Published in final edited form as:

J Pathol. 2007 December ; 213(4): 420–428. doi:10.1002/path.2241.

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. μ CT 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*.

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 κ B (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 glycosylphosphatidylinositol (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 over-expression 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 BamHI and PmeI digestion. The resulting DNA fragment (430 bp) encoding the complete coding sequence of OIP-1 was sub-cloned into the pKCR3 Δ R1-mTRAP gene promoter containing plasmid (Figure 1A). The transgene fragment from the plasmid construct (pKCR3 Δ R1-mTRAP-OIP-1#4) was micro-injected into the male pronucleus of fertilized one-cell mouse embryos at 3 μ g/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 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 (μ CT) analysis

The bones collected from 4-week-old mice were fixed in 70% ethanol and the distal metaphyses were scanned with a Skyscan 1072 μ CT 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- μ m 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- μ m 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 μ g/ml ascorbic acid for an indicated period (0–12 days). The total RNA (2 μ g) 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 radio-opacity 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). μ CT 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, μ CT 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 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 the number of TRAP-positive multinucleated OCLs formed in these cultures was scored. As shown in Figure 5B, the number of OCLs formed in OIP-1 No 5 and OIP-1 No 13 mouse bone marrow cultures was significantly decreased, by 39% and 42% respectively, compared with control mice in response to RANKL (100 ng/ml) treatment. Immunocytochemical staining further confirmed OIP-1 transgene expression in OCLs formed in OIP-1 mouse bone marrow cultures (Figure 5C). As is evident from the osteopetrotic bone phenotype in OIP-1 mice, it is likely that OIP-1 inhibits the bone resorption capacity of OCLs in these mice. We therefore further examined the bone resorption capacity of OCLs formed in OIP-1 mouse bone marrow cultures. As shown in Figures 5D and 5E, OCLs formed in OIP-1 No 5 and OIP-1 No 13 mouse bone marrow cultures demonstrated a significant decrease (39%) in resorption area on dentine slices compared with wild-type mice when cultured in the presence of M-CSF (10 ng/ml) and RANKL (100 ng/ml). These data indicate that targeted over-expression of OIP-1 to the OCL lineage results in 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.

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 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, μ CT 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 (FcR γ) 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 calvaria 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.

Earlier 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.

Acknowledgments

This work was supported by the National Institute of Health grant DE 12603, a DOD Medical Research Award, and grant C06RR015455 from the Extramural Research Facilities Program of the National Center for Research Resources.

References

1. Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr Rev* 1999;20:345–357. [PubMed: 10368775]
2. Hsu H, Lacey DL, Dunstan CR, Solovyev I, Colombero A, Timms E, et al. Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc Natl Acad Sci U S A* 1999;96:3540–3545. [PubMed: 10097072]
3. Choi SJ, Devlin RD, Mena C, Chung H, Roodman GD, Reddy SV. Cloning and identification of human Sca as a novel inhibitor of osteoclast formation and bone resorption. *J Clin Invest* 1998;102:1360–1368. [PubMed: 9769328]
4. Palfree RG. Ly-6-domain proteins — new insights and new members: a C-terminal Ly-6 domain in sperm acrosomal protein SP-10. *Tissue Antigens* 1996;48:71–79. [PubMed: 8883295]
5. Capone MC, Gorman DM, Ching EP, Zlotnik A. Identification through bioinformatics of cDNAs encoding human thymic shared Ag-1/stem cell Ag-2. A new member of the human Ly-6 family. *J. Immunol* 1996;157:969–973. [PubMed: 8757598]
6. Koide M, Kurihara N, Maeda H, Reddy SV. Identification of the functional domain of osteoclast inhibitory peptide-1/hSca. *J Bone Miner Res* 2002;17:111–118. [PubMed: 11771657]
7. Kosugi A, Saitoh S, Narumiya S, Miyake K, Hamaoka T. Activation-induced expression of thymic shared antigen-1 on T lymphocytes and its inhibitory role for TCR-mediated IL-2 production. *Int Immunol* 1994;6:1967–1976. [PubMed: 7696214]
8. Kosugi A, Saitoh S, Noda S, Miyake K, Yamashita Y, Kimoto M, et al. Physical and functional association between thymic shared antigen-1/stem cell antigen-2 and the T cell receptor complex. *J Biol Chem* 1998;273:12301–12306. [PubMed: 9575182]
9. Bonyadi M, Waldman SD, Liu D, Aubin JE, Grynblas MD, Stanford WL. Mesenchymal progenitor self-renewal deficiency leads to age-dependent osteoporosis in Sca-1/Ly-6A null mice. *Proc Natl Acad Sci U S A* 2003;100:5840–5845. [PubMed: 12732718]
10. Hogan, BBR.; Costantini, F.; Lacy, E. *Manipulating the Mouse Embryo*. Plainview, NY: Cold Spring Harbor Laboratory Press; 1994.
11. Richard S, Torabi N, Franco GV, Tremblay GA, Chen T, Vogel G. Ablation of the Sam68 RNA binding protein protects mice from age-related bone loss. *PLoS Genet* 2005;1:e74. [PubMed: 16362077]
12. Horn DA, Garrett IR. A novel method for embedding neonatal murine calvaria in methyl methacrylate suitable for visualizing mineralization, cellular and structural detail. *Biotech Histochem* 2004;79:151–158. [PubMed: 15621887]
13. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res* 1987;2:595–610. [PubMed: 3455637]
14. Koide M, Maeda H, Roccisana JL, Kawanabe N, Reddy SV. Cytokine regulation and the signaling mechanism of osteoclast inhibitory peptide-1 (OIP-1/hSca) to inhibit osteoclast formation. *J Bone Miner Res* 2003;18:458–465. [PubMed: 12619930]
15. Zhou S, Yates KE, Eid K, Glowacki J. Demineralized bone promotes chondrocyte or osteoblast differentiation of human marrow stromal cells cultured in collagen sponges. *Cell Tissue Bank* 2005;6:33–44. [PubMed: 15735899]

16. Wada T, Nakashima T, Hiroshi N, Penninger JM. RANKL-RANK signaling in osteoclastogenesis and bone disease. *Trends Mol Med* 2006;12:17–25. [PubMed: 16356770]
17. Horowitz MC, Fields A, DeMeo D, Qian HY, Bothwell AL, Trepman E. Expression and regulation of Ly-6 differentiation antigens by murine osteoblasts. *Endocrinology* 1994;135:1032–1043. [PubMed: 7520861]
18. Xing L, Venegas AM, Chen A, Garrett-Beal L, Boyce BF, Varmus HE. Genetic evidence for a role for Src family kinases in TNF family receptor signaling and cell survival. *Genes Dev* 2001;15:241–253. [PubMed: 11157779]
19. Mocsai A, Humphrey MB, Van Ziffle JA, Hu Y, Burghardt A, Spusta SC, et al. The immunomodulatory adapter proteins DAP12 and Fc receptor gamma-chain (FcRgamma) regulate development of functional osteoclasts through the Syk tyrosine kinase. *Proc Natl Acad Sci U S A* 2004;101:6158–6163. [PubMed: 15073337]
20. Kanazawa K, Kudo A. TRAF2 is essential for TNF-alpha-induced osteoclastogenesis. *J Bone Miner Res* 2005;20:840–847. [PubMed: 15824857]
21. Kanazawa K, Azuma Y, Nakano H, Kudo A. TRAF5 functions in both RANKL- and TNFalpha-induced osteoclastogenesis. *J Bone Miner Res* 2003;18:443–450. [PubMed: 12619928]
22. Lee SE, Woo KM, Kim SY, Kim HM, Kwack K, Lee ZH. The phosphatidylinositol 3-kinase, p38, and extracellular signal-regulated kinase pathways are involved in osteoclast differentiation. *Bone* 2002;30:71–77. [PubMed: 11792567]
23. Ikeda F, Nishimura R, Matsubara T, Tanaka S, Inoue J, Reddy SV, et al. Critical roles of c-Jun signaling in regulation of NFAT family and RANKL-regulated osteoclast differentiation. *J Clin Invest* 2004;114:475–484. [PubMed: 15314684]
24. Crotti TN, Flannery M, Walsh NC, Fleming JD, Goldring SR, McHugh KP. NFATc1 regulation of the human beta3 integrin promoter in osteoclast differentiation. *Gene* 2006;372:92–102. [PubMed: 16513293]
25. Matsuo K, Galson DL, Zhao C, Peng L, Laplace C, Wang KZ. Nuclear factor of activated T-cells (NFAT) rescues osteoclastogenesis in precursors lacking c-Fos. *J Biol Chem* 2004;279:26475–26480. [PubMed: 15073183]

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 PKCR3 Δ R1 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 1×10^6 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 $\pm$ SD ($n = 5$; $p < 0.05$)

Figure 2.

μ CT analysis of bones from 4 week old wild-type (Wt) and OIP-1 mice. (A) μ CT 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) μ CT 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 $\pm$ SD ($n = 5$; $p < 0.05$)

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: $\times 20$. (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: $\times 200$. (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 $\pm$ 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: $\times 20$. (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

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 $\times 20$. (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 ($p < 0.05$)

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