# Supplementary Data

## Establishment of Vitro Bone Resorption Model

### *Materials and methods*

RAW264.7 cell culture and differentiation. Preosteoclast RAW264.7 cells (ATCC, Cell Bank of Chinese Academy of Sciences) were cultured in the Dulbecco's minimum essential medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (Gibco) and antibiotics (10 U/L penicillin and 100 mg/L streptomycin) in a humidified atmosphere containing 5% $CO_2$/95% air at 37°C and the medium was changed every two days. Exponentially growing cells were seeded in 24-well plates ($2\times10^3$/well) for tartrate-resistant acid phosphatase (TRAP) staining, TRAP activity analysis and establishment of the bone resorption model or in six-well plates ($2\times10^4$/well) for reverse transcriptase–polymerase chain reaction (RT-PCR) analysis. Cells were treated with the receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) (50 ng/mL each; Peprotech) to promote differentiation into an osteoclast phenotype.

TRAP staining. Cells treated with RANKL and M-CSF were stained for TRAP (Sigma) on days 1, 3, 5, and 7 using a previously described method.[1] Observation of more than two nuclei, blue nuclei and red cytoplasm were considered to be indicative of active osteoclasts.[2]

TRAP activity assay. The secreted TRAP activity is a marker of the osteoclast resorption capacity.[3] After 3, 7, 10, and 14 days of stimulation with RANKL and M-CSF, the culture medium was removed and 0.2%Triton X-100 was used to lyse the cells. The relative TRAP activity compared with the control group was determined using the TRAP detection solution (Sigma) as described previously.[4] The total protein content was measured at 570 nm using the BCA assay kit (Pierce). The culture medium was discarded and a radio-immunoprecipitation assay buffer (RIPA; Beyotime) was added to lyse the cells. The supernatant was collected and centrifuged (14,000 rpm, 15 min, 4°C). Protein concentrations were determined using the BCA assay kit (Pierce) and the concentration was calculated according to the standard curve. The relative TRAP activity was normalized against the level of total protein content.

RT-PCR analysis of osteoclast-specific genes. The expression of osteoclast-specific genes (*TRAP* and *cathepsin K*) was analyzed by RT-PCR. Total RNA was extracted from RAW264.7 cells using the TRIzol reagent (Invitrogen), and an aliquot (2 μg) was used as a template for cDNA synthesis using the Murine Moloney Leukemia Virus-Reverse Transcriptase (Promega). The PCR conditions were as follows: 35 cycles of preheating at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 68°C for 60 s, followed by 5 min at 70°C and 30 min at 4°C. Amplified samples were analyzed by 2% agarose gel electrophoresis and observed under UV light (Gel Doc 2000; Bio-Rad). The band intensity on PCR photographs was quantified by ImageJ 1.44p. The PCR product was normalized to the intensity of the band for the house-keeping gene *β-actin* and expressed as a ratio of the relative band intensity. The primer sets used were *TRAP* (forward: AGCAGCCAAGGAGGACTACGTT; reverse: TCGTTGATGTCGCACAGAGG), CtsK (forward: TTAATTTGGGAGAAAAACCT; reverse: AGCCGCCTCCA CAGCCATAAT), *β-actin* (forward: TTGTTACCAACTGGG ACGACATGG; reverse: GATCTTGATCTTCATGGTGCT AGG).

Preparation of cortical bone slices. Cortical bone slices (5 mm diameter) of fresh bovine femur slices (0.1 mm thick) were cut with an Isomet low-speed saw and emery stone, cleaned by sonication (×3, 10 min), and sterilized at 120°C, 0.4 MPa before incubation in the regular DMEM at the temperature of 4°C.

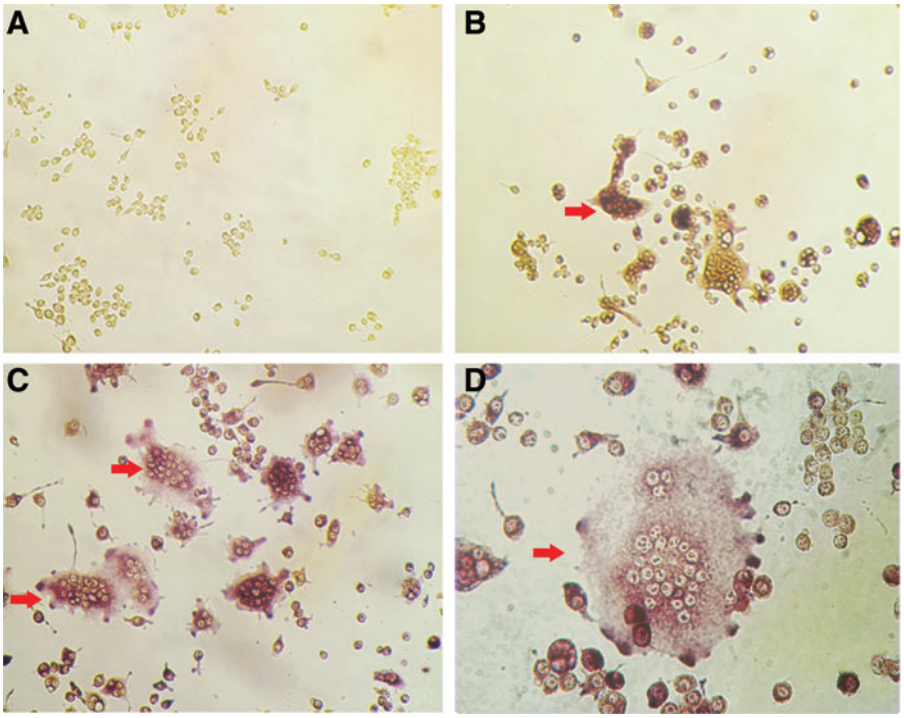
Establishment of bone resorption models *in vitro*. RAW264.7 cells were seeded in 24-well culture plates (Corning, USA) and cultured with the prepared cortical bone slices. After 24 h, cells were treated with RANKL and M-CSF (50 ng/mL each). The culture medium was changed on days 3, 5, 7, 10, 12, and 14. Supernatants were centrifuged (5000 rpm, 4°C, 10 min), filtered through a 0.22-μm membrane filter (polyether sulfone; Millipore), and stored at −20°C.

Scanning electron microscopy analysis of cortical bone slices. Scanning electron microscopy (SEM) was used to confirm the formation of resorption lacunae within osteoclasts cultured with bovine cortical bone slices. The bone slices from the bone resorption models on days 7 and 14 were fixed with 2.5% glutaraldehyde in a 0.1 mol/L cacodylate buffer (pH 7.3) (2 h, 4°C). Subsequently, bone slices were dehydrated with serially graded ethanol solutions (50%, 70%, 80%, 90%, 95%, 100%), critical point dried, coated with gold: platinum (90:10), and viewed using a scanning electron microscope (PHILIPS XL30 ESEM).

## Results

### *Establishment of the bone resorption model* in vitro

TRAP-positive, multinucleated cells with strong Alizarin Red S staining of the cytoplasm were observed on day 3. The number of these cells was increased on day 7 following treatment with RANKL and M-CSF. Similar cells were not detected in the negative control group (Supplementary Fig. S1).



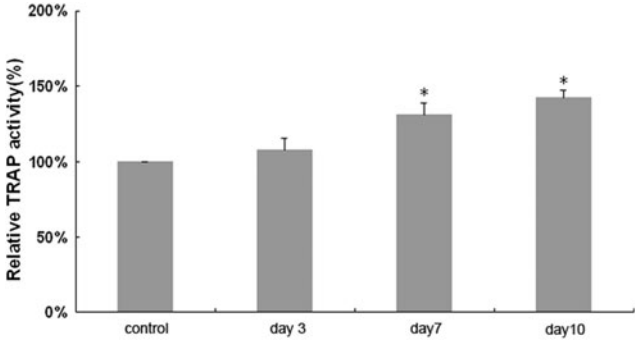

**SUPPLEMENTARY FIG. S1.** Tartrate resistant acid phosphatase (TRAP) staining of the RAW264.7 cells cultured with RANKL and M-CSF (50 ng/mL each). TRAP-positive multinucleate cells in: **(A)** the negative control group (×100); **(B)** day 3; **(C)** day 7; **(D)** day 7 (×400). Arrow indicates multinucleate cells.

The secreted TRAP activity is a marker of the osteoclast resorption capacity.[5] After 3 days of stimulation, the TRAP activity was increased significantly compared with the negative control group. The TRAP activity increased steadily from day 7 to 14 (Supplementary Fig. S2).

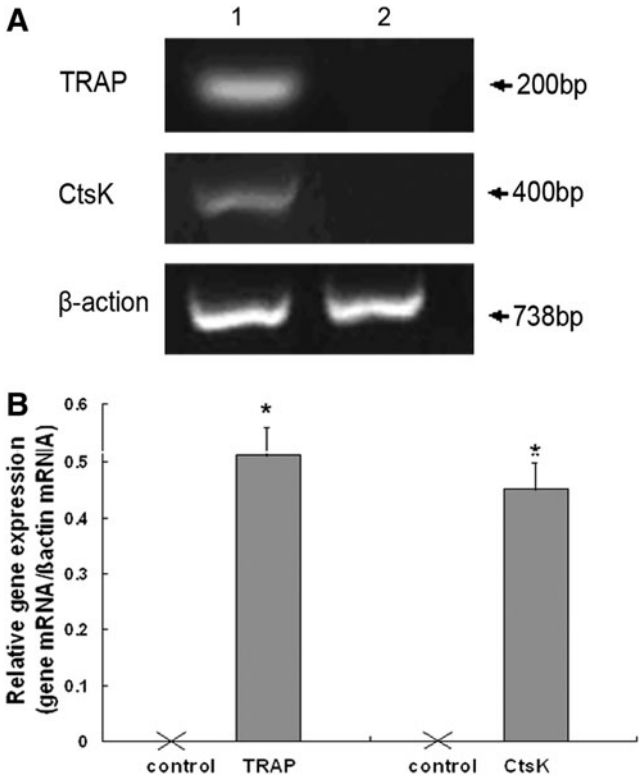
Expression of *cathepsin K* (*CtsK*) and *TRAP* gene expression, which are markers of the osteoclast activity, was analyzed to characterize osteoclast induction.[6] RANKL and M-CSF induced *CtsK* and *TRAP* expression in RAW264.7 cells, while expression of these genes was not detected in uninduced control cells (Supplementary Fig. S3).

### *Bone resorption lacunae*

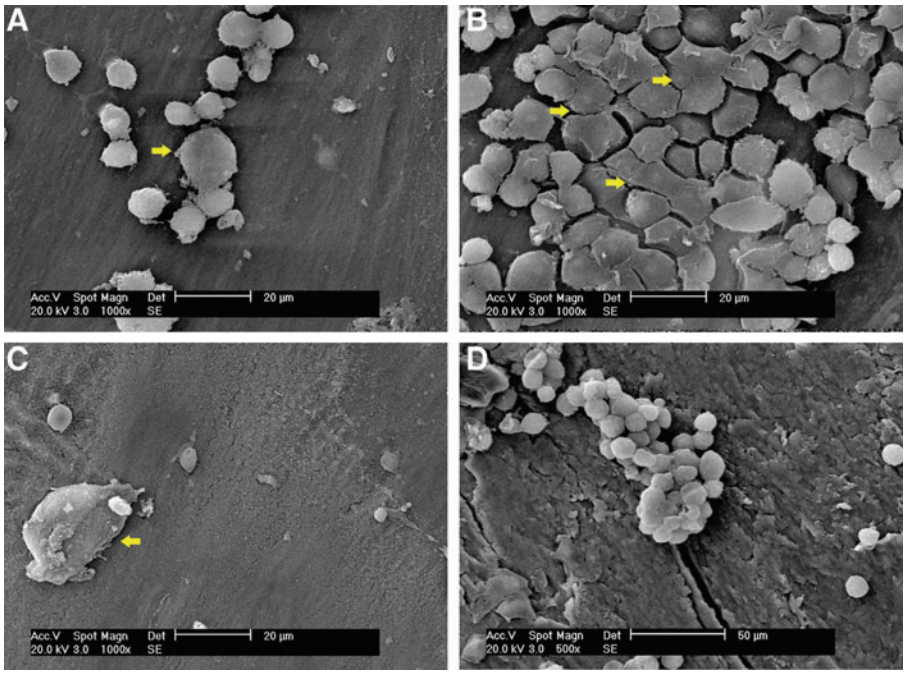
Multinucleate cells, as well as many mononuclear cells, were observed by SEM following culture of RAW264.7 cells




**SUPPLEMENTARY FIG. S2.** The relative changes in TRAP activity in RAW264.7 cells stimulated with RANKL and M-CSF (50 ng/mL each) for 3, 7 and 10 days. Values represent the means ± SD (n = 4). (*, P < 0.05 vs. control).




**SUPPLEMENTARY FIG. S3.** RT-PCR analysis of *TRAP* and *CtsK* expression. Representative results of three repeated experiments. **(A)** Lane 1: RAW264.7 cells treated with RANKL and M-CSF; Lane 2: negative control. **(B)** Densitometric quantification of mRNAs expressed as the ratio of *β-actin* expression in the same sample.




**SUPPLEMENTARY FIG. S4.** Scanning electron microscope images of slices of calcified bone. Preosteoclast multinucleate giant cells were observed in bone cortical slices on **(A)** day 7 (×1000) of co-culture with RAW264.7 cells supplemented with RANKL and M-CSF. **(B)** The number of multinucleated cells was significantly increased on day 14 (×1000). **(C)** Resorption lacunae were observed under the multinucleate cells (×1000). **(D)** Negative control (×1000). Arrow indicates multinucleate cells.

and bone cortical slices in a medium containing RANKL and M-CSF. Some resorption lacunae under the multinucleated cells were observed on the bone slices. No multinucleate cells and resorption lacunae were detected in the negative control (untreated RAW264.7 cells) (Supplementary Fig. S4).

## References


1. Galli, C., Passeri, G., and Macaluso, G.M. FoxOs, Wnts and oxidative stress-induced bone loss: new players in the periodontitis arena? J Periodont Res **46,** 397, 2011.
2. Zheng Y., Wu G., Zhao J., Wang L., Sun P., and Gu Z. rhBMP2/7 heterodimer: anosteoblastogenesis inducer of not higher potency but lower effective concentration compared with rhBMP2 and rhBMP7 homodimers. Tissue Eng PartA **16,** 879, 2010.
3. Martin, T.J., and Sims, N.A. Osteoclast-derived activity in the coupling of bone formation to resorption. Trends Mol Med **11,** 76, 2005.
4. Kubota, K., Sakikawa, C., Katsumata, M., Nakamura, T., and Wakabayashi, K. Platelet-derived growth factor BB secreted from osteoclasts acts as anosteoblastogenesis inhibitory factor. J Bone Miner Res **17,** 257, 2002.
5. Nomura, M., Yoshimura, Y., Kikuiri, T., Hasegawa, T., Taniguchi, Y., Deyama, Y., Koshiro, K., Sano, H., Suzuki, K., and Inoue, N. Platinum nanoparticles suppress osteoclastogenesis through scavenging of reactive oxygen species produced in RAW264.7 cells. J Pharmacol Sci **117,** 243, 2011.
6. Hoemann, C.D., El-Gabalawy, H., and McKee, M.D. *In vitro* osteogenesis assays: influence of the primary cell source on alkaline phosphatase activity and mineralization. Pathol Biol **57,** 318, 2009.