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TITLE: Measles Virus Nucleocapsid (MVNP) Gene Expression and RANK Receptor Signaling in Osteoclast Precursors, Osteoclast Inhibitors Peptide Therapy for Pagets Disease

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Paget's disease (PD) of bone occurs in 3-4% of population over the age of 50. We have identified expression of measles virus nucleocapsid transcripts in osteoclast (OCL) precursors and that MVNP expression induces pagetic phenotype in osteoclasts with increased bone resorption activity as seen in patients with Paget's disease. We previously cloned and identified osteoclast inhibitory peptide-1 (OIP-1/hSca) which inhibits osteoclast formation and bone resorption. We hypothesize that MVNP expression in osteoclast precursors modulates RANK receptor signaling leading to Pagetic OCL development. OIP-1 blocks these signaling events and inhibits MVNP induced osteoclastogenesis and elevated bone resorption activity. We demonstrated that MVNP increases TNF-alpha induced OCL differentiation and activation by increasing NF-kB signaling through increased expression of p62, and IKK-gamma and increased MAPK signaling. Our results also suggest that MVNP's effects on TNF-alpha signaling contribute to the increased OCL formation in PD. Furthermore, expression of MVNP gene in OCL in vivo induces a pagetic-like phenotype. RANKL stimulation of OIP-1 mice derived bone marrow cells resulted in significantly decreased osteoclast formation. Furthermore, OIP-1 transgenic mouse bones demonstrated an osteopetrotic phenotype. These data suggest that OIP-1 is an important physiologic regulator of osteoclast development and bone resorption in vivo and may have therapeutic utility to control excess bone turnover in patients with Paget's disease.
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INTRODUCTION:

Paget’s disease affects approximately 2-3 million people in the United States and is the second most common bone disease after osteoporosis. We shown that bone marrow cells from patients with Paget’s disease express measles virus nucleocapsid protein (MVNP) transcripts and further demonstrated that expression of the Edmonston MVNP gene in normal osteoclast (OCL) precursors results in formation of OCL that share many of the characteristics of OCL from Paget’s patients. The MVNP gene contained several sense mutations, which constituted 1% of the nucleotide sequence. The pathologic significance of MVNP and associated mutations to induce abnormal OCL formation and activity in Paget’s disease, is unknown (1). RANKL is a member of Tumor necrosis factor (TNF) family member that is expressed on stromal/osteoblast cells and RANK receptor is expressed on committed osteoclast precursor cells. RANKL/RANK signaling is critical for osteoclast differentiation and bone resorption activity in vitro and in vivo (2,3). We have recently cloned and identified the Ly-6 family member, osteoclast inhibitory peptide-1 (OIP-1/hSca) which inhibits osteoclast formation and bone resorption activity. We have further demonstrated that OIP-1 significantly inhibits TNF receptor associated factor-2 (TRAF-2) and c-Jun kinase activity in osteoclast precursor cells (4). Our hypothesis is that MVNP expression in osteoclast precursors modulates the status of RANK receptor signaling molecules leading to Pagetic OCL development in Paget’s disease. OIP-1 blocks RANK receptor signaling events and inhibits MVNP induced osteoclastogenesis and elevated bone resorption activity in Paget’s patients.

BODY:

The progress on Task-3 (35-45 months) in the statement of work is as follow:

Task 1. Determine the sensitivity of MVNP transduced osteoclast precursors to RANK Ligand (RANKL) and TNF-alpha stimulation to form pagetic osteoclasts (Months 1-24): 

Completed

Task 2. Determine the RANK receptor signaling in MVNP transduced osteoclast precursors (Months 24-36).

Completed

Task 3. Determine the effects of OIP-1 on MVNP altered RANK receptor signaling in osteoclast precursor cells (Months 29-48).

(a) Determine the effect of OIP-1 on osteoclast differentiation of MVNP transduced osteoclast precursors (Months 29-35).

Completed
Mutations in the sequestosome 1 (p62) gene has been reported in patients with Paget’s disease. In previous report, we have shown that a mutant p62 (P392L) modulates NF-kB signaling during osteoclast (OCL) differentiation. We further identified that both wild-type p62 and mutant p62P392L transduced OCL precursors formed significantly larger osteoclasts compared to empty vector (EV) transduced cells (Fig.1). However, our results concluded that mutation of the p62 gene increases osteoclastogenesis but do not induce Paget disease (article appended).

In the present study period, we have characterized transgenic mice targeted with osteoclast inhibitory peptide-1 (OIP-1) expression to the cells of osteoclast lineage using the mouse tartrate resistant acid phosphatase gene promoter. The OIP-1 mice demonstrated osteopetrotic bone phenotype (article appended). We have performed ex vivo experiments using bone marrow cells isolated from the OIP-1 and wild-type mice to further assess OIP-1 inhibition of OCL formation. Non-adherent bone marrow cells derived from the wild-type and OIP-1 mice were cultured in the presence of hGM-CSF (10 ng/ml) in methyl cellulose (1.2%) to form CFU-GM colonies. As shown in Fig.2A, OIP-1 transgenic mouse lines #5 and #13 derived cells showed a significant decrease in the number of CFU-GM colonies by 35% and 41% respectively, compared to control mice. We further examined the capacity for OCL differentiation and bone resorption by OIP-1 transgenic mouse derived bone marrow cells. OIP-1 and wild-type mice derived non-adherent bone marrow cells were stimulated with various concentrations of RANKL (5-100 ng/ml) with M-CSF (10 ng/ml), and the number of TRAP positive multinucleated OCLs formed in these cultures were scored. As shown in Fig.2B, the number of OCLs formed in OIP-1 #5 and OIP-1 #13 mouse bone marrow cultures was significantly decreased by 39% and 42% respectively, compared to control mice in response to RANKL (100 ng/ml) treatment. We further examined the effects of MVNP on RANK-RANKL signaling in wild-type and OIP-1 transgenic mice derived preosteoclast cells. The non-adherent bone marrow cells from wild-type and OIP-1 mice were transduced with EV or MVNP and cultured with 10 ng/ml M-CSF and 100 ng/ml RANKL for 48 hours to induce osteoclast differentiation. Western blot analysis of total cell lysates obtained revealed that OIP-1 did not affect the levels of RANK receptor expression in these cells. However, there is a significant increase (2.5 fold) in the levels of RANK adaptor protein TRAF2 expression in MVNP transduced wild-type mouse preosteoclast cells, but not TRAF 6. Interestingly, OIP-1 mice derived preosteoclast cells demonstrated no significant increase in the levels of TRAF2 in response to MVNP expression. In addition, transcription factors such as c-Fos, NFATc1 critical for OCL differentiation were significantly decreased in OIP-1 transgenic mice derived preosteoclast cells compared to wild-type mice (Fig.3A). We further examined the c-Jun N-terminal kinase (JNK) activation in response to MVNP stimulation in OIP-1 mouse derived preosteoclast cells. As shown in Fig.3B, OIP-1 mouse derived preosteoclast cells transduced with MVNP showed a significant inhibition of JNK phosphorylation in response to RANKL stimulation compared to wild-type mice. Furthermore, the JNK activators such as Rac1 and ASK1 expression was also inhibited in the OIP-1 derived preosteoclast cells stimulated with MVNP when compared with wild-type mice (Fig.3C). Taken together, these results suggest that OIP-1 inhibits MVNP stimulated RANK-RANKL signaling during osteoclast differentiation.
Fig. 1. OCL formation by p62 and p62P392L transduced human OCL precursors. GM-CFU-derived cells transduced with p62, p62P392L, or EV were cultured with RANKL.

Fig. 2. Inhibition of osteoclastogenesis in OIP-1 mouse bone marrow cultures. (A) CFU-GM formation in OIP-1 mouse bone marrow cultures. Wild-type (Wt) and OIP-1 mouse derived non-adherent bone marrow cells (4 × 10⁵/ml) were cultured with hGM-CSF (10 ng/ml) in 1.2% methyl cellulose to form CFU-GM colonies. At the end of a 7 day culture period, CFU-GM colonies (aggregates >50 cells) formed in these cultures were scored using a light microscope. (B) Wild-type (Wt) and OIP-1 transgenic mouse bone marrow derived non-adherent cells were stimulated with RANKL (5-100 ng/ml) and M-CSF (10 ng/ml) for 5 days and the TRAP (+) multinucleated OCLs formed in these cultures were scored. The results represent quadruplicate cultures of three independent experiments (p<0.05).
Fig. 3 Western blot analysis of RANK receptor signaling molecules in wild type (Wt) and OIP-1 transgenic mice derived bone marrow cells transduced with empty vector (EV) or MVNP retrovirus. (A-C) Bone marrow cells from Wt and OIP-1 mice were transduced with MVNP or EV and cultured in presence of 10 ng/ml mMCSF and 100 ng/ml mRANKL for 2 days total cell lysates prepared were subjected to western blot analysis.

(c) Determine the potential of OIP-1 to block MVNP stimulated osteoclast formation and bone resorption in vivo (41-48).

Not yet initiated
KEY RESEARCH ACCOMPLISHMENTS:

We identified that OIP-1 expression significantly decreased MVNP enhanced levels of TRAF2, c-Fos, p-c-Jun and NFATc1. However no change in the levels of RANK and TRAF6 expression in RANKL stimulated mouse bone marrow cultures.

REPORTABLE OUTCOMES:

Published articles relevant to the proposal:


Abstracts:


CONCLUSIONS:

- In conclusion, OIP-1 inhibits MVNP stimulated Pagetic osteoclast formation/activity through suppression of RANK signaling. OIP-1 may have therapeutic utility against excess bone turnover associated with Paget’s disease.

REFERENCES:


APPENDICES:

Reprints enclosed for two relevant articles as noted under outcomes.
Original Paper

Transgenic mice with OIP-1/hSca overexpression targeted to the osteoclast lineage develop an osteopetrosis bone phenotype

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Abstract

Regulatory mechanisms operative in bone-resorbing osteoclasts are complex. We previously defined the Ly-6 gene family member OIP-1/hSca as an inhibitor of osteoclastogenesis in vitro; however, a role in skeletal development is unknown. In this study, we developed transgenic mice with OIP-1/hSca expression targeted to the osteoclast lineage that develop an osteopetrotic bone phenotype. Humeri from OIP-1 mice showed a significant increase in bone mineral density and bone mineral content. µCT analysis showed increased trabecular thickness and bone volume. OIP-1 mice have dense sclerotic cortical bone with absence of spongiosa and inadequate formation of marrow spaces compared to wild-type mice. Moreover, complete inhibition of osteoclasts and marrow cavities in calvaria suggests defective bone resorption in these mice. OIP-1 mouse bone marrow cultures demonstrated a significant decrease (41%) in osteoclast progenitors and inhibition (39%) of osteoclast differentiation/bone resorption. Western blot analysis further demonstrated suppression of TRAF-2, c-Fos, p-c-Jun, and NFATc1 levels in RANKL-stimulated osteoclast precursors derived from OIP-1 mice. Therefore, OIP-1 is an important physiological inhibitor of osteoclastogenesis and may have therapeutic value against bone loss in vivo.

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Keywords: osteoclast inhibitory peptide-1/human Sca; osteoclast; osteopetrosis; bone resorption; osteoblast

Introduction

The osteoclast (OCL) is the bone-resorbing cell derived from the monocyte/macrophage lineage. OCL formation and bone resorption are regulated by local factors present in the bone marrow micro-environment. Tumour necrosis factor (TNF) gene family member, receptor activator of nuclear factor κB (RANK), expressed on OCL precursors, and RANK ligand (RANKL), expressed on osteoblast/stromal cells, interaction is critical for OCL differentiation and bone resorption [1]. RANK–RANKL signalling promotes the binding of TNF receptor associated factor (TRAF) family proteins such as TRAF-6 to RANK, which results in the activation of Jun N-terminal kinase (JNK) pathways [2]. We have previously identified and characterized a novel autocrine/paracrine inhibitor of OCL formation termed osteoclast inhibitory peptide-1 (OIP-1/hSca) [3]. OIP-1/hSca is a Ly-6 gene family member expressed on immature thymocytes and thymic epithelial cells [4]. OIP-1/hSca is a glycosphatidylinositol (GPI)-linked membrane protein (16 kD) containing a 79-amino acid extracellular peptide and a 32-amino acid carboxy-terminal GPI-linked peptide (c-peptide). OIP-1/hSca is a human homologue of mouse Sca-2 with 65% identity at the nucleotide level, with a conserved pattern of cysteine residues in protein structure [5]. Previously, we have demonstrated that the OIP-1 c-peptide region is critical for OCL inhibitory activity and that a neutralizing antibody against the c-peptide completely blocks OIP-1 activity to inhibit OCL formation in vitro; however, a role in skeletal development is unknown [6]. We have also shown that OIP-1/hSca mRNA is highly expressed in osteoblastic cells, OCLs, and bone marrow cells, and that the hSca protein is cleavable from the OCL surface [3]. It has been demonstrated that Sca-2 functions as a modulator of the T-cell receptor (TCR) signalling pathway [7]. In addition, it has been shown to be physically and functionally associated with CD3 ζ chains of the TCR complex [8]. Furthermore, Ly-6A knock-out mice demonstrated a significant decrease in bone mineral density and bone mineral content compared with wild-type mice [9].
These studies implicate an essential role for the Ly-6 gene family in normal bone remodelling. In this study, we show that transgenic mice with OIP-1/hSca overexpression targeted to the osteoclast lineage develop an osteopetrotic bone phenotype through inhibition of OCL formation/activity in vivo.

**Materials and methods**

**Development of OIP-1 transgenic mice**

Human OIP-1 cDNA was excised from the CDS 5-3 plasmid by BamH1 and Pmel digestion. The resulting DNA fragment (430 bp) encoding the complete coding sequence of OIP-1 was sub-cloned into the pKCR3ΔR1-mTRAP gene promoter containing plasmid (Figure 1A). The transgene fragment from the plasmid construct (pKCR3ΔR1-mTRAP-OIP-1#4) was micro-injected into the male pronucleus of fertilized one-cell mouse embryos at 3 µg/ml concentration and re-implanted into the oviducts of pseudo-pregnant female mice as previously described [10]. The presence of the transgene was identified in resulting offspring by polymerase chain reaction (PCR) screening with OIP-1 gene-specific primers [6] using template genomic DNA purified from a small piece of tail taken from each animal at the time they were

![Figure 1](image_url)

**Figure 1.** OIP-1 transgenic mice exhibit focal osteopetrotic bone phenotype. (A). mTRAP-OIP-1 transgene plasmid map. The mTRAP-OIP-1 transgene fragment (4 Kb) was excised by Sal-I-Sac I digestion from the plasmid PKCR3ΔR1 mTRAP-OIP#4 for micro-injection into the mouse embryos to develop OIP-1 transgenic mice. (B). PCR screening of potential founder mice with OIP-1 gene specific primers as described in methods. PCR product amplified using the transgene plasmid served as a positive control (+). We thus identified two potential founder mouse lines #5 and#13. (C) Western blot analysis of OIP-1 expression in transgenic mouse derived preosteoclast cells. Non-adherent bone marrow cells obtained from wild-type (Wt) and OIP-1 transgenic mice were cultured at a density of 1 x 10⁶ cells for 4 days in the presence of 10 ng/ml M-CSF and 100 ng/ml RANKL to obtain preosteoclast cells. Total cell lysates obtained were subjected to Western blot analysis using rabbit anti-OIP-1 antibody. (D) Radiological analysis of the wild-type (Wt) and OIP-1 transgenic mice were performed using a Faxitron MX20 equipped with a FPX-2 Imaging system. Magnified radiographs of left and right humerus of Wt and OIP-1 mice (4 week old) showed increased radiodensity in the proximal humeral region as pointed by arrows. (E) Bone mineral density (BMD) and Bone mineral content (BMC) of Wt and OIP-1 mice humerus bones at 4 weeks of age as measured by dual energy X-ray absorptiometry (DEXA) and values represent mean ± SD (n = 5; p < 0.05)


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weaned. We thus identified two potential founder lines (Nos 5 and 13) of OIP-1 transgenic mice (Figure 1B). Western blot analysis further confirmed high levels of OIP-1 expression in pre-osteoclast cells derived from these founder lines compared with wild-type mice (Figure 1C). All experiments were performed using OIP-1 mice, line 13 (3 to 4 weeks old), unless otherwise specified, with appropriate littermate controls, following the Institutional Animal Care and Use Committee (IACUC) procedure approved by the Institutional Review Board.

**Micro-computed tomography (μCT) analysis**

The bones collected from 4-week-old mice were fixed in 70% ethanol and the distal metaphyses were scanned with a Skyscan 1072 μCT instrument and analysed by CT-Analyzer software (SkyScan). Two-dimensional images were used to generate three-dimensional reconstructions and to calculate morphometric parameters.

**Histology and histomorphometric analysis**

The wild-type and OIP-1 mouse bone specimens were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), decalcified in 0.5 M EDTA (pH 7.4) for a 1 to 3-week period, and processed for paraffin embedding. Serial 5-μm sections were cut on a modified Leica RM 2155 rotary microtome (Leica Microsystems, Ontario, Canada) and stained with haematoxylin and eosin [11].

In order to perform histochemical staining, bone specimens were fixed overnight in 70% ethanol and embedded in methyl methacrylate (MMA). Serial 4 to 6-μm sections of MMA embedded calvaria were sectioned from anterior to posterior through frontal and parietal bone tissues and humerus sections were stained for tartrate resistant acid phosphatase (TRAP) activity using a histochemical kit (Sigma). Alkaline phosphatase activity and Goldner trichrome staining were performed as previously described [12,13]. Histomorphometric analysis was performed with OsteoMeasure version 2.2 software.

**Osteoclast culture and bone resorption activity assays**

Wild-type and OIP-1 transgenic mouse bone marrow cells were cultured to form OCLs as previously described [6]. Bone marrow-derived non-adherent cells (1.3 × 10^6 per ml) were cultured in 24-well plates in the presence of mRANKL (5–100 ng/ml) and M-CSF (10 ng/ml) for 5 days. At the end of the culture period, the cells were fixed with 2% glutaraldehyde in PBS for 20 min and stained for TRAP activity. TRAP-positive multinucleated OCLs containing three or more nuclei were scored microscopically.

To determine the bone resorption activity, wild-type and OIP-1 transgenic mouse bone marrow cells treated with 10 ng/ml mM-CSF for 12 h, and non-adherent bone marrow mononuclear cells (1 × 10^6 cells per well) were cultured to form OCLs on sterile dentine slices for 10 days as previously described [6]. The cells were removed using 1 M NaOH and stained with 0.1% toluidine blue. The areas of resorption lacunae on the digital images were quantified. The percentage of resorbed area was calculated relative to the total dentine disc area.

Western blot analysis for RANK receptor signalling molecules in OIP-1 transgenic mouse-derived pre-osteoclasts and immunocytochemical staining of OIP-1 expression in osteoclast cells were performed using rat anti-mouse TSA-1/Sca MAb (PharMingen, CA, USA) [14].

**Osteoblast differentiation**

Wild-type and OIP-1 transgenic mouse bone marrow-derived stromal/pre-osteoblast cells were cultured with medium containing 10 mM glycerophosphate and 50 μg/ml ascorbic acid for an indicated period (0–12 days). The total RNA (2 μg) isolated was reverse-transcribed using random hexamers and Moloney murine leukaemia virus reverse transcriptase (Applied Biosystems, CA, USA). The resulting cDNAs were then amplified by PCR using osteoblast marker gene-specific primers for osteocalcin, alkaline phosphatase, collagen type I, and β-actin control primers as previously described [15].

**Statistical analysis**

Data are presented as mean and the statistical analysis between the wild-type and OIP-1 transgenic mice for a given parameter was established by Student’s t-test, with p < 0.05 considered statistically significant. Statistical analysis of skeletal parameters was also applied by one-way ANOVA.

**Results**

**OIP-1 mice show osteopetrotic bone phenotype**

We have previously identified and characterized the osteoclast inhibitory peptide-1 (OIP-1/hSca), which inhibits osteoclast formation and bone resorption activity in vitro; however, a role for OIP-1 in skeletal development is unknown [6]. To define a functional role for OIP-1 in bone remodelling, we developed transgenic mice in which OIP-1 expression is targeted to the osteoclast lineage using the mouse TRAP gene promoter as described in the Materials and methods section. OIP-1 transgenic mice that are heterozygous were born normal and fertile; however, there was no significant difference in overall body size compared with non-transgenic littermates. OIP-1 mice showed normal tooth eruption and no visible deformity, or in the growth of the skeleton, compared with wild-type mice. Interestingly, the radiographic analysis of OIP-1 transgenic mice showed an increased
radiodensity of the proximal humerus when compared with wild-type mice of 4-week-old littermates, indicating an osteopetrotic bone phenotype in OIP-1 mice (Figure 1D). To confirm the increased radiopacity in the humerus of OIP-1 mice, bone mineral density (BMD) and bone mineral content (BMC) were measured by DEXA. The humeral region of 4-week-old OIP-1 mice showed a significant increase in BMD (58.36%) and in BMC (79.41%) (Figure 1E). µCT analysis also demonstrated a significant increase in trabecular thickness, bone volume, and bone surface in the humeral region of OIP-1 mice (Figures 2A–2D) compared with wild-type littermates. Furthermore, µCT analysis of the vertebral region of OIP-1 mice showed an increased trabecular thickness, trabecular bone volume, and bone surface compared with wild-type mice (Figures 2E–2H).

To analyse the osteopetrotic bone phenotype further, we examined the humeri from the OIP-1 mice histologically. The altered growth zone at the proximal end in the humerus of 4-week-old OIP-1 transgenic mice is shown in Figure 3A. OIP-1 mice exhibited absence of primary and secondary spongiosa and inadequate formation of marrow spaces. Much of the epiphysis consisted of dense sclerotic cortical bone, suggesting defective bone resorption in OIP-1 mice. The solid block of bone below the growth plate obliterating the marrow cavity varied in severity in the majority (>85%) of OIP-1 mice at 3 to 4 weeks old. The growth plate region appeared distorted due to the decreased marrow space rather than a significant change in the height or premature closure. To clarify further that the osteopetrotic bone phenotype in OIP-1 mice was due to OIP-1 inhibition of OCL development, we evaluated the OCL numbers in histological specimens by staining for TRAP activity (Figure 3B). Histomorphometric analysis identified a significant decrease (46.16%) in the number of TRAP-positive OCLs in the humeri from OIP-1 mice compared with wild-type littermates (Figure 3C). However, the number of osteoblast cells per bone perimeter area was not affected in humeri from OIP-1 mice compared with wild-type mice (Figure 3D). In addition, calvaria from the OIP-1 mice demonstrated inhibition of TRAP-positive OCLs at the endosteal bone surfaces and underdeveloped bone marrow spaces compared with wild-type littermates (Figure 4A). Furthermore, as evident from the alkaline phosphatase activity staining, there was no significant change in osteoblastic activity on periosteal and endosteal bone surfaces (Figure 4B). Also, calvarial sections showed normal osteoid seams.

**Figure 2.** µCT analysis of bones from 4 week old wild-type (Wt) and OIP-1 mice. (A) µCT images showed increased bone mass in the humeral region of OIP-1 mice compared to wild-type littermates. (B) Quantification of trabecular thickness (TbTh). (C) trabecular bone volume (bone volume per tissue volume, BV/TV) and (D) bone surface/volume (BS/BV) in the humeral region of wild-type and OIP-1 mice. (E) µCT images of the lumbar vertebrae from OIP-1 mice compared to wild-type littermates. (F) Quantification of trabecular thickness (TbTh). (G) trabecular bone volume (BV/TV) and (H) bone surface/volume (BS/BV) in the vertebral region of wild-type and OIP-1 mice. Values represent mean ± SD (n = 5; p < 0.05)
OIP-1/hSca is a novel physiological inhibitor of OCL development and bone resorption in vivo

Figure 3. Histological and immunohistochemical analysis of humeri from wild-type (Wt) and OIP-1 mice. (A) Haematoxylin and eosin staining of the growth zone of the proximal end of the humerus of OIP-1 and Wt mice. OIP-1 mice show the osteopetrotic-like bone phenotype with inadequate formation of marrow spaces (as shown by an arrow). Original magnification: ×20. (B) Histochemical staining for TRAP-positive OCLs present at the growth plate region of humeri from Wt and OIP-1 mice (4 weeks old). The TRAP(+) OCLs are indicated by arrows. Original magnification: ×200. (C) Histomorphometric analysis of the number of osteoclasts/bone perimeter (N.OCL/B.Pm/100 mm) and (D) the number of osteoblast/bone perimeter (N.OB/B.Pm/100 mm) in humeri of Wt and OIP-1 mice; values represent mean ± SD (n = 5; p < 0.05).

Figure 4. Histological analysis of wild-type (Wt) and OIP-1 transgenic mouse calvaria. (A) Wt mice show TRAP(+) OCLs on the endosteal bone surface of marrow cavities, as indicated by arrows. OIP-1 mouse calvaria showed absence of TRAP(+) OCLs and underdeveloped bone marrow spaces (n = 10). (B) Calvaria stained for alkaline phosphatase activity. (C) Goldner’s trichrome staining shows osteoid seams on periosteal and endosteal bone surfaces. Original magnification: ×20. (D) Semi-quantitative RT-PCR analysis of osteoblast marker genes, alkaline phosphatase (ALP), collagen type IA (COL IA), and osteocalcin (OC) expression levels in Wt and OIP-1 mouse bone marrow-derived osteoblast cultures. Total RNA isolated from the Wt and OIP-1 mouse bone marrow-derived stromal/pre-osteoblast cells cultured with osteogenic medium for the period indicated (0–12 days) was subjected to RT-PCR analysis for osteoblast differentiation markers as described in the Materials and methods section. ELISA analysis of serum (E) osteocalcin and (F) TRACP5b levels in OIP-1 and Wt mice as noted in the Materials and methods section.
on the periosteal and endosteal bone surfaces of both OIP-1 and wild-type mice (Figure 4C).

RT-PCR analysis of the total RNA isolated from osteoblast cells derived from the OIP-1 mouse bone marrow cultures did not show a significant difference in the expression levels of osteoblast differentiation marker genes such as alkaline phosphatase, type I collagen, and osteocalcin gene expression compared with wild-type littermates (Figure 4D). Furthermore, ELISA analysis demonstrated no significant change in the serum osteocalcin levels in OIP-1 mice (Figure 4E). However, the osteoclast activity marker, serum TRACP5b, was significantly reduced in the OIP-1 transgenic mice, compared with wild-type littermates (Figure 4F). Collectively, these results indicate an osteopetrotic bone phenotype in OIP-1 mice due to inhibition of OCL formation/activity in vivo, and that OIP-1 does not modulate bone formation.

**Inhibition of osteoclastogenesis in OIP-1 mouse bone marrow cultures**

We next performed ex vivo experiments using bone marrow cells isolated from the OIP-1 and control mice to assess OIP-1 inhibition of OCL formation. Non-adherent bone marrow cells derived from the wild-type and OIP-1 mice were cultured in the presence of hGM-CSF (10 ng/ml) in methyl cellulose (1.2%) to form CFU-GM colonies. As shown in Figure 5A, OIP-1 transgenic mouse line 5 and 13-derived cells showed a significant decrease in the number of CFU-GM colonies, by 35% and 41% respectively, compared with control mice. We further examined the capacity for OCL differentiation and bone resorption by OIP-1 transgenic mouse-derived bone marrow cells. OIP-1 mouse and wild-type mouse derived non-adherent bone marrow cells were stimulated with various concentrations of RANKL (5–100 ng/ml) with M-CSF (10 ng/ml) and RANKL (100 ng/ml) for 48 h. Western blot analysis of total cell lysates obtained from these pre-osteoclast cells identified no significant changes in the levels of RANK receptor expression. In contrast, RANK adaptor protein TRAF-2 expression, but not TRAF-6, was significantly decreased (ten-fold) in OIP-1 transgenic mouse-derived cells compared with wild-type mice. OIP-1 mouse-derived pre-osteoclasts also demonstrated a three-fold decrease in the levels of c-Fos and NFATc1 (Figure 6A).

We further examined the activation status of extra-cellular signal-regulated kinases (ERK) and c-Jun N-terminal kinase (JNK) molecules in response to RANKL stimulation of OIP-1 mouse bone marrow cells. As shown in Figure 6B, OIP-1 mouse-derived pre-osteoclast cells showed a time-dependent inhibition of ERK phosphorylation in response to RANKL stimulation, compared with wild-type mice. Similarly, JNK activation was significantly decreased in OIP-1 mouse pre-osteoclast cells compared with wild-type mice (Figure 6C). Taken together, OIP-1 inhibits OCL formation and bone resorption activity through the suppression of RANK receptor signalling molecules. These results further support the osteopetrotic bone phenotype in OIP-1 transgenic mice.

**Discussion**

We have previously reported on OIP-1/hSca as an autocrine/paracrine inhibitor of OCL formation and bone resorption activity [3,6]. Expression of OIP-1 mRNA in early and more committed OCL cell lineages and osteoblasts [17] suggested that OIP-1/hSca may play an important role in OCL formation. Immune cell products such as interferon (IFN)-γ are potent inhibitors of OCL formation. We have previously shown that IFN-γ stimulates OIP-1/hSca expression in OCL precursor cells [14]. It is more likely, therefore, that OIP-1 is an important physiological regulator of osteoclast development and bone resorption activity in vivo. Previously it has been shown significant inhibition of osteoclastogenesis and bone resorption activity in vivo.

**RANK receptor signalling in pre-osteoclast cells from OIP-1 mice**

RANKL–RANK signalling plays a critical role in OCL differentiation and bone resorption activity. We have also demonstrated that OIP-1 c-peptide treatment of RAW 264.7 mouse macrophage cells inhibits OCL formation through the suppression of molecules associated with RANKL–RANK signalling during OCL differentiation [14]. We therefore further examined the status of RANK receptor signalling molecules such as TRAF-2, ERK, JNK, c-Fos, and NFATc1 which are responsive to RANKL stimulation of OIP-1 mouse bone marrow cultures [16]. Non-adherent bone marrow cells derived from the wild-type and OIP-1 transgenic mice were stimulated with M-CSF (10 ng/ml) and RANKL (100 ng/ml) for 48 h. Western blot analysis of total cell lysates obtained from these pre-osteoclast cells identified no significant changes in the levels of RANK receptor expression. In contrast, RANK adaptor protein TRAF-2 expression, but not TRAF-6, was significantly decreased (ten-fold) in OIP-1 transgenic mouse-derived cells compared with wild-type mice. OIP-1 mouse-derived pre-osteoclasts also demonstrated a three-fold decrease in the levels of c-Fos and NFATc1 (Figure 6A).

We further examined the activation status of extra-cellular signal-regulated kinases (ERK) and c-Jun N-terminal kinase (JNK) molecules in response to RANKL stimulation of OIP-1 mouse bone marrow cells. As shown in Figure 6B, OIP-1 mouse-derived pre-osteoclast cells showed a time-dependent inhibition of ERK phosphorylation in response to RANKL stimulation, compared with wild-type mice. Similarly, JNK activation was significantly decreased in OIP-1 mouse pre-osteoclast cells compared with wild-type mice (Figure 6C). Taken together, OIP-1 inhibits OCL formation and bone resorption activity through the suppression of RANK receptor signalling molecules. These results further support the osteopetrotic bone phenotype in OIP-1 transgenic mice.
OIP-1/hSca is a novel physiological inhibitor of OCL development and bone resorption in vivo

Figure 5. Inhibition of osteoclastogenesis in OIP-1 mouse bone marrow cultures. (A) CFU-GM formation in OIP-1 mouse bone marrow cultures. Wild-type (Wt) and OIP-1 mouse derived non-adherent bone marrow cells (4 × 10^5/ml) were cultured with hGM-CSF (10 ng/ml) in 1.2% methyl cellulose to form CFU-GM colonies. At the end of a 7 day culture period, CFU-GM colonies (aggregates > 50 cells) formed in these cultures were scored using a light microscope as described earlier [7]. (B) Wild-type (Wt) and OIP-1 transgenic mouse bone marrow derived non-adherent cells were stimulated with RANKL (5–100 ng/ml) and M-CSF (10 ng/ml) for 5 days and the TRAP (+) multinucleated OCLs formed in these cultures were scored. The results represent quadruplicate cultures of three independent experiments (p < 0.05). (C) Immune staining for OIP-1/hSca expression in OCL formed in Wt and OIP-1 mouse bone marrow cultures was performed using rat anti-mouse TSA-1/Sca-2 antibody at a concentration of 1 µg/ml. Photomicrographs were taken at magnification ×20. (D) Wt and OIP-1 mouse bone marrow derived non-adherent cells (1 × 10^6) were cultured to form OCL as described on dentine slices for 10 days. The cells were removed by treating the disc with 1 M NaOH and stained with 0.1% toluidine blue. Resorption lacunae formed on dentine slices were identified by light microscopy. (E) The percentage of resorbed area on dentine was quantified as described in the methods. The results represent quadruplicate cultures of three independent experiments (p < 0.05).

that c-Src, a proto-oncogene deficiency, causes severe osteopetrosis in mice which lacks tooth eruption. The OCL development is normal in Src-deficient mice; however, the OCLs cannot form ruffled borders to resorb bone [18]. In contrast, OIP-1 mice show normal tooth eruption and no significant difference in body size. However, μCT analysis indicated an osteopetrosis bone phenotype in OIP-1 mice. Also, DEXA analysis showed a significant increase in BMD and BMC in the humeri from OIP-1 mice. Recently it has been reported that mice lacking the immunomodulatory adapter proteins DAP12 and Fc receptor gamma chain (FcγR) exhibit severe osteopetrosis; however, they develop teeth, distinguishing their phenotype from Src−/− and RANKL-deficient mice [19]. Histological analysis indicated inadequate formation of marrow spaces in calvaria and humeri of OIP-1 mice. Histomorphometric analysis of humeri and...
Figure 6. Western blot analysis of RANK receptor associated signaling molecules during OCL differentiation. (A) Wild-type (Wt) and OIP-1 mouse derived bone marrow cells were cultured in the presence of 10 ng/ml M-CSF for 24 h. The non-adherent cells were treated with M-CSF (10 ng/ml) and stimulated with or without RANKL (100 ng/ml) for 2 days. Total cell lysates prepared from the preosteoclast cells were subjected to Western blot analysis for TRAF2, TRAF6, c-Fos and NFATc1. β-actin expression levels were also analyzed to normalize the protein loading onto the gels in all the samples. (B) The preosteoclast cells were incubated with RANKL (100 ng/ml) for an indicated period and expression of pERK was determined. (C) JNK activity in the preosteoclast cells derived from Wt and OIP-1 mice further demonstrated a significant decrease in OCL development. In contrast, femurs from OIP-1 mice had no significant change in the numbers of TRAP-positive osteoclasts; however, they demonstrated a significant change in bone volume (data not shown). These results indicate OIP-1-specific inhibition of osteoclast formation/activity in vivo. Furthermore, our results that the number of osteoblasts per bone perimeter area was not affected in OIP-1 mice compared with control mice and that there was a lack of significant differences in the levels of serum osteocalcin and osteoblast-specific gene expression in OIP-1 mice compared with wild-type mice suggest that bone formation is not affected in these mice.

Mouse bone marrow culture studies ex vivo further suggested that the osteopetrotic bone phenotype is due to a significant decrease in OCL number and activity compared with control mice. These results are consistent using bone marrow cells derived from two of the founder lines established with comparable levels of OIP-1 expression. It is possible that genomic integration of the OIP-1 transgene may influence the severity of the osteopetrotic bone phenotype in these mice. We have further confirmed OIP-1-specific inhibition of OCL differentiation of RAW cells in vitro through constitutive overexpression (data not shown). Our results are therefore consistent with an osteopetrotic phenotype due to inhibition of osteoclastogenesis and bone resorption activity in OIP-1 mice. Previously we demonstrated that OIP-1 significantly decreased RANKL-induced JNK activity in RAW 264.7 cells. Furthermore, OIP-1 decreased TRAF-2 expression in pre-osteoclast cells, but had no significant effect on TRAF-6 and RANK expression in these cells [14]. These data suggest that targeted overexpression of OIP-1 to the OCL lineage in vivo results in inhibition of OCL differentiation through suppression of TRAF-2 and JNK activity. Although TRAF-2 is essential for TNF-α-induced osteoclastogenesis and TRAF-5 functions in both RANKL and TNF-α-induced osteoclastogenesis [20,21], our results indicate that OIP-1 suppresses RANK receptor signalling through TRAF-2, but not TRAF-6, to inhibit OCL differentiation. It is possible that TRAF-2 interaction with other signalling molecules may play an important role in downstream signalling that is necessary to activate JNK activity during OCL differentiation. ERK has been implicated in cellular proliferation, migration, and survival [22]. Thus, inhibition of ERK activation in OIP-1 mouse-derived pre-osteoclast
cells further supports our results indicating a significant decrease in the growth of early OCL precursors, CFU-GM. NFATc1 is a critical transcription factor that modulates OCL-specific genes such as the calcitonin receptor and TRAP expression during OCL differentiation [23–25]. OIP-1 mouse-derived pre-osteoclast cells demonstrated a significant decrease in the level of NFATc1 expression in response to RANKL stimulation compared with wild-type mice. It is therefore possible that NFATc1 may play a critical role in OIP-1 function as a downstream effector to suppress gene expression during OCL differentiation.

In summary, OIP-1/hSca represents a novel physiological inhibitor of osteoclast development and bone resorption in vivo. Thus, OIP-1 may have therapeutic utility for bone disease with high bone turnover, such as osteoporosis and Paget’s disease of bone.

Acknowledgements

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References

Mutation of the sequestosome 1 (p62) gene increases osteoclastogenesis but does not induce Paget disease

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Paget disease is the most exaggerated example of abnormal bone remodeling, with the primary cellular abnormality in the osteoclast. Mutations in the p62 (sequestosome 1) gene occur in one-third of patients with familial Paget disease and in a majority of patients with sporadic Paget disease, with the P392L amino acid substitution being the most commonly observed mutation. However, it is unknown how p62P392L mutation contributes to the development of this disease. To determine the effects of p62P392L expression on osteoclasts in vitro and in vivo, we introduced either the p62P392L or WT p62 gene into normal osteoclast precursors and targeted p62P392L expression to the osteoclast lineage in transgenic mice. p62P392L-transduced osteoclast precursors were hyper-responsive to receptor activator of NF-κB ligand (RANKL) and TNF-α and showed increased NF-κB signaling but did not demonstrate increased 1,25-(OH)2D3 responsiveness, TAFII-17 expression, or nuclear number per osteoclast. Mice expressing p62P392L developed increased osteoclast numbers and progressive bone loss, but osteoblast numbers were not coordinately increased, as is seen in Paget disease. These results indicate that p62P392L expression on osteoclasts is not sufficient to induce the full pagetic phenotype but suggest that p62 mutations cause a predisposition to the development of Paget disease by increasing the sensitivity of osteoclast precursors to osteoclastogenic cytokines.

Introduction

Paget disease (PD) is the second most common bone disease in persons of Anglo-Saxon descent over the age of 55 (1). It is the most exaggerated example of disordered bone remodeling, with abnormalities in all phases of the bone remodeling process (2). The primary cellular abnormality resides in the osteoclast (OCL). OCLs in PD are increased in number and size and have increased numbers of nuclei (3). In addition, they are hyperresponsive to 1,25-(OH)2D3 and receptor activator of NF-κB ligand (RANKL) (4, 5) and show increased expression of TAFII-17, a member of the TFIID transcription complex, which acts as a coactivator of vitamin D receptor-mediated transcription and is upregulated in pagetic osteoclasts (6).

Both genetic and environmental factors have been proposed as contributing to the etiology of PD, and multiple families with an autosomal dominant mode of inheritance have been described (7, 8). Recently, mutations in the p62 (sequestosome 1) gene have been linked to approximately 30% of patients with familial PD and to a minority of patients with sporadic PD (8). All of the mutations identified to date lie within or near the ubiquitin-binding domain in the carboxyterminal region of the protein, with the P392L amino acid substitution representing the most frequently observed mutation (8). p62 plays a critical role in NF-κB activation induced by TNF-α, CD40, and IL-1 through its interactions with the atypical protein kinases ζPKC and ζPKC (9, 10). However, the role of p62 mutations in PD is unclear since not all individuals carrying a p62 mutation have PD (11–13).

It is our hypothesis that both genetic and nongenetic factors are required for the development of PD and that the genetic factors, such as p62 mutations, function to increase OCL formation but are not sufficient to induce the abnormal OCLs or pagetic bone lesions characteristic of PD. To test this hypothesis, we characterized OCL precursors from Paget patients carrying the p62P392L mutation as well as control human OCL precursors transduced with either p62 or p62P392L expression vectors. We also determined whether the p62P392L gene can induce pagetic-like OCLs or bone lesions in vivo when expressed in OCL precursors of transgenic mice.

Results

OCL precursors from PD patients carrying the p62P392L gene are hyper-responsive to RANKL, TNF-α, and 1,25-(OH)2D3 and express increased levels of TAFII-17. OCL precursors from PD patients carrying the p62P392L mutation or from controls were compared for their capacity to form OCLs over a range of concentrations of RANKL, TNF-α, and 1,25-(OH)2D3. OCL precursors from patients carry-
The p62P392L mutation was detected in GM-CFU without treatment with RANKL. In contrast, cells with a monoclonal antibody that recognizes both WT and mutant p62 by immunoblotting extracts from transduced GM-CFU–derived empty vector normal human OCL precursors with the WT presence of p62 WT or p62P392L transduced cells were much more responsive to TNF-α than WT p62 cells. Both p62- and p62P392L-transduced OCL precursors formed significantly larger OCLs than EV cells (Figure 2D), although nuclear number per OCL was not increased in p62- or p62P392L-derived OCLs regardless of treatment (Table 1). Also, a 2-fold increase in bone resorption was observed when OCLs formed by p62P392L-transduced human GM-CFU were treated with RANKL (50 ng/ml) and cultured on dentin, as compared with RANKL-treated EV-transduced OCLs (Figure 3, A and B).

In contrast with the results with RANKL and TNF-α, neither p62- nor p62P392L-transduced OCL precursors were hyperresponsive to 1,25-(OH)2D3 (Figure 2C) or formed increased numbers of OCLs compared with EV-transduced cells. In addition, neither p62- nor p62P392L-transduced GM-CFU–derived cells showed elevated expression of TAFII-17 in the presence or absence of 10–10 M 1,25-(OH)2D3 (Figure 4).

Effects of p62P392L expression on OCLs in vivo. Since OCLs harbor the primary cellular abnormality in PD, we used the tartrate-resistant acid phosphatase (TRAP) promoter to target expression of the human p62P392L gene to the OCL lineage in transgenic mice (TRAP-p62P392L mice) to determine the effects of p62P392L expression in OCLs in vivo. Eight lines of TRAP-p62P392L transgenic mice were generated from independent founder mice, and each of these was characterized with regard to transgene expression level, TNF-α responsiveness, and histology. The results described below were obtained from a single line (Trp62m2), although similar results were observed in multiple other lines as well. The expression levels of the total p62 protein in mice of the Trp62m2 line, as determined by immunoblotting of extracts from bone marrow cells with an antibody that detects both murine and human p62, were found to be approximately 2.5-fold higher than p62 levels in WT mice (data not shown).

Histomorphometric evaluation of vertebral bones from TRAP-p62P392L mice at 4, 8, 12, and 16 months of age revealed an increase in OCL perimeter (the amount of bone surface covered with TRAP-positive, mono-, and multinuclear cells) and a progressive reduction in cancellous bone volume when compared with that of age-matched WT controls (Table 2 and Supplemental Figure 1; supplemental material available online with this article).
The decrease in cancellous bone volume was associated with decreases in both trabecular width and number. Although OCL perimeter was elevated, there was no coupled increase in osteoblast perimeter, as is seen in PD lesions.

Electron microscopic examination of OCLs from TRAP-p62P392L mice demonstrated that the cells did not contain the nuclear inclusions characteristic of pagetic OCLs. OCLs were similar to those in WT controls in terms of nuclear and cytoplasmic ultrastructure as well in the morphology of the ruffled border (data not shown).

OCL precursors from TRAP-p62P392L mice are hyperresponsive to RANKL and TNF-α but not 1,25-(OH)2D3. When marrow cells from TRAP-p62P392L mice were cultured with RANKL and TNF-α to induce OCL formation, they were found to be hyperresponsive to both cytokines, and they formed increased numbers of OCLs as compared with nontransgenic littermates (Figure 5, A and B). Similarly, treatment of TRAP-p62P392L mice with TNF-α (0–1.5 μg/d) significantly increased OCL formation compared with that of WT mice at all concentrations tested (Figure 5, E and G). Further, the dose-response curves for NF-κB reporter gene activity in TRAP-p62P392L OCL precursors for both RANKL and TNF-α were shifted to the left compared with cells from WT mice (Figure 5, D and E). However, OCL precursors from TRAP-p62P392L mice were not hyperresponsive to 1,25-(OH)2D3 (Figure 5C) and did not express detectable TAFII-17 (data not shown). They also did not have increased nuclear number per OCL (Table 1). These results are consistent with our results from p62P392L-transduced human OCL precursors and indicate that expression of p62P392L induces a subset of pagetic characteristics, including hyperresponsivity to the osteoclastogenic cytokines RANKL and TNF-α, but does not result in development of OCLs that express the complete pagetic phenotype or development of pagetic-like lesions.

To further examine the mechanisms responsible for the increased levels of OCL formation in marrow cultures from TRAP-p62P392L mice, we determined the time course for OCL formation, the rates of proliferation of OCL precursors and of OCL apoptosis, and the expression levels of OCL differentiation markers. As shown in Figure 6A, OCL formation in marrow cultures from TRAP-p62P392L

Table 1
Nuclear number per OCL

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Vehicle</th>
<th>1,25-(OH)2D3 (10⁻⁸ M)</th>
<th>RANKL (50 ng/ml)</th>
<th>TNF-α (50 pg/ml)</th>
</tr>
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<tbody>
<tr>
<td>Control human</td>
<td>10 ± 3</td>
<td>13 ± 2</td>
<td>12 ± 2</td>
<td>13 ± 2</td>
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<tr>
<td>Familial PD</td>
<td>24 ± 3</td>
<td>52 ± 13⁴</td>
<td>32 ± 5⁴</td>
<td>25 ± 4⁴</td>
</tr>
<tr>
<td>Human GM-CFU-EV</td>
<td>6 ± 3</td>
<td>7 ± 2</td>
<td>12 ± 5</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>Human GM-CFU-p62P392L</td>
<td>5 ± 3</td>
<td>7 ± 2</td>
<td>10 ± 2</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>WT mice</td>
<td>5 ± 3</td>
<td>6 ± 2</td>
<td>9 ± 2</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>TRAP-p62P392L mice</td>
<td>5 ± 3</td>
<td>6 ± 2</td>
<td>9 ± 2</td>
<td>5 ± 2</td>
</tr>
</tbody>
</table>

The number of nuclei per OCL was determined in 20 random 23c6-positive or TRAP-positive OCLs for each treatment group. Results are expressed as mean ± S.D. *Significantly different from the same treatment as the normal donor, P < 0.01.

Figure 2
OCL formation by p62- and p62P392L-transduced human OCL precursors. GM-CFU–derived cells (10⁵ cells/well) transduced with p62, p62P392L, or EV were cultured with RANKL (A), TNF-α (B), or 1,25-(OH)2D3 (C). After 3 weeks of culture, cells were fixed and stained with the 23c6 monoclonal antibody, which identifies OCLs. Results are expressed as the mean ± SEM for quadruplicate cultures from a typical experiment. *Significant differences (P < 0.001) compared with results of EV-transduced cell cultures treated with the same concentration of individual factors. A similar pattern of results was seen in 3 independent experiments. (D) Morphology of OCLs formed by p62P392L- and EV-transduced GM-CFU–derived cells. Original magnification, ×100.
mice was maximum after 6 days of culture while OCL formation in WT cultures was maximum at 9 days of culture. Further, OCL precursor proliferation was significantly increased in marrow cultures from p62P392L mice compared with WT cultures but followed a similar time course (Figure 6B). In contrast, although the number of apoptotic OCLs was increased in TRAP-p62P392L marrow cultures compared with WT marrow cultures, the percentages of apoptotic OCLs were similar (18% versus 10%; P > 0.05) in TRAP-p62P392L and WT cultures after 9 days (Figure 6C). Apoptotic OCLs were not detected in cultures of mice from either genotype at day 3 or 6 of culture. Marrow cultures from TRAP-p62P392L mice expressed relatively higher levels of TRAP, cathepsin K, and calcitonin receptor mRNA compared with WT cultures (Figure 7).

Since OCL precursors from Paget patients carrying the P392L mutation were hyperresponsive to 1,25-(OH)2D3 and also expressed MVNP, we transduced normal human OCL precursors with MVNP and determined their responsivity to 1,25-(OH)2D3. As shown in Figure 8, OCL precursors from TRAP-p62P392L mice transfected with MVNP were hyperresponsive to 1,25-(OH)2D3 and formed OCL at concentrations that were 1 to 2 logs lower than those of EV-transfected cells.

To further delineate the mechanisms responsible for the enhanced OCL formation in TRAP-p62P392L mice, we examined inhibitor of NF-κB (IκB), p38 MAPK, and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling in nonadherent marrow cells from WT and TRAP-p62P392L mice. As shown in Supplemental Figure 2A, p-ERK1/2 was increased in marrow cells treated with RANKL or TNF-α while only modest changes were seen in p38 MAPK and p-IκB activity. Transfection of GM-CFU from WT and TRAP-p62P392L mice with the MVNP gene further increased levels of NF-κB in TRAP-p62P392L. OCL precursors compared with those of WT precursors (Supplemental Figure 2B). Expression of MVNP in OCL precursors from WT or TRAP-p62P392L mice did not increase expression of c-Fos.

Discussion

Mutations in the p62 gene have been linked to PD in approximately one-third of patients with familial PD and a minority of patients with sporadic PD. However, it is unlikely that these mutations are sufficient to induce the OCL abnormalities and bone lesions that are characteristic of PD, since pagetic lesions are focal even in patients carrying germline p62 mutations and some individuals harboring p62 mutations fail to develop PD (11–13). To determine the role of p62 mutation in PD, we characterized OCL precursors from familial PD patients carrying the most common PD-associated p62 mutation (P392L) and compared them with normal OCL precursors. OCL precursors from p62P392L-positive PD patients formed OCLs at lower concentrations of RANKL, TNF-α, and 1,25-(OH)2D3 than controls (Figure 1, A–C) and had increased TAFII-17 expression (Figure 1D), all characteristic of PD; this was similar to our previous findings in OCL precursors from patients with sporadic PD (3–6). However, OCL precursors from these patients also expressed MVNP, which we have previously reported results in increased OCL formation in response to RANKL and 1,25-(OH)2D3 and to increased expression of TAFII-17 both in vitro and in vivo (6, 14).

To further characterize the contributions of p62P392L to PD in the absence of MVNP, we transduced normal human OCL precursors with vectors encoding WT p62 or p62P392L or with EV. As with OCL precursors from PD patients, p62P392L-transduced OCL precursors showed increased TNF-α and RANKL sensitivity (Figure 2, A and B) but, in contrast, did not demonstrate increased 1,25-(OH)2D3 responsiveness (Figure 2C) or increased expression of TAFII-17 (Figure 4). In addition, nuclear number per OCL was not increased in p62P392L-transduced OCLs (Table 1). Thus, these OCLs express only a subset of the characteristics of pagetic OCLs (15). Similar results were obtained when expression of the p62P392L gene was targeted to cells in the OCL lineage in vivo. OCL precursors from TRAP-p62P392L mice were hyperresponsive to RANKL and TNF-α but not to 1,25-(OH)2D3 (Figure 5, A–C) and did not express increased levels of TAFII-17. Further, OCL formation in TRAP-p62P392L mice was significantly increased in vivo by treatment with TNF-α (Figure 5, F and G).

Both RANKL and TNF-α activate signaling pathways involving p62 that ultimately lead to the activation of NF-κB, p38 MAPK, and ERK1/2, which are important for OCL formation and function.
Our results demonstrate elevated NF-κB activation as well as p38 MAPK and ERK1/2 signaling in OCLs expressing p62P392L, strongly suggesting that Paget-associated mutations in p62 lead to increased osteoclastogenesis by stimulating signaling pathways that activate NF-κB (Supplemental Figure 2A). However, the detailed mechanisms by which these p62 mutations activate signaling remain to be determined. All of the PD-associated p62 mutations reside in or near the ubiquitin-binding domain and result in loss of the ubiquitin-binding capacity of p62 (16). Thus, it is possible that the ubiquitin-binding domain of p62 normally mediates a protein/protein interaction that dampens NF-κB signaling in OCLs in response to inflammatory cytokines, such that loss of this interaction leads to increased activation of these pathways.

It is interesting to note that in both transduced human OCL precursors and in transgenic mouse OCL precursors, expression of p62P392L had a much more dramatic effect on responsivity to TNF-α than to RANKL. Consistent with our results, Duran et al. have reported that the P392L mutation in p62 increased NF-κB reporter activity (17). These results suggest that TNF-α, in addition to RANKL and 1,25-(OH)2D3, may be involved in the increased osteoclastogenesis in PD and should be studied further.

Histomorphometric analysis of vertebral cancellous bone in the TRAP-p62P392L transgenic mice revealed a phenotype that was characterized by low bone volume with reduced trabecular number and width. OCL perimeter was increased at all ages examined, but there was no coupled increase in osteoblast perimeter. This marked imbalance between OCL formation and new bone formation is analogous to imbalance in inflammatory diseases of bone, such as rheumatoid arthritis and lytic bone metastases, in which bone formation is suppressed, rather than PD, in which OCL activity is closely coupled to new bone formation. This phenotype is very different from that observed in TRAP-MVNP transgenic mice, which persistently express the gene encoding the MVNP (14). In contrast with TRAP-p62P392L mice, TRAP-MVNP mice displayed a bone phenotype that closely resembled PD in humans. This included a coupled increase in both bone resorption and new bone formation and enlarged OCLs with increased nuclear number (14). Moreover, a subset (30%) of 12-month-old TRAP-MVNP mice displayed pagetic-like lesions, with increased bone volume and dramatically thickened and disorganized trabecular composing primarily of woven bone. No such lesions were observed in the TRAP-p62P392L mice, which were examined up until 18 months of age. In addition, OCL precursors from the TRAP-MVNP mice showed increased sensitivity to 1,25(OH)2D3 and expressed increased levels of TAFII-17, findings that were not observed in the TRAP-p62P392L mice unless the cells were transfected with MVNP (Figure 8). Finally, transfection of OCL precursors from TRAP-p62P392L mice with MVNP further increased levels of NF-κB, suggesting that MVNP can increase the enhanced OCL formation induced by the p62P392L mutation (Supplemental Figure 2B).

One possible explanation for the failure of the TRAP-p62P392L mice to display increased osteoblast activity is that expression of p62P392L is restricted to cells of the OCL lineage in this model while familial PD patients carrying a p62 mutation express the mutant protein in all cell types. It remains to be determined whether the p62 mutation plays a direct role in other cell types besides OCL in PD. It will therefore be of interest to evaluate the bone phenotype in knockin mice carrying the analogous p62 mutation in the germ-line, which are currently being developed.

Taken together, these results demonstrate that the expression of p62P392L in OCLs increases OCL formation but is not sufficient to induce PD. Thus, p62 mutations may predispose to the development of PD by increasing basal OCL activity, but 1 or more additional factors are required for development of the full PD phenotype.

### Table 2

<table>
<thead>
<tr>
<th>Variable</th>
<th>WT 4 mo</th>
<th>p62 4 mo</th>
<th>WT 8 mo</th>
<th>p62 8 mo</th>
<th>WT 12 mo</th>
<th>p62 12 mo</th>
<th>WT 16 mo</th>
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<td>BV/TV (%)</td>
<td>19.1±0.8</td>
<td>15.4±1.3</td>
<td>15.3±1.0</td>
<td>13.6±0.7</td>
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<td>Tb.Wi (μm)</td>
<td>36.6±1.0</td>
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<td>32.9±0.7</td>
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<td>35.3±3.7</td>
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<td>Tb.N (per mm²)</td>
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<td>4.4±0.4</td>
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<td>Tb.Sp (μm)</td>
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<tr>
<td>Oc.Pm (%)</td>
<td>19.4±0.8</td>
<td>22.7±1.7</td>
<td>18.0±0.9</td>
<td>20.8±2.0</td>
<td>17.8±0.7</td>
<td>27.4±3.9</td>
<td>16.8±1.7</td>
<td>20.6±2.3</td>
</tr>
<tr>
<td>Ob.Pm (%)</td>
<td>12.3±1.3</td>
<td>10.8±1.6</td>
<td>8.4±0.9</td>
<td>7.4±0.8</td>
<td>8.8±1.0</td>
<td>4.5±1.9</td>
<td>8.9±1.7</td>
<td>13.0±0.9</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. P62, TRAP-p62P392L mice; BV/TV, cancellous bone volume; Tb.Wi, trabecular width; Tb.N, trabecular number; Tb.Sp, trabecular separation; Oc.Pm, OCL perimeter; and Ob.Pm, osteoblast perimeter. Data were analyzed using 2-way ANOVA. Significant differences are indicated as follows: *P < 0.05 and **P < 0.01 versus WT; †P < 0.05 and ‡P < 0.01 versus 4-month group. A significant interaction (P < 0.05) between the factors of treatment (p62 or WT) and age (4, 8, 12, and 16 months) is noted in the variable of osteoblast perimeter.
was seen in 2 independent experiments. (Journal of Investigation Clinical (sense) and 5 specific primers for 5 were 5′-GTTGTTTTCCG-3′ and 5′-GAACCAGTTTGGCCCTTCA-3′ were cultured for 2 days and subjected to RT-PCR analysis for expression of scope by an observer without knowledge of the treatment group. 23c6-positive multinucleated cells were scored using an inverted microscope. Original magnification, ×100. (G) TRAP-positive OCL perimeter on the endosteal surface of calvaria in WT and TRAP-p62<sup>P392L</sup> mice. *Significant differences (P < 0.01) between TRAP-p62<sup>P392L</sup> and WT mice were found by 2-way ANOVA.

pooled colonies were cultured in the presence of RANKL, TNF-α, or 1,25-(OH)<sub>2</sub>D<sub>3</sub> to induce OCL formation as described below.

Long-term cultures for OCL formation. For experiments employing highly purified OCL precursors, GM-CFU–derived cells, prepared as described above, were cultured in 96-well plates in α-MEM containing 20% horse serum and varying concentrations of RANKL, TNF-α, or 1,25-(OH)<sub>2</sub>D<sub>3</sub>. Every 3 days, half the medium was replaced, and after 21 days of culture, the cells were fixed with 1% formaldehyde and tested using a VECTASTAIN ABC-AP kit (Vector Laboratories) for cross-reactivity with the monoclonal ECTASTAIN. The cells were fixed and stained for TRAP activity. Results are expressed as mean ± SEM for quadruplicate cultures from a typical experiment. *Significant differences (P < 0.001) compared with results with WT cultures. A similar pattern of results was seen in 2 independent experiments. (F) Six-month-old TRAP-p62<sup>P392L</sup> and WT mice were injected over the calvaria for 5 days with TNF-α (0.375 μg, and 1.5 μg/day). Calvaria were harvested and tissue sections stained for TRAP activity (red color). Original magnification, ×100. (G) TRAP-positive OCL perimeter on the endosteal surface of calvaria in WT and TRAP-p62<sup>P392L</sup> mice. *Significant differences (P < 0.01) between TRAP-p62<sup>P392L</sup> and WT mice were found by 2-way ANOVA.

Polymerase chain reaction amplification of RT-PCR. GM-CFU–derived cells were cultured for 2 days and subjected to RT-PCR analysis for expression of MVNP and TAFII-17. The gene-specific primers for human MVNP were 5′-CAGATTAT-GAACCAGTTTGGCCCTTCA-3′ (sense) and 5′-CCTGTGTTATTTCTTGG-GTTGTATTTCCTC-3′ (antisense). The gene-specific primers for human TAFII-17 were 5′-CATGCCATGCTATGAAACCAGTTTGGCCCTTCA-3′ (sense) and 5′-AACCATGCTATGAAACCAGTTTGGCCCTTCA-3′ (antisense). The gene-specific primers for β-actin were 5′-GGCCGGTACACTGGCATCCTGATG-3′ (sense) and 5′-CTTGGGCGTCAGGCAGCTCGTAGC-3′ (antisense). Conditions for amplification were as follows: 94°C for 5 minutes, 35 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, followed by extension at 72°C for 7 minutes. PCR products were separated by 2% agarose gel electrophoresis and were visualized by ethidium bromide staining with ultraviolet light illumination (6).

Production of WT p62 and p62<sup>P392L</sup> vectors. A plasmid containing the full-length human p62 cDNA was kindly provided by J. Moscat (University of Cincinnati, Cincinnati, Ohio, USA), and the P392L mutation (a C-to-T transition) was introduced by PCR-based site-directed mutagenesis. The mutagenized cDNA was fully sequenced to verify correct introduction of the P392L mutation. Retroviral constructs containing the p62 or p62<sup>P392L</sup> cDNAs under the control of the CMV promoter were prepared and transfected into normal human OCL precursors as previously described (15). In brief, the p62 or p62<sup>P392L</sup> cDNAs were inserted into the XhoI site of the p-LXSN retroviral vector, and the recombinant plasmid constructs were transfected into the PT67 amphotropic packaging cell line using calcium phosphate. Stable cloned cell lines producing recombinant retrovirus at 10<sup>8</sup> virus particles/ml were established by selecting for resistance to neomycin (600 μg/ml). Similarly, a control retrovirus producer cell line was also established by transfecting the cells with the p-LXSN.EV. Producer cell lines were maintained in DMEM containing 10% FBS, 100 U/ml streptomycin/penicillin, 4 mM l-glutamine,
and high glucose (4.5 g/l). Retroviral supernatants from the producer cell cultures were collected and filtered (0.45 μm) for immediate use. The retrovirus stocks were demonstrated to be helper free by a marker assay. Viral titers present in the culture supernatants were determined by testing for multiplicity of infection with serial dilutions of the supernatants on NIH3T3 cells and scoring the number of G418-resistant CFUs formed (following exposure to 250 μg/ml G418) as described (15).

Overexpression of p62 and p62 P392L in normal human OCL precursors. After obtaining informed consent, we obtained bone marrow aspirates from normal volunteers as previously described (15). These studies were approved by the Institutional Review Board at the University of Pittsburgh. Human bone marrow mononuclear cells were cultured for 2 days in α-MEM containing 10% FBS and 10 ng/ml each of IL-3, IL-6, and SCF. The bone marrow cells were cultured for an additional 48 hours with varying amounts of viral supernatant (1–10% v/v) containing the p62 or p62P392L vectors or EV. Cultures were supplemented with 4 μg/ml of polybrene, 20 ng/ml of IL-3, 50 ng/ml of IL-6, and 100 ng/ml of SCF. We previously determined that this was the optimum cytokine combination that supported the highest transduction efficiency. After 24 hours, cells were centrifuged, spent supernatant was removed, and freshly prepared viral supernatant supplemented with 4 μg/ml of polybrene and growth factors was added; then the cultures were continued for an additional 24 hours. After 48 hours, cells were harvested for short-term GM-CFU assays in methylcellulose as previously described (15, 19), and an aliquot of the cells was evaluated for p62 expression by immunostaining with an anti-p62 monoclonal antibody (BD Biosciences).

Bone resorption assays. GM-CFU–derived cells transduced with p62P392L or EV (105 cells/well) were cultured with RANKL (50 ng/ml) or TNF-α (100 pg/ml) on mammoth dentin slices (Wako). After 3 weeks of culture, cells were removed, dentin slices were stained with acid hematoxylin, and areas of dentin resorption were determined using image analysis techniques, as previously described (20).

Construction of the TRAP-p62 P392L hybrid transgene. We have previously described construction of the p-BSmTRAP5′ plasmid, which contains 1294 bp of the 5′ flanking sequence as well as the entire 5′ untranslated region of the murine TRAP gene (21). The full-length human p62P392L cDNA described above was inserted into the unique EcoRI site of p-B5pKCR3 (21), which contains part of the second exon, the second intron, and the third exon, including the polyadenylation site of the rabbit β-globin gene. There are
no AUG initiation codons within the β-globin sequences upstream of the cDNA insertion site, so translation of the p62P392L protein starts at the normal p62 initiation codon. The murine TRAP promoter was then inserted into the multiple cloning site immediately upstream of the rabbit β-globin sequences. The TRAP-p62P392L transgene was excised by XhoI digestion from the resulting plasmid, p-KCR3-TRAP-p62P392L, and was agarose gel purified before microinjection.

Production and identification of TRAP-p62P392L transgenic mice. These studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. The TRAP-p62P392L transgene was microinjected at a concentration of 3 μg/ml into the male pronucleus of fertilized 1-cell mouse embryos by standard methods (22). The embryos were obtained from mating CB6F1 (C57BL/6 × BALB/c) males and females (Harlan). The injected embryos were then reimplanted into the oviducts of pseudopregnant CD-1 female mice. The presence of the transgene was identified in resulting offspring by Southern blot analysis of genomic DNA prepared from a small tail-tip biopsy taken at the time of weaning. Probes for Southern blot analysis were generated by random oligonucleotide labeling (Amersham Biosciences) using [α-32P] dCTP (DuPont/NEN). Transgenic mice of subsequent generations were identified by PCR analysis using transgene-specific primers. The upstream primer was derived from the murine TRAP5′ untranslated region: 5′-GTCCTCACCAGACTCT-GAACTC-3′ (sense); and the downstream primer was derived from the human p62 cDNA: 5′-TGAGCGACGCCATAGCGAGCGG-3′ (antisense).

The conditions for amplification were as follows: 94°C for 2 minutes, 35 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 2 minutes, followed by extension at 72°C for 7 minutes. PCR products were separated by 1.25% agarose gel electrophoresis and were visualized by ethidium bromide staining with ultraviolet light illumination.

Histologic analysis of TRAP-p62P392L vertebral bones. The first through fourth lumbar vertebrae from 4-, 8-, 12-, and 16-month-old TRAP-p62P392L and WT littermates were fixed in 10% buffered formalin for 24–48 hours, then completely decalcified in 10% EDTA at 4°C, processed through graded alcohols, and embedded in paraffin. Longitudinal sections of 5 μm were cut and mounted on glass slides. Deparaffinized sections were stained for TRAP as described by Liu et al. (23). OCLs containing active TRAP were stained red. Another set of sections was stained with 0.1% toluidine blue. Histomorphometry was performed on the region of cancellous bone between the cranial and caudal growth plates of the third lumbar vertebral body under bright field and polarized light at a magnification of x200, using the OsteoMeasure 4.00C morphometric program (OsteoMeasure; OsteoMetrics). OCL perimeter was defined as the length of bone surface covered with TRAP-positive and mono- and multinuclear cells. Osteoblast perimeter, cancellous bone volume, trabecular width, trabecular number, and trabecular separation were also quantified and calculated. All variables were expressed and calculated according to the recommendations of the American Society for Bone and Mineral Research Nomenclature Committee (24, 25).

Figure 7
Expression of markers of OCL differentiation by TRAP-p62P392L and WT OCL precursors. OCL precursors (10⁶ cells) from WT and TRAP-p62P392L littermates were cultured for 2, 4, or 6 days with 50 ng/ml of RANKL or 100 pg/ml TNF-α. RNA was prepared and subjected to RT-PCR analysis for cathepsin K, MMP-9, calcitonin receptor, and TRAP as described in Methods. Ratio of marker mRNA expression to β-actin is shown below each lane. Lane 1, WT M-CSF (10 ng/ml); lane 2, WT M-CSF (10 ng/ml) plus RANKL (50 ng/ml); lane 3, WT M-CSF (10 ng/ml) plus TNF-α (100 pg/ml); lane 4, p62P392L M-CSF (10 ng/ml); lane 5, p62P392L M-CSF (10 ng/ml) plus RANKL (50 ng/ml); lane 6, p62P392L M-CSF (10 ng/ml) plus TNF-α (100 pg/ml).

Figure 8
MVNP-transduced TRAP-p62P392L mouse GM-CFU cells are hyper-responsive to 1,25-(OH)₂D₃. OCL precursors (5 × 10⁵ cells/well) from MVNP- or EV-transduced TRAP-p62P392L mouse GM-CFU were cultured in the presence of 1,25-(OH)₂D₃. After 9 days of culture, cells were fixed and stained for TRAP activity. Results are expressed as the mean ± SEM for quadruplicate cultures. *P < 0.001 compared with results of cultures of EV-transduced GM-CFU treated with the same concentration of 1,25-(OH)₂D₃.
NF-kB gene reporter activity in p62\textsuperscript{2935}- and WT marrow cells. For reporter gene assays, GM-CFU-derived cells from TRAP-p62\textsuperscript{2935} or WT littermate controls were cotransfected with a luciferase reporter plasmid containing an NF-kB–responsive promoter (Clontech; Cambrex) and a β-gal expression plasmid using the FuGENE 6 Reagent (Roche Diagnostics). Sixteen hours after transfection, varying concentrations of RANKL or TNF-α were added. Twenty-four hours later, cells were harvested and lysed in the cell lysate solution provided with the luciferase assay kit (Promega). The luciferase activities of the cell lysates were measured with the luciferase assay kit according to the manufacturer’s instructions and were normalized to β-gal activities of the same cell lysates using a β-gal assay kit (Promega).

Measurement of OCL differentiation markers in TRAP-p62\textsuperscript{2935} and WT marrow cells by polymerase chain reaction amplification of RT-PCR. Marrow cells from TRAP-p62\textsuperscript{2935} or WT mice were cultured for 2, 4, or 6 days with M-CSF (10 ng/ml) alone, RANKL (50 ng/ml) and M-CSF, or TNF-α (100 pg/ml) and M-CSF. Total RNA was extracted using RNeasy B solution (Tel-Test Inc.) and reverse transcribed as follows: 5% of the first-strand cDNA pool was subjected to PCR amplification using gene-specific PCR primers following standard PCR protocols. The gene-specific primers for m\textit{ouse cattexipin} were 5’-GCAGAACAGGAGTCATGAC-3’ (sense) and 5’-TGCCGGTAACATCTCCTGG-3’ (antisense). The gene-specific primers for mouse m\textit{mp locus} were 5’-TTGGTTTCTGCCCAGTTATG-3’ (sense) and 5’-TGCCCAAGGACAGCAAGG-3’ (antisense). The gene-specific primers for mouse c\textit{alcitonin receptor} were 5’-CCCAAGAATCCAGAAAAG-3’ (sense) and 5’-CAGGACATCGAGCCATC-3’ (antisense). The gene-specific primers for mouse b\textit{eta-actin} were 5’-GGGCGGTACACTGGCATGTTG-3’ (sense) and 5’-CCTGCGGCAGCATGCTGAC-3’ (antisense). The condi\textit{tions for amplification were as follows:} 94°C for 5 minutes, 35 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, followed by extension at 72°C for 7 minutes. PCR products were separated by 2% agarose gel electrophoresis and were visualized by ethidium bromide staining with ultraviolet light illumination.

Transduction of OCL precursors from TRAP-p62\textsuperscript{2935} or WT marrow cells with MVNP. Bone marrow cells were obtained by flushing the femurs of TRAP-p62\textsuperscript{2935} or WT mice with α-MEM containing 10% FBS and cultured in the α-MEM and cells were collected by centrifugation at 300×g for 10 minutes. The cells were resuspended at 2.5×10\textsuperscript{6} cells/ml and cultured in 96-well plates in α-MEM containing 10% FBS and cultured in the presence of 1,25-(OH)\textsubscript{2}D\textsubscript{3} to induce OCL formation. The cultures were fed every 3 days by replacing half the medium, and after 6 days of culture, the cells were fixed with 1% formaldehyde and stained for TRAP using a leukocyte acid phosphatase kit (Sigma-Aldrich). The TRAP-positive multinucleated cells were scored using an inverted microscope.

Immunoblotting of OCL precursors from TRAP-p62\textsuperscript{2935} or WT mice. Cytokine-treated or control OCL precursors from TRAP-p62\textsuperscript{2935} or WT mice were washed twice with ice-cold PBS. Cells were lysed in the buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM NaVO\textsubscript{4}, 1 mM NaF, and 1× protease inhibitor mixture. Fifty micrograms of cell lysates were boiled in the presence of SDS sample buffer (0.5 M Tris-HCl, pH 6.8, 10% [w/v] SDS, 10% glycerol, 0.05% [w/v] bromphenol blue) for 5 minutes and subjected to electrophoresis on 7.5% SDS-PAGE. Proteins were transferred to nitrocellulose membranes using a semi-dry blotter (Bio-Rad) and incubated in blocking solution (5% nonfat dry milk in TBS containing 0.1% Tween-20) for 1 hour to reduce nonspecific binding. Membranes were then exposed to primary antibodies overnight at 4°C, washed 3 times, and incubated with secondary goat anti-mouse or rabbit IgG HRP-conjugated antibody for 1 hour. Membranes were washed extensively, and enhanced chemiluminescence detection assay was performed following the manufacturer’s directions (Bio-Rad). All blots were densitometrically quantitated and the results expressed relative to control and normalized to β-actin.

Statistics. Significance was evaluated using a 2-tailed, unpaired Student’s \( t \) test, with \( P < 0.05 \) considered to be significant.

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