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Graphical abstract

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Osteoclast-derived IGF1 is required for pagetic lesion formation in vivo

Kazuaki Miyagawa,1 Yasuhisa Ohata,1 Jesus Delgado-Calle,1 Jumpei Teramachi,1 Hua Zhou,2 David D. Dempster,3 Mark A. Subler,4 Jolene J. Windle,4 John M. Chirgwin,1 G. David Roodman,1,5 and Noriyoshi Kurihara1

1Division of Hematology and Oncology, Department of Medicine, Indiana University (IU), Indianapolis, Indiana, USA. 2Regional Bone Center, Helen Hayes Hospital, West Haverstraw, New York, USA. 3Department of Clinical Pathology and Cell Biology, Columbia University, New York, New York, USA. 4Department of Human and Molecular Genetics, Virginia Commonwealth University (VCU), Richmond, Virginia, USA. 5Roudebush VA Medical Center, Indianapolis, Indiana, USA.

We report that transgenic mice expressing measles virus nucleocapsid protein (MVNP) in osteoclasts (OCLs) (MVNP mice) are Paget’s disease (PD) models and that OCLs from patients with PD and MVNP mice express high levels of OCL-derived IGF1 (OCL-IGF1). To determine OCL-IGF1’s role in PD and normal bone remodeling, we generated WT and MVNP mice with targeted deletion of igf1 in OCLs (igf1-cko) and MVNP/igf1-cko mice, and we assessed OCL-IGF1’s effects on bone mass, bone formation rate, EphB2/EphB4 expression on OCLs and osteoblasts (OBs), and pagetic bone lesions (PDLs). A total of 40% of MVNP mice, but no MVNP/igf1-cko mice, had PDLs. Bone volume/tissue volume (BV/TV) was decreased by 60% in lumbar vertebrae and femurs of MVNP/igf1-cko versus MVNP mice with PDLs and by 45% versus all MVNP mice tested. Bone formation rates were decreased 50% in igf1-cko and MVNP/igf1-cko mice versus WT and MVNP mice. MVNP mice had increased EphB2 and EphB4 levels in OCLs/OBs versus WT and MVNP/igf1-cko, with none detectable in OCLs/OBs of igf1-cko mice. Mechanistically, IL-6 induced the increased OCL-IGF1 in MVNP mice. These results suggest that high OCL-IGF1 levels increase bone formation and PDLs in PD by enhancing EphB2/EphB4 expression in vivo and suggest OCL-IGF1 may contribute to normal bone remodeling.

Introduction

Paget’s disease (PD) is the most exaggerated example of coupled bone remodeling, with highly localized areas of increased bone resorption, accompanied by exuberant new bone formation (1). The rapid, excessive, focal deposition of new bone in PD results in woven bone that is much weaker, can bow and cause bone deformity, can fracture, exhibits skull thickening, causes bone pain, shows signs of secondary osteoarthritis, and shows signs of nerve root compression (2). The primary cellular abnormality in PD resides in the osteoclast (OCL) (3). OCLs in PD (PD-OCLs) are increased in number and size, are hypermultinucleated, and have increased 1,25-(OH)2D3 and RANKL responsivity compared with normal OCLs (4–7). Furthermore, PD-OCLs secrete high levels of IL-6, which are detectable in the marrow and peripheral blood of patients with PD (8). OCLs drive the increased bone formation in PD because treatment with bisphosphonates, which decreases OCL activity, blocks new pagetic bone formation (2). These findings suggest that high PD-IGF1 levels increase bone formation and PDLs in PD by enhancing EphB2/EphB4 expression in vivo and suggest OCL-IGF1 may contribute to normal bone remodeling.
The high levels of OCL-derived IL-6 induced IGF1 and EphB2 in OCLs, and EphB4 on OBs, to increase OB differentiation in vitro (13). Furthermore, we previously reported that EphB4-Fc treatment of OCL precursors from WT and MVNP mice inhibited OCL formation, decreased bone resorption, and abrogated the effects of MVNP on the expression of a pagetic phenotype by the OCLs that formed. In addition, EphB2-Fc treatment inhibited OB differentiation, decreased Rho signaling in both WT and MVNP OB precursors, and blocked the increased OB differentiation induced by coculture with MVNP-expressing OCLs (MVNP-OCLs) (13). We found that OCLs, rather than OBs, are the major producer of local IGF1 in PD and that the amount of IGF1 produced by MVNP-expressing OCLs is considerable. Several studies reported a role for IGF1 in coupling bone resorption and formation, but they focused on the effects of bone matrix–derived IGF1, released by osteoclastic bone resorption, on OB differentiation and new bone formation (14, 15). These reports showed that IGF1 signaling in OBs enhanced the expression and secretion of RANKL (16) and that new bone formation induced by IGF1 occurred at bone resorption sites to maintain normal bone remodeling (14). However, the role of OCL-derived IGF1 (OCL-IGF1) in PD and physiologic bone remodeling in vivo is unknown.

In this study, we examined the mechanisms responsible for the effects of OCL-IGF1 on the increased bone formation and development of pagetic bone lesions (PDLs) in PD and on physiologic bone remodeling in vivo. We generated potentially novel mouse models with conditional deletion of Igf1 in OCLs of WT and MVNP mice by breeding TrACP-Cre or MVNP/TrACP-Cre with Igflfl/fl mice. PDLs were detected in 18- to 24-month-old MVNP mice but not in the other genotypes. Notably, bone volume/tissue volume (BV/TV) of MVNP/Igf1–conditional KO (VNP/Igf1-cKO) vertebrae and femurs was decreased by 60% versus MVNP mice with PDL and by 45% versus BV/TV in all MVNP mice tested. Furthermore, bone formation rates (BFRs) in Igf1-cKO and MVNP/Igf1-cKO mice were decreased 50% compared with WT and MVNP mice, respectively. Additionally, expression of EphB2 and EphB4 in MVNP/Igf1-cKO mice was very low compared with WT and MVNP mice, respectively. Thus, our results suggest OCL-IGF1 (a) increases bone formation, (b) is required for development of PDL in PD through increased EphB2/EphB4 expression in OCLs and OBs, and (c) possibly contributes to adult physiological bone remodeling in vivo.

Results

IGF1 expression in OCLs and BFRs are increased in bone samples from a patient with PD. To determine if IGF1 expression was increased in OCLs of patients with PD in vivo, we performed histologic analysis of bone samples from a PD patient and a healthy donor. The OCLs in the PD bone sample were large, hyper multinucleated, and resided in deep resorption cavities characteristic of PD (Figure 1A). In contrast, OCLs in the normal bone specimen were smaller, had fewer nuclei/OCL, and resided in shallower resorption pits. In addition, immunostaining of the bone sections with anti-IGF1 antibody showed that OCLs from the PD patient had much stronger staining compared with OCLs in normal bone (Figure 1B). Furthermore, dynamic histomorphometric analyses of these calcein-labeled bone specimens showed that the mineral apposition rate (MAR) and BFR were higher in the PD patient than the healthy donor sample (Figure 1C), suggesting a potential link between high OCL-IGF1 and increased bone formation in PD.

Specificity of TRACP-Cre expression in OCL lineage cells in bone. To characterize the role of increased OCL-IGF1 in the increased bone formation in PD, we bred TRACP-Cre transgenic mice to Igfl floxed mice to delete Igfl in the OCL lineage. However, we first confirmed the specificity of the TRACP promoter to OCLs in bone by breeding TRACP-Cre mice to the Ai9 reporter mouse strain that contains a CAG promoter, followed by a loxP-flanked STOP cassette and the coding region of a red fluorescent protein variant (tdTomato) (17). In cells expressing Cre, the STOP cassette is deleted, resulting in strong Tomato red fluorescence. The localization of TRACP+ OCLs and anti–Tomato immunopositive OCLs is shown in Supplemental Figure 1 (supplemental material available online with this article: https://doi.org/10.1172/jci.insight.133113DS1). Using sequential sections, we found that TRACP and Tomato staining colocalized in OCLs from TrACP-Cre(+)/Tomato mice, as shown by the arrows, whereas OCLs in TrACP-Cre(−)/Tomato bones were not stained by the anti-Tomato antibody. The anti-Tomato antibody also weakly stained occasional osteocytes (OCys) in bone.

Since targeted disruption of the Igfl gene in OCys impairs developmental bone growth in mice, we examined if OCy IGF1 expression was affected in OCys in Igfl-cKO mice. Immunostaining of OCys with an anti-IGF1 antibody showed similar levels of IGF1 staining of OCys from 4 genotypes (Supplemental Figure 2). Furthermore, Western blotting of lysates and ELISA assays of OCy conditioned media
of WT, Igfl-cKO, MVNP, and MVNP/Igfl-cKO showed that IGF1 expression levels were similar for all 4 genotypes (Supplemental Figure 2). The amounts of IGF1 secreted by OCys from the 4 genotypes were much lower than these secreted by MVNP-OCLs (20 pg/mL/2 × 10⁵ OCys versus 200 pg/mL/5 × 10⁴ OCLs). Based on these data, we concluded that cKO of Igfl using TRACP-Cre did not significantly affect IGF1 expression by OCys.

Effects of Igfl deletion in OCLs on the size, weight, femoral length, IGF1, and IGFBP3 levels in 4 genotype mice. To examine the effects of Igfl deletion in OCLs of WT and MVNP mice, we generated cohorts of mice of the following 4 genotypes: (a) Igfl fl/fl (WT), (b) TRACP-Cre+/Igfl fl/fl (Igfl-cKO), (c) TRACP-MVNP/Igfl fl/fl (MVNP), and (d) TRACP-MVNP/TRACP-Cre/Igfl fl/fl (MVNP/Igfl-cKO). Lack of OCL-Igfl had no effect on the size, weight, and femur length of WT or MVNP mice at 12–24 months of age. In addition, serum IGF1 and IGFBP3 levels were unchanged by the absence of OCL-Igfl (Supplemental Figure 3).

TRACP expression levels in liver, which is the major source of IGF1 and can express TRACP, were almost undetectable compared with OCLs, as assessed by Western blot (data not shown). Interestingly, mice with targeted deletion of Igfl in OCLs, either with or without MVNP, were born at much-lower-than-expected numbers (~25% instead of the expected 50%). Since TRACP is expressed in the kidney, we also analyzed if 1,25-(OH)₂D₃ produced by kidney is affected in the Igfl-cKO mice. The serum levels of 1,25-(OH)₂D₃ in WT mice (38.2 ± 5.2 pg/mL, n = 5), Igfl-cKO mice (39.3 ± 5.8 pg/mL, n = 5), MVNP mice (43.4 ± 3.3 pg/mL, n = 5), and MVNP/Igfl-cKO mice (45.6 ± 7.3 pg/mL, n = 5) were not significantly different.

Genetic deletion of Igfl in OCLs prevents PDL formation in MVNP mice. WT, Igfl-cKO, MVNP, and MVNP/Igfl-cKO mice (18–24 months of age) were examined for the presence of PDLs. Mice of this age were examined because we most consistently find PDLs in MVNP mice at 12 months of age or older (9). PDLs were detectable in 40% of MVNP mice (4 of 10) by μCT (Table 1) and histological analysis, and they were found in vertebrae, femurs, and tibiae at 18–24 months of age (Supplemental Figure 4 and Supplemental

Figure 1. OCL phenotype and remodeling in Paget’s patient and healthy donor bone. (A) OCLs were stained with TRACP. (B) Detection of IGF1. IGF1 expression was determined by immunostaining using anti-IGF1 antibody as described in Methods. TRACP and IGF1 staining were performed on consecutive sections. (C) New bone formation. Mineral apposition rates and new bone formation rates were measured as described in Methods. Scale bars: 100 μm. All data shown are typical of 3 technical replicates. The information on the PD patient and healthy donor is described in Methods. Arrows denote an osteoclast.
Table 1. The number of mice and PDLs in transgenic or WT mice

<table>
<thead>
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<th>WT</th>
<th>Igfl-cKO</th>
<th>MVNP</th>
<th>MVNP/Igf1-cKO</th>
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<td>8</td>
<td>10</td>
<td>6</td>
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<tr>
<td>Pagetic Lesions</td>
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PDLs were screened by μCT as described in Methods. Markedly abnormal bone structure was seen 4 of the 10 MVNP mice (40%) at 18–24 months of age.

Table 1. In contrast, no PDLs were detected in any of the MVNP/Igf1-cKO, Igfl-cKO, or WT mice examined at the same age as the MVNP mice.

Characterization of the skeletal consequences of conditional deletion of Igf1 in WT and MVNP mice. We next determined bone volume (BV) and trabecular bone parameters in vertebrae and femurs from mice of the 4 genotypes at 20 months of age. μCT images of vertebrae and femurs of MVNP mice with PDLs showed increased bone mass compared with the other genotypes (Figure 2A and Supplemental Figure 5B). BV/TV in PDLs of vertebrae from MVNP mice was increased 1.5-fold compared with vertebrae of MVNP mice without PDLs and 2.5-fold compared with WT mice (P < 0.01). BV/TV of vertebrae from MVNP/Igf1-cKO mice was decreased by 60% compared with vertebrae with PDL from MVNP mice and was decreased by 45% compared with BV/TV in vertebrae of all MVNP mice tested (Figure 2B). Notably, BV/TV in Igfl-cKO mice was also decreased compared with WT mice and lower than MVNP/Igf1-cKO mice. Trabecular number (Tn.N) in vertebrae with PDL from MVNP mice was significantly increased compared with vertebrae from WT mice and MVNP mice without PDLs (P < 0.01) (Figure 2B). MVNP/Igf1-cKO and Igfl-cKO mice had similar Tn.N, which was lower than Tn.N in vertebrae with PDLs from MVNP mice (P < 0.01). Conditional deletion of OCL-IGF1 decreased trabecular width (Tb.Wi) and increased trabecular separation (Tb.Sp) in vertebrae of both WT and MVNP mice (P < 0.05) and was significantly different in MVNP mice with/without PDLs compared with the other genotypes (Figure 2B). Similar effects on bone parameters in femurs were also found (Supplemental Figure 5A).

We then examined if OCL-IGF1 affected cortical bone structure and volume in the same mice used for trabecular bone analyses. We measured cortical bone structures in the midshaft of the tibia from the mice according to guidelines for assessment of bone microstructure in rodents using μCT (18). No differences were detected among the 4 genotypes at 20 months of age (Figure 2C). Similarly, cortical area fraction, average cortical thickness, periosteal perimeter, and endocortical perimeter were not significantly different among the 4 genotypes (Figure 2D). We determined if our μCT results shown in Figure 2 significantly differed in male versus female mice. The μCT results for male versus female WT mice, MVNP mice without PDL, and MVNP/Igf1-cKO mice did not differ significantly, but results of male versus female MVNP mice with PDLs and MVNP/Igf1-cKO could not be analyzed because we only had 1 male MVNP mouse with a PDL and 2 male Igf1-cKO mice.

Histomorphometric analysis and BFRs in WT and MVNP mice with conditional deletion of Igf1 in OCLs. TRACP staining of OCLs in MVNP mice showed that the OCLs were larger in size and hypermultinucleated compared with WT mice analogous to PD-OCLs. As expected, the resorption cavities in MVNP mice appeared deeper than in the other genotypes. OCLs present in bones from WT, Igf1-cKO, and MVNP/Igf1-cKO mice displayed normal morphology and were smaller than OCLs found in MVNP mice (Figure 3A). Histomorphometric analyses of vertebrae for mice of the 4 genotypes at 20 months of age are shown in Figure 3, B and D. OCL surface (osteoclast surface/bone surface [Oc.S/BS]) and number (number of osteoclasts/bone perimeter [N.Oc/B.Pm]) and OB surface (osteoblast surface/bone surface [Ob.S/BS]) and number (number of osteoblasts/bone perimeter [N.Ob/B.Pm]) were significantly increased in MVNP mice compared with the other genotypes. Deletion of Igf1 in OCLs significantly decreased these parameters in both WT and MVNP mice. We then determined the ratio of Oc.S/BS and Ob.S/BS in vertebrae with PDLs from MVNP mice and in vertebrae of MVNP mice without PDLs (Figure 3B). In vertebrae with PDLs from MVNP mice, Oc.S/BS was increased 1.44-fold, N.Oc/B.Pm was increased 1.34-fold, Ob.S/BS was increased 1.48-fold, and N.Ob/B.Pm was increased 1.52-fold, compared with vertebrae from MVNP mice without PDLs. Histomorphometric analyses of femurs also showed that femoral PDLs in MVNP mice had higher bone mass compared femurs of MVNP mice without PDLs and mice of the other genotypes (Supplemental Figure 5C). These results show that increased numbers of OCLs and OBs are present in PDLs of MVNP mice compared with bones from MVNP mice without PDLs.
We next examined BFRs in calcein-labeled bones from mice of the 4 genotypes at 20 months of age. Fluorescence images of vertebral bone from these mice are shown in Figure 3C. The width of newly formed bone was markedly increased in MVNP mice compared with the other genotypes. Mineralizing surface (MS/BS) and MAR in MVNP mice were also significantly higher than in mice with cKO of OCL-
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Igf1 or WT controls (P < 0.01) (Figure 3D). Furthermore, MAR and BFR/BS in PDLs of MVNP mice were greater than in MVNP mice without PDLs (P < 0.01). Importantly, conditional deletion of Igf1 in OCL restored these parameters to control levels in MVNP mice and decreased them in WT mice. These results suggest that OCL-IGF1 contributes to new bone formation in both MVNP and control mice.

Figure 3. Histomorphometric analysis and bone formation rates in WT, Igf1-cKO, MVNP, and MVNP/Igf1-cKO mice. (A) OCL morphology. OCLs in vertebra sections were stained with TRACP. Scale bars: 100 μm. (B) Bone morphometric analysis. Results are expressed as the mean ± SEM for WT (2 male, 4 female, 19 ± 2 months), Igf1-cKO (1 male, 4 female, 16 ± 3 months), MVNP with PDLs (1 male, 2 female, 21 ± 2 months), MVNP without PDLs (3 male, 1 female, 21 ± 2 months), and MVNP/Igf1-cKO (3 male, 1 female, 25 ± 2 months). The data were analyzed using 1-way ANOVA with Tukey test. *P < 0.05 as compared with each indicated group. (C) Photomicrograph of new bone formation in vertebra (L5). Scale bars: 50 μM. (D) Mineralized perimeter, mineral apposition, and new bone formation rates. Data are expressed as the mean ± SEM WT (2 male, 4 female, 19 ± 2 months), Igf1-cKO (1 male, 4 female, 16 ± 3 months), MVNP with PDLs (1 male, 2 female, 21 ± 2 months), MVNP without PDLs (3 male, 1 female, 21 ± 2 months), and MVNP/Igf1-cKO (3 male, 1 female, 25 ± 2 months). The data were analyzed using 1-way ANOVA with Tukey test. *P < 0.05, **P < 0.01 as compared with each indicated group. Results in B and D are representative of 4–6 biological replicates. Mice of similar age were used in each experiment.
IGF1 deletion in OCLs decreases EphB2 and EphB4 expression of OCLs and OBs in WT and MNVP mice. To determine the mechanisms responsible for the effects of OCL-IGF1 on bone formation, femurs from 20-month-old mice were immunostained with anti-IGF1, anti-EphB2, and anti-EphB4 antibodies as described in Methods. OCLs from MVNP mice were clearly stained for IGF1 and EphB2 compared with OCLs from the other genotype mice, which showed minimal or negative staining. OBs from MVNP mice strongly stained positively for EphB4 compared with OBs from the other genotype mice. Determination of staining intensity (positive or negative) was performed by blinded observers. Arrows point to OCLs, and arrowheads point to OBs. Bones of MVNP mice stained with control IgG. Scale bars: 50 μm. Results are representative of WT (17 months), Igf1-cKO (21 months), MVNP-PDL (24 months), MVNP-No PDL (21 months), and MVNP/Igf1-cKO (23 months old mice). Results are representative of 3 biological replicates. Mice of similar age were used in each experiment.

Characterization of the role of OCL-Igf1 on OCL differentiation and function. To examine the effects of IGF1 in cells of the OCL lineage on the function of OCL precursors, we cultured CD11b+ BM mononuclear cells from 12-month-old mice of each genotype as described in Methods. The number of OCLs formed from OCL precursors obtained from MVNP/Igf1-cKO mice treated with RANKL was significantly decreased compared with OCL precursors from MVNP mice (Figure 5A). Similarly, OCL formation from OCL precursors derived from Igf1-cKO mice was significantly decreased in response to RANKL treatment compared with OCL precursors from WT mice (Figure 5A). Furthermore, the OCLs formed from MVNP mice with/without PDL exhibited strong EphB4 staining compared with OBs from the other genotypes. Plump, cuboidal OBs were also more common in MVNP mice than in mice of the other genotypes, including WT mice. Of note, EphB2 and EphB4 staining of OCLs/OBs in MVNP mice with or without PDLs was similar.

To further assess the role of OCL-Igf1 in OCL function, we determined the expression levels of cathepsin K and NFATc-1 in purified OCLs formed in vitro. OCLs formed in bone marrow cultures of MVNP/Igf1-cKO and Igf1-cKO mice expressed lower levels of cathepsin K and NFATc-1 than OCLs formed in cultures from MVNP and WT mice. Cathepsin K levels in OCLs from MVNP/Igf1-cKO mice were decreased by 50% compared with OCLs derived from MVNP mice (Figure 5D). We then tested the bone resorption capacity of these OCLs by plating the same number of mature OCLs on bone slices in the presence of RANKL (50 ng/mL) for 72 hours. OCLs formed in MVNP mouse bone marrow cultures formed numerous large resorption pits. However, OCLs from mice lacking OCL-Igf1 or WT mice formed low numbers of small pits (Figure 5E). The bone resorption capacity of MVNP/Igf1-cKO or Igf1-cKO OCLs was significantly decreased compared with OCLs formed in MVNP or WT mouse bone marrow cultures (Figure 5F). In addition, treatment of purified OCL precursors from Igf1-cKO or MVNP/Igf1-cKO mice with 10 ng/mL of mouse recombinant IGF-1 restored OCL number and nuclear number/OCL to levels present in WT or MVNP-OCLs (Supplemental Fig-
Figure 5. Characterization of OCLs formed in marrow cultures of WT, Igf1-cKO, MVNP, and MVNP/Igf1-cKO mice. All experiments used BM mononuclear cells from 12-month-old mice. (A) OCLs formation in 4 genotype mice. Results are expressed as the mean ± SEM from 4 technical replicates from 4 genotype mice. The data were analyzed using a 1-way ANOVA with Tukey test. *P < 0.05, **P < 0.01 with OCLs from 4 genotype mice. The results are representative of 4 biological replicates. (B) OCL morphology, photomicrographs of OCLs in marrow cultures treated with RANKL (50 ng/mL) and stained with TRACP. Scale bar: 50 μm. (C) Nuclear numbers per OCL. Results are expressed as the mean ± SEM of 25 randomly counted OCLs from 4 genotype mice. The data were analyzed using 1-way ANOVA with Tukey test, *P < 0.01 as compared with each indicated group. (D) Expression of cathepsin K and NFATc-1. The expression of cathepsin K and NFATc-1 were examined by Western blotting as described in Methods. GAPDH was used as the loading control. Protein expression levels were quantitated by ImageJ software (NIH). The basal ratio for each protein/loading control for OCLs from WT mice was set at 1.0. Results are representative of 3 biological replicates of 4 genotype mice. (E) Photomicrographs of bone resorption area. Bone slices were stained with hematoxylin. Scale bar: 50 μm. (F) Bone resorption capacity was assayed as described in Methods. Results are expressed as the mean ± SEM (representative of 3 biological replicates for 4 genotype mice). The data were analyzed using a 1-way ANOVA with Tukey test. *P < 0.01 as compared with each indicated group. The results are representative of 4 biological replicates.
Furthermore, Igf1-cKO and MVNP/Igf1-cKO mice had decreased OCL formation, and these OCLs that formed showed decreased PI3 and Erk kinase activation (Supplemental Figure 7).

IL-6 expression in OCLs is not regulated by IGF1 expression in OCLs (9). Since IL-6 is a major driver of the abnormal OCL formation in PD (9), we determined if loss of IGF1 in OCLs affected IL-6 expression in OCLs. Therefore, we measured IL-6 production and secretion by highly purified OCLs formed in bone marrow cultures derived from the 4 genotypes. IL-6 levels in conditioned media (CM) from WT, Igf1-cKO, MVNP, and MVNP/Igf1-cKO OCLs were < 50 pg/mL (< 0.01) (Figure 6C). Since deletion of Igf1 in OCLs did not affect IL-6 expression in OCLs, these results show that increased OCL–IL-6 levels in MVNP mice are not dependent on enhanced IGF1 expression in OCLs.

As previously shown (13), EphB2 expression on purified OCLs from MVNP mice was increased compared with the other genotypes and was much lower in MVNP/Igf1-cKO and Igf1-cKO compared with MVNP and WT mice, respectively (Figure 6D). IGF1-receptor expression on OCLs was similar in all genotypes (data not shown).
Characteristics of OBs derived from long bones and their OB differentiation capacity in cocultures with OCLs from MVNP mice. We next determined the differentiation potential of OB precursors derived from bones of WT, Igf1-cKO, MVNP, and MVNP/Igf1-cKO mice (Figure 7A). OBs from MVNP mice displayed higher alkaline phosphatase (ALP) activity compared with OBs from the other genotypes. We then determined expression of OB differentiation markers in cell lysates from outgrowth cells of bones from 12-month-old mice cultured for 14 days. Cells were stained ALP and staining was quantitated by ImageJ software (NIH). Results are representative of 3 biological replicates. (B) Expression of OB differentiation maker proteins. The expression of EphB4, osterix, and Col-1A were assessed by Western blotting using anti-EphB4 antibody, anti-osterix antibody, and anti-Col-1A antibody as described in Methods. β-Actin was used as the loading control. The expression levels of EphB4, osterix, and Col-1A were quantitated by ImageJ software (NIH). The basal ratio for every protein/loading control for OBs from WT mice was set at 1.0. Results are representative of 3 biological replicates. (C) OB differentiation in cocultures with MVNP-OCLs. OCLs (5 × 10⁴/well) derived from MVNP mice were scraped with a rubber policeman as described in Methods and were then replated and cultured with 50 ng/mL RANKL overnight. The OBs (1 × 10⁵/well) described above were plated on top of the OCLs the next day, and the cells were cocultured for 72 hours. The expression levels of EphB4, osterix, and Col-1A was determined by Western blotting using these antibodies as above. β-Actin was used as the loading control. The expression levels of EphB4, osterix, and Col-1A were quantitated by ImageJ software. The basal ratio of every protein/loading control for OBs from WT mice was set at 1.0. Results are expressed as the mean ± SEM (representative of 3 biological replicates for 4 genotype mice). The data were analyzed using 1-way ANOVA with Tukey test. *P < 0.01 compared with each indicated group. These results of experiment were similarly in 2 different biological replicates.

Characteristics of OBs derived from long bones and their OB differentiation capacity in cocultures with OCLs from MVNP mice. We next determined the differentiation potential of OB precursors derived from bones of WT, Igf1-cKO, MVNP, and MVNP/Igf1-cKO mice (Figure 7A). OBs from MVNP mice displayed higher alkaline phosphatase (ALP) activity compared with OBs from the other genotypes. We then determined expression of OB differentiation markers in cell lysates from outgrowth cells of bones from 12-month-old mice cultured for 14 days. Levels of EphB4, osterix, and Col-1A in OBs from MVNP/Igf1-cKO and Igf1-cKO mice were decreased compared with MVNP and WT OBs, respectively (Figure 7B). However, IGF1 receptor levels on OBs derived from these genotypes or WT mice were not significantly different (data not shown).

We found that MVNP-OCLs produced high levels of IL-6 and IGF1 (Figure 6, B and C) and induced EphB2 expression in OCLs and EphB4 in OBs in MVNP mice in vivo to enhance OB differentiation (Figure 6A and Figure 7B) (13). However, we do not know if loss of OCL-IGF1 in vivo affected the capacity of OBs derived from these mice to differentiate, since these OBs express lower levels of EphB4.

Therefore, we tested the effects of coculturing WT OBs with WT, MVNP, or MVNP/Igf1-cKO OCLs.
MVNP-OCLs enhanced OB differentiation and increased EphB4 expression on WT OBs compared with coculture with MVNP/Igf1-cKO and WT-OCLs. These results support our model that increased OCL-IGF1 produced by MVNP-OCLs induces upregulation of EphB4, osterix, and Col-1A to increase OB differentiation (Supplemental Figure 8). We then cocultured OCLs from MVNP mice with OBs derived from bones of the 4 genotypes for 72 hours. EphB4, osterix, and Col-1A expression in OBs was measured by Western blot. As shown in Figure 7, EphB4, osterix, and Col-1A expression levels in OBs from MVNP/Igf1-cKO and Igf1-cKO mice were lower than those of MVNP and WT OBs, respectively (P < 0.05). EphB4, osterix, and Col-1A levels in OBs were only upregulated in OBs of MVNP but not OBs from MVNP/Igf1-cKO mice (Figure 7C). These results suggest that the low EphB4 expression by OB precursors from the other genotypes impaired their capacity to respond, in the 72-hour cocultures, to the increased IL-6, IGF1, and EphB2 levels expressed by MVNP-OCLs.

Discussion

We previously reported that MVNP expression in OCLs from MVNP mice and patients with PD induce high levels of IL-6, which upregulate expression of IGF1 and EphB2 in OCLs and EphB4 on OBs (13). These studies provided insights into OCL regulation of OB activity in PD and the potential mechanisms responsible for the enhanced bone formation induced by PD-OCLs. Our in vitro findings suggested that enhanced IL-6 expression and increased IGF1 production by pagetic OCLs may induce the rapid bone formation that occurs in PD. However, the roles of these factors in WT and MVNP mice in vivo were unclear. In the current study, we examined the mechanisms responsible and effects of OCL-IGF1 on the increased bone formation and development of bone lesions in PD, as well as in physiologic bone remodeling in vivo.

The liver is the major source of IGF1, and approximately 80% of circulating IGF1 is bound to IGFBP3. OBs and OCys also produce IGF1, which acts as a paracrine and autocrine factor to enhance bone formation. Studies of tissue-specific genetic KO or overexpression of Igf1 have provided further insights into the importance of local sources of IGF1 in bone. For example, studies of deletion of Igf1 (19) and overexpression of Igf1 (20, 21) in OBs or deletion of Igf1 in OCys (22) showed that IGF1 markedly affects bone formation. These results suggest that changes in local levels of IGF1 in bone are important and not compensated by the high circulating levels of hepatic-derived IGF1. We found that IGFBP3 was also expressed by OCLs (13). However, the effects of conditional deletion of Igf1 in OCLs in vivo have not been reported, although the effects of global Igf1 (23) or Igf1 receptor (24) KO in mice on OCL activity were reported. Therefore, to determine the contribution of increased OCL-IGF1 that occurs in MVNP mice to bone formation in PD, we generated WT and MVNP mice with conditional deletion of Igf1 in OCLs. We confirmed that our Tracp-Cre transgene targeted Cre to cells in the OCL lineage in bone by generating Tracp-Cre/Tomato mice. We found that Tracp-Cre(+) /Tomato mice showed colocalization of TRACP and Tomato staining in OCLs and, at much lower levels, in scattered OCys in bone (Supplemental Figure1). Immunostaining OCys with an anti-IGF1 antibody showed similar levels of IGF-expressing OCys in bone sections from the 4 genotypes. Furthermore, we did not find differences either by Western blotting or ELISA assays in IGF1 expression by bone-derived OCys from WT, Igf1-cKO, MVNP, or MVNP/Igf1-cKO mice (Supplemental Figure 2). These results suggest that changes in IGF1 expression by OCys are not responsible for our findings. Since TRACP expression is also detected in the kidney, we measured 1,25(OH)2D3, a calcitropic hormone produced by the kidney. Serum levels of 1,25(OH)2D3 in all 4 genotypes were similar. Thus, although TRACP is expressed in the kidney, bowel, and other tissues (25), these mice provide a useful model for determining the effects of deletion of Igf1 in OCLs in bone.

Igf1-cKO and MVNP/Igf1-cKO mice were born at much-lower-than-expected numbers. We do not have any data to explain this finding. Possibly, since TRACP is expressed in the placenta and syncytiotrophoblasts (26), the lower numbers of cKO mice may result from insufficient IGF1 expression in the placenta, which affected fetal development.

We found that cKO of Igf1 in OCLs of MVNP or WT mice did not affect femur length, body weight, cortical BV, and serum levels of IGF1 and IGFBP3 (at 20 months of age). These results are in contrast to results of mice with targeted deletion of Igf1 in the liver. Mice with hepatic Igf1-cKO had a 75% decrease in serum IGF1 concentration, with a 26% decrease in cortical BV, mainly affecting the periosteum (27, 28). These results suggest that serum concentrations of IGF1 contribute to earlier acquisition of peak bone mass but do not result in an overall change in bone mass. Since we did not find changes in serum levels of IGF1, or effects on mouse size, body weight, or femoral length, our results suggest that our models reflect the local effects of loss of OCL-IGF1 on bone remodeling — effects that are not compensated for by systemic IGF1 in serum.
In this study, we also examined IGF1 expression in OCLs and BFR in bone specimens from a PD patient and a healthy donor. We confirmed that the abnormal OCLs in the PD patient expressed increased levels of IGF1 and had an increased BFR compared with the healthy donor (Figure 1). These findings supported examining the contribution of increased IGF1 expressed by PD-OCLs to the rapid bone formation in PD and supported determining if IGF1 from normal OCLs also contributes to physiologic bone remodeling.

We found that loss of IGF1 in MVNP-OCLs has major effects on the pagetic phenotype of MVNP mice. We detected PDLs in 40% of MVNP mice, but PDLs were not detected in MVNP/Igf1-cKO mice. Furthermore, BV/TV was decreased by 60% in MVNP/Igf1-cKO mice compared with MVNP mice with PDLs (Figure 2B), and bone mass and OCL and OB surface and numbers in MVNP/Igf1-cKO mice were similar to levels in WT mice. Thus, conditional deletion of Igf1 in OCLs from MVNP mice abrogated the pagetic phenotype in MVNP mice (Figure 2). Interestingly, our data suggest that OCL-IGF1 mainly affects trabecular bone formation by increasing OCL and OB numbers and surfaces. Furthermore, conditional deletion of OCL-Igf1 in MVNP mice decreased BFR by 80% compared with MVNP mice (Figure 3D). These results support that OCL-IGF1 is a major contributor to the pathologic rapid new bone formation that occurs in PD.

Next, we determined if OCL-IGF1 is responsible for the increase in EphB2 and EphB4 in OCLs/OBs of MVNP mice. OCLs from MVNP mice with/without PDLs displayed much higher IGF1 expression levels compared with OCLs from WT mice. IGF1 was not detected in OCLs from Igf1-cKO and MVNP/Igf1-cKO mice (Figure 4). EphB2 and EphB4 were increased in OCLs and OBs, respectively, in MVNP mice but not in MVNP/Igf1-cKO and Igf1-cKO mice. These results show that increased OCL-IGF1 expression in MVNP mice increased EphB2 expression on OCLs and enhanced EphB4 expression on OBs. Similar to PDLs in patients with PD, PDLs in MVNP mice showed increases in OB and OCL parameters (Oc.S/BS, N.Oc/B.Pm, Ob.S/BS and N.O/B.Pm), mineralized perimeters, MAR, BFR/BS, and larger, more hyper-multinucleated OCLs in bone (Figure 3) compared with MVNP mice without PDLs. These results suggest that PDLs may only form in areas of bone with increased numbers of highly active abnormal OCLs and that the high bone turnover state induced by expression of OCL-IGF1 and EphB2 in PD-OCLs contributes to formation of PDLs. The basis for the localized increase in OCL and OB numbers that result in PDLs in PD is currently unknown. Consistent with our results, Wang et al. found that mice lacking the IGF1R in OCLs have decreased EphB2 levels (23). However, their experiments did not determine if IGF1 is expressed by OCLs or if OCL-IGF1 induces EphB2 on OCLs.

Surprisingly we found that cKO of Igf1 in OCLs does not affect cortical thickness and volume, since these parameters were similar in mice of the 4 genotypes tested (Figure 2, C and D). These results are consistent with those of Zhang et al., who reported that OB-specific Igfr1 KO in mice resulted in a striking decrease in cancellous bone parameters but did not change cortical bone parameters (24).

IGF1 has been shown to be essential for OCL differentiation and to maintain OCL function (29, 30). We found that OCL formation, nuclear number/OCL, and bone resorption capacity of OCLs from MVNP/Igf1-cKO and Igf1-cKO mice were significantly decreased compared with WT and MVNP mice (Figure 5). These results suggest the OCL-derived IGF1 may have autocrine effects on OCL formation in PD and normal bone.

We next examined the mechanism responsible for the increased IGF1 expression in MVNP-OCL. We previously found that loss of IL-6 resulted in loss of the pagetic phenotype in MVNP mice (9). OCLs formed in bone marrow cultures of MVNP/IL-6-KO mice did not display the characteristics of pagetic OCLs. Furthermore, histomorphometric analysis of bones from MVNP/IL-6-KO mice showed that loss of IL-6 also abrogated the increase in MAR found in MVNP mice and reduced mineralizing perimeter and BFR. However, we did not know if IGF1 increased IL-6 expression. We show here that OCLs from MVNP/Igf1-cKO and MVNP mice produced similar levels of IL-6 (Figure 6B). Taken together, these results — with our previous findings that showed OCLs from MVNP/IL6-/- mice express much lower levels of IGF1 than OCLs from MVNP mice (13) — support that IL-6 is an inducer of the pagetic OCL phenotype and contributes to PD. They also suggest that increased IL-6 induced by MVNP gene expression in OCLs drives the increased IGF1 expression in OCLs, which in turn is required to increase EphB2 expression in OCLs in MVNP mice in vivo. Thus, IL-6 appears to regulate the increased OCL-IGF1 in OCLs from MVNP mice in vivo, which plays an important role in the abnormal bone remodeling in PD.

We then determined if OCLs from MVNP mice could stimulate the differentiation of OBs from WT, Igf1-cKO, MVNP, and MVNP/Igf1-cKO mice. When OCLs from MVNP mice were cocultured with OBs from mice of the 4 genotypes, EphB4, osterix, and Col-1A levels were upregulated to a greater extent in
coclultures containing OBs from MVNP mice compared with OBs from WT mice. This increase was lost in cultures containing OBs from MVNP/Igf1-cKO mice (Figure 7C). These results further suggest that OCL-IGF1, in addition to IL-6 from PD-OCLs, is necessary to increase bone formation in PD by enhancing bidirectional signaling through increased EphB2/EphB4 in vivo.

Finally, we suggest that OCL-IGF1 may play a role in physiologic bone formation. Histological analysis of bones from Igf1-cKO mice showed they had lower trabecular BV associated with reduced numbers and size of OCLs compared with WT mice (Figure 2). In vitro experiments indicated that loss of OCL-IGF1 was responsible for the decreased OCL generation and bone formation (Figure 5 and Figure 7). These results suggest that OCL-IGF1 may contribute to the maintenance of bone mass in normal adult bone by stimulating OCL differentiation and function that is coupled to bone formation in vivo. However, further studies are needed to confirm a role for OCL-Igf1 in normal bone formation because we only analyzed a small number of Igf1-cKO mice and used only 1 Cre model for our study.

In conclusion, our results suggest that OCL-IGF1 increases bone formation and development of PDLs in PD by enhancing bidirectional signaling between OCLs and OBs through increased EphB2/EphB4 in vivo.

Methods

Blinding. To avoid bias, all data were collected in a blinded fashion, where the observer was unaware of the experimental group. Key studies were performed by more than 1 individual to determine if there was consistency in the observations.

PD patient and healthy control. The resin embedded bone sections from a PD patient and healthy donor were provided in house. A transiliac crest bone biopsy was taken from a 58-year-old female patient with PD and from a 30-year-old female patient as a healthy control; both were treated with calcine and tetracycline before sampling. The biopsy sample was embedded without decalcification in methyl methacrylate. The sections were stained for TRACP, and IGF1 was detected by an anti-IGF1 antibody (R&D Systems, MAB2911). Histological evaluation was performed under bright-field, fluorescent light, and polarized light. The detailed histological examinations of bone sections from this PD patient and healthy donor were previously reported (9). MAR and BFR/BS were calculated and expressed as recommended to the ASBMR Nomenclature Committee (31–33).

Animal studies. Animals were housed at VCU in individually ventilated cages in a barrier vivarium, which excludes all known mouse viruses and parasites and most bacteria (including helicobacter). The mice were fed standard mouse diet (irradiated Teklad LM-485 diet) and autoclaved water. Mice of both sexes and multiple ages were euthanized under isoflurane anesthesia, followed by cervical dislocation, for collection of bone tissues, which were shipped overnight to IU in DMEM + 10% FBS + penicillin/streptomycin (Sigma Aldrich) at 20°C.

Generation of mice with cKO of Igf1 in OCLs. Mice with cKO of Igf1 in OCLs were generated by breeding Igf1<sup>fl/fl</sup> mice that carry loxP sequences flanking exon 4 of the gene (17) (The Jackson Laboratory, 016831) with transgenic mice expressing Cre recombinase under the control of a 2.3-kb murine TRACP promoter (34) to generate TRACP-Cre<sup>+/Igf1<sup>fl/fl</sup></sup> mice. These were further bred to TRACP-MVNP mice (35, 36), to generate mice of the following 4 genotypes: (a) WT, (b) Igf1-cKO, (c) MVNP, and (d) MVNP/Igf1-cKO. All mice were on a C57BL/6J background. At each generation, only 1 parent carried the TRACP-MVNP transgene and only 1 carried the TRACP-Cre transgene, so offspring carrying either were homozygous for the transgene. At 12 months of age, body weight was determined and serum IGF1 and IGFBP3 were measured by IGF1-ELISA kit (Abcam, ab100695) and IGFBP3-ELISA kit (Abcam, ab100692). All experiments were performed in mice of both sexes.

TRACP-Cre/Tomato mice. TRACP-Cre mice were bred to a reporter line that contains a CAG promoter-loxSTOPlox-dTomato transgene inserted into the ROSA26 locus (A19, The Jackson Laboratory, 007909), to generate double-transgenic TRACP-Cre/Tomato mice in which only TRACP-expressing cells should also express the tdTomato protein. We examined the colocalization of TRACP<sup>+</sup> and Tomato<sup>+</sup> OCLs by performing TRACP staining and anti-Tomato immunostaining (anti-Tomato antibody from Thermo Fisher Scientific, NC9590225) on serial sections.

μCT and histomorphometry. Femurs, tibiae, and vertebrae from WT, Igf1-cKO, MVNP, and MVNP/Igf1-cKO at 20 months of age were fixed in 10% buffered formalin at 4°C. Bone microstructure analyses were performed using a μCT scanning system (viva CT 40, Scanco Medical) with an isotropic voxel size of 10.5 μm and the scanner settings of 55 kVp, 25 μA, and 350 ms integration time. Structural parameters were ana-
lyzed in reconstructed 3-dimensional images using evaluation software (μCT v1.6, Scanco Medical) according to the recommended guideline (18). The regions of interest were defined using previously described methods (37, 38). The cancellous bone and marrow compartments of the L5 vertebral body were examined between the cranial and caudal growth plates. The cortical bones parameters were analyzed in 100 slices at the tibial midshaft, starting 5.5 mm from the proximal metaphysis. The μCT data were then exported as a sequence of 8-bit DICOM grayscale images, and simultaneous multiplanar reconstructed (MPR) images were viewed using ImageJ software (NIH).

The lumbar vertebrae were decalcified in 10% EDTA at 4°C and embedded in paraffin. The decalcified sections were stained for TRACP, and OCLs containing active TRACP were stained red as described by Liu et al. (39). OCL perimeter (Oc.S/BS) was defined as the length of bone surface covered with TRACP+ multinucleated cells. OB perimeter (Ob. S/BS) was also measured in the same field.

Bone formation parameter analysis. To examine bone formation parameters, animals were injected i.p. with calcein (10 mg/kg) 7 days and 2 days before euthanasia. The fifth lumbar vertebra was embedded without decalcification in methyl methacrylate. The undecalcified sections were left unstained for the evaluation of fluorescent labels. The MS/BS, MAR, and BFR/BS were quantified. All variables were expressed and calculated according to the recommendations of the ASBMR Nomenclature Committee (31–33).

Immunohistochemical analysis. The femurs from 20-month-old mice were fixed in 10% buffered formalin and completely decalcified in 10% EDTA at 4°C and embedded in paraffin. Longitudinal sections (5 μm) were cut and mounted on glass slides. Deparaffinized sections were treated with 1% horse serum for 1 hour, followed by primary antibodies against IGF1 (Abcam, ab9572), EphB2 (Abcam, ab75868), EphB4 (Abcam, ab64820), and control IgG rabbit (Santa Cruz Biotechnology Inc., sc-2027). The sections were incubated overnight and then stained with anti–rabbit IgG conjugated to peroxidase (Vector Laboratories).

Scoring was based on staining intensity (positive or negative) and was performed by a blinded observer.

OCL formation from purified OCL precursors. Nonadherent bone marrow cells were harvested and enriched for CD11b+ mononuclear cells using CD11b microbeads (MACS, 120-000-300) and a Miltenyi Biotec MACS magnetic cell-sorting system. OCL formation from CD11b+ cells was then performed as described previously (13) in the presence of RANKL (R&D Systems) to generate OCLs. The cells were stained for TRACP, and TRACP+ multinucleated cells (≥3 nuclei/cell) were scored as OCLs.

Isolation of mature OCLs from BM cultures. BM cells flushed from long bones of WT, Igf1-cKO, MVNP, and MVNP/Igf1-cKO mice were cultured in a 10-cm dish (2.5 × 10^7 cells/dish) with M-CSF (10 ng/mL) (R&D Systems) for 3 days and RANKL (50 ng/mL) (R&D Systems) for 4 days as previously described (13). At the end of culturing, trypsin-EDTA (Corning) was added, and the cultures were incubated for 3 minutes to remove nonosteoclastic cells and enrich the concentration of OCLs in the cultures.

Bone resorption assays. OCLs generated as described above were cultured on bovine bone slices (Immunodagnostik Systems, DT-IBON1000-96) in αMEM (Thermo Fisher Scientific) containing 10% FCS (Sigma-Aldrich) and RANKL (50 ng/mL). After 7 days of culture, the cells were removed, the bone slices were stained with acid hematoxylin, and the areas of dentin resorption were determined as previously described (40).

Isolation and culture of primary OBs. After flushing the BM from tibiae and femurs of WT, Igf1-cKO, MVNP, and MVNP/Igf1-cKO mice, the tibiae and femurs were cultured in αMEM containing 10% FCS for 7–10 days. The bones were then placed in 60-mm dishes, and the cultures continued in αMEM containing 10% FCS until cells growing out of the bones formed a confluent monolayer. The original bone was removed, and the outgrowth cells from the bone were treated with 0.25% trypsin and 0.05% EDTA for 10 minutes at 37°C. These cells were used as primary OBs without further passage. The primary OBs (2 × 10^6 cells/well in 6-well plates) were cultured in αMEM containing 10% FCS for 7–14 days. Cells were either stained for ALP or alizarin red, or cell lysates were collected and analyzed for protein expression, as previously described (13).

Coculture of OCLs and OBs. OCLs (2 × 10^6 cells/well in 12-well plates) derived from cultures of MVNP-OCL precursors were scraped with a rubber policeman as described above, replated, and cultured with 50 ng/mL RANKL overnight. The OBs (1 × 10^6 cells/well) described above were plated on top of the OCLs the next day, and the cells were cocultured for 72 hours.

Isolation and culture of primary osteocyte-like cells. Primary OBs (1 × 10^6 cells/mL/well in 12-well plates) isolated as described above were cultured in αMEM containing 10% FCS for 30 days. Cells were analyzed for OC markers sclerostin, DMP1, and ORP150 (Supplemental Figure 2) by Western blotting and used to determine IGF1 production by OCs isolated from femurs of the 4 genotypes.

Immunoblotting. Total proteins were extracted from OCLs, OBs, or OCs, with RIPA buffer and the cell
lysates (10 μg/lane) loaded on SDS gels using Bio-Rad Mini-PROTEAN Precast Gels. The resolved proteins were transferred onto nitrocellulose membranes (TGX Membrane; Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad); then, the membranes were stained with Ponceau S and cut into strips based on the molecular weight markers. Membranes were then exposed to primary antibodies overnight at 4°C and incubated with IgG HRP–conjugated antibody for 1 hour. The blots were washed and visualized by a Super Signal West Dura Extended Duration System (Cell Signaling). Antibodies used for detection of EphB2 (Abcam, ab75868), EphB4 (Abcam, ab64820), IGF1 (Abcam, ab63926), IGF1R (Abcam, ab39398), osterix (Abcam, ab22552), β-actin (Abcam, ab49900), NFATc-1 (Santa Cruz Biotechnology Inc., sc-7294), cathepsin K (Santa Cruz Biotechnology Inc., sc-48353), IL-6 (Santa Cruz Biotechnology Inc., sc-7920), Col-1A (MilliporeSigma, AB765P), DMP1 (Novus Biological, NRP1-45525), Sclerostin (Abcam, ab63097), ORP150 (Santa Cruz Biotechnology Inc., sc-398224), and GAPDH (Cell-Signaling Technology, 3683).

**IGF1 and IL-6 ELISA assays.** Mouse OCLs were isolated as described above, and OCLs (5 × 10^4 cells/mL) were cultured with RANKL for 3 days. OCys (2 × 10^5 cells/mL) were cultured in αMEM 10%FCS for 3 days. Conditioned media from these cultures were harvested at the end of the culture period, and the concentration of IGF1 was measured using an ELISA kit for murine IGF1 (Abcam, ab100695). IL-6 was measured in OCL-CM using an ELISA kit for murine IL-6 (R&D system, M600B) according to the manufacturer's instructions.

**Measurement of serum 1,25-(OH)2D3.** Mouse serum 1,25-dihydroxyvitamin D3 levels were measured with an ELISA Kit (CUSABIO, CSB-E13697m) according to the manufacturer's instructions.

**Statistics.** Significance was evaluated using a 1-way ANOVA with a Tukey test. Differences with P < 0.05 were considered significant.

**Study approvals.** All animal studies were performed as described in approved IACUC protocols from VCU and IU and ACURO protocol from the Department of Defense, in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Academies Press, 2011).

**Author contributions.** JMC, GDR, and NK designed the study, interpreted the data, and wrote the manuscript. KM, YO, JT, JDC, and NK performed the experiments. JJW and MAS generated the transgenic mice, and JJW helped write the manuscript. KM, HZ, and DDD performed histological studies and analyses. All authors approved the submission of the manuscript.

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Address correspondence to: G. David Roodman, Department of Medicine, Hematology/Oncology, School of Medicine, Indiana University, 980 West Walnut Street, Suite C312, Indianapolis, Indiana 46204, USA.

Phone: 317.278.6255; Email: groodman@iu.edu.


