Pseurotin A inhibits osteoclastogenesis and prevents ovariectomized-induced bone loss by suppressing reactive oxygen species.

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Supplementary Results

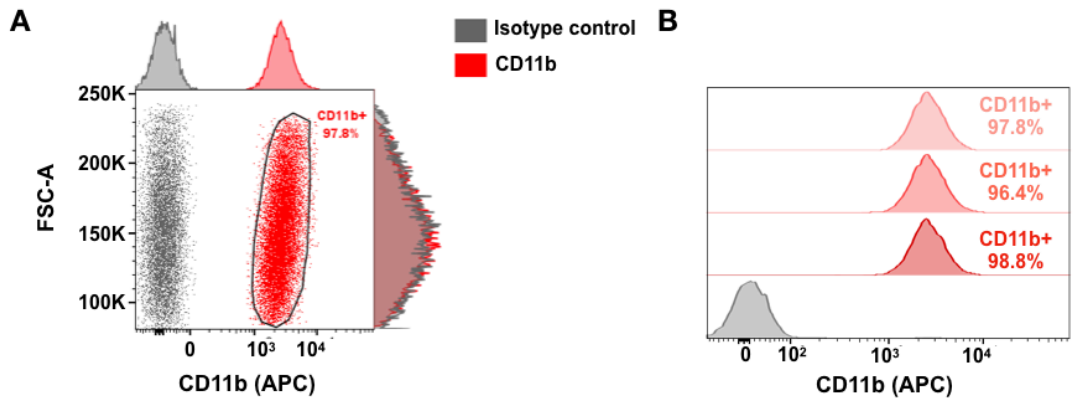

Figure S1. Flow cytometric analysis for bone marrow-derived macrophages. (A) CD11b expression in isotype control stained cells (grey) and APC conjugated CD11b stained cells (red) within single cell population. **(B)** The percentage of CD11b+ cells were determined in triplicate.

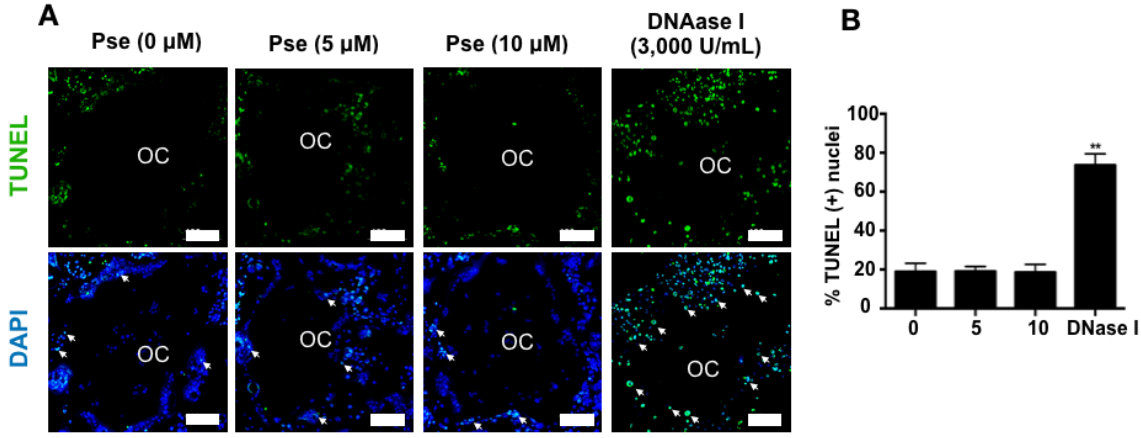

Figure S2. Pse showed no effect on osteoclast apoptosis. (A) After mature osteoclasts induced by RANKL, different concentrations of Pse (0, 5, 10 μM) were used to treat the cells for additional 2 days. As a positive control for apoptosis, DNase I treatment (3,000 U/mL, 30 min) was included. Apoptotic osteoclasts were then assessed by TUNEL assay (green). Nuclei were visualized by DAPI staining. The TUNEL-positive apoptotic nuclei are indicated by white arrows. **(B)** The quantification of the percentage of TUNEL-positive nuclei in each group (n=5 per group). All bar graphs are presented as mean ± SD. **p<0.01 compared with the group without Pse and DNase I treatment. Scale bar=100 μm. DAPI, 4,6-diamidino-2-phenylindole; Pse, Pseurotin A; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; OC, osteoclast

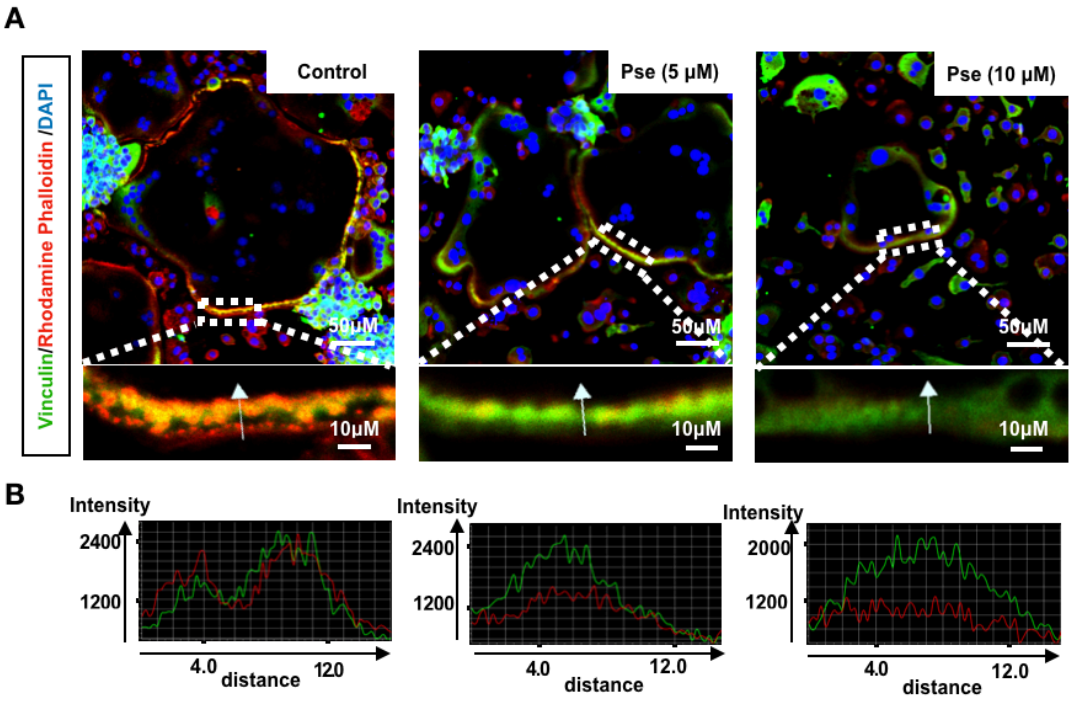

Figure S3. Confocal images of podosome belts of osteoclasts. (A) High magnification confocal images showing podosome belts formation in osteoclasts treated with or without Pse. **(B)** Fluorescence intensity profile through podosome belts (indicated by the white arrow in A). DAPI, 4,6-diamidino-2-phenylindole; Pse, Pseurotin A

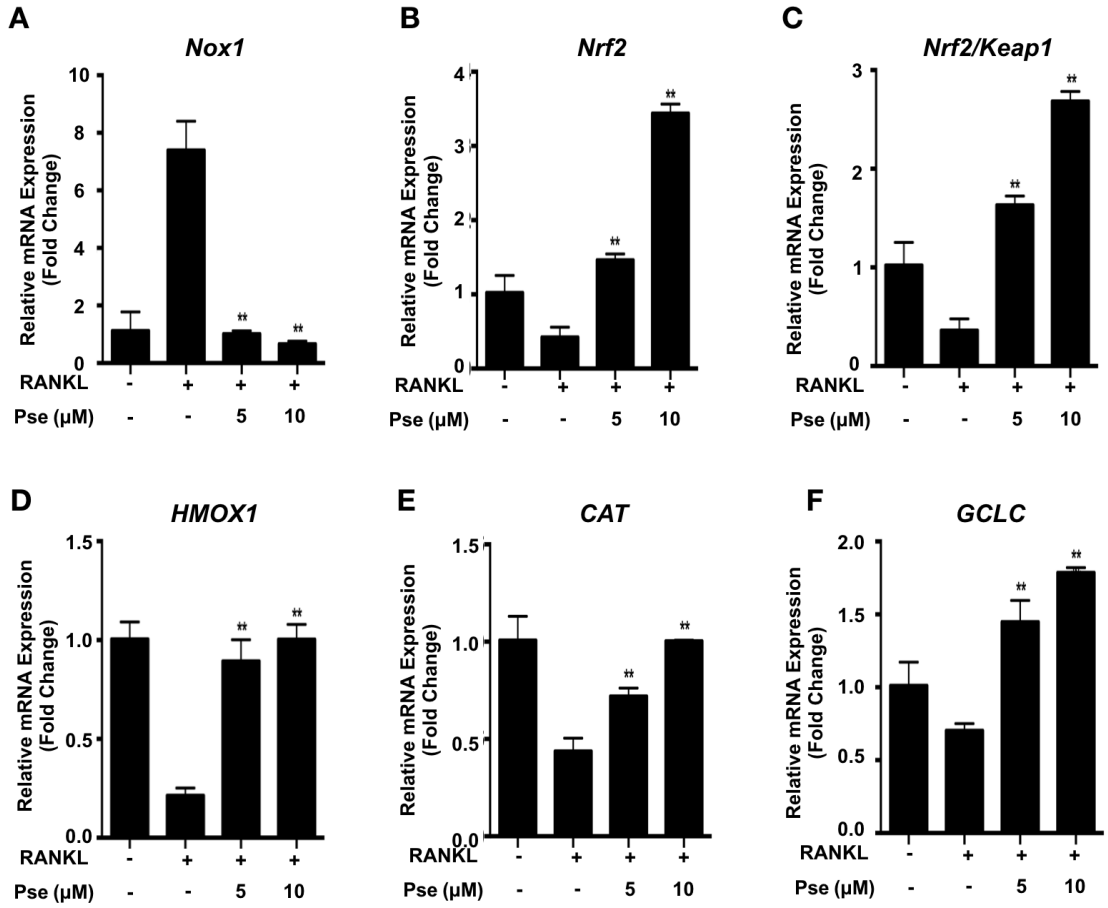

Figure S4. ROS-related genes expression in RANKL-stimulated BMMs. The expressions of *Nox1* **(A)**, *Nrf2* **(B)**, Nrf2/Keap1 **(C)**, *HMOX1* **(D)**, *CAT* **(E),** and *GCLC* **(F)** were examined by qPCR after 2-day RANKL stimulation in the absence or presence of Pse (5, 10 μM). Gene expression was calibrated using the *Hprt1* housekeeping gene. **p<0.01 compared with the group with only RANKL treatment. CAT, catalase; *GCLC,* gamma-glutamylcysteine synthetase; *HMOX1,* heme oxygenase 1; *Hprt1,* hypoxanthine phosphoribosyltransferase 1; *Keap1*, kelch like ech associated protein 1; *Nox1*, nicotinamide adenine dinucleotide phosphate oxidase 1; *Nrf2,* nuclear factor- erythroid 2 related factor 2; Pse, Pseurotin A; RANKL, receptor activator of nuclear factor-κB (NF-κB) ligand

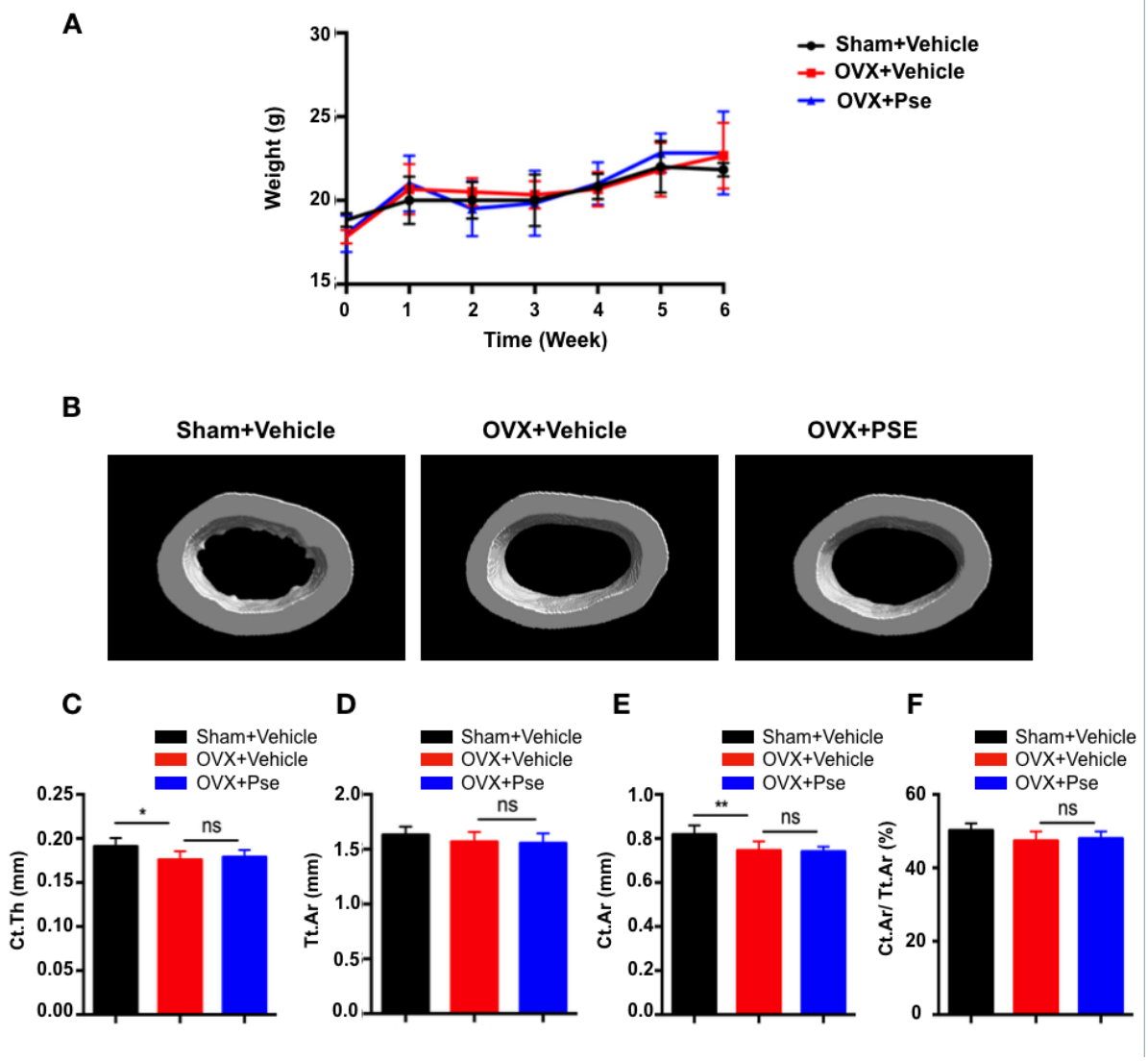

Figure S5. Body weights and cortical bone parameters of all mice in each group. (A) Body weights of all mice recorded at indicated time points. **(B)** Representative μCT images of femur cortical bone in each group. **(C-F)** Quantitative analyses of cortical bone related parameters, including cortical thickness (Ct.Th), total cross-sectional area (Tt.Ar), cortical bone area (Ct.Ar), and cortical area fraction (Ct.Ar/Tt.Ar) (N=6 per group). All bar graphs are presented as mean ± SD. *P<0.05, **P<0.01 relative to the OVX group. OVX, ovariectomized; Pse, Pseurotin A; ns, non-significant.

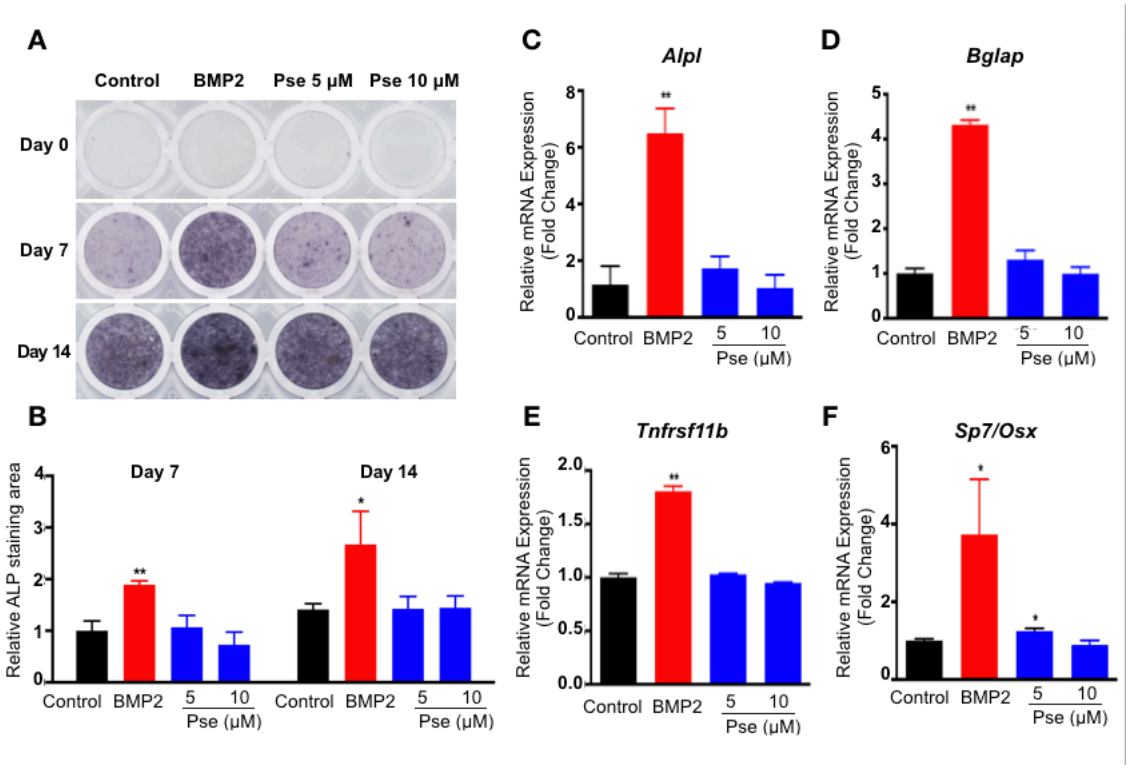

Figure S6. Pse has no effect on osteoblast differentiation. (A) MC3T3-E1 cells were induced osteogenic differentiation for 0, 7, and 14 days. ALP staining was carried out using BCIP/NBT liquid substrate system. **(B)** Quantitative analyses of ALP staining area (n=3 per group). **(C-F)** qPCR analysis of osteoblast genes expression of *Alpl, Bglap*, *Tnfrsf11b*, and *Sp7/Osx* relative to *Actb* in MC3T3-E1 cells induced in osteogenic medium for 14 days in each group (n=3 per group). All bar graphs are presented as mean ± SD. *p<0.05 **p<0.01 compared with control. *Actb,* actin beta; *Alpl,* alkaline phosphatase; *Bglap*, bone gamma-carboxyglutamate protein (osteocalcin); BMP2, bone morphogenetic protein 2; *Sp7/Osx,* sp7 transcription factor; *Tnfrsf11b*, tumor necrosis factor receptor superfamily member 11b

Supplementary Methods

Flow cytometric analysis for macrophage marker.

Bone marrow was flushed from the long bone (femur and tibia) of mice and then cultured in complete αMEM for 24h. Non-adherent cells were then collected and cultured in complete αMEM containing M-CSF (50 ng/mL) for 3 days culture. The attached cells were used for flow cytometric analysis. To investigate the purity of bone marrow macrophages (BMMs), cell suspension (5 x 10^5 cells) were incubated with APC conjugated monoclonal antibody for mouse CD11b (Thermo Fisher Scientific, Scoresby, VIC, Australia) or isotype-matched control IgGs for 30 min on ice. After washing 3 times, cells were subjected to flow cytometric analysis using FACSCantoII (BD Biosciences, New Jersey, USA). Data were processed using FlowJo software (FlowJo LLC, Ashland, Oregon, USA).

TUNEL assay

TUNEL assays were performed using an In Situ Cell Death Detection kit (Sigma-Aldrich, Sydney, NSW, Australia). 6 x 10^3 BMMs were seeded onto coverslips in 96-well plates and were induced into osteoclasts. To determine whether Pse can induce osteoclast apoptosis,

cells were cultured with M-CSF and RANKL for further 2 days after mature osteoclasts formation. Cells were treated with different concentrations of Pse (0, 5, 10 μM). DNase I (3,000 U/mL, 30 min) treated cells were used as positive control. Cells were fixed with 4% formaldehyde and permeabilized with 0.1% Triton X-100 for 15 minutes. Cells were then processed following the manufacturer's protocol in order to label fragmented DNA. Nuclei were stained with DAPI. The percentage of nuclei was used to evaluate Pse's effect on apoptosis.

Osteoblast differentiation

To determine whether Pse has effects on osteoblast, MC3T3-E1 cells (ATCC, Manassas, Virginia, USA) were cultured with α-MEM, 10% FBS, and 1% penicillin-streptomycin at 37℃ in a humidified atmosphere of 5% CO_2. To induce osteogenic differentiation, cells (1.0 x 10^4/well) were seeded in 48-well plates overnight and then changed into osteogenic medium (10 mM β-glycerophosphate and 50 μg/mL ascorbic acid) with different concentrations of Pse (5, 10 μM). BMP-2 (50 ng/mL) treatment was used as positive control; Group without Pse and BMP-2 was negative control. Medium was changed every 3 days. After incubation for 0, 7, and 14 days, cells in each group were fixed in 4% paraformaldehyde for 10 min, washed with PBS for 3 times, and stained by BCIP/NBT liquid substrate system (Sigma-Aldrich, Sydney, NSW, Australia) for alkaline phosphatase (ALP) at 37℃ for 30 min.

Supplementary Table 1. Primers Sequences

Gene	Forward (5'-3')	Reverse (5'-3')
Alpl	CAGGCCGCCTTCATAAGCA	AATTGACGTTCCGATCCTGC
Bglap	GCGCTCTGTCTCTCTGACCT	ACCTTATTGCCCTCCTGCTT
Tnfrsf11b	GCCACGCAAAAGTGTGGAAT	TTTGGTCCCAGGCAAACTGT
Sp7/Osx	GTCCTCTCTGCTTGAGGAAGAA	GGGCTGAAAGGTCAGCGTAT
Actb	CACCCGCGAGCACAGCTTCTT	CCACCATCACACCCTGGTGCCT
Nox1	AAGTTTCTCTCCCGAAGGACC	CCAACCAGGAAACCAGAAACAA
Nrf2	TCTCCTCGCTGGAAAAAGAA	AATGTGCTGGCTGTGCTTTA
CAT	CTCGCAGAGACCTGATGTCC	GACCCCGCGGTCATGATATT
HMOX1	GAGCAGAACCAGCCTGAACT	AAATCCTGGGGCATGCTGTC
Keap1	TGCCCCTGTGGTCAAAGTG	GGTTCGGTTACCGTCCTGC
GCLC	GGGGTGACGAGGTGGAGTA	GTTGGGGTTTGTCCTCTCCC