



Tectorigenin Promotes Osteoblast Differentiation and *in vivo* Bone Healing, but Suppresses Osteoclast Differentiation and *in vivo* Bone Resorption

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Although tectorigenin (TG), a major compound in the rhizome of *Belamcanda chinensis*, is conventionally used for the treatment of inflammatory diseases, its effects on osteogenesis and osteoclastogenesis have not been reported. The objective of this study was to investigate the effects and possible underlying mechanism of TG on *in vitro* osteoblastic differentiation and *in vivo* bone formation, as well as *in vitro* osteoclast differentiation and *in vivo* bone resorption. TG promoted the osteogenic differentiation of primary osteoblasts and periodontal ligament cells. Moreover, TG upregulated the expression of the BMP2, BMP4, and Smad-4 genes, and enhanced the expression of Runx2 and Osterix. *In vivo* studies involving mouse calvarial bone defects with μ CT and histologic analysis revealed that TG significantly increased new bone formation. Furthermore, TG treatment inhibited osteoclast differentiation and the mRNA levels of osteoclast markers. *In vivo* studies of mice demonstrated that TG caused the marked attenuation of bone resorption. These results collectively demonstrated that TG stimulated osteogenic differentiation *in vitro*, increased *in vivo* bone regeneration, inhibited osteoclast differentiation *in vitro*, and suppressed inflammatory bone loss *in vivo*. These novel findings suggest that TG may

be useful for bone regeneration and treatment of bone diseases.

Keywords: bone remodeling, differentiation, osteoblast, osteoclast, tectorigenin

INTRODUCTION

The balance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation maintains bone homeostasis (Rho et al., 2004). Osteolytic diseases such as osteoporosis, Paget's disease, and multiple myeloma are caused by a decoupling of bone remodeling as a result of increased activity of osteoclasts and decreased activity of osteoblasts (Marie et al., 2011). These lesions constitute a major healthcare problem, because of not only their high incidence but also the resultant high levels of disability and costs to the healthcare system (Muraki et al., 2014). Estrogen and other anti-osteoporosis drugs (e.g., bisphosphonates and calcitonin) function as inhibitors of bone resorption, but their ability to increase or recover bone mass is minimal

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(Riggs et al., 2003; 2005). Moreover, use of the FDA-approved anabolic agent parathyroid hormone is limited because it is quite expensive and difficult to administer (Augustine et al., 2013). Therefore, specific suppressors of bone resorption and stimulators of bone formation are of therapeutic importance in osteolytic diseases, such as osteoporosis.

Traditional Chinese medicines or natural products, including flavonoids, are a potential source of agents for preventing and treating bone disease (Chen et al., 2005; Kim et al., 2004). Active natural compounds, including dioscin, can reduce ovariectomy-induced bone loss by enhancing osteoblastogenesis and inhibiting osteoclastogenesis (Tao et al., 2016). Recently, we demonstrated that 2,4,5-trimethoxyldalbergiquinol, isolated from *D. odorifera*, promoted osteoblastic differentiation and mineralization (Yun et al., 2015). Tectorigenin (TG), an O-methylated isoflavone, isolated from the rhizome of *Belamcanda chinensis* (Iridaceae), is a Traditional Chinese Medicine for the treatment of inflammatory diseases such as asthma and tonsillitis (Kim, 1996). Previous studies have reported that TG has anticancer (Kapoor 2013; Lee et al., 2001; Liu et al., 2012; Thelen et al., 2005; Yang et al., 2012), antiproliferative, and pro-apoptotic effects on hepatic stellate cells (Wu et al., 2010). In addition, it showed antioxidant (Han et al., 2012), hypoglycemic, and hypolipidemic effects (Ma et al., 2014), and various anti-inflammatory

activities such as against lung injury (Ma et al., 2014), carrageenan-induced rat paw edema (Ha et al., 2013), and *in vitro* macrophage RAW 264.7 cells (Kim et al. 1999A; 1999B; Pan et al., 2008). We recently reported that TG has antibacterial activity against methicillin-resistant *Staphylococcus aureus* (Jeong et al., 2007). Furthermore, TG inhibits adipogenic differentiation and adipocytokine secretion (Li et al., 2015).

Although TG is considered a promising candidate drug, the effects of this compound on bone metabolism have not been reported to date. Therefore, this study evaluated the effects and underlying mechanism of TG on *in vitro* osteoblastic differentiation and *in vivo* bone regeneration, as well as *in vitro* osteoclast formation and *in vivo* bone resorption.

MATERIALS AND METHODS

Isolation and purification of TG

TG (Fig. 1A) was deposited in the Standardized Material Bank for New Botanical Drugs (number NNMBP000017) at Wonkwang University (Korea). It was isolated from the rhizome of *B. chinensis* as previously described (Jeong et al., 2007). The purity (99.46%) of TG was determined by HPLC.

Cell culture

Primary osteoblasts were isolated from calvariae of 1-day-old

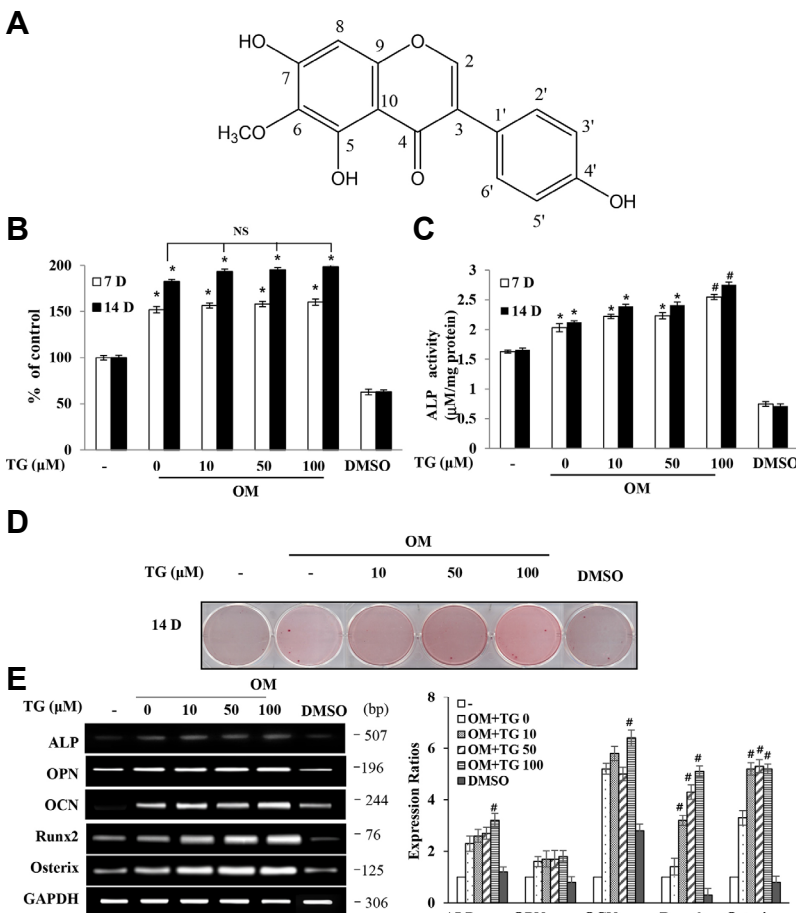


Fig. 1. Effects of TG on cytotoxicity and osteoblastic differentiation in primary osteoblasts. (A) Chemical structure of TG. (B) Cell viability was determined using the MTT assay. (C-E) Differentiation was assessed by ALP activity (C), Alizarin red staining (D), and RT-PCR for marker genes (E). Cells were treated with osteogenic medium (OM) containing growth medium, 50 μg/mL L-ascorbic acid and 10 mM β-glycerophosphate for 14 days (B-E). The histogram (right) shows the quantification of mRNA expression by densitometry; the data are expressed relative to non-stimulated control cells (E). **p* < 0.05 vs. control. #*p* < 0.05 vs. OM control. NS = not significant. The data are representative of three independent experiments.

ICR mice after aseptic dissection and treated with 0.2% collagenase-dispase enzyme solution (Sigma-Aldrich, USA). Cells from digestions 6-8 ($10\text{--}25 \times 10^6$ cells) were pooled and seeded at a density of 2×10^6 cells/ 175 cm^2 in culture flasks containing α -minimum essential medium (α -MEM) supplemented with 10% FBS and antibiotics. The cells were cultured for 4-6 days, with a change of medium every 2 or 3 days, at 37°C in a humidified atmosphere containing 5% CO₂ in air. The Ethics Committee for Animal Experiments at Kyung Hee University (Seoul, Korea) approved the study. To induce differentiation, cells were cultured with sulfuretin or rh-BMP2 (Calbiochem Co., USA) and osteogenic supplement (OS: 50 $\mu\text{g/ml}$ ascorbic acid and 10 mM β -glycerophosphate) as previously described (Lee et al., 2013; 2015). Immortalized human dental periodontal ligament cells (PDLCs) (Kitagawa et al, 2006) transfected with the human telomerase catalytic component (hTERT) were kindly provided by Professor Takashi Takata (Hiroshima University, Japan). Cells were cultured at 37°C in α -MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin in a humidified atmosphere of 5% CO₂.

Primary cultures of mouse bone marrow-derived macrophages (BMMs) were used for osteoclast differentiation, as previously described (Bae et al., 2015; Shin et al., 2015). For the generation of BMMs, monocytes were isolated from the tibiae of 6-week-old ICR mice (Charles River Laboratories, Korea). Cells were seeded in 100 mm plates and cultured in the presence of 30 ng/ml M-CSF for 72 h. RAW 264.7 cells were obtained from the Korean Cell Line Bank (KCLB). All of the cells were cultured in α -MEM (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO) and 1% antibiotic-antimycotic in a 5% CO₂ atmosphere. The culture medium was exchanged for fresh medium every 3 days. All animal experiments were carried out in accordance with the relevant guidelines.

Cytotoxicity

Cytotoxicity of TG was measured by an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. BMMs were plated in 96-well plates at a density of 2×10^4 cells/well with indicated concentrations of TG for 5 days. Primary osteoblasts and PDLCs were treated with indicated concentration for 14 days. Fifty microliters of MTT solution (5 mg/ml, Sigma, USA) was added to the cells, and then incubated for 2 h. The absorbance of each well was measured at 570 nm using enzyme linked immunosorbent assay (ELISA) reader (Beckman Coulter, USA). The cell viability was calculated as the percentages of absorbance of the treatment divided by the absorbance of the control.

TRAP staining

BMMs were seeded in 48-well plates at a density of 2×10^4 cells per well and were incubated with RANKL (100 ng/ml) and M-CSF (30 ng/ml). After 5 days, cells were fixed by soaking in 3.7% formaldehyde for 15 min. Cells were washed and then incubated for 30 min at 37°C in the dark using a mixture of the solutions in the Leukocyte Acid Phosphatase Assay kit following the manufacturer's instructions. TRAP-positive multinucleated cells containing three or more

nuclei were considered to be osteoclasts.

Alkaline phosphatase activity

Alkaline phosphatase activity (ALP) activity was measured in 0.7 M 2-aminomethyl-1-propanol (pH 10.3) and 6.7 mM MgCl₂ using p-nitrophenyl phosphate (3 mM final concentration) as the substrate. The absorbance at a wavelength of 405 nm was measured using an ELISA reader.

Alizarin Red S staining

Cells were stained with 40 mM Alizarin Red S (pH 4.2) for 10 min with gentle agitation. Alizarin Red S staining was visualized by light microscopy.

RNA isolation and reverse transcriptase-polymerase chain reaction

Total RNA was extracted from cells with TRIzol reagent (Life Technologies, USA), according to the manufacturer's instructions. RNA (1 μg) was reverse-transcribed using oligo (dT)₁₅ primers and AccuPower RT PreMix (iNtRON Biotechnology, Korea). The cDNAs generated were amplified using AccuPower PCR PreMix (Bioneer Corporation, Korea). PCR products were subjected to electrophoresis in 1.5% agarose gels and stained with ethidium bromide. Densitometric analysis of each band was performed using a computerized image processing system (Quantity One; Bio-Rad, USA).

Western blot analysis

Western blot analysis was performed by lysing cells in 20 mM Tris-HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM phenylmethanesulfonyl fluoride, 5 mg/ml aprotinin, 5 mg/ml pepstatin A, and 1 mg/ml chymostatin). Protein concentration was determined using the Lowry protein assay kit (P5626; Sigma). An equal amount of protein for each sample was resolved on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and then electrophoretically transferred to a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Bio-Rad, USA). The membrane was blocked in 5% skim milk and sequentially incubated with primary antibody and horseradish peroxidase-conjugated secondary antibody, followed by ECL detection (Amersham Pharmacia Biotech, USA).

Surgical procedure for calvarial bone defects in mice

Female 8-week-old ICR mice (Samtako, Korea) were maintained in accordance with National Institute of Toxicological Research of the Korea Food and Drug Administration guidelines for the humane care and use of laboratory animals. After acclimation for 2 weeks, the mice were anesthetized and a 5 mm diameter calvarial critical-sized defect was created on each side of the calvarial bone using a dental bur attached to a slow-speed handpiece with minimal invasion of the dura mater. The critical-sized defects in mice were randomly divided into three groups to receive the following surgery: (1) sham-surgery (empty) controls ($n = 6$); (2) TRR (3 $\mu\text{g/g}$ mouse weight, $n = 6$); (3) rh-BMP-2 (1 $\mu\text{g/g}$ mouse weight, $n = 6$). Animals were sacrificed 6 weeks post-surgery, and the calvarial bone was carefully excised, cleaned, and

fixed immediately in 10% formalin. Tissues were decalcified in 10% EDTA for 14 days, embedded in paraffin, and sectioned at 5 μm . Sections were stained with hematoxylin and eosin (H&E). All animal experiments were carried out in accordance with the relevant guidelines (Kyung Hee University, approval number: KHMC-IACUC 2015-002).

Micro-computed tomography

Micro-computed tomography (microCT) was performed at the Advanced Institutes of Convergence Technology (Genoss Co., Ltd., Korea). MicroCT data of calvaria were acquired using a Skyscan 1173 scanner (Bruker-microCT, Kontich, Belgium). Scanning was performed at 75 kV/106 μA for 500 ms. In total, 800 projections were collected at a resolution of 9.94 $\mu\text{m}/\text{pixel}$. Reconstruction of sections was performed using the software associated with the scanner (Nrecon), with the beam-hardening correction set to 40%. The Realistic 3D-Visualization software (Bruker-microCT) was used to reconstruct the CT images three-dimensionally from data acquired on ~2,000 cross-sections.

In vivo bone resorption

To evaluate the *in vivo* effects of TG on bone loss, mice were injected subcutaneously over the calvariae with LPS (12.5

$\mu\text{g}/\text{g}$ body weight) or TG (3 $\mu\text{g}/\text{g}$ body weight) on days 0 and 3. Mice were sacrificed 6 days after the first injection of LPS. The calvarial bone of the mice was analyzed by microCT. Quantitative analysis of the relative percentage of bone resorption (BV/TV) was performed with 3D microCT imaging. This study was approved by the Institutional Animal Care and Use Committee of Kyung Hee University (Korea) and performed in accordance with the criteria defined by the committee.

Statistical analysis

Data were analyzed using GraphPad Prism version 4 software (GraphPad Software, Inc., USA) and are presented as means \pm standard deviation. Comparisons between groups were performed by *t*-tests (two-sided) or ANOVA for experiments with more than two subgroups. Probability values of $P < 0.05$ were considered statistically significant.

RESULTS

TG stimulates osteoblastic differentiation of primary osteoblasts without cytotoxicity

Initially, MTT assays were performed over a 14-day cultivation period to assess the effects of TG on the cytotoxicity of

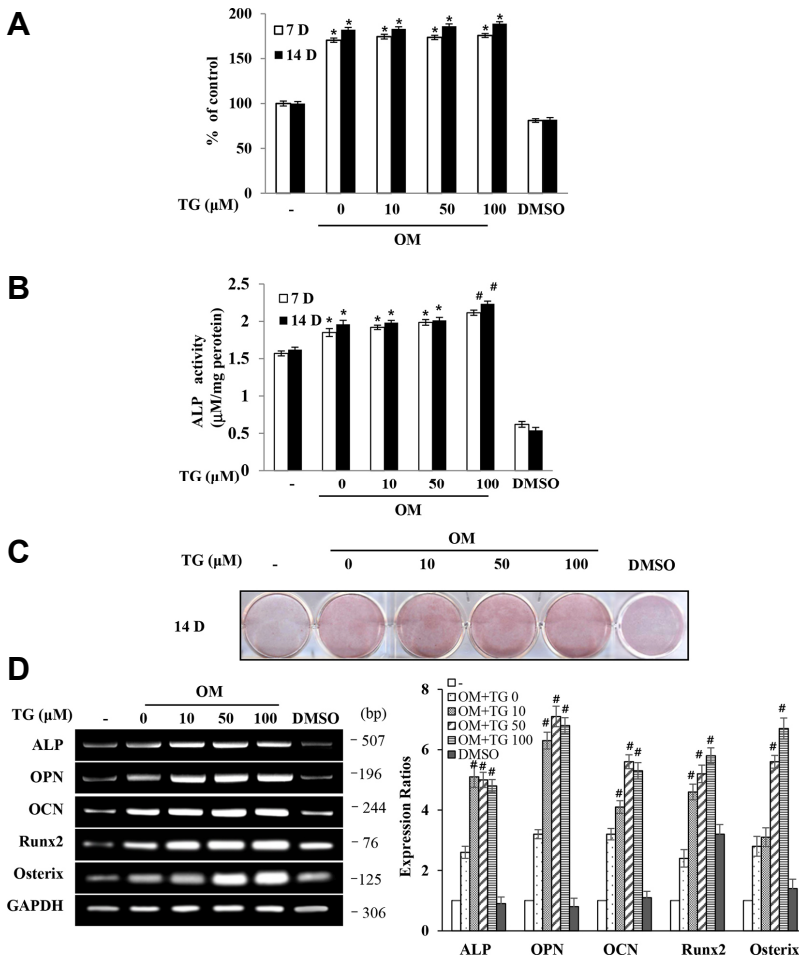


Fig. 2. Effects of TG on cytotoxicity and osteoblastic differentiation in human periodontal ligament cells (PDLCs). (A) Cell viability was determined using the MTT assay. (B-D) Differentiation was assessed by ALP activity (B), Alizarin red staining (C), and RT-PCR for marker genes (D). Cells were treated with osteogenic medium (OM) containing growth medium, 50 $\mu\text{g}/\text{mL}$ L-ascorbic acid and 10 mM β -glycerophosphate with the indicated concentrations of TG for 14 days (B-D). The histogram (right) shows the quantification of mRNA expression by densitometry; the data are expressed relative to non-stimulated control cells (D). * $p < 0.05$ vs. control. # $p < 0.05$ vs. OM control. The data are representative of three independent experiments.

primary osteoblasts. As shown in Fig. 2B, TG at 10-100 μM did not exhibit any cytotoxicity in primary osteoblasts. To examine the effects of TG on osteogenic property, ALP activity, mineralization, and the mRNA levels of various markers were assessed. Treatment of primary cultured osteoblasts with TG for 14 days increased the expression of early (ALP activity) and terminal differentiation markers (calcium deposition) in a concentration-dependent manner (Figs. 2B and 2C). Furthermore, TG upregulated the mRNA of differentiation markers such as ALP, osteocalcin (OCN), and osteopontin (OPN), as well as major bone-specific transcription factors such as Runx2 and osterix, in a concentration-dependent manner (Fig. 2D).

TG stimulates osteoblastic differentiation without cytotoxicity in PDLCs

To determine whether TG-induced osteoblastic differentiation is of broader importance for cells involved in bone regeneration, we examined the effects of TG in PDLCs, which contributes to periodontal tissue regeneration (Murakami et al., 2003). Treatment with TG increased ALP activity, mineral nodule formation, and the mRNA levels of markers, but did not affect cell growth (Figs. 2A-2D).

TG activates the BMP and MAPK pathways during osteoblastic differentiation

To determine the mechanism underlying the influence of TG on osteoblastic differentiation in osteoblasts, its effects on BMP and mitogen-activated protein kinase (MAPK) signaling

were examined. TG administration enhanced mRNA levels of *Bmp2*, *Bmp4*, and *Smad4* in primary osteoblasts (Fig. 3A). In addition, TG increased phosphorylated Smad1/5/8, ERK, and JNK levels, which are downstream molecules of BMP signaling, in primary osteoblasts (Figs. 3B and 3C).

TG promotes bone regeneration in a calvarial defect model

To assess the ability of TG to induce bone regeneration *in vivo*, mouse calvarial defects were treated with control, TG, or BMP-2, and were analyzed by microCT and histology. MicroCT images demonstrated increasing bone formation throughout a 6-week period for both TG and BMP-2-treated defects, whereas the control group exhibited minimal bone formation (Fig. 4A). Densitometric microCT analysis revealed that the new bone volume fraction in the TG group was significantly higher than that in the control group ($P < 0.05$), but was lower than that induced by treatment with rh-BMP2 (Fig. 4B). Histologic examination confirmed bone formation in the defect sites treated with TG, compared to mostly fibrous tissue in the control sites (Fig. 4C). Moreover, in the BMP group, the large bone mass between skull defects contained dense cancellous bone.

TG inhibits RANKL-induced osteoclastogenesis and mRNA levels of osteoclast-specific genes in mouse BMMs without cytotoxicity

To identify the nature of TG on osteoclast differentiation from mouse BMMs, the cells were cultured with 0-100 μM TG for 5 days in the presence of 30 ng/ml M-CSF and 100

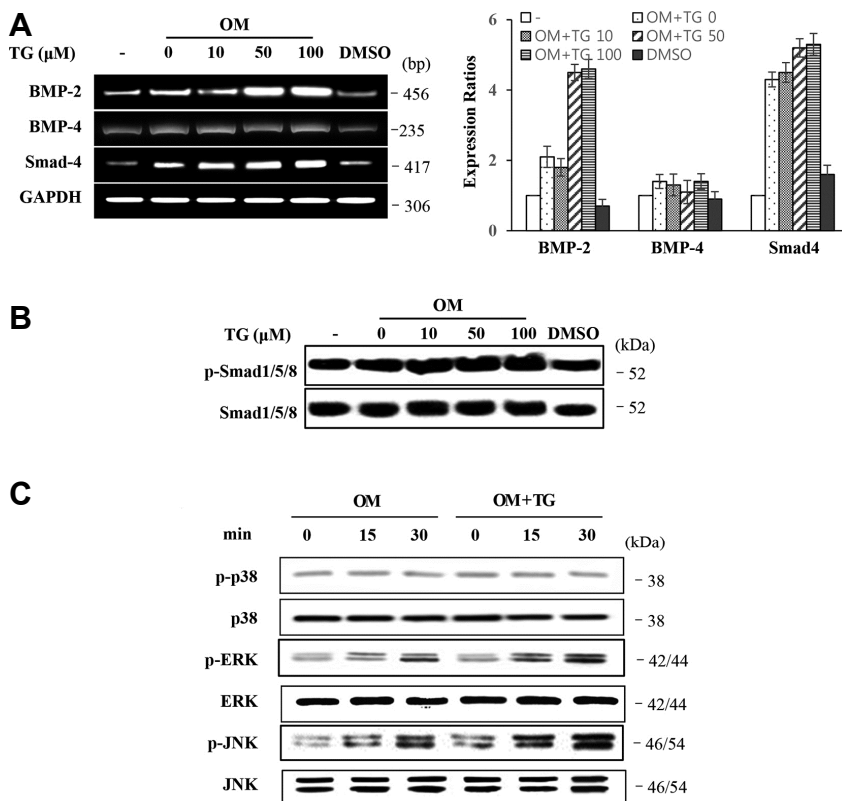


Fig. 3. Effects of TG on activation of BMP (A, B) and MAP kinase (C) pathways in osteoblasts. (A-C) Cells were cultured in OM with the indicated concentrations of TG for 3 days (A), 60 min (B), and 30 min (C). mRNA and protein levels were determined by RT-PCR and Western blotting, respectively. The histogram (right) shows the quantification of mRNA expression by densitometry; the data are expressed relative to non-stimulated control cells (A). Results are representative of three independent experiments.

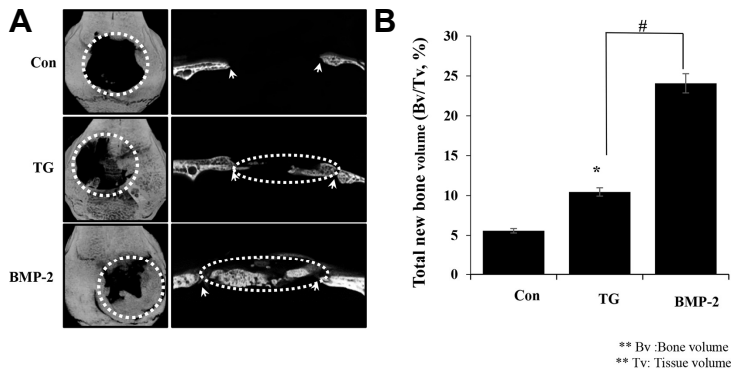


Fig. 4. Effects of TG on *in vivo* bone regeneration. (A) Representative microCT images of the calvarial defect at 6 weeks. (B) Quantitative analysis of new bone formation by 3D microCT. (C) Representative H&E-stained histologic images of calvarial bone defect (*left*), and higher-magnification images (*right*). Data are means \pm standard deviation (SD) (n = 6). The data are representative of three independent experiments. * $p < 0.05$ vs. control, # $p < 0.05$ vs. each group.

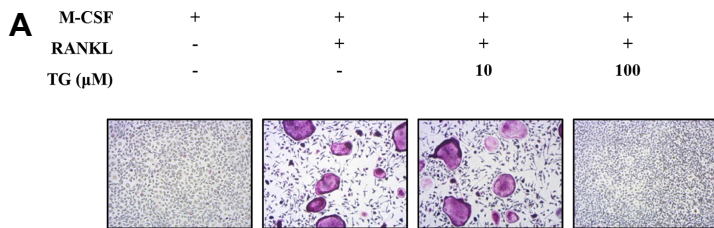
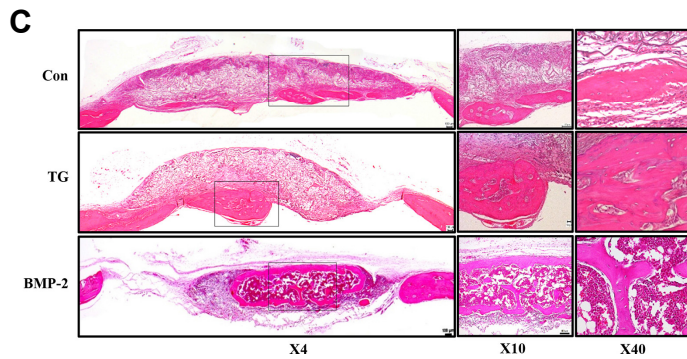
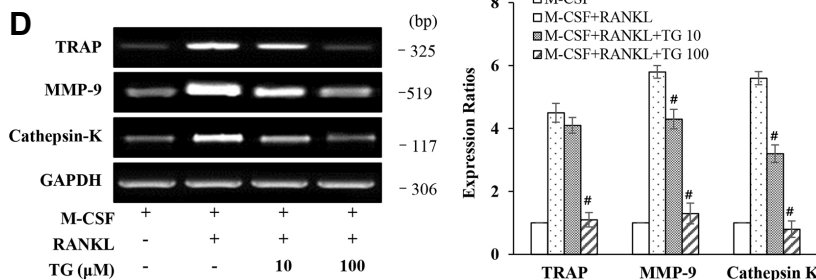
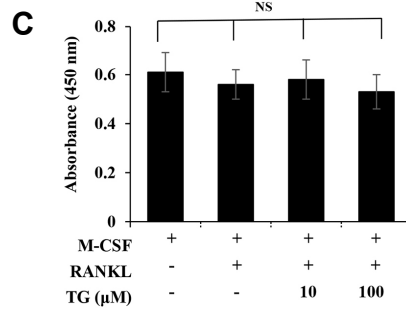
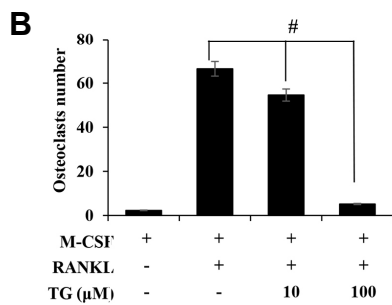


Fig. 5. Effects of TG on RANKL-mediated osteoclast differentiation in mouse BMMs. BMMs were cultured with the indicated concentrations of TG in the presence of RANKL, as described in the “Materials and Methods”. After 5 days, cells were fixed and stained for TRAP. (A) Representative images of TRAP staining. (B) TRAP-positive multinuclear cells (MNC) containing more than three nuclei were scored. (C) Cytotoxicity was determined using the MTT assay. (D) Expression of osteoclast-specific marker genes was assessed in BMM cells by RT-PCR. The histogram (right) shows the quantification of mRNA expression by densitometry; data are expressed relative to non-stimulated control cells (D). NS = not significant, # $p < 0.05$ vs. M-CSF and RANKL group.



ng/mL RANKL. RANKL-mediated osteoclast differentiation was suppressed by combined treatment with TG (Fig. 5A). The number of tartrate-resistant acid phosphatase (TRAP)-positive cells was significantly reduced by TG (Fig. 5B). To determine whether the reduction in osteoclast generation by TG was due to effects on the viability of precursor cells, a cytotoxicity assay was performed. As shown in Fig. 5C, TG at the concentrations tested showed no cytotoxicity. In addition, treatment of cells with TG inhibited the expression of osteoclastogenesis marker genes, such as TRAP, cathepsin-K, and MMP-9 (Fig. 5D).

TG inhibited RANKL-induced activation of MAPK and transcription factors

To gain insight into the molecular mechanisms underlying the inhibition of osteoclastogenesis by TG, we examined the MAPK and transcription factors involved in osteoclast differentiation. TG inhibited RANKL-stimulated phosphorylation of p38 and ERK, but not JNK, in mouse BMMs (Fig. 6A). The increased NFATc1 and c-Fos protein levels induced by RANKL were abolished in the presence of TG (Fig. 6B).

TG inhibits bone resorption *in vivo*

To investigate the *in vivo* effects of TG in an experimental animal model of bone erosion, mice were injected subcutaneously over calvariae with LPS with or without TG. microCT

images showed that LPS-induced bone loss was clearly recovered in the skull of TG-treated, LPS-injected mice (Fig. 7A). The microstructural indices of trabecular bone density (BV/TV) were significantly reduced by LPS induction, and this reduction was significantly recovered in the TG-treated group (Fig. 7B).

DISCUSSION

Osteoclast-mediated bone resorption and inhibition of osteoblast-mediated bone formation lead to bone loss. An alternative to classical hormone replacement therapy (HRT) that aims to avoid its related risks is the use of phytoestrogens or isoflavonoids, which are plant-derived nonsteroidal compounds that bind to estrogen receptors (ERs) and have estrogen-like activity (Dastmalchi et al., 2015; Ferretti et al., 2010). Moreover, identification of mechanisms of action and cellular targets of isoflavonoids are important in understanding both their beneficial and adverse effects in bone homeostasis. Based on a previous report showing that TG had the highest anti-inflammatory effects among seven isoflavonoids in a macrophage cell line (Kim et al., 1999A), we hypothesized that osteoblast or osteoclast differentiation

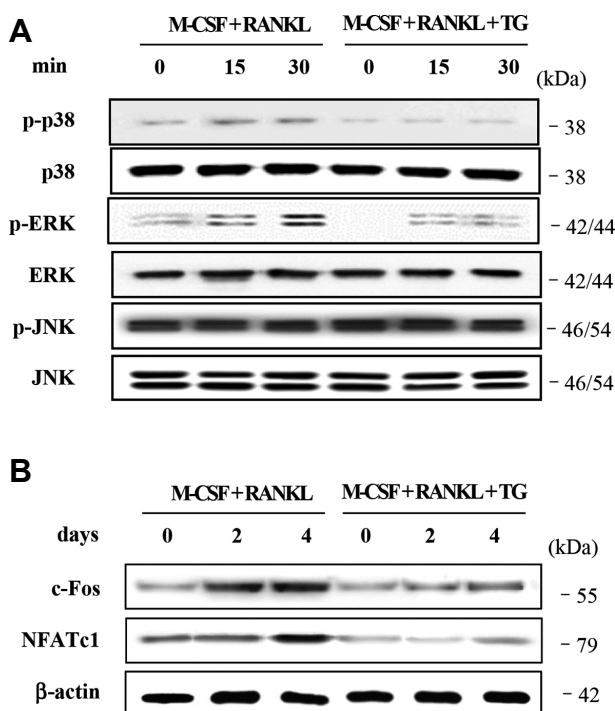


Fig. 6. Effects of TG on RANKL-mediated MAPK (A) and transcription factor expression. BMMs were cultured with 100 μ M TG in the presence of RANKL for 30 min (A) and 6 h (B). The activation statuses of signal pathways were determined by Western blotting. Results are from three independent representative experiments.

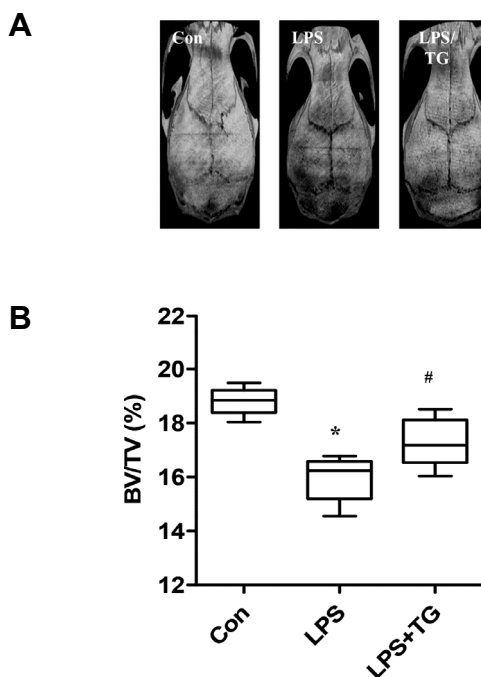


Fig. 7. Effects of TG on *in vivo* LPS-induced bone resorption. Mice were injected subcutaneously over calvariae with TG (3 μ g/g body weight) or PBS and LPS (12.5 μ g/g body weight) for 6 days. microCT images of the calvarial bone were obtained to visualize and quantify calvarial bone resorption (A). Quantitative analysis of the relative percentage of bone resorption (BV/TV) was performed by 3D microCT imaging (B). Data are representative of three independent experiments. * P < 0.05, versus the vehicle-injected group, # P < 0.05, versus the LPS-injected group. BV: bone volume, TV: tissue volume.

and function would be affected by TG. To the best of our knowledge, this is the first study to examine the effects of TG on the differentiation of primary osteoblasts and osteoclasts, as well as related mechanisms *in vitro*. We also utilized mouse calvarial defect and bone resorption models to explore the regulatory effects of TG on bone regeneration and resorption *in vivo*.

Because mouse primary cultured calvarial osteoblasts are frequently used to analyze differentiation and mineralization *in vitro* (Wang et al., 2015; Yun et al., 2015), these cells were used to investigate the effects of TG on cytotoxicity and osteoblastic differentiation by evaluating the expression of markers of the early (ALP, ON), middle (OPN), and late (OCN and mineralization) stages of differentiation. Our results showed that TG was not cytotoxic, and induced marked osteogenesis and mineralization by primary osteoblasts, as evidenced by the increased ALP activity, and expression of middle- and late-stage markers. Runx2 and osterix are the major transcription factors required for activation of osteoblast differentiation, and are crucial for the regulation of genes encoding bone-specific proteins (Prince et al., 2001). Our results showed that TG upregulated Runx2 and osterix mRNA levels in primary cultured osteoblasts, suggesting that TG regulates osteoblast differentiation via activation of the Runx2 pathway. We used TG at concentrations of 10-100 μM because concentrations of 25-75 μM have been shown to inhibit adipogenic differentiation in 3T3-L1 preadipocytes (Li et al., 2015). Moreover, TG at 200 μM was not cytotoxic, and it inhibited interferon- γ and lipopolysaccharide-induced inflammatory responses in macrophages cells (Pan et al., 2008). This suggests that TG may have favorable activity toward differentiation of osteoblasts and osteoclasts at doses greater than 100 μM .

PDLCs can differentiate into osteoblast-like cells *in vitro*, and contribute to bone repair *in vivo* (Shin et al., 2015). Moreover, successful periodontal regeneration may be accomplished by selective migration, proliferation, and differentiation of cells from the PDL and bone tissue (Herr et al., 1995). In this study, TG promoted osteoblastic differentiation in PDLCs, as evidenced by the induction of ALP activity, formation of mineralized nodules, and upregulation of marker genes. Our results suggest that the effects of TG on differentiation may be relevant to other cell types involved in bone regeneration.

BMP and MAPK pathways play an important role in *in vitro* osteoblast differentiation and *in vivo* bone remodeling (Cao et al., 2005; Lee et al., 2015). BMP pathway signaling is initiated by ligation of BMP-2 to the BMP receptor, which results in phosphorylation of Smad1/5/8 or MAPKs (Nohe et al., 2004). Phosphorylated Smad1/5/8 forms a complex with Smad4 and translocates into the nucleus, where it activates the transcription of bone-specific genes, stimulating bone formation (Nohe et al., 2004). Our data showed that treatment of primary osteoblasts with TG enhanced *Bmp2*, *Bmp4*, and *Smad4* mRNA levels as well as phosphorylation of Smad1/5/8, the central molecules in BMP signaling, and increased the expression of Runx2 and *Osx*, downstream target genes of BMP signaling. In addition, TG-treated osteoblasts showed activation of the ERK and JNK signaling

pathways. These results suggest that TG might regulate osteoblast differentiation through activation of BMP/MAPK signaling in primary osteoblasts.

In this study, a 5 mm diameter critical-sized cranial defect mouse model was utilized to determine whether TG exhibited greater bone-regeneration capabilities than the control and rh-BMP-2. Our microCT results of significantly increased new bone volume in defects treated with TG correlated well with the histological analysis. Therefore, promotion by TG of osteoblast differentiation *in vitro* was correlated with an increase in bone regeneration *in vivo*. Although TG was inferior to the *in vivo* bone repair induced by rh-BMP-2, TG can provide a substantial challenge to osteoconductive and osteoinductive properties during bone regeneration.

Osteoclasts are responsible for bone lysis in several bone diseases, such as osteoporosis, arthritis, and periodontitis. Natural products that suppress osteoclast differentiation may benefit the treatment of bone diseases involving osteoclasts (Kim et al., 2004). We found that TG inhibited the differentiation of primary BMM precursor cells into TRAP-positive multinucleated osteoclasts. Moreover, TG downregulated the mRNA levels of TRAP, cathepsin-K, and MMP-9, which are markers of osteoclast differentiation (Faccio et al., 2005).

RANKL binds to its receptor RANK on osteoclast precursors and activates many signaling pathways, including MAPK pathways, which can regulate transcription factors such as c-Fos and NFATc1 (Lee et al., 2002; Takayanagi et al., 2002). In studies of the mechanisms by which TG inhibits osteoclast differentiation, we found that it reduced the protein levels of two transcription factors associated with osteoclastogenesis, c-Fos and NFATc1, in BMM osteoclast precursor cells stimulated with RANKL. In addition, TG inhibited RANKL-induced phosphorylation of p38 and ERK MAPKs in BMMs. Therefore, TG may suppress osteoclastogenesis by downregulating RANKL-induced transcription factors and MAPKs.

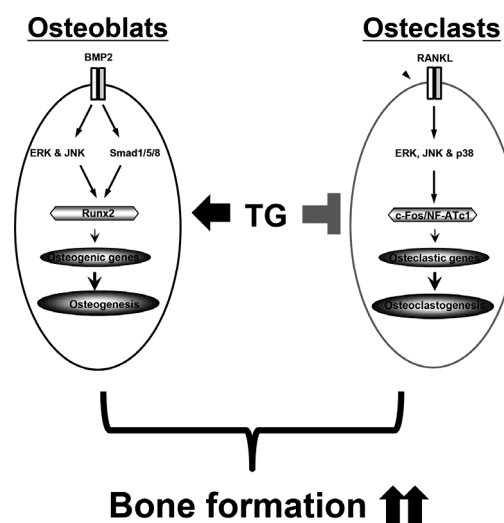


Fig. 8. Schematic diagram showing the dual mode of action of TG: induction of osteoblastic differentiation and suppression of osteoclastic differentiation.

LPS induces bone resorption and signals via TRAF6, similar to RANKL (Sakuma et al., 2000). In the *in vivo* experiments using LPS-injected mice, TG treatment markedly decreased LPS-induced bone resorption. This finding is consistent with the effects of TG on RANKL-induced osteoclastic differentiation, and suggests that TG may be a useful herbal medicine for preventing and treating disorders associated with bone loss.

In summary, this study is the first to demonstrate that TG promotes osteogenic differentiation via BMP and MAPK pathways *in vitro*, enhances bone regeneration *in vivo*, reduces *in vitro* osteoclastogenesis by suppressing RANKL-induced MAPK, c-Fos, and NFATc1 expression, and suppresses *in vivo* bone loss. Schematic diagrams of the potential mechanisms underlying the effects of TG signaling on osteogenesis and osteoclastogenesis are shown in Fig. 8. Thus, TG may be useful as an herbal medicine for bone or periodontal tissue regeneration as well as bone-destructive diseases.

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