Inorganic Pyrophosphate Promotes Osteoclastogenic Commitment and Survival of Bone Marrow Derived Monocytes mediated by *Egr-1* up-regulation and MITF phosphorylation

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CONFLICT OF INTEREST

The authors have no conflict of interests

ABSTRACT

Several reports emphasized the importance of inorganic pyrophosphate (PP_i) in hindering osteoblast differentiation and bone matrix mineralization. Its ubiquitous presence is thought to prevent “soft” tissue calcification, whereas its degradation to P_i in bones and teeth by alkaline phosphatase (ALP) may facilitate crystal growth. While the inhibiting role of PP_i on osteoblast differentiation and function is largely understood, less is known about its effects on osteoclast determination and activity. In this study, we investigated the role of PP_i in bone resorption using calverial organ cultures *ex vivo*. We present an evidence that PP_i stimulated calvarial bone resorption marked by calcium (Ca²⁺) release in the condition media (CM). We then examined PP_i effects on osteoclast differentiation using mouse bone marrow-derived monocytes (BMMs). Our results revealed that PP_i enhanced osteoclast differentiation *ex vivo*, marked by increased number and size of TRAP-stained mature osteoclasts. Moreover, PP_i stimulated osteoclastogenesis in BMMs co-cultured with osteoblasts. These data supported the increased osteoclast activity in bone resorption using functional osteo-assays. The finding of PU.1-Egr-1 dependent up-regulation of c-FMS and RANK receptors in BMMs supported the enhanced pre-osteoclast commitment and differentiation. Moreover, osteoclast survival was enhanced by activation of MITF-BCL-2 pathway that was mediated by MAPK-ERK1/2 signaling. Last, our data showed that PP_i up-regulated ANK; PP_i transporter, during osteoclast differentiation through ERK1/2 phosphorylation whereas mutation of ANK inhibited osteoclastogenesis. Collectively, our data suggest that PP_i promotes osteoclast differentiation, survival, and function through PU.1 up-regulation and MITF phosphorylation whereas ANK loss-of-function inhibited osteoclastogenesis.

INTRODUCTION

Mineralization of the mammalian skeleton is a tightly regulated process, in which the key regulators play critical roles in defining the site, timing and the extent of mineral deposition. In bone, calcium (Ca^{2+}) and phosphate-based apatite crystal formation is initiated by membrane bound matrix vesicles, deposited to the type I collagen-rich organic matrix and propagate along the collagen fibrils (4, 35). Unlike in the highly insoluble exoskeleton of numerous invertebrates, the calcium phosphate apatite crystal in mammalian skeleton is not a perfect crystalline with its stability subjected to a local pH change (46). A slight shift from neutral to acidic pH converts bone apatite crystals to soluble ions, and vice versa. The dynamic nature of vertebral skeleton is important not only to support homeostasis of serum mineral ions but also to facilitate bone remodeling, allowing enlargement, contouring and repair of bones (46).

Inorganic pyrophosphate (PP_i), which consists of two molecules of inorganic phosphates connected by a high-energy ester bond, is best known for its ability to inhibit tissue calcification (49). In mammalian cells, PP_i does not appear to be synthesized *de novo*. Instead, it is generated as an abundant byproduct or metabolic intermediate of numerous intracellular and extracellular biochemical reactions (55). Intracellular PP_i is generated and stored largely in mitochondria, but it is also detected in endoplasmic reticulum and Golgi (27). The extracellular PP_i concentration in the skeletal tissue is determined by several types of plasma membrane proteins: ectonucleotide pyrophosphatase (ENPP), which generates PP_i from ATP (51), tissue non-specific alkaline phosphatase (TNAP), which hydrolyzes PP_i into two inorganic phosphates (P_i) (58), and progressive ankyloses (ANKH) transporter; which is involved in PP_i in- and efflux (8).

While the functional role of intracellular PP_i in mammalian cells remains elusive, extracellular PP_i has been extensively studied for its inhibitory role in tissue calcification (28). Extracellular PP_i directly binds to the surface of basic calcium phosphate hydroxyapatites and

interferes with the propagation of crystal formation, contributing to the formation of poorly ordered bone crystal structure (3). In addition, exogenous PP_i at micro-molar concentrations stimulates the expression of osteopontin; a negative regulator of mineralization that also inhibits the PP_i hydrolyzing activity of TNAP in MC3T3-E1 osteoblast cultures (3).

The physiological significance of extracellular PP_i in tissue calcification is observed in various human pathologies and genetically modified mouse strains with dysregulated PP_i metabolism, where a reduction of extracellular PP_i is associated with ectopic calcification of arteries (48), joint tissues (65), renal stone formation (43), and hyperostosis of craniofacial bones (50). Under physiological concentrations, it is well documented that the mineral crystals formed of extracellular PP_i are mainly the basic calcium phosphate crystals (29). Increased extracellular PP_i concentrations, on the other hand, is associated with hypo-mineralization of the bone matrix as in *Tnap*^{-/-} mice or patients with deficient TNAP activities (20). Moreover, higher concentrations of PP_i results in formation of calcium pyrophosphate dihydrate (CPPD) crystals with spontaneous deposition in the articular tissues leading to an arthritis condition called; chondrocalcinosis (29).

Another syndrome characterized by PP_i metabolic disorder is the craniometaphyseal dysplasia (CMD) with genetic nonsense mutation of *ankh* that underlie increased intracellular and decreased extracellular PP_i (11). The ankh protein is type II transmembrane with 10–12 helices, spanning the outer cell membrane, and is associated with PP_i efflux. Most of the *ANKH* mutations are located in cytoplasmic domains close to the C-terminus (44). Previous study reported *ANKH* gene mutation impairs osteoclast differentiation and function that will disrupt bone remodeling (50). Reduced bone turnover can contribute to the bone thickening characteristic of CMD. Another study showed that *ANKH* gene mutation increases osteoblast activity rather than a defect in bone remodeling (53). Moreover, *ANKH* gene mutation reduces

PP_i transportation out of the cells. A shortage of extracellular PP_i will increase bone mineralization, which also contribute to the bone overgrowth of CMD (53). Earlier studies of CMD point to the important regulatory role of PP_i in bone modeling/remodeling process (6). Given the expanding functional implication of PP_i in bone remodeling, it is imperative to test the role of PP_i in bone resorption. We hypothesize that extracellular PP_i has a direct stimulating effect on osteoclasts, promoting differentiation of bone marrow-derived monocytes (BMMs) into mature osteoclasts that are active in bone resorption. In this study, murine calvarial organ cultures and BMMs have been essentially used to determine the potential role of exogenous PP_i in pre-osteoclast differentiation, and function. Here, we report that PP_i enhances commitment and survival of BMMs into mature osteoclasts in a PU.1 dependent up-regulation of EGR-1, and MAPK-dependent phosphorylation of MITF. Thus, our data suggest PP_i works as a positive regulator of osteoclastogenesis and osteoclast-mediated bone resorption.

EXPERIMENTAL PROCEDURES

Materials.

Tetrasodium pyrophosphate (NaPP_i), sodium phosphate (NaP_i); monobasic and dibasic, phosphonoformic acid (PFA), 1 alpha 25 Dihydroxy vitamin D3, levamisole, Hydroxytamoxifen, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human TNF- α , RANKL, and MCSF were purchased from Peprotech EC (London, UK). Disodium clodronate and quinazoline were purchased from Calbiochem (La Jolla, CA,). Probenecid, DMEM, α -MEM, IMDM, recombinant mouse IL-3, L-glutamine, β -mercaptoethanol, Penicillin- Streptomycin, and Fungizone® Antimycotic were purchased from Invitrogen (Carlsbad, CA). L-Ascorbic acid, MEM Non-essential Amino Acid Solution (100X), and bovine serum albumin, Fraction V (BSA) were purchased from Sigma-Aldrich. Fetal bovine

serum (FBS) was purchased from Gemini Bio-Products (West Sacramento, CA). Calf serum (CS) was purchased from Thermo Scientific ATLC (Rockford, IL). U0126 (MAPK inhibitor) and SB203580 (p38 MAPK inhibitor) were purchased from Calbiochem (Gibbstown, NJ).

Antibodies.

All antibodies were used for protein blotting as described below. Rabbit anti-mouse phospho-p38 MAPK (Thr180/Tyr182) (Cat# 9211), p38 MAPK (Cat# 9212), and GAPDH antibodies (Cat# 97166) were purchased from Cell Signaling (Boston, MA). Rabbit anti-mouse phospho-ERK 1/2 (Thr202, Tyr204) (Cat# sc-136521) and ERK 1/2 (Cat# sc-292838) were purchased from Santa Cruz (Santa Cruz, CA). Anti-mouse phospho-MITF-S307 was gift from Dr Michael Ostrowski (Ohio State University, Columbus, OH)). Rabbit anti-mouse MITF (Cat# ab20663) was purchased from abcam (Cambridge, MA). Horseradish Peroxidase (HRP)-conjugated anti-mouse antibody (Cat# NA931) or anti-rabbit antibody (Cat# NA934) was purchased from GE Healthcare (Buckinghamshire, UK). ank-specific antibody was raised in a rabbit against C-terminal peptide sequence TEEVTDIVEMRENE (Proteintech, Chicago, IL), purified from the antiserum and confirmed for its specificity on immunoblots as described previously (63).

Calvarial Organ Culture.

Intact cranial vaults that include frontal, parietal and interparietal bones were dissected from C57BL/6J mice, postnatal day 6. They were placed with the concave side down on a stainless steel grids in 12-well, and cultured in α -MEM containing 0.1% BSA, 50 μ g/ml ascorbic acid, and antibiotics-antimycotic (50 μ g/ml penicillin, 50 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B) for 24 hours. Murine calvaria were washed with phosphate buffered saline solution (PBS) and cultured for additional three days in the fresh medium containing various combinations of chemical and biochemical reagents, as indicated in the Result section. The calvarial organs were then assessed for osteoclast bone resorption, as described below.

ANK-mutant Mice.

C3FeB6 A/A^{W-J} - ANK^{ank}/J : (ank/ank) mice, and their wild-type: C3FeB6 A/A^{W-J} were obtained from the Jackson Laboratory, JAX stock #000200 (Bar Harbor, ME). Heterozygote breeders were used to generate and study ank/ank littermates, with genotypes analyzed by RT-PCR, as described previously (23). All mouse colonies were maintained in The Children Hospital of Philadelphia facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), under veterinary supervision and according to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell Culture.

Mouse macrophage cell line RAW 264.7 (ATCC, Manassas, VA) was maintained in DMEM containing 10% FBS supplemented with 1% nonessential amino acids, 1% L-glutamine and antibiotics-antimycotic. For osteoclast differentiation, the RAW cells were cultured for 7 days in the medium supplemented with 35 ng/ml RANKL.

RAW 264.7/C4 cells are stably transfected cell line for MITF expression (RAW^{MITF}) was a kind gift from Dr. David Hume (University of Edinburgh). RAW^{MITF} cells were maintained in a complete DMEM media as described above with geneticin (450 μ g/ml), added for selection.

Mouse bone marrow-derived monocytes (BMMs) were isolated from 8-week old C57BL/6J, C3FeB6A/ A^{W-J} - ANK^{ank}/J : (ank/ank) male mice, and their wild-type: C3FeB6A/ A^{W-J} , as described previously (2). Briefly, Mice were euthanized, and their tibiae and femurs were dissected using sterile technique, scraped off adherent soft tissue, and placed in ice-chilled complete media (α -MEM containing 10% FBS, and antibiotics-antimycotic). The ends of the bone were cut off, and the bone marrow was flushed out from the shaft, using PBS in syringe with a 26-gauge needle. The extruded bone marrow was triturated by repeatedly pipetting up and

down, and centrifuged. The cell pellet was re-suspended in complete medium and cultured overnight. Next day, non-adherent BMMs were collected, passed through a 40 μm nylon mesh cell strainer, counted, and plated for future experiments.

For the experiments requiring NaPP_i pretreatment, BMMs were plated at 2×10^5 cells/well in a 96-well and cultured for 48 hours in a complete medium supplemented with 25 ng/ml MCSF and PP_i (100-1000 μM). The pretreated cells were washed with PBS and switched to a complete medium supplemented with 35 ng/ml RANKL in addition to 25 ng/ml MCSF. Cells were fed every two days by replacing 50% of the medium with a fresh complete medium containing 2X concentration of RANKL and MCSF. After 7-day culture period, cells were analyzed for TRAP activity and TRAP⁺ osteoclasts as described below.

For the co-culture experiments requiring NaPP_i pretreatment, primary calvarial osteoblasts were isolated as described previously (1). Osteoblasts were plated at 1.8×10^4 cell/ cm^2 in 48-well plate with or without PP_i (100-1000 μM) or vitamin D_3 (10^{-8}M). The next day, BMM cells were plated at 2.8×10^5 cells/ cm^2 on top of osteoblasts. Co-cultured cells were fed on day 6, replacing half of the media with fresh media containing 2x concentration of PP_i or vitamin D_3 . Large osteoclasts were evident by day 9, then cells were fixed for measurement of TRAP activity, cell count, and size of TRAP⁺ osteoclasts. In parallel experiments, condition media (CM) were collected from osteoblasts cultures treated with PP_i (1000 μM) or vitamin D_3 (10^{-8}M) for 48 hours. The CM were concentrated using Amicon Ultra-15 centrifugal filter device (MilliporeSigma, St. Louis, MO) to collect proteins with molecular weight < 3K. BMMs were cultured in 96-well plate and treated with the CM. Culture media were replaced with fresh CM every 48 hours for total of 9 days cultures. Cells were fixed for measurement of TRAP activity and TRAP⁺ osteoclasts as described below.

For the experiments requiring the immunoblot detection of the phosphorylated forms of ERK, P38 MAPK and MITF, non-adherent BMMs were collected, plated in 6-well (1.6×10^6 cells/well) and cultured with 25 ng/ml MCSF for 48 hours. BMMs were now called bone marrow-derived macrophages. The BMM were serum and MCSF deprived overnight. BMMs were treated with NaPP_i (1000 μ M) and/or U0126 (10 μ M) or SB203580 (10 μ M) in serum free-medium for 0, 5, 10, 30, 60, and 90 minutes, and total proteins were isolated at the specific time points.

For cell survival assay experiments, BMMs and RAW^{MITF} were harvested as described previously, counted and plated in 24-well (4×10^5 cells/well) with 25 ng/ml MCSF for BMMs only, for 48 hours. Bone marrow-derived macrophages were treated with NaPP_i (1000 μ M) and/or U0126 (10 μ M) or SB203580 (10 μ M) in MCSF and serum deprived medium for 48 hours.

PU.1 cell culture - PU.1 low (PU.1^{lo}), and PU.1 high (PU.1^{hi}) fetal liver progenitor cells were a kind gift from Dr Harinder Singh (University of Chicago). PU.1-hematopoietic progenitors were maintained in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% calf serum, 50 μ M β -mercaptoethanol, 2 mM L-glutamine, IL-3 (5 ng/ml) and antibiotics-anti-mycotics. As PU.1 cell line comes from murine fetal liver hematopoietic cells, they stay floating for days in IMDM media. The PU.1 cells don't adhere to the culture plate unless cells were passaged for few times. Only the adherent PU.1 cells differentiate and the rest of floating cells became apoptotic. PU.1-cells were collected, counted, divided into 10 cm plates (3.5×10^6) and treated with NaPP_i (1000 μ M) and/or Hydroxytamoxifen (100 nM) in complete medium for 72 hours. The PU.1-cells treated versus untreated with NaPP_i and/or Hydroxytamoxifen were collected and centrifuged. Cell pellets were washed in 1X HBSS and total RNA was extracted from the cells, using TRIzol® Reagent (Invitrogen).

Calcium Measurement.

Calcium (Ca^{2+}) levels in conditioned media (CM) from cell and organ cultures were determined using the QuantiChrom™ Calcium Assay Kit (Bioassay System, Hayward, CA), as instructed by the manufacturer. Calcium concentrations have been calculated by measuring the net change (Δ) of total calcium in the CM minus calcium concentration in the initial culture media. Each treatment group consisted of 3-replicates.

Enzyme Immunoassay (EIA) for Collagen Helical Peptide (CTX-1).

The MicroVue™ Helical Peptide EIA kit (Quidel, San Diego, CA) was used to measure the level of a helical peptide containing the residues 620-633 of the type I collagen $\alpha 1$ chain or more formally carboxy-terminal collagen crosslinks, known by the acronym (CTX-1) in conditioned media from cell and organ cultures, as instructed by the manufacturer. Each treatment group consisted of 3-replicates.

Quantitative TRAP Activity Solution Assay and TRAP Staining.

BMM and osteoclasts cultures in a 96 well plate were fixed with 10% formalin for 15 minutes, washed in PBS, permeabilized with a mixture of methanol and acetone (1:1) for 5 minutes, and then air-dried. For TRAP; Tartrate-resistant acid phosphatase activity assays, the formalin-fixed cells were incubated with a TRAP buffer (52 mM of Na-tartrate in 0.1 M Na-acetate buffer, pH 5.2) containing 0.1 mg/ml of *p*-nitrophenyl phosphate (*p*-NPP) for one hour at 37°C. The reaction was stopped by adding 50 μl of 0.1 N NaOH to 100 μl of the reaction mixture and read for optical density at 405 nm. For TRAP staining, cell and organ cultures were incubated with the TRAP buffer containing 1.5 mM naphthol AX-MX phosphate (Sigma) and 0.5 mM Fast Red Violet LB salt (Sigma) for one hour at 37°C. TRAP positive cells with ≥ 3 nuclei were counted as mature osteoclasts. TRAP activity and staining data are averages of 3-replicates per condition.

ex vivo Assays for Osteoclastic Bone Resorption.

BMMs were cultured for 48 hours in the OsteoAssay™ Human Bone Plate (Lonza, Walkersville, MD) coated with a thin layer of adherent human bone particles in complete medium containing 25 ng/ml MCSF and PP_i (100-1000 μM). BMMs were then switched to RANKL and MCSF for additional 10 days as described above. Bone resorption was assessed by measuring Ca²⁺ and type I collagen helical peptide 620-633 (CTX-1) in conditioned media harvested at the end of culture. In parallel experiments, murine BMMs were cultured with MCSF and NaPP_i for 48 hours and then differentiated with RANKL for additional 8 days on BD BioCoat™ Osteologic™ MultiTest Slides (BD Biosciences, Bedford, MA) coated with a synthetic calcium phosphate film. The cell culture condition was same as that for the OsteoAssay™ Human Bone Plate. At the end of culture, multi-well slides were treated with 1 M NH₄OH to remove cells, washed 3 times with ddH₂O, and processed for von Kossa staining and visualized using Olympus IX71 microscope. The total number and area of resorption pits were captured and analyzed using image J program as described below.

In parallel experiments, co-culture of murine BMMs and osteoblasts were established on dentin discs (OsteoSite, Boldon, UK) in 96-well and treated as described above. On day 12, cells were removed with 1 M NH₄OH and discs were stained with 1% toluidine blue. The total bone resorption area was captured and analyzed using image J program as described below.

Cell survival Assays - Cell survival of BMMs and RAW^{MITF} cells were measured after 48 hours treatment with NaPP_i and/or U0126 or SB203580 in complete or serum-free medium as described previously. Twelve mM of MTT substrate (Thiazolyl Blue Tetrazolium Bromide) (Sigma) was added to the 24-well plate and was incubated at 37°C. Dimethyl sulfoxide (DMSO); (200 μl/well) was added to solubilize the formazan reaction and read for optical density at 570 nm.

Northern Blot Analysis.

Total RNA was isolated from cells using TRIzol® Reagent (Invitrogen) and confirmed for RNA integrity on an agarose gel. Five µg of RNA was separated on a 1% denaturing agarose gel, containing 2.2 M formaldehyde, and transferred onto a nylon membrane (Maximum Strength Nytran, Schleicher & Schuell, Keene, NH). The membrane was hybridized with cDNA probes for mouse *ANK* labeled with [α -³²P] dCTP using Rediprime™II (Amersham Biosciences, Piscataway, NJ), washed, and exposed on X-ray film (Denville Scientific, Metuchen, NJ).

RT-PCR.

BMM cultures were treated with NaPP_i at various concentrations (100-1000 µM) for 48 hours, as described above. Total RNA was isolated from cells using TRIzol® Reagent (Invitrogen) and confirmed for integrity on an agarose gel. Three µg of total RNA were reverse transcribed to cDNA in a volume of 10 µl containing the following: 150 ng Random Hexamers, 10 mM dNTP (Invitrogen), ddH₂O and incubated at 65°C for 5 minutes. Ten µl of the reaction mixture that contain 10X RT buffer, 25 mM MgCl₂, 0.1 mM DTT and 1 µl of SuperScript II RT (Invitrogen) was added to each RNA/primer sample and incubated at 25°C for 10 minutes and at 42°C for 50 minutes. The reaction was terminated at 70°C for 15 minutes then 1 µl of RNase H (Invitrogen) was added to each reaction and incubated at 37°C for 20 minutes. Two microliters aliquots of the generated cDNA was amplified in 50 µl of PCR reaction mixture containing 2X GoTaq® green master mix (Promega, Madison, WI), 100 nM of each primer and ddH₂O. PCR cycles consisted of the initial cycle of 95°C for 2 minutes followed by a three step-program of 95°C for 45 seconds, 56°C for 45 seconds and 72°C for 45 seconds for 25 cycles. Twenty µl of RT-PCR reactions were mixed with 2X DNA dye and loaded on 1% agarose gel, run for 30 minutes in ENDURO gel XL electrophoresis system (Labnet, Edison, NJ). Gel images were captured using SafeBlue imager system (MajorScience, Saratoga, CA)

Quantitative Real Time RT-PCR.

BMM and osteoblast cultures were treated with NaPP_i at various concentrations (100-1000 μM) for 48 hours, as described above. Total RNA was extracted from the cells, using TRIzol® Reagent (Invitrogen). After confirming the RNA integrity on an agarose gel, cDNA was prepared using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems™, Foster City, CA). PCR was performed with the 7500 Fast Real-Time PCR system (Applied Biosystems™) in duplicate of 20 μl reactions, each containing 1 μl cDNA reaction mix, 100 nM of each primer and 10 μl 2x SYBR® Green PCR Master Mix (Applied Biosystems™). qPCR cycles consisted of the initial cycle of 50°C for 2 minutes and the second cycle of 95°C for 10 minutes, followed by a two-step program of 95°C for 15 seconds and 60°C for 1 minute for 40 cycles. Using GAPDH as the internal control, relative gene expression among samples was calculated by comparison of C_t values. A dissociation curve was checked for each PCR run to confirm specific amplification of target RNA. The PCR primer sets used in this study are listed in Table 1.

Immunoblot Analysis - Protein was isolated in RIPA buffer (Sigma) and subjected to SDS-PAGE as described previously. Briefly, proteins isolated from primary cultures were mixed with Laemmli denature buffer (Bio-Rad, Hercules, CA) and heated at 100°C for 5 minutes. Samples were subjected to 10% SDS-PAGE in 1× TGS (Bio-Rad) for one and half hour with current settings at 30 mA per gel. Gel was then transferred to Hybond ECL nitrocellulose membrane (Amersham Bioscience, UK) by semi-dry transfer apparatus (Bio-Rad) for two hours with current settings at 50 mA per gel. The blot was incubated in blocking buffer (5% skim milk in TTBS) (Bio-Rad) for one hour. The blot was then incubated with the primary antibody diluted in fresh blocking buffer (1:1000) overnight at 4°C. The blot was washed in 1X TTBS for 3 times, 5 minutes each, and then incubated with HRP-conjugated secondary anti-mouse or anti-rabbit antibody diluted in fresh blocking buffer (1:5000), for one hour. The blot was washed in TTBS

for 3 times and the signal was developed using Western Lightening Plus-ECL kit (PerkinElmer, Waltham, MA) and detected on HyBlot CL X-ray films (Denville).

Morphometric Image Analysis.

All images were captured using a Canon EOS 5D 12.8 MP digital SLR camera with EF 24-105 mm f/4 L IS USM Lens. All images were analyzed with Java-based image-processing program developed at the NIH and the Laboratory for Optical and Computational Instrumentation, Image J (v. 1.49o). The percent area fraction of TRAP positive staining was calculated for calvarial organ cultures as follow: dividing six randomly selected area fraction (The percentage of pixels in the selected image that have been highlighted in red), at or above the limit of threshold, by the same area fraction for total number of pixels through the entire field. Each treatment group consisted of three different murine calvaria.

Osteoclast activity of cells on synthetic calcium phosphate and dentin discs was assessed by counting resorption pits from six randomly selected fields per well, three wells per treatment group. The total resorption area was computed using the area measurement function in the image J program.

Statistical Analysis.

Statistical analyses were performed using Prism 4 (GraphPad Software, La Jolla, CA). one-way ANOVA was used to analyze quantitative PCR, semi-quantitative RT-PCR, Northern, and Western blot densitometry results for differences in analytes between groups. Then, post hoc analyses were carried out using the Bonferroni test for multiple comparisons and adjusted p values are reported. An adjusted p value of < 0.05 was considered significant for all analyses. A *p*-value of less than 0.05 was considered statistically significant. Data are presented as the mean + standard error (SE) of at least three independent assays.

RESULTS

Pyrophosphate (PP_i) stimulates bone resorption *ex vivo*. Previous reports investigated the role of PP_i in skeletal hypo-mineralization (21). In this study we asked whether exogenous PP_i has a direct impact on bone resorption, using *ex vivo* murine calvarial bone dissected from C57BL/6 mice at postnatal day 6. The organ cultures were treated with sodium pyrophosphates (Na PP_i) for 72 hours at final concentrations of (0-1000) μM and assayed for the Ca²⁺ level in the conditioned media (CM). Lower doses of Na PP_i <100 μM have no effect in calvarial bone resorption (data not shown), however higher doses of Na PP_i ≥100 μM promoted osteolysis. As shown in Fig-1A, PP_i treatment increased the Ca²⁺ release from calvarial bone cultures into the media in a dose dependent manner ($p < 0.01$), compared to the untreated controls, suggesting that exogenous PP_i has a calciotropic role. To verify the previous data, we measured CTX-1; an indicator for osteoclast activity, in conditioned media. PP_i treatment increased the level of CTX-1 ($p < 0.01$), compared to the untreated controls (Fig-1B). These data were confirmed by the whole mount TRAP staining of calvarial organ cultures. PP_i treatment increased the TRAP staining intensity (Fig-1C). Calculated percent area fraction of the stained calvaria confirmed the increased TRAP staining of PP_i treated calvaria in a dose dependent manner ($p < 0.01$) (Fig-1D). Moreover, calvarial organs treatment with PP_i (1000 μM) increased size of TRAP positive multinucleated osteoclasts in the whole mount calvaria, compared to untreated control (Fig-1E). Sagittal sections of TRAP stained calvaria and counterstained with von Kossa showed larger multinucleated osteoclasts in large and deep resorption pit compared to untreated control (Fig-1F). To gain a further insight of PP_i effects on osteoclast activity in bone resorption, calvarial organs were treated with PP_i and/or quinazoline; an inhibitor of NFκB activation (59). Quinazoline inhibited PP_i-mediated bone resorption marked by reduced Ca²⁺ levels in a dose dependent manner ($p < 0.01$), compared to calvarial treatment with PP_i alone (Fig-1G). Taken

together, these data provide an evidence that exogenous PP_i stimulates osteoclast activity in bone resorption.

To investigate the calciotropic role of PP_i on cytokines-mediated bone resorption, we measured Ca^{2+} levels in conditioned media of calvaria treated with cytokines and/or PP_i . Interestingly, PP_i enhanced $\text{TNF}\alpha$ -mediated Ca^{2+} release in a dose dependent manner ($p < 0.01$), compared to $\text{TNF}\alpha$ or PP_i treated controls (Fig-2A). In contrast, the pyrophosphate analogue; disodium clodronates inhibited $\text{TNF}\alpha$ -mediated Ca^{2+} release in a dose dependent manner ($p < 0.01$), compared to $\text{TNF}\alpha$ treated controls (Fig-2B).

As previously indicated that transmembrane alkaline phosphatase; TNAP hydrolyzes PP_i into two inorganic phosphates (P_i) (58), we sought that Levamisole; TNAP inhibitor (14) will enhance PP_i effects. Levamisole has a no significant effect on PP_i -mediated Ca^{2+} release from calvarial bones ($p > 0.05$), compared to PP_i treated controls (Fig-2C). Moreover, treatment of calvarial organs with phosphonoformic acid (PFA); P_i transport inhibitor (17) and PP_i has no additive effects on PP_i -mediated Ca^{2+} release, compared to PP_i treated controls (Fig-2D).

To exclude the possibility of P_i action in PP_i -stimulated bone resorption, calvarial bones were treated with sodium phosphates (Na P_i) for 72 hours and assayed for the Ca^{2+} level in conditioned media. Interestingly, Na P_i treatment shifted the Ca^{2+} off media in a dose dependent manner ($p < 0.001$), compared to controls (Fig-2E). This effect was reversed when PFA was added to P_i -treated calvarial bones (Fig-2F). These data restrain the possibility of PP_i hydrolysis into P_i in the culture media, and also constrain the effect of P_i in PP_i -mediated Ca^{2+} release.

To further confirm the potential role of PP_i in bone resorption, calvarial organ cultures were treated with probenecid; an anion transport inhibitor, that decrease PP_i entrance intracellular. Interestingly, probenecid inhibited PP_i -mediated Ca^{2+} release in a dose dependent

manner ($p < 0.001$), compared to PP_i treated controls (Fig-2G). Taken together, these results unleash the authentic role of Na PP_i in stimulation of bone resorption.

Pyrophosphate enhances osteoclast differentiation and function *ex vivo*.

In the following study, we investigated whether PP_i has an effect on bone marrow-derived monocytes (BMMs) to differentiate into mature osteoclasts. Quantitative PCR analysis showed an increased TRAP expression in BMMs treated with exogenous PP_i in a dose dependent manner ($p < 0.01$), compared to control (Fig-3A). Next, BMMs were treated with PP_i (100-1000) μ M and stained for TRAP. As shown in Fig-3B, BMMs treated with PP_i demonstrated an increased number of TRAP positive mononuclear cells (osteoclast precursors; pre-osteoclasts) compared to control. Consistent with the previous data, TRAP activity was higher in BMMs treated with PP_i ($p < 0.001$) (Fig-3C). Next, we examined osteoclast differentiation in BMMs treated with PP_i . Interestingly, BMMs pretreated with PP_i showed a significant osteoclast differentiation, in response to RANKL, as indicated by larger TRAP-positive osteoclasts, compared to untreated counterparts (Fig-3D). These findings were supported with significant increase in TRAP activity, number and size of osteoclasts differentiated from PP_i -treated BMMs ($p < 0.001$) compared to control (Fig-3E, F, G, and H). To further support our data, BMMs were treated with PP_i and differentiated on glass slides then stained with Rhodamine for actin cytoskeleton. Consistently, the size of osteoclast was larger in BMMs pretreated with PP_i then differentiated with RANKL compared to untreated controls (Fig-3I). Moreover, the acting ring was sharp and prominent in osteoclasts treated with PP_i compared to control. These data suggest that PP_i treatment enhance osteoclastogenesis.

Given that PP_i promoted osteoclast differentiation, we explored the osteolytic activity of those mature osteoclasts. BMMs treated with PP_i were differentiated into osteoclasts in response to RANKL, over human bone chips. Photomicrographs of bone chips showed large TRAP-positive

osteoclasts with decrease of bone chips fragments in BMMs treated with PP_i compared to untreated controls (Fig-4A). These findings were confirmed with marked increase in TRAP activity, Ca²⁺ and CTX-1 levels in the CM of osteoclasts promoted from BMMs treated with PP_i ($p < 0.001$) in a dose dependent manner (Fig-4B, C, and D).

These data were supported by testing the osteolytic activity of PP_i promoted osteoclasts on bone discs. Photomicrographs of bone discs, after removal of osteoclasts, showed large pit area by PP_i-promoted osteoclasts (Fig-4E). These findings were supported by increased number of resorption pits, total resorbed surface area, and the average pit size in PP_i-promoted osteoclasts ($p < 0.01$) in a dose dependent manner (Fig-4F, G, and H). Collectively, these results show that PP_i-promoted-osteoclasts have a robust activity in bone resorption. Taken together, these data provide a pivotal evidence on the regulatory role of PP_i in osteoclast differentiation and function.

Pyrophosphate stimulates osteoclast differentiation and function in co-culture system.

As PP_i stimulated bone resorption in murine calvarial organs in the data described above, we investigated the contribution of osteoblasts in PP_i-enhanced pre-osteoclasts and osteoclastogenesis. Co-culture system of murine osteoblasts and BMMs was established and treated with vitamin D₃ (positive control) or PP_i (100-1000) μ M and stained for TRAP. Interestingly, BMMs co-cultured with osteoblasts and treated with PP_i demonstrated obvious osteoclast differentiation indicated by large TRAP⁺ mature osteoclasts compared to untreated control (Fig-5A). These data were supported with marked increase in TRAP activity, number and size of osteoclasts differentiated from PP_i-treated co-cultures ($p < 0.001$) compared to control (Fig-5B, C, and D). Surprisingly, the number and size of TRAP⁺ osteoclasts was not different ($p > 0.05$) in PP_i-treated co-culture, compared to vitamin D₃ positive control.

Given that PP_i promoted osteoclast differentiation in co-culture system, we test the osteolytic activity of those mature osteoclasts. The co-culture system of murine Osteoblasts and BMMs was established on dentin discs and treated with vitamin D₃ or PP_i (1000) μM. Photomicrographs of dentin discs displayed an obvious bone resorption marked by large resorption pits and tracks in PP_i-treated co-cultures compared to untreated controls (Fig-5E). The results were confirmed with marked increase ($p < 0.001$) in CTX-1 levels in the CM (Fig-5F) and the average total resorption area of dentin discs (Fig-5G) in PP_i-treated co-cultures compared to untreated control. Interestingly, the dentin discs displayed more complex bone resorption tracks in PP_i-stimulated osteoclasts, compared to vitamin D₃ positive control in the co-culture system (Fig-5E). These data suggest that PP_i-stimulated osteoclastogenesis and bone resorption is mediated at least in part by osteoblasts.

To gain insight on the underlying mechanism of PP_i-promoted osteoclasts in co-culture, we examined RANKL and OPG gene expressions in murine calvarial osteoblasts treated with PP_i (100-1000) μM or vitamin D₃ positive control. Quantitative (q)RT-PCR showed up-regulation of RANKL mRNA ($p < 0.01$) and RANKL/OPG ratio ($p < 0.001$) in osteoblasts treated with PP_i (1000) μM, compared to the untreated control (Fig-5H and I). Interestingly, the RANKL expression and RANKL/OPG ratio were not different ($p > 0.05$) in (1000) μM PP_i-treated osteoblasts compared to vitamin D₃ positive control. Although RANKL mRNA expression was not different, RANKL/OPG ratio was increased ($p < 0.05$) in lower dose (100) μM of PP_i-treated osteoblasts compared to the untreated control. In parallel experiments, we differentiated BMMs with concentrated condition medium (CM) from osteoblasts treated with PP_i. Our results revealed increased TRAP activity and TRAP⁺ mature osteoclasts treated with concentrated osteoblast CM compared to the untreated control (data not shown). Taken together, these data

showed a clear evidence that PP_i increased RANKL by osteoblasts that is responsible, at least in part, for PP_i-stimulated osteoclastogenesis and bone resorption.

Activation of RANK-RANKL pathway in Pyrophosphate-enhanced pre-osteoclasts.

Next, we explored the down-stream signaling pathway that mediates enhanced osteoclastogenesis in PP_i-treated BMMs. In this study, BMMs-treated PP_i were analyzed for the early transcription factors responsible for BMM commitment, using semi-quantitative RT-PCR (RT-PCR) analysis. The transcription factors; PU.1 and MITF responsible for hematopoietic stem cells commitment into osteoclast progenitors (47), showed an up-regulation in PP_i-treated BMMs in a dose dependent manner (Fig-6A). Similarly, the cFMS (MCSF receptor) and RANK receptor gene expressions were also increased in PP_i-treated BMMs (Fig-6A). These data were confirmed by quantitative (q)RT-PCR that showed up-regulation ($p < 0.01$) of cFMS and RANK in PP_i-treated BMMs (Fig-6B and C). Moreover, the transcription factors; c-Fos and c-Jun (AP-1 heterodimeric complex); downstream of MAPK signaling, also showed higher expression in PP_i-treated BMMs (Fig-6D and E). By examining the NF- κ B mRNA, our findings showed a ~2-fold increase of NF- κ B (P50/P65) expression in PP_i-treated BMMs compared to untreated control (Fig-6F).

Next, we examined activation of MAP-Kinase (ERK1/2 and P38) which is downstream of RANK receptor in osteoclast differentiation (37). BMMs were treated with PP_i and/or ERK1/2 inhibitor (U0126) and/or P38 inhibitor (SB203580) for up to 90 minutes. Western blot data showed increased phosphorylation of ERK1/2 (pERK) in PP_i-treated BMMs reaching the maximum at 10 minutes, compared to untreated control (Fig-6G). On the other hand, U0126 inhibited PP_i effect on ERK phosphorylation at 5 and 10 minutes (Fig-6G). Densitometric analysis confirmed ~3-fold inhibition of ERK phosphorylation in U0126-treated conditions, compared to PP_i treatment alone (Fig-6H). Similarly, phosphorylation of P38 was increased

significantly in PP_i-treated BMMs, compared to untreated control, whereas SB203580 inhibited PP_i effect on P38 phosphorylation (Fig-6I). Densitometry showed approximately ~2-fold decrease of P38 phosphorylation in SB203580-treated conditions, compared to PP_i treatment alone (Fig-6J). Collectively, these data support the activation of downstream signaling cascade of RANK and MAPK in PP_i-treated BMMs.

Pyrophosphate regulates PU.1 signaling pathway in BMMs.

As PU.1 is an early transcription factor in BMM pre-osteoclasts and has a pivotal role in regulation of cFMS; MCSF receptors (38), we investigated the role of PP_i in the downstream signaling pathway of cFMS in PP_i-treated BMMs. Interestingly, qRT-PCR showed that PU.1 is up-regulated (~2.5-fold) in PP_i-treated BMMs in a dose dependent manner (Fig-7A). Next, we examined the transcription factors; Egr-1 and Egr-2 that have been reported as downstream mediators of PU.1 in BMMs (16). qRT-PCR analysis of Egr-1 gene expression was ~2.5-fold higher in PP_i-treated BMMs in a dose dependent manner (Fig-7B). In contrast, Egr-2 expression was not different in PP_i-treated BMMs and control (Fig-7C). To support our data and define the precise role of PP_i on PU.1, we treated PUER cells with PP_i and examined cFMS and RANK, Egr-1, and Egr-2 expressions. PUER cells are murine hematopoietic precursor cells that have been retro-virally transduced to express PU.1 fused to the estrogen receptor (ER) (57). PUER^{hi} cells was used as appositve control to PUER^{lo} cells. RT-PCR data showed marked up-regulation of cFMS and RANK expressions, when hydroxytamoxifen (OHT) is added to PUER^{hi} cells, compared to untreated PUER^{hi} cells (Fig-7D). On the other hand, PUER^{lo} treated with PP_i showed relative up-regulation of cFMS and RANK expressions compared to untreated PUER^{lo} control by RT-PCR (Fig-7D). Densitometric analyses of RT-PCR data demonstrated ~2 to 6-fold increased levels of cFMS and RANK in PP_i-treated PUER^{lo} cells compared to untreated PUER^{lo}

control (Fig-7E, F). Interestingly, expression levels of cFMS and RANK were relatively similar ($p > 0.05$) in PP_i-treated PUER^{lo} cells and untreated PUER^{hi} cells (Fig-7D, E, F).

Next, we characterized expression of Egr-1 and -2 in PUER^{lo} and PUER^{hi} cells. Interestingly, Egr-1 expression was up-regulated in PP_i-treated PUER^{lo} cells, compared to untreated PUER^{lo} control (Fig-7D). These data were verified by densitometric analysis as ~3-fold increased level of Egr-1 in PP_i-treated PUER^{lo} cells (Fig-7G). However, Egr-2 expression no significant difference in PP_i-treated PUER^{lo} cells, untreated PUER^{lo} cells, and OHT treated or untreated PUER^{hi} cells (Fig-7D). These data were confirmed by densitometric analysis that showed relatively equal level of Egr-2 ($p > 0.05$) in all treated and untreated conditions (Fig-7H). Taken together, we conclude that PP_i stimulates PU.1 and its downstream signaling in osteoclast progenitors.

Pyrophosphate enhances differentiation and survival of pre-osteoclasts in MITF-dependent pathway.

Because we showed above that PP_i up-regulated MITF expression in BMM differentiation, we investigated if MITF signaling pathway might be involved in BMM survival as well. MITF is a key transcription factor, responsible for BMM commitment into osteoclast progenitors (41), we verified MITF expression in PP_i-treated BMMs. qRT-PCR data showed a dose-dependent increase of MITF expression ($p < 0.01$) in PP_i-treated BMMs, compared to untreated control (Fig-8A). MITF has several isoforms where MITF-A and -E have been reported to regulate macrophage polarization and osteoclastogenesis, respectively (5). Semi-quantitative RT-PCR data showed relevant higher expressions of MITF-A and -E in PP_i-treated BMMs in a dose dependent manner, compared to untreated control (Fig-8B). These data were confirmed by densitometry analysis that showed ~6 to 10-fold increase in MITF-A and MITF-E in PP_i-treated BMMs compared to untreated control (Fig-8C, D). Next, we examined survival of osteoclast

progenitors, where BMMs were primed with MCSF then treated with PP_i in serum deprived conditions for 48 hours. Interestingly, BCL-2, a pro-survival transcription factor, showed a dose-dependent increase ($p < 0.01$) in PP_i-treated osteoclast progenitors compared to untreated control (Fig-8E). To study the downstream mechanism by which PP_i stimulates BCL-2 expression, we examined BCL-2 in RAW 264.7 macrophage-cell lines, treated with PP_i in comparison to RAW cells over-expressing MITF. Interestingly, PP_i stimulated ~2-fold increase of BCL-2 in wild-type RAW cells compared to untreated control (Fig-8F). Moreover, BCL-2 expression in PP_i-treated wild-type RAW cells was comparable to its expression in RAW cells over-expressing MITF. Interestingly, ERK-1 and -2 inhibitor (U0126) inhibited BCL-2 expression in PP_i-treated wild-type RAW cells compared to PP_i alone or RAW overexpressing MITF controls (Fig-8F). Collectively, these data provide a supportive evidence that PP_i stimulates survival of osteoclast progenitors, MITF and BCL-2, an effect mediated by MAPK signaling pathway.

Several studies affirmed the regulatory effects of MAP-Kinase on MITF in osteoclast progenitors (41). In this study, we examined if activation of MAPK controls survival of osteoclast progenitors, BMMs were primed with MCSF then treated with PP_i and/or ERK-1 and -2 inhibitor (U0126), and/or P38 inhibitor (SB203580), in serum deprived conditions. Survival of osteoclast progenitor was enhanced ($p < 0.01$) when treated with PP_i compared to untreated control (Fig-8G). However, treatment of osteoclast progenitors with PP_i and U0126 decreased osteoclast survival significantly ($p < 0.01$) compared to PP_i alone and comparable to untreated control (Fig-8G). On the other hand, SB203580 has no effect on PP_i-enhanced survival of osteoclast progenitors, compared to PP_i treatment alone. Moreover, U0126 has a dominant inhibitory effect ($p < 0.05$) on survival of osteoclast progenitors when treated with PP_i and SB203580 (Fig-8G). To confirm that MAPK regulates MITF-mediated survival of osteoclast progenitors, we treated wild-type RAW cells and RAW cells overexpressing MITF with PP_i

and/or U0126, and/or SB203580 in serum deprived conditions. Consistent with the previous data, PP_i treatment increased survival of wild-type RAW cells ($p < 0.05$) compared to untreated control and comparable to RAW overexpressing MITF (Fig-8H). Moreover, U0126 inhibited survival of PP_i-treated wild-type RAW cells ($p < 0.05$) compared to PP_i alone or RAW overexpressing MITF controls. However, U0126 didn't inhibit survival of RAW overexpressing MITF. Following, SB203580 had no effects neither on PP_i-treated wild-type RAW cells nor on RAW overexpressing MITF (Fig-8H). Combined treatment with U0126 and SB203580 reduced survival of PP_i-treated wild-type RAW cells ($p < 0.05$) compared to untreated control. However, U0126 and SB203580 didn't affect survival of RAW overexpressing MITF (Fig-8H). To support our conclusions, we tested MITF phosphorylation in osteoclast progenitors by Western blot. Osteoclast progenitors were primed with MCSF then serum starved and treated with PP_i and/or U0126 for the indicated time points. Interestingly, PP_i treatment increased MITF phosphorylation on the Serine 307 residue at all-time points reaching the maximum at 90 minutes, compared to 0-minute control (Fig-8I). In accordance with the previous data, U0126 inhibited PP_i effects on MITF phosphorylation. Treatment of RAW cells with PP_i and/or U0126 showed similar results (data not shown). Densitometric analysis of the Western blot demonstrated ~4- to 8-fold increase in MITF phosphorylation at 60 and 90 minutes in PP_i-treated osteoclast progenitors compared to 0-minute control (Fig-8J). Moreover, U0126 inhibited MITF phosphorylation significantly compared to PP_i alone at 60 and 90 minutes. Taken together, we conclude that PP_i stimulates survival of osteoclast progenitors and MITF phosphorylation through ERK-1/2 signaling pathway.

Role of ANK in Pyrophosphate-mediated osteoclastogenesis.

The data above described the roles of PP_i in BMM survival, differentiation, and osteoclast function, so we pursued to examine the importance of ANK in regulation of PP_i effects on

osteoclastogenesis. ANK has been reported in various studies to transport inorganic PP_i across the cell membrane (64). In the beginning, we examined ank mRNA during osteoclast differentiation by Northern blot. Expression of ank mRNA demonstrated a temporal increase during osteoclast differentiation, coming from BMMs and RAW 264.7 cells, respectively (Fig-9A). Densitometry showed a significant increase of ank expression during osteoclast differentiation reaching the maximum at 11 ($p < 0.01$) with BMMs and 6 days ($p < 0.05$) with RAW cells, respectively (Fig-9B). Western blot supported the significant increase in ANK levels in BMMs differentiated into osteoclasts (data not shown). Consistent with the previous data, ANK protein showed a temporal increase in osteoclasts differentiated from RAW cells reaching the highest level at day 6 ($p < 0.01$), compared to 0-undifferentiated control (Fig-9C, D). Next, we tested if PP_i regulates ank in osteoclast progenitors. Interestingly, qPCR analysis of PP_i-treated BMMs showed a dose dependent increase in ank expression ($p < 0.01$), (Fig-9E).

To characterize the calciotropic role of PP_i transporter; ANK, we treated calvarial bone organs with PP_i and/or probenecid; an anion ANK inhibitor (12). PP_i stimulated bone resorption marked by Ca²⁺ release ($p < 0.01$), compared to untreated control (Fig-9F). However, probenecid inhibited PP_i-mediated Ca²⁺ release in a dose dependent manner ($p < 0.001$). To support these data, we treated BMMs with PP_i and/or probenecid then osteoclast progenitors were differentiated with RANKL. TRAP activity was increased ($p < 0.01$) in osteoclast differentiated from PP_i-treated BMMs, compared to RANK-L alone (Fig-9G). However, combined treatment of BMMs with PP_i and probenecid decreased TRAP activity of osteoclasts, compared to PP_i treatment alone ($p < 0.05$). To confirm the selective role of ANK in PP_i transport during osteoclast differentiation, we used BMMs isolated from *ank/ank* mice and their wild-type littermates. *ank/ank* mice are characterized with nonsense mutation of *ank* gene leading to loss of function in active PP_i transport (15). Treatment of *ank/ank* BMMs with PP_i didn't have an effect,

compared to upstroke increase ($p < 0.01$) in osteoclast differentiation in their wild-type PP_i-treated BMMs (Fig-9H). To investigate if ANK-PP_i transport regulates ERK-1/2 phosphorylation, WT-*ank* BMMs were treated with PP_i and/or probenecid in serum-deprived conditions. probenecid inhibited PP_i-mediated ERK-1/2 phosphorylation in WT-*ank* BMMs, compared to PP_i-treatment alone (Fig-9I). Densitometry showed a significant reduction ($p < 0.05$) of ERK-1/2 phosphorylation in probenecid/ PP_i treated WT-*ank* BMMs at 5 and 10 minutes (Fig-9J). Taken together, these data provide a strong evidence that ANK transporter regulates PP_i effects during osteoclast differentiation.

DISCUSSION

The role of pyrophosphates (PP_i) in tissue remodeling has been controversial. PP_i preserve the differentiated phenotype of articular chondrocytes, whereas several studies have documented PP_i generation, transport, and activity *in vivo*, most notably for chondrocytes and the tissues of joints (8). It is well established that pyrophosphates (PP_i) inhibit tissue calcification in normal conditions (45, 56). In bone, several studies reported PP_i as a negative regulator of osteoblast differentiation and matrix mineralization (3, 24). PP_i was reported to inhibit mineralization by direct binding to hydroxyapatite crystals and inhibiting alkaline phosphatase activity (ALP) (3). However, the significance of PP_i in osteoclast differentiation and function was yet to be determined. This led us to further investigate the impact of PP_i on osteoclastogenesis and so bone resorption.

In this study, we examined the direct effect of PP_i on calvarial bone resorption and osteoclast differentiation *ex vivo*. Concentration of PP_i reaches the saturation level $\geq 100 \mu\text{M}$ and form CPPD crystals in culture media. We hypothesized that CPPD microcrystals promoted bone resorption *in vitro*. Interestingly, only higher doses of PP_i that form CPPD microcrystals are

effective inducers of osteolysis. CPPD microcrystals stimulated osteolysis in calvarial organs over 3 days, as indicated by Ca^{2+} and CTX-1 data. It could be explained by CPPD microcrystals regulate pre-osteoclast differentiation at simultaneous stages in the context of cell milieu of calvaria. Another possibility is CPPD microcrystals increase RANKL expression by calvarial osteoblasts, promoting BMMs to differentiate to osteoclasts. The CPPD-mediated bone resorption might also due to direct activation of mature calvarial osteoclasts. These possibilities plus others warrant further analysis in future studies.

It is important to distinguish the effect of PP_i from bisphosphonates on osteoclast-stimulated bone resorption. Although bisphosphonates are stable analogue of pyrophosphates (PP_i), previous reports well documented bisphosphonates to inhibit bone resorption and treat patients with osteoporosis (33).

Consistent with the previous reports, indeed bisphosphonates decreased calvarial bone resorption. These data yet provide a clear evidence on the unique positive role of PP_i in osteolysis. Next, pyrophosphates anions (PP_i) are formed of two phosphate (P_i) ions linked by ester bond and it is the simplest condensed phosphate. In contrast to PP_i bone resorptive effects, P_i was found to inhibit osteolysis in consistent with the previous published reports of the inhibitory role of P_i on osteoclast differentiation and function (62). Although, P_i are released from PP_i by hydrolysis upon stimulation of the ectoenzyme alkaline phosphatase (ALP) on osteoblasts, however, inhibition of ALP was not different to PP_i effect on bone resorption. Suppression of ALP activity not only inhibit osteoblast function, but also decrease osteoclast differentiation factors and the overall bone remodeling (62). That may explain the trivial decline in osteoclast mediated Ca^{2+} release in PP_i -stimulated bone resorption. Another possibility is suppression of ALP has no role in formation of CPPD microcrystals. The PP_i effects on calvarial bone resorption was also clarified by its indirect effect on osteoblasts and release of osteoclast

differentiation factors. The note of presence of large mature osteoclasts when differentiated in co-culture with calvarial osteoblasts *ex vivo*, was explained, at least in part, by RANKL overexpression in PP_i-treated osteoblasts.

Initial depiction of BMMs showed hyper-responsiveness to RANKL, which can be confirmed by the high expression of RANK and cFMS receptor mRNA in pre-osteoclasts treated with PP_i. Vast reports have shown that overexpression of RANK receptors in osteoclast precursors, accelerated osteoclast differentiation in response to RANKL (30) whereas BMMs isolated from RANK ^{-/-} mice failed to respond to RANKL because of the apparent block in osteoclast differentiation (13). In addition, we have observed a marked increase in osteoclast number and size in BMMs treated with PP_i. Our data suggest that RANKL-induced signaling in PP_i-treated BMMs is enhanced. It is well documented that RANK receptors are regulated by the transcription factors PU.1 and MITF in osteoclasts (34). Moreover, Egr-1 and Egr-2 transcription factors are reported to work as downstream mediators of PU.1 in BMMs (16). Our study showed an increased expression of PU.1 in PP_i-treated-BMMs and -PUER^{lo} cells, which is correlated with increased RANK expression. Knowing that Egr-1 regulates osteoclastogenesis and Egr-1 deficiency block pre-osteoclast differentiation and osteoclast function (9). In our study, up-regulation of Egr-1 was evidenced in PP_i-treated BMM cultures. Conversely, Egr-2 expression was not altered in PP_i-treated BMMs. One study identified Egr-2 as a negative modulator of osteoclast differentiation using gain-of-function and loss-of-function approaches (31). On the other hand, Bradley and her group claimed the importance of Egr-2 in promotion of osteoclast survival through activation of MEK/ERK-dependent pathway (7). Our data suggest that stimulation of PU.1 dependent pathway in PP_i-treated BMMs, resulted in osteoclast differentiation in response to RANKL. As enhanced OCL survival can possibly add to the increased number and size of osteoclasts, we investigated BMM survival programs in these PP_i-

treated BMMs. Our data showed that survival of BMMs is enhanced and mediated by MAPK-MITF phosphorylation. Expression analysis of overall MITF mRNA was also high in PP_i-treated BMMs. There are 7 isoforms of MITF (40). Previous studies showed that MITF-A isoform is expressed in similar amounts in macrophages (BMMs) and osteoclasts, however MITF-E isoform is almost non-noticeable in BMMs, and its expression is considerably up-regulated during osteoclastogenesis (39). Moreover, MITF activation by serine phosphorylation at residue 307 was reported to recruit P38 MAPK and NFATc1 target genes during osteoclastogenesis (52). Because enhanced osteoclast survival could possibly contribute to increased osteoclast number and size, we investigated osteoclast survival pathway in these PP_i-treated conditions. Our data showed that PP_i stimulated pre-osteoclast survival and BCL-2 mediated by MAPK signaling pathway. It is well supported the crucial role of BCL-2 as anti-apoptotic in osteoclastogenesis and osteoclast survival (42). Indeed, our data supported up-regulation of BCL-2 in PP_i-treated pre-osteoclasts. Yamashita et al., reported the critical importance of BCL-2 in osteoclast survival using *Bcl-2*^{-/-} mice with increased bone mass due to at least in part declined osteoclastogenesis (61). Moreover, our study showed BCL-2 was downregulated in pre-osteoclasts treated with PP_i and ERK inhibitor (U0126). In previous study, Subramanian and Shaha reported ERK phosphorylation up-regulates BCL-2 expression, associated with increased human macrophage survival (54). The overall cell survival of PP_i treated pre-osteoclasts was decreased with ERK inhibitor, but not with P38 inhibitor. Interestingly, P38 was shown to induce the intrinsic apoptotic (Bim) molecule in human osteoclasts downstream of TGFβ1-Smad-2 signaling pathway. Furthermore, TGFβ1-induced osteoclast apoptosis was declined by inhibiting the Smad pathway with abolished Bim up-regulation following TGF-β stimulation (25). Collectively, survival of PP_i-treated pre-osteoclasts is enhanced, perhaps due to controlled up-regulation of MAPK-BCL-2 pathway.

Next, our data showed an increased RANK signaling in PP_i-treated BMMs resulted in activation of MAP kinase pathways through ERK (pERK) and P38 (pP38) phosphorylation. Indeed, ERK inhibitor (U0126) and P38 inhibitor (SB280) decreased ERK and P38 phosphorylation in PP_i-treated pre-osteoclasts. ERK1 was reported to stimulate osteoclast differentiation and their bone resorptive activity, as genetic disruption of ERK1 reduced numbers of osteoclast progenitors, and compromised pit formation (22). Moreover, it was reported that P38-MAPK activation is required for inducing osteoclast differentiation but not for their function (37).

Next, several reports well documented that RANK signaling activates MAP kinase, which in-turn activates AP-1 transcription factors (members of the Fra, Fos, and Jun) that activate downstream MITF, and thus, regulate osteoclastogenesis (36). Interestingly, our qPCR data showed up-regulation of c-Fos, c-Jun, and NFκB in PP_i-treated pre-osteoclasts. A previous report has demonstrated that deletion of the *c-FOS* gene resulted in osteopetrosis by arresting osteoclast differentiation at the macrophage stage and this defect was completely rescued by expressing Fos protein (18). The role of PP_i in activation of AP-1 warrant further investigation in future studies.

Next, it is well documented that PP_i molecules are transported across the cell membrane through ank transporter (8). Specific rare genetic bone disorder known as craniometaphyseal dysplasia (CMD), characterized by progressive sclerosis and hyperostosis of the craniofacial bones has been linked to *ANKH* nonsense mutations resulting in increased intracellular and decreased extracellular PP_i (19). Recent studies of CMD pointed to the role of PP_i in regulation of bone modeling/remodeling process (26). Thus, a decrease in extracellular PP_i may hinder normal bone remodeling, for instance, by inhibiting osteoclast differentiation or activity. In support of this notion, bone marrow-derived monocytes (BMMs) from a CMD knock-in mouse

(*ank^{ki}/ank^{ki}*) poorly differentiated to osteoclasts in cultures, compared to those from wild type mice (10). Consistent with the mouse data, the number of bone marrow-derived osteoclast-like cells from a CMD patient was only 40% of a normal individual, and they lacked osteoclast-specific vacuolar proton pump and the ability to absorb a dentin slice (60). Interestingly, our results showed temporal increase of ANK levels during osteoclast differentiation. Moreover, the ANK expression was escalated in PP_i enhanced pre-osteoclasts that possibly indicate a positive feedback loop between PP_i and ANK. In addition, probenecid; ANK inhibitor abrogated osteoclast differentiation and their function in PP_i enhanced pre-osteoclasts. These data are supported by previous study showing PP_i as a positive regulator of osteoclast differentiation events toward an osteoclast phenotype (32). Kim et al., reported the diminished osteoclast differentiation and activity of BMMs coming from *ank/ank* mutant mice (32). Since PP_i stimulated temporal expression of ANK during osteoclast differentiation, and on the other hand probenecid limited PP_i induced phosphorylation of ERK1/2. We conclude there's an increased possibility that PP_i induces ANK expression through MAPK phosphorylation that will require further investigations. Interestingly, we showed that extracellular PP_i was not able to rescue osteoclast differentiation in BMMs coming from *ank/ank* mutant mice compared to their wildtype littermates. These findings indicate that ANK is essential to transport PP_i intracellular that up-regulate signaling pathways critical for osteoclast differentiation and thereby function. This notion is supported by the decreased osteoclastogenesis in knock-in *ank^{ki}/ank^{ki}* mice probably due to reduced pre-osteoclast fusion and migration capability; disrupted osteoclast actin ring formation; and abnormal osteoblast–osteoclast communication. In conclusion, our findings provide an evidence that PP_i enhances pre-osteoclast commitment leading to increased osteoclast differentiation, survival, and function. The positive effects of PP_i on pre-osteoclast commitment are mediated by PU.1-dependent up-regulation of c-FMS, RANK and MAPK-

dependent phosphorylation of MITF signaling pathways (Fig-10). The PP_i-enhanced pre-osteoclasts may be at least in part due to temporal increase of ANK expression during osteoclast differentiation, despite the fact that extracellular PP_i did not rescue the osteoclast phenotype in *ank/ank* mutant mice. Generation of other genetically engineered mouse models, such as an osteoclast-specific knockout of *ank*, will help us to understand and identify the distinct signaling pathway(s) of ANK in osteoclast differentiation and thereby activity in bone resorption.

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FIGURE LEGENDS

Table-1.

Oligonucleotide primer sequences used in quantitative (q) real time polymerase chain reaction

Figure-1. Pyrophosphate activates bone resorption *ex vivo*. Murine calvaria were treated with PP_i (100-1000 μM) for 72hrs. The conditioned media (CM) were then harvested for calcium (Ca^{2+}) (A) and collagen peptide (CTX-1) (B) measurements and calvaria were stained for TRAP. (C) Macroscopic images of a whole representative calvarium treated with and without PP_i . Scale bars, 2500 μm . (D) Percent fraction area of the TRAP-stained-calvarial surfaces in PP_i -treatment and control. (E) Microscopic images of the TRAP-stained-calvaria showing multinucleated osteoclast on the calvarial surfaces (arrows) in PP_i -treatment versus control conditions. (F) Microscopic images of sagittal sections in TRAP-stained-calvaria and counterstained with von Kossa showing osteoclast in their resorption lacunae (arrows), in PP_i -treatment versus control. Scale bars, 200 μm . (G) Ca^{2+} assay of murine calvarial bones treated with PP_i (1000 μM) and/or quinazoline; NF κ B inhibitor (0.01-1) μM . Results are shown as mean and SEM. Experiments were repeated at least 3 times and showed comparable results. N=3 per group. The Mean value is represented in all graphs + SEM. The data points ($\diamond$) are represented in all graphs. *, $p < 0.05$; **, $p < 0.01$.

Figure-2. Pyrophosphate action on bone resorption is selective and specific. Ca^{2+} measurements in the 72-hour CM of murine calvaria treated with TNF α (20 ng/ml) and/or PP_i (500-1000) μM in (A), TNF α (20 ng/ml) and/or bisphosphonate (25-50) μM in (B), PP_i (1000) μM and/or levamisole (10-50) μM in (C), PP_i (1000) μM and/or PFA (100-500) μM in (D), P_i (500-2000) μM alone in (E), P_i (2000) μM and/or PFA (100-500) μM in (F). Experiments were

repeated at least 3 times and showed similar results. N=3 per group. The Mean value is represented in all graphs + SEM. The data points (◇) are represented in all graphs. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$.

Figure-3. Pyrophosphate enhances osteoclastogenesis *ex vivo*. (A) BMMs were treated with PP_i (100-1000) μM for 48 hours and examined for TRAP mRNA expression by quantitative (q)RT-PCR. (B-C) BMMs were treated with PP_i (100-1000) μM and stained for TRAP. (B) Microscopic images show higher number of TRAP-positive mono-nuclear cells in PP_i-treated BMMs (arrows), compared to control. (C) TRAP activity was significantly higher in the PP_i-treated conditions. (D-I) BMMs were treated with PP_i (100-1000) μM then differentiated with RANK-L (35 ng) for 7 days. (D) Microscopic images show more number and larger size osteoclasts in PP_i-treated BMMs (arrows), compared to control. Parameters of osteoclast differentiation that include; TRAP activity (E); count of TRAP-positive osteoclasts (≥ 3 nuclei) (F); differential count of osteoclasts ≥ 20 nuclei, and (G); size of TRAP-positive osteoclasts (H) are significantly increased in PP_i-treated BMMs, compared to control. (I) BMMs were treated with PP_i (1000) μM then differentiated with RANK-L on glass slides and immunostained with rhodamin phalloidin for the actin ring (red) and counterstained with DAPI for the nuclei (blue). Microscopic images show larger size osteoclasts with distinct acting ring in PP_i-treated BMMs. Experiments were repeated at least 3 times and showed comparable results. N=6 per group. The Mean value is represented in all graphs + SEM. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$. Scale bars, 200 μm.

Figure-4. Pyrophosphate stimulates osteoclast function *ex vivo*. (A-D) BMMs were treated with PP_i (100-1000) μM then differentiated with RANK-L (35 ng) over human OsteoAssay bone chips and examined for TRAP. (A) Microscopic images show larger TRAP+ osteoclasts (arrows) and less bone chips (asterisk) in PP_i-treated BMMs, compared to control. (B) TRAP activity of

differentiated osteoclasts are significantly up in PP_i-treated BMMs. Parameters of osteoclast function that include; Ca²⁺ (C), and CTX-1 (D) in the CM are increased in PP_i-treated BMMs, compared to control. (E-H) BMMs were treated with PP_i (100-1000) μM then differentiated with RANKL (35 ng) over OsteoAssay discs. (E) Microscopic images of von kossa stained discs, after removal of osteoclasts, show increased number and size of resorption pits in PP_i-treated BMMs. Parameters of bone resorptions that include; number of resorption pits (N. pits) (F), total resorption area (Tt. Ar) (G), and average pit size per osteoclast (pit Ar) are higher in PP_i-treated BMMs, compared to control. Experiments were repeated at least 3 times and showed similar results. N= 6 per group. N= 6 per group. The Mean value is represented in all graphs + SEM. *, $p < 0.05$; **, $p < 0.01$. Scale bars, 200 μm.

Figure-5. Pyrophosphate stimulate osteoclastogenesis in BMMs-osteoblast co-cultures.

Murine BMMs were co-cultured with calvarial osteoblasts and with treated with vitamin D₃ (10⁻⁸ M); positive control or PP_i (100-1000) μM for 9 days and examined for TRAP+ osteoclasts. (A) Microscopic images show more number and larger size osteoclasts in vitamin D₃ and PP_i-treated BMMs (blue arrows), compared to control. Parameters of osteoclast differentiation that include; TRAP activity (B); count of TRAP+ osteoclasts (≥ 3 nuclei) (C); size of TRAP+ osteoclasts (D) are significantly increased in PP_i-treated BMMs, compared to control. (E-G) BMMs were co-cultured with osteoblasts on dentin discs and treated for 12 days, as described above. (E) Microscopic images show active osteoclasts (yellow arrow heads) in their lacunae. Note the osteoclast resorption tracks, stained with toluidine blue (yellow arrows) that extend from lacuna. Parameters of osteoclast function that include; CTX-1 in the CM (F) and average bone resorption area (G) are significantly higher in BMMs treated with vitamin D₃ and PP_i (1000) μM, compared to (-) control. (H and I) comparative quantitative (q)RT-PCR analysis of murine calvarial osteoblasts, treated with vitamin D₃ (10⁻⁸ M) or PP_i (100-1000 μM) showing

increased RANKL mRNA expression (**H**) and RANKL/OPG ratio (**I**) in vitamin D₃ and PP_i-treated osteoblasts, compared to (-) control. Experiments were repeated at least 3 times and showed similar results. Data presented in all graphs represent Mean + SEM in duplicate per sample. N=4 per group. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Scale bars, 200 μ m.

Figure-6. Pyrophosphate stimulates osteoclast markers and MAP-K pathway. (A) Semi-quantitative RT-PCR analysis of PU.1, MITF transcription factors, cFMS, RANK receptors in PP_i-treated BMMs. **(B-F)** Comparative qRT-PCR analyses of mRNA expressions from three replicates were performed for cFMS (**B**), RANK (**C**), c-Fos (**D**), c-Jun (**E**), and NF κ B (**F**). Note the significant increase in all gene expressions with PP_i treatment. **(G-J)** BMMs were serum starved and treated with PP_i for the indicated time points. **(G)** Representative Western blot of the phosphorylated (p) ERK1/2 in BMMs treated with PP_i and/or ERK inhibitor (U0126). **(H)** Densitometric analyses of the pERK1/2 (44 and 42KDa) from three replicate Western blots after normalization to ERK1/2 (44 and 42KDa) levels show the ratio of pERK1/2 to ERK1/2 at 5 and 10 minutes. **(I)** Representative Western blot of the phosphorylated (p) P38 in BMMs treated with PP_i and/or P38 inhibitor (SB203580). **(J)** Densitometric analyses of the pP38 (41KDa) from three replicate Western blots after normalization to P38 (41KDa) levels show the ratio of pP38 to P38 at 5 and 10 minutes. Experiments were repeated at least 3 times and showed comparable results. N=3 per group. The Mean value is represented in all graphs + SEM. *, $p < 0.05$; **, $p < 0.01$.

Figure-7. Pyrophosphate-enhanced osteoclastogenesis is mediated by PU.1. (A-C)

Comparative qRT-PCR analyses of mRNA collected from PP_i-treated BMMs (three replicates) were performed for the following genes: PU.1 (**A**), Egr-1 (**B**), and Egr-2 (**C**). Note the PP_i-dose dependent increase in all gene expressions except Egr-2. **(D-H)**. PUER^{lo} and PUER^{hi} stem cells were treated with PP_i (1000) μ M and/or OHT for 48 hours. **(D)** Semi-quantitative RT-PCR analysis of cFMS, RANK, Egr-1 and Egr-2 gene expressions in PUER^{lo} treated with PP_i and

PUER^{hi} treated with OHT compared to untreated PUER^{lo} and PUER^{hi} cells. Densitometric analyses show the following gene expressions in PP_i-treated PUER^{lo} and OHT-treated PUER^{hi} compared to untreated PUER^{lo} cells: cFMS (**E**), RANK (**F**), Egr-1 (**G**), and Egr-2 (**D**).

Experiments were repeated at least 3 times and showed similar results. Data presented in all graphs represent Mean + SEM in duplicate per sample. * = $p < 0.05$; ** = $p < 0.01$.

Figure-8. Pyrophosphate-enhanced pre-osteoclast survival is mediated by MITF. (**A**) qRT-PCR analysis of total MITF in PP_i-treated BMMs. (**B-D**) Semi-quantitative RT-PCR analysis of MITF-A and -E isoforms in PP_i-treated BMMs. Densitometric analyses of the RT-PCR from three replicates show higher expressions of MITF-A (**C**), and MITF-E (**D**), compared to untreated control. (**E**) qRT-PCR analysis of BCL-2 in BMMs treated with PP_i (100-1000 μ M) in serum-free conditions. (**F**) qRT-PCR analysis of BCL-2 in RAW^{MITF} or RAW^{WT} cells with PP_i (1000 μ M) and/or U0126 in serum-free conditions. (**G**) Survival assay of BMMs treated with PP_i (1000 μ M) and/or U0126 and/or SB203580 for 48 hours in serum-free conditions. Notice the lower cell viability with PP_i and U0126. (**H**) Survival assay of RAW 264.7^{WT} or RAW^{MITF} cells treated with PP_i and/or U0126, and/or SB203580 for 48 hours in serum-free conditions. Notice the decreased cell viability of RAW^{WT} with PP_i and U0126, compared to no effect on RAW^{MITF} cells. (**I**) Representative Western blot of the phosphorylated (p) MITF in BMMs treated with PP_i and/or U0126 in serum-free conditions. (**J**) Densitometric analyses of the pMITF (52KDa) from three replicate Western blots after normalization to MITF (52KDa) levels show the ratio of pMITF to MITF at 60 and 90 minutes. Experiments were repeated at least 3 times and showed similar results. N=3 per group. Data presented in all graphs represent Mean + SEM in duplicate per sample. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Figure-9. ANK regulates Pyrophosphate effects in osteoclast differentiation and function.

(**A**) Northern blot of ank mRNA in osteoclasts differentiated from BMMs or RAW 264.7 cells

for the indicated time points. **(B)** Densitometry analyses of the ank from three replicate Northern blots show the ratio of ank mRNA to internal control rRNA. Note the highest ank mRNA in osteoclasts at 11 days for BMMs and 6 days for RAW. **(C)** Representative Western blot of ANK protein in osteoclasts differentiated from RAW cells. **(D)** Densitometric analyses of the ANK (48KDa) from three replicate Western blots after normalization to GAPDH (37KDa) levels. **(E)** qRT-PCR analysis of ank in BMMs treated with PP_i (100-1000) μ M for 48 hours. **(F)** Ca²⁺ measurements in the 72-hour condition medium (CM) of murine calvaria treated with PP_i (1000 μ M) and/or probenecide (500-1000) μ M. **(G)** TRAP activity of BMMs treated with PP_i (1000 μ M) and/or probenecide then differentiated with RANK-L (35 ng) for 7 days. **(H)** TRAP activity of *ank/ank* and WT BMMs treated with PP_i (100-1000) μ M for 48 hours then differentiated with RANK-L. **(I)** Representative Western blot of pERK in WT BMMs treated with PP_i (1000 μ M) and/or probenecide (1000 μ M). **(J)** Densitometric analyses of the pERK1/2 (44 and 42KDa) from three replicate Western blots after normalization to ERK (44 and 42KDa) levels show the ratio of pERK to ERK at 5 and 10 minutes. Experiments were repeated at least 3 times and showed similar results. N=3 per group. Data presented in all graphs represent Mean + SEM in duplicate per sample. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Figure-10. Schematic diagram of Pyrophosphate effects on osteoclast differentiation, survival and function. Pyrophosphate promotes osteoclast differentiation mediated by PU.1, Egr-1, and up-regulation of cFMS and RANK receptors. Pyrophosphate also enhances osteoclast survival through MAPK and MITF phosphorylation and up-regulation of the downstream BCL2. Pyrophosphate promotes osteoclastogenesis indirectly by stimulating RANKL production by osteoblasts. Pyrophosphate improves the OCL activity in bone resorption perhaps by up-regulation of ank and modulating the actin cytoskeleton through unknown mechanism yet need to be determined.

Figure-1

Figure-2

Figure-3

Figure-4

Figure-5

Figure-7

Figure-8

Figure-9

Figure-10

Gene name	Primer sequence (5'-3')	Amplicon size (bp)	GeneBank accession	Amplicon T _m (°C)
TRAP	F: GACCACCTTGGCAATGTCTCTG R: TGGCTGAGGAAGTCATCTGAGTTG	176	NM_001102405	59.9 54.9
RANKL	F: CACACCTCACCATCAATGCTGC R: GAAGGGTTGGACACCTGAATGC	194	NM_011613.3	58.9 58.4
OPG	F: AGTCCGTGAAGCAGGAGTG R: CCATCTGGACATTTTTTGCAA	171	NM_008764	57 52.5
cFMS	F: GAGAGCATCTTTGACTGCGTCTAC R: ACAGGCTGGGCCATTTGGTA	164	NM_001037859	57.4 59.4
RANK	F: CGACTGGTTCCTGCTCCTAATC R: TTCTATTTCTTTTGTCTAGGTGCTTTTC	112	NM_009399	57.3 54.4
cFOS	F: GGAGCCAGTCAAGAGCATCAG R: GGGTAGGTGAAGACAAAGGAAGAC	179	NM_010234	57.8 57.1
cJun	F: GCGTTAGCATGAGTTGGCAC R: CGCATGAGGAACCGCATCGC	150	NM_010591	55.7 56.8
NFκB	F: GCCAGCTTCCGTGTTTGTTTC R: TCAGGGTAGTAGAGAAAGGGTTTCG	80	NM_008689	57.2 57.6
PU.1	F: AGAAGCTGATGGCTTGGAGC R: GCGAATCTTTTTCTTGCTGCC	70	NM_011355	57.7 55.4
EGR1	F: GACGAGTTATCCCAGCCAAA R: GGCAGAGGAAGACGATGAAG	202	NM_007913	54.8 55.3
EGR2	F: GAAGGAACGGAAGAGCAGTG R: ATCTCACGGTGTCTTGGTTC	194	NM_001373987	55.7 56.8
MITF	F: GGACTTTCCTTATCCCATCCA R: GTTCTTGCTTGATGATCCGATTC	70	NM_001113198	56.3 54.2
BCL2	F: CTGGCATCTTCTCCTTCCAG R: GACGGTAGCGACGAGAGAAG	183	NM_009741	55.1 57
ANK	F: TAAACTGGCGAACACAAGCAA R: AAGAAGGAGAAGATACGCAGAGGAA	201	NM_020332	55.4 57.3
GAPDH	F: CCGGTGCTGAGTATGTCGTGGAG R: GCAGAAGGGGCGGAGATGAT	100	NM_008084.2	56 56

Table-1