Deficiency of SHP-1 Protein-Tyrosine Phosphatase Activity Results in Heightened Osteoclast Function and Decreased Bone Density

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Mice homozygous for the motheaten (*Hcph^{me}*) or viable motheaten (*Hcph^{me-v}*) mutations are deficient in functional SHP-1 protein-tyrosine phosphatase and show severe defects in hematopoiesis. Comparison of femurs from *me^v/me^v* mice revealed significant decreases in bone mineral density (0.33 ± 0.03 mg/mm³ for *me^v/me^v* versus 0.41 ± 0.01 mg/mm³ for controls) and mineral content (1.97 ± 0.36 mg for *me^v/me^v* versus 10.64 ± 0.67 for controls) compared with littermate controls. Viable motheaten mice also showed reduced amounts of trabecular bone and decreased cortical thickness. These bone abnormalities were associated with a 14% increase in numbers of multinucleated osteoclasts and an increase in osteoclast resorption activity. In co-cultures of normal osteoblasts with mutant or control bone marrow cells, numbers of osteoclasts developing from mutant mice were increased compared with littermate control mice. Although *me^v/me^v* osteoclasts develop in the absence of colony-stimulating factor (CSF)-1, nevertheless cultured osteoclasts show increased size in the presence of CSF-1. CSF-1-deficient osteopetrosis (*op/op*) mutant mice develop severe osteosclerosis. However, doubly homozygous *me^v/me^v op/op* mice show an expansion of bone marrow cavities and reduced trabecular bone mass compared with *op/op* mice. Western blot analysis showed that several proteins that were markedly hyperphosphorylated on tyrosine residues were detected in the motheaten osteoclasts, including a novel 126-kd phosphotyrosine protein. The marked hyperphosphorylation of a 126-kd protein in motheaten osteoclasts suggests that this protein depends on SHP-1 for dephosphorylation. These find-

ings demonstrate that the decreased SHP-1 catalytic activity in *me/me* and *me^v/me^v* mice results in an increased population of activated osteoclasts and consequent reduction in bone density. (*Am J Pathol* 1999, 155:223–233)

Osteoporosis is a major public health problem and is characterized by fragility fractures of the skeleton, most notably of the spine, wrist, and hip.¹ The maintenance of bone mass is a dynamic process requiring a balance between bone resorption and bone formation.² This process requires the coordinated regulation of bone-forming cells (osteoblasts) and bone-resorbing cells (osteoclasts). Increased osteoclast activity and/or decreased osteoblast activity may contribute to the development of osteoporosis. Osteoclasts are derived from hematopoietic progenitors and are members of the monocyte/macrophage family, whereas osteoblasts are derived from mesenchymal stem cells.^{3–6}

The autosomal recessive motheaten mutation (*Hcph^{me}*) and the less severe allelic viable motheaten mutation (*Hcph^{me-v}*) cause aberrant splicing of the hematopoietic cell phosphatase (*Hcph*) gene transcript. The *Hcph* gene encodes the cytoplasmic protein-tyrosine phosphatase (PTP) Src-homology domain-2 phosphatase 1 (SHP-1) (also known as hematopoietic cell phosphatase, PTP-1C, src homology PTP-1, or PTP nonreceptor type 6).^{7–11} The finding that the *me* and *me^v* mutations disrupt the *Hcph* structural gene encoding SHP-1 has illustrated the role of SHP-1 as a negative regulator in many signaling pathways in the hematopoietic and immune systems.^{12,13} SHP-1 is a member of the family of PTPs that contain SH2 domains and is a cytoplasmic PTP expressed primarily in hematopoietic cells.^{7,8,10,14,15} The *me* and *me^v* mutations result in a complete or partial loss, respectively, of SHP-1 catalytic activity.^{16,17} These two mutant alleles generate phenotypes that are qualitatively similar but of different severity (Table 1).^{18–21}

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Table 1. Phenotypes of Mutant Mice

Mutation (gene symbol)	Affected gene	Characteristic phenotype
Moth eaten (<i>Hcph^{me}</i>)	Hematopoietic cell phosphatase	Complete loss of SHP-1 catalytic activity Skin lesions appear at 3 to 5 days of age Mean life span of 3 weeks Impaired immunological function: Reduced proliferative response to B cell and T cell mitogens Absence of cytotoxic T cell responses Severely reduced NK cell function Systemic autoimmunity: Polyclonal B cell activation Expression of multiple autoantibodies
Viable moth eaten (<i>Hcph^{me-v}</i>)	Hematopoietic cell phosphatase	Partial activity of SHP-1 (10–20%) Skin lesions appear at 3 to 5 days of age Mean life span of 9 weeks Impaired immunological function, similar to <i>me/me</i> mice Systemic autoimmunity but develops in a chronic fashion
Osteopetrosis (<i>Csfm^{op}</i>)	Macrophage CSF	Complete loss of CSF-1 activity Osteopetrosis Absence of incisors Monocyte/macrophage deficiency Osteoclast deficiency

Our preliminary experiments showed that *me/me* and *me^v/me^v* mice had fragile bones. Although previous studies demonstrated hematopoietic abnormalities in these mice, little is known about the effect of deleterious alleles at the moth eaten mutation on osteoclast development or function. The work described in this paper was carried out to test the hypothesis that SHP-1 protein-tyrosine phosphatase activity is required for the regulation of osteoclastogenesis and osteoclast function. First, we assessed bone density in *me^v/me^v* mice by radiography and by peripheral quantitative computed tomography (pQCT). Second, we carried out light microscopic and histochemical studies of bone tissue in *me/me* and *me^v/me^v* mice. Third, we measured the function of osteoclasts by pit formation in dentine slices and examined osteoclast differentiation in co-culture using bone marrow cells from *me/me* and *me^v/me^v* mice. Fourth, to define the potential role and mechanism of SHP-1 in the regulation of osteoclastogenesis and osteoclast function, we localized SHP-1 in osteoclasts from normal and *me^v/me^v* mice and analyzed possible SHP-1 substrates in these cells.

Colony-stimulating factor 1 (CSF-1 or macrophage colony-stimulating factor (M-CSF)) is known to be essential in the development and differentiation of osteoclasts and certain macrophages. The essential role of CSF-1 in development and differentiation of osteoclasts and certain macrophages is evidenced by the effects of the osteopetrosis mutation (*csfm^{op}*, hereafter termed *op*), which causes a total absence of CSF-1 production.²² The CSF-1 deficiency in *op/op* mice causes widespread defects in development of the monocyte/macrophage lineage, including defects in osteoclast development. The failure of osteoclast development and differentiation in osteopetrosis (*op/op*) mice results in impaired bone resorption and leads to systemic osteopetrosis (Table 1).^{6,23–25} Immunohistochemical staining and flow cytometry analyses revealed increased numbers of macrophages in the spleen, thymus, lungs, and liver of *me^v/me^v*

mice.²⁶ It has been reported that macrophages from SHP-1-deficient mice show increased proliferation in response to CSF-1.²⁷ We also produced *me^v/me^v* mice that genetically lacked CSF-1 because of homozygosity for the osteopetrosis mutation to determine the role of CSF-1 in the bone disease observed in *me^v/me^v* mice. The doubly homozygous *me^v/me^v op/op* mice displayed less severe osteopetrosis than *op/op* mice.

This article shows that SHP-1-deficient mice develop osteoporosis that is due to increased numbers of osteoclasts and heightened osteoclast function. Thus, SHP-1 plays an important role as a negative regulator in osteoclastogenesis and osteoclast function.

Materials and Methods

Mice

C57BL/6J-*me/me* and *me^v/me^v* mice were produced in our research colony from matings of *+/me* or *+/me^v* heterozygotes, respectively. Homozygous *me/me* and *me^v/me^v* mice were identified by their characteristic skin lesions at 3 to 5 days of age. Littermate controls included both heterozygotes (*+/me* or *+/me^v*) and *+/+* mice. The (C57BL/6J × B6C3FeJ)F2-*op/op me^v/me^v* mice were produced as follows: (C57BL/6J × B6C3FeJ)F1-*+/op +/me^v* doubly heterozygous mice were made from C57BL/6J-*+/me^v* × B6C3FeJ-*+/op* matings. The (C57BL/6J × B6C3FeJ)F2-*op/op me^v/me^v* mice were produced from matings of (C57BL/6J × B6C3FeJ)F1-*+/op +/me^v* double heterozygotes. Homozygous *op/op me^v/me^v* mice were identified by the characteristic (moth eaten) skin lesions and characteristic (osteopetrosis) absence of incisors at 10 days of age. The femurs of *op/op me^v/me^v* and *op/op +/?* mice were studied by light microscopy and histochemical analyses.

Radiography

For conventional radiography, mice were anesthetized by intraperitoneal injection with tribromoethanol (0.2 ml/10 g body weight of 1.2% solution).²⁸ The mice were radiographed at 45 kV for 20 seconds at a focal distance of 35 cm with a cabinet x-ray system (Hewlett Packard, Wilmington, DE).

Bone Densitometry

Isolated femurs from 2-month-old *me^y/me^y* and littermate control mice were analyzed by peripheral quantitative computed tomography (pQCT) with a Stratec XCT 960M (Norland Medical Systems, Ft. Atkinson, WI) specifically modified for use on small bone specimens to measure bone mineral and volume as described previously.²⁹ Briefly, isolated femurs from *me^y/me^y* and littermate control mice were scanned at 2-mm intervals over their entire lengths, and the unit volume within which mineral was measured was set at 0.1 mm³. The density values were calculated by dividing the mineral content by volume. The femur lengths were divided by body weights.

Light Microscopy, Histochemistry, and Immunohistochemistry

Femurs and tibias from 2-month-old *me^y/me^y* and littermate control mice and from 3- to 4-week-old *me^y/me^y op/op*, *me^y/me^y +/?*, *+/? op/op*, and *+/? +/?* mice were fixed and demineralized in Bouin's fixative, processed routinely, and then embedded in paraffin. Sections were cut at 6- μ m thickness and stained with hematoxylin and eosin (H&E) for histological examination. For detection of osteoclasts, paraffin sections were processed for the histochemical localization of tartrate-resistant acid phosphatase (TRAP) as described previously.⁶ Numbers of TRAP-positive cells per millimeter of bone edge were counted in the distal metaphysis of femurs. The bone surface lengths were measured by using the Quantimet Q600HR system (Leica, Deerfield, IL). In addition, tissue sections in *me/me* and normal littermate mice were stained by indirect immunoperoxidase using rabbit polyclonal antibody against SHP-1.^{25,30} Briefly, the paraffin sections were incubated for 15 minutes in 10% hydrogen peroxide to quench endogenous peroxidase activity. After blocking for 10 minutes with donkey serum, the sections were incubated with rabbit anti-SHP-1 polyclonal antibody. Anti-rabbit immunoglobulin horseradish-peroxidase-linked whole antibody (Amersham, Little Chalfont, UK) was used as secondary antibody. After visualization with 3,3'-diaminobenzidine, the sections were counterstained with hematoxylin and coverslipped.

Measurement of Bone Resorption by Osteoclasts

Dentine slices from elephant tusk were the kind gift of Dr. I. Itonaga (Oita, Japan).^{31,32} Dentine slices (4 mm diam-

eter $\times$ 0.5 mm) were cut with a low-speed saw. The slices were sterilized by autoclaving and placed in 96-well culture plates. Bone marrow cells were harvested by scraping from the distal metaphysis of femurs and proximal metaphysis of tibias in 1-month-old *me/me*, *me^y/me^y*, and littermate control mice. A suspension (10^4 cells) of bone marrow cells from mutant or control mice was added to individual wells of 96-well culture plates. After 48 hours, the dentine slices were sonicated to remove the osteoclasts, and resorption lacunae formed on the slices were detected using a JSM-35C scanning electron microscope (Jeol, Tokyo, Japan). Four to six pits were measured for each animal, and six mice were examined. Pit areas were measured by using NIH Image 1.60 (National Institutes of Health, Bethesda, MD).

Co-Culture of Osteoblasts and Bone Marrow Cells

Osteoblasts were isolated from the calvariae of 3-day-old C57BL/6J-+/+ mice. The calvariae were collected and digested with a solution of phosphate-buffered saline (PBS) containing 0.1% collagenase (Wako Chemical, Dallas, TX) and 0.2% dispase (Boehringer Mannheim, Indianapolis, IN). Isolated cells were cultured until they became confluent in α -minimal essential medium (α MEM) containing 10% heat-inactivated fetal bovine serum (FBS; GIBCO, Grand Island, NY) in 25-cm² culture flasks at 10^6 cells/flask. Cells were then detached from the flasks by the addition of 0.05% trypsin in PBS, collected by centrifugation (1500 rpm for 5 minutes), suspended in α MEM containing 10% FBS, and plated in 75-cm² flasks (Corning Labware and Equipment, Corning, NY) or eight chamber Lab Tek chambers (Nalge Nunc International, Naperville, IL) at 1×10^4 cells/cm². The *me/me*, *me^y/me^y*, and littermate control mice (8 weeks old) were killed by CO₂ asphyxiation, and tibias and femurs were aseptically removed. The bone ends were cut off with scissors, and marrow cavities were flushed with 1 ml of α MEM by using syringes and 27-gauge needles. The bone marrow cells were then filtered through nylon mesh and washed once with α MEM. Bone marrow cells (2.5×10^4 /cm²) from the mutant or control mice were cultured with C57BL6J-+/+ osteoblasts in α MEM containing 10% FBS and 10 nmol/L 1,25(OH)₂D₃ (Calbiochem, San Diego, CA) without or with 1.0 μ g/ml human CSF-1 (Cetus, Emeryville, CA) or 10 μ g/ml anti-mouse CSF-1 receptor antibody.³³ Medium was replaced every 3 days. After 8 days, the cells cultured in eight-well Lab Tek chambers were washed twice with PBS and fixed with 10% neutral buffered formalin, permeabilized with 1:1 (v/v) acetone/ethanol for 1 minute, and stained for histochemical localization of TRAP. Numbers of TRAP-positive cells (>2 nuclei/cell) per square millimeter were counted. The cells cultured in 75-cm² flasks were collected after treating with trypsin and prepared for immunoblotting.

Antibodies, Immunoprecipitation, Binding Assays, and Western Blotting

Antibodies against SHP-1 have been described previously.³⁰ Antibodies against phosphotyrosine (anti-ptyr; UBI, Lake Placid, NY) and β -actin (Amersham) were purchased from commercial sources. Immunoprecipitation and Western blotting were performed as described previously.^{16,34,35} Briefly, total cell lysates (TCLs) were prepared by lysing the cell samples in cold lysis buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 0.2 mmol/L Na_3VO_4 , 20 mmol/L NaF, 1% Nonidet P-40, 2 mmol/L phenylmethylsulfonyl fluoride, 20 $\mu\text{g}/\text{ml}$ aprotinin, and 10% glycerol). The cell lysates were clarified by centrifugation, and Western blotting for SHP-1 protein and phosphotyrosine proteins was carried out as previously described.¹⁶ For immunoprecipitation experiments, the TCLs were incubated with antibodies at 4°C for 60 minutes. Immune complexes were then collected with protein A-Sepharose beads (Pharmacia, Piscataway, NJ). TCLs and immune complexes were separated by SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH), probed with specific antibodies, and detected using an enhanced chemiluminescence kit (ECL, Amersham).

Statistical Analyses

Data are presented as means $\pm$ SEM. Statistical analyses were performed with Stat View software for Macintosh. All data were analyzed first by ANOVA to detect major effects due to genotype. When a significant F ratio was identified, groups were compared using Fisher's protected least significant difference *post hoc* test. Differences were judged as statistically significant when $P < 0.05$.

Results

Assessment of the Bone Density by Radiography and by pQCT

Four pairs of me^y/me^y and littermate control mice at 2 months of age were examined by radiography. The me^y/me^y mice at 2 months of age were smaller than littermate control mice, and there was no marked disproportion of the limbs and tail. However, absorption of x-rays by bone in me^y/me^y mice was markedly decreased, and the cortical bone was thinner compared with littermate control mice (Figure 1). The mid-diaphyseal scans for femurs

Figure 1. Femoral bone from me^y/me^y mice shows reduced cortical density by radiography of a femur in a 2-month-old littermate control mouse (A) and a me^y/me^y mouse (B). A: The femur of the littermate control mouse has clearly distinguishable cortical areas. B: In contrast, the femoral bone mass of the me^y/me^y mouse is decreased, and cortical bone density is reduced compared with that of the littermate control mouse.

from littermate control (Figure 2A) and me^y/me^y (Figure 2B) mice were obtained simultaneously and correspond to the XCT 960M measurements of bone parameters. High-density bone is represented by white and blue-green color; low-density bone and trabecular bone appear yellow and red in these images. The scan from the femur of a littermate control mouse (Figure 2A) shows more higher-density bone than the scan from a me^y/me^y mouse (Figure 2B). The thickness of cortical bone measured at the mid diaphysis of femurs in me^y/me^y and normal littermate control mice was 0.20 ± 0.01 mm and 0.25 ± 0.02 mm, respectively ($P < 0.02$). The body weight and femur parameters (length, density, mineral content, volume, and proportion of femur length to body weight) obtained from 2-month-old me^y/me^y and littermate control mice are shown in Table 2. There was no effect of sex on femur parameters in me^y/me^y or in littermate control mice (data not shown). The mineral content and bone volume of me^y/me^y mice were significantly

Figure 2. Decreased levels of high density bone in me^y/me^y mice, as shown by mid-diaphyseal pQCT tomograms of femurs from a 2-month-old littermate control mouse (A) and me^y/me^y mouse (B). High-density bone is represented by white and blue-green color, whereas low-density bone is represented by red/yellow color. Littermate control femur (A) shows substantially more high-density cortical bone compared with femur from a me^y/me^y mouse (B).

Table 2. Body Weight and Femur Measurements from 2-Month-Old Mice

Genotype	Body weight (g)	Length (mm)	Density (mg/mm ³)	Mineral (mg)	Volume (mm ³)	Length (mm)/body weight (g)
me^y/me^y (n=11)	10.80 $\pm$ 0.52	12.55 $\pm$ 0.18	0.33 $\pm$ 0.03	1.97 $\pm$ 0.36	5.78 $\pm$ 0.84	1.18 $\pm$ 0.05
+/? (n=12)	20.28 $\pm$ 0.69*	14.77 $\pm$ 0.12*	0.41 $\pm$ 0.01†	10.64 $\pm$ 0.67*	25.60 $\pm$ 1.35*	0.74 $\pm$ 0.20*

Mean $\pm$ SEM are presented for groups.

* $P < 0.0001$.

† $P < 0.02$.

Figure 3. Reduced cortical and trabecular bone accompanied by increased numbers of multinucleated osteoclasts in bones from *me^v/me^v* mice, as shown by histological analyses of bone tissue in mice at 2 months of age: littermate control (A, C, and E) and *me^v/me^v* (B and D) and *me/me* (F) mice. **A:** Sagittal section of the femur in littermate control mouse shows abundant trabecular bone and thick cortical bone (H&E). Scale bar, 0.5 mm. **B:** Prominent loss of trabecular bone is seen in the metaphysis and the thickness of cortical bone is reduced in *me^v/me^v* mouse (H&E). Scale bar, 0.5 mm. **C and D:** Transverse section of the tibia in littermate control (C) and *me^v/me^v* mouse (D) were stained for TRAP. TRAP-positive cells are observed on the endosteal surfaces as indicated by arrows. TRAP-positive cells in *me^v/me^v* mouse (D) are larger in size and have increased numbers of nuclei compared with those from littermate control mouse (C). Scale bar, 0.03 mm. **E and F:** Immunohistochemical stain with anti-SHP-1 polyclonal antibody. SHP-1-positive multinucleated osteoclasts are detected on the endosteal surface in littermate control mouse (E). There are no SHP-1-positive multinucleated cells in *me/me* mouse, which lack SHP-1 protein (F). Scale bar, 0.03 mm. The arrows indicate multinucleated osteoclasts.

lower than in littermate control mice ($P < 0.001$). Bone density of femurs in *me^v/me^v* mice were also lower than in littermate control mice ($P < 0.02$). Finally, the femur lengths were divided by body weight. The femur length/body weight ratios in *me^v/me^v* mice were larger than in littermate control mice.

Histopathological Changes and Distribution of Osteoclasts in *me^v/me^v* Mice

Histological analysis of femurs revealed a marked reduction of trabecular bone and reduced thickness of cortical bone in *me^v/me^v* mice compared with littermate control

mice (Figure 3, A and B). Similar histological changes were detected in femurs of *me/me* mice (data not shown). To determine the numbers and distribution of osteoclasts in *me^v/me^v* mice, we prepared sagittal sections of the distal metaphysis in femurs and transverse sections of proximal metaphysis in tibias for enumeration of TRAP-positive cells. Numbers of TRAP-positive cells per millimeter of bone edge in the distal femur of 8-week-old *me^v/me^v* mice were significantly increased compared with littermate control mice ($P < 0.05$; Figure 4A). Transverse sections of the proximal metaphysis in tibias from mutant and control mice were made to compare numbers of multinucleated TRAP-positive cells (Figure 3, C and D).

Figure 4. Increased numbers of multinucleated TRAP-positive cells in bones from *me^v/me^v* mice. Shown are numbers of TRAP-positive cells per millimeter in the distal femur (A) and percentage of TRAP-positive multinucleated (>4 nuclei) cells in the proximal tibia (B). **A:** Numbers of TRAP-positive cells per millimeter in the distal femur of 8 week-old *me^v/me^v* mice were significantly increased compared with littermate control mice. **B:** The percentage of multinucleated TRAP-positive cells was significantly increased in *me^v/me^v* mice compared with littermate control mice. Data are presented as mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.0001$, significantly different from the control group.

These data show the multinucleated TRAP-positive cells (>4 nuclei/cell) as a percentage of total TRAP-positive cells (Figure 4B). Numbers of multinucleated TRAP-positive cells were significantly increased in *me^v/me^v* mice compared with littermate control mice ($P < 0.0001$). Similar increased numbers of multinucleated TRAP-positive cells were observed in the distal femurs of *me^v/me^v* mice (data not shown). Immunohistochemical staining using anti-SHP-1 polyclonal antibody demonstrated the expression of SHP-1 in osteoclasts in the littermate control mice (Figure 3E). However, as expected, we could not detect SHP-1-positive osteoclasts in *me/me* mice, which lack SHP-1 protein and serve as a negative control for SHP-1 localization (Figure 3F).

Determination of Osteoclast Function by Analyses of Pit Formation in Dentine Slices

Histological data suggested that osteoclast activities in *me^v/me^v* and *me/me* mice were increased compared with littermate control mice. We examined osteoclast function directly by the isolated osteoclast resorption pit assay on dentine slices. Table 3 shows that pit area/osteoclast in dentine slices incubated with osteoclasts from *me/me*

Table 3. Osteoclast Pit Area in Bone Slices Incubated with Osteoclasts from *me/me*, *me^v/me^v*, or Normal Control Mice at 1 Month of Age

Genotype	Pit area ($\times 10^3 \mu\text{m}^2$ /osteoclast)
<i>me^v/me^v</i>	13.25 $\pm$ 1.08
<i>me/me</i>	7.97 $\pm$ 2.16
+/?	1.15 $\pm$ 0.41

Mean $\pm$ SEM are presented for groups; $n = 6$. $P < 0.0001$ between all groups.

mice and *me^v/me^v* mice were significantly larger than pit area/osteoclast in dentine slices incubated with osteoclasts from littermate control mice ($P < 0.0001$).

Assay of Osteoclastogenesis by Osteoblast/Bone Marrow Cell Co-Cultures

As *me^v/me^v* mice showed increased numbers of TRAP-positive cells and increased percentages of multinucleated TRAP-positive cells compared with littermate control mice, we next examined osteoclastogenesis *in vitro* from bone marrow cells isolated from *me/me*, *me^v/me^v*, or littermate control mice. Osteoclastogenesis was determined by measuring numbers of osteoclasts produced in co-culture using normal C57BL/6J-+/+ osteoblasts co-cultured with bone marrow cells from *me/me*, *me^v/me^v*, or littermate control mice. The results are reported as numbers of TRAP-positive multinucleated cells (>2 nuclei/cell) per square millimeter of eight-chamber Lab Tek chambers. TRAP-positive cells from *me^v/me^v* mice were larger in size compared with those from littermate control mice (Figure 5, A and B). Numbers of TRAP-positive cells in *me^v/me^v* mice were significantly increased compared with those in littermate control mice. However, there was no significant difference in numbers of TRAP-positive cells between *me/me* and littermate control mice (Table 4).

CSF-1 or neutralizing monoclonal antibody to mouse CSF-1 receptor was added to stimulate or inhibit osteoclast formation, respectively. Addition of anti-CSF-1 receptor antibody completely inhibited the formation of multinucleated TRAP-positive cells from bone marrow cells of *me/me*, *me^v/me^v*, or littermate control mice (Table 4). Addition of CSF-1 stimulated development of TRAP-positive cells in *me/me*, *me^v/me^v*, and littermate control mice, but there was no significant difference in numbers of CSF-1-stimulated TRAP-positive cells among *me/me*, *me^v/me^v*, and littermate control mice (Table 4). TRAP-positive cells from *me/me* and *me^v/me^v* mice were larger in size and had increased numbers of nuclei compared with those from littermate control mice (Figure 5, C and D).

Histopathological Changes of Femurs in Doubly Homozygous *me^v/me^v* op/op Mice

We produced doubly homozygous *me^v/me^v* op/op mice to determine the effects of CSF-1 deficiency *in vivo* on osteoporosis in *me^v/me^v* mice. Histological analysis of femurs from *me^v/me^v* op/op mice showed increased bone thickness compared with *me^v/me^v* mice. However, an expansion of bone marrow cavities and reduced trabecular bone were exhibited *me^v/me^v* op/op compared with +/? op/op mice (Figure 6, A and B). Although there were no observable TRAP-positive osteoclasts in +/? op/op mice (Figure 6C), osteoclasts on the endosteal surface of femoral bones in *me^v/me^v* op/op mice were detected by positive histochemical reaction for TRAP (Figure 6D). However, these endosteal TRAP-positive cells were small and mononuclear.

Figure 5. Increased size of *me/me* osteoclasts. Osteoclast cultures from littermate control (A and C) and *me/me* mice (B and D) were fixed and stained for TRAP. A and B were cultured without CSF-1. C and D were cultured with CSF-1. The arrows indicate TRAP-positive cells. A and B: Cultured osteoclasts from *me/me* mice (B) in the absence of exogenous CSF-1 are larger than those from littermate control mice (A). Numbers of TRAP-positive cells in *me/me* mice were increased compared with those in controls. C and D: After addition of CSF-1, osteoclasts from *me/me* mice (D) are larger in size than that from littermate control mice (C) and have increased numbers of nuclei (arrowhead). Scale bar, 0.05 mm.

Western Blotting Analysis of SHP-1 Protein

Osteoclasts isolated from osteoblast/bone marrow cell co-cultures were analyzed for the expression of SHP-1 protein. Osteoclasts from littermate control and *me^y/me^y* bone marrow cells treated with 1,25(OH)₂D₃ expressed SHP-1 protein (Figure 7A). Although *me^y/me^y* mice are deficient in SHP-1 functional activity, these mice, nevertheless, express normal levels of SHP-1 protein. As is shown in Figure 7A, lane 3, those mice produce two SHP-1 bands on Western blotting. One band is slightly lower than normal and is slightly smaller. However, as

Table 4. Numbers of TRAP-Positive Cells in Co-Culture in the Presence or Absence of CSF-1 Growth Factor or Neutralizing Anti-CSF-1 Receptor Antibody

Genotype	Numbers of cells × 10 ² /mm ²		
	No treatment	CSF-1	Anti-CSF-1
+/?	1.74 ± 0.26	2.81 ± 0.32*	0 ± 0
<i>me^y/me^y</i>	3.56 ± 0.61 [†]	3.85 ± 0.87	0 ± 0
<i>me/me</i>	2.66 ± 0.45	2.98 ± 0.54	0 ± 0

Data are presented as mean ± SEM. In the no-treatment group, there was no significant difference between +/? and *me/me* or between *me/me* and *me^y/me^y*. In the CSF-1 treatment group, there was no significant difference between +/? and *me^y/me^y* or *me/me*. There was no significant difference between the presence and absence of CSF-1 in *me/me* and *me^y/me^y* mice.

*P < 0.05 versus no treatment.

[†]P < 0.05 versus +/? group.

expected, osteoclasts isolated from *me/me* mice did not express SHP-1 (Figure 7A). This is consistent with our previous finding that *me^y/me^y* hematopoietic cells express SHP-1 protein with severely reduced catalytic activity whereas *me/me* hematopoietic cells lack SHP-1 protein.¹⁶ To identify potential SHP-1 substrates, we examined the total cell lysate (TCL) of cultured osteoclasts from *me/me*, *me^y/me^y*, and littermate control mice. Several distinct protein bands in TCLs from *me/me* and *me^y/me^y* osteoclasts were found to be hyperphosphorylated on tyrosine residues, including proteins of approximately 47, 60, and 126 kd (Figure 7A). We next looked for phosphotyrosine proteins that were associated with SHP-1 in osteoclasts. A phosphotyrosine protein of approximately 126 kd (p126) was detected in the anti-SHP-1 immune complex from *me^y/me^y* osteoclasts (lane 3) but not in osteoblasts (lane 1), littermate control osteoclasts (lane 2), or *me/me* osteoclasts, which lack SHP-1 protein (lane 4; Figure 7B).

Discussion

This study shows that the absence or severe reduction of SHP-1 activity in *me/me* and *me^y/me^y* mice, respectively, caused marked abnormalities in the development and activity of osteoclasts *in vitro* and *in vivo*. Histological studies demonstrated increased numbers of TRAP-posi-

Figure 6. Reduction of osteopetrosis in CSF-1-deficient me^v/me^v op/op mice, as shown by histological analyses of bone tissue in mice at 4 weeks of age. **A** and **C**: $+/?$ op/op mice. **B** and **D**: me^v/me^v op/op mice. **A** and **C**: Sagittal section of $+/?$ op/op femur shows prominent osteosclerosis, marked reduction of bone marrow cellularity, and lack of TRAP-positive cells. **B** and **D**: Decreased trabecular bone and increased bone marrow cellularity are seen in me^v/me^v op/op mouse. Although TRAP-positive cells are detected, they are small and mononuclear. **Inset**: A high magnification of TRAP-positive mononuclear cell indicated by box. Scale bar, 0.01 mm. The **arrows** indicate the TRAP-positive cells. H&E (**A** and **B**); TRAP stain (**C** and **D**). Scale bar, 0.1 mm (**A** and **B**) and 0.03 mm (**C** and **D**).

tive cells and increased percentages of multinucleated osteoclasts (>4 nuclei/cell) in me^v/me^v mice. When osteoclasts from me/me , me^v/me^v , and littermate control mice were incubated on dentine slices, the areas of pit formation caused by osteoclasts from the mutant mice were substantially increased compared with pit areas from littermate control osteoclasts. SHP-1 was found to be expressed in osteoclasts, but not in osteoblasts as determined by immunohistochemical staining and Western blotting with anti-SHP-1 antibody. In our previous studies, it was shown that treatment of motheaten mice with 500 R γ -irradiation followed by reconstitution with normal bone marrow cells resulted in increased life span.³⁶ The density of femoral bone in these treated mutant mice was normal (unpublished data). This result indicates a primary defect in hematopoietic progenitor cells. It has been reported that the turnover rate of bone in me/me mice was increased compared with that in littermate control mice.³⁷ As bone turnover rate is increased in the me/me mutant mice, osteoblast activity is not likely to be impaired, and increased osteoclast activity is likely the major factor contributing to osteoporosis in the mutant mice. These data clearly suggest that the osteoporotic changes

observed in these mutant mice resulted from increased osteoclast numbers and heightened osteoclast function.

To determine the role of SHP-1 in osteoclastogenesis, we have used *in vitro* culture systems. Co-culture using normal osteoblasts and bone marrow cells from me/me , me^v/me^v , or littermate control mice showed that numbers of TRAP-positive cells generated from bone marrow cells from me/me and me^v/me^v mice were increased compared with those from littermate control mice. These data suggest that the abnormal growth of osteoclasts in the mutant mice may be due to impaired negative regulation of cytokine signaling by SHP-1 *in vitro*. Similarly, in the erythroid lineage, activation of SHP-1 by binding to the erythropoietin receptor plays a major role in terminating proliferative signals.^{18,20,38,39} Likewise, absence or reduction of enzymatic activity of SHP-1 results in hyperresponsiveness to erythropoietin. Our data show that absence or reduction of functional SHP-1 in me/me and me^v/me^v mice, respectively, results in increased osteoclastogenesis. First we examined effects of CSF-1, which play an important role in osteoclast development and differentiation. It has been reported that SHP-1 is rapidly phosphorylated in macrophages after binding of

Figure 7. Hyperphosphorylation of phosphotyrosine proteins in *me^v/me^v* osteoclasts, as shown by Western blotting analysis of SHP-1 and proteins that were hyperphosphorylated on tyrosine residues (A) and immunoprecipitation with anti-SHP-1 antibody (B). Osteoclasts were obtained from co-culture of normal osteoblasts (+/+) and bone marrow cells from *me/me*, *me^v/me^v*, or littermate control mice (+/?). **A:** The cells were lysed to prepare total cell lysate (TCL). The positions of protein size markers, SHP-1, β-actin, and several hyperphosphorylated proteins are indicated. The arrow indicates a 126-kd protein. **B:** TCL was used for immunoprecipitation with anti-SHP-1 antibody. The immune complexes were analyzed by SDS-PAGE/Western blotting with anti-phosphotyrosine protein. A phosphotyrosine protein of 126 kd was detected in TCL anti-SHP-1 immunoprecipitates from *me^v/me^v* osteoclasts (lane 3) but not in osteoblasts (lane 1), littermate control osteoclasts (lane 2), or *me/me* osteoclasts (lane 4). Lane 1 shows the +/+ osteoblast culture without bone marrow cells.

CSF-1 to its receptor.²⁷ Addition of neutralizing monoclonal antibody to CSF-1 receptor completely blocked multinucleated TRAP-positive cell development *in vitro*. We expected that osteoclasts from *me/me* and *me^v/me^v* mice would show increased proliferation in response to CSF-1. Although addition of CSF-1 (1.0 μg/ml) stimulated increased numbers of TRAP-positive cells in littermate control mice, there were no significant effects of adding exogenous CSF-1 on numbers of TRAP-positive cells in *me/me* and *me^v/me^v* mice. We found that TRAP-positive cells in *me/me* and *me^v/me^v* mice were larger than those in normal mice and were multinucleated after incubation with CSF-1. In a recent *in vitro* study, it was shown that CSF-1 induced the formation of large multinucleated osteoclasts in rats.⁴⁰ This may explain why there were increased TRAP-positive multinucleated cells in *me/me* and *me^v/me^v* mice and suggests that SHP-1 is a negative regulator of CSF-1 signaling in osteoclasts.

We performed genetic crosses with CSF-1-deficient osteopetrosis (*op*) mutant mice to determine the role of CSF-1 in the bone disease observed in *me^v/me^v* mice. Doubly homozygous *me^v/me^v op/op* mice showed poor survival associated with overgrowth of granulocytes in

the lungs, skin, and elsewhere. However, *me^v/me^v op/op* mice manifested improved development of lymphoid follicles compared with *me^v/me^v +/+* mice, suggesting that the overgrowth of macrophages in the spleen of *me^v/me^v* mice may play a suppressive role in follicle development (data not shown). Although *me^v/me^v op/op* mice displayed osteopetrosis, mononucleated TRAP-positive cells developed in these mice, and osteopetrosis in *me^v/me^v op/op* mice was less severe than in *+/+ op/op* mice. These observations suggest that differentiation of osteoclasts is supported by CSF-1-independent mechanisms in *me^v/me^v op/op* mice to compensate for the absence of functional CSF-1 activity. It is known that osteopetrosis in aged *op/op* mice is partially reversed with a spontaneous increase of numbers of mononuclear osteoclasts and expansion of bone marrow cavities.^{41,42} Bcl-2 overexpression in monocytes of *op/op* mice results in replenishment of tissue macrophages and partial reversal of long bone osteopetrosis.⁴³ The partial reversal of osteopetrosis with age in *op/op* mice may be associated with increased levels of granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-3 as these cytokines are increased in aged *op/op* mice.⁴² Although

studies by Wiktor-Jedrzejczak et al⁴⁴ suggested that short-term administration of GM-CSF to *op/op* mice failed to cure osteopetrosis, studies by Myint et al⁴² suggested that injection of rmGM-CSF induced osteoclast development in *op/op* femurs. In previous *in vitro* studies, the effects of GM-CSF on osteoclast formation were controversial. In contrast to most known cytokines, osteoprotegrin ligand (OPGL) stimulates osteoclast differentiation directly.⁴⁵ The signaling pathways of the OPGL receptor are not clear. We sought to define the potential role and mechanisms of SHP-1 in osteoclasts.

It was reasoned that protein tyrosine phosphorylation, which is down-regulated by SHP-1 in normal mice, would be altered in osteoclasts from SHP-1-deficient motheaten mice. To identify potential SHP-1 substrates, we examined proteins that were hyperphosphorylated on tyrosine residues in the total cell lysates (TCLs) of osteoclast co-cultures from *me/me* and *me^v/me^v* mice. Several distinct protein bands were found to be hyperphosphorylated on tyrosine residues compared with littermate control TCLs, indicating that dephosphorylation depends on SHP-1. The identities of these hyperphosphorylated proteins have not yet been determined. We also examined phosphotyrosine proteins associated with SHP-1 in osteoclasts. A phosphotyrosine protein of approximately 126 kd (p126) was detected in the anti-SHP-1 immune complexes from *me^v/me^v* osteoclasts. We have previously reported that p126 is a novel phosphoprotein in macrophages.³⁰ The marked hyperphosphorylation of p126 in *me^v/me^v* osteoclasts suggests that it is a major SHP-1 substrate in these cells. One of the most significant advances in the study of osteoclast differentiation was the development of a co-culture system for production of osteoclasts in tissue culture. Although the murine osteoclast-like cells produced in this manner are well characterized, it is difficult to obtain purified osteoclast preparations. Although we used a co-culture system to produce osteoclasts, there is a possibility that the lysates were contaminated by a proportion of non-osteoclastic cells, including osteoblasts and adherent bone marrow cells. Thus, our findings reported here must be considered as preliminary. Recently, it has been reported that OPGL stimulates osteoclast differentiation directly without osteoblasts.⁴⁵ OPGL would provide improvement in the study of osteoclast biochemical characterization.

In conclusion, *me/me* and *me^v/me^v* mice show markedly lowered bone density due to increased numbers and function of multinucleated osteoclasts. Thus, SHP-1 plays an important role in the regulation of osteoclastogenesis and osteoclast function. The association of p126 with SHP-1 suggests that p126 plays an important role in osteoclast signaling pathways. However, it is not clear from the current data whether p126 mediates the heightened osteoclastogenesis or osteoclast function. Additional studies to identify and characterize the hyperphosphorylated proteins in motheaten osteoclasts are not only important for defining SHP-1 substrates but will also help to elucidate the signaling pathways for osteoclastogenesis and osteoclast function.

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References

1. Khosla S, Riggs L, Melton L: Clinical spectrum. Osteoporosis: Etiology, Diagnosis and Management. Edited by L Riggs, L Melton. New York, Raven Press, 1995, pp 133–160
2. Parfitt AM: The cellular basis of bone remodeling: the quantum concept reexamined in light of recent advances in the cell biology of bone. *Calcif Tissue Int* 1984, 36(Suppl 1):S37–S45
3. Walker DG: Osteopetrosis cured by temporary parabiosis. *Science* 1973, 180:875
4. Kahn AJ, Simmons DJ: Investigation of cell lineage in bone using a chimera of chick and quail embryonic tissue. *Nature* 1975, 258:325–327
5. Ibbotson KJ, Roodman GD, McManus LM, Mundy GR: Identification and characterization of osteoclast-like cells and their progenitors in cultures of feline marrow mononuclear cells. *J Cell Biol* 1984, 99:471–480
6. Umeda S, Takahashi K, Naito M, Shultz LD, Takagi K: Neonatal changes of osteoclasts in osteopetrosis (*op/op*) mice defective in production of functional macrophage colony-stimulating factor (M-CSF) protein and effects of M-CSF on osteoclast development and differentiation. *J Submicrosc Cytol Pathol* 1996, 28:13–26
7. Shen SH, Bastien L, Posner BI, Chretien P: A protein-tyrosine phosphatase with sequence similarity to the SH2 domain of the protein-tyrosine kinases. *Nature* 1991, 352:736–739
8. Plutzky J, Neel BG, Rosenberg RD: Isolation of a src homology 2-containing tyrosine phosphatase. *Proc Natl Acad Sci USA* 1992, 89:1123–1127
9. Plutzky J, Neel BG, Rosenberg RD, Eddy RL, Byers MG, Jani-Sait S, Shows TB: Chromosomal localization of an SH2-containing tyrosine phosphatase (PTPN6). *Genomics* 1992, 13:869–872
10. Yi TL, Cleveland JL, Ihle JN: Protein tyrosine phosphatase containing SH2 domains: characterization, preferential expression in hematopoietic cells, and localization to human chromosome 12p12–p13. *Mol Cell Biol* 1992, 12:836–846
11. Zhao Z, Bouchard P, Diltz CD, Shen SH, Fischer EH: Purification and characterization of a protein tyrosine phosphatase containing SH2 domains. *J Biol Chem* 1993, 268:2816–2820
12. McCulloch J, Siminovitch KA: Involvement of the protein tyrosine phosphatase PTP1C in cellular physiology, autoimmunity and oncogenesis. *Adv Exp Med Biol* 1994 365:245–254
13. Ihle JN: Cytokine receptor signalling. *Nature* 1995, 377:591–594
14. Matthews RJ, Bowne DB, Flores E, Thomas ML: Characterization of hematopoietic intracellular protein tyrosine phosphatases: description of a phosphatase containing an SH2 domain and another enriched in proline-, glutamic acid-, serine-, and threonine-rich sequences. *Mol Cell Biol* 1992, 12:2396–2405
15. Adachi M, Fischer EH, Ihle J, Imai K, Jirik F, Neel B, Pawson T, Shen S, Thomas M, Ullrich A, Zhao Z: Mammalian SH2-containing protein tyrosine phosphatases. *Cell* 1996, 85:15
16. Shultz LD, Schweitzer PA, Rajan TV, Yi T, Ihle JN, Matthews RJ, Thomas ML, Beier DR: Mutations at the murine motheaten locus are within the hematopoietic cell protein-tyrosine phosphatase (*Hcph*) gene. *Cell* 1993, 73:1445–1454
17. Tsui HW, Siminovitch KA, de-Souza L, Tsui FW: Motheaten and viable motheaten mice have mutations in the haematopoietic cell phosphatase gene. *Nat Genet* 1993, 4:124–129

18. Green MC, Shultz LD: Motheaten, an immunodeficient mutant of the mouse. I: Genetics and pathology. *J Hered* 1975, 66:250–258
19. Shultz LD: Pleiotropic effects of deleterious alleles at the "motheaten" locus. *Curr Top Microbiol Immunol* 1998, 137:216–222
20. Shultz LD, Coman DR, Bailey CL, Beamer WG, Sidman CL: "Viable motheaten," a new allele at the motheaten locus. I. Pathology. *Am J Pathol* 1984, 116:179–192
21. Shultz LD: Hematopoiesis and models of immunodeficiency. *Semin Immunol* 1991, 3:397–408
22. Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Sudo T, Shultz LD, Nishikawa S: The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* 1990, 345:442–444
23. Marks SC, Lane PW: Osteopetrosis, a new recessive skeletal mutation on chromosome 12 of the mouse. *J Hered* 1976, 67:1–18
24. Naito M, Hayashi S-I, Yoshida H, Nishikawa S-I, Shultz LD, Takahashi K: Abnormal differentiation of tissue macrophage populations in 'osteopetrosis' (op) mice defective in the production of macrophage colony-stimulating factor. *Am J Pathol* 1991, 139:657–667
25. Umeda S, Takahashi K, Shultz LD, Naito M, Takagi K: Effects of macrophage colony-stimulating factor on macrophages and their related cell populations in the osteopetrosis mouse defective in production of functional macrophage colony-stimulating factor protein. *Am J Pathol* 1996, 149:559–574
26. Nakayama K, Takahashi K, Shultz LD, Miyakawa K, Tomita K: Abnormal development and differentiation of macrophages and dendritic cells in viable motheaten mutant mice deficient in haematopoietic cell phosphatase. *Int J Exp Pathol* 1997, 78:245–257
27. Chen HE, Chang S, Trub T, Neel BG: Regulation of colony-stimulating factor 1 receptor signaling by the SH2 domain-containing tyrosine phosphatase SHPTP1. *Mol Cell Biol* 1996, 16:3685–3697
28. Papaioannou VE, Fox JG: Efficacy of tribromoethanol anesthesia in mice. *Lab Anim Sci* 1993, 43:189–192
29. Beamer WG, Donahue LR, Rosen CJ, Baylink DJ: Genetic variability in adult bone density among inbred strains of mice. *Bone* 1996, 18:397–403
30. Jiao H, Yang W, Berrada K, Tabrizi M, Shultz L, Yi T: Macrophages from motheaten and viable motheaten mutant mice show increased proliferative responses to GM-CSF: detection of potential HCP substrates in GM-CSF signal transduction. *Exp Hematol* 1997, 25:592–600
31. Fujikawa Y, Shingu M, Torisu T, Itonaga I, Masumi S: Bone resorption by tartrate-resistant acid phosphatase-positive multinuclear cells isolated from rheumatoid synovium. *Br J Rheumatol* 1996, 35:213–217
32. Raynal C, Delmas PD, Chenu C: Bone sialoprotein stimulates in vitro bone resorption. *Endocrinology* 1996, 137:2347–2354
33. Sudo T, Nishikawa S, Ogawa M, Kataoka H, Ohno N, Izawa A, Hayashi S, Nishikawa S: Functional hierarchy of c-kit and c-fms in intramarrow production of CFU-M. *Oncogene* 1995, 11:2469–2476
34. Tabrizi M, Yang W, Jiao H, DeVries EM, Platanias LC, Arico M, Yi T: Reduced Tyk2/SHP-1 interaction and lack of SHP-1 mutation in a kindred of familial hemophagocytic lymphohistiocytosis. *Leukemia* 1998, 12:200–206
35. Yang W, Tabrizi M, Berrada K, Yi T: SHP-1 phosphatase C-terminus interacts with novel substrates p32/p30 during erythropoietin and interleukin-3 mitogenic responses. *Blood* 1998, 91:3746–3755
36. Shultz LD, Bailey CL, Coman DR: Hematopoietic stem cell function in motheaten mice. *Exp Hematol* 1983, 11:667–680
37. Vignery A, Silverglate A, Horowitz M, Shultz L, Baron R: Abnormal bone remodeling activity in the immunodeficient nude (nu/nu) and motheaten (me/me) mutant mice. *Calcif Tissue Int* 1981, 33:301
38. Klingmuller U, Lorenz U, Cantley LC, Neel BG, Lodish HF: Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. *Cell* 1995, 80:729–738
39. Van Zant G, Shultz L: Hematologic abnormalities of the immunodeficient mouse mutant, viable motheaten (me^v). *Exp Hematol* 1989, 17:81–87
40. Amano H, Yamada S, Felix R: Colony-stimulation factor-1 stimulates the fusion process in osteoclasts. *J Bone Miner Res* 1998, 13:846–853
41. Takatsuka H, Umezu H, Hasegawa G, Usuda H, Ebe Y, Naito M, Shultz LD: Bone remodeling and macrophage differentiation in osteopetrosis (op) mutant mice defective in the production of macrophage colony-stimulating factor. *J Submicrosc Cytol Pathol* 1998, 30:239–247
42. Myint YY, Miyakawa K, Naito M, Shultz LD, Oike Y, Yamamura K-I, Takahashi K: Granulocyte/macrophage colony-stimulating factor, and interleukin-3 correct osteopetrosis in mice with osteopetrosis mutation. *Am J Pathol* 1999, 154:553–566
43. Lagasse E, Weissman IL: Enforced expression of Bcl-2 in monocytes rescues macrophages and partially reverses osteopetrosis in op/op mice. *Cell* 1997, 89:1021–1031
44. Wiktor-Jedrzejczak W, Urbanowska E, Szperl M: Granulocyte-macrophage colony-stimulating factor corrects macrophage deficiencies, but not osteopetrosis, in the colony-stimulating factor-1-deficient op/op mouse. *Endocrinology* 1994, 134:1932–1935
45. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ: Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 1998, 93:165–176