

**Dual effects of liquiritigenin on the proliferation of bone cells: promotion of osteoblast differentiation and inhibition of osteoclast differentiation**

Kaho Uchino\*, Kuniaki Okamoto\*, Eiko Sakai, Erika Yoneshima, Mayumi Iwatake, Yutaka Fukuma, Kazuhisa Nishishita, and Takayuki Tsukuba<sup>§</sup>

Department of Dental Pharmacology, Nagasaki University Graduate School of Biomedical Sciences, Sakamoto 1-7-1, Nagasaki 852-8588, Japan

\*These authors equally contributed to this work.

<sup>§</sup>Corresponding author

Department of Dental Pharmacology, Nagasaki University Graduate School of Biomedical Sciences, Sakamoto 1-7-1, Nagasaki 852-8588, Japan

Tel.: +81-95-819-7654; Fax: +81-95-819-7655

E-mail address: tsuta@nagasaki-u.ac.jp

**Short title:** Dual effects of liquiritigenin on osteoblasts and osteoclasts

**Abbreviations:** Osteoblasts, (OBLs); Osteoclasts, (OCLs); mesenchymal stem cells, (MSCs); transforming growth factor-beta, (TGF- $\beta$ ); bone morphogenic protein, (BMP); mitogen-activated protein kinase pathway, (MAPK); Runt-related transcription factor 2, (Runx2); macrophage colony-stimulating factor, (M-CSF); receptor activator of nuclear factor kappa-B ligand, (RANKL); nuclear factor kappa B, (NF- $\kappa$ B); phosphatidylinositol 3-kinase, (PI3K); Akt, (Protein kinase B); c-Jun N-terminal kinase, (JNK); extracellular signal-regulated kinase, (Erk); mitogen-activated protein kinase, (MAPK); nuclear factor of activated T cells cytoplasmic-1, (NFATc1); Antibodies, (Abs); bone marrow-derived macrophages, (BMMs); phenylmethanesulfonylfluoride, (PMSF); Recombinant human bone morphogenetic protein-2 (rhBMP-2);  $\alpha$ -minimal essential medium, ( $\alpha$ -MEM); fetal bovine serum, (FBS); tartrate-resistant acid phosphatase, (TRAP); phosphate-buffered saline, (PBS) Poly-Acrylamide Gel Electrophoresis, (PAGE); Tris Buffered Saline with 0.1% Tween 20, (TBST); standard deviations, (SD)

## **Abstract**

Bone is constantly controlled by a balance between osteoblastic bone formation and osteoclastic bone resorption. Liquiritigenin is a plant-derived flavonoid and has various pharmacological effects, such as anti-oxidative, anti-tumor, and anti-inflammatory effects. Here, we show that liquiritigenin has dual effects on the proliferation of bone cells, regarding the promotion of osteoblast differentiation and the inhibition of osteoclast differentiation. Liquiritigenin-treated murine osteoblastic MC3T3-E1 cells showed an increased alkaline phosphatase activity and enhanced phosphorylation of Smad1/5 compared to untreated cells. Moreover, liquiritigenin inhibited osteoclast differentiation, its bone-resorption activity through slightly decreased the phosphorylation of extracellular signal-regulated kinase, c-Jun N-terminal kinase, and inhibitor of nuclear factor kappa B- $\alpha$ ; however, the phosphorylation of Akt and p-38 slightly increased in bone marrow-derived osteoclasts. The expression levels of the osteoclast marker proteins nuclear factor of activated T-cells cytoplasmic-1, Src, and cathepsin K diminished. These results suggest that liquiritigenin may be useful as a therapeutic and/or preventive agent for osteoporosis or inflammatory bone diseases.

**Keywords:** osteoclasts, osteoblasts, liquiritigenin, differentiation

## **Introduction**

Bone is constantly remodeled by a controlled balance between bone formation by osteoblasts (OBLs) and bone resorption by osteoclasts (OCLs). OBLs are mononucleated bone-forming cells that are derived from mesenchymal stem cells (MSCs). The differentiation of MSCs into OBLs is regulated by seven important signaling networks (Soltanoff et al., 2009). Among them, the transforming growth factor-beta (TGF- $\beta$ )/bone morphogenic protein (BMP) signaling pathway facilitates the activation of two pathways: canonical Smad-dependent pathways and non-canonical Smad-independent signaling pathways, including the p38 mitogen-activated protein kinase pathway (MAPK) (Chen et al., 2012). Both the Smad and p38 MAPK-dependent pathways result in the activation of Runt-related transcription factor 2 (Runx2), which is a key transcription factor for OBL differentiation (Komori, 2008). Meanwhile, OCLs are multinucleated bone-resorbing cells that mature from hematopoietic precursors of monocyte/macrophage lineage (Chambers, 2000; Teitelbaum, 2000). OCL differentiation is regulated by two important factors, macrophage colony-stimulating

factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL). In addition, recent studies have shown that oxidative stress plays important roles in osteoclastogenesis (Lee et al., 2005).

Liquiritigenin is an aglycone of liquiritin, and it is one of the flavonoids present in *Glycyrrhiza radix*. Liquiritigenin has been shown to have various pharmacological effects, such as anti-oxidative, anti-tumor, and anti-inflammatory effects (Kupfer et al., 2008) (Zhou et al., 2010; Jiang et al., 2013). Moreover, liquiritigenin has been reported to be a plant-derived, selective estrogen receptor  $\beta$  agonist, indicating that it is probably useful for the treatment of breast cancer and osteoporosis (Mersereau et al., 2008). Although several studies have reported the potential utility of liquiritigenin in the treatment of breast cancer, there is little information about the effects of liquiritigenin on osteoporosis. In a previous study, liquiritigenin was shown to exert the following pharmacological effects: increased cell growth, increased alkaline phosphatase activity, promotion of collagen synthesis, and the mineralization of osteoblastic MC3T3-E1 cells (Choi, 2012). Further, liquiritigenin has been shown to protect MC3T3-E1 cells from oxidative damage induced by pharmacological inhibitors of the mitochondria (Choi et al., 2014).

Based on these findings, a strong possibility that liquiritigenin has osteogenic activity exists, but such an effect remain to be elucidated. In this study, the effects of liquiritigenin on OBL proliferation and on OCL differentiation were examined.

## **Materials and methods**

### *2.1. Reagents*

Liquiritigenin was purchased from Wako Pure Chemicals (Osaka, Japan). M-CSF was purchased from Kyowa Hakko Kogyo (Tokyo, Japan). Recombinant RANKL was prepared as described previously (Hu et al., 2008). Antibodies (Abs) were purchased as follows:  $\beta$ -actin (Cat. No. A5060, rabbit polyclonal Ab, 1:20000; Sigma-Aldrich, St. Louis, MO, USA), Src (Cat. No. 05-184, mouse monoclonal Ab, 1:1000; Upstate Biotechnology, Lake Placid, NY, USA), and heme oxygenase (HO)-1 (rabbit polyclonal Ab, 1:10000; Stressgen, Ann Arbor, MI, USA). Anti-c-fms (Cat. No. sc-692, rabbit polyclonal Ab, 1:1000), anti-RANK (Cat. No. sc-9072, rabbit polyclonal Ab, 1:1000), and anti-NFATc1 (Cat. No. sc-7294, mouse monoclonal Ab, 1:1000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Abs specific for

phospho-ERK1/2 (Cat. No. 9101S, Thr202/Tyr204, rabbit polyclonal Ab, 1:1000), phospho-JNK (Cat. No. 9751S, Thr183/Tyr185, rabbit polyclonal Ab, 1:1000), phospho-p38 (Cat. No. 9211S, Thr180/Tyr182, rabbit polyclonal Ab, 1:1000), phospho-inhibitor of nuclear factor kappa B alpha (I $\kappa$ B $\alpha$ ) (Cat. No. 2859S, Ser32, rabbit polyclonal Ab, 1:1000), and phospho-Akt (Cat. No. 9271S, Ser473, rabbit polyclonal Ab, 1:1000) were purchased from Cell Signaling Technology (Danvers, MA, USA). Cathepsin K Ab was prepared as described previously (Kamiya et al., 1998). The Osteo Assay Plate was purchased from Corning (Corning, New York, NY, USA). All other reagents, including phenylmethanesulfonylfluoride (PMSF) and the protease inhibitor cocktail, were obtained from Sigma-Aldrich. Recombinant human bone morphogenetic protein-2 (rhBMP-2) was purchased from R & D systems.

## 2.2. Cell culture

Murine osteoblastic MC3T3-E1 cells (RBRC-RCB1126) were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. MC3T3-E1 cells were cultured in  $\alpha$ -minimal essential medium ( $\alpha$ -MEM) (Wako Pure Chemicals, Code: 135-15175; bicarbonate buffered with L-glutamine) containing 10%

fetal bovine serum (FBS) with 100 U/mL penicillin and 100 µg/mL streptomycin. When cells were differentiated into OBLs, cells were incubated with  $\alpha$ -MEM containing 50 µg/mL ascorbic acid, 5 mM  $\beta$ -glycerolphosphate, and 1 µM dexamethasone, or 100 µM rhBMP-2.

The isolation of bone marrow-derived macrophages (BMMs) was performed as described previously (Sakai et al., 2012). Briefly, five-week-old male C57BL/6 mice were obtained from CLEA Japan, Inc. (Tokyo, Japan) and handled in our facilities under the approved protocols of the Nagasaki University Animal Care Committee. The BMMs were replated in culture plates and incubated in  $\alpha$ -MEM containing 10% FBS with 100 U/mL penicillin and 100 µg/mL streptomycin in the presence of M-CSF (50 ng/mL) and RANKL (50 ng/mL) for 60 h or 72 h, until the cells differentiated into multinucleated mature OCLs.

The cells were fixed with 4% paraformaldehyde and stained for tartrate-resistant acid phosphatase (TRAP) activity using a previously described method (Sakai et al., 2013). TRAP-positive red-colored cells with 3 or more nuclei were considered mature OCLs. Murine monocytic cell line RAW-D cells were kindly provided by Prof. Toshio Kukita (Kyushu University, Japan) and cultured in  $\alpha$ -MEM containing 10% FBS with

RANKL (50 ng/mL) (Watanabe et al., 2004). For bone resorption pit formation, BMMs were seeded onto Osteo Assay Plates coated with thin calcium phosphate films (Corning, New York, NY, USA) and incubated with M-CSF and RANKL for 5 days, until mature OCL resorbed the calcium phosphate film. Cells were dissolved in 5% sodium hypochlorite. Images of the resorption pit were taken with a reverse phase microscope (Olympus, Tokyo, Japan). The ratios of the resorbed areas to the total areas were calculated using Image J image-analysis software (<http://rsbweb.nih.gov/ij/>) as described previously (Narahara et al., 2012).

### *2.3. Cell viability assay*

Cells seeded in 96-well cell culture plates were incubated with the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) for 1 h, and then the absorbance at 450 nm was measured with a microplate reader (Bio-Rad iMark<sup>TM</sup>, Hercules, CA, USA).

### *2.4. Western blot analysis*

Cells were rinsed twice with ice-cold phosphate-buffered saline (PBS), and lysed in a cell lysis buffer (50 mM Tris-HCl [pH 8.0], 1% Nonidet P-40, 0.5% sodium

deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 1 mM PMSF, and a proteinase inhibitor cocktail). The protein concentration of each sample was measured with BCA Protein Assay Reagent (Thermo Pierce, Rockford, IL, USA). Lysate protein (5 µg) was applied to each lane. After SDS-PAGE, proteins were electroblotted onto a polyvinylidene difluoride membrane. The blots were blocked with 3% skim milk/ Tris Buffered Saline with 0.1% Tween 20 (TBST) for 1 h at room temperature, probed with various Abs overnight at 4°C, washed, incubated with horseradish peroxidase-conjugated secondary Abs (anti-rabbit IgG, Cat. No. 7074, 1:2000, and anti-mouse IgG, Cat. No. 7076, 1:2000; Cell Signaling Technology), and finally detected with ECL-Plus (GE Healthcare Life Sciences, Tokyo, Japan). The immunoreactive bands were analyzed by LAS4000-mini (Fuji Photo Film, Tokyo, Japan).

## 2.5. Statistical analysis

All values were expressed as means ± standard deviations (SD) for 3 independent experiments. The data were analyzed using one-way ANOVA and differences were considered significant at \* $P < 0.05$  or \*\* $P < 0.01$ .

### 3. Results

#### *3.1. Effects of liquiritigenin on the differentiation of osteoblastic MC3T3-E1 cells*

Fig. 1A shows the structure of liquiritigenin. We first examined the effects of liquiritigenin on the differentiation of osteoblastic MC3T3-E1 cells under two different induced conditions. Firstly, upon stimulation of MC3T3-E1 cells with  $\beta$ -glycerophosphate, ascorbic acid, and dexamethasone, alkaline phosphatase staining analysis showed that liquiritigenin dose-dependently promoted the differentiation of osteoblastic MC3T3-E1 cells (Fig. 1B). Secondly, in MC3T3-E1 cells stimulated with BMP-2, alkaline phosphatase staining also showed that liquiritigenin dose-dependently increased osteoblast differentiation (Fig. 1C). These results confirm that liquiritigenin promotes OBL differentiation of MC3T3-E1 cells.

#### *3.2. Effects of liquiritigenin on the intracellular signaling mediating the differentiation of osteoblastic MC3T3-E1 cells*

Next, the effects of liquiritigenin on the intracellular signaling mediating the

differentiation of MC3T3-E1 cells induced by BMP-2 were investigated (Fig. 1D). The effects of liquiritigenin on BMP2-induced early intracellular signaling pathways, specifically the phosphorylation of Smad1/5 were examined by western blotting, during OBL differentiation of MC3T3-E1 cells. As shown in Fig. 1D, phosphorylation of Smad1/5 in liquiritigenin-treated cells clearly increased at 15 - 120 min compared to untreated cells, although the protein levels of Smad1/5 were unchanged between liquiritigenin-treated and untreated cells. Taken together, these findings indicate that liquiritigenin induces the differentiation of osteoblastic MC3T3-E1 cells through the Smad1/5-dependent pathway.

### 3.3. *Liquiritigenin inhibits osteoclastogenesis in vitro*

To evaluate the effects of liquiritigenin on RANKL-induced osteoclastogenesis, the effects of liquiritigenin on OCL differentiation of RAW-D cells with RANKL (50 ng/mL) were investigated by TRAP staining, which is a standard method to test OCL formation. Liquiritigenin inhibited the formation of mononuclear and multinuclear OCLs derived from RAW-D cells (Fig 2A). The number of TRAP-positive, multinucleated OCLs after liquiritigenin treatment decreased in a dose-dependent

manner (Fig. 2B). The cell viability of OCLs treated with 1 - 25  $\mu\text{M}$  liquiritigenin was indistinguishable from that of untreated cells, although the viability of cells treated with deltamethrin at 50  $\mu\text{M}$  or 100  $\mu\text{M}$  significantly decreased (Fig. 2C).

To examine further whether similar results could also be observed in native cells, the effects of liquiritigenin on RANKL-induced osteoclastogenesis in BMMs were tested. The TRAP staining indicated that 10 and 50  $\mu\text{M}$  liquiritigenin treatment decreased multinucleated OCL formation (Fig 2D). The number of TRAP-positive BMM-derived OCLs significantly decreased after liquiritigenin treatment (Fig. 2E). The viability of OCLs treated with 2 - 10  $\mu\text{M}$  liquiritigenin was comparable to that of untreated cells; however, 50 - 100  $\mu\text{M}$  liquiritigenin treatment slightly increased cell viability (Fig. 2F). These results indicate that liquiritigenin significantly inhibits the osteoclastogenesis of RAW-D cells and BMMs, although liquiritigenin shows different effects on cell viability in RAW-D cells and BMMs.

#### *3.4. Effects of liquiritigenin on the bone-resorption activity of OCLs*

To determine whether liquiritigenin decreases the bone-resorption activity of OCLs, a pit formation assay with BMM-derived OCLs was performed. Bone resorption activity under two different conditions was tested. In the first condition, liquiritigenin was added at the beginning of stimulation with RANKL (50 ng/mL) and M-CSF (30 ng/mL) (Fig. 3A, showing a). In the second condition, liquiritigenin was added three days after activation, when BMMs were differentiated into active OCLs (Fig. 3A, showing b).

In order to compare the resorption pit area, when untreated OCLs had moderate resorbing activity, the OCLs treated with liquiritigenin at the beginning exhibited reduced resorbing activity (Fig. 3B). Moreover, the OCLs treated with liquiritigenin after OCL activation also showed a reduced bone-resorption activity (Fig. 3C). These results indicate that liquiritigenin impairs the bone-resorption activity of OCLs.

### *3.5. Effects of liquiritigenin on the expression levels of OCL marker proteins and intracellular signaling*

To examine the effects of liquiritigenin on OCLs, the expression levels of OCL marker proteins by western blotting were determined. As shown in Fig. 4A, although

the expression levels of c-fms, c-fos, and RANK were unchanged at lower concentrations (up to 10  $\mu$ M) of liquiritigenin, they decreased at higher concentrations (50 - 100  $\mu$ M) of liquiritigenin. However, the protein levels of NFATc1 considerably decreased upon 10  $\mu$ M liquiritigenin treatment. Consistent with these results, the expression levels of Src and cathepsin K, which are transcriptionally regulated by NFATc1, were also found to be decreased by liquiritigenin treatment. Thus, liquiritigenin inhibits the expression of various OCL marker proteins.

Therefore, the expression levels of HO-1, a phase II antioxidant cytoprotective enzyme against oxidative stress, were examined after liquiritigenin treatment. HO-1 levels increased upon 50 or 100  $\mu$ M liquiritigenin treatment, indicating that liquiritigenin induced a weak expression of HO-1 in OCLs (Fig. 4A).

Finally, the effects of liquiritigenin on RANKL-induced early intracellular signaling pathways, including the phosphorylation of p38 MAPK, JNK, I $\kappa$ B $\alpha$ , Erk, and Akt were analyzed by western blotting, during the OCL differentiation of BMMs, since these signaling cascades are important for osteoclastogenesis (Boyle et al., 2003). BMMs were pre-incubated with 50  $\mu$ M liquiritigenin for 24 h and subsequently stimulated with RANKL according to a previous protocol (Lee et al., 2010). Liquiritigenin slightly

inhibited the phosphorylation of Erk, JNK, and I $\kappa$ B $\alpha$  (Fig. 4B). However, the phosphorylation of p38 and Akt was enhanced after liquiritigenin. These results indicate that liquiritigenin inhibits some RANKL-induced signaling cascades such as Erk, JNK, and I $\kappa$ B $\alpha$ , but conversely increases p38 and Akt signaling.

#### **4. Discussion**

A previous study has reported that liquiritigenin induced enhanced cell growth, increased alkaline phosphatase activity, increased collagen synthesis, and the mineralization of osteoblastic MC3T3-E1 cells (Choi, 2012). However, the detailed mechanisms of the liquiritigenin-mediated proliferation of osteoblastic cells have not yet been determined. In this study, we found that the phosphorylation of Smad1/5 in liquiritigenin-treated MC3T3-E1 cells clearly increased compared to untreated cells. We further demonstrated that liquiritigenin inhibited osteoclast differentiation from BMMs and RAW-D cells into mature OCLs *in vitro*. Liquiritigenin markedly prevented the bone-resorption activity of OCLs. Upon liquiritigenin treatment, the phosphorylation of Erk, p38 MAPK, Akt, and JNK was abolished, and there were slight effects on I $\kappa$ B $\alpha$ -dependent pathways. Moreover, the expression of NFATc1 and its regulated

proteins such as Src and cathepsin K were down-regulated in OCLs. Thus, liquiritigenin has dual effects on the proliferation of bone cells, regarding the promotion of osteoblast differentiation and the inhibition of osteoclast differentiation.

The effects of liquiritigenin on cell viability are most likely to be rather mild. The previous study reported that liquiritigenin increased cell growth, alkaline phosphatase activity, and collagen synthesis in osteoblastic MC3T3-E1 cells (Choi, 2012). Consistent with these results, this study also showed that liquiritigenin increased cell growth and alkaline phosphatase activity. Moreover, liquiritigenin caused significantly increased cell viability of RANKL-stimulated BMMs at higher concentrations (50 - 100  $\mu$ M), although it inhibited the cell viability of RANKL-stimulated RAW-D cells at the same concentrations. The detailed mechanisms behind the discrepancy between BMMs and RAW-D cells are presently unknown. Nevertheless, it is most likely that liquiritigenin has mild effects on the viability of bone cells, such as osteoblastic and osteoclastic cells. Therefore, this study raises the possibility that liquiritigenin may be a safe therapeutic agent for maintaining bone, including stimulation of bone formation, during late life.

In addition to cell viability, liquiritigenin has been shown to significantly decrease

the production of OCL differentiation factors, such as tumor necrosis factor- $\alpha$ , interleukin-6, and receptor RANKL in osteoblastic MC3T3-E1 cells (Choi, 2012). Moreover, this study showed that liquiritigenin prevented the OCL differentiation and bone-resorption activity of OCLs (Choi, 2012). Thus, liquiritigenin has effects on decreasing bone resorption.

Estrogen receptors are speculated to be target molecules of liquiritigenin in bone cells. Since liquiritigenin has been shown to selectively bind to estrogen receptor  $\beta$ , it is most likely that liquiritigenin works as a kind of “phytoestrogen” (Mersereau et al., 2008). Phytoestrogens are plant-derived bioactive compounds that have structural and physiological effects similar to that of estradiol. Estrogen receptor  $\alpha$  regulates the proliferative effects of estrogens in the target tissues, whereas estrogen receptor  $\beta$  has rather suppressive effects on estrogen receptor  $\alpha$  (Jiang et al., 2013). Generally, phytoestrogens have dual effects on bone cells, such as the promotion of OBL maturation and the inhibition of OCL differentiation. For example, genistein, a representative soy isoflavone, has been shown to have effects on the enhancement of OBL differentiation (Chen et al., 2003) and the inhibition of OCL differentiation (Bitto et al., 2010). Since genistein prevents trabecular bone loss in ovariectomy mouse *in vivo*,

which is a model of osteoporosis (Wu et al., 2001), determining whether liquiritigenin can promote bone formation *in vivo* is of interest.

During preparation of this study, Chen *et al.* have reported that several dietary phenolic acids stimulate OBL differentiation and proliferation, and it inhibits adipocyte differentiation *in vitro*, leading to increased bone mass *in vivo* (Chen et al., 2014). Considering these similarities, it will be of interest to determine whether liquiritigenin has inhibitory effects on adipocyte differentiation *in vitro*, resulting in increased bone mass *in vivo*.

## **5. Conclusions**

This study determined that liquiritigenin promotes OBL differentiation of MC3T3-E1 cells via increased phosphorylation of Smad1/5. Conversely, liquiritigenin prevented OCL differentiation through multiple pathways, such as Erk, p38 MAPK, and JNK. These results indicate that liquiritigenin has dual effects on the proliferation of bone cells, regarding the promotion of OBL differentiation and the inhibition of OCL differentiation. Liquiritigenin may be considered an effective therapeutic and/or preventive agent for bone cells, and further investigations in animal models are warranted.

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## Figure legends

**Fig. 1.** (A). The structure of liquiritigenin. (B, C) The effects of liquiritigenin on osteoblast differentiation of MC3T3-E1 cells. MC3T3-E1 cells were incubated with  $\alpha$ -MEM containing 50  $\mu\text{g}/\text{mL}$  ascorbic acid, 5 mM  $\beta$ -glycerolphosphate, and 1  $\mu\text{M}$  dexamethasone for 7days (B), or 100  $\mu\text{M}$  rhBMP-2 for 4days (C) in the absence or

presence of liquiritigenin (2.5, 5, 10, 25, 50 and 100  $\mu\text{M}$ ). After fixation, alkaline phosphatase staining was performed. (D) The effects of liquiritigenin on the signaling underlying osteoblast (OBL) differentiation. MC3T3-E1 cells were incubated with 100  $\mu\text{M}$  rhBMP-2 in the absence or presence of 50  $\mu\text{M}$  liquiritigenin. Cell lysates with equal amounts of protein were subjected to SDS-PAGE, followed by western blotting with Abs to p-Smad1/5 or total Smad1/5.  $\beta$ -actin was used as a loading control. The data are representative of 3 independent experiments.

**Fig. 2.** The effects of liquiritigenin on osteoclast (OCL) differentiation from RAW-D cells. (A) RAW-D cells were cultured for 72 h with the indicated concentrations of liquiritigenin in the presence of receptor activator of nuclear factor kappa-B ligand (RANKL; 50 ng/mL). TRAP staining was performed after fixation. The data are representative of 3 independent experiments. (B) The number of TRAP-positive multinucleated OCLs at 72 h of culture was counted. (C) Cell viability of the RAW-D-cell-derived-OCLs at 72 h of culture was analyzed using the Cell Counting Kit. The data shown in panels B and C are the mean  $\pm$  SD of 3 independent experiments. The asterisks indicate statistical significance compared to the control cells without

liquiritigenin, \*\*  $P < 0.01$ . (D) The effects of liquiritigenin on osteoclast (OCL) differentiation from bone marrow-derived macrophages (BMMs). BMMs were cultured for 72 h with the indicated concentrations of liquiritigenin in the presence of macrophage colony stimulating factor (M-CSF; 30 ng/mL) and receptor activator of nuclear factor kappa-B ligand (RANKL; 50 ng/mL). TRAP staining was performed after fixation. The data are representative of 3 independent experiments. (E) The number of TRAP-positive multinucleated OCLs at 72 h of culture was counted. (F) Cell viability of the BMM-derived-OCLs at 72 h of culture was analyzed using the Cell Counting Kit. The data shown in panels E and F are the mean  $\pm$  SD of 3 independent experiments. The asterisks indicate statistical significance compared to the control cells without liquiritigenin, \*  $P < 0.05$ , \*\*  $P < 0.01$ .

**Fig. 3.** The effects of liquiritigenin on the bone-resorption activity of osteoclasts (OCLs). Bone marrow-derived macrophages (BMMs) were cultured for 7 days in the presence of macrophage colony stimulating factor (M-CSF; 30 ng/mL) and receptor activator of nuclear factor kappa-B ligand (RANKL; 50 ng/mL). (A) Addition of liquiritigenin was performed under two different conditions; before differentiation (at the beginning time)

or after differentiation (at 3 days after differentiation). (B) Photographs of addition of liquiritigenin before differentiation (at the beginning time). The data are representative of 3 independent experiments. (C) Photographs of addition of liquiritigenin after differentiation (at 3 days after differentiation). The data are representative of 3 independent experiments.

**Fig. 4.** (A) The effects of liquiritigenin on the protein expression of osteoclast (OCL) marker proteins. Bone marrow-derived macrophages (BMMs) were cultured with macrophage colony stimulating factor (M-CSF; 30 ng/mL) and receptor activator of nuclear factor kappa-B ligand (RANKL; 50 ng/mL) for 60 h in the presence of liquiritigenin at the indicated concentrations (0, 2, 5, 10, 50, and 100  $\mu$ M). The same protein amounts of cell lysates were subjected to SDS-PAGE, followed by western blotting with Abs specific to c-fms, RANK, NFATc1, Src, and cathepsin K.  $\beta$ -Actin was used as a loading control. The data are representative of 3 independent experiments.

(B) The effects of liquiritigenin on the essential signaling of osteoclast (OCL) differentiation. Bone marrow-derived macrophages (BMMs) were cultured with macrophage colony stimulating factor (M-CSF; 30 ng/mL) for 24 h in the presence or

absence of 10  $\mu$ M liquiritigenin. After 2 h of culture with serum-free media, the cells were subsequently stimulated with receptor activator of nuclear factor kappa-B ligand (RANKL; 300 ng/mL) for the indicated times (0, 5, 10, 15, and 30 min). Cell lysates with equal amounts of protein were subjected to SDS-PAGE, followed by western blotting with Abs to p-Erk, p-p38 MAPK, p-JNK, p-Akt, and p- I $\kappa$ B $\alpha$ .  $\beta$ -actin was used as a loading control. The data are representative of 3 independent experiments.

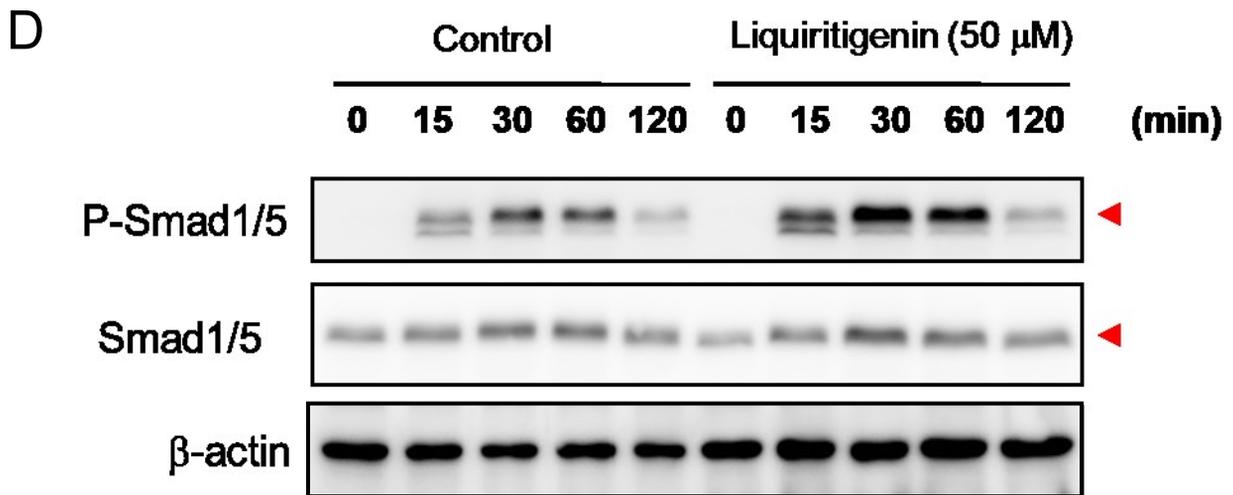
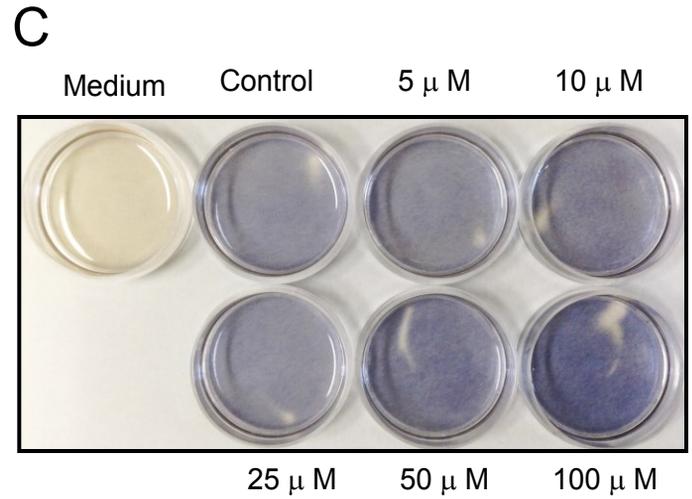
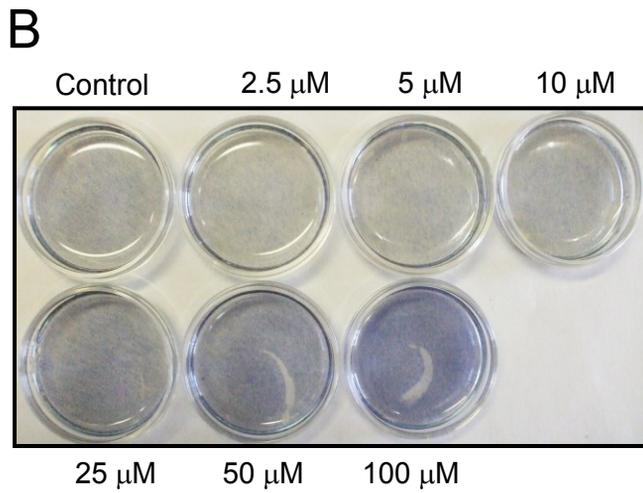
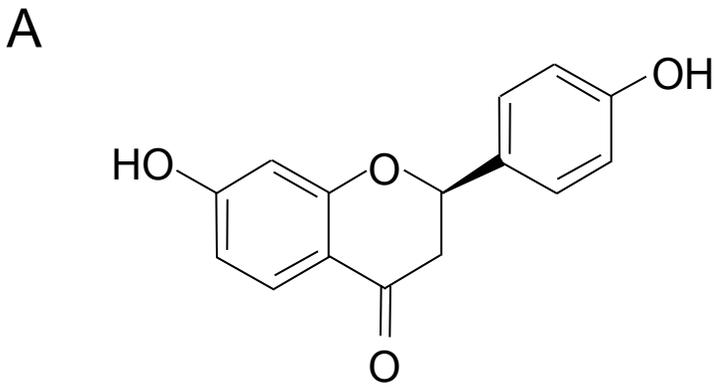


Figure 1

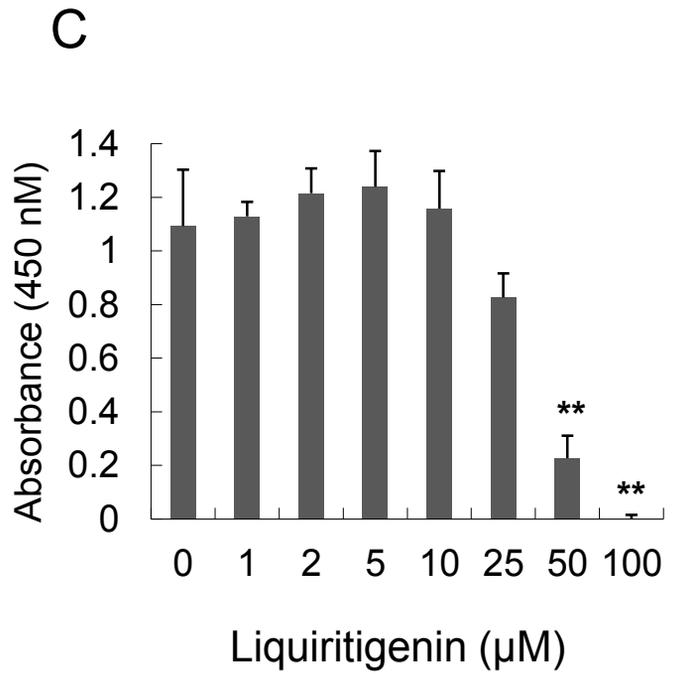
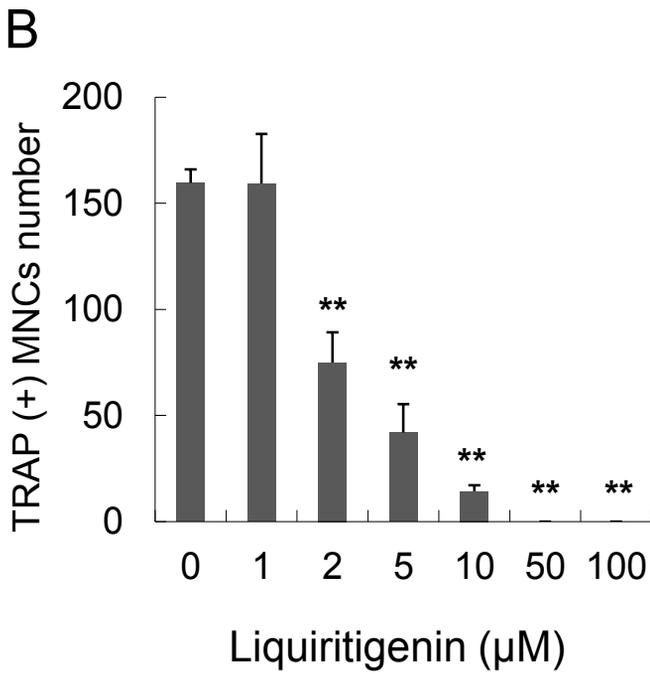
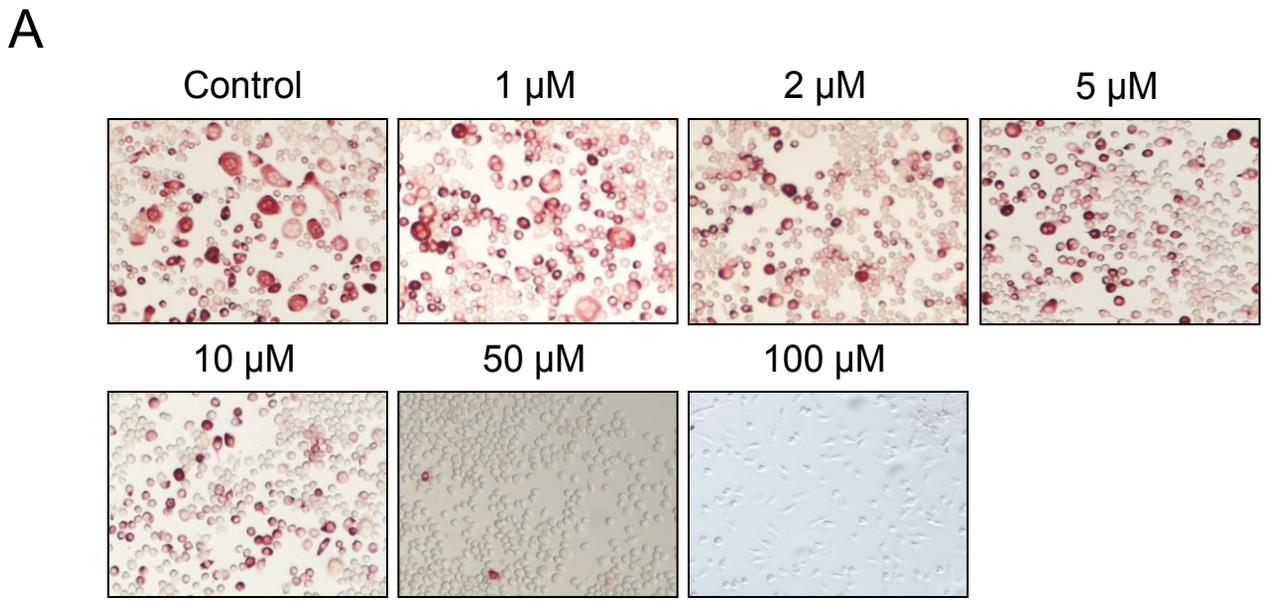
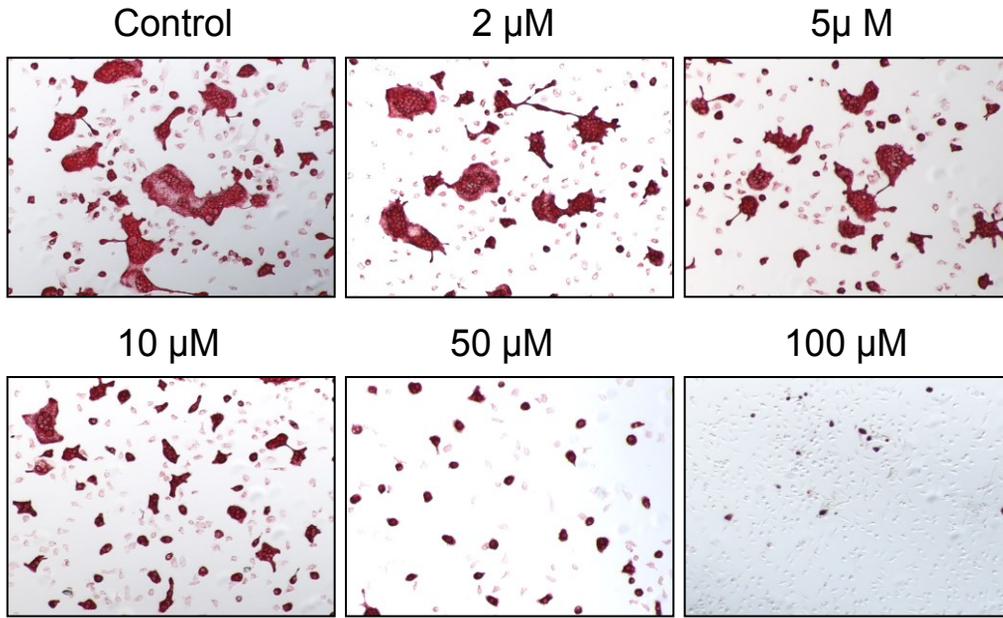
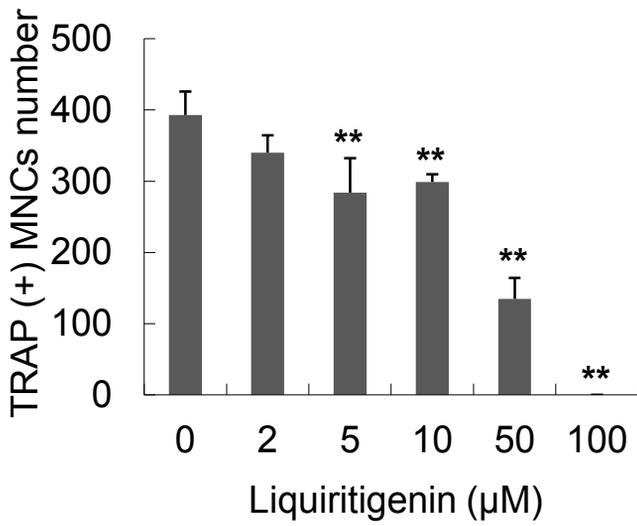
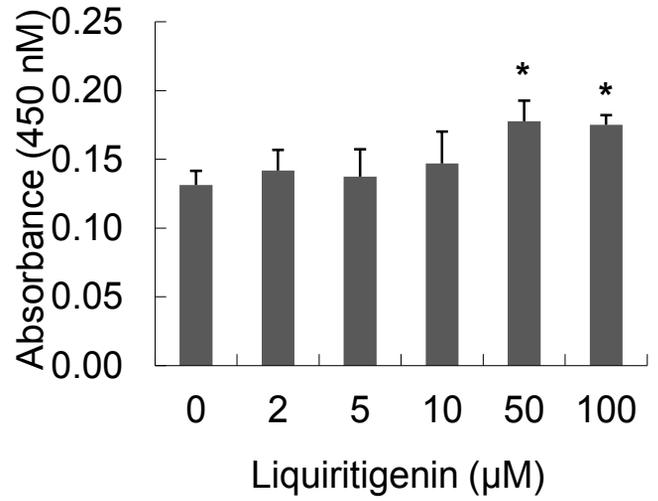
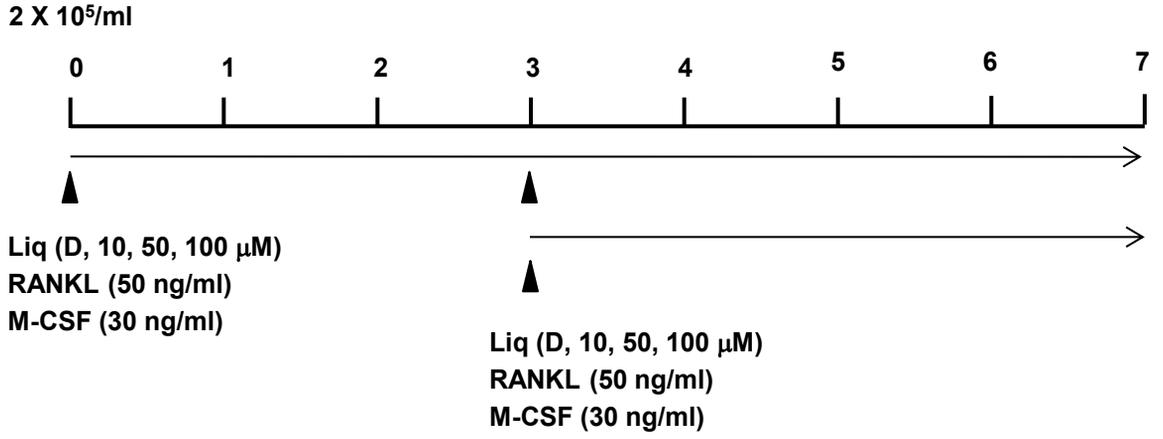


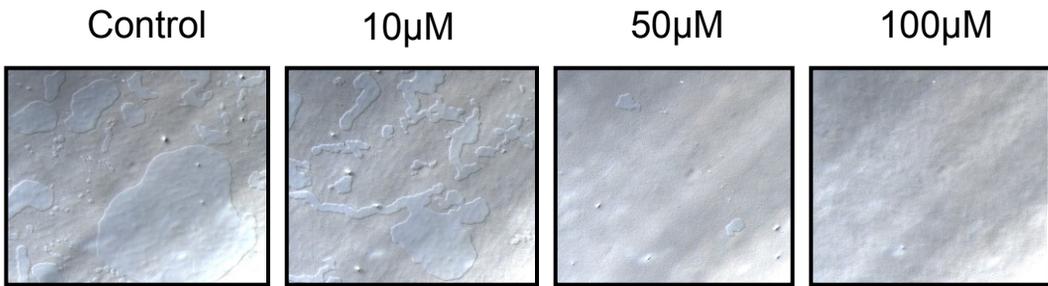
Figure 2 (continued)

**D****E****F****Figure 2**

A



B



C

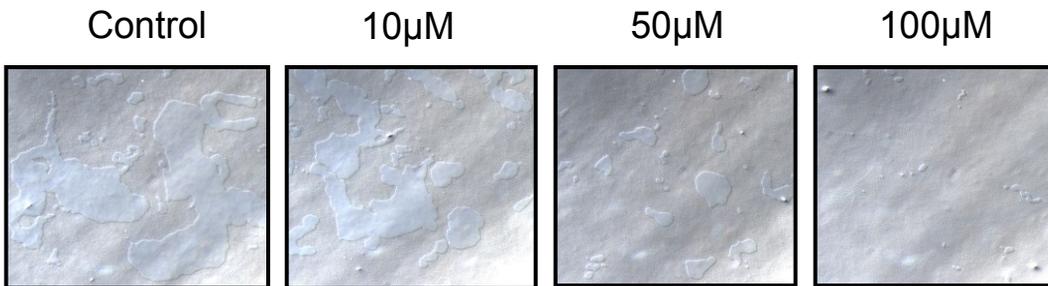


Figure 3

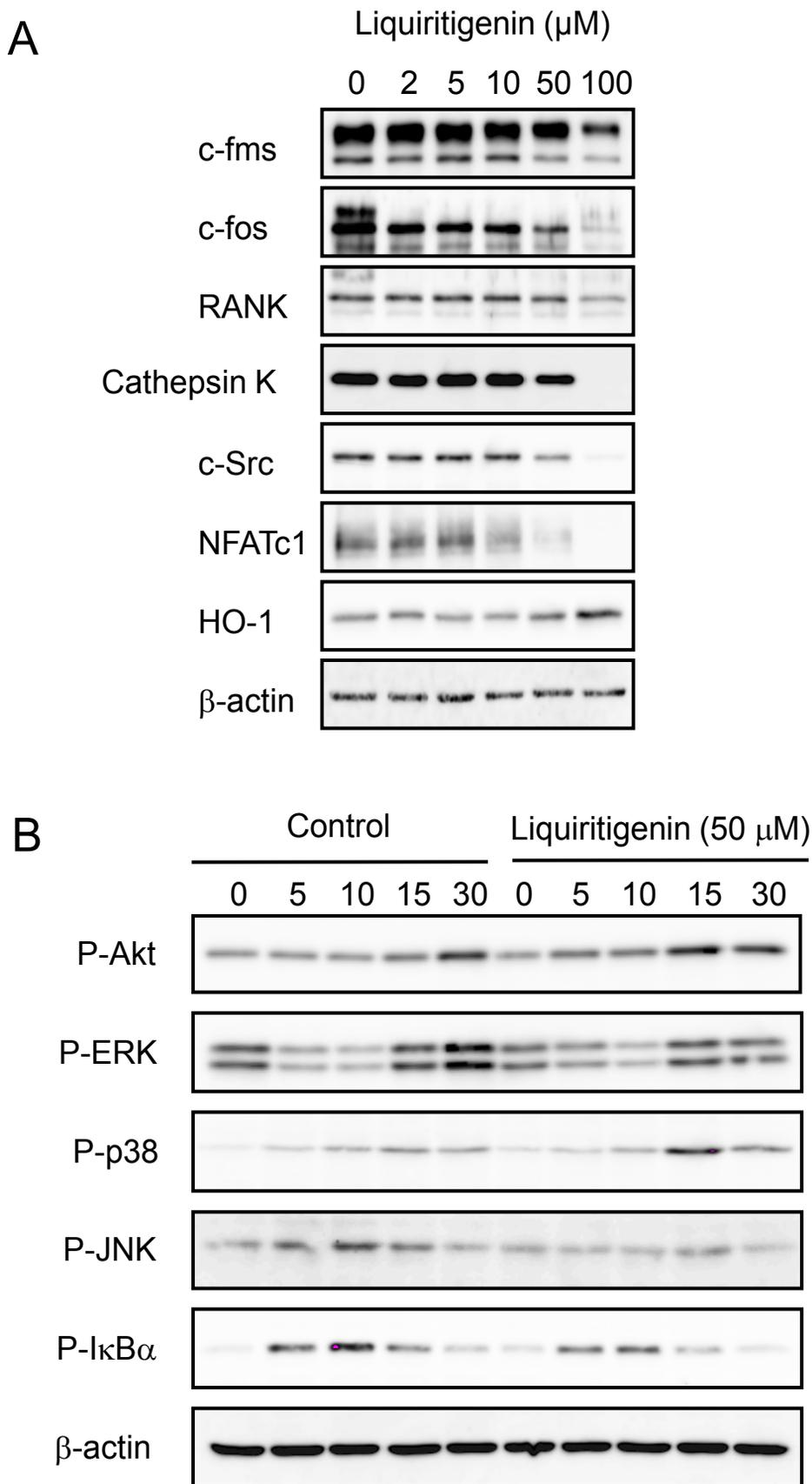


Figure 4