Chondrocytes-Specific Expression of Osteoprotegerin Modulates Osteoclast Formation in Metaphyseal Bone

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Bone marrow stromal cells/osteoblasts were originally thought to be the major player in regulating osteoclast differentiation through expressing RANKL/OPG cytokines. Recent studies have established that chondrocytes also express RANKL/OPG and support osteoclast formation. Till now, the in vivo function of chondrocyte-produced OPG in osteoclast formation and postnatal bone growth has not been directly investigated. In this study, chondrocyte-specific Opg transgenic mice were generated by using type II collagen promoter. The Col2-Opg transgenic mice showed delayed formation of secondary ossification center and localized increase of bone mass in proximal metaphysis of tibiae. TRAP staining showed that osteoclast numbers were reduced in both secondary ossification center and proximal metaphysis. This finding was further confirmed by in vitro chondrocyte/spleen cell co-culture assay. In contrast, the mineral apposition rates were not changed in Col2-Opg transgenic mice. TUNEL staining revealed more apoptotic hypertrophic chondrocytes in the growth plate of Col2a1-Opg mice. Flow cytometry analysis showed fewer RANK-expressing cells in the marrow of Col2a1-Opg mice, suggesting the role of OPG in blocking the differentiation of early mesenchymal progenitors into RANK-expressing pre-osteoclasts. Our results demonstrated that OPG expression in chondrocyte increases bone mass in the proximal metaphysis of tibiae through negative regulation of osteoclast formation.

The growth plate chondrocytes are a major player in the process of endochondral ossification that contributes to the longitudinal growth of the skeleton. This process is initiated when progenitor cells in the resting zone are stimulated to proliferate and then proceed through stages of maturation and hypertrophy. Hypertrophic chondrocytes produce a matrix that undergoes calcification, forming a calcified cartilaginous template for new bone formation. Hypertrophic chondrocytes also undergo apoptosis at the lower region of the hypertrophic zone as new blood vessels begin to invade the calcified cartilage and bring in osteoblast precursors, forming trabecular bone. Although the principles and overall mechanisms of endochondral bone formation are well established, many details remain to be defined. For example, the mechanisms how chondrocytes signal to adjacent osteoclasts (or chondroclasts) to trigger bone resorption remains poorly understood.

It has been established that osteoclast formation and activation require two molecules: macrophage colony-stimulating factor (M-CSF) and RANKL. Both of them are necessary to activate gene

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transcription to allow osteoclast differentiation. As a member of TNF super family, RANKL is originally believed to be produced by osteoblast lineage cells and activated T-cells and functions differently from M-CSF. While M-CSF increases the pool of osteoclast precursors, RANKL binds to its receptor RANK expressed on osteoclast precursors and mature osteoclasts, signaling to downstream molecules leading to enhanced osteoclast differentiation and activation and reduced apoptosis. RANK, the receptor of RANKL, is a member of the TNF receptor superfamily. In vivo data showed that deletion of either Rank or Rankl in mice resulted in the same phenotypes, including the profound defects in osteoclast formation, lymph-node formation, and B-cell development. Osteoprotegerin serves as the decoy receptor of RANKL. It is an atypical member of the TNF receptor superfamily as it is a secreted protein without transmembrane domain. It contains four homologous domains and competes with RANK for binding the same target RANKL, but does not transmit the signal to the osteoclast lineage cells, thus exerting an inhibitory effect on osteoclast differentiation and activation. In support of this, Opg knockout mice displayed severe osteoporosis, whereas Opg overexpressing mice showed osteopetrosis. The originally identified cells that express OPG include osteoblasts, endothelial cells, vascular smooth muscle, and lymphoid cells. Recently published immunohistochemistry (IHC) and in situ hybridization data have shown that RANKL and OPG are also expressed by hypertrophic chondrocytes in murine and rat growth plates. Further studies demonstrate a novel role for chondrocytes in supporting osteoclast formation by expressing RANKL, an effect regulated by 1, 25-(OH)_{2} vitamin D_{3} and BMP-2. However, the role of chondrocyte-produced OPG in regulation of osteoclast formation was not implicated in the previous studies.

We have recently reported that inactivation of canonical Wnt/β-catenin signaling in chondrocytes decreases proximal metaphyseal bone mass through enhancing osteoclast formation, which is mediated by the down-regulation of OPG and up-regulation of RANKL. To investigate the role of chondrocyte-produced OPG in osteoclast formation and postnatal bone growth, we generated chondrocyte-specific Opg transgenic mice using a 1.0-kb type II collagen promoter (Col2a1). Our results demonstrated that chondrocyte-produced OPG plays a significant role in regulation of osteoclast formation during bone growth and bone remodeling.

**Results**

**Opg transgene was expressed in chondrocytes in Col2-Opg transgenic mice.** In this study, we generated Col2-Opg transgenic mice in which expression of the Opg-Flag transgene was targeted to chondrocytes using the 1.0 kb type II collagen promoter (Col2a1) (Fig. 1A). Two independent lines of Col2-Opg transgenic mice were established and both of them displayed similar phenotypes. Col2-Opg transgenic mice are viable, fertile with normal body size and have no any gross, physical, or behavioral abnormalities.

Expression of the OPG-Flag protein was detected by Western blotting using an anti-Flag antibody in primary sternal chondrocytes isolated from Col2-Opg transgenic mice but not in those from WT mice (Fig. 1B). To determine the specificity of the Opg-Flag transgene expression, total RNA was extracted from multiple tissues and expression of Opg-Flag mRNA was examined by RT-PCR using transgene specific primers (Opg Fw and Flag-Rev). Strong expression of Opg-Flag was detected in femur epiphysis that spans from the articular surface to the lower border of the growth plate. Weaker expression of the transgene was detected in other bone tissues involving cartilages like calvaria and rib and in the brain, but was not detected in other tissues, including heart, kidney, and muscle (Fig. 1C). Interestingly, the transgene was not detected in femur metaphysis and diaphysis that were free of cartilages. This is consistent with our previous findings in Col2CreER²² transgenic mice. We also cultured the primary sternal chondrocytes, calvarial pre-osteoblasts and bone marrow stromal cells (BMSCs) and compared the mRNA and protein expression of Opg in the Col2-Opg transgenic mice to that in the WT control littersmates (Fig. 1D–G). The results showed that the Opg mRNA expression in chondrocytes was increased by 232-fold in the Col2-Opg transgenic mice (Fig. 1D). ELISA measurement of chondrocyte conditioned medium also revealed a significant increase in the OPG protein levels in Col2-Opg mice (Fig. 1G). In contrast, the mRNA expression of Opg did not increase in BMSCs and only increased by 40% in calvarial pre-osteoblasts (Fig. 1E,F) in Col2-Opg mice. No significant change of OPG protein level was observed in either primary calvarial pre-osteoblasts or BMSCs (Fig. 1G). Moreover, the serum OPG was increased by 73% in the 5-week old transgenic mice.

**Expression of Opg transgene in chondrocytes increased trabecular bone mass.** We then examined changes in bone mass of the transgenic mice by X-ray radiographic, histological and μCT analyses. X-ray radiographic results showed that the radiodensity in the proximal metaphyseal regions of tibiae was higher in the 5-week-old Col2-Opg mice than in their WT littersmates. In contrast, the radiodensity in the mid-shaft of the bones was not changed (Fig. 2A). We then examined if there are defects in bone or cartilage development in the embryonic stage. Alcian blue/Alizarin red staining of E18.5 skeleton revealed no significant differences between WT and Col2a1-Opg embryos (Fig. 2B).

Histological sections were stained with Alcian blue/Hematoxylin & Orange G. The Col2-Opg mice did not show significant changes in bone mass at E18.5 embryos (Fig. 2C) and P7 mice (Fig. 2D). The Col2-Opg mice had normal columnar arrangement of the growth plate chondrocytes. However, the Col2-Opg mice showed delay in the formation of the secondary ossification center. At P14 in WT mice,
mineralized cartilage in secondary ossification centers has been removed and replaced by trabecular bone; in contrast, mineralized regions have only partly been replaced by trabecular bone in Col2-Opg mice. The trabecular bone volume in the metaphyseal region was not significantly altered (Fig. 2E). At P28 and P35, the trabecular bone volumes in the proximal metaphyseal region were significantly increased in Col2-Opg mice (Fig. 2F, G). The transgenic mice maintained the typical columnar structure of growth plate chondrocytes, but the thickness of the growth plate, especially the hypertrophic zone, was 60% greater in the transgenic mice than in WT controls (Fig. 2H).

Consistent with this, μCT imaging revealed the bone mass increase in the proximal metaphysis beneath the growth plate in 4-week-old Col2-Opg mice (Fig. 3A). The trabecular bone volume (% BV/TV) was 41% greater (Fig. 3B) and the bone mineral density was 29% greater (Fig. 3C) in Col2-Opg mice. The trabecular number (Tb. N., 1/mm) was 20% higher (Fig. 3D), and the connectivity density (Conn. D.) was 60% higher (Fig. 3E), while the trabecular separation (Tb.Sp.) was 24% lower (Fig. 3F) in Col2-Opg mice. The structural model index (SMI, a measure of the shape of trabeculae; 0 for plates and 3 for cylindrical rods) was significantly decreased by 21% in Col2-Opg mice (Fig. 3G).

Osteoclast formation was altered in Col2-Opg mice. We performed the TRAP staining in tibiae from E18.5 embryos and P14 and P28 postnatal mice. The numbers of TRAP-positive osteoclasts and osteoclast surfaces were significantly decreased in Col2-Opg mice at all these time points (Fig. 4A–F). Of note, in 14-day-old Col2-Opg mice, the osteoclast numbers and osteoclast surfaces were lower not only in the primary metaphyseal region (Fig. 4B,E,F), but also in the secondary ossification center region, where the osteoclast numbers and osteoclast surfaces were reduced by 79% and 75%, respectively, compared...
to WT controls (Fig. 4C,E,F). This may explain why the formation of the secondary ossification center was delayed in Col2-Opg transgenic mice. We also examined changes in mineral apposition rate (MAR), the indicator of osteoblast activity, and found that the MAR was not significantly changed in Col2-Opg mice (Fig. 4G,H).

**In vitro osteoclast formation was reduced in Col2-Opg mice.** To determine the role of chondrocyte-produced OPG in osteoclast formation, we performed chondrocyte-spleen cell co-culture experiments in the presence of 10^{-8} M 1,25-(OH)_{2} vitamin D_{3}, TRAP-positive osteoclast formation was completely abolished when spleen cells were cultured with chondrocytes derived from Col2-Opg mice (Fig. 5A). To further identify if the overexpression of the Opg transgene alters the osteoclast formation from bone marrow cells, we cultured bone marrow cells in the presence of M-CSF and RANKL. Osteoclast formation was significantly reduced when bone marrow cells derived from Col2-Opg transgenic mice were cultured for osteoclast formation assay (Fig. 5B,C), suggesting that bone marrow cells were affected in chondrocyte-specific Col2-Opg mice. The Opg overexpression might have changed bone marrow cell populations. To examine this, we performed flow cytometric analysis using monoclonal antibodies against RANK and CD11b. The results showed that the RANK positive cells were reduced in Col2-Opg mice compared to WT mice; while the CD11b positive cells did not change significantly in Col2-Opg mice (Fig. 5D). These results suggest that RANK-expressing pre-osteoclasts may also be involved in OPG-mediated osteoclast inhibition.

**Changes in growth plate chondrocyte function in Col2-Opg mice.** To evaluate chondrocyte apoptosis, we performed TUNEL staining in 4-week-old WT and Col2-Opg mice and counted percentage of green fluorescent cells by normalizing to DAPI-positive cells. We found that the number of apoptotic hypertrophic chondrocytes was significantly increased in Col2-Opg mice (Fig. 6A). This result suggests that the expanded width of the hypertrophic zone could be owing to fewer chondrocytes being resorbed by osteoclasts after entering the apoptosis phase.

To further clarify if OPG affects chondrocyte development, mRNA expression of several chondrocyte marker genes were examined by real-time PCR assay. Expression levels of collagen type II (Col2a1), collagen type X (Col10a1), matrix metalloproteinase 13 (Mmp13), osteocalcin (OC), and alkaline phosphatase
were not significantly altered in Col2-Opg chondrocytes (Fig. 6B). These findings demonstrated that the OPG does not play a significant role in regulation of chondrocyte maturation.

Slight changes in osteoblast and adipocyte formation in Col2-Opg mice. We then examined osteoblast differentiation in BMSCs of Col2-Opg mice. ALP and Alizarin red staining showed similar osteoblast differentiation tendency in Col2-Opg mice compared to WT controls (Fig. 7A,B). Interestingly, results of real-time PCR assay revealed that the mRNA expression of Runx2 was not changed, while expression levels of Col1a1, Alp, OC, and Bsp were slightly reduced ($p < 0.05$) when BMSCs of Col2-Opg mice were cultured with osteoblast differentiation medium (Fig. 7C). These results likely reflect the coupling between osteoclast-mediated resorption and osteoblast-mediated bone formation.

We also examined in vitro adipocyte formation and adipocyte marker gene expression. When cells were cultured with adipogenic medium, mild increase in adipocyte numbers was observed from BMSCs of Col2-Opg mice, measured by Oil red O staining (Fig. 7D,E). An up-regulation of C/EBPa and aP2 mRNA expression in BMSCs from transgenic mice was observed whereas PPARγ mRNA was not significantly changed (Fig. 7F).

Discussion
OPG is known to be produced by osteoblasts and BMSCs in bone. Recent studies reported that RANKL and OPG are also expressed in growth plate chondrocytes. RANKL/OPG expressed in chondrocytes may modulate osteoclast (or chondroclast) differentiation by a mechanism similar to osteoblast-dependent osteoclastogenesis. So far, several groups have reported the in vitro and in vivo chondrocyte-dependent osteoclastogenesis. This implicates that the growth plate chondrocytes might be the direct regulator of bone mass at the chondro-osseus junction region. Our group further demonstrated that bone morphogenetic protein-2 enhances osteoclast formation by promoting RANKL expression in chondrocytes via Runx2/Smad1 pathway.

The crucial role of OPG in bone modeling and remodeling has been demonstrated in animal models using conventional deletion or non-tissue specific overexpression strategies. Targeted deletion of Opg in mice results in severe, early-onset osteoporosis due to excessive osteoclastogenesis. On the contrary, transgenic mice overexpressing Opg in the liver have high levels of OPG protein in their circulation and exhibit a marked increase in bone density. Recently, we created chondrocyte specific β-catenin conditional KO mice showing severe bone loss due to the enhanced osteoclast formation in proximal metaphysis. Detail in vitro investigations demonstrated that the β-catenin signaling in chondrocytes
inhibits osteoclast formation by up-regulation of OPG and down-regulation of RANKL expression in chondrocytes.

Several recent reports suggest that Col2a1, Col10a1 and Aggrecan promoters could drive Cre recombinase expression in hypertrophic chondrocytes during embryonic and postnatal skeletal development. A subset of the Cre targeting chondrocyte population could transform into osteoblast precursors or osteoblast-like cells. To determine the targeting efficiency and specificity of Col2-CreER mice, we have bred these mice with RosamT/mG reporter mice and found that some of Col2-positive cells could be detected at primary spongiosa area underneath the growth plate, suggesting that these cells may be derived from hypertrophic chondrocytes. These cells could directly contact osteoclast precursors and promote osteoclast formation and differentiation. Although a subset of chondrocyte-derived cells in primary spongiosa area could contribute to osteoclast formation, our ELISA assay revealed over 10-fold increase in OPG protein by in vitro cultured chondrocytes isolated from the Col2-Opg transgenic mice, suggesting that chondrocytes are the major source of OPG production in Col2-Opg transgenic mice and may play a major role in regulation of osteoclast formation.

Comparing to the generalized osteopetrosis phenotype observed in the liver-specific Opg transgenic mice, our Col2-Opg mice showed that bone mass increase is only localized to the proximal metaphysis in Col2-Opg mice. This may represent local OPG production by chondrocytes without systemic effect of OPG in Col2-Opg mice. At P28 and P35, the trabecular bone volume in the proximal metaphyseal region was greater in the Col2-Opg transgenic mice than in the WT littermates. This is due to the reduction of osteoclast formation in transgenic mice. The thickness of the growth plate, especially the hypertrophic zone, was increased in transgenic mice as a result of the reduced osteoclast formation. TUNEL staining results showed that numbers of apoptotic hypertrophic chondrocytes were significantly increased in Col2-Opg transgenic mice, suggesting that the expanded width of hypertrophic zone could be owing to fewer chondrocytes being resorbed by osteoclasts after entering the apoptosis phase. However, transgenic mice maintained the typical columnar arrangement of growth plate chondrocytes, suggesting that OPG...
protein does not alter the biological phenotype and behavior of growth plate chondrocytes. We did not observe significant changes in bone mass in older mice and this is probably due to reduced Col2a1 promoter activity in older mice.

Figure 5. Osteoclast differentiation was reduced in Col2-Opg mice. (A) Primary chondrocytes derived from WT and Col2-Opg mice were co-cultured with WT spleen cells in the presence of $10^{-8}$ M 1,25-(OH)$_2$ D$_3$ (blue arrows: multinuclear osteoclasts). (B,C) Osteoclast formation was also studied in bone marrow cells derived from Col2a1-Opg transgenic mice in the presence of 50 ng/ml sRANKL and 10 ng/ml M-CSF. (D) Flow cytometry analysis was performed using monoclonal antibodies against RANK and CD11b (n = 3 per group). Data are presented as means ± SD.

Figure 6. Changes in growth plate chondrocyte function in Col2-Opg mice. (A) TUNEL staining was performed using 4-week-old WT and Col2-Opg mice (red arrows: apoptotic chondrocytes). (B) Real-time PCR was performed to determine changes in expression of Col2a1, Col10a1, Mmp13, ostocalcin, and Alp in sternal chondrocytes derived from WT and Col2-Opg mice. Data are presented as means ± SD (n = 3 per group).
In vitro chondrocyte-spleen cell co-culture experiment demonstrated that the Opg-overexpressing chondrocytes inhibited WT spleen cells to form TRAP-positive osteoclast. However, by then it still remained unknown if in vivo the overexpression of the Opg transgene in chondrocytes changed the percentage of osteoclast lineage in bone marrow. Thus, we cultured bone marrow cells and found that osteoclast formation was significantly reduced with bone marrow cells derived from Col2a1-Opg transgenic mice, suggesting that bone marrow cells were affected in Col2a1-Opg mice. The Opg transgene might have changed cell populations of bone marrow. To further confirm this, we performed flow cytometric analysis and revealed that the Col2a1-Opg mice have fewer RANK positive cells but similar percentage of the CD11b positive cells compared to the WT littermates. This suggests that OPG is able to down-regulate the RANK expression in mononucleated cells of bone marrow or decrease the number of RANK-expressing cells. This is interesting because it is previously not known if RANKL/OPG has direct regulatory effect on RANK expression until recent report showing that RANKL induces RANK expression in bone marrow cells and RAW264.7 cells before the formation of osteoclasts 32. Our present study provides the in vivo data that support this finding. Thus it is clear that RANKL/OPG acts on not only RANK-expressing preosteoclasts, but early mesenchymal progenitor cells as well to trigger/block the differentiation of osteoclasts.

In our study, decreased osteoclast formation were not accompanied by dramatic changes in osteoblastic activities in the Opg-overexpressing tibial epiphyses, as indicated by the results of calcein double labeling, which did not reveal any change in MAR in Col2a1-Opg mice. This disagrees with Bucay’s study in which they reported the presence of abundant calcein deposition in the Opg−/− mice11–14, indicating that bone mineralization was enhanced and osteoblast activity was increased in their mutant mice. Our findings suggest that the locally produced OPG protein by the growth plate chondrocytes mainly affects adjacent osteoclasts (or chondroclasts) and does not contribute much to bone remodeling. To further identify the events at the cellular and molecular level, we examined osteoblast differentiation from bone marrow cells. Although ALP and Alizarin red staining showed similar osteoblast differentiation tendency in bone marrow cells from the transgenic mice, real-time PCR analysis revealed that the mRNA expression of Col1a1, osteocalcin, and bone sialoprotein (Bsp) was mildly reduced in bone marrow cells from Col2a1-Opg mice, likely reflecting the coupling between osteoclast-mediated resorption and

Figure 7. Osteoblast and adipocyte differentiation was slightly altered in Col2-Opg mice. (A,B) ALP and Alizarin red O staining was performed using BMSCs from WT and Col2-Opg mice. (C) Real-time PCR was performed to examine expression of osteoblast marker genes. (D) Oil red O staining was performed to determine adipocyte differentiation from BMSCs derived from WT and Col2-Opg mice (green arrows: Oil red O staining-positive cells). (E) Real-time PCR was performed to examine expression of adipocyte marker genes. Data are presented as means ± SD (n = 4 per group).
osteoblast-mediated bone formation. Consistent with this, when bone marrow cells were cultured under adiopogenic conditions, adipocyte formation and the mRNA expression of adiopogenic genes, including C/EBPα and aP2, were slightly increased in the Col2-Opg transgenic mice. The significance of regulation of osteoblast and adipocyte formation by chondrocyte produced OPG needs to be further investigated.

In this study we overexpressed Opg transgene in Col2-expressing chondrocytes. Although this is an overexpression study, it clearly demonstrates that chondrocyte produced OPG could regulate osteoclast formation in vivo. This study also provides evidence that pathological upregulation of OPG expression in chondrocytes could lead to osteopetrosis phenotype in metaphyseal bone.

**Methods**

**Generation of Col2-Opg transgenic mice.** To generate Col2a1-Opg transgenic mice, a DNA fragment encoding murine osteoprotegerin fused with a 3′ Flag tag was cloned into the NotI site of an expression vector PKN185 in which the transgene expression is under the control of Col2a1 promoter and enhancer33,34. The expression cassette of Opg-Flag was excised by Ndel and HindIII digestion. The Opg-Flag transgene was then purified and injected into pronuclei of fertilized eggs. Positive transgenic founder mice were identified by PCR and confirmed by Southern blot analysis. The sequences of PCR primers for genotyping Col2-Opg mice are as follows: Fw, 5′- GACCATATAACTGGAGCCTC-3′ and Rev, 5′-CACACAGGTTGACATCTATC -3′. The 1120-bp PCR product was detected in the transgenic mice. The animal protocol and experimental methods were approved by the Institutional Animal Care and Use Committee (IACUC) of University of Rochester (New York) and all animal experiments were carried out in accordance with the approved protocol and guidelines.

**Micro-CT analysis.** Quantitative analysis of bone samples was performed in tibiae of 5-week-old Col2-Opg transgenic mice and their WT littermates at 10μM resolution on a micro-CT Scanner (VivaCT 40; Scanco Medical AG, Bassersdorf, Switzerland) as previously described35.

**Histology.** Histological analyses were performed in tibiae from Col2a1-Opg transgenic mice and their WT littermates. To reveal patterning changes in cartilage and bone of the entire skeleton, whole embryos (E18.5) were sequentially stained with Alcian blue (for cartilage) and Alizarin red (for bone) as described in previous studies36. In short, after fixation with 95% ethanol for 48–72 hours, embryos were stained with 0.03% (w/v) Alcian blue in 80% ethanol and 20% acetic acid solution for 1–3 days, followed by 0.03% (w/v) Alizarin red in 1% KOH solution for another 12–24 hours, and then maintained in a solution of 2% KOH:glycerol (20:80) until analysis.

To reveal bone mass changes prenatally and postnatally, bone samples were dissected, fixed in 10% formalin, decalcified in 5% formic acid or 14% EDTA, and embedded in paraffin. Serial sections of tibiae were cut every 3 μm from both the medial and lateral compartments. The formic acid-treated sections were stained with Alcian blue/Hematoxylin and counterstained with Orange G to evaluate the structures and volumes of cartilage and bone. The EDTA-treated sections were stained for tartrate acid resistant phosphatase (TRAP) and counterstained with Hematoxylin. Histomorphometric analyses were performed on proximal tibial metaphyses to quantify the osteoclast numbers and osteoclast surfaces using the OsteoMeasure system (Osteometrics, Atlanta, GA). Osteoclast numbers and osteoclast surfaces were expressed per mm in longitudinal sections.

The mineral apposition rate (MAR) was determined by calcein double labeling. Col2a1-Opg transgenic mice and their WT littermates were injected with calcein (10mg/kg) 7 days and 2 days prior to sacrifice. Following dissection, calvariae and tibiae were fixed in 70% ethanol and embedded in polyethylene methacrylate resin for sectioning. Coronal sections of comparable anatomic position were examined by fluorescence microscopy. The mean distance between the calcein double labels was measured using AxioVision 3.0 image software.

**Enzyme-linked immunosorbent assay (ELISA).** Blood was collected right before sacrifice and the serum was prepared. The levels of mouse OPG were measured using an ELISA kit (RND systems, Minneapolis, MN). The conditioned medium was collected from cultures of primary sternal chondrocytes, calvarial osteoblasts and bone marrow stromal cells and subjected to ELISA assays, following the manufacturer’s recommendation.

**Chondrocyte/spleen cell co-culture assay.** 3-day-old neonatal mice were genotyped and sacrificed. Primary mouse sternal chondrocytes were isolated from the anterior rib cage of 3-day-old neonatal mice as described previously36,37. Spleen cells were isolated from 2-month-old WT mice by disaggregation through a wire sieve and seeded in a 48-well culture plate at the density of 4 × 105 cells/well. Primary sternal chondrocytes were seeded into the culture at the density of 2,000 cells/well and co-cultured with the spleen cells for 2 days in minimum essential medium-α (a-MEM) supplemented with 10% fetal bovine serum (FBS) in the presence of 20ng/ml M-CSF (R&D Systems, Minneapolis, MN, USA). Cells were then treated with 20ng/ml M-CSF and 10-8 M 1,25-(OH)2 vitamin D3 (Sigma, St. Louis, CA, USA) for additional 5 days. The adherent cells were fixed and stained for TRAP, a marker of mature osteoclasts. The numbers of TRAP-positive cells with 3 or more nuclei were counted.
Osteoclast formation from bone marrow. Bone marrow cells were isolated from 4-week-old mice according to the previously published protocol. The cells were seeded in a 48-well plate at the density of $5 \times 10^5$ cells/well in minimum essential medium-α (α-MEM) supplemented with 10% fetal bovine serum (FBS). The cells were treated with M-CSF (20 ng/ml) for 2 days and then cultured in the presence of 20 ng/ml M-CSF and 50 ng/ml recombinant human sRANKL (R&D Systems, Minneapolis, MN, USA) for additional 5 days. TRAP staining was performed and the TRAP-positive multi-nucleated osteoclasts were counted.

In vitro osteoblast formation assay. Bone marrow cells were isolated from femurs and tibiae of 4-week-old mice and cultured with α-MEM supplemented with 10% FBS in a 10-cm culture dish. When cells reached 80% confluence, they were digested with 0.25% trypsin and 0.02% EDTA. Cells were plated into 6 well culture plates at the density of $4 \times 10^5$ cells/well in α-MEM containing 10% FBS, 50 μg/mL ascorbic acid and 5 mmol/L β-glycerophosphate. Cells were cultured for 7 days followed by alkaline phosphatase (ALP) staining, or 21 days followed by Alizarin red staining (nodule formation assay), or 3 days followed by RNA extraction and real-time RT-PCR assay.

Oil red O staining. Fully differentiated adipocytes were gently washed twice with phosphate-buffered saline (PBS), and then fixed in 4% paraformaldehyde for 10 min. The samples were then washed twice with deionized water, and 60% saturated oil-red O staining was carried out for 5 min. For oil-red O quantification, 4% IGEPAL CA 630 (Sigma) in isopropanol was added to each well. Light absorbance was measured at 520 nm.

RNA extraction and real-time PCR assay. Total cellular and tissue RNA was prepared by PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. 1 μg of total RNA was used to synthesize cDNA using iScripts cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time PCR amplifications were performed using specific primers and SYBR green real-time PCR kit. The primers used for the PCR are listed in Table 1. The thermal cycling conditions were as follows: an initial denature at 95°C for 3 minutes, followed by 40 cycles of 20 seconds of denature at 95°C, 20 seconds of annealing at 58°C, and 20 seconds of extension at 72°C. The levels of the target gene expression were normalized to that of β-actin in the same cDNA sample.

Transferase-mediated digoxigenin-dUTP nick-end labeling (TUNEL). A TUNEL staining kit (DeadEnd Fluorometric TUNEL System, Promega, Madison, WI) was used to assess cell apoptosis by catalytically incorporating fluorescein-12-dUTP at 3’-OH DNA ends using the terminal deoxynucleotidyl transferase and recombinant enzyme (rTDT).

| Primer name | Sequence |
|-------------|----------|
| Opg, forward | 5’- CAGAGCAGAAACACAGTTTG-3’ |
| Opg, reverse | 5’- CACACAGGGTGACATCTATTC-3’ |
| Runx2, forward | 5’- CCTGAAGCTGACACAAAGCTC-3’ |
| Runx2, reverse | 5’- TCATCAGGTCAGATGAGGAGG-3’ |
| Alp, forward | 5’- CCAGAAGACACCTTGACGGTG-3’ |
| Alp, reverse | 5’- TCTTGTCGCTGCTGCTC-3’ |
| Col1a1, forward | 5’- CTTCAAGGGTTTCCCTTTCAC-3’ |
| Col1a1, reverse | 5’- CAGAGAAGACCTGTTGGCAGG-3’ |
| OC, forward | 5’- GCAAATAGTGAGGAACAGCTCC-3’ |
| OC, reverse | 5’- CCATAGATGCGTTTGGTAGGGG-3’ |
| BSP, forward | 5’- GAGCCAGGACTGCCGAAGGAA-3’ |
| BSP, reverse | 5’- CCCTTTGACGCCACCTGCT-3’ |
| Coll10a1, forward | 5’- ACCCAGGACCTAAGGAA-3’ |
| Coll10a1, reverse | 5’- CCCCAGGATACCCGTGTTT-3’ |
| Mmp13, forward | 5’- TTTAGAAGCGGGAGAAGAA-3’ |
| Mmp13, reverse | 5’- ACTTTTGTTGCAATTCAGG-3’ |
| β-actin, forward | 5’- GACACCTAAGGCCAACCTG-3’ |
| β-actin, reverse | 5’- ACCAGAGCCTACAGGAGAAG-3’ |

Table 1. Names and sequences of PCR primers.
Flow cytometry analysis. BMSCs were isolated from 4-week-old mice. 1 × 10^6 cells per sample were collected and fixed with 1% paraformaldehyde (15 min at 4°C), permeabilized with 0.05% Tween 20 (15 min at 37°C). Cells were first blocked with the Fc blocker in FACS buffer for 15 min, followed by incubation for 20 min on ice with mouse RANK monoclonal antibody (clone 9A725, Thermo Scientific, Rockford, IL) conjugated with PE and rat anti-mouse CD11b antibody conjugated with APC (BD Biosciences, San Jose, CA), and washed by FACS buffer. Data were acquired with a FACSCalibur flow cytometer (BD Biosciences), and analyzed with CellQuest software version 3.5.1 (BD Biosciences).

Statistical analysis. Data were presented as mean ± standard deviation (SD). Independent samples t-test was used to determine statistical difference between two groups using the SPSS program. P values less than 0.05 were considered significant.

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**Author Contributions**
B.W., H.J., B.S and R.R.M. performed experiments. D.C. conceived of the study and supervised the work. B.W. and D.C. analyzed data and drafted the manuscript. All authors approved the final version of the manuscript to be published.

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