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# Daidzein promotes proliferation and differentiation in osteoblastic OCT1 cells *via* activation of the BMP-2/Smads pathway

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Daidzein, the most widely studied soy phytoestrogen, is not only a potential antiosteoporosis agent owing to its possible osteogenic activity, but also shows anticancer activity. However, the mechanisms through which daidzein affects osteoblast function have not been well understood. Here, we show that daidzein stimulated cell proliferation and differentiation of osteoblasts, demonstrated by upregulation of XTT activity, enhancement of alkaline phosphatase (ALP) activity, and upregulation of osteoblast-specific marker genes, including Runt-related transcription factor 2 (Runx2) and Smad1, as well as up-regulation of Runx2 and Smad1 protein expression. To determine the mechanisms underlying daidzein's effects on osteoblast differentiation, we first tested the role of daidzein in bone morphogenetic protein (BMP)-2 gene expression in OCT1 cells, and found that it significantly upregulated the expression of BMP-2. Furthermore, it significantly enhanced the phosphorylated protein level of Smad1/5/8and protein expression of Osterix (Osx, a direct target gene of BMP signaling) and increased the activity of BMP signaling reporter (12xSBE-OC-Luc). Finally, we demonstrated that daidzein stimulated Col I, Runx2, and ALP expression, while these effects were significantly blocked by the BMP signaling inhibitor noggin. Thus, our data indicate that daidzein acts through stimulating the activation of BMP-2/Smads pathway to promote osteoblast proliferation and differentiation.

## 1. Introduction

Osteoporosis is a systemic skeletal disease with bone loss and microstructure destruction. This disease is caused by an increase in bone resorption and a reduction of bone formation. Bone extracellular matrix is destroyed in patients with osteoporosis. Without the normal structure, the osteoblast was incapable of normal bone remodeling. Although estrogen replacement therapy has been shown to be effective in increasing the activity of osteoblast, it may increase the incidence of uterine and breast cancers (Christiansen 1993).

Phytoestrogens raise lots of concerns about their potential benefits in the prevention and treatment for osteoporosis. Daidzein, the most widely studied soy phytoestrogen, shows promise as a potential antiosteoporosis agent. Many reports have shown that daidzein has an effect not only on osteogenic activity, but also shows anticancer activity (Strong et al. 2013; Adjaklyet al. 2013; Chen et al. 2015). Daidzein-enhanced osteoblast growth might be mediated through the upregulation of BMP expression in primary osteoblast cells (Jia et al. 2003). Daidzein stimulated cell differentiation and mineralization in mouse osteoblast-like MC3T3-E1 cells (Ge et al. 2006). The effects of daidzein on osteoblasts were mediated through the ERbeta pathway (De Wilde et al. 2004, 2006). However, in order to fully exploit its antiosteoporotic properties, more studies are needed to better understand and elucidate all the pathways affected by daidzein.

In the present study, we found the explicit molecular mechanisms involved in daidzein's effect on the function of osteoblast by performing the in vitro experiments with osteoblastic OCT1 cells. Our data indicated that daidzein promoted osteoblast proliferation and differentiation through stimulating the activation of the BMP-2/Smads pathway.

## 2. Investigations and results

### *2.1. Daidzein promotes osteoblast proliferation dose-dependently*

We examined the role of daidzein in the proliferation of OCT1 cells by the assay of XTT. We found that daidzein promoted the proliferation of osteoblastic cells dose-dependently, with a maximal role at the dose of 10 μg/mL after 48-h treatment ($P<0.05$, Fig. 1).

### *2.2. Daidzein enhances osteoblast differentiation dose-dependently*

To test if daidzein also plays a role in the differentiation of osteoblasts, ALP activity assay, RT-PCR assay and Western-blot assay were performed. Because ALP is a key marker for the differentiation of osteoblast, we firstly examined the activity of ALP. We found that daidzein significantly enhanced ALP activity dose-dependently, with a maximal role at a dose of 10 μg/mL ($P<0.05$) in OCT1 cells (Fig. 2A). We found that daidzein promoted osteoblast differentiation in a dose-dependent manner, demonstrated by up-regulation of osteoblastic marker genes such as Runx2 ($P<0.05$) and Smad1 ($P<0.05$) after 48-h treatment at a concentration of 10 μg/mL (Fig. 2B–C). We also found that daidzein significantly increased the protein levels of Runx2 and Smad1 at a concentration of 10 μg/mL (Fig. 2D). Together, our data indicate that daidzein enhances the differentiation of osteoblast.

### *2.3. Daidzein activates BMP-2/Smads pathway*

To clarify the effect of daidzein on the differentiation of osteoblast, we first detected the role of daidzein in the expression of BMP-2 gene in OCT1 cells. We showed that daidzein significantly

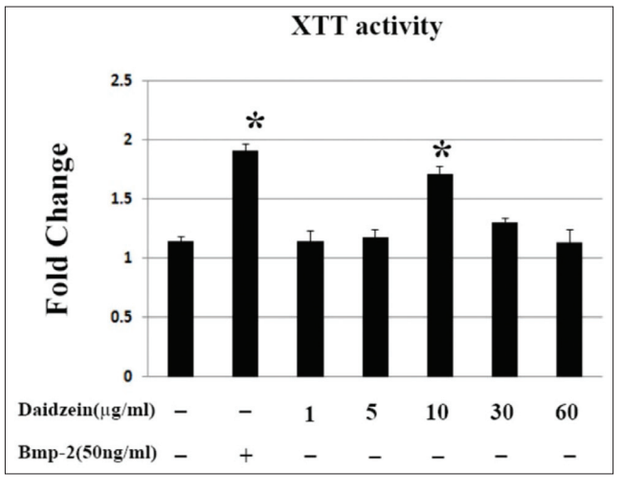


Fig. 1: Daidzein promoted osteoblast proliferation dose-dependently. XTT assay showed that daidzeinsignificantly promoted the proliferation of osteoblastic OCT1cells at a concentration of 10 μg/mLafter 48-h treatment. *P <0.05, compared with control.

Smad1/5/8, while its effect was significantly blocked by noggin, a specific BMP signaling inhibitor (Fig. 3B). We also tested the role of daidzein in the expression of osterix (Osx), a direct target gene of BMP pathway. We found that daidzein significantly increased Osx protein level by quantified analysis (P<0.05). However, its effect was significantly blocked by noggin (P<0.05) (Fig. 3 C,D). Finally, to further determine the role of daidzein in the BMP pathway, we detected the efficacy of daidzein on the activity of 12xSBE-OC-Luc, a BMP signaling reporter. We found that daidzein enhanced the 12xSBE-OC-Luc activity with a 3.1-fold increase in OCT1 cells (P<0.05), while its efficacy was significantly eliminated by noggin (P<0.05) (Fig. 3E). Together, our data demonstrate that daidzein may activate BMP-2/Smads pathway.

### *2.4. Daidzein-enhanced osteoblast proliferation and differentiation is mediated through stimulating the activation of BMP-2/Smads pathway*

To further explore whether daidzein-enhanced osteoblast proliferation and differentiation occurs *via* the BMP-2/Smads pathway, we first performed a XTT assay to test the effect of daidzein (10 μg/mL) on cell proliferation with noggin, a specific inhibitor of

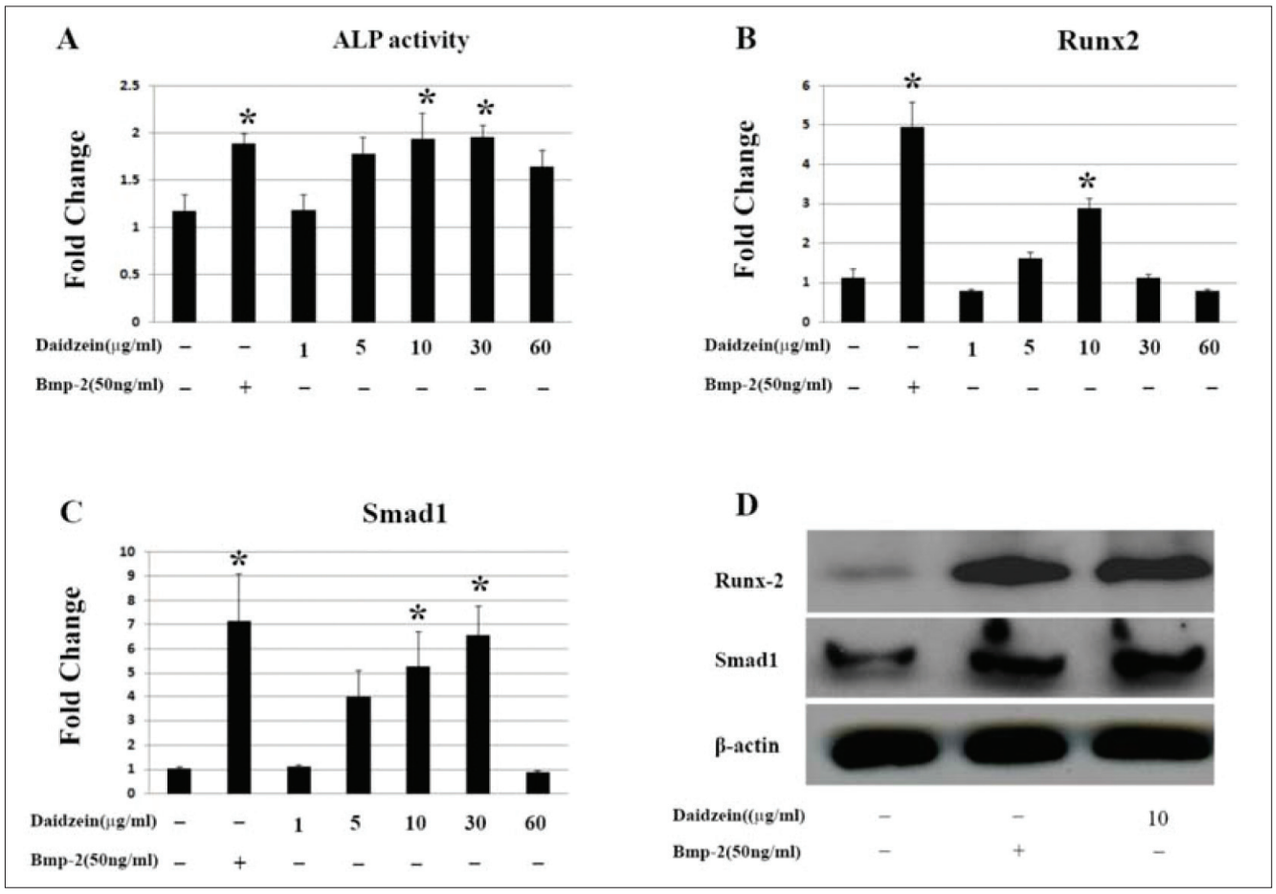


Fig. 2: Daidzein promoted osteoblast proliferation dose-dependently. XTT assay showed that daidzeinsignificantly promoted the proliferation of osteoblastic OCT1cells at a concentration of 10 μg/mLafter 48-h treatment. *P <0.05, compared with control.

upregulated the gene expression of BMP-2 (P<0.05), particularly at a concentration of 10 μg/mL, which showed a 12.7-fold increase (Fig. 3A). We then treated cells for only 2 h and examined the effect of daidzein (10 μg/mL) on the Smad1/5/8 proteins, the key molecules of BMP pathway. Although we did not find any difference in the total Smad1/5/8 protein levels, we found that daidzein significantly upregulated the phosphorylated protein levels of BMP signaling. We found that daidzein significantly stimulated osteoblast proliferation (P<0.05), while its effect was significantly eliminated by noggin (P<0.05) (Fig. 4A). We next detected the roles of daidzein (10 μg/mL) in the expression of some osteoblastic marker genes with or without co-treatment of noggin. We found that daidzein remarkably increased the mRNA level of type I collagen in osteoblasts with a 9.9-fold change (P<0.05), while

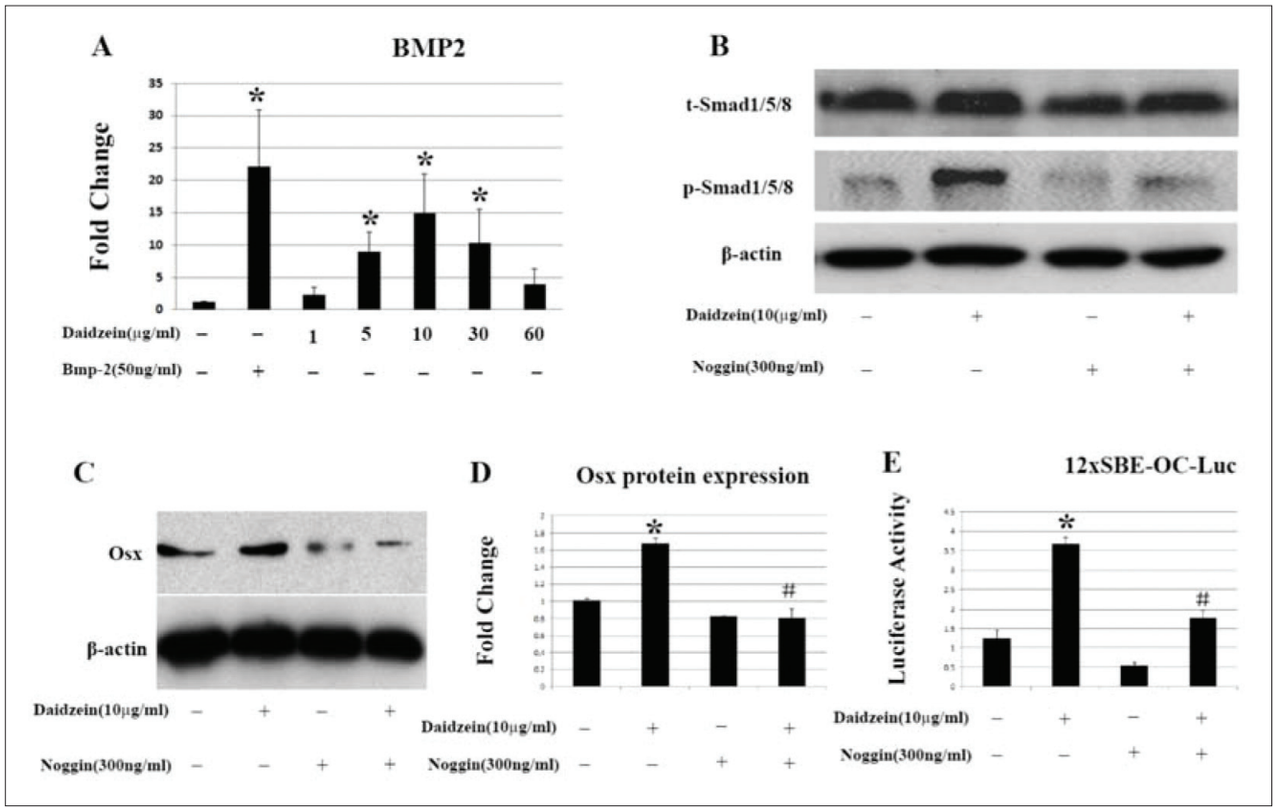


Fig. 3: Daidzein activated BMP-2/Smads signaling.(A) Daidzein significantly upregulated BMP-2mRNA expression in OCT1 cells after 48-h treatment. (B) Daidzein significantly increased the phosphorylated protein levels of Smad1/5/8 in OCT1 cells after 2-h treatment, and its effect was significantly blocked by noggin. (C) Daidzein significantly increased the protein level of Osx expression in OCT1 cells after 48-h treatment, and its effect was significantly blocked by noggin. (D) The quantitative data of C. (E) Daidzein significantly increased the activity of 12xSBE-OC-Luc in OCT1 cells after 48-h treatment, and its effect was significantly blocked by noggin. *P <0.05, compared with control; #P <0.05, compared with the group of daidein.

its role was significantly eliminated by noggin (P<0.05) (Fig. 4B). We also found a significant increase in the mRNA level of Runx2 expression in osteoblasts with a 2.9-fold change (P<0.05) after the treatment of daidzein, which effect that was also remarkably eliminated by noggin (P<0.05) (Fig. 4C). We also found that daidzein significantly upregulated ALP gene expression in osteoblasts with an 8.1-fold increase (P<0.05), and this effect was also eliminated by noggin (P<0.05) (Fig. 4D). Finally, ALP staining showed that daidzein significantly enhanced ALP expression in osteoblasts, and this effect was significantly reversed by noggin (Fig. 4E). These findings indicate that daidzein induces osteoblast proliferation and differentiation through stimulating the activation of the BMP-2/Smads pathway, and that a BMP signaling inhibitor can block these effects.

## 3. Discussion

Daidzein is present in a number of plants and herbs such as *Pueraria mirifica* and *Pueraria lobata*. Previous studies have shown that daidzein has an anti-osteoporosis effect. Jia et al. (2003) found that daidzein increased the viability of osteoblasts, ALP activity, osteocalcin synthesis and BMP-2 expression in primary osteoblastic cells. Strong et al. (2013) reported that daidzein enhanced the expression of some ostegenic genesin human bone marrow-derived mesenchymal stem cells (MSCs), suchas Osx, ALP, and OPN. The stimulatory role of daidzein on the osteoblast differentiation and mineralization in MC3T3-E1 cells has also been reported (Ge et al. 2006). In this study, we showed that daidzein stimulated osteoblast proliferation and differentiation in OCT1 cells. Treatment of osteoblastic cells with daidzein increased the activity of cell proliferation and the expression of some osteogenic differentiation marker genes, such as ALP, Smad1, Runx2, Osx, Col I, etc. It has been reported that the effect of daidzein on osteoblast differentiation was mediated by the expression of BMP-2. However, the detailed molecular mechanism remains unknown (Jia et al. 2003).

It is well known that BMPs are very important for the cell proliferation and differentiation of osteoblasts (Sykaras and Opperman 2003). BMP-2 binds to their cell surface receptors, and subsequently induces the phosphorylation of Smad1/5/8, then acts on bone cells. Phosphorylated Smad1/5/8 and Smad4forms a protein complex, translocates into the nucleus to activate the bone-specific-genes transcription, which in turn promote osteoblast differentiation (Sykaras and Opperman 2003; Chen et al. 2004; Cao et al. 2005; Miyazono et al. 2005; Wan and Cao 2005; Bilican et al. 2008). It has been reported that several small molecular weight compounds can be able to activate BMP-2 signaling (Mundy et al. 1999; Garrett et al. 2003; Tang et al. 2010, 2011; Li et al. 2013). To clarify the mechanism by which daidzein promotes cell proliferation and differentiation of osteoblasts, we tested if it can activate BMP-2 signaling. First, we found that the intervention of osteoblastic cells with daidzein increased the gene expression of BMP-2. Second, we found that daidzein increased the phosphorylated protein level of Smad1/5/8, the key molecules in BMP pathway, as well as the gene expression of Osx, a direct target gene of BMP signaling. We further demonstrated that daidzein increased the 12xSBE-OC-Luc activity, which is a BMP signaling reporter. Moreover, our data also showed that daidzein-activated BMP signaling could be blocked by noggin, a specific BMP signaling inhibitor.

Further investigations were carried out to confirm whether daidzein-promoted osteoblast differentiation is mediated by BMP signaling. We found that noggin, a specific BMP signaling inhibitor, blocked a daidzein-induced increase in gene expression of Col I, Runx2, and ALP, suggesting that daidzein promotes osteoblast differentiation by stimulating the activation of the BMP signaling pathway.

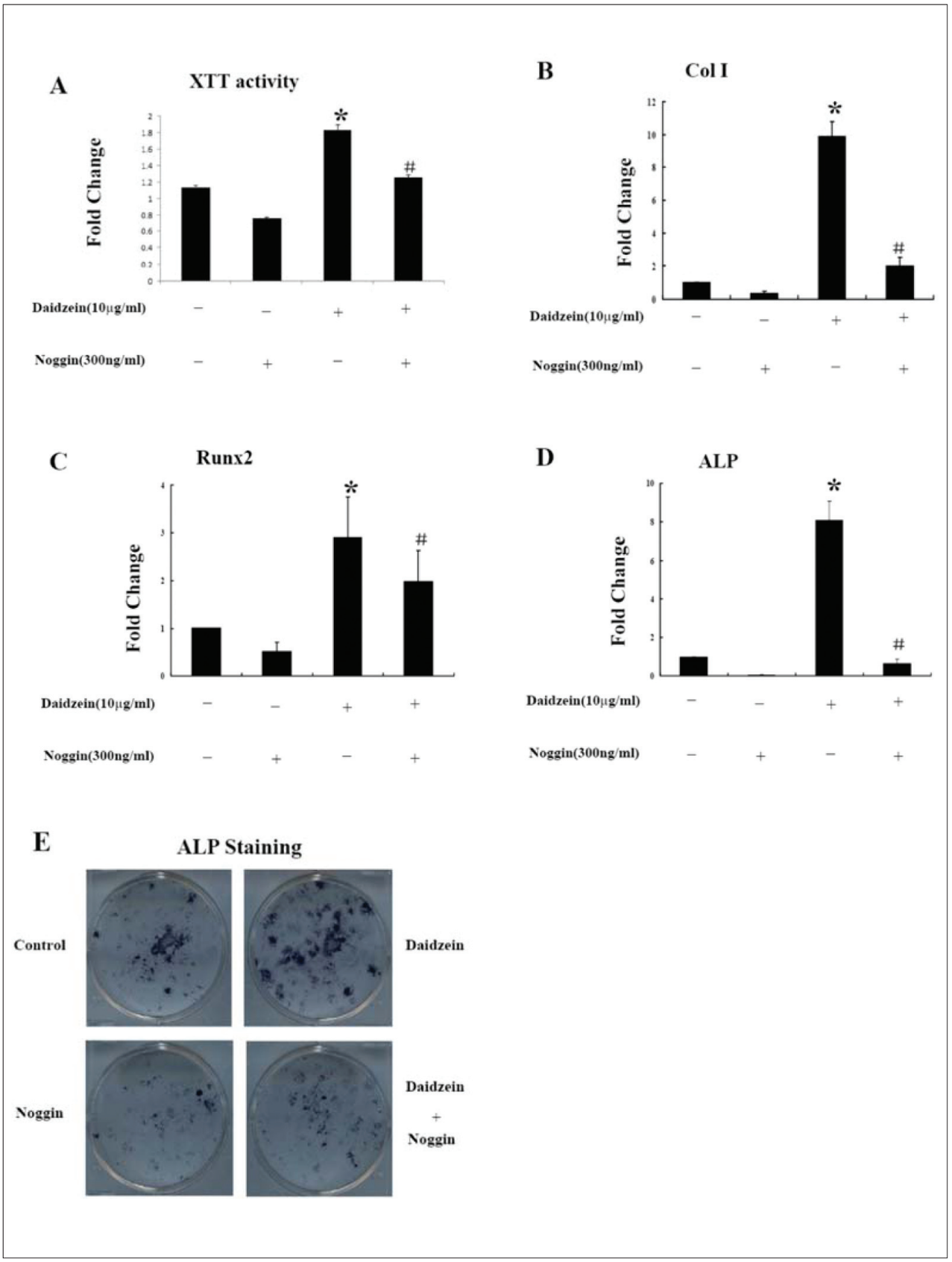


Fig. 4: Daidzein-induced osteoblast proliferation and differentiation was mediated through simulating the activation of the BMP-2/Smads signaling. (A) Daidzein significantly increased the XTT activity in OCT1 cells after 48-h treatment, and its effect was significantly blocked by noggin. (B) Daidzein significantly increased mRNA expression of Col I in OCT1 cells after 48-h treatment, and its effect was significantly blocked by noggin. (C) Daidzein significantly increased mRNA expression of Runx2 in OCT1 cells after 48-h treatment, and its effect was significantly blocked by noggin. (D) Daidzein significantly increased mRNA expression of ALP in OCT1 cells after 48-h treatment, and its effect was significantly blocked by noggin. (E) ALP staining results showed that daidzeinsignificantly enhanced ALP expression in OCT1 cells after 48-h treatment, and its effect was significantly blocked by noggin. *P <0.05, compared with control; #P <0.05,compared with the group of daidein.

In addition to effecting osteoblasts, daidzein was also reported to inhibit osteoclast differentiation by increasing ERalpha expression (Garcia et al. 2005). Here, we did not investigate the efficacy of daidzein on osteoclasts, focusing instead only on its effects on osteoblasts. In future studies, we will analyze the effects and detailed molecular mechanisms of daidzein on osteoclastogenesis. In conclusion, our findings demonstrate that daidzein promotes osteoblast proliferation and differentiation by stimulating the activation of the BMP-2/Smads signaling.

## 4. Experimental

### *4.1. Cell cultures*

OCT1 osteoblast precursor cells, derived from the osteocalcin (OC-TAg) promoter SV-40 transgenic mice calvarias, were cultured in low-glucose α-MEM medium with 10% fetal bovine serum as previously described (Bian et al. 2011).

### *4.2. Cell proliferation assay*

The XTT assay was performed to measure the effects of daidzein on cell proliferation. OCT1 cells were seeded in 96-well culture plates ($1x10^4$ cells/well). After cultured for 24 h, cells were intervened with different concentrations (1 μg/mL, 5 μg/mL, 10 μg/mL, 30 μg/mL,and 60 μg/mL) of daidzein or BMP-2 (50 ng/mL), with control groups, for 48 h. XTT testing solution, which was prepared by mixing 5 mLXTT-labeling reagents (Sigma, U.S.) with 100 μL electron coupling reagents, was added to each well. After incubating for 4 h, we used an ELISA Plate reader (Thermo Electron Corporation, Waltham, MA) to detect the absorbance at a test wavelength of 492 nm and a reference wavelength of 690 nm.

### *4.3. Assay of ALP activity*

Cells cultured in 96-well culture plates ($1x10^4$ cells/well) for 24 h, were treated with different concentrations (1 μg/mL, 5 μg/mL, 10 μg/mL, 30 μg/mL, and 60 μg/mL) of daidzein or BMP-2 (50 ng/mL), including controls, for 48 h. Then we washed cells for three times using 0.9% NaCl buffer and collected the cell lysates with M-Per (Pierce, UA). Substrate p-nitrophenol phosphate was prepared by dissolving in AMP buffer (Bio-Rad, Hercules, CA, U.S.). Total lysates (20 μL) were added to 1 mL substrate. After incubating for 5 min, we added 0.5 mL stop solution (0.3 M $Na_3PO_4$) to the reaction system and used an ELISA Plate reader (Thermo Electron Corporation, Waltham, MA) to detect the absorbance at a test wavelength of 410 nm for the measure of ALP activity. We normalized the absorbance values to the total protein levels which were quantified by using a BCA kit (Pierce, UA).

### *4.4. ALP staining assay*

After incubation for 48 h with or without daidzein (10 μg/mL) and noggin (300 ng/mL), we fixed cells with 10% neutral buffered formalin for 15 min and washed cells with 0.9 % NaCl buffer for three times. Then cells were stained at 37 °C for 30 min with ALP staining buffer, NBT-BCIP (Bio-Rad, Hercules, CA, USA).

### *4.5. Transfection and luciferase activity assay*

For the luciferase activity assay, we transfected the constructs of 12xSBE-OC into OCT1 cells by using FUGENE HD reagents. We also co-transfected an SV40-Renilla luciferase construct with the above reporter construct to normalize the result for detecting the efficiency of transfection. Then cells were cultured for another 48 h with or without daidzein (10 μg/mL) and noggin (300 ng/mL). Cell lysates were extracted after treatment for 48 h, and the activity of luciferase was detected with a Promega luciferase assay kit. Luciferase activity in the cell lysate was determined using a luminometer (Thermo Electron Corporation, Waltham, MA).

### *4.6. Real-time qPCR analysis*

Total cellular RNA was isolated from cultured OCT1 cells after 48 h treatment with or without different concentrations (1 μg/mL, 5 μg/mL,10 μg/mL, 30 μg/mL and 60 μg/mL) of daidzein or BMP-2 (50 ng/mL) or noggin (300 ng/mL). Total RNA was extracted using the Qiagen RNAeasy mini kit (Valencia, CA, USA) and the first-strand cDNA was synthesized using 1 μg of RNA with a Clontech RT kit primed by an oligo primer (Mountain View, CA, USA). Real-time PCR analysis was performed with mouse specific primers, which were listed in the Table. The expression levels of these genes were normalized to β-actin.

### *4.7. Western blot analysis*

Total cellular protein was extracted from cultured OCT1 cells after treatment with different concentrations (10 μg/mL) of daidzein or BMP-2 (50 ng/mL) or noggin (300 ng/mL), and controls, for 48 h or 2 h. Cells were lysed using protein isolation buffer , including 150 mM sodium chloride, 50 mM TRIS, 10% protease inhibitor cocktail, and 1% IGEPAL. We then used a BCA protein assay kit (Pierce, Rockford, IL, USA) for quantification. Proteins were fractionated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, incubated with anti-Runx2, anti-Osx (Cell signaling Tech, MA, USA), anti-p-Smad1/5/8, anti-Smad1, and anti-β-actin (Oncogene Research Products, San Diego, CA, USA) antibodies overnight at 4 °C. After washing, membranes were incubated with secondary HRP-conjugated antibodies (Jackson Immunoresearch) for 1 h at room temperature. Bands were detected by enhanced chemiluminescence-mediated visualization kit (Amersham Biosciences, Piscataway, NJ, USA).

### *4.8. Statistical analysis*

All quantitative experiments were repeated three times independently and data were expressed as means±standard deviation (SD). For multiple-group comparison, one-way ANOVA followed by the Dunnett's test was performed. For two-group comparison, the unpaired Student's *t*-test was performed. A value of $P< 0.05$ was considered as significant.

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Conflicts of interest: None declared.

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**Table: Mouse primers for qPCR assays**

| Genes | Forward primers | Reverse primers |
|---|---|---|
| β-actin | CTGTCCCTGTATGCCTCTG | ATGTCACGCACGATTTCC |
| BMP-2 | GATCTGTACCGCAGGCACTC | TTCCCACTCATCTCTGGAAGTT |
| Runx2 | CATTTGCACTGGGTCACACGTA | GAATCTGGCCATGTTTGTGCTC |
| Smad1 | CACCTGCTTACCTGCCTCCT | TGCCTGAACATCTCCTCTGCT |
| Col I | TGATCACTCCCACGTTTTCA | CTGGGCCTGGTAGTTGTTGT |
| ALP | TGACCTTCTCTCCTCCATCC | CTTCCTGGGAGTCTCATCCT |

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