



Eucalyptol induces osteoblast differentiation through ERK phosphorylation in vitro and in vivo

Do-Won Lee^{1,2,3} · Kyeong-Min Kim^{1,3} · Seulki Park² · Sang-Hyun An² · Young-Ju Lim^{1,3} · Won-Gu Jang^{1,3}

Received: 27 March 2023 / Revised: 27 June 2023 / Accepted: 28 June 2023 / Published online: 20 July 2023
© The Author(s), under exclusive licence to Springer-Verlag GmbH Germany, part of Springer Nature 2023

Abstract

Eucalyptol (EU) is monoterpene oxide that is the main component of the essential oil extracted from aromatic plants such as *Eucalyptus globules*. EU has therapeutic effects such as antibacterial, anti-inflammatory and antioxidant in chronic diseases including inflammation disorder, respiratory disease, and diabetic disease. However, the effects of EU on osteoblast differentiation and bone diseases such as osteoporosis have not been studied. The present study investigated the effects of EU on osteoblast differentiation and bone formation. EU induces mRNA and protein expression of osteogenic genes in osteoblast cell line MC3T3-E1 and primary calvarial osteoblasts. EU also promoted alkaline phosphatase (ALP) activity and mineralization. Here, the osteoblast differentiation effect of EU is completely reversed by ERK inhibitor. These results demonstrate that osteoblast differentiation effect of EU is mediated by ERK phosphorylation. The efficacy of EU on bone formation was investigated using surgical bone loss-induced animal models. EU dose-dependently promoted bone regeneration in zebrafish caudal fin rays. In the case of ovariectomized mice, EU increased ERK phosphorylation and ameliorated bone loss of femurs. These results indicate that EU ameliorates bone loss by promoting osteoblast differentiation through ERK phosphorylation. We suggest that EU, plant-derived monoterpenoid, may be useful for preventing bone loss.

Key messages

- Eucalyptol (EU) increases osteoblast differentiation in pre-osteoblasts.
- EU up-regulates the osteogenic genes expression via ERK phosphorylation.
- EU promotes bone regeneration in partially amputated zebrafish fin rays.
- Oral administration of EU improves ovariectomy-induced bone loss and increases ERK phosphorylation.

Keywords Eucalyptol · Osteoblast differentiation · Bone formation · Osteoporosis · ERK

Introduction

Osteoblasts derived from mesenchymal stem cells are differentiated by various hormones, growth factors, cytokines and cell signaling mediators [1, 2]. The bone morphogenetic protein

(BMP) pathway, a major signaling cascades in bone formation, is involved in stimulating the expression of osteogenic transcription factors such as Runt-related transcription factor 2 (Runx2) through the activation of Smad1/5/8 and Mitogen-activated protein kinases (MAPKs) pathway [3]. MAPKs are important signaling mediators that regulate cellular responses to various biological stimuli [4, 5]. Extracellular signal-regulated kinase (ERK) is part of MAPK family, and participates in cellular functions such as cell proliferation, differentiation, survival, and senescence [6, 7]. In addition, ERK plays an essential role in bone development by regulating growth factor activation and osteoblast differentiation [8, 9]. When ERK is inactivated, osteoblast proliferation, differentiation and mineralization is inhibited [10]. ERK phosphorylates Runx2, which is highly expressed in osteoblasts of all bones during the early stages of bone formation

✉ Won-Gu Jang
jangwg@daegu.ac.kr

¹ Department of Biotechnology, College of Engineering, Daegu University, Gyeongsbuk 38453, Republic of Korea

² Preclinical Research Center, Daegu-Gyeongbuk Medical Innovation Foundation (DGMIF), Daegu 41061, Republic of Korea

³ Research Institute of Anti-Aging, Daegu University, Gyeongsbuk 38453, Republic of Korea

and stimulates transcriptional activity to enhance the expression of genes specific for osteoblast differentiation [11, 12].

Bone is a dynamic tissue; the process of bone remodeling is controlled by the balance of activity between osteoblasts and osteoclasts [13]. The interaction between osteoblast-mediated osteogenesis and osteoclast-mediated bone resorption becomes imbalanced due to various stresses including aging, and hormones [14, 15]. If the imbalance persists, bone remodeling fails and bone density decreases, which can lead to bone diseases such as osteoporosis [16]. The number of patients suffering from bone diseases such as osteoporosis and the duration of treatment is expected to increase as the human lifespan increases in an aging society [17]. In particular, postmenopausal women may be at an increased risk of bone disease due to hormone declines [18]. Currently, antiresorptive therapies such as estrogen and bisphosphonates are widely used for treatment of osteoporosis [19]. However, these therapies have side effects during long-term treatment [20–22]. Therefore, it is important to develop new therapeutic agents and functional health foods to better treat bone diseases [23].

Some natural products have few side effects and have beneficial effects on bone remodeling [24–26]. Essential oils extracted from plants contain highly fragrant monoterpene compounds as their main component. Monoterpene compounds are widely distributed in the plant kingdom, and have antibacterial, anti-inflammatory, and immunomodulatory properties [27]. They are also reported to have a positive effect on bone metabolism, but the exact mechanism is not well understood [28, 29]. Some monoterpene compounds prevent bone resorption and potentially modulate bone metabolism in mice by inhibiting of osteoclast activity [30, 31]. Eucalyptol (EU), the major organic compound in the essential oil extracted from *Eucalyptus globulus*, is a monoterpene oxide [32]. EU is found in various plant species such as *Salvia*, *Psidium*, and *Croton*, and has a fresh mint flavor and a cooling taste. Furthermore, EU exhibits anti-inflammatory and antioxidant effects in respiratory diseases such as bronchitis, sinusitis, chronic rhinitis, and asthma [33, 34]. However, the effects of EU on osteoblast differentiation and bone formation have not yet been studied. Therefore, the present study investigated the effect of EU on osteoblast differentiation and bone formation.

Materials and methods

Reagents and chemicals

EU and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffered saline (PBS), α -minimum essential medium (α -MEM), penicillin–streptomycin, and 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Atlas

Biologicals (Fort Collins, CO, USA). PD98059 was purchased from Calbiochem (San Diego, CA, USA). Emerald Amp GR PCR master mix was purchased from TaKaRa (Shiga, Japan), and Ampigene qPCR green mix Hi-Rox was purchased from Enzo (Farmingdale, NY, USA).

Cell culture

MC3T3-E1 cells were obtained from ATCC (Manassas, VA, USA). All cells were maintained in α -MEM supplemented with 10% FBS and 1% antibiotics (100 U/mL penicillin, 100 g/mL streptomycin) at 37 °C with 5% CO₂. The culture medium was changed every 2 days.

Isolation of primary mouse calvarial cells

Primary cells were isolated from the calvaria of 2-day-old ICR mice. Isolated calvaria were incubated in PBS supplemented with 0.1% collagenase (Gibco) and 0.2% dispase (Gibco). After 20 min incubation, the primary cells were isolated by centrifugation for 8 min. The incubation and centrifugation processes were repeated five times. Cells were maintained in α -MEM supplemented with 10% FBS and 1% antibiotics (100 U/mL penicillin, 100 g/mL streptomycin) at 37 °C with 5% CO₂. All animal procedures used to isolate the primary cells were carried out in accordance with the guidelines and regulations approved by the Committee for Laboratory Animal Care and Use of Daegu University (approval number: DUIACC-12020/4–0313-006).

Cell viability assay

MC3T3-E1 cells were seeded in 48-well plates at a density of 2×10^4 cells/well and cultured in medium containing EU of 1–50 M concentration. Cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma-Aldrich) for 1 h. Formed formazan was dissolved by adding DMSO to each well. The absorbance was measured at 570 nm using an Infinite M200 Pro multiplate reader (Tecan, Mannedorf, Switzerland).

Reverse-transcriptase PCR (RT-PCR) and quantitative PCR (qPCR)

RT-PCR and qPCR were performed to confirm the mRNA expression levels of osteogenic genes for EU. Total RNA was extracted from cultured cells using TRI-solution (Bio Science Technology, Daegu, Korea) according to the manufacturer's protocols. The cDNA was synthesized by reverse transcription from equal amounts of total RNA (3 g) with TOPscript RT Dry-MIX (Enzynomics, Daejeon, Korea). Each reaction consisted of initial denaturation at 95 °C for 5 min followed by a three-step cycle: denaturation at 95 °C for 30 s, annealing at the optimal temperature for each primer pair for 30 s, and extension at 72 °C

for 30 s. After 30–35 cycles, a final extension step was carried out at 72 °C for 5 min. The relative expression level of each gene was normalized relative to the expression of β -actin. Primer sequences used for PCR are listed in Table S1 and Table S2.

Western blot analysis

Cultured cells were lysed in RIPA buffer (ATTO Technology, Tokyo, Japan) supplemented with protease and phosphatase inhibitors (Roche, Mannheim, Germany). Total protein was quantified using Bradford protein assay reagent (Sigma-Aldrich). Quantified proteins were boiled in loading buffer and separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes, which were then blocked with 5% skim milk prepared in Tris-buffered saline (TBS) containing Tween-20. Membranes were then incubated with a specific primary antibody for overnight at 4°C. After washing, membranes were incubated with a secondary antibody at room temperature for 2 h. Signals were detected using ECL reagent (Advansta, Menlo Park, CA, USA) according to the manufacturer's protocol. The amount of detected signal was visualized as a band using a Fusion Solo analyzer system (Vilber Lourmat, Eberhardzell, Germany) and densitometry was quantified through ImageJ. Antibodies used for western blot are listed Table S3.

Alkaline phosphatase (ALP) activity

Measurement of ALP activity was performed using ALP staining. Cells were seeded in 24-well plates at a density of 5×10^4 cells/well and cultured for 10 days. Cultured cells were fixed with 4% formaldehyde (Duksan Pure Chemicals, Gyunggi-do, Korea). The fixed cells were washed with distilled water and stained with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) solution (Sigma-Aldrich) at room temperature. ALP activity was evaluated by imaging the stained area with an Epson Perfection V37 scanner (Seiko Epson, Suwa, Japan) and quantifying with the ImageJ program.

Mineralization

Measurement of extracellular matrix mineralization was performed using alizarin red S (ARS) staining. Cells were seeded in 24-well plates at a density of 5×10^4 cells/well and cultured for 3 weeks. Cultured cells were fixed with 4% formaldehyde, washed with distilled water, and stained with 2% ARS solution (Sigma-Aldrich) at room temperature. The levels of mineralization were evaluated by imaging the stained area with an Epson Perfection V37 scanner (Seiko Epson, Suwa, Japan) and quantifying with the ImageJ program.

Quantitative analysis of zebrafish caudal fin ray regeneration

Wild-type zebrafish (*Danio rerio*) (length = 3.4–4.4 cm) were maintained under standard conditions in tanks (temperature $30 \pm 1^\circ\text{C}$, 300 mL: 3 fish per tank) with 14 h light/10 h dark cycle. Zebrafish were fed daily with commercial flakes (Tetra Bits Complete, Tetra, Melle, Germany) and *Artemia* nauplii (*Artemia salina*). Zebrafish were stunned in cold water, and approximately half of the caudal fin ray (lepidotrichia) was amputated using a scalpel. Zebrafish were divided into three groups (control, 5 M EU, 50 M EU; $n=3$ per group). EU was added to the tank water according to the relevant concentration for each group, and zebrafish were incubated for 10 days. The tank water was changed every 2 days. All zebrafish were sacrificed and fixed in 10% neutral-buffered formalin for 24 h. Fixed specimens were treated with 25% saturated sodium tetraborate for 2 h. After washing, specimens were stained with 1% KOH and 1 mg/mL ARS. To visualize the mineralized structures, specimen tissues were cleared with 1% KOH and 3% H_2O_2 for 12 h. The caudal fins bone rays of the zebrafish were imaged through a scanner and microscope. The level of bone regeneration was measured by quantifying the images through the ImageJ program. Zebrafish studies were carried out in accordance with the guidelines and regulations approved by the Committee for Laboratory Animal Care and Use of Daegu University (approval number: DUIACC-12020/4- 0313–006).

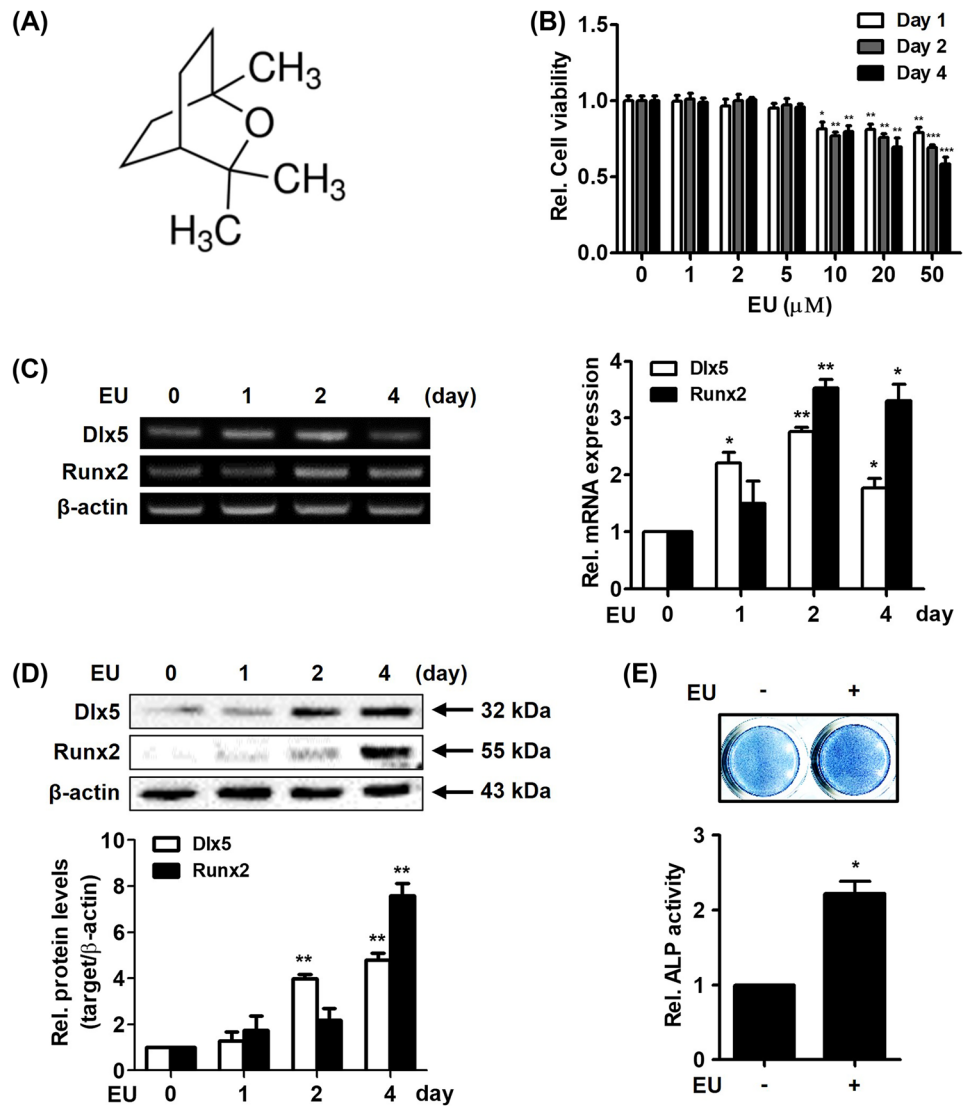
Ovariectomy (OVX) mouse model

Female 7-week-old C57BL/6 mice (average weight = 18 g) were purchased from Koatech (Pyeongtaek, Korea). Mice were maintained under specific pathogen-free conditions (12 h light/dark cycle, temperature $22 \pm 3^\circ\text{C}$, humidity $50 \pm 20\%$), and had free access to water and food. After acclimatization for 1 week, mice ($n=16$) either underwent bilateral ovariectomy ($n=12$, OVX group) or sham surgery ($n=4$, sham group) in which the ovaries were not removed. The ovariectomized mice were divided into three groups (OVX, OVX + EU 10 mg/kg, OVX + EU 50 mg/kg; $n=4$ per group). After inducing osteoporosis for 4 weeks, the test substances were orally administered to mice every day for 8 weeks. Sham group and OVX control group were given the same amount of vehicle. All procedures were carried out in accordance with the guidelines and regulations approved by the Institutional Animal Care and Use Committee of Laboratory Animal Center of Daegu-Gyeongbuk Medical Innovation Foundation (approval number: DGMIF-20060304–02).

Micro-computed tomography (micro-CT) image analysis

The mouse femurs were examined using a microcomputed tomography system. Scanned images were obtained at a tube voltage of 90 kVp and tube current of 180 μA using

Fig. 1 Effect of EU on osteogenic differentiation in MC3T3-E1 cells. **A** The chemical structure of EU. **B** The cytotoxicity of EU on cell viability of MC3T3-E1 cells was measured using an MTT assay after incubation with different concentrations of EU (1, 2, 5, 10, 20, and 50 μ M) for 1, 2, and 4 days. **C** Effect of EU (5 μ M) on the mRNA expression of osteogenic genes was measured by RT-PCR and qPCR. **D** The protein levels of osteogenic markers were measured by western blotting. Relative intensity is shown in the lower panel. **E** ALP staining of cells cultured for 10 days in the absence or presence of EU (5 μ M). The upper panel represents the image of staining, and the lower panel indicates the relative intensity. Data are presented as the mean \pm SEM of three individual experiments. The statistical significance was determined relative to the control by the Student's t-test (* P < 0.05, ** P < 0.01, *** P < 0.001)



a Quantum FX micro-CT scanner (Perkin Elmer, Waltham, MA, USA), set at an image voxel size of 19 μ m and field of view of 10 mm. After scanning, the regions of interest (ROI) were selected at a 0.3 mm interval from the growth plate reference. Trabecular bone 3D images were visualized based on the ROI. Morphometric parameters including bone mineral density (BMD), bone volume/tissue volume (BV/TV), trabecular thickness (Tb. Th), and trabecular number (Tb. N) were measured using Analyze 12.0 software (AnalyzeDirect, Stilwell, KS, USA).

Histological and immunohistochemical analyses

Mice were sacrificed, and femurs were isolated. The isolated femurs were fixed with 10% neutral-buffered formalin and decalcified in 10% EDTA. The femur tissue was embedded in

a paraffin block and sectioned to a thickness of 5 μ m. Paraffin sections were then deparaffinized, and hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining was performed. H&E staining was conducted according to standard procedures. For IHC staining, sections were incubated with an antibody to P-ERK (#4370, Cell Signaling Technology). The 3,3-diaminobenzidine (DAB) reaction was used to detect the antibody, and hematoxylin was used as a counterstain. Stained sections of tissue were scanned using an AxioScan.Z1 slide scanner (ZEISS, Thuringia, Germany).

Statistical analysis

All experiments were performed at least three times and data are expressed as mean \pm SEM. Comparisons between two groups were analyzed by unpaired Student's t test.

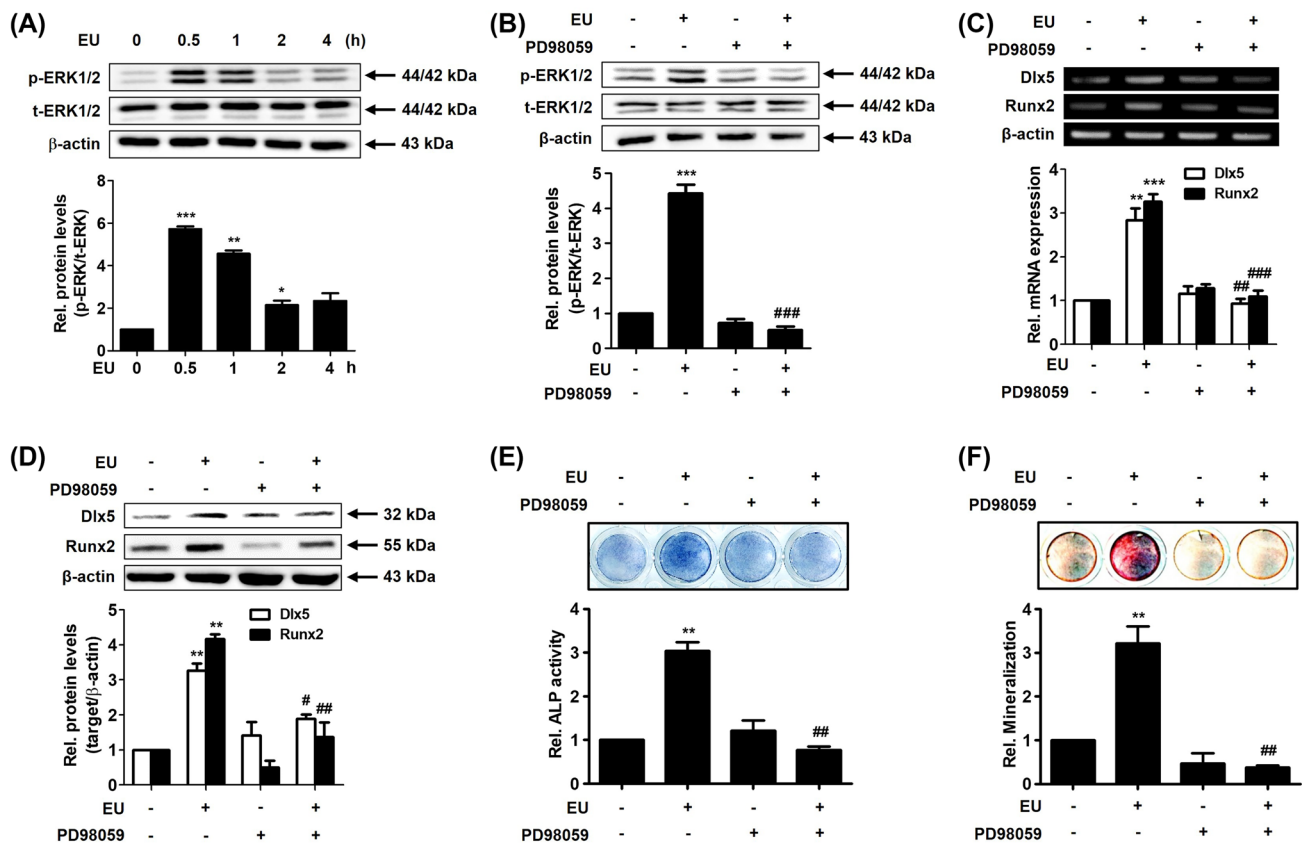


Fig. 2 EU increases osteogenic differentiation via ERK phosphorylation in MC3T3-E1 cells. **A** MC3T3-E1 cells were treated with EU (5 μ M) for the indicated times. **B** MC3T3-E1 cells were treated with or without EU (5 μ M) and PD98059 (10 μ M) for 0.5 h. **A** and **B** Phosphorylation of ERK were determined by western blotting. Relative intensity is shown in the lower panel. **C**, **D**, **E** and **F** MC3T3-E1 cells were cultured according to the specified conditions. **C** mRNA expression of osteogenic genes measured by RT-PCR and qPCR. **D** Protein expression levels of

osteogenic genes measured by western blotting. Relative intensity is shown in the lower panel. **E** Cells cultured for 10 days were subjected to ALP staining. **F** Cells cultured for 3 weeks were stained with ARS. The upper panel represents the image of staining, and the lower panel indicates the relative intensity. Data are presented as the mean \pm SEM of three individual experiments (* P < 0.05, ** P < 0.01, *** P < 0.001 compared with the control group; # P < 0.05, ## P < 0.01, ### P < 0.001 compared with the EU-treated group)

Comparisons between multiple groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. All analyzes were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA), and statistical significance was considered at P value < 0.05.

Results

EU induces osteoblast differentiation of MC3T3-E1 cells

The chemical formula of EU is $C_{10}H_{18}O$, and the structure is shown in Fig. 1A. MC3T3-E1 cells were exposed to various concentrations (1–50 M) of EU for 1, 2 or 4 days and the potential cytotoxicity of EU was determined by MTT

assay. The results showed that EU was not cytotoxic at concentrations below 5 M but was cytotoxic at concentrations above 10 M (Fig. 1B). Therefore, EU was used at a concentration of 5 M in the subsequent experiment. To investigate whether EU can induce osteoblast differentiation, we measured the mRNA and protein expression levels of osteogenic genes such as distal-less homeobox5 (Dlx5) and Runx2 during osteoblast differentiation. As the results, EU significantly increased the mRNA expression of osteogenic genes in MC3T3-E1 cells (Fig. 1C). Western blot analysis showed that EU significantly increased the protein levels of osteogenic genes in a time-dependent manner (Fig. 1D). Also, ALP activity in MC3T3-E1 cells treated with EU for 10 days significantly increased (Fig. 1E). These results indicate that EU induces osteoblast differentiation by increasing the expression of osteogenic genes.

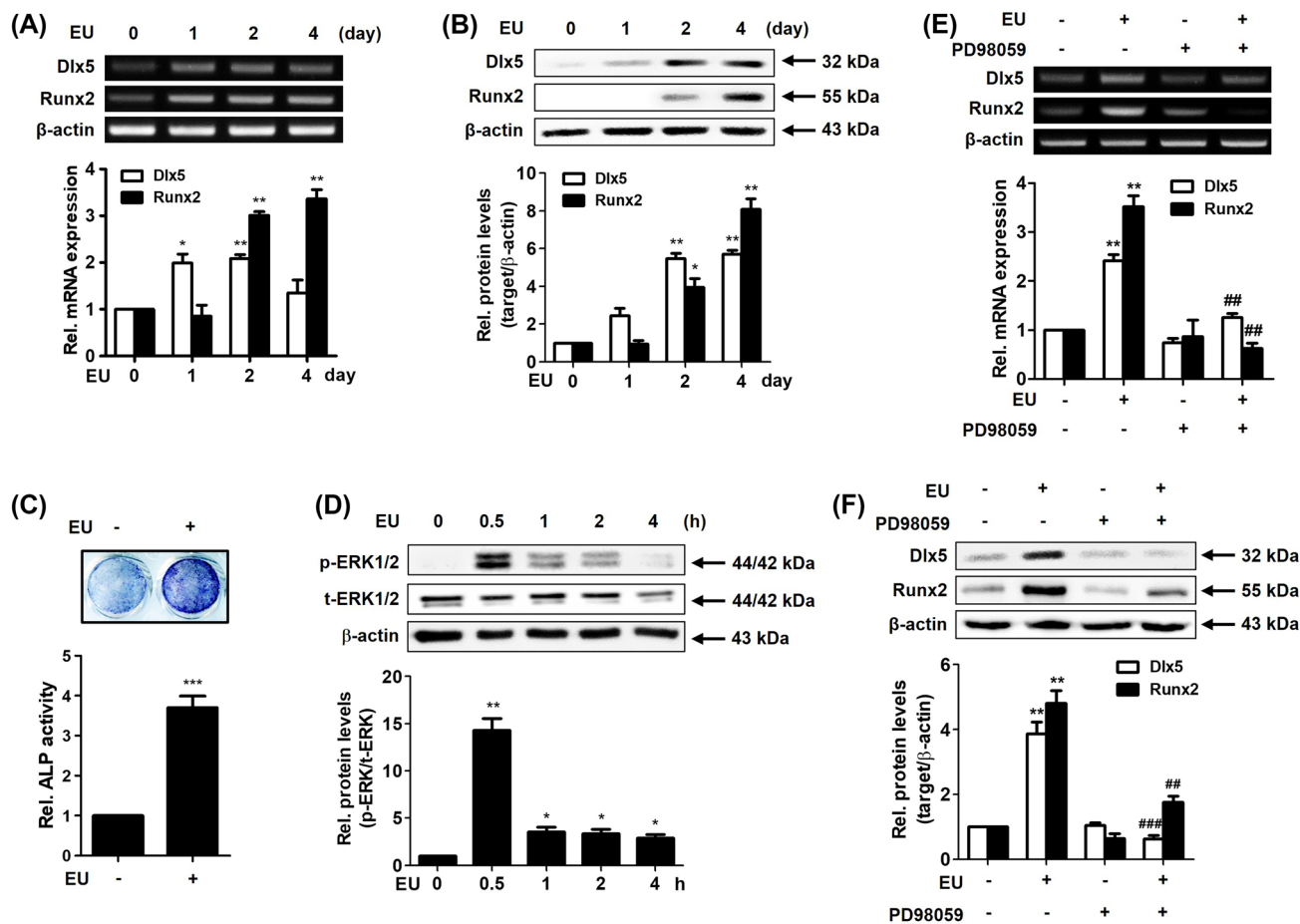


Fig. 3 Effect of EU on osteogenic differentiation in primary mouse calvarial cells. **A** and **B**) Primary cells were treated with EU (5 μ M) for 1, 2, 4 days. **A** Effect of EU on the mRNA expression of osteogenic genes was measured by RT-PCR and qPCR. **B** The protein levels of osteogenic markers were measured by western blotting. **C** ALP staining of cells cultured for 10 days in the absence or presence of EU (5 μ M). **D** Primary cells were treated with EU (5 μ M) for the indicated times. Phosphorylation of ERK were determined by western blotting. Relative inten-

sity is shown in the lower panel. **E**, **F** Primary cells were treated with or without EU (5 μ M) and PD98059 (10 μ M). **E** The mRNA expression of osteogenic genes measured by RT-PCR and qPCR. **F** The protein expression levels of osteogenic genes measured by western blotting. Relative intensity is shown in the lower panel. Data are presented as the mean \pm SEM of three individual experiments (* P < 0.05, ** P < 0.01, *** P < 0.001 compared with the control group; # P < 0.05, ## P < 0.01, ### P < 0.001 compared with the EU-treated group)

EU regulates osteogenic differentiation via ERK phosphorylation in MC3T3-E1 cells

The molecular mechanism behind the osteogenic effect of EU in osteoblast was investigated via evaluation of ERK. MC3T3-E1 cells were treated with EU for the specified times. EU significantly increased the protein level of phosphorylated ERK (Fig. 2A). ERK phosphorylation by EU is blocked by an ERK inhibitor (PD98059) (Fig. 2B). We measured mRNA and protein expression of osteogenic genes using PD98059 to determine whether osteoblast differentiation by EU was regulated through ERK phosphorylation or independently. As a result, mRNA expression of EU-induced osteogenic genes was suppressed by the PD98059 (Fig. 2C). The protein expression of EU-induced osteogenic genes was completely reversed by

PD98059 (Fig. 2D). Also, EU significantly increased ALP activity and mineralization, which are representative markers of bone formation, but these effects were reversed by PD98059 (Fig. 2E, F). These results demonstrate that EU regulates osteogenic differentiation through ERK phosphorylation.

EU induces osteogenic differentiation via ERK phosphorylation in primary calvarial osteoblasts

The effect of EU on osteoblast differentiation was confirmed in primary calvarial osteoblasts isolated from neonatal mice. Primary calvarial osteoblasts were treated with 5 μ M EU for 1, 2 and 4 days. EU treatment significantly increased the mRNA and protein expression levels of Dlx5 and Runx2 (Fig. 3A, B). In addition, EU treatment for

10 days increased ALP activity in primary calvarial osteoblasts (Fig. 3C). We next determined whether EU regulates the expression of osteogenic genes through ERK phosphorylation. Primary calvarial osteoblasts were treated with EU for the indicated times. Western blot analysis showed that EU treatment for 0.5 h increased ERK phosphorylation (Fig. 3D). In addition, PD98059 suppressed the EU-induced increase in the mRNA and protein expression of osteogenic genes (Fig. 3E, F). These results suggest that EU induces osteogenic differentiation via ERK phosphorylation in primary calvarial osteoblasts.

EU promotes bone regeneration of zebrafish fin rays

The zebrafish injury model is mainly used for regeneration research because it can regenerate various tissues including bone tissue [35–37]. In the current study, the injury model indicates the partial amputation of zebrafish fin rays for bone regeneration studies. Amputations of zebrafish fin rays and EU treatment were performed as described in the materials and methods section and are depicted in Fig. 4A, B. To investigate the effect of EU on

bone regeneration, amputated zebrafish were treated with EU. The results of our quantitative analysis focusing on ARS staining images, showed that EU dose-dependently increased the level of bone regeneration (Fig. 4D, E). These results show that EU promotes bone regeneration in adult zebrafish.

EU ameliorates bone loss via ERK phosphorylation in OVX mice

We investigated whether EU could improve bone loss in the OVX-induced postmenopausal mouse model. C57BL/6 mice underwent ovarian resection, and then bone loss was allowed to develop for 4 weeks. Figure 5A shows a schematic diagram of EU evaluation in OVX mice. Each group (sham + vehicle, OVX + vehicle, OVX + EU 10 mg/kg, OVX + EU 50 mg/kg) was orally administered EU or vehicle for 8 weeks. OVX mice had a significantly higher body weight than the sham group, and the weight gain in the EU-treated group was lower than that in the OVX group at 12 weeks (Fig. 5B). The effect of EU on bone loss was assessed by micro-CT analysis in the distal femur. OVX

Fig. 4 Effect of EU on the regeneration of zebrafish fin rays. **A** The schedule and **B** procedure used for EU treatment in adult zebrafish (n = 3, per group). **D** The caudal fin rays of zebrafish were amputated **C** on day 0 and were treated with or without EU (5 or 50 μ M) for 10 days. Images of fin ray were scanned after ARS staining. **E** The level of regeneration of caudal fin rays was quantified using the scanned image. Scale bar, 200 μ m (** P < 0.01, *** P < 0.001 compared with the control group)

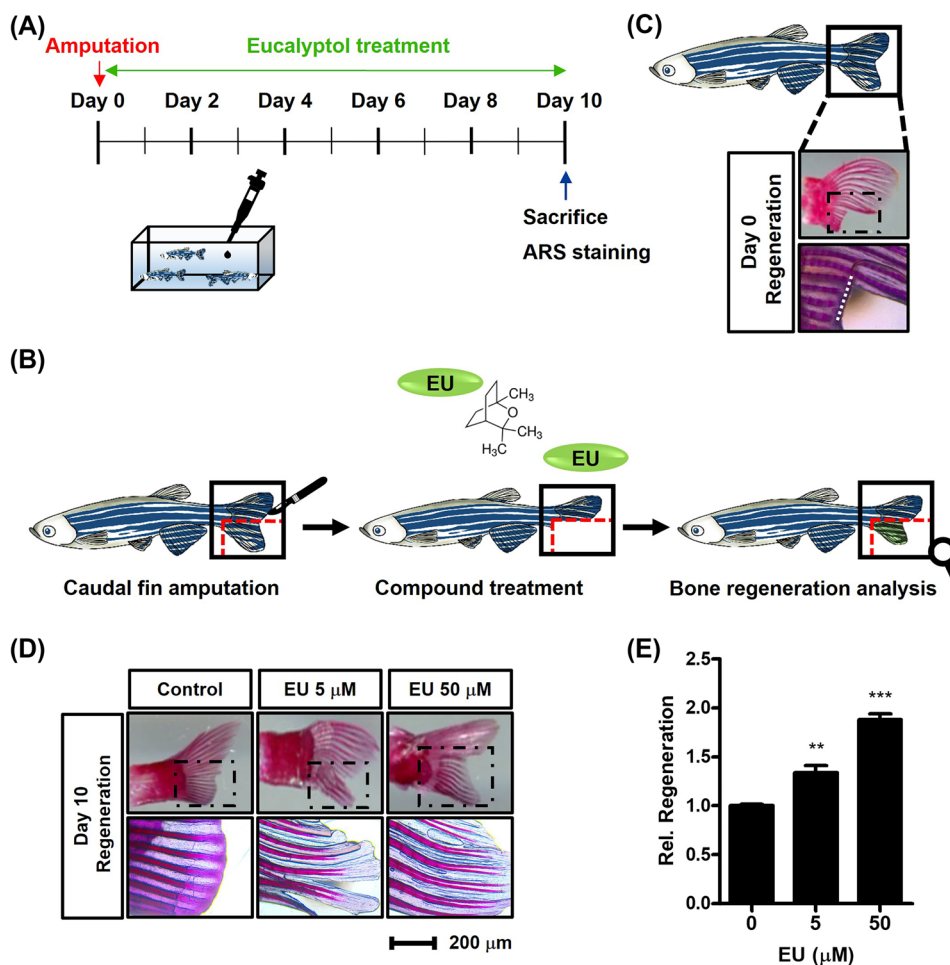
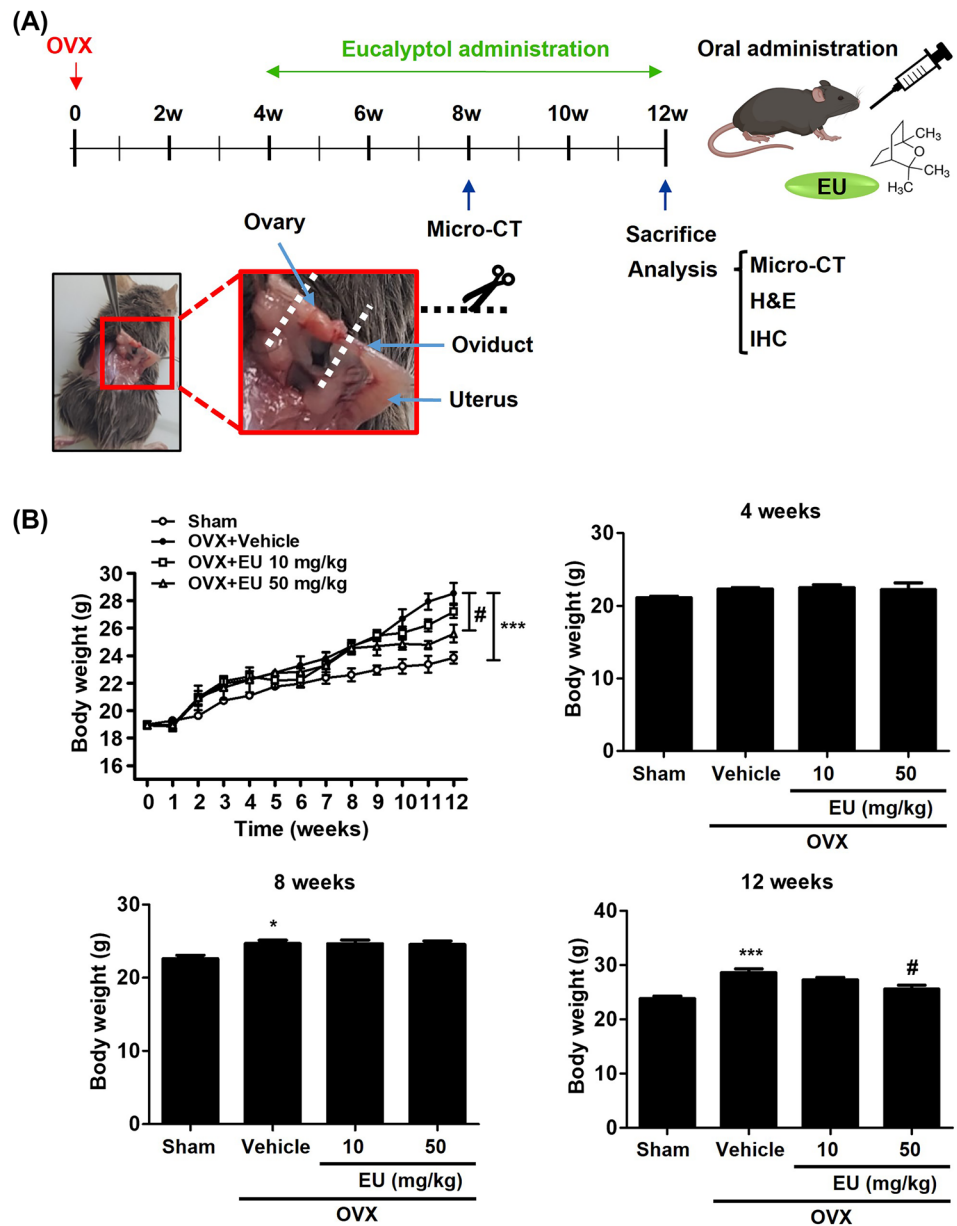


Fig. 5 Effect of EU on the body weight of OVX mice. **A** Schematic diagram of experimental design used for EU treatment in OVX mice. Bone loss was induced for 4 weeks after surgical removal of the ovaries. OVX mice were orally administered with EU daily for 8 weeks. Mice were sacrificed, and the effect of EU on bone loss was analyzed. **B** Changes of body weight after ovariectomy and EU treatment in C57BL/6 mice (n = 4, per group) (* $P < 0.05$, *** $P < 0.001$ compared with the sham group, # $P < 0.05$ compared with the OVX + vehicle group)



surgery significantly decreased trabecular bone mass, but the OVX + EU 50 mg/kg group at 8 weeks had significantly less bone loss (Fig. 6A). As a result of quantitative analysis of trabecular morphological parameters, bone mineral density (BMD) was significantly increased in the OVX + EU 50 mg/kg group compared to the OVX group. BV/TV, Tb.N and Tb.Th showed no statistical significance (Fig. 6B-E). In addition, we confirmed histological morphology of the femurs through H&E staining images. Distal femoral tissues of OVX mice showed lower trabecular bone density and higher number of fat droplets compared to the sham group, but these results were reversed in the OVX + EU 50 mg/kg group (Fig. 7A). H&E staining images showed consistent results with micro-CT scanning. In addition, the

expression of phosphorylated ERK was significantly lower in the OVX group than in the sham group but increased in the EU 50 mg/kg treatment group (Fig. 7B). These results suggest that EU ameliorated bone loss by promoting bone formation in OVX mice, and this effect is mediated through ERK phosphorylation.

Discussion

EU is a monoterpene compound present in the form of monoterpene oxide. Naturally occurring terpenes, such as EU, are lipophilic molecules that increase the intracellular penetration of drugs, so they may be potentially important

drugs in the treatment strategy of chronic diseases [38]. EU has antibacterial, anti-inflammatory and antioxidant effects in chronic diseases such as inflammation, respiratory disease, diabetic disease, and contractile cardiovascular disease [34, 38]. Oxidative stress and inflammation are important processes that act as major etiologies in bone disease [39]. Several plant-derived natural products with anti-inflammatory and antioxidant properties have been reported to be effective in osteoblast differentiation and bone formation [26, 40, 41]. We hypothesized that EU would have a potentially beneficial effects on bone and verified new efficacy on

osteoblast differentiation and bone formation. This study is an initial study to identify the new functions of EU. Therefore, it is unclear whether EU mediates the effects of bone through anti-inflammatory and antioxidant effects, and further studies are needed.

In this study, we used pre-osteoblast cell line MC3T3-E1 and primary mouse calvarial cells and showed that EU induces osteoblast differentiation by increasing the mRNA and protein levels of *Dlx5* and *Runx2*. Osteogenic genes such as *Dlx5* and *Runx2* are essential regulators in osteoblast differentiation. *Dlx5* mediates expression of several

Fig. 6 Effect of EU on trabecular morphometric parameters in the distal femurs of OVX mice. After 4 and 8 weeks of EU oral administration, micro-CT analysis was performed on the distal femurs. **A** Representative 3D images of the trabecular bone. Micro-CT images were obtained from sham group, OVX + vehicle group, OVX + EU 10 mg/kg group and OVX + EU 50 mg/kg group ($n=4$, per group). Comparative analysis of the bone structural parameters: **B** bone mineral density (BMD), **C** bone volume/tissue volume ratio (BV/TV), **D** trabecular number (Tb.N), and **E** trabecular thickness (Tb.Th) (** $P < 0.001$ compared with the sham group; # $P < 0.05$ compared with the OVX + vehicle group)

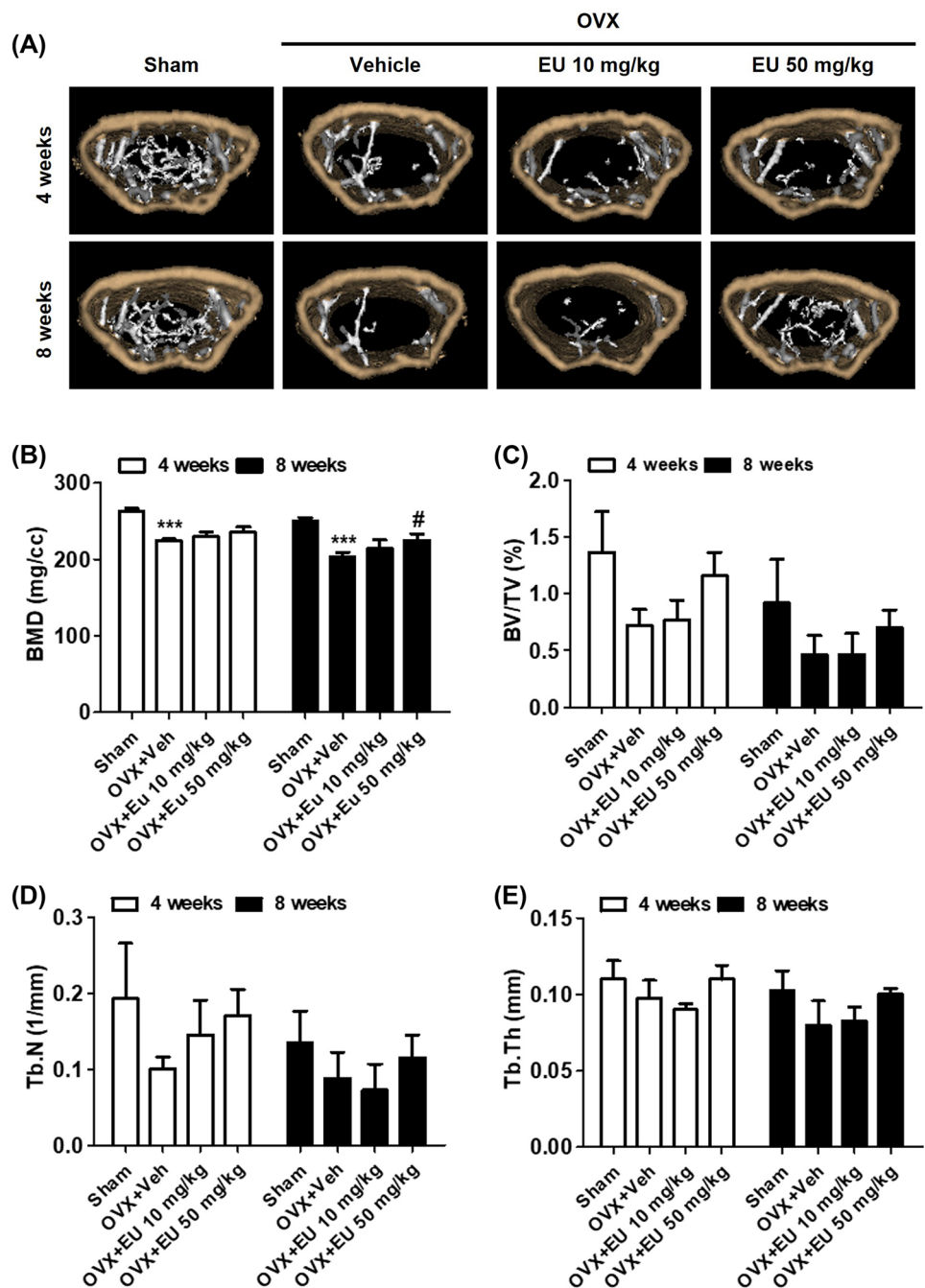
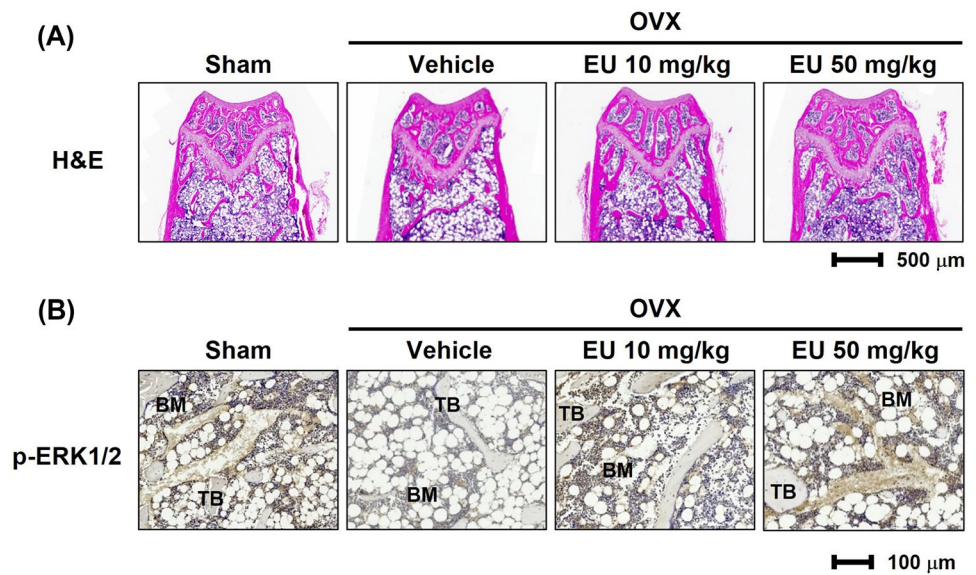


Fig. 7 Histological and immunohistochemical evaluation in distal femurs of OVX mice. After all the treatment procedures, mice were sacrificed and femur tissues were isolated (n = 4, per group). **A** Representative images of H&E staining from femur bone sections of each group. Scale bar, 500 μ m. **B** Representative images of IHC staining with p-ERK antibody from femur bone sections of each group. Scale bar, 100 μ m. Abbreviations: BM, bone marrow; TB, trabecular bone

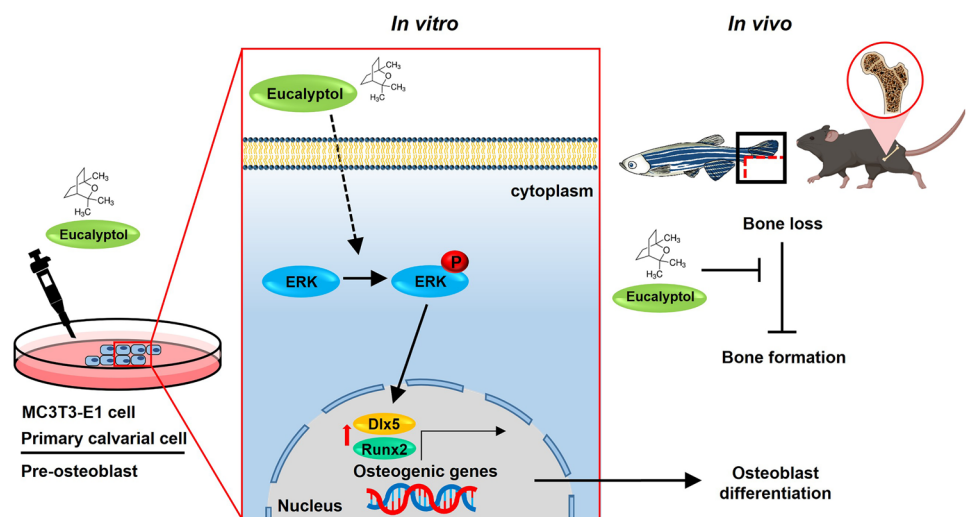


osteogenic markers to induce osteoblast differentiation [13]. Runx2 produces bone matrix proteins and plays a central role in osteoblast differentiation [42]. Our results indicate that EU regulates osteoblast differentiation by ERK phosphorylation. According to several previous reports, MAPK promotes osteoblast differentiation by inducing phosphorylation of osteogenic proteins such as Dlx5 or Runx2 to increase their stability [43, 44]. EU has been reported to regulate MAPK/ERK signaling in macrophages and podocytes, but the molecular mechanisms for osteoblast differentiation and bone formation have not been studied [38, 45]. MAPK/ERK signaling is an important pathway in promoting osteoblast differentiation and bone formation [9]. As a result of confirming the correlation between osteoblast differentiation and ERK by EU treatment, it was found that PD98059, an ERK inhibitor, suppressed the expression of EU-induced Dlx5 and Runx2. In addition, blockade of

ERK phosphorylation by PD98059 reduced ALP activity and extracellular matrix mineralization. This suggests that ERK can not only increase the stability of Dlx5 and Runx2 but also regulate their expression. However, further studies will be needed on how ERK increases the expression of these genes.

Next, we conducted an *in vivo* study based on the *in vitro* results. Bony fish such as zebrafish can regenerate various organs and tissues after injury [46]. In particular, the zebrafish caudal fin rays regenerate and maintain the bone rays through a series of dedifferentiation and redifferentiation [35, 36]. Therefore, the regenerative capacity of the caudal fin is a useful system for compounds screening that promote bone formation [47]. In this study, EU promoted the regeneration of the caudal fin rays in a dose-dependent manner. Although zebrafish have high regenerative capacity, complete regeneration is impossible if the injury exceeds

Fig. 8 Schematic diagram showing the effects of EU on osteoblast differentiation and its ameliorative effects on bone loss-induced animal models such as zebrafish and OVX mice. As depicted, EU induces osteoblast differentiation through ERK phosphorylation. EU promotes bone rays' regeneration in amputated zebrafish caudal fin and ameliorates bone loss in OVX mice through ERK phosphorylation



a critical size [48]. This suggests that EU promotes osteoblast differentiation in zebrafish caudal fin ray. The OVX mouse model is used mainly to study osteoporosis caused by estrogen deficiency after the menopause [31]. Estrogen promotes the differentiation of osteoblasts from the same embryonic cells and inhibits the differentiation of adipocytes [49]. In other words, ovariectomy activates the differentiation of hematopoietic stem cell-derived osteoclasts and mesenchymal stem cell-derived adipocytes by deficient estrogen secretion in mice. Therefore, weight gain by estrogen deficiency is caused by adipose tissue accumulation, and weight change in OVX mice can be an important predictor of osteoporosis disease because there is a correlation between bone resorption and weight gain [50]. In this study, it was confirmed that the body weight of osteoporosis model mice induced by ovariectomy increased significantly compared to normal mice, but the weight gain of EU-administered mice decreased at 12 weeks. In addition, we found that oral administration of EU ameliorated bone loss in OVX mice. However, while high concentration of EU (50 mg/kg) protected against bone loss, low concentration of EU (10 mg/kg) had little effect. These results suggest that 50 mg/kg is the standard concentration for EU to improve metabolically induced osteoporosis. The MAPK/ERK pathway is essential for skeletal development and homeostasis [9]. Several studies have shown that the ERK pathway promotes osteoblast differentiation and bone formation [7, 8]. Herein, administration of EU (50 mg/kg) prevented the OVX-mediated reduction of ERK phosphorylation in OVX mice femurs. ERK phosphorylation was mainly detected in the trabecular region below the growth plate, which has high bone remodeling activity. We thought that EU prevents bone loss by increasing ERK phosphorylation in femur tissue.

In summary, this study investigated the effect of EU on osteoblast differentiation and an *in vivo* model for surgically induced bone loss (Fig. 8). EU induced osteoblast differentiation by regulating ERK phosphorylation. In addition, EU promoted regeneration of caudal fin bony rays in amputated zebrafish. In addition, EU increased ERK phosphorylation and ameliorated bone loss in the distal femur of osteoporosis induced OVX mice. We demonstrated that EU treatment induced osteoblast differentiation and osteogenesis via phosphorylation of ERK *in vitro* and *in vivo*. Therefore, we suggest that EU is an organic compound that may be useful in the prevention of bone loss.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00109-023-02348-x>.

Author contributions Conceptualization: Do-Won Lee; methodology: Do-Won Lee, Kyeong-Min Kim, Seulki Park; formal analysis: Do-Won Lee; investigation: Do-Won Lee, Kyeong-Min Kim, Seulki Park, Young-Ju Lim; writing – original draft preparation: Do-Won Lee; resources: Sang-Hyun An and Won-Gu Jang; funding acquisition and supervision: Won-Gu Jang; All authors read and approved the final manuscript.

Funding This research was supported by the Basic Science Research Program of the National Research Foundation of Korea (NRF) funded by the Ministry of Education (No. 2021R1F1A1062502).

Data availability The data presented in this work are available upon request from the corresponding author.

Declarations

Ethics approval and consent to participate Zebrafish studies were carried out in accordance with the guidelines and regulations approved by the Committee for Laboratory Animal Care and Use of Daegu University (approval number: DUIACC-12020/4-0313-006). All procedures for OVX studies were carried out in accordance with the guidelines and regulations approved by the Institutional Animal Care and Use Committee of Laboratory Animal Center of Daegu-Gyeongbuk Medical Innovation Foundation (approval number: DGMIF-20060304-02). This study does not involve human subjects.

Competing interests The authors declare no competing interests.

References

1. Heino TJ, Hentunen TA (2008) Differentiation of osteoblasts and osteocytes from mesenchymal stem cells. *Curr Stem Cell Res Ther* 3:131–145
2. Birmingham E, Niebur G, McHugh PE (2012) Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche. *Eur Cell Mater* 23:13–27
3. Rosen V (2009) BMP2 signaling in bone development and repair. *Cytokine Growth Factor Rev* 20:475–480. <https://doi.org/10.1016/j.cytogfr.2009.10.018>
4. Ballif BA, Blenis J (2001) Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. *Cell Growth Differ* 12:397–408
5. Raman M, Chen W, Cobb M (2007) Differential regulation and properties of MAPKs. *Oncogene* 26:3100–3112
6. Murphy LO, Smith S, Chen RH, Fingar DC, Blenis J (2002) Molecular interpretation of ERK signal duration by immediate early gene products. *Nat Cell Biol* 4:556–564. <https://doi.org/10.1038/ncb822>
7. Shen M-j, Wang G-g, Wang Y-z, Xie J, Ding X (2018) Nell-1 enhances osteogenic differentiation of pre-osteoblasts on titanium surfaces via the MAPK-ERK signaling pathway. *Cell Physiol Biochem* 50:1522–1534
8. Ge C, Xiao G, Jiang D, Franceschi RT (2007) Critical role of the extracellular signal-regulated kinase-MAPK pathway in osteoblast differentiation and skeletal development. *J Cell Biol* 176:709–718
9. Kim J-M, Yang Y-S, Park KH, Oh H, Greenblatt MB, Shim J-H (2019) The ERK MAPK pathway is essential for skeletal development and homeostasis. *Int J Mol Sci* 20:1803
10. Lai C-F, Chaudhary L, Fausto A, Halstead LR, Ory DS, Avioli LV, Cheng S-L (2001) Erk is essential for growth, differentiation, integrin expression, and cell function in human osteoblastic cells. *J Biol Chem* 276:14443–14450
11. Franceschi RT, Ge C, Xiao G, Roca H, Jiang D (2009) Transcriptional regulation of osteoblasts. *Cells Tissues Organs* 189:144–152. <https://doi.org/10.1159/000151747>
12. Ge C, Xiao G, Jiang D, Yang Q, Hatch NE, Roca H, Franceschi RT (2009) Identification and functional characterization of ERK/

- MAPK phosphorylation sites in the Runx2 transcription factor. *J Biol Chem* 284:32533–32543
13. Olsen BR, Reginato AM, Wang W (2000) Bone development. *Annu Rev Cell Dev Biol* 16:191–220
 14. Donaubaue A-J, Deloch L, Becker I, Fietkau R, Frey B, Gaip US (2020) The influence of radiation on bone and bone cells—differential effects on osteoclasts and osteoblasts. *Int J Mol Sci* 21:6377
 15. Pham QP, Kasper FK, Baggett LS, Raphael RM, Jansen JA, Mikos AG (2008) The influence of an in vitro generated bone-like extracellular matrix on osteoblastic gene expression of marrow stromal cells. *Biomaterials* 29:2729–2739
 16. Seeman E (2003) Reduced bone formation and increased bone resorption: rational targets for the treatment of osteoporosis. *Osteoporos Int* 14:2–8
 17. Boonen S, Dejaeger E, Vanderschueren D, Venken K, Bogaerts A, Verschueren S, Milisen K (2008) Osteoporosis and osteoporotic fracture occurrence and prevention in the elderly: a geriatric perspective. *Best Pract Res Clin Endocrinol Metab* 22:765–785
 18. Jilka R (1998) Cytokines, bone remodeling, and estrogen deficiency: a 1998 update. *Bone* 23:75–81
 19. Srivastava M, Deal C (2002) Osteoporosis in elderly: prevention and treatment. *Clin Geriatr Med* 18:529–555
 20. Lobo RA (1995) Benefits and risks of estrogen replacement therapy. *Am J Obstet Gynecol* 173:982–989. [https://doi.org/10.1016/0002-9378\(95\)90247-3](https://doi.org/10.1016/0002-9378(95)90247-3)
 21. Janovská Z (2012) Bisphosphonate-related osteonecrosis of the jaws. A severe side effect of bisphosphonate therapy. *Acta Medica (Hradec Kralove)* 55:111–115
 22. Skjodt MK, Frost M, Abrahamson B (2019) Side effects of drugs for osteoporosis and metastatic bone disease. *Br J Clin Pharmacol* 85:1063–1071. <https://doi.org/10.1111/bcp.13759>
 23. Li H, Xiao Z, Quarles LD, Li W (2021) Osteoporosis: Mechanism, Molecular Target and Current Status on Drug Development. *Curr Med Chem* 28:1489–1507. <https://doi.org/10.2174/0929867327666200330142432>
 24. Anderson JJ, Garner SC (1998) Phytoestrogens and bone. *Baillieres Clin Endocrinol Metab* 12:543–557. [https://doi.org/10.1016/s0950-351x\(98\)80003-7](https://doi.org/10.1016/s0950-351x(98)80003-7)
 25. Putnam SE, Scutt AM, Bicknell K, Priestley CM, Williamson EM (2007) Natural products as alternative treatments for metabolic bone disorders and for maintenance of bone health. *Phytotherapy research* : PTR 21:99–112. <https://doi.org/10.1002/ptr.2030>
 26. Kim MB, Song Y, Hwang JK (2014) Kirenel stimulates osteoblast differentiation through activation of the BMP and Wnt/ β -catenin signaling pathways in MC3T3-E1 cells. *Fitoterapia* 98:59–65. <https://doi.org/10.1016/j.fitote.2014.07.013>
 27. Mahmoud AL (1994) Antifungal action and antiaflatoxicogenic properties of some essential oil constituents. *Lett Appl Microbiol* 19:110–113. <https://doi.org/10.1111/j.1472-765x.1994.tb00918.x>
 28. Sabbieti MG, Agas D, Maggi F, Vittori S, Marchetti L (2011) Molecular mediators involved in *Ferulago campestris* essential oil effects on osteoblast metabolism. *J Cell Biochem* 112:3742–3754. <https://doi.org/10.1002/jcb.23306>
 29. Muhlbaue RC, Lozano A, Palacio S, Reinli A, Felix R (2003) Common herbs, essential oils, and monoterpenes potently modulate bone metabolism. *Bone* 32:372–380. [https://doi.org/10.1016/s8756-3282\(03\)00027-9](https://doi.org/10.1016/s8756-3282(03)00027-9)
 30. Dolder S, Hofstetter W, Wetterwald A, Muhlbaue RC, Felix R (2006) Effect of monoterpenes on the formation and activation of osteoclasts in vitro. *J Bone Miner Res* 21:647–655. <https://doi.org/10.1359/jbmr.060111>
 31. Azmy Abd El-Motelp B, Tarek Ebrahim M, Khairy Mohamed H (2021) *Salvia officinalis* Extract and 17 β -Estradiol Suppresses Ovariectomy Induced Osteoporosis in Female Rats. *Pak J Biol Sci* 24:434–444. <https://doi.org/10.3923/pjbs.2021.434.444>
 32. Santos FA, Rao VS (2000) Antiinflammatory and antinociceptive effects of 1,8-cineole a terpenoid oxide present in many plant essential oils. *Phytotherapy research* : PTR 14:240–244. [https://doi.org/10.1002/1099-1573\(200006\)14:4%3c240::aid-ptr573%3e3.0.co;2-x](https://doi.org/10.1002/1099-1573(200006)14:4%3c240::aid-ptr573%3e3.0.co;2-x)
 33. Juergens UR (2014) Anti-inflammatory properties of the monoterpene 1,8-cineole: current evidence for co-medication in inflammatory airway diseases. *Drug Res (Stuttg)* 64:638–646. <https://doi.org/10.1055/s-0034-1372609>
 34. Gondim FL, Serra DS, Cavalcante FSA (2019) Effects of Eucalyptol in respiratory system mechanics on acute lung injury after exposure to short-term cigarette smoke. *Respir Physiol Neurobiol* 266:33–38. <https://doi.org/10.1016/j.resp.2019.04.007>
 35. Knopf F, Hammond C, Chekuru A, Kurth T, Hans S, Weber CW, Mahatma G, Fisher S, Brand M, Schulte-Merker S et al (2011) Bone regenerates via dedifferentiation of osteoblasts in the zebrafish fin. *Dev Cell* 20:713–724
 36. Geurtzen K, Knopf F, Wehner D, Huitema LF, Schulte-Merker S, Weidinger G (2014) Mature osteoblasts dedifferentiate in response to traumatic bone injury in the zebrafish fin and skull. *Development* 141:2225–2234
 37. Kishimoto N, Shimizu K, Sawamoto K (2012) Neuronal regeneration in a zebrafish model of adult brain injury. *Dis Model Mech* 5:200–209
 38. Seol GH, Kim KY (2016) Eucalyptol and Its Role in Chronic Diseases. *Adv Exp Med Biol* 929:389–398. https://doi.org/10.1007/978-3-319-41342-6_18
 39. Ginaldi L, Di Benedetto MC, De Martinis M (2005) Osteoporosis, inflammation and ageing. *Immun Ageing* 2:14. <https://doi.org/10.1186/1742-4933-2-14>
 40. Min HY, Son HE, Jang WG (2020) Alpha-pinene promotes osteoblast differentiation and attenuates TNF α -induced inhibition of differentiation in MC3T3-E1 pre-osteoblasts. *Clin Exp Pharmacol Physiol* 47:831–837. <https://doi.org/10.1111/1440-1681.13245>
 41. Zhao L, Wang Y, Wang Z, Xu Z, Zhang Q, Yin M (2015) Effects of dietary resveratrol on excess-iron-induced bone loss via anti-oxidative character. *J Nutr Biochem* 26:1174–1182. <https://doi.org/10.1016/j.jnutbio.2015.05.009>
 42. Komori T (2017) Roles of Runx2 in skeletal development. *Adv Exp Med Biol* 83–93
 43. Rodriguez-Carballo E, Gamez B, Ventura F (2016) p38 MAPK Signaling in Osteoblast Differentiation. *Front Cell Dev Biol* 4:40. <https://doi.org/10.3389/fcell.2016.00040>
 44. Greenblatt MB, Shim JH, Bok S, Kim JM (2022) The Extracellular Signal-Regulated Kinase Mitogen-Activated Protein Kinase Pathway in Osteoblasts. *J Bone Metab* 29:1–15. <https://doi.org/10.11005/jbm.2022.29.1.1>
 45. Kim DY, Kang MK, Lee EJ, Kim YH, Oh H, Kang YH (2018) Eucalyptol Inhibits Advanced Glycation End Products-Induced Disruption of Podocyte Slit Junctions by Suppressing RAGE-Erk-C-Myc Signaling Pathway. *Mol Nut Food Res* 62:e1800302. <https://doi.org/10.1002/mnfr.201800302>
 46. Gemberling M, Bailey TJ, Hyde DR, Poss KD (2013) The zebrafish as a model for complex tissue regeneration. *Trends Genet* 29:611–620
 47. Thatcher EJ, Paydar I, Anderson KK, Patton JG (2008) Regulation of zebrafish fin regeneration by microRNAs. *Proc Natl Acad Sci U S A* 105:18384–18389
 48. Uemoto T, Abe G, Tamura K (2020) Regrowth of zebrafish caudal fin regeneration is determined by the amputated length. *Sci Rep* 10:1–11
 49. Okazaki R, Inoue D, Shibata M, Saika M, Kido S, Ooka H, Tomiyama H, Sakamoto Y, Matsumoto T (2002) Estrogen promotes

- early osteoblast differentiation and inhibits adipocyte differentiation in mouse bone marrow stromal cell lines that express estrogen receptor (ER) alpha or beta. *Endocrinology* 143:2349–2356. <https://doi.org/10.1210/endo.143.6.8854>
50. Khor VK, Dhir R, Yin X, Ahima RS, Song WC (2010) Estrogen sulfotransferase regulates body fat and glucose homeostasis in female mice. *Am J Physiol Endocrinol Metab* 299:E657–664. <https://doi.org/10.1152/ajpendo.00707.2009>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.