bone mass was reduced in $Wnt1^{-/-}_{Prrx}$ mice compared to control mice, as demonstrated by decreased bone volume, trabecular number, trabecular thickness, and increased trabecular separation in $Wnt1^{-/-}_{Prrx}$ mice. Mechanistically, histomorphometric analyses showed that the reduced subchondral bone mass in $Wnt1^{-/-}_{Prrx}$ mice was due to impaired bone formation and enhanced bone resorption. *In vitro*, exogenous Wnt1 inhibited chondrogenesis and chondrocyte hypertrophy in both cell autonomous and juxtacrine manners, while matrix mineralization and the expression of *Mmp13*, *Mmp9* and *Opn* were induced in a juxtacrine manner. Taken together, mesenchymal cell-derived Wnt1 is an important regulator of subchondral bone remodeling, although it has no effect on the regulation of growth plate or articular cartilage.

1. Introduction

Endochondral ossification is a critical process in the formation and growth of long bones in vertebrates [1–3]. There are two ossification centers in endochondral ossification: primary ossification center (POC) and secondary ossification center (SOC). The formation of POC occurs in the diaphysis of long bones during embryonic development. It begins with mesenchymal condensation, in which the stem cells differentiate into chondrocytes, followed by proliferation and hypertrophy. Blood vessels then invade the hypertrophic cartilage, bringing along osteoprogenitors that differentiate into osteoblasts to form trabecular bone. SOC forms at the epiphyses of long bones during postnatal development *via* similar process as POC: chondrocyte hypertrophy, maturation, matrix mineralization, vascular invasion, and osteoblast differentiation [4]. The processes of endochondral ossification are regulated by multiple signaling molecules, including IHH, PTHrP, BMPs, WNTs, and FGF, and transcription factors, such as SOX9, RUNX2, and osterix (OSX/SP7) [5–9]. Dysregulation of these factors can cause skeletal diseases, including osteoporosis and osteoarthritis (OA) [10,11].

Wnt signaling is a highly conserved signal transduction cascade that plays essential roles in cell proliferation, differentiation, polarization, and migration during development. Genetically modified animal studies

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https://doi.org/10.1016/j.bone.2022.116497
Received 5 January 2022; Received in revised form 13 May 2022; Accepted 13 July 2022
Available online 18 July 2022

have demonstrated that Wnt family proteins also regulate chondrogenesis, endochondral ossification and joint homeostasis. Ectopic expression of *Wnt1, Wnt7, Wnt4 or Wnt8* in the developing limbs of chick embryos inhibits chondrogenesis, while overexpression of *Wnt5a or Wnt5b* promotes early chondrogenesis [12–14]. Moreover, *Wnt16* overexpression specifically in chondrocytes in mice significantly inhibited chondrocyte hypertrophy during skeletal development [15].

In adult tissues, the expression level of WNT1 is generally extremely low or absent [16]. We have previously demonstrated that targeted deletion of *Wnt1* in mesenchymal progenitors in mice ($Wnt1^{-/-}_{Prrx}$) led to spontaneous fractures and severe osteopenia due to impaired osteoblast function and increased bone resorption [17], suggesting that Wnt1 is a key regulator of bone metabolism. As both osteoblasts and chondrocytes originate from mesenchymal stem cells, we hypothesized that Wnt1 might also have functions within articular cartilage and growth plate, as well as in the chondrocyte differentiation. To address this, we generated limb bud mesenchymal cell-targeted knockout mice and analyzed the phenotype of articular cartilage of knee joint, growth plate, and subchondral bone. Further, we studied the potential molecular mechanisms of Wnt1 in primary chondrocytes by performing cell culture experiments.

2. Materials and methods

2.1. RT-PCR and quantitative RT-PCR analysis

Articular cartilage samples of C57BL/6 N mice were obtained under sterile conditions using a stereomicroscope. Samples were taken during embryonic phase at E16.5-E18.5 and postnatally at 5 days, 10 days and at 3 months of age. Samples were frozen in liquid nitrogen crushed into powder, and homogenized in TRIsure reagent (Bioline). Total RNA was isolated from epiphyseal cartilage using TRIsure reagent followed by RNeasy Mini Kit (Qiagen). Random hexamer-primed cDNA was synthesized with M-MuLV RNase H+ reverse transcriptase with RNase inhibitor (DyNAmo cDNA Synthesis Kit, Thermo Scientific). Tissue expression analysis of *Wnt1* was run using DyNAzyme II DNA polymerase (Thermo Scientific) on MJ-Research® PTC-2000 Thermal Cycler and visualized with agarose gel electrophoresis.

Expression of cartilage specific genes in knee joint of 4-week-old mice was determined using Dynamo Flash SYBR Green qPCR kit (ThermoFisher) (Supplementary Table 1). RNA expression in primary chondrocytes was also determined by Dynamo Flash SYBR Green qPCR kit (ThermoFisher) (Supplementary Table 1). Messenger RNA levels were normalized to β-actin expression by using $2^{-\Delta\Delta CT}$ method.

2.2. Immunohistochemistry

Paraffin embedded sections of adult murine C57BL/6N tibias were routinely prepared for immunohistochemistry. The sections were boiled in 0.01 M sodium citrate (pH 6.0) for 2x10min to reveal the epitopes. Endogenous peroxidase was inactivated with 0.3 % hydrogen peroxide in methanol for 30 min. Expression of WNT1 was detected using a rabbit polyclonal primary antibody (ab15251, 1:75, Abcam), followed by Histostain-*Plus* secondary antibody and biotinylated horseradish peroxidase-conjugated streptavidin (Invitrogen). Peroxidase activity was visualized using 3,3′-diaminobenzidine tetrahydrochloride-plus kit (Invitrogen). Images were captured using Zeiss Axioimager microscope and attached High End Image Analysis XEON workstation.

2.3. Generation of conditional $Wnt1^{-/-}_{Prrx}$ mice

Generation of Wnt1 conditional knockout mice on C57BL/6N background has been described recently [17]. Briefly, exon 2–4 of the *Wnt1* gene is flanked by loxP sites in the $Wnt1^{flox/+}$ mice. The following primer pairs were used for genotyping of wild-type and $Wnt1^{flox/+}$ alleles: 5′-TGCATTGTGACTTCACATCC-3′ and 5′-TTAAATGGGAATGGTCTCTG-3′. To specifically delete *Wnt1* in early limb bud mesenchyme, $Wnt1^{flox/+}$ female mice were crossed with $Wnt1^{flox/+}$ male mice expressing Cre recombinase driven by the Paired Related Homeobox (Prrx) promoter (Tg(Prrx-cre)1cjt/J mice) (#005584, Jackson Laboratory, Bar Harbor, ME, USA). The presence of *Prrx-cre* gene was determined by the primer pair: 5′-CCAATTTACTGACCGTACACC-3′ and 5′-CCCGGCAAAA-CAGGTAGTTA-3′.

All mouse studies were approved by the National Animal Experiment Board ELLA (Project license number: 5186/04.10.07/2017), complying with the international guidelines on the care and use of laboratory animals. *Wnt1* deficient mice were crossbred with C57BL/6 N background. Animals were maintained in the Central Animal Laboratory of University of Turku following 3Rs principles. $Wnt1^{-/-}_{Prrx}$ and their control age- and gender-matched littermates were used in all studies. $Wnt1^{-/-}_{Prrx}$ mice must be euthanized before/at 12-weeks old due to spontaneous fractures in long bone.

2.4. Histological analysis of tibial growth plate and articular cartilage

Tibias of 4-, 6- and 12-weeks old control and $Wnt1^{-/-}_{Prrx}$ mice were fixed in 4 % formaldehyde and decalcified in 10 % EDTA, and 5-μm-thick paraffin-embedded sagittal sections in the middle of lateral condyle were obtained. The sections were deparaffinized, rehydrated and stained with Hematoxylin and eosin (H&E) and Safranin O/Fast green for epiphyseal cartilage. The average thickness of one medial and two marginal lines of proliferative and hypertrophic zones in growth plate was measured (Supplementary Fig. 1). Image J software was used for analysis. Cell density was analyzed by calculating the average cell number in 10–13 measurement squares (each with an area of 10,000μm^2) of proliferative zone and in 19–22 measurement squares (each with an area of 2500μm^2) of hypertrophic zone in tibial growth plate (Supplementary Fig. 1).

In tibial articular cartilage, the average thickness of one medial and two marginal lines was measured. Cell density was analyzed by calculating the average cell number in 18–22 measurement squares (each with an area of 2500 μm^2).

2.5. Micro-computed tomography

Micro-computed tomography (μCT) analyses were performed on the proximal tibia using a Skyscan 1070 μCT scanner (Skyscan, Kontich, Belgium) and imaged with an X-ray tube voltage of 72 kV and current of 138 μA, with a 0.25 mm aluminum filter. The scanning angular rotation was 182.45 degrees, and the angular increment was 0.45 degrees. Cross sectional images were reconstructed with NRecon 1.4 software. For the analysis of subchondral trabecular bone in tibia, a volumetric region of interest (ROI) excluding subchondral plate was defined at the epiphysis of the tibia starting 50 layers (488 μm) above the lower surface of the growth plate and extending 20 layers (195 μm) proximally.

2.6. Isolation of chondroprogenitor cells and micromass cultures

Newborn mice (5 days old) were euthanized by decapitation according to the recommendation of the National Animal Experiment Board ELLA. For isolation of articular cartilage, mouse hind limbs were dissected and flushed first in 70 % ethanol, then in sterilized phosphate-buffered saline (PBS). The skin and soft tissues of hind limbs were removed to expose distal femoral head and tibial plateau at knee joint. The obtained articular cartilage pieces were then digested in 3 mg/ml collagenase D (Gibco) for 2 h followed by an overnight digestion with 0.5 mg/ml of collagenase D. All collagenase solutions were diluted in Dulbecco's modified Eagle's medium (DMEM) (Gibco), supplemented with 1 % penicillin/streptomycin (P/S) (Gibco).

Digested cartilage pieces were filtered through a 100-μm Falcon™ cell strainer (Corning Life Sciences) followed by 1500 rpm centrifugation for 5 min at room temperature. Cell pellet was resuspended in

DMEM, supplemented with 1 % P/S, 10 % fetal bovine serum (FBS), 2 mM glutamine and Gibco® Insulin-Transferin-Selenium (ITS-G) (Gibco), and a 20 μL drop of cell suspension was pipetted into each well of a 6-well plate. The cells were kept at 37 °C for 3 h to attach and then 2 ml of culture medium was added. After culturing for 6 or 12 days, wells were rinsed twice with PBS, fixed with 4 % formaldehyde for 15 min at room temperature, washed with 0.1 N HCl and covered with Alcian blue solution (1 % Alcian blue 8 GS in 0.1 N HCl). After overnight staining, the stained wells were washed extensively with 0.1 N HCl and observed using an image scanner.

2.7. Proliferation assay

Primary articular chondrocytes isolated from control and $Wnt1_{Prrx}^{-/-}$ mice were seeded in 96-well plates at a density of 1×10^3 cells/well. The CellTiter 96® Non-radioactive Cell Proliferation Assay (Promega) was used to detect cell viability at time points of 4, 6 and 8 days.

2.8. Histomorphometric analyses

Bone histomorphometry was performed as previously described [17]. In short, mice were subcutaneously injected with calcein (20 mg/kg body weight) and demeclocycline (40 mg/kg body weight) (both from Sigma-Aldrich) at 9 and 2 days for 12-week-old mice and at 7 and 2 days for 6-week-old mice prior to euthanasia, respectively. The tibias were fixed in 70 % ethanol for 3 days. The fixed bones were embedded in methyl methacrylate. For analyses of subchondral trabecular bone, undecalcified 5-μm-thick sagittal sections were cut with microtome and stained with von Kossa method for mineralized bone, with 2 % Toluidine Blue for the analysis of osteoblasts, osteoid and osteoclasts or first with tartrate-resistant acid phosphatase (TRAP) and then counterstained with Toluidine Blue for confirming the analysis of osteoclasts. All parameters were measured using the OsteoMeasure histomorphometry system (OsteoMetrics, USA) following the guideline of the American Society for Bone and Mineral Research [18].

2.9. Viral package and infection

Viral packaging and infection was performed as previously described [17]. Briefly, human *WNT1* coding region was cloned to pBABE-puro vector (Cell Biolabs, Inc.). *WNT1* and empty viruses were produced in human embryonic kidney (HEK) 293 Phoenix cells. Chondroprogenitor cells were incubated with viral media containing 4 μg/mL polybrene (Sigma-Aldrich) overnight. The infected chondroprogenitor cells were selected by medium with 3 μg/mL puromycin for 2–3 days. The puromycin-resistant cell pools were plated for subsequent experiment.

Wnt1 overexpressing (Cho-*WNT1*) and Cho-EV (empty virus) chondroprogenitor cells were seeded in a 20μL drop at 5×10^6 cells/mL in DMEM and 10 % FBS into 6-well plates. The cells were kept at 37 °C for 3 h to attach and then 2 mL of chondrogenic medium containing DMEM, 10 % FBS, 1 % P/S and 50μg/mL ascorbic acid was added. After 7 days of culture, micromass cultures were stained with Alcian blue for chondrogenic nodules, as described above. After 28 days of culture, von Kossa staining was performed to detect matrix calcification. The cells were fixed with 4 % formaldehyde for 15 min at room temperature. The fixed cells were washed with distilled water for 3 times and then stained with 5 % silver nitrate (Sigma-Aldrich) for 30 min at room temperature. The stained cells were rinsed with distilled water 2 times and observed using an image scanner.

2.10. Co-cultures of WNT1-overexpressing C3H10T1/2 cells and chondroprogenitor cells

Wnt1-overexpressing C3H10T1/2 cells were generated as previously described [17]. C3H10T1/2-EV or C3H10T1/2-*WNT1* cells were seeded in 12-well plates at the density of 1×10^4 cells/well. After attachment to the bottom of the wells, C3H10T1/2 cells were treated with 10 μg/mL mitomycin C for 2 h, followed by careful rinsing by PBS for 3 times. Then chondroprogenitor cells isolated from articular cartilage of knee joint of 3–5 days newborn mice were plated on top of growth arrested C3H10T1/2 cells at a density of 3×10^4 cells/well. The cells were cultured in DMEM, supplemented with 10%FBS, 1 % P/S, 2 mM glutamine, 50μg/mL ascorbic acid and 10 mM β-glycerophosphate, and 1:1000 dilution of Dimethyl sulfoxide (DMSO) (Sigma-Aldrich) as a control or 10 μM Wnt signaling inhibitor XAV939 (Tocris Bioscience) was added. Culture medium was changed every 2 days. After 10 days of culture, the cells were fixed with 4 % formaldehyde and then stained for alkaline phosphatase (ALP) using Fast Blue RR Salt (Sigma-Aldrich) and Alizarin Red S (Sigma-Aldrich).

2.11. Co-cultures of WNT1-overexpressing C3H10T1/2 cells and mouse $CD115^+$ monocytes

C3H10T1/2-EV or C3H10T1/2-*WNT1* cells were seeded in 96-well plates at the density of 1.68×10^4 cells/well. After attachment to the bottom of the wells, C3H10T1/2 cells were treated with 10 μg/mL mitomycin C (Sigma-Aldrich) for 2 h followed by careful washing with PBS. Bone marrow cells obtained from both femurs and tibias of 8 to 10 weeks old male C57BL/6 N mice were centrifuged and resuspended in ammonium-chloride-potassium buffer for 5 min to remove red blood cells. $CD115^+$ cells were labeled with CD115 MicroBeads (Cat#130–096-354, Miltenyi Biotec) and isolated using a MACS column according to manufacturer's instructions (Miltenyi Biotec). $CD115^+$ cells were then seeded on top of C3H10T1/2 cells at the density of 1.0×10^4 cells/well. The cells were stimulated with 10 ng/mL macrophage colony-stimulating factor (M-CSF) (PeproTech) and 50 ng/mL receptor activator of nuclear factor kappa-B ligand (RANKL) (PeproTech) to induce osteoclast differentiation. The culture medium was changed every 2 days and TRAP staining was performed at day 7.

2.12. Statistical analyses

Values are given as mean ± SD. Two-tailed Student's *t*-test was performed for the comparing groups with equal size. Welch's *t*-test was applied for the comparing groups with unequal size. P value of <0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc.)

3. Results

3.1. Wnt1 expression in murine articular cartilage and growth plate

To analyze Wnt1 expression in murine articular cartilage during development and postnatally, we collected samples of epiphyseal cartilage from E16.5 to 3-month-old mice. RT-PCR analysis showed that *Wnt1* was expressed from the earliest obtained epiphyseal cartilage of E16.5 mouse embryos until birth (Fig. 1A). Distribution of WNT1 protein was studied by immunohistochemistry in sagittal sections of proximal tibia of adult wild-type mice. In articular cartilage, WNT1 protein was expressed in chondrocytes of the superficial zone (Fig. 1B, upper left). In the growth plate, WNT1 expression was concentrated in the early proliferative and hypertrophic zones (Fig. 1B, upper right). In pre-hypertrophic/hypertrophic and resting chondrocytes, the staining was detected only in nucleus (Fig. 1B, lower left and right).

3.2. Wnt1 deletion in limb bud mesenchymal cells has no effect on the development of growth plate

To investigate the function of Wnt1 in postnatal bone growth, we specifically deleted *Wnt1* in limb bud mesenchymal cells using Prrx-Cre/LoxP-deleter mice. *Wnt1* deletion was observed in knee joint cartilage of $Wnt1_{Prrx}^{-/-}$ mice by PCR of genomic DNA [Supplementary Fig. 2A]. As

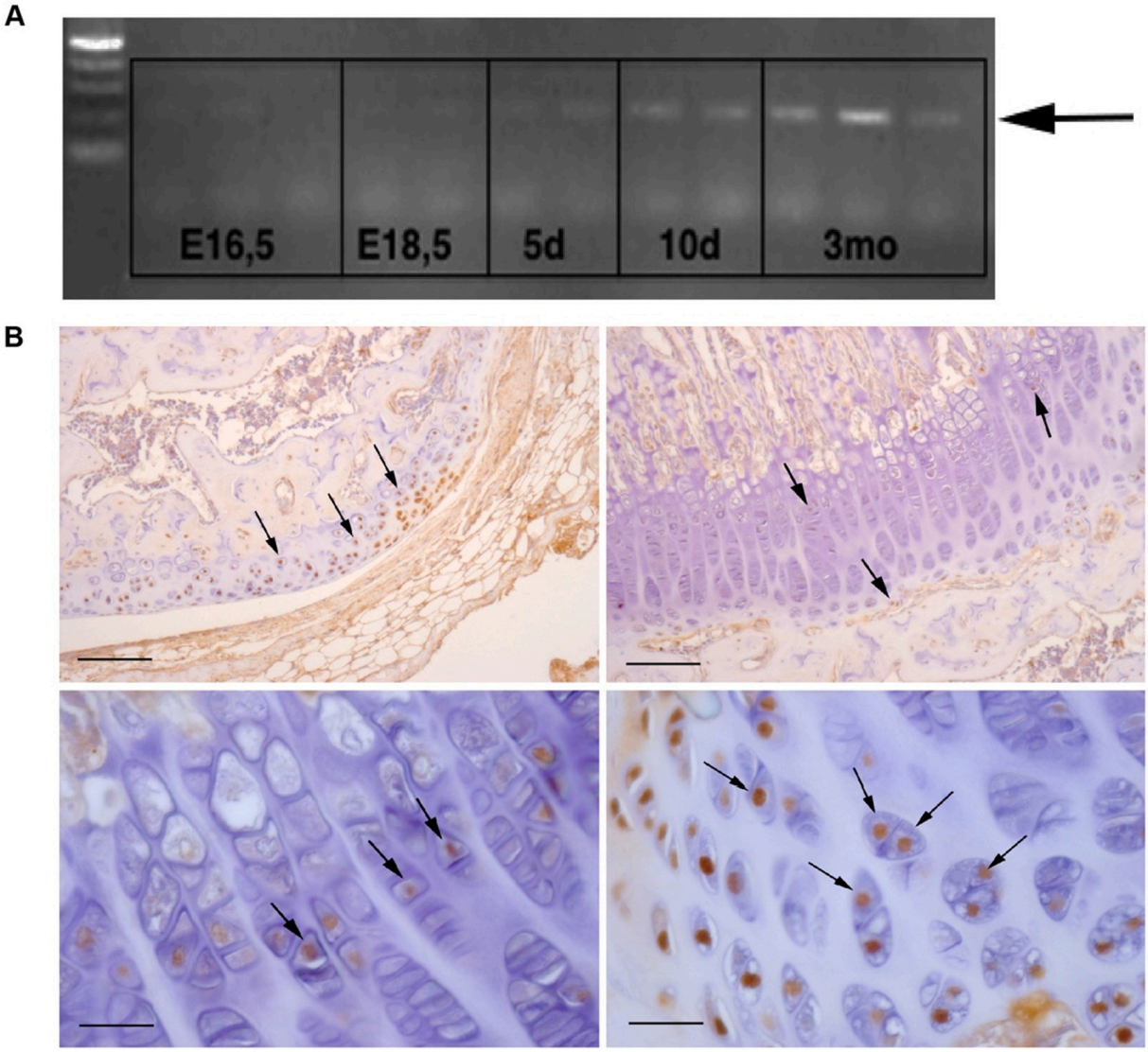

Fig. 1. Expression of Wnt1 in mouse tissues.
(A) *Wnt1* expression at mRNA level in murine articular cartilage during the development and postnatally. RNA samples were isolated from epiphyseal cartilage of wildtype mice at different time points and *Wnt1* expression was detected by RT-PCR. The RT-PCR products were analyzed by agarose gel electrophoresis. *Wnt1* band (210 bp) is indicated with an arrow. (B) WNT1 protein expression was detected in articular cartilage and growth plate of 5 μm paraffin-embedded sagittal sections of lateral condyle. Strong positive staining is seen in the lateral superficial cartilage (upper left panel). Growth plate cartilage shows positive staining in resting chondrocytes and in prehypertophic/hypertrophic zone (upper right panel). Prehypertrophic/hypertrophic zone is on the left side with a region of WNT1 expressing chondrocytes (lower left). Resting zone chondrocytes with nuclear WNT1 staining (lower right). Examples of WNT1 positive cells are indicated by arrows. Scale bar corresponds 100 μm in upper panel and 25 μm in lower panel.

previously reported [17], $Wnt1^{-/-}_{Prrx}$ mice developed spontaneous long-bone fractures, which were most frequent in mid-tibia. The lengths of femur and tibia were measured by vernier caliper. Femurs and tibias of $Wnt1^{-/-}_{Prrx}$ mice were significantly shorter when compared to control mice at 6 weeks of age. The femurs were 7.5 % ($P = 0.032$) and the tibias 31.4 % ($P < 0.0001$) shorter in $Wnt1^{-/-}_{Prrx}$ *vs.* control mice (Fig. 2A).

The average thicknesses and chondrocyte densities of proliferative and hypertrophic zones of proximal tibial growth plate were measured in Safranin O/Fast green stained sections (Fig. 2B–D and Supplementary Fig. 1). The average thickness was slightly reduced in the proliferative zone (−12.9 %, $P = 0.161$), while it was increased in the hypertrophic zone of $Wnt1^{-/-}_{Prrx}$ mice compared to control mice (+11.1 %, $P = 0.408$), although the differences were not statistically significant (Fig. 2B and C). Furthermore, no significant differences were observed between genotypes in cell density, neither in proliferative ($P = 0.308$) nor in hypertrophic zone ($P = 0.211$) (Fig. 2B and D).

3.3. *Wnt1* deletion has no effect on the development of articular cartilage

To assess whether deletion of *Wnt1* in limb bud mesenchymal cells affects development of articular cartilage, we measured the average thicknesses and cell densities of articular cartilage in sagittal hind limb sections of 6-week-old $Wnt1^{-/-}_{Prrx}$ mice. Although *Wnt1* deletion was observed in knee joint cartilage of $Wnt1^{-/-}_{Prrx}$ mice by PCR of genomic DNA (Supplementary Fig. 2A), there were no significant differences between genotypes in articular cartilage thickness ($P = 0.844$) or cell density ($P = 0.392$) (Fig. 3A and B). In line with that, mRNA expression levels of chondrocyte-specific genes were similar between genotypes in knee epiphyses of 4-week-old mice (Fig. 3C).

We also analyzed the proliferation and differentiation of $Wnt1^{-/-}_{Prrx}$ chondroprogenitor cells isolated from knee epiphyses of newborn mice. *Wnt1* was indeed deleted in $Wnt1^{-/-}_{Prrx}$ chondroprogenitor cells (Supplementary Fig. 2B) but no difference was observed in the differentiation

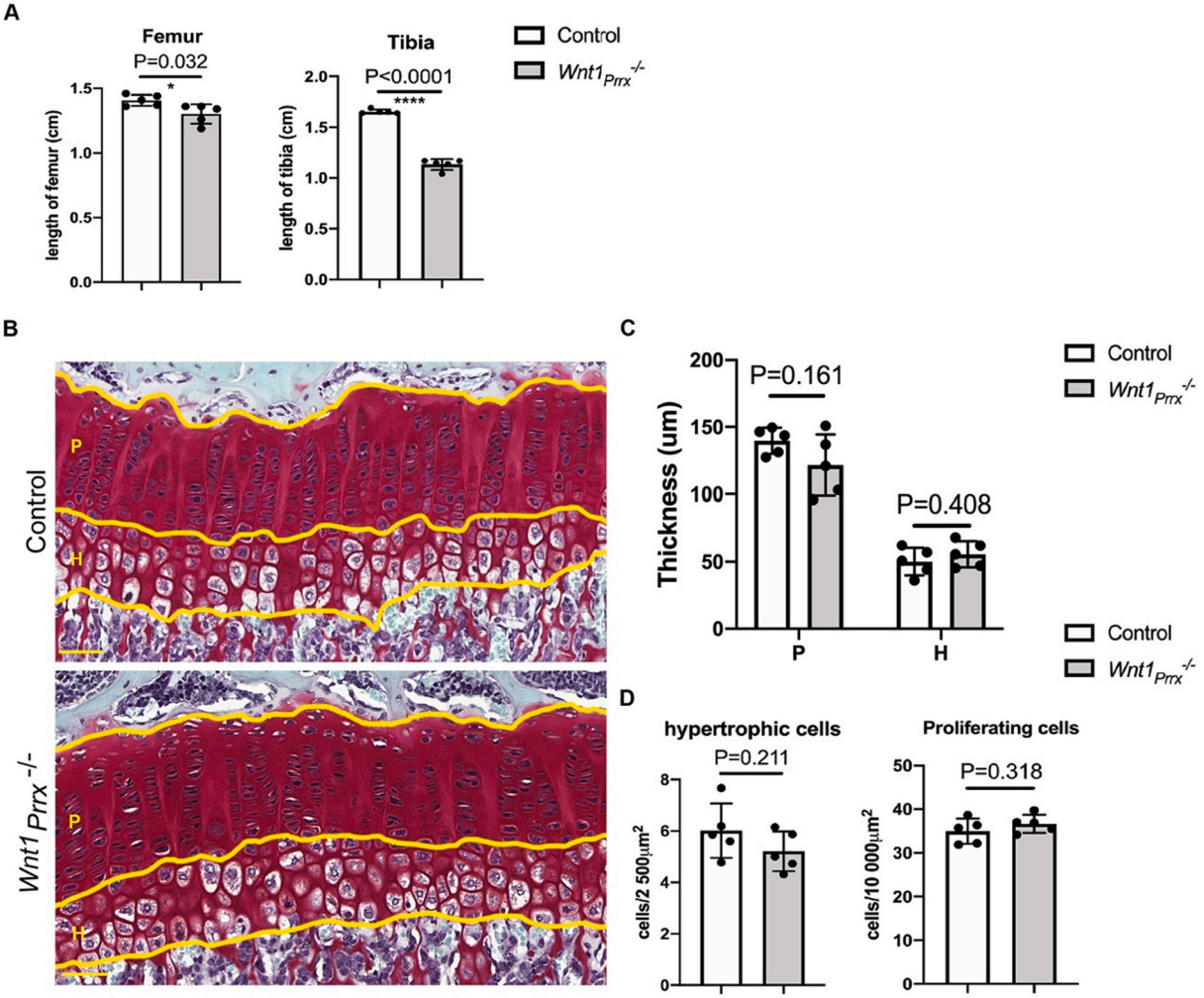

Fig. 2. The effect of Wnt1 on the development of growth plate.
(A) Femur and tibia lengths were measured in control and $Wnt1^{-/-}_{Prrx}$ male mice at the age of 6 weeks (n = 5). (B) Histological analysis of Safranin O/Fast green stained sections of proximal tibia epiphysis of control and $Wnt1^{-/-}_{Prrx}$ male mice was performed at the age of 6 weeks. Solid yellow lines delineate the proliferative (P) and hypertrophic (H) zones of growth plate, which were used for the analysis of thickness and cell density. Scale bar corresponds to 50 μm. (C) Thicknesses of proliferative and hypertrophic zones in the growth plate of control and $Wnt1^{-/-}_{Prrx}$ mice (n = 5). (D) Cell densities in proliferative and hypertrophic zones in the growth plate of control and $Wnt1^{-/-}_{Prrx}$ mice (n = 5). In (A), (C) and (D), each symbol represents an individual mouse; bar show the mean ± SD. Statistical significance was tested by two-tailed Student's *t*-test. P-values for significant differences between genotypes are presented as *P < 0.05, **** P < 0.0001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

potential of $Wnt1^{-/-}_{Prrx}$ chondroprogenitor cells when compared to wild-type (Fig. 3D). In agreement with this, no significant differences were observed in the mRNA expression of chondrocyte-specific genes in cultured cells between genotypes (Fig. 3E). As PTHrP signaling is one of the important pathways in regulating chondrocyte hypertrophy [19], we also analyzed the expression of *Pthrp* and *Pth1r*. There was a trend for increased mRNA expression of *Pthrp* (+75.8 %, P = 0.068) in $Wnt1^{-/-}_{Prrx}$ chondroprogenitor cells, which however did not reach statistical difference (Fig. 3E). As assessed by non-radioactive cell proliferation assay, $Wnt1^{-/-}_{Prrx}$ chondroprogenitor cells showed similar proliferation potential as wild-type cells (Fig. 3F).

3.4. *Wnt1 deletion leads to decreased subchondral bone mass*

Extensive evidence supports the essential role of Wnt signaling in the regulation of subchondral bone [20,21]. To address the role of Wnt1 in subchondral bone formation, we analyzed the subchondral bone phenotype of control and $Wnt1^{-/-}_{Prrx}$ mice by histology and μCT. Subchondral trabecular bone mass was significantly decreased in $Wnt1^{-/-}_{Prrx}$ at 4 weeks, 6 weeks and 12 weeks of age (Fig. 4A and Supplementary Fig. 3). Also, μCT analysis of tibia subchondral trabecular bone of male $Wnt1^{-/-}_{Prrx}$ revealed 64.7 % (P < 0.0001) and 39.8 % (P < 0.0001) reduction in bone volume per tissue volume (BV/TV) compared to control mice at 6 weeks and 12 weeks of age, respectively. The trabecular number (Tb.N) was also decreased (−45.2 %, P < 0.001 at 6 weeks old and −21.1 %, P = 0.001 at 12 weeks old), accompanied by reduced trabecular thickness (Tb.Th) (−36.4 %, P < 0.001 at 6 weeks old and -23.8 %, P < 0.001 at 12 weeks old), while the trabecular separation (Tb.Sp) was markedly increased (+38.5 %, P < 0.001 at 6 weeks old and + 53.2 %, P < 0.0001 at 12 weeks old) in male $Wnt1^{-/-}_{Prrx}$ mice compared to control mice (Fig. 4B and C).

Female $Wnt1^{-/-}_{Prrx}$ mice exhibited similar phenotype of subchondral trabecular bone as male mice. BV/TV was reduced by 57.8 % (P < 0.001) and 51.0 % (P < 0.0001) in $Wnt1^{-/-}_{Prrx}$ female mice compared to control mice at 6 and 12 weeks of age, respectively. The Tb.N was significantly decreased (−39.1 %, P = 0.002 at 6 weeks old and − 39.0 %, P < 0.001 at 12 weeks old mice), accompanied by decreased Tb.Th (−31.3 %, P < 0.0001 at 6 weeks old and −19.8 %, P = 0.002 at 12 weeks old), while the Tb.Sp was highly increased (+23.4 %, P = 0.01 at 6 weeks old and + 53.8 %, P < 0.0001 at 12 weeks old) in female $Wnt1^{-/-}_{Prrx}$

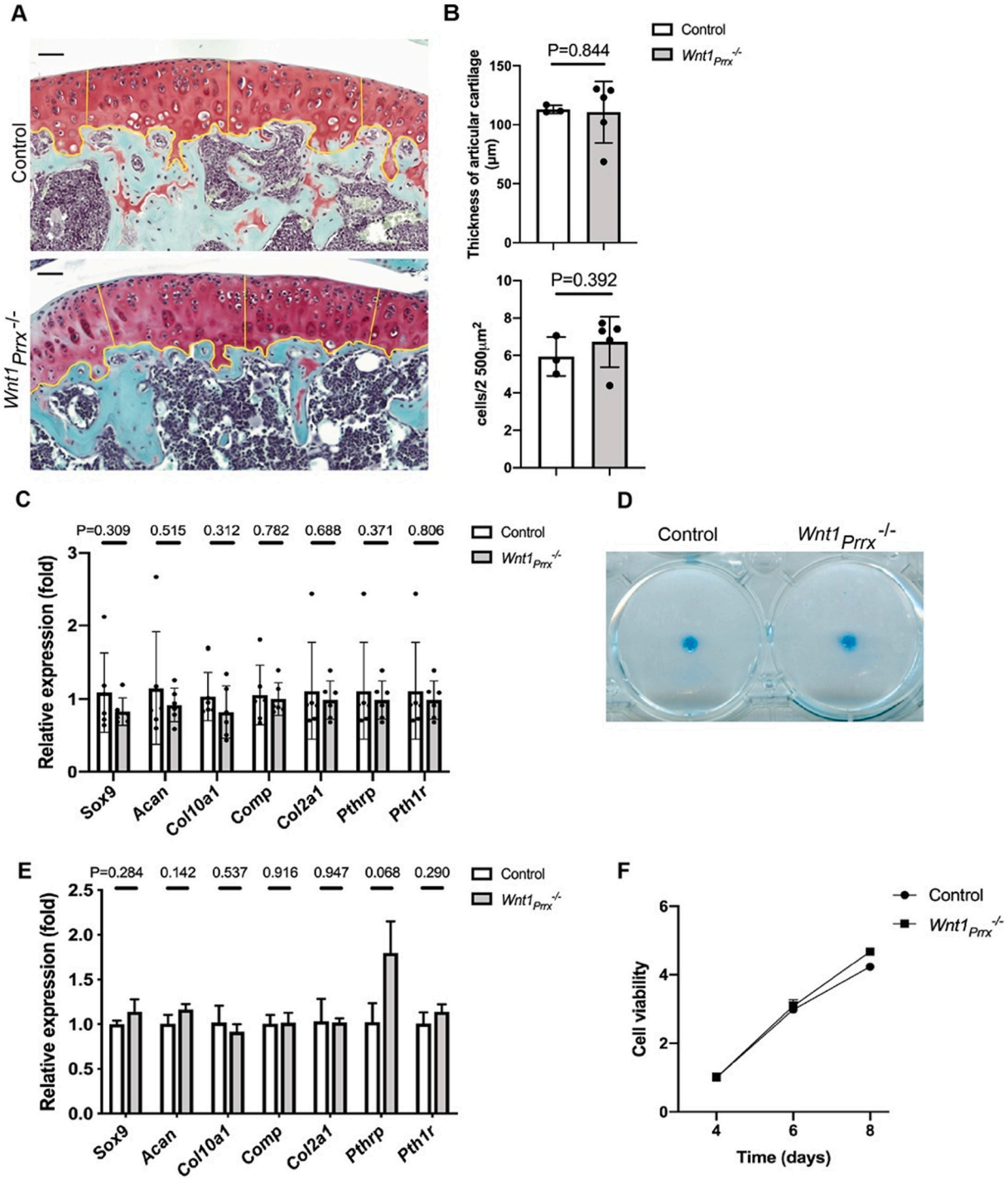

Fig. 3. The effect of Wnt1 on the development of articular cartilage.
(A) Safranin-O/Fast green stained sections of superficial articular cartilage in proximal tibia of control and $Wnt1^{-/-}_{Prrx}$ male mice at the age of 6 weeks. (B) Thicknesses and cell densities of articular cartilage was measured (control: n = 3, $Wnt1^{-/-}_{Prrx}$: n = 5). Scale bar corresponds to 50 μm. (C) mRNA expression level of cartilage specific genes, *Pthrp* and *Pth1r* was analyzed by qRT-PCR in knee joint of control and $Wnt1^{-/-}_{Prrx}$ mice at the age of 4 weeks (n = 6). (D) Alcian blue staining of chondroprogenitor cells isolated from control and $Wnt1^{-/-}_{Prrx}$ newborn mice after differentiation for 6 days. (E) mRNA expression level of chondrocyte specific genes, *Pthrp* and *Pth1r* was analyzed by qRT-PCR in chondroprogenitor cells isolated from control and $Wnt1^{-/-}_{Prrx}$ newborn mice after 6 days of culture**.** (F) Cell Proliferation Assay was used to detect cell viability of chondroprogenitor cells isolated from control and $Wnt1^{-/-}_{Prrx}$ newborn mice at indicated time points. In panel (B) and (C), each symbol represents an individual mouse tibia; bar shows the mean ± SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$^{-}$ mice compared to control mice (Fig. 4D).

3.5. *Wnt1 deletion impairs bone formation and enhances bone resorption in subchondral trabecular bone*

Histomorphometric analyses confirmed the reduction of BV/TV

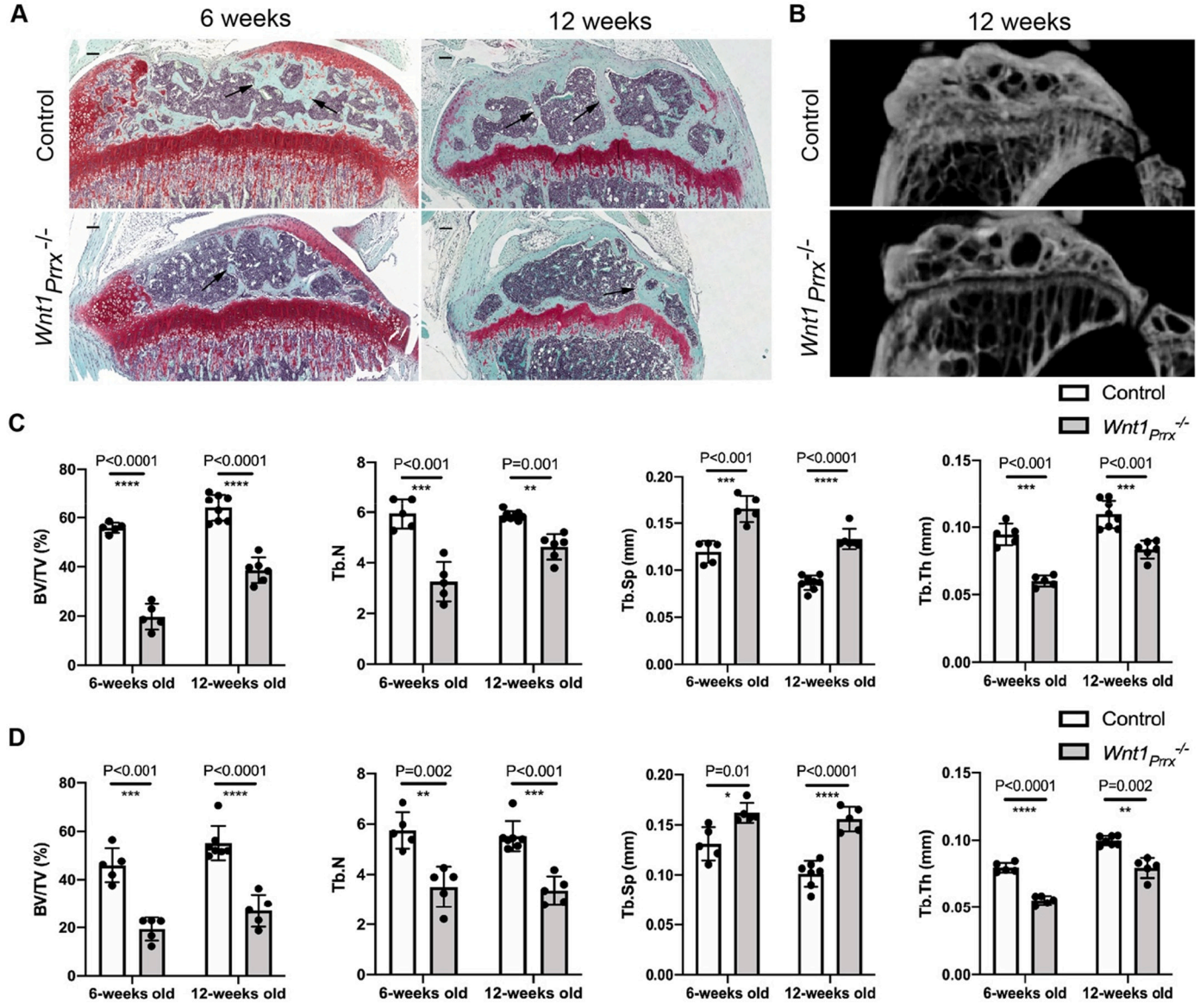

Fig. 4. The regulation of subchondral bone mass by Wnt1.
(A) Paraffin embedded sections of tibias of 6-weeks and 12-weeks old control and $Wnt1^{-/-}_{Prrx}$ male mice were stained with Safranin O/Fast green. Arrows indicate subchondral trabecular bone tissue. Scale bar corresponds to 100 μm. (B) Sagittal 3D μCT images of metaphyseal bone in control and $Wnt1^{-/-}_{Prrx}$ male mice at 12 weeks of age. (C) μCT analysis of subchondral bone of male control and $Wnt1^{-/-}_{Prrx}$ mice at 6 weeks (n = 5) and 12 weeks of age (control: n = 8, $Wnt1^{-/-}_{Prrx}$: n = 6). (D) μCT analysis of subchondral bone of female control and $Wnt1^{-/-}_{Prrx}$ mice at 6 weeks (n = 5) and 12 weeks of age (control: n = 7, $Wnt1^{-/-}_{Prrx}$: n = 5). In (C) and (D), each symbol represents an individual mouse; bar show the mean ± SD. Statistical significance was tested by Welch's *t*-test. P-values for significant differences between genotypes are presented as *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(−61.8 %, P < 0.001), Tb.N (−39.7 %, P = 0.017) and Tb.Th (−35.3 %, P = 0.001) in subchondral trabecular bone of $Wnt1^{-/-}_{Prrx}$ male mouse tibia at the age of 12 weeks (Supplementary Table 2). However, Wnt1 deficiency did not alter the number of osteoblasts per bone perimeter (P = 0.402) (Supplementary Table 2), while the number of osteoclasts in subchondral trabecular bone was increased in $Wnt1^{-/-}_{Prrx}$ mice compared to control mice (Supplementary Table 2).

In subchondral bone plate (SBP) of tibia, tissue area (T.Ar) (−32.9 %, P < 0.001), bone area (B·Ar) (−39.4 %, P = 0.002) and bone perimeter (B·Pm) (−23.8 %, P = 0.01) were significantly decreased in $Wnt1^{-/-}_{Prrx}$ mice (Supplementary Table 3). No difference was observed in SBP BV/TV (P = 0.187), while SBP thickness was slightly but significantly decreased (−19.7 %, P = 0.007) in $Wnt1^{-/-}_{Prrx}$ mice at 12 weeks of age (Supplementary Table 3).

We have in our previous study demonstrated that Wnt1 directly induced osteoblast differentiation in C3H10T1/2 cells in a juxtacrine manner. We also showed that Wnt1 directly suppressed osteoclastogenesis in murine monocyte/macrophage-like cell line, Raw264.7 cells [17]. To confirm the suppression of osteoclastogenesis by Wnt1, we performed a co-culture of growth-arrested *WNT1* or control C3H10T1/2 cells with purified $CD115^{+}$ mouse bone marrow monocytes and induced osteoclast differentiation with M-CSF and RANKL. *WNT1*-overexpressing cells directly inhibited osteoclastogenesis in this model (Supplementary Fig. 4A), which was accompanied with decreased expression of osteoclast markers *Nfatc1*, *Ctsk* and *Acp5* (Supplementary Fig. 4B). Notably, Wnt1 also inhibited the mRNA expression of cell-cell fusion markers *Atp6v0d2* and *Oscar*, indicating that WNT1 might inhibit osteoclastogenesis of $CD115^{+}$ monocytes by suppressing the cell fusion

(Supplementary Fig. 4B).

3.6. WNT1 inhibits chondrogenesis and chondrocyte hypertrophy through cell autonomous and juxtacrine manner

It has been reported that Wnt/β-catenin signaling is involved in chondrocyte maturation, generation of ossification center, and cartilage-to-bone conversion [4,5,22]. As Prrx is expressed in both osteoblasts and chondrocytes during the development and growth [23], the reduced subchondral bone mass in $Wnt1^{-/-}_{Prrx}$ mice might be partially due to the defect of chondrocyte maturation, hypertrophy and matrix mineralization. To study whether Wnt1 regulates chondrocyte differentiation *in*

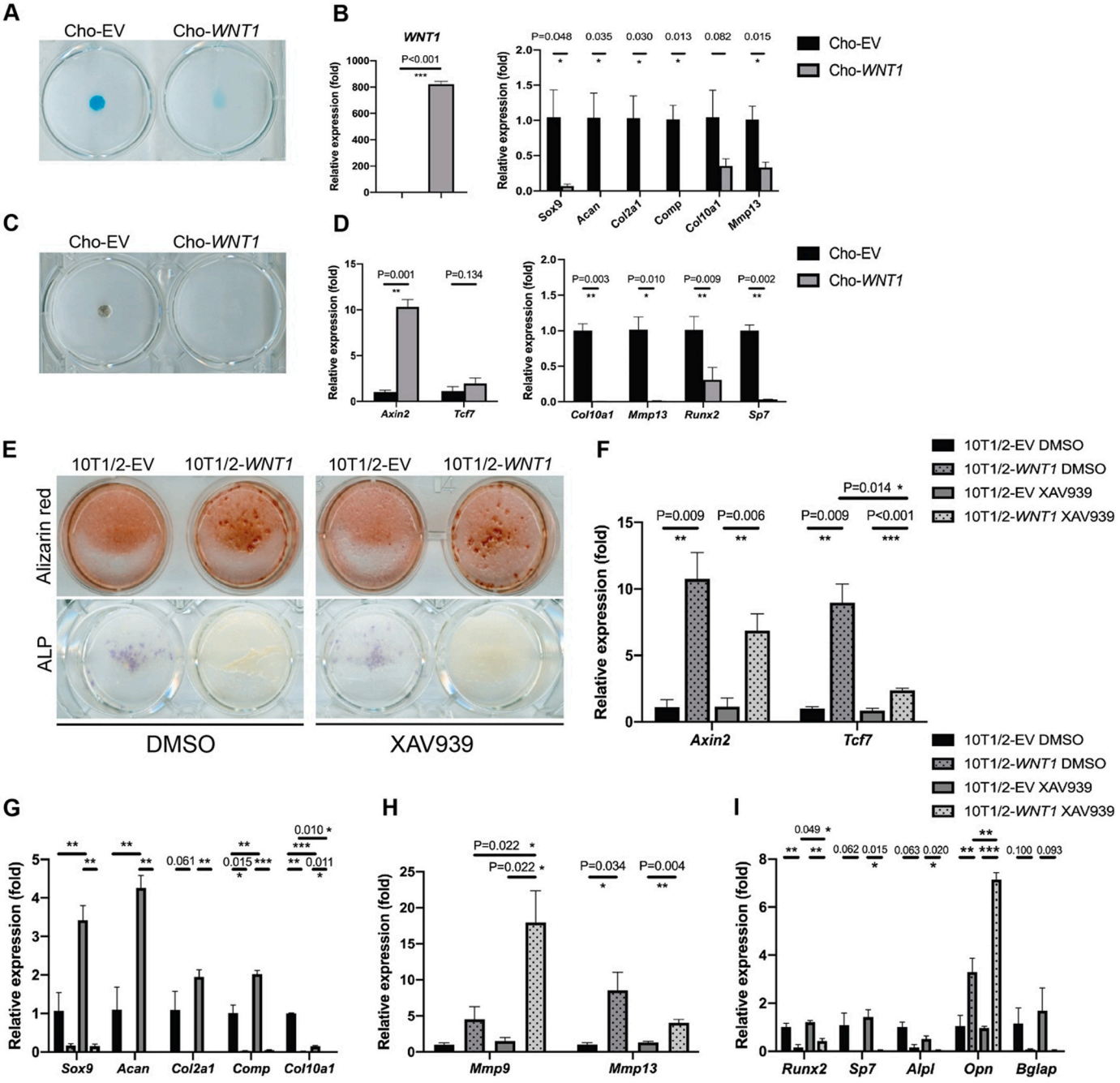

Fig. 5. The effect of Wnt1 on chondrogenesis, chondrocyte hypertrophy and matrix mineralization.
(A) Micromass culture of *WNT1* overexpressing and control EV-transduced chondroprogenitor cells with Alcian blue staining at 7 days. (B) mRNA expression of *WNT1*, chondrocyte and hypertrophic markers in *WNT1* overexpressing and control EV-transduced chondroprogenitor cells at 7 days. (C) Micromass culture of *WNT1* overexpressing and control EV-transduced chondroprogenitor cells with von Kossa staining at 28 days. (D) mRNA expression of Wnt target genes, hypertrophic markers and osteoblastic genes in *WNT1* overexpressing and control EV-transduced chondroprogenitor cells at 28 days. (E) Co-culture of C3H10T1/2 control or *WNT1*-overexpressing C3H10T1/2 with chondroprogenitor cells with Alizarin red and ALP staining at 10 days. Cells were cultured in osteogenic medium and treated with DMSO or 10 μM XAV939. (F)–(I), mRNA expression of Wnt target genes (F), chondrocyte specific genes (G), hypertrophic markers (H) and osteoblastic genes (I). All *in vitro* experiments were repeated three independent times with three replicate wells per condition. Staining and mRNA data are presented from one representative experiment as mean ± SD (n = 3). Statistical significance was tested by Student's *t*-test. P-values for significant differences between genotypes are presented as *P < 0.05, **P < 0.01, ***P < 0.001. EV = empty virus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vitro, we overexpressed *WNT1* in wild-type chondroprogenitor cells (Fig. 5). First, we confirmed the overexpression of *WNT1* in chondroprogenitor cells by qRT-PCR (Fig. 5B). As expected, the expression of Wnt signaling related genes, *Axin2* and *Tcf7* were also upregulated by *WNT1* overexpression (Fig. 5D). Interestingly, *WNT1* overexpression in chondroprogenitor cells strongly suppressed chondrocyte differentiation and hypertrophy (Fig. 5A and C), which was accompanied with decreased expression of chondrocyte-specific genes, such as *Sox9*, *Acan*, *Col2a1* and *Comp*, as well as markers of hypertrophic chondrocytes, such as *Col10a1* and *Mmp13* (Fig. 5B and D). Moreover, the transcriptional factors regulating osteoblast proliferation and differentiation, *Runx2* and *Sp7*, were also downregulated by *WNT1*.

Our previous study suggested that WNT1 is not secreted into culture medium, but functions in a juxtacrine manner, requiring cell-cell contact [17]. To verify that the suppression of chondrogenesis and hypertrophy by WNT1 in primary chondrocytes did not occur only in a cell autonomous manner, we co-cultured growth-arrested *WNT1*-overexpressing or control C3H10T1/2 cells with chondroprogenitor cells and induced chondrogenic differentiation. *WNT1*-overexpressing cells directly inhibited chondrogenesis in a juxtacrine manner, which was revealed by downregulated expression of chondrocyte specific genes *Sox9*, *Acan*, *Col2a1*, *Comp* and *Col10a1* (Fig. 5G). XAV939 is a small molecular inhibitor of Wnt/β-catenin signaling, stimulating β-catenin degradation by stabilizing AXIN. As expected, XAV939 significantly suppressed the expression of Wnt1 targeted genes *Axin2* and *Tcf7* in *WNT1*-overexpressing co-cultures (Fig. 5F). Inhibition of canonical Wnt/β-catenin signaling enhanced the expression of chondrogenic markers in control cultures, while it did not rescue the suppression in *WNT1*-overexpressing co-cultures (Fig. 5G), suggesting that under this experimental condition WNT1 directly inhibits chondrogenesis independent of canonical Wnt/β-catenin signaling.

Similar to chondrogenesis, the hypertrophy of primary chondrocytes was also suppressed by *WNT1*-overexpressing cells in a juxtacrine manner, as demonstrated by decreased ALP staining as well as mRNA expression of *Alpl* gene (Fig. 5E and I). In addition, the expression of osteoblast-specific genes *Runx2*, *Sp7* and *Bglap*, which are also expressed by hypertrophic chondrocytes, was downregulated by Wnt1 (Fig. 5I). The inhibition of Wnt/β-catenin by XAV939 failed to rescue the suppression of chondrocyte hypertrophy in Wnt1-overexpressing co-cultures (Fig. 5E and I). Taken together, Wnt1 inhibited chondrogenesis and chondrocyte hypertrophy both in a cell autonomous manner and in a juxtacrine manner. Furthermore, the suppression of chondrogenesis and chondrocyte hypertrophy by Wnt1 appears to be independent of canonical Wnt/β-catenin signaling.

3.7. *Wnt1 promotes matrix mineralization through juxtacrine manner*

Further analysis of *WNT1*-overexpressing or control C3H10T1/2 cells and chondroprogenitor cells co-cultures revealed that WNT1 strongly induced matrix mineralization in chondroprogenitor cells in a juxtacrine manner (Fig. 5E). Consistent with this, the mRNA expression of matrix metalloproteinase-9 (*Mmp9*) and *Mmp13,* as well as an osteoblastic marker, osteopontin (*Opn*) known to regulate mineralization process, was significantly upregulated by *WNT1* (Fig. 5H and I). The inhibition of Wnt/β-catenin by XAV939 was not able to alter WNT1-induced mineralization in the co-culture system (Fig. 5E). However, XAV939 suppressed *WNT1*-upregulated mRNA expression of *Mmp13* (Fig. 5H), suggesting that *WNT1*-overexpressing C3H10T1/2 cells may stimulate *Mmp13* expression in chondroprogenitor cells *via* Wnt/β-catenin signaling pathway. In contrast, XAV939 did not alter the *WNT1*-induced expression of *Mmp9* and *Opn*, but rather enhanced their expression in both control and *WNT1*-overexpressing co-cultures (Fig. 5H and I), indicating that *WNT1* could stimulate chondrocytes to express *Mmp9* and *Opn via* Wnt/β-catenin-independent pathway.

It is known that MMPs are important mediators of cartilage canal and secondary ossification center (SOC) formation [24], and that both MMP9 and MMP13 play critical roles in extracellular matrix remodeling, blood vessel invasion, and osteoblast migration [25,26]. Since our data showed that *WNT1* promotes the expression of *Mmp9* and *Mmp13* in a juxtacrine manner in co-cultures, it was of interest to evaluate, whether Wnt1 deletion in limb bud mesenchymal progenitor cells affects SOC vascularization (Supplementary Fig. 5). Histological analysis showed that vessel area per total SOC area was decreased by 30.1 % ($P = 0.119$) in 4-week-old $Wnt1^{-/-}_{Prrx}$ mice compared to control mice, even though the difference was not statistically significant (Supplementary Fig. 5A and B). Moreover, the total vessel area in SOC was significantly reduced by 40.1 % ($P = 0.024$) in $Wnt1^{-/-}_{Prrx}$ mice compared to control mice. These data indicated that Wnt1 might be a regulator of blood vessel invasion in SOC, thus regulating SOC formation.

4. Discussion

It has been documented that Wnt/β-catenin signaling has an important role in regulation of chondrocyte function during long bone formation and growth [21,27]. Excessive activation or deficiency of this signaling causes severe abnormal cartilage formation, growth plate organization and function in mice [28–30]. In human genetic studies, the alterations in Wnt-related genes have been suggested as risk factors for OA development and progression. For example, the level of Wnt1-inducible-signaling pathway protein 1 (WISP1) are increased in plasma and synovial fluid in OA patients, while the levels of Dickkopf 1 (DKK1), an inhibitor of Wnt/β-catenin signaling are significantly lower [31,32]. Although we observed shorter femur and tibia in $Wnt1^{-/-}_{Prrx}$ mice compared to control mice in the current study, the data surprisingly also shows that the *Wnt1* deficiency in limb bud mesenchymal progenitor cells does not affect the development of growth plate in mice. This indicates that the decreased femur and tibia lengths in $Wnt1^{-/-}_{Prrx}$ mice were rather due to fractures, which caused bending of long bones. In addition, we demonstrate here that Wnt1 deficiency in limb bud mesenchymal progenitor cells does not affect the development of articular cartilage. Our findings are supported by a recent study by Yorgan and colleagues, who reported that the growth plate morphology at proximal tibia was unaltered in mice with *Wnt1* mutation [33]. Moreover, Lehtovirta and colleagues reported that patients with *WNT1* mutation did not differ in cartilage thickness compared to mutation negative group [34]. Taken together, the WNT1 deficiency in cartilage might be compensated by other WNT ligand/s, which could robustly induce Wnt/β-catenin signaling pathway.

Abnormal remodeling of subchondral trabecular bone is a hallmark in the development of OA [35,36]. It has been reported that the expression of *WNT1* in the subchondral bone is significantly correlated with the alteration of bone structure [37]. In this study, we show that homozygous deletion of *Wnt1* in mesenchymal progenitors led to decreased bone mass in both subchondral trabecular bone and subchondral bone plate due to impaired bone formation and enhanced bone resorption. The data is consistent with our previous report showing the effects on trabecular and cortical bone in the same animal model [17] and suggest that Wnt1 is a key regulator of bone remodeling both at POC and SOC.

It is well-established that some WNT proteins regulate chondrocyte differentiation *in vitro*. For example, overexpression *Wnt1* or *Wnt7a* in micromass cultures of chick limb bud mesenchymal cells inhibited chondrogenesis and chondrocyte maturation [14]. In addition, treatment of primary porcine articular chondrocytes with WNT3A recombinant protein resulted in stimulated proliferation and de-differentiation of chondrocytes [38], while administration of WNT16 recombinant protein in mouse primary chondrocytes had no effect on proliferation or chondrogenesis, but significantly inhibited chondrocyte maturation and mineral deposition [15]. Our cell culture data confirmed that WNT1 inhibited chondrogenesis of primary mouse chondrocytes. Furthermore, we demonstrate here that both overexpression of *WNT1* in chondroprogenitor cells and co-cultures of *WNT1*-overexpressing C3H10T1/

2 cells with chondroprogenitor cells result in suppression of chondrogenesis. Our previous study revealed that WNT1 could not be secreted into culture medium, but functions in a juxtacrine manner in bone cells requiring physical contact to its target cells [17]. The current data however implies that WNT1 inhibits chondrogenesis not only through a cell autonomous manner, but also in a juxtacrine manner.

Hypertrophic chondrocytes are actively involved in the regulation of matrix mineralization, remodeling, and vascular invasion during endochondral ossification. In addition to expressing gene *Col10a1*, hypertrophic chondrocytes also express numerous osteoblast markers, such as *Alpl*, *Sp7*, *Runx2*, bone sialoprotein *(Bsp)*, *Bglap*, and *Opn* [39–41]. Wnt/β-catenin signaling has been demonstrated to positively regulate chondrocyte hypertrophy in multiple animal models. In contrast, we show here that Wnt1 inhibited chondrocyte hypertrophy, as demonstrated by down-regulated expression of the genes mentioned above (*Alpl, Runx2, Sp7, Opn,* and *Ocn*). This implies that in addition to canonical Wnt/β-catenin signaling, WNT1 can also activate non-canonical Wnt signaling pathway, which suppresses chondrocyte hypertrophy. Furthermore, inhibition of Axin-dependent canonical Wnt/β-catenin signaling by small molecule inhibitor XAV939 did not rescue the inhibition of chondrogenesis or chondrocyte hypertrophy in *WNT1*-overexpressing co-cultures, confirming that the juxtacrine Wnt1 signaling suppresses chondrogenesis and chondrocyte hypertrophy through Wnt/β-catenin-independent pathway.

Knockout mouse models and human genetic diseases have revealed that many MMPs, such as MMP9, MMP13 and MMP14 are important mediators of endochondral ossification during long bone development [24–26,42]. MMP9 and MMP13, secreted by hypertrophic chondrocytes, are required for chondrocyte apoptosis, degradation of cartilage matrix, vascular invasion and osteoblast recruitment. A recent study reported that mice with β-catenin (*Ctnnb1*) overexpression in cartilage displayed early SOC formation and vascular invasion compared to littermate controls [4]. Moreover, the expression of *Mmp9*, *Mmp13* and *Mmp14* in epiphysis of β-catenin gain-of-function also occurred earlier than in controls, suggesting that cartilage-specific β-catenin regulates vascular invasion in SOC by driving the expression of these *Mmp* genes [4]. The co-culture experiments presented in our current study show that *WNT1*-overexpressing cells significantly upregulated the expression of *Mmp9* and *Mmp13*, although they clearly suppressed chondrocyte hypertrophy. Taken together, the reduced vessel area observed in subchondral region of $Wnt1^{-/-}_{Prrx}$ mice might be due to impaired secretion of MMP9 and MMP13 by WNT1 deficient chondrocytes, so that it affects the degradation of cartilage matrix and invasion by blood vessels.

One limitation of our study is that the specificity of *Wnt1* knockout in the current mouse model is insufficient. As both osteoblasts and chondrocytes originate from mesenchymal progenitor cells, the deletion of *Wnt1* in $Wnt1^{-/-}_{Prrx}$ mice occurs in both osteoblasts and chondrocytes at SOC. This limits the possibility to investigate the role of chondrocyte-specific *Wnt1* in the regulation of SOC formation. To address this limitation, it would be of interest to generate chondrocyte-specific *Wnt1* knockout mice by *e.g.*, *Col2a1*-Cre mice and find out whether chondrocyte-derived *Wnt1* has effects on SOC formation.

5. Conclusions

In conclusion, our data show that mesenchymal cell-derived *Wnt1* regulates subchondral bone remodeling by promoting osteoblast activity and by suppressing osteoclastogenesis, while it does not affect the development of growth plate or articular cartilage *in vivo*. *In vitro*, WNT1 inhibits chondrogenesis and hypertrophy of chondroprogenitor cells in both cell autonomous and juxtacrine manners, while inducing matrix mineralization only through juxtacrine manner. Further understanding of the role of *Wnt1* in the regulation of subchondral bone and SOC formation could provide novel therapeutic strategies for the patients with OA and other skeletal diseases.

Funding

This study was funded by the Academy of Finland (R.K.: 298625, 268535, 139165), Novo Nordisk Foundation, Sigrid Juselius Foundation, the Finnish Cultural Foundation, Orion Research Foundation, Jalmari ja Rauha Ahokkaan Foundation, Maud Kuistila Memorial Foundation, Finnish-Norwegian Medical Foundation and the work at TCDM by funding provided by the University of Turku, Finland.

CRediT authorship contribution statement

R.K. conceived the idea for the study and acquired the funding. R.K. and F.W. designed the experiments. F.W. collected mouse samples. F.W., P.R. and M.P. performed the experiments. F.W., P.R. and R.K. analyzed the data. R.K. and T.J.H. supervised the work. F.W. wrote the first draft. F.W., A-M. S., T.J.H. and R.K. edited the manuscript.

All authors contributed to the final version of the manuscript. F.W. had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors have read, provided critical feedback on intellectual content and approved the final manuscript.

Declaration of competing interest

The authors have no conflict of interest.

Acknowledgements

The authors thank Merja Lakkisto, Dr. Fuping Zhang and the staff of Turku Central Animal Laboratory for their excellent technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2022.116497.

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