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Original Article

ORIGINAL ARTICLE¹

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RAR γ ACTIVITY IN MSCs: ENDOCHONDRAL BONE, ANGIOGENESIS, B
LYMPHOPOIESIS

**Retinoic Acid Receptor γ Activity in Mesenchymal Stem Cells Regulates Endochondral
Bone, Angiogenesis, and B Lymphopoiesis**

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ABSTRACT

Retinoic acid receptor (RAR) signaling regulates bone structure and hematopoiesis through intrinsic and extrinsic mechanisms. This study aimed to establish how early in the osteoblast lineage loss of RAR γ (*Rarg*) disrupts the bone marrow microenvironment. Bone structure was analyzed by micro-computed tomography (μ CT) in *Rarg*^{-/-} mice and mice with *Rarg* conditional deletion in *Osterix-Cre*-targeted osteoblast progenitors or *Prrx1-Cre*-targeted mesenchymal stem cells. *Rarg*^{-/-} tibias exhibited less trabecular and cortical bone and impaired longitudinal and radial growth. The trabecular bone and longitudinal, but not radial, growth defects were recapitulated in *Prrx1:Rarg* ^{Δ/Δ} mice but not *Osx1:Rarg* ^{Δ/Δ} mice. Although both male and female *Prrx1:Rarg* ^{Δ/Δ} mice had low trabecular bone mass, males exhibited increased numbers of trabecular osteoclasts and *Prrx1:Rarg* ^{Δ/Δ} females had impaired mineral deposition. Both male and female *Prrx1:Rarg* ^{Δ/Δ} growth plates were narrower than controls and their epiphyses contained hypertrophic chondrocyte islands. Flow cytometry revealed that male *Prrx1:Rarg* ^{Δ/Δ} bone marrow exhibited elevated pro-B and pre-B lymphocyte numbers, accompanied by increased *Cxcl12* expression in bone marrow cells. *Prrx1:Rarg* ^{Δ/Δ} bone marrow also had elevated megakaryocyte-derived *Vegfa* expression accompanied by smaller sinusoidal vessels. Thus, RAR γ expression by *Prrx1-Cre*-targeted cells directly regulates endochondral bone formation and indirectly regulates tibial vascularization. Furthermore, RAR γ expression by *Prrx1-Cre*-targeted cells extrinsically regulates osteoclastogenesis and B lymphopoiesis in male mice. © 2018 American Society for Bone and Mineral Research

KEY WORDS: GENETIC ANIMAL MODELS; OSTEOIMMUNOLOGY; OSTEOBLASTS; STROMAL/STEM CELLS; TISSUE SIGNALING—OTHER (RETINOIC ACID RECEPTOR SIGNALING)

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Additional Supporting Information may be found in the online version of this article.

Introduction

The skeleton is a dynamic organ that is constantly remodeled to maintain its structural integrity. The medullary cavity of bones contains bone marrow (BM), which is the primary postnatal site of hematopoiesis. Microenvironments in the BM are comprised of multiple cell types that influence each other to regulate both hematopoiesis and bone formation and resorption. These cell types include mesenchymal stem cells (MSCs), osteoblast-lineage cells, perivascular cells, endothelial cells, adipocytes, and hematopoietic cells including osteoclasts and immune cells. Previous studies have found that deletion of retinoic acid receptor γ (RAR γ) resulted in mice with low bone mass and hematopoietic defects that were, at least in part, induced by nonhematopoietic cells.^(1,2) Thus, although RAR γ has direct roles in hematopoiesis (intrinsic regulation), RAR γ signaling in nonhematopoietic cells in the BM microenvironment also influences hematopoiesis (extrinsic regulation).

RARs are nuclear receptors that regulate transcription of genes and have important roles in the proliferation and differentiation of many cell lineages. There are three subtypes of RARs; α , β , and γ , that can have either distinct or equivalent functions in different cell lineages.⁽³⁾ *Rara*^{-/-} and *Rarg*^{-/-} mice have defects in multiple tissues and were initially reported to exhibit neonatal lethality.^(4,5) The housing of these mice in a clean animal facility improved survival, especially for *Rara*^{-/-} mice. Although *Rarg*^{-/-} mice still showed significant postnatal lethality in cleaner housing conditions, some survived up to 12 months of age.⁽⁶⁾ Eight-week-old *Rara*^{-/-} mice displayed no defects in bone⁽¹⁾ or hematopoiesis.^(6,7) In comparison, histomorphometric analysis of tibias from 8-week-old *Rarg*^{-/-} mice revealed they had less trabecular bone when compared to wild-type littermates, and in males this was associated with increased osteoclastogenesis.⁽¹⁾

In addition to lower bone mass, *Rarg*^{-/-} mice developed a myeloproliferative-like syndrome (MPS) characterized by significantly more granulocytes in the peripheral blood (PB), BM, and spleen.⁽²⁾ *Rarg*^{-/-} mice also exhibited defects in erythropoiesis and B lymphopoiesis,^(2,8) with lower numbers of pro-B and pre-B lymphocytes in the BM.⁽⁹⁾ When BM from wild-type mice was transplanted into mice with a *Rarg*^{-/-} microenvironment, the transplanted mice rapidly

developed MPS-like disease and erythrocyte and B lymphocyte defects. In comparison, when *Rarg*^{-/-} BM was transplanted into wild-type mice these hematopoietic defects were resolved. This indicated that RAR γ expression in microenvironment cells (extrinsic) was essential for normal hematopoiesis, and that the mature hematopoietic cell defects observed in *Rarg*^{-/-} mice were not due to intrinsic loss of RAR γ in those cells.

We have recently shown that deletion of *Rarg* in primitive *nestin-Cre*-targeted neural crest-derived MSCs and their progeny impaired BM B lymphopoiesis and thymic T lymphopoiesis.⁽⁹⁾ The defects observed in *nestin-Cre:Rarg* ^{$\Delta\Delta$} mice did not fully recapitulate all hematopoietic phenotypes exhibited by *Rarg*^{-/-} mice, suggesting that additional cells within the microenvironment regulate hematopoiesis via the actions of RAR γ . Because osteoblast-lineage cells play a key role in supporting B lymphopoiesis,⁽¹⁰⁻¹²⁾ and *Rarg* is expressed throughout osteoblast differentiation,⁽¹³⁾ we sought to investigate the role of RAR γ at different stages of osteoblast commitment in the regulation of bone structure and hematopoiesis.

To determine whether committed osteoblast-lineage cells were involved in inducing changes to bone mass and hematopoiesis we conditionally deleted *Rarg* in osteoblast progenitors (OPs) and their progeny using *Osx1-Cre* and compared their phenotype to mice with targeted deletion in more primitive limb bud-derived MSCs and their progeny using *Prrx1-Cre* (also known as *Prr1*). Here we report a role for *Rarg* signaling in *Prrx1-Cre*-targeted limb bud-derived mesenchymal cells, but not in *Osx1-Cre*-targeted osteoprogenitor cells, in the regulation of endochondral bone, sinusoidal vessels, and B lymphopoiesis.

Subjects and Methods

Mice

All experiments were approved by the St Vincent's Health Melbourne Animal Ethics Committee and conducted in strict compliance to the regulatory standards of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Tibias were obtained from 8-week-old and 12-week-old male and female *Rarg*^{-/-} [*Rarg*^{tm1Ipc}] and *Rarg*^{+/+} littermate mice.⁽⁴⁾ *Rarg* was conditionally deleted in OPs using *Osx1-GFP::Cre* [B6.Cg-Tg(Sp7-tTA,tetO-EGFP/cre)1Amc/J]⁽¹⁴⁾ and in limb bud-derived MSCs using *Prrx1-Cre* mice [B6.Cg-Tg(Prrx1-cre)1Cjt/J]⁽¹⁵⁾ each of these crossed with *Rarg*^{fl/fl} mice [*Rarg*^{tm3Ipc}].⁽¹⁶⁾ In all studies 12-week-old *Cre*⁺:*Rarg*^{+/+} mice were used as controls for 12-week-old *Cre*⁺:*Rarg*^{fl/fl} mice. *Osx1-GFP::Cre*⁺:*Rarg*^{fl/fl} mice are referred to as *Osx1:Rarg* ^{$\Delta\Delta$} , *Osx1-GFP::Cre*⁺:*Rarg*^{+/+} as

Osx1:Rarg^{+/+}, *Prrxl1-Cre*⁺:*Rarg*^{fl/fl} as *Prrxl1:Rarg*^{Δ/Δ}, and *Prrxl1-Cre*⁺:*Rarg*^{+/+} as *Prrxl1:Rarg*^{+/+}. All mice were fully backcrossed onto the C57BL/6 strain prior to use in these studies.

Prrxl1:Rarg mice were crossed with *Rosa26-loxP-stop-loxP*-enhanced yellow fluorescent protein (*R26-stop-EYFP*; B6.129X1-*Gt(ROSA)26Sor*^{tm1(EYFP)Cos/J}) mice for these studies. *Prrxl1-Cre* mice occasionally exhibit germline activation of Cre.⁽¹⁵⁾ To exclude these mice from analysis germline activation was identified by EYFP expression in peripheral blood (PB) cells by flow cytometry for all *Prrxl1-Cre*⁺ mice. In *Prrxl1:Rarg*^{Δ/Δ} mice, excision of *Rarg* exon 8 was also confirmed to be specific to nonhematopoietic tissues by genomic PCR of PB (forward primer: tcttacctgctgagtgccaaccaa, reverse primer: agaagaaagcagttacagggcagg).

Micro-computed tomography

Bone structure of tibias from 8-week-old *Rarg* mice (to compare to previous work⁽¹⁾) and adult 12-week-old *Rarg*, *Osx1:Rarg*, and *Prrxl1:Rarg* mice were analyzed by μ CT. Tibias were dissected, cleaned of muscle and connective tissue, fixed in 2% paraformaldehyde overnight at 4°C, then stored in 70% ethanol. The SkyScan 1076 system (Bruker-microCT, Kontich, Belgium) was used to scan tibias, following standard guidelines.⁽¹⁷⁾ The image acquisition parameters used were: 9 μ m voxel resolution, 0.5 mm aluminum filter, 45 kV voltage, 100 μ A current, 2300 ms exposure time, 0.5-degree rotation step, and frame averaging of 1.

Reconstruction and analysis of images was performed using SkyScan software NRecon (1.6.9.8), DataViewer (1.5.0), and CTAnalyser (1.13.11.0). The region of interest (ROI) for tibial trabecular bone was identified as a 3% distal offset from the growth plate and a height of 13.5% of total tibial length. Trabecular bone was analyzed utilizing adaptive thresholding for all samples; minimum values were 45 (*Rarg*), 44 (*Osx1:Rarg*), 51 (*Prrxl1:Rarg*), with a maximum of 225. ROIs for cortical bone were defined as a 40% distal offset from the growth plate and 13.5% region of total tibial length. Global automatic thresholding was used for cortical bone analysis with a minimum of 95 (*Rarg*, *Osx1:Rarg*) or 100 (*Prrxl1:Rarg*) and a maximum of 225.

Histomorphometry, immunohistochemistry, and immunofluorescence

Tibias were embedded in methylmethacrylate and 5- μ m sections were stained with toluidine blue as described.^(1,18) Histomorphometry of the secondary spongiosa was performed using OsteoMeasure7 v4.1.1.2 (OsteoMetrics, Atlanta, GA, USA) using standard guidelines.⁽¹⁹⁾ The region analyzed was 370 μ m distal to the base of the growth plate using a 370- μ m² box from the edge of the marrow cavity excluding all cortical bone in three nonoverlapping rows.

Cartilage and sinusoidal vessels were analyzed on decalcified tibial sections as described.⁽²⁰⁾ Tibias were fixed in 2% paraformaldehyde overnight, decalcified by replacing the EDTA solution twice over 2 weeks. Samples were processed and embedded in paraffin, 5- μ m sections were dewaxed and rehydrated. To analyze sinusoidal vessels, samples were stained for VEGFR-3 (eBioscience, Santa Clara, CA, USA) at [1:400] as described.⁽²⁰⁾ To analyze the growth plate, samples were stained with Safranin-O and Fast Green as described.⁽²¹⁾ The numbers and sizes of VEGFR-3+ sinusoidal vessels and sinusoid perimeter in the secondary spongiosa, in addition to growth plate height and the number of epiphyseal hypertrophic chondrocyte islands, were measured using OsteoMeasure software.⁽²⁰⁾ The growth plate proliferative versus hypertrophic zones could not be distinguished, so separate samples were collected at 20 days of age to allow assessment of these zones according to standard protocols using OsteoMeasure software.⁽²²⁾

Mouse tibias were prepared following the protocol described by Poulton and colleagues⁽²³⁾ and Parra and colleagues.⁽²⁴⁾ Immunofluorescence labeling protocol for formalin-fixed, paraffin-embedded tissue was adapted from Nolan and colleagues.⁽²⁵⁾ Antibodies, detection, and labeling conditions are found in Supplemental Table 2. Endogenous peroxidase activity was blocked with 3% H₂O₂ (Merck, Burlington, MA, USA) for 30 min, washed in Tris-buffered saline (TBS), followed by a protein block with TNB blocking solution (Perkin Elmer, Waltham, MA, USA) for 1 hour prior to primary antibody incubation (Supplemental Table 2). All washes were performed three times with TBS for 5 min each. Multiplexed immunofluorescence labeling was done through sequential staining as described.⁽²⁴⁾ After antibody staining, sections were counterstained with DAPI (1:1000) for 5 min and mounted in Citifluor hard set mounting media CFPVOH plus AF100 (Citifluor, Hatfield, PA, USA). Whole slide scans were imaged on the Vectra Polaris Quantitative Pathology Imaging System (Perkin Elmer).

Hematological analysis

PB was obtained by retro-orbital bleeding, BM was flushed from one femur, spleens were crushed and filtered to single-cell suspensions. Hematopoietic cellularity was assessed with a Sysmex KX-21N analyzer (Sysmex, Kobe, Japan). BM cells were stained with fluorophore-conjugated antibodies (Supplemental Table 1),⁽²⁶⁾ all flow cytometry data were collected using a Becton Dickinson (BD) LSR Fortessa (BD Biosciences, San Jose, CA, USA) and data analyzed

using FlowJo 8.8.7 (Treestar, Ashland, OR, USA).

Analysis of primary osteoblast-lineage cells

Osteoblast-lineage cells were isolated from *Prrxl1:Rarg* long bones as described.⁽¹³⁾ Briefly, bones were cleaned, cut into 1-mm² pieces and gently crushed using a mortar and pestle. Bone marrow was removed by washing fragments with 2% FBS and 5mM EDTA in PBS. Bone fragments (containing trabecular and cortical bone) were digested with 3 mg/mL collagenase type 1 (Worthington) in PBS for 45 min at 37°C with agitation and stained with fluorophore-conjugated antibodies (Supplemental Table 1). Nonviable, hematopoietic (CD45, Ter119) and endothelial (CD31) cells were omitted, MSCs (Sca-1⁺, CD51⁺) and osteoblast-lineage cells (Sca-1⁻, CD51⁺) were sorted on a BD Influx (BD Biosciences).⁽¹³⁾

RNA isolation and qPCR

Total RNA was isolated using an Isolate II RNA Micro Kit (Bioline USA, Taunton, MA, USA) and cDNA synthesized using AffinityScript qPCR cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA, USA) as per the manufacturers' instructions. qPCR was performed using Brilliant II SYBR green (Agilent Technologies) and 5 pmol of primers (all reported previously^(1,26,27)) using a MxPro 3000P Stratagene system and software (Stratagene California, La Jolla, CA, USA).

Statistics

Data are presented as mean ± SD and statistical analysis was performed using an unpaired two-tailed Student's *t* test in Prism 7 (GraphPad Software, La Jolla, CA, USA).

Results

Rarg^{-/-} mice have less trabecular bone

μCT analysis of 8-week-old and 12-week-old *Rarg* mice confirmed the findings of our previous study that used histomorphometric analysis.⁽¹⁾ In 8-week-old and 12-week-old *Rarg*^{-/-} tibias, trabecular bone volume was 72% and 62% lower in males, respectively, and 42% and 58% lower in females, respectively, compared to *Rarg*^{+/+} mice (Fig. 1 and Supplemental Fig. 1 show 12-week-old and 8-week-old data, respectively). There were no changes in trabecular thickness in *Rarg*^{-/-} mice (Fig. 1B, Supplemental Fig. 1B). However, trabecular separation was significantly higher (Fig. 1C, Supplemental Fig. 1C) and trabecular number was significantly lower in both male and female *Rarg*^{-/-} mice compared to their sex-matched controls (Fig. 1D, Supplemental Fig. 1D). The lower trabecular bone mass in *Rarg*^{-/-} mice is shown in reconstructed images of

3D trabecular bone structure (Fig. 1E–H, Supplemental Fig. 1E–H).

<Insert Figure 1>

Rarg^{-/-} mice have impaired bone growth

Tibias of 8-week-old and 12-week-old *Rarg*^{-/-} mice were >12% shorter compared to *Rarg*^{+/+} mice (Supplemental Fig. 1I, Fig. 1I), indicating impaired longitudinal growth of tibias. μ CT analysis found no change in cortical bone parameters in 8-week-old female mice, consistent with our previous histomorphometric analysis⁽¹⁾ (Supplemental Fig. 1J–M). However, 8-week-old male and all 12-week-old *Rarg*^{-/-} mice had thinner cortical bone compared to *Rarg*^{+/+} mice (Supplemental Fig. 1J, Fig. 2A). These mice also had a significantly smaller cortical area and periosteal perimeter (Supplemental Fig. 1L–M; Fig. 2C, D), indicating impaired radial growth. Twelve-week-old *Rarg*^{-/-} females also had a greater marrow area (Fig. 2B).

<Insert Figure 2>

Osx1:Rarg ^{Δ/Δ} mice have no change in bone volume

We next sought to identify which osteoblast precursor population is important for maintaining trabecular and cortical bone mass via RAR γ signaling. *Rarg*^{-/-} male mice exhibited increased osteoclastogenesis in vivo, yet RAR inhibition blocked osteoclast differentiation of mouse whole BM cells and RAW264.7 cells in vitro.⁽¹⁾ Osteoclast formation is commonly regulated by osteoblast-lineage cells via their production of RANKL and osteoprotegerin (OPG)⁽²⁸⁾; therefore, we investigated the bone phenotype in mice with *Rarg* deletion in the osteoblast lineage, directed with *Osx1-Cre*. Analysis of 12-week-old male tibias by μ CT found no changes in trabecular bone structure in the secondary spongiosa (Fig. 3A–F). Analysis of cortical bone similarly identified no significant differences between *Osx1:Rarg* ^{Δ/Δ} and *Osx1:Rarg*^{+/+} mice and tibial length was unchanged by *Rarg* targeting (Supplemental Fig. 2A–G). Hence loss of *Rarg* from *Osx1-Cre*-targeted OPs and their progeny is not the cause of the bone defects observed in *Rarg*^{-/-} mice.

<Insert Figure 3>

Prrx1:Rarg ^{Δ/Δ} mice have reduced trabecular bone

We deleted *Rarg* in primitive limb bud-derived MSCs and their progeny using *Prrx1-Cre* to determine if an earlier mesenchymal cell might mediate the impaired bone growth and lower trabecular bone mass observed with global loss of *Rarg*. *Prrx1:Rarg* ^{Δ/Δ} male and female mice had significantly less trabecular bone compared to age- and sex-matched *Prrx1:Rarg*^{+/+} mice (Fig. 4A, E–H). Trabecular thickness was unchanged (Fig. 4B), but *Prrx1:Rarg* ^{Δ/Δ} males had

significantly greater trabecular separation (Fig. 4C), and both male and female *Prrx1:Rarg $\Delta\Delta$* mice had lower trabecular number than controls (Fig. 4D). These changes to trabecular bone mimicked those observed in *Rarg $^{-/-}$* mice, although the difference in bone mass was more variable in males. The low trabecular bone mass was more pronounced in *Prrx1:Rarg $\Delta\Delta$* males than females, consistent with observations in *Rarg $^{-/-}$* mice (Fig. 1)⁽¹⁾ The tibias were shorter in *Prrx1:Rarg $\Delta\Delta$* compared to *Prrx1:Rarg $^{+/+}$* mice (Fig. 4I); however, no differences in cortical bone structure were observed between *Prrx1:Rarg $\Delta\Delta$* and *Prrx1:Rarg $^{+/+}$* mice (Supplemental Fig. 3), indicating that longitudinal, but not radial, growth was impaired.

<Insert Figure 4>

Prrx1:Rarg $\Delta\Delta$ females exhibit more trabecular osteoblasts and males have more trabecular osteoclasts

In female *Prrx1:Rarg $\Delta\Delta$* mice, histomorphometric analysis revealed that the low bone volume was accompanied by a greater proportion of unmineralized bone (osteoid) in the trabecular bone (Fig. 5A). Female *Prrx1:Rarg $\Delta\Delta$* mice had a significantly higher percentage of bone comprising osteoid due to a greater proportion of osteoid-covered bone surface (Fig. 5A, B) rather than a significant change in osteoid thickness (Fig. 5C). Female *Prrx1:Rarg $\Delta\Delta$* mice also had more osteoblasts than controls (Fig. 5E), and although most *Prrx1:Rarg $\Delta\Delta$* females had a higher osteoblast surface this was not statistically significant (Fig. 5D).

<Insert Figure 5>

In contrast to female mice, male *Prrx1:Rarg $\Delta\Delta$* mice displayed no significant changes in trabecular osteoid surface, osteoblast numbers, or osteoblast surface compared to age-matched *Prrx1:Rarg $^{+/+}$* mice (Fig. 5A–E). Instead, male *Prrx1:Rarg $\Delta\Delta$* mice exhibited greater osteoclast surface and osteoclast numbers in trabecular bone compared to *Prrx1:Rarg $^{+/+}$* males (Fig. 5F, G). These data have revealed that *Rarg* deletion in very primitive cells of the osteoblast lineage is the major contributor to the bone phenotype in *Rarg $^{-/-}$* mice and that the cellular mechanisms by which RAR γ regulates trabecular bone mass are either different in males and females or predominate to different extents in males and females.

Further investigation of tibial sections revealed islands of hypertrophic chondrocytes in the epiphyseal trabecular bone of both male and female *Prrx1:Rarg $\Delta\Delta$* mice (Fig. 5H–K). When quantified, chondrocyte islands were present in the majority of *Prrx1:Rarg $\Delta\Delta$* mice but only in one *Prrx1:Rarg $^{+/+}$* male mouse. This suggests their impaired growth relates to a delay in

establishment of the secondary ossification center. Furthermore, the growth plates of *Prrxl:Rarg*^{ΔΔ} male and female mice were 14.8% and 16.3% shorter than controls (Fig. 5L), respectively, correlating with the impaired bone growth in these mice and the high level of RAR γ expression in the murine growth plate.⁽²⁹⁾ Surprisingly, when the growth plates of young (20-day-old) *Prrxl:Rarg*^{ΔΔ} male and female mice were assessed, this difference in growth plate width was not present, but the proliferative zone (Fig. 5M) was of normal width and the hypertrophic zone width was extended (Fig. 5N). The hypertrophic chondrocytes also appeared larger in size (Fig. 5O, P). This suggested that both the reduction in bone length, and the presence of cartilage islands within the secondary ossification center may relate to an extension in the period of hypertrophy. This was clearly transient, and was followed by an early reduction in growth plate width.

Prrxl:Rarg^{ΔΔ} male mice showed altered bone marrow B lymphopoiesis

We sought to determine whether *Prrxl:Rarg*^{ΔΔ} mice also had microenvironment-induced alterations in hematopoietic cells. Hematological analysis revealed that *Prrxl:Rarg*^{ΔΔ} males, but not females, exhibited a significant increase in BM leukocytes (Fig. 6A). To further investigate the altered leukocyte numbers in *Prrxl:Rarg*^{ΔΔ} males, BM samples were profiled by flow cytometry (Fig. 6B, C). In *Prrxl:Rarg*^{ΔΔ} males there was a significant increase in the numbers of BM-derived B220⁺IgM⁻ cells, with no changes in the numbers of B220⁺IgM⁺ B lymphocytes (identifying a mix of BM-derived immature B lymphocytes and recirculating mature B lymphocytes) (Fig. 6D). Further investigation revealed that within the B220⁺IgM⁻ population (Fig. 6C) there was an increase in the numbers of both CD19⁺CD43⁺ pro-B lymphocytes and CD19⁺CD43⁻ pre-B lymphocytes, but no change in the numbers of CD19⁻CD43⁺ pre-pro-B lymphocytes in *Prrxl:Rarg*^{ΔΔ} compared to *Prrxl:Rarg*^{+/+} male mice (Fig. 6E). Thus the enhanced formation of early B lymphocyte precursors originated at the pro-B lymphocyte stage but did not affect maturing (B220⁺IgM⁺) B lymphocyte numbers (Fig. 6F). The hematopoietic phenotype was sex-specific as there were no changes in BM B lymphocytes in female *Prrxl:Rarg*^{ΔΔ} mice (Supplemental Fig. 4). We also confirmed that *Prrxl-Cre* did not target the B lymphocytes, with no co-expression of B220 and EYFP observed in immunofluorescence studies of tibias or flow cytometry analysis of the peripheral blood of the mice (Supplemental Fig. 5). Furthermore, there were no differences in the numbers of PB (Supplemental Fig. 4) or splenic (data not shown) B lymphocytes in male or female *Prrxl:Rarg*^{ΔΔ} mice. Aside from an

increase in BM granulocytes in female *Prrx1:Rarg $\Delta\Delta$* mice, no other significant differences were detected in the other hematopoietic cell populations (data not shown).

<Insert Figure 6>

Our data suggested that *Prrx1-Cre* targets cells that are involved in the regulation of B lymphopoiesis, thus we investigated the gene expression of key factors involved in this process^(30,31) in whole BM cells. The expression of *Cxcl12*, tumor necrosis factor receptor superfamily member 11b (*Tnfrsf11b*; OPG), and *Vegfa* were significantly higher in *Prrx1:Rarg $\Delta\Delta$* mice, *Flt3l* was unchanged (Fig. 6G–J) and *Tnfsf11* (RANKL), *Kitl*, and *Il7* could not be detected. To determine if mesenchymal cells were the sources of the altered transcripts, osteoblastic cells were purified by FACS into *Prrx1:EGFP⁺* MSCs (lineage, CD45 and CD31 negative, Sca-1⁺, CD51⁺) and osteoblast-lineage cells (lineage, CD45 and CD31 negative, Sca-1⁻, CD51⁺) from *Prrx1:Rarg^{+/+}* and *Prrx1:Rarg $\Delta\Delta$* mice. qPCR revealed different changes in gene expression in purified *Prrx1:Rarg $\Delta\Delta$* osteoblastic populations compared to whole BM cells. The expression of *Cxcl12*, the secreted and membrane forms of Kit ligand (*Kitl*) and *Flt3l* were significantly lower in *Prrx1:Rarg $\Delta\Delta$* MSCs compared to *Prrx1:Rarg^{+/+}* MSCs (Fig. 6K). All other transcripts were unaltered in MSCs (Fig. 6K), with the exception of *Il7* and *Tnfsf11*, which were not detected. Furthermore, *Cxcl12*, secreted *Kitl*, and *Il7* were significantly reduced in *Prrx1:Rarg $\Delta\Delta$* osteoblast-lineage cells whereas all other transcripts were unaltered (Fig. 6L), with the exception of *Tnfsf11*, which could not be detected (data not shown).

Prrx1:Rarg $\Delta\Delta$ mice altered BM vascularization

Endochondral bone formation is tightly linked to BM angiogenesis and sinusoidal vessels are important constituents of the BM microenvironment. Given that *Vegfa* expression was increased in the BM of *Prrx1:Rarg $\Delta\Delta$* mice and is a key regulator of BM angiogenesis, we quantified the numbers of sinusoidal vessels in the BM of the secondary spongiosa by histomorphometry. The number of sinusoids in the BM was increased in *Prrx1:Rarg $\Delta\Delta$* females (Fig. 7A, G, H). In both male and female *Prrx1:Rarg $\Delta\Delta$* mice the sinusoids were smaller (Fig. 7B, E–H). The sinusoids occupied less space within the BM in male *Prrx1:Rarg $\Delta\Delta$* mice (Fig. 7B, C), reflecting the sinusoidal vessel constriction. In contrast, the sinusoid perimeter per marrow area was unchanged (Fig. 7D). The vessel phenotype was reflected throughout the BM including in the primary spongiosa (data not shown).

<Insert Figure 7>

To determine the source of the increased *Vegfa* observed in the BM we performed immunofluorescence studies, investigating the co-expression of the *Prrxl1-Cre*-targeted cells (using an antibody against EYFP), and *Vegf-a* in tibia sections from *Prrxl1:Rarg Δ/Δ* and *Prrxl1:Rarg $^{+/+}$* mice. *Vegf-a* was highly expressed in the growth plate of *Prrxl1:Rarg $^{+/+}$* mice (Fig. 7I), consistent with a previous report showing *Vegf-a* expression in this region.⁽²³⁾ In contrast, the production of *Vegf-a* was significantly reduced in the growth plate of *Prrxl1:Rarg Δ/Δ* mice, but increased in the bone marrow megakaryocytes (Fig. 7J). Flow cytometry-based quantitation studies showed that the proportions of CD41+ megakaryocytes were unaltered in the bone marrow of *Prrxl1:Rarg Δ/Δ* compared to *Prrxl1:Rarg $^{+/+}$* mice (Fig. 7K).

Discussion

This study has identified that RAR γ activity in *Prrxl1-Cre*-targeted cells is a key regulator in the formation of a healthy BM microenvironment, significantly influencing the development of normal trabecular bone mass, angiogenesis, and B lymphopoiesis.

Loss of *Rarg* in *Prrxl1-Cre*-targeted cells and their progeny resulted in similar (albeit less severe) changes to trabecular bone and longitudinal bone growth as *Rarg $^{-/-}$* mice. The bone phenotypes were not observed when *Rarg* was deleted in *Osx1-Cre*-targeted early osteoblast progenitor cells (and also a subset of hypertrophic chondrocytes), confirming that this role of *Rarg* is mediated by a nonoverlapping population of immature mesenchymal cells targeted by *Prrxl1-Cre*. Deletion of *Rarg* in *Prrxl1-Cre*-targeted cells also influenced growth plate chondrocyte hypertrophy preceding the trabecular bone defects and abnormal BM vascularization in *Prrxl1:Rarg Δ/Δ* mice.

Prrxl1:Rarg Δ/Δ , but not *Osx1:Rarg Δ/Δ* , mice had defects in trabecular bone mass, similar to that observed in *Rarg $^{-/-}$* mice. *Prrxl1-Cre* and *Osx1-Cre* both target cells that give rise to the osteoblast lineage; however, their expression patterns vary between anatomical sites and the other mesenchymal lineages they form. In *Prrxl1-Cre* mice, Cre recombinase (Cre) is expressed in the multipotent cells of the limb bud mesenchyme, flank mesoderm and craniofacial mesenchyme; ie, in the appendicular skeleton, some calvarial bones, and the sternum, but not in the vertebrae.⁽¹⁵⁾ This means that genes deleted from *Prrxl1-Cre $^{+}$* multipotent cells will remain deleted in their progeny. In the BM of long bones, 50% of *Prrxl1-Cre $^{+}$* cells are PDGFR α^{+} Sca-1 $^{+}$ (P α S) cells (a population enriched with MSCs) and *Prrxl1-Cre $^{+}$* cells give rise to osteoblasts/osteocytes, CXCL12-abundant reticular (CAR) cells, adipocytes, and chondrocytes,

but not hematopoietic or endothelial cells.^(32,33) Thus, the changes observed in *Prrx1:Rarg*^{Δ/Δ} bones could be due to loss of *Rarg* in any of these cell types or combinations of cells. In comparison, *Osx1-Cre* only targets a small proportion of PαS cells (~5%)⁽³²⁾ and is expressed in more differentiated early OPs that give rise to osteoblasts residing in the perichondrium, periosteum, endosteum, and trabecular bone, as well as osteocytes.^(14,34,35) In addition to the osteoblast lineage, *Osx1-Cre* is expressed in some growth plate prehypertrophic and hypertrophic chondrocytes, perivascular cells (including CAR cells), and BM adipocytes postnatally.^(32,34,36) The lack of a bone phenotype in *Osx1:Rarg*^{Δ/Δ} mice suggests that the bone defects following deletion of *Rarg* are not due to lack of *Rarg* in cells committed to the osteoblast lineage. Thus, the regulation of bone length and trabecular bone mass via RARγ activity appears to occur in MSCs (or potentially their immature progeny).

Deletion of RARs using *Col2a1-Cre*, which targets chondrocytes, osteoblasts, and synovial fibroblasts,^(37,38) has also been shown to alter endochondral bone growth.⁽²⁹⁾ *Col2a1-Cre*⁺:*Rara*^{fl/fl}*Rarg*^{fl/fl} mice and *Col2a1-Cre*⁺:*Rarb*^{fl/fl}*Rarg*^{fl/fl} mice were reported to have low trabecular bone mass, but this was not quantitated,⁽²⁹⁾ so we cannot reliably compare that aspect of the phenotype to our models. However, *Col2a1-Cre*⁺:*Rara*^{fl/fl}*Rarg*^{fl/fl} mice and *Col2a1-Cre*⁺:*Rarb*^{fl/fl}*Rarg*^{fl/fl} mice, but not *Col2a1-Cre*⁺:*Rara*^{fl/fl}*Rarb*^{fl/fl} mice, had narrow growth plates and exhibited impaired long bone growth.⁽²⁹⁾ In the growth plates of these mice the hypertrophic zone appeared largely unaffected, but chondrocyte proliferation was impaired. This aberrant functioning of the growth plate impaired endochondral bone formation, resulting in a lower trabecular bone mass in *Col2a1-Cre*⁺:*Rara*^{fl/fl}*Rarg*^{fl/fl} mice and *Col2a1-Cre*⁺:*Rarb*^{fl/fl}*Rarg*^{fl/fl} mice. The bone phenotype of *Col2a1-Cre*⁺:*Rarg*^{fl/fl} mice was not assessed; however, given that mice lacking *Rara* and *Rarb* in *Col2a1-Cre*-targeted chondrocytes appeared phenotypically normal, loss of RARγ is likely to be the main contributor to the phenotypes observed in the *Col2a1-Cre*⁺:*Rara*^{fl/fl}*Rarg*^{fl/fl} and *Col2a1-Cre*⁺:*Rarb*^{fl/fl}*Rarg*^{fl/fl} mice.

If *Rarg* expression in differentiating chondrocytes is required for normal growth plate function and subsequent endochondral bone formation and long bone growth, this could contribute to both the trabecular bone and longitudinal growth defects observed in *Prrx1:Rarg*^{Δ/Δ} mice, because chondrocytes are targeted by *Prrx1*. Indeed, in *Prrx1:Rarg*^{Δ/Δ} mice the growth plate showed early abnormalities in hypertrophy, and in adult bones was shorter than that of controls, suggesting a potential cause of the impaired longitudinal growth. Intriguingly,

Prrxl:Rarg^{Δ/Δ} mice also exhibited islands of hypertrophic chondrocytes trapped in the trabecular bone in the epiphysis of tibias; this also suggests an extension of the hypertrophic stage. Cartilage remnants have similarly been observed in diaphyseal trabecular bone of *Osx1-Cre:EphrinB2*^{Δ/Δ} mice and occur because of defective osteoclastic resorption in the primary ossification center.⁽³⁹⁾ Similarly, children with osteogenesis imperfecta also contain cartilage within their bones after treatment with antiresorptive bisphosphonate therapy.⁽⁴⁰⁾ The presence of an extended hypertrophic zone at the growth plate and hypertrophic chondrocyte islands in *Prrxl:Rarg*^{Δ/Δ} mice thus suggests that chondrocyte hypertrophy may progress more slowly leading to impaired resorption of the secondary ossification center.

One factor that plays a role in endochondral ossification and therefore long bone growth and trabecular bone formation is Vegf-a, which may contribute to the phenotype in *Prrxl:Rarg*^{Δ/Δ} mice. Vegf-a plays an essential role in the neovascularization of cartilage, facilitating endochondral bone formation.^(41,42) In the BM, Vegf-a is expressed by osteoblast-lineage cells, hypertrophic chondrocytes, and some proliferating chondrocytes,^(41,43) and some hematopoietic cells, including myeloid progenitors and megakaryocytes.⁽⁴⁴⁾ Vegf-a stimulates the recruitment of endothelial cells for vessel formation, which in turn recruits osteoclasts and osteoblasts to the growth plate to resorb and replace cartilage with mineralized bone.⁽⁴⁵⁾ As expected, the increased *Vegfa* expression in the BM *Prrxl:Rarg*^{Δ/Δ} female mice was accompanied by significantly more sinusoidal vessels in the BM secondary spongiosa; however, the sinusoids were constricted, resulting in significantly reduced overall vessel area in the BM compared to *Prrxl:Rarg*^{+/+} mice. This gain of function effect is the opposite of, and hence consistent with, reports of mice lacking two of the three VEGF isoforms; ie, VEGF₁₆₄ and VEGF₁₈₈, but still expressing VEGF₁₂₀. These mice have fewer, more dilated blood vessels, indicating that the VEGF₁₆₄ and VEGF₁₈₈ isoforms are essential for BM vascularization.⁽⁴⁶⁾ The sinusoidal vessels were similarly constricted in male *Prrxl:Rarg*^{Δ/Δ} mice; however, the vessel number was not increased. The normal number of sinusoidal vessels in the males may indicate that the excess Vegf-a is acting elsewhere in the BM. Furthermore, given *Vegfa* expression was unchanged in *Prrxl:Rarg*^{Δ/Δ} MSCs and osteoblast-lineage cells isolated from collagenase-digested bone, the increased *Vegfa* in the BM must be derived from an alternate cell type. Indeed, our studies revealed that Vegf-a was upregulated in the megakaryocytes in the bone marrow of the *Prrxl:Rarg*^{Δ/Δ} mice. Megakaryocytes and megakaryocyte cell lines have

previously been shown to produce and secrete VEGF.^(47,48) It is currently unclear as to why Vegf-a was increased in the megakaryocytes of *Prrxl1:Rarg*^{Δ/Δ} mice. However, the increased Vegf-a observed throughout the BM correlated with the vascular changes we observed throughout the BM, whereas localized disruption to Vegf-a expression in chondrocytes of mice lacking leukemia inhibitory factor (LIF) results in enhanced vascularization localized to the growth plate.⁽²³⁾

Vegf-a also has direct effects on osteoclast precursors, increasing their expression of RANK⁽⁴⁹⁾ and enhancing osteoclastic differentiation, survival, and resorptive activity.⁽⁵⁰⁾ Furthermore, Gerber and colleagues⁽⁴³⁾ found that inhibition of Vegf-a signaling in 24-day-old mice impaired vascularization and cartilage resorption. Thus, in *Prrxl1:Rarg*^{Δ/Δ} males, it is likely that the increase in *Vegfa* contributed to the elevated trabecular osteoclasts and lower trabecular bone mass through the stimulation of osteoclast differentiation and recruitment, stimulation of cartilage and bone resorption, and/or prolonging the survival of trabecular osteoclasts. This was consistent with the reduction in trabecular number accompanied by increased trabecular spacing but unaltered trabecular thickness in the *Prrxl1:Rarg*^{Δ/Δ} male mice, which is indicative of enhanced resorption. Although the osteoclast numbers were unchanged in *Prrxl1:Rarg*^{Δ/Δ} females, the enhanced vascularization may have increased the osteoblast recruitment to the bone surface, contributing to the increased numbers of osteoblasts observed in these female mice.

Prrxl1:Rarg^{Δ/Δ} male mice also displayed altered B lymphopoiesis, indicating that RAR γ signaling in *Prrxl1-Cre*-targeted cells and/or their progeny are important BM microenvironment regulators of B lymphopoiesis. Male *Prrxl1:Rarg*^{Δ/Δ} mice had increased numbers of B lymphocyte precursors in the BM, with elevated pro-B and pre-B lymphocytes, but no changes in the more primitive pre-pro-B lymphocytes. Interestingly, the increase in immature B lymphocyte numbers were not reflected in mature B lymphocyte populations, because the numbers of immature B220⁺IgM⁺ B lymphocytes, splenic and mature PB B lymphocytes were unchanged. The upregulated *Cxcl12* expression in *Prrxl1:Rarg*^{Δ/Δ} male BM was consistent with the B lymphocyte phenotype observed in these mice. *Cxcl12* is a positive regulator of B lymphopoiesis from the pre-pro-B to pre-B cell stages, hence could be contributing to the increased numbers of pro-B and pre-B lymphocytes observed in *Prrxl1:Rarg*^{Δ/Δ} male mice. The BM is the site of early B lymphopoiesis and distinct niches regulate different stages of development, including osteoblasts, osteoclasts, CAR cells, IL-7-expressing cells, and endothelial cells. MSCs and

osteoblast lineage cells play important roles in regulating early B lymphopoiesis.⁽³¹⁾ To determine if the increased expression of *Cxcl12*, or other potential B lymphocyte regulators, originated in *Prrx1*-derived mesenchymal cells, we FACS-isolated populations of MSCs (Sca-1⁺, CD51⁺) and osteoblast-lineage cells (Sca-1⁻, CD51⁺) from bone and examined their expression of known key B lymphocyte regulators. Surprisingly, many regulatory genes were reduced in both MSCs (eg, *Cxcl12*, *Kitl*, and *Flt3*) and osteoblast-lineage cells (eg, *Cxcl12*, *Kitl*, and *Il7*), despite pro-B and pre-B cells being higher in *Prrx1:Rarg*^{Δ/Δ} mice. This is the opposite of what we would expect if these cell types were regulating B lymphopoiesis in *Prrx1:Rarg*^{Δ/Δ} mice because they are all identified as positive regulators of early B lymphopoiesis.^(31,32,51–54) This implies that other cell type(s) in the BM microenvironment are responsible for the increased B lymphocyte phenotype in these mice. These cells are likely to be the source of the increased *Cxcl12* expression in the BM in *Prrx1:Rarg*^{Δ/Δ} male mice. Some candidates are chondrocytes⁽⁵⁵⁾ and endothelial cells,⁽³²⁾ which have both been shown to express *Cxcl12*.

Another possible contributor to the increased pro-B and pre-B cells in the male *Prrx1:Rarg*^{Δ/Δ} mice was the increased osteoclasts observed in these mice.⁽³¹⁾ Blin-Wakkach and colleagues⁽⁵⁶⁾ showed that osteopetrotic *oc/oc* mice had impaired production of BM B lymphocytes from the pro-B cell stage which was rescued by transplanting normal BM containing osteoclast precursors.⁽⁵⁷⁾ Furthermore, administration of the antiresorptive zoledronic acid (ZA) led to impaired B lymphopoiesis from the pre-pro-B lymphocyte stage, with no direct effect of ZA observed on the B lymphocyte cells.⁽⁵⁷⁾ ZA treatment also dramatically reduced *Cxcl12* and *Il7* expression in CD45⁻ BM cells, which are devoid of most hematopoietic cells.⁽⁵⁷⁾ Thus, altered osteoclast function modifies the regulation of B lymphopoiesis in the BM.⁽³¹⁾ This is complementary to our studies showing that *Prrx1:Rarg*^{Δ/Δ} male, but not female, mice had increased osteoclastogenesis and increased pro-B and pre-B lymphocyte numbers, suggesting that enhanced osteoclastogenesis may contribute to the B lymphocyte phenotype.

The increase in B lymphocyte precursors in male *Prrx1:Rarg*^{Δ/Δ} mice (Fig. 6) contrasts to the reduced pre-B lymphocytes observed in male *Rarg*^{-/-} and *nestin-Cre*⁺:*Rarg*^{Δ/Δ} mice, reduced pro-B cells in *Rarg*^{-/-} mice, and unchanged pro-B cells in *nestin-Cre*⁺:*Rarg*^{Δ/Δ} mice.⁽⁹⁾ Furthermore, *Osx1:Rarg*^{Δ/Δ} mice had no alterations in the numbers of B lymphocytes in their BM, spleen, or PB.⁽⁹⁾ *Prrx1-Cre*-expressing and *nestin*-expressing cells are distinct, nonoverlapping populations in the BM, targeting limb bud and neural crest mesenchyme,

respectively.^(9,32,58) As such, RAR γ signaling appears to differentially regulate B lymphopoiesis in *Prrx1-Cre*-targeted and *nestin-Cre*-targeted cells and these cells may form distinct B lymphocyte niches.

In this study, we identified that RAR γ signaling in *Prrx1-Cre*-targeted mesenchymal cells is required for BM microenvironment development. Loss of *Rarg* in *Prrx1-Cre*-targeted cells impaired tibial longitudinal growth and caused a lower trabecular bone mass; however, the normal bone phenotype in *Osx1:Rarg Δ/Δ* mice suggested that this was not due to loss of *Rarg* in osteoblast progenitors or osteoblasts. *Prrx1:Rarg Δ/Δ* mice also exhibited a smaller growth plate, retained hypertrophic chondrocytes in epiphyseal trabecular bone, and had defective endochondral bone formation with increased angiogenesis and altered trabecular osteoclastogenesis (in males) and osteoblastogenesis (in females). Furthermore, the increased osteoclastogenesis observed in *Prrx1:Rarg Δ/Δ* males was accompanied by increased numbers of pro-B and pre-B lymphocytes in the male mice, suggesting another link between osteoclasts and B lymphocytes. Collectively, our data identify that in limb bud-derived MSCs, RAR γ is a key direct regulator of endochondral bone formation, and is an extrinsic regulator of angiogenesis, osteoclastogenesis, and immature B lymphopoiesis in the BM.

Disclosures

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Authors' roles: ACG and LEP designed the study. Experiments were performed and analyzed by ACG, VRS, BCI, LS, GT, MW, NAS, and LEP. Data interpretation was performed by ACG, VRS, TJM, NAS, and LEP. ACG and LEP drafted the manuscript. ACG, VRS, TJM, NAS, and LEP revised the manuscript. All authors approved the final manuscript.

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Fig. 1. *Rarg*^{-/-} mice have less trabecular bone and shorter tibias than *Rarg*^{+/+} mice. Tibial trabecular bone structure and length was analyzed by μ CT in 12-week-old male and female *Rarg*^{+/+} versus *Rarg*^{-/-} mice. The graphs show (A) BV/TV, (B) Tb.Th, (C) Tb.Sp, and (D) Tb.N.

Representative reconstructed tibias images for male (*E, F*) and female (*G, H*) mice. (*I*) Graph shows tibial lengths for 12-week-old mice. Male ($n = 10$), female *Rarg*^{+/+} ($n = 8$), and *Rarg*^{-/-} ($n = 6$) mice. ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ (unpaired two-tailed Student's *t* test). BV/TV = trabecular bone volume; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation; Tb.N = trabecular number.

Fig. 2. *Rarg*^{-/-} mice have thinner cortical bone and narrower tibias than *Rarg*^{+/+} mice. Tibial cortical bone structure was analyzed by μ CT in 12-week-old male and female *Rarg*^{+/+} versus *Rarg*^{-/-} mice. The graphs show (A) Ct.Th, (B) Ma.Ar, (C) Ct.Ar, and (D) Ps.Pm. Males ($n = 10$), female *Rarg*^{+/+} ($n = 8$), and *Rarg*^{-/-} ($n = 6$) mice. ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ (unpaired two-tailed Student's *t* test). Ct.Th = cortical bone thickness; Ma.Ar = marrow area; Ct.Ar = cortical area; Ps.Pm = periosteal perimeter.

Fig. 3. Twelve-week-old *Osx1:Rarg* ^{Δ/Δ} mice do not exhibit a defect in trabecular bone. Tibial trabecular bone structure was analyzed by μ CT in 12-week-old male *Osx1:Rarg*^{+/+} ($n = 7$) versus *Osx1:Rarg* ^{Δ/Δ} ($n = 12$) mice. The graphs show (A) BV/TV, (B) Tb.Th, (C) Tb.Sp, and (D) Tb.N. Representative reconstructed images of trabecular bone are shown for *Osx1:Rarg*^{+/+} (*E*) and *Osx1:Rarg* ^{Δ/Δ} (*F*). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (unpaired two-tailed Student's *t* test). BV/TV = trabecular bone volume; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation; Tb.N = trabecular number.

Fig. 4. *Prrxl:Rarg* ^{Δ/Δ} mice have less trabecular bone than *Prrxl:Rarg*^{+/+} mice. Tibial trabecular bone structure was analyzed by μ CT in 12-week-old male and female *Prrxl:Rarg*^{+/+} versus *Prrxl:Rarg* ^{Δ/Δ} mice. The graphs show (A) BV/TV, (B) Tb.Th, (C) Tb.Sp, (D) Tb.N, and (I) tibial length. Representative reconstructed images of trabecular bone are shown for males (*E, F*) and females (*G, H*). Male *Prrxl:Rarg*^{+/+} ($n = 11$) and *Prrxl:Rarg* ^{Δ/Δ} ($n = 12$), female *Prrxl:Rarg*^{+/+} ($n = 13$) and *Prrxl:Rarg* ^{Δ/Δ} ($n = 15$) mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (unpaired two-tailed Student's *t* test). BV/TV = trabecular bone volume; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation; Tb.N = trabecular number.

Fig. 5. *Prrxl:Rarg* ^{Δ/Δ} male mice have greater osteoclastogenesis and females have more osteoid surface and osteoblasts compared to *Prrxl:Rarg*^{+/+} mice. Histomorphometry of the tibial secondary spongiosa was performed on nondecalcified plastic-embedded bones (*A–G*), and decalcified paraffin-embedded bones (*H–L*). The graphs show (A) OV/BV, (B) OS/BS, (C) O.Th, (D), Ob.S/BS, (E) N.Ob/B.Pm, (F) Oc.S/BS, and (G) N.Oc/B.Pm. Male *Prrxl:Rarg*^{+/+} ($n = 9$),

Prrxl:Rarg^{Δ/Δ} (*n* = 10), female *Prrxl:Rarg*^{+/+} (*n* = 8), and *Prrxl:Rarg*^{Δ/Δ} (*n* = 10) mice. Graph (H) shows the number of hypertrophic chondrocyte islands in epiphyseal bone, representative images (I–K) of male *Prrxl:Rarg*^{+/+} (I) and *Prrxl:Rarg*^{Δ/Δ} (J, K) tibias showing chondrocyte islands (black arrowheads) and graph (L) shows growth plate width in tibias; scale bars = 500 μm (I, J) and 100 μm (K). Graph (M) shows the width of the proliferative zone and graph (N) shows the width of the hypertrophic zone in 20-day-old tibias, representative images (O, P) of male *Prrxl:Rarg*^{+/+} (O) and *Prrxl:Rarg*^{Δ/Δ} (P) tibias. Male *Prrxl:Rarg*^{+/+} (*n* = 7), *Prrxl:Rarg*^{Δ/Δ} (*n* = 6), female *Prrxl:Rarg*^{+/+} (*n* = 6), and *Prrxl:Rarg*^{Δ/Δ} (*n* = 9) mice. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 (unpaired two-tailed Student's *t* test). Ov/BV = osteoid volume/bone volume; OS/BS = osteoid surface/bone surface; O.Th = osteoid thickness; Ob.S/BS = osteoblast surface/bone surface; N.Ob/B.Pm = number of osteoblasts/bone perimeter; Oc.S/BS = osteoclast surface/bone surface; N.Oc/B.Pm = number of osteoclasts/bone perimeter; HZ = hypertrophic zone, PZ = proliferative zone, GZ = germinal zone.

Fig. 6. *Prrxl:Rarg*^{Δ/Δ} male mice have increased pro-B and pre-B lymphocytes in the bone marrow compared to *Prrxl:Rarg*^{+/+} male mice. (A) shows the number of BM leukocytes per femur in *Prrxl:Rarg*^{+/+} and *Prrxl:Rarg*^{Δ/Δ} mice. (B, C) Representative flow cytometry plots of B lymphocytes subsets. The graphs show the numbers of cells per femur for: B220⁺IgM⁻ and B220⁺IgM⁺ B lymphocytes (D), CD19⁻CD43⁻ pre-pro-B lymphocytes, CD19⁺CD43⁺ pro-B lymphocytes, and CD19⁺CD43⁻ pre-B lymphocytes (E). Male *Prrxl:Rarg*^{+/+} (*n* = 10), *Prrxl:Rarg*^{Δ/Δ} (*n* = 12), female *Prrxl:Rarg*^{+/+} (*n* = 13), and *Prrxl:Rarg*^{Δ/Δ} (*n* = 12) mice. Schematic (I) shows stages of B lymphopoiesis highlighting pro-B and pre-B stages altered in *Prrxl:Rarg*^{Δ/Δ} males; HSC, CLP. qPCR of *Cxcl12* (G), *Flt3l* (H), *Tnfrsf11b* (I), and *Vegfa* (J) gene expression in whole BM cells from *Prrxl:Rarg*^{+/+} and *Prrxl:Rarg*^{Δ/Δ} male mice (*n* = 4 mice/group). The expression of these transcripts, including secreted (sec) and membrane-bound (mem) forms of *Kitl* are also shown in FACS-sorted MSCs (K) and osteoblast lineage cells (L) obtained from *Prrxl:Rarg*^{Δ/Δ} male mice, with expression normalized to cells sorted from *Prrxl:Rarg*^{+/+} male mice (*n* = 3 to 5 separate experiments/group). **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 (unpaired two-tailed Student's *t* test). BM = bone marrow; HSC = hematopoietic stem cell; CLP = common lymphoid progenitor; Hprt1 = _____; b2m = _____.

Fig. 7. *Prrxl:Rarg*^{Δ/Δ} mice have smaller BM sinusoidal vessels and females have more sinusoids and increased Vegf-a expression in megakaryocytes. Immunohistochemistry of VEGFR-3+

sinusoidal vessels in the secondary spongiosa of *Prrxl:Rarg^{+/+}* and *Prrxl:Rarg^{Δ/Δ}* mice. The graphs show (A) N.sinusoids/Ma.Ar, (B) Sinusoid.Ar/sinusoid, (C) sinusoid Ar/Ma.Ar, and (D) sinusoid Pm./Ma.Ar. Representative images (E–H) of VEGFR-3-stained BM sections, showing sinusoids (s), bone marrow (bm), and trabecular bone (tb). Scale bars = 100 μm. Representative images showing EYFP and Vegf-a stained BM sections obtained from (I) *Prrxl:Rarg^{+/+}* and (J) *Prrxl:Rarg^{Δ/Δ}* mice. The images show growth plate and bone marrow regions separately, with enlargements of each region in the insets. Scale bars = 100 μm. (K) Flow cytometry quantitation of the proportion of CD41⁺ megakaryocytes in the BM of *Prrxl:Rarg^{+/+}* and *Prrxl:Rarg^{Δ/Δ}* mice. Male *Prrxl:Rarg^{+/+}* (n = 8), *Prrxl:Rarg^{Δ/Δ}* (n = 8); female *Prrxl:Rarg^{+/+}* (n = 8) and *Prrxl:Rarg^{Δ/Δ}* (n = 9) mice. *p < 0.05 and **p < 0.01 (unpaired two-tailed Student's t test). N.sinusoids/Ma.Ar = number of sinusoids/marrow area; Sinusoid.Ar/sinusoid = sinusoid area/sinusoid; Sinusoid Ar/Ma.Ar = sinusoid area/marrow area; Sinusoid Pm./Ma.Ar = sinusoid perimeter/marrow area.

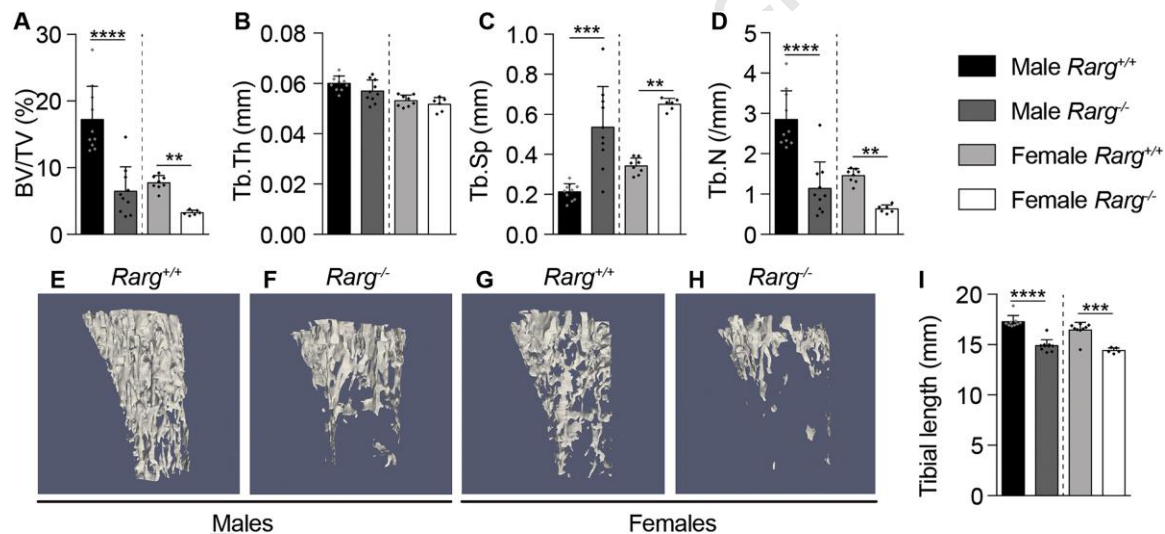


Figure 1

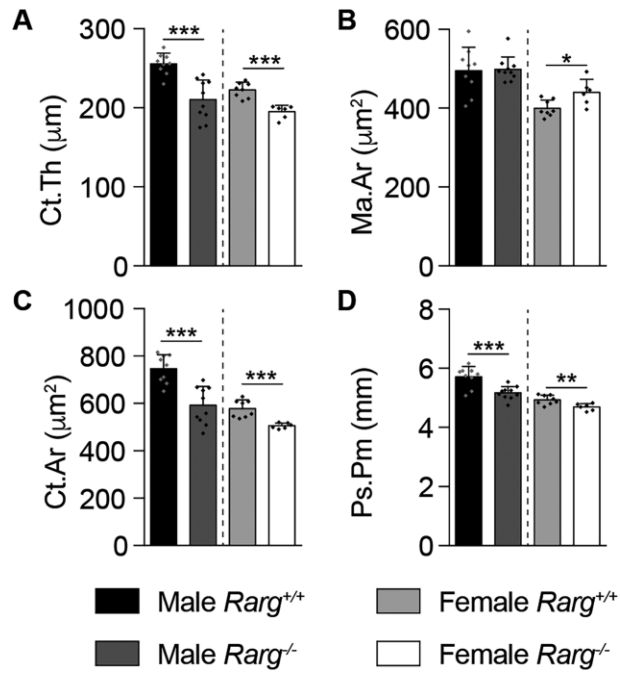


Figure 2

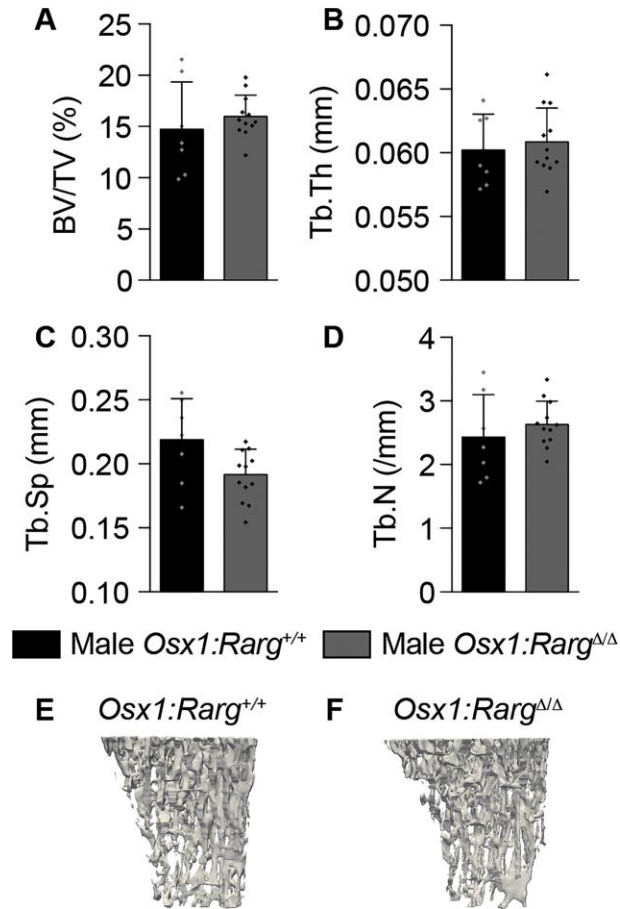


Figure 3

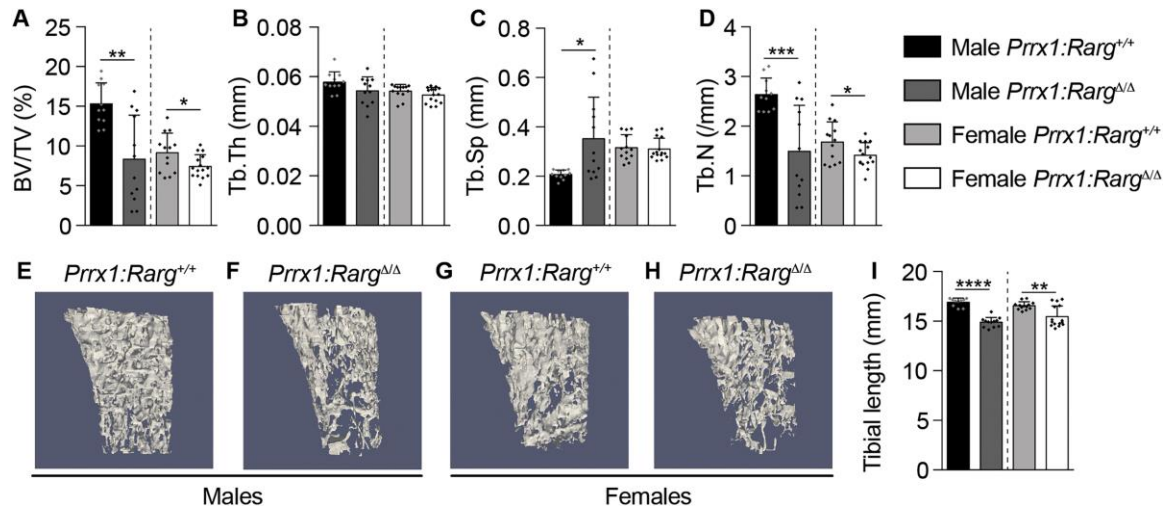


Figure 4

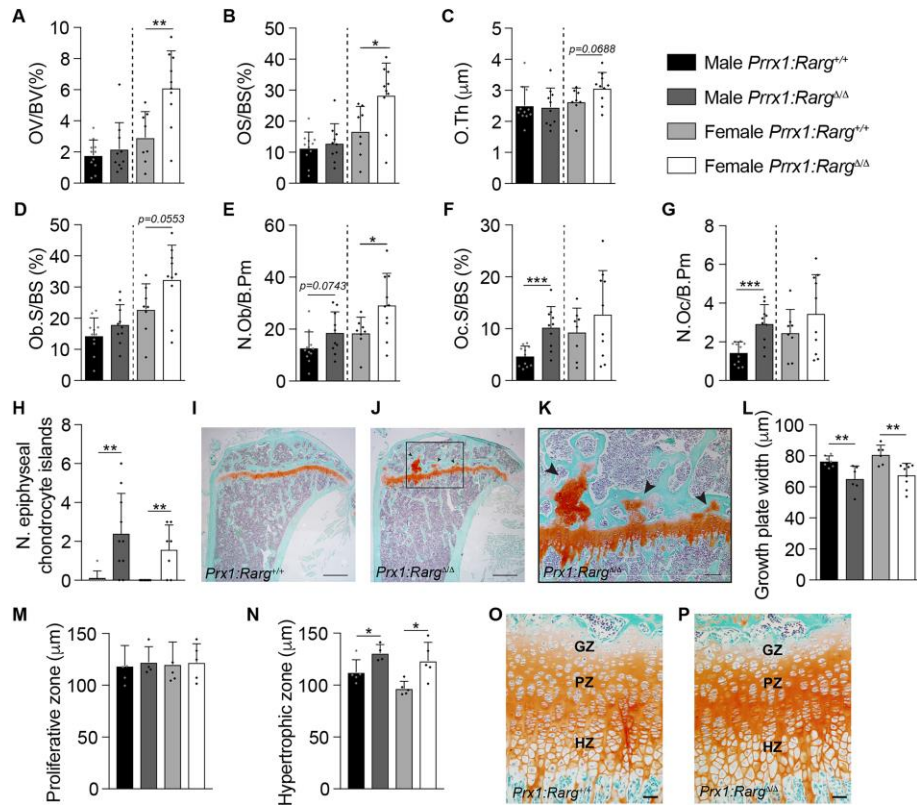


Figure 5

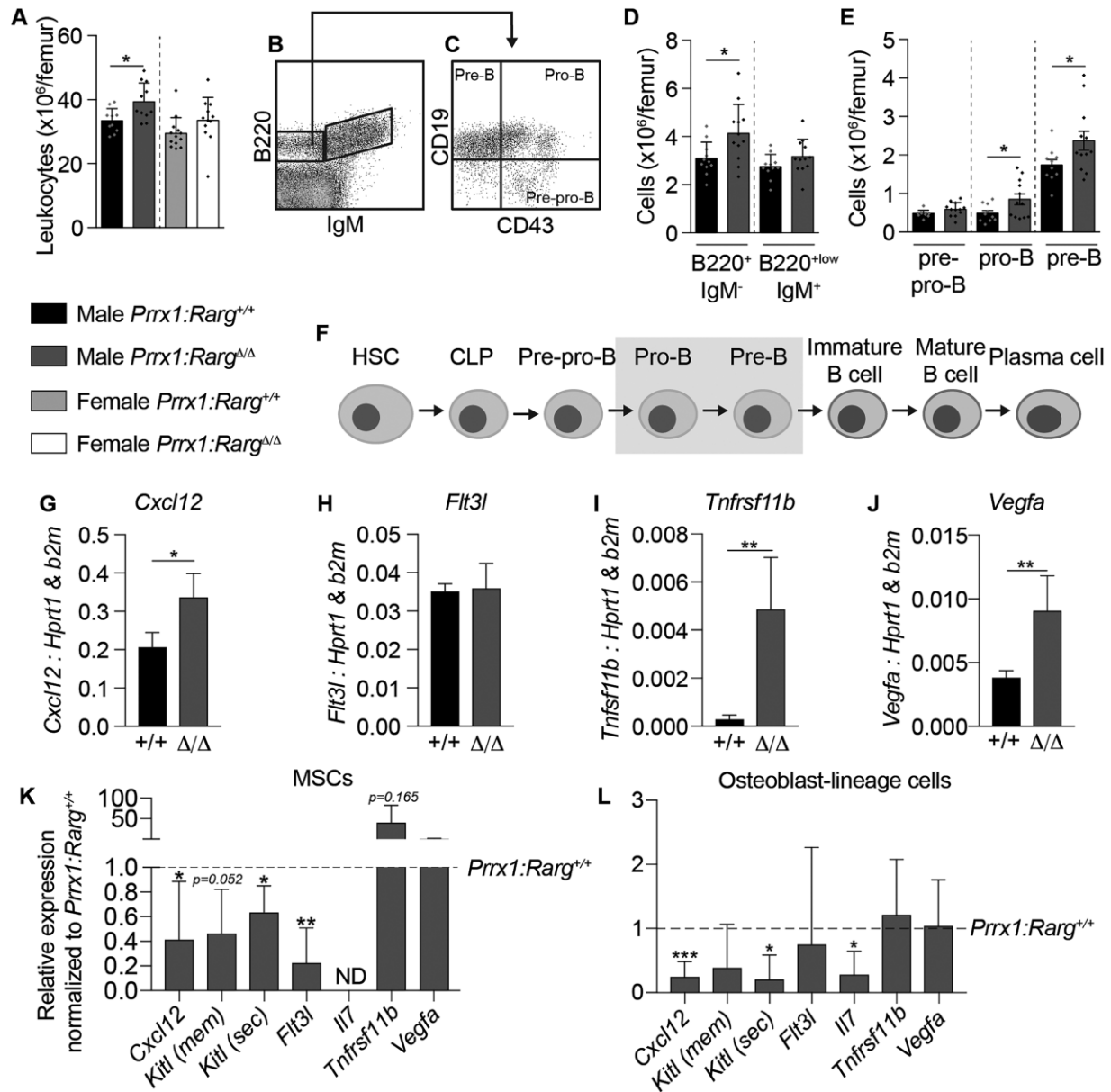


Figure 6

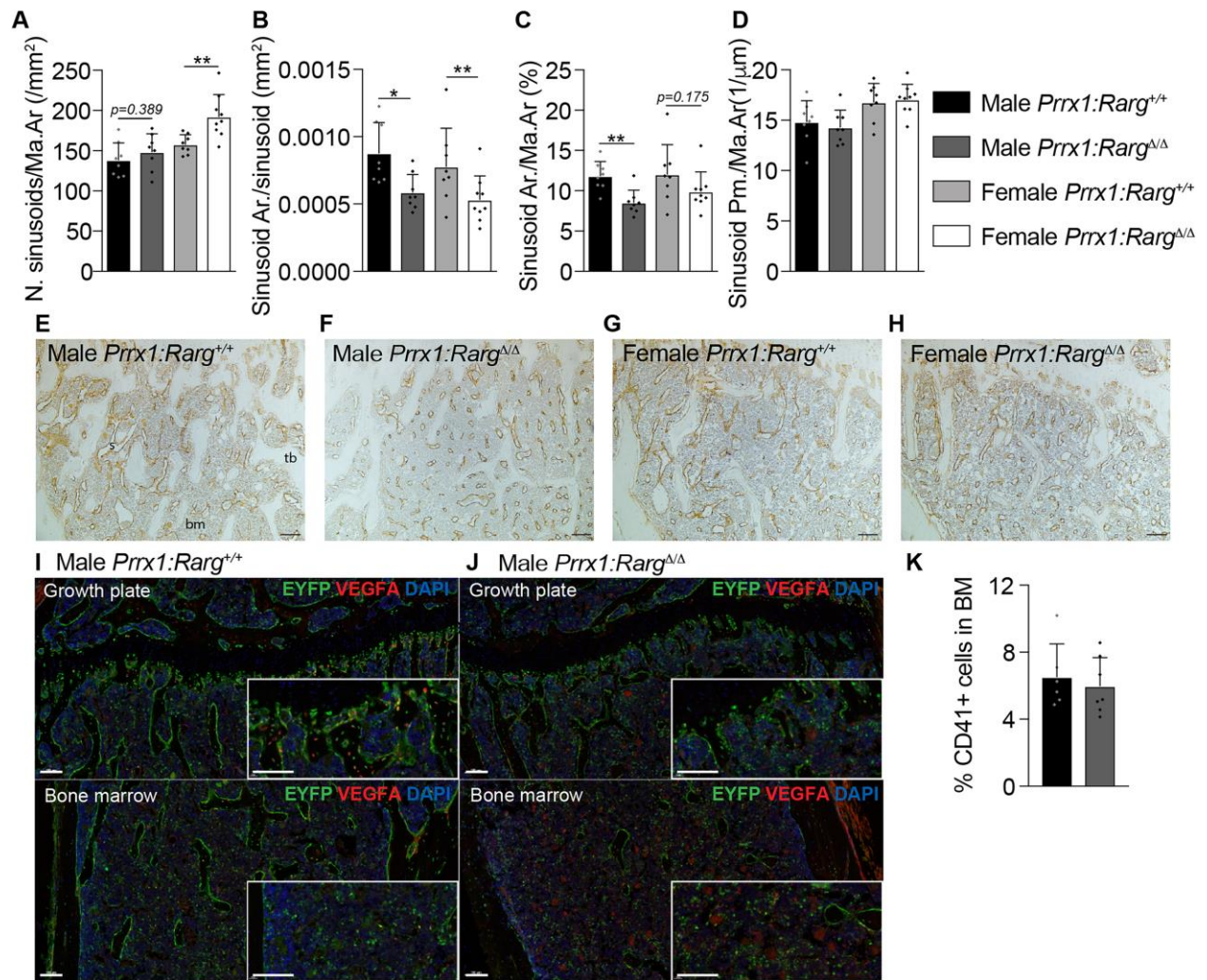


Figure 7

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