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Dehydrodiconiferyl alcohol promotes BMP-2-induced osteoblastogenesis through its agonistic effects on estrogen receptor

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ABSTRACT

Estrogen deficiency results in an imbalance between the levels of bone-resorping osteoclasts and bone-forming osteoblasts, eventually leading to overall bone loss. Dehydrodiconiferyl alcohol (DHCA), a lignan compound originally isolated from *Cucurbita moschata*, has been shown to bind to estrogen receptor, and indeed exhibits various activities of estrogen, such as anti-inflammatory and anti-oxidative stress effects. In this study, we tested whether synthetic DHCA could affect the BMP-2-induced osteoblastogenesis in vitro. In MC3T3-E1 cells, DHCA promoted BMP-2-induced differentiation of osteoblasts. Consistently, the expression of three osteoblastogenic genes known to be induced by BMP-2, ALP, osteocalcin and OPG, was up-regulated by DHCA treatment. DHCA was also shown to activate the production of RUNX2 by activating Smad1/5/9 and AMPK. Data from transient transfection assays suggested that DHCA might activate the estrogen receptor signaling pathway. Effects of DHCA on BMP-2-induced osteoblastogenesis were reduced when cells were treated with a specific siRNA to ERα or ERβ. Taken together, our results suggest that DHCA may be developed as an efficient therapeutic for osteoporosis by regulating osteoblastogenesis through its estrogenic effects.

1. Introduction

Postmenopausal osteoporosis is a skeletal disease characterized by weakened bone strength and reduced bone mineral density [1,2]. Estrogen deficiency results in the imbalance between the levels of bone-resorping osteoclasts and bone-forming osteoblasts, eventually leading to overall bone loss [3,4]. Bone resorption inhibitors such as bisphosphonates are widely used to treat osteoporosis, but their long-term use is known to generate side effects such as severe musculoskeletal pain and hypocalcemia [5]. Therefore, there is a significant unmet medical need for the development of alternative treatment methods with fewer side effects for managing osteoporosis.

Bone formation is mediated by osteoblasts, which is a terminally differentiated form of mesenchymal stem cells [6]. Osteoblastogenesis, namely the differentiation process of osteoblasts from precursor cells, is regulated by endocrine and paracrine factors such as estrogen and growth factors that activate a variety of intracellular signaling pathways [7]. In osteoblastogenesis, BMP-2 (bone morphogenetic protein-2) plays a master role in the regulation of various genes involved in osteoblast functions such as RUNX2 (runt-related transcriptional factor 2), ALP (alkaline phosphatase), osteocalcin and OPG (osteoprotegerin), by activating the Smad signaling pathway [8–11]. The AMPK (AMP-activated protein kinase) signaling pathway has also been shown to play an important role(s). Indeed, AMPK activators such as AICAR and metformin has been shown to increase bone nodule formation in vitro [12].

Dehydrodiconiferyl alcohol (DHCA) is a lignan compound isolated from the water-soluble extracts of *Cucurbita moschata* [13]. DHCA was originally found to contain anti-adipogenic [13], anti-inflammatory [14] and anti-oxidative stress activities [15]. More recently, DHCA has been shown to bind to the estrogen receptor, and similar to estrogen, this lignan molecule did indeed exert anti-osteoclastogenic effects by acting as an estrogen receptor agonist (submitted for publication). Based on these observations, it was hypothesized that DHCA might promote osteoblastogenesis through its estrogenic activities. In this study, we investigated the effects of DHCA on BMP-2-induced osteoblastogenesis at molecular levels using MC3T3-E1 cells.

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2. Materials and methods

2.1. Cell culture and reagents

Synthetic DHCA was produced by previously described methods [16] and obtained from Biochemnet (Seoul, Korea). MC3T3-E1 cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in Minimum Essential Medium Alpha (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) at 37 °C under 5% CO_2. BMP-2 and 17β-Estradiol were purchased from Sigma (St Louis, MO, USA).

2.2. Osteoblast differentiation in vitro

For the osteoblast differentiation experiments, MC3T3-E1 cells were plated at 2×10^3 cells per well in 96-well culture plates containing α-MEM with 10% FBS. Twenty-four hours later, cells were treated with 25 ng/mL of BMP-2 and various concentrations of DHCA. After 5 days in culture, the cells were subjected to Leukocyte Alkaline Phosphatase (ALP) Kit (Sigma, St Louis, MO, USA) according to the manufacturer's instructions.

2.3. Measuring ALP activity

After osteoblast differentiation, ALP activities of osteoblasts were measured in the well by incubation for 30 min at 37 °C with 100 μl of Alkaline Phosphatase Yellow (pNPP) Liquid Substrate System for ELISA (Sigma, St Louis, MO, USA) containing 1% Tween-20. The reaction was terminated by the addition of 50 μl of NaOH (300 mM), and activities were measured at 405 nm.

2.4. MTT assay

MTT assay was performed as described previously [14]. Briefly, MC3T3-E1 cells were treated with BMP-2 (25 ng/mL) or various concentrations of DHCA for 24–72 h. Cells were then incubated with an MTT labeling reagent for 4 h followed by the addition of solubilization solution. After 24 h, cytotoxicity was determined by measuring the OD at 550 nm using an ELISA microplate reader.

2.5. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

After 24 h in osteoblast differentiation, total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized using an oligodT primer (Qiagen, Valencia, CA) and AMV reverse transcriptase (TaKaRa, Shiga, Japan). One microliter of this cDNA per sample was used for quantitative polymerase chain reaction using SYBR Premix Ex TaqTM (TaKaRa, Shiga, Japan). The primer sequences used in this study were [forward, CCAACTCTTTTGTGCCAGAGA; reverse, GGCTACATTGGTGTTGAGCTTTT] for ALP, [forward, CTGACCTCACAGATGCCAAG; reverse, GTAGCGCCGGAGTCTGTTC] for osteocalcin, and [forward, GTTTCCCGAAGGACCACAAT; reverse, CCATTCAATGATGTCCAGGAG] for OPG. Conditions for PCR were denaturation at 95 °C for 5 s, followed by annealing and extension at 60 °C for 20 s.

2.6. Western blot analysis

MC3T3-E1 cells were plated in 100 mm culture dishes. Twenty-four hours later, cells were treated with BMP-2 (25 ng/mL) and various concentrations of DHCA for 30 min. After treatment, cells were washed with cold PBS and lysed with phosphosafe extraction buffer (Novagen, Madison, WI, USA). Total proteins were separated by SDS-PAGE and electrophoretically transferred to PVDF membranes. The membranes were incubated with primary antibodies against RUNX2 (1:1000, Cell Signaling, Beverly, MA), p-Smad1/5/9 (1:1000, Cell Signaling), Smad1/5/9 (1:1000, Abcam, Cambridge, MA, USA), p-AMPKα1 (1:1000, Cell Signaling), AMPKα1 (1:1000, Cell Signaling) and β-actin (1:5000, Sigma). Membranes were then treated with horse radish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:100,000, Sigma) and visualized in films using ECL solution (Milipore, Billerica, MA).

2.7. Luciferase reporter plasmid assay

Inducible estrogen responsive element (ERE)-responsive luciferase reporter plasmid was purchased from QIAGEN (Valencia, CA). Luciferase reporter plasmid assay was performed as described previously [14]. Briefly, MC3T3-E1 cells were transiently transfected with ERE-reporter plasmid and a β-galactosidase plasmid (1 μg, Invitrogen), using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were treated with 17β-estradiol (10 nM) and various concentrations of DHCA for 24 h. Cell lysates were prepared, and a luciferase activity assay was performed using the Luciferase Reporter kit according to the manufacturer's protocol (Promega, Madison, WI, USA) with a microplate luminometer (MicroLumat Plus LB96V, Berthold, Germany). Luciferase activity was normalized to β-gal activity.

2.8. siRNA transfection

The siRNA specific for ERα, ERβ and scrambled siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The siRNA was transfected into MC3T3-E1 cells using the RNAiMAX (ThermoFisher Scientific, Woburn, MA, USA) according to the manufacturer's protocol. After 48 h, the cells were subjected to the analysis. Knock-down efficiency was evaluated using primary antibodies against ERα (1:1000, Cell Signaling) and ERβ (1:1000, Cell Signaling).

3. Results

3.1. DHCA promotes BMP-2-induced osteoblastogenesis with no cytotoxic effect

MC3T3-E1 is a murine pre-osteoblast cell line that can differentiate into osteoblasts when stimulated with BMP-2 [17]. To test the effects of DHCA on osteoblastogenesis, MC3T3-E1 cells were treated with BMP-2 (25 ng/mL) and three different concentrations of DHCA (10, 20 and 40 μM) for 5 days followed by measuring the number of ALP-positive cells and the activity of ALP. As shown in Fig. 1A, the number of ALP-positive cells, as determined by ALP staining, was highly increased by treatment with DHCA in a dose-dependent manner. The effect of DHCA on actual ALP activity was also measured using cellular extracts and pNPP as a substrate. The level of ALP activity was enhanced as DHCA concentration increased (Fig. 1B).

The effects of DHCA alone, (that is, in the absence of BMP-2), were also measured. MC3T3-E1 cells were treated with 10, 20, 40 and 80 μM of DHCA. Interestingly, neither the number of ALP-positive cells (Fig. 1C) nor the level of ALP activity (Fig. 1D) was changed, suggesting that DHCA works only when cells are differentiated by BMP-2.

To be certain, the effects of DHCA on cell viability were measured. MC3T3-E1 cells were cultured with or without BMP-2 in the presence of DHCA followed by MTT assay. As shown in Fig. 1E, DHCA had little effect on cell viability throughout all concentrations used in this study, regardless of the presence of BMP-2 during the

72-h period. Taken together, these data indicated that DHCA might promote the BMP-2-induced osteoblastogenesis without cytotoxic effects.

3.2. DHCA regulated the expression of genes associated with osteoblast differentiation

It has previously been reported that stimulation of MC3T3-E1 cells by BMP-2 up-regulates the expression of ALP, osteocalcin and OPG, which all play important roles in the differentiation and function of osteoblasts [9,10,18]. To study the effects of DHCA on the expression of these genes, MC3T3-E1 cells were treated with BMP-2 (25 ng/mL) and DHCA (10, 20 and 40 μM) for 24 h, and the RNA level was determined by quantitative RT-PCR. In all three cases, BMP-2 treatment increased their RNA levels by 2–3 fold. When cells were co-treated with 40 μM of DHCA, their levels were further enhanced by 2 fold in a dose-dependent manner (Fig. 2).

3.3. DHCA promoted BMP-2-induced RUNX2 production via Smad and AMPK activation

During BMP-2-induced osteoblastogenesis, RUNX2 becomes activated by the Smad signaling pathway. This is a critical step in the differentiation of osteoblasts [19], while AMPK has also been shown to play a positive role in this process [20]. To test the effects of DHCA on the BMP-2-induced expression of RUNX2, MC3T3-E1 cells were co-treated with BMP-2 and three different concentrations of DHCA (10, 20 and 40 μM) for 24 h, and the protein level of RUNX2 was measured by Western blot. When cells were treated with BMP-2, the protein level of RUNX2 was increased (Fig. 3A, compare lanes 1 and 2), and co-treatment with DHCA further enhanced the protein level of RUNX2 (Fig. 3A, compare lanes 2 and 5).

We also measured the effects of DHCA on other signaling proteins involved in the BMP-2-induced signaling pathway. MC3T3-E1 cells were co-treated with BMP-2 and DHCA for 30 min, and the phosphorylation status of Smad1/5/9 and AMPK were each determined by Western blot. When cells were treated with BMP-2, the level of phosphorylated Smad1/5/9 was highly increased (Fig. 3B, compare lanes 1 and 2), and co-treatment with DHCA further enhanced the amount of this phosphorylated protein (Fig. 3B, compare lanes 2 and 5). Similarly, phosphorylation of AMPK was also up-regulated by DHCA treatment (Fig. 3B). However, DHCA alone, namely in the absence of BMP-2, did not have any effect on the level of RUNX2 and phosphorylated Smad1/5/9 (Fig. 3C and D). These data indicated that DHCA could up-regulate the RUNX2-related signaling pathways, but only when cells were already in an activated status by BMP-2.

3.4. Effects of DHCA on osteoblastogenesis were mediated by ERα and ERβ

It is well known that estrogen promotes early osteoblast differentiation [21]. To test the effects of DHCA on the estrogen-induced signaling pathway, MC3T3-E1 cells were transfected with a luciferase reporter plasmid containing the nucleotide sequences for estrogen responsive element (ERE). Twenty-four hours later, transfected cells were treated with estradiol or DHCA for 6 h. Total proteins were extracted, and the relative level of luciferase activity was measured. When cells were treated with DHCA, the level of luciferase activity was increased in a dose-dependent manner (Fig. 4A), by 2.6-fold at 40 μM, indicating that DHCA might interact with the estrogen receptor in MC3T3-E1 cells.

There are two different types of estrogen receptors, ERα and ERβ, and each has different functions due to its difference in their affinity for ligands [22,23]. To investigate which of the two estrogen receptors interacts with DHCA to exert the observed effects, MC3T3-E1 cells were transfected with siRNA against ERα or ERβ followed by treatment with BMP-2 and DHCA. First, specificity of siRNA was measured. Cells were transfected with 30 pmole of siRNA for each receptor, and the protein level of ERα and ERβ was measured by Western blot. In both cases, the protein level was highly reduced (Fig. 4B). Next, the effect of siRNAs on the osteoblastogenesis was determined by measuring the number of ALP-positive cells and the level of ALP activity when cells were treated with BMP-2 (25 ng/mL) and DHCA (40 μM). As shown in Fig. 4C and D, both parameters were highly decreased when cells were transfected with siRNAs for ERα or ERβ. The effect of siRNAs was also measured on the BMP-2/DHCA-mediated activation of three osteoblastogenic genes (ALP, osteocalcin and OPG), and the RNA levels of all three genes were highly reduced (Fig. 4E–G). Taken together, these data indicated that DHCA might interact with both ERα and ERβ to promote BMP-2-induced osteoblast differentiation.

4. Discussion

DHCA is a lignan compound isolated from the water-soluble extracts of *Cucurbita moschata* [13]. It was previously shown to contain anti-adipogenic activities in 3T3-E1 cells, and also anti-inflammatory [14] and anti-oxidative activities [15] in macrophage and lymphocyte cell types. In this study, we investigated the effects of DHCA on BMP-2-induced osteoblastogenesis using the MC3T3-E1 pre-osteoblast cell line. DHCA increased the number of ALP-positive cells as well as the level of ALP activity. This lignan molecule further up-regulated the BMP-2 mediated activation of Smad1/5/9 and AMPK signaling pathways, involving the expression of RUNX2 and subsequently that of genes such as ALP, osteocalcin and OPG. These osteoblastogenic effects of DHCA were attenuated by inhibiting both ERα and ERβ using specific siRNAs.

DHCA alone, that is, in the absence of BMP-2, did not affect either osteoblast differentiation and RUNX2-related Smad signaling pathway. The fact that DHCA does not work when cells contain unactivated, null Smad 1/5/9, means that DHCA exerts its effects by regulating another molecule(s) involved in the control of BMP-2 mediated activation. One possible explanation is that DHCA affects PPM1H, which is a Smad1/5/9-specific phosphatase [24]. It has been shown that the ectopic expression of PPM1H inhibits BMP signaling, while suppression of PPM1H by siRNA promotes the expression of those genes controlled by BMP-2 and enhances osteoblast differentiation [24]. Therefore, it may be possible that DHCA regulates osteoblast differentiation through the inhibition of the PPM1H-related actions. To our knowledge, there has been no report on the relationship between estrogen and PPM1H. It remains to be seen whether DHCA indeed controls PPM1H.

We previously reported that DHCA could suppress the expression of PPARγ, inhibiting adipocyte differentiation [13]. Peroxisome proliferator-activated receptor γ (PPARγ) is a transcription factor activated by long chain fatty acid or peroxisome proliferators which play a key role(s) in adipocyte differentiation [25]. PPARγ was recently found to be involved in osteoblast differentiation [26]. For example, when mice were treated with PPARγ activator, TZD, significant bone loss was observed due to an increase in marrow adipocytes together with a decrease in osteoblasts, while in PPARγ deficient mice, bone formation and osteoblastogenesis were enhanced [27]. Therefore, it may be possible that DHCA controls osteoblast differentiation through PPARγ-related actions as well as estrogen receptor-mediated actions.

Wnt/β-catenin signaling is well known to play important roles in embryonic and postnatal developmental processes [28,29]. It

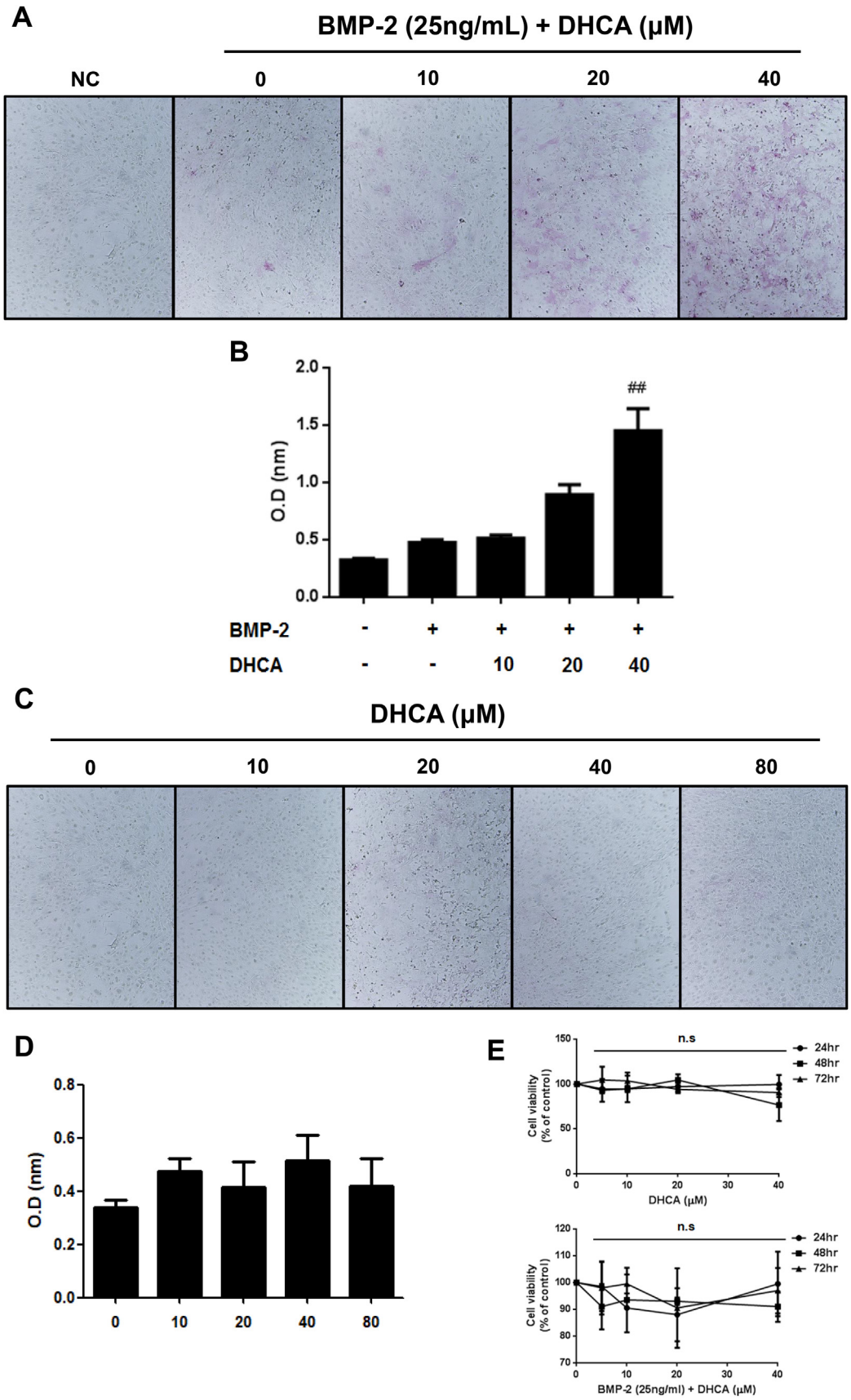

Fig. 1. Effects of DHCA on osteoblast differentiation in MC3T3-E1 cells. MC3T3-E1 cells were treated with or without BMP-2 (25 ng/mL) in the presence of different concentrations of DHCA (10, 20, 40 and 80 μM) for 5 days. ALP-positive cells were visualized by ALP stain, while ALP activity was measured at 450 nm following ALP activity assay as described in Materials and Methods. (A) Effects of DHCA on the number of ALP-positive cells; (B) Effects of DHCA on the level of ALP activity; (C) Effects of DHCA on the number of ALP-positive

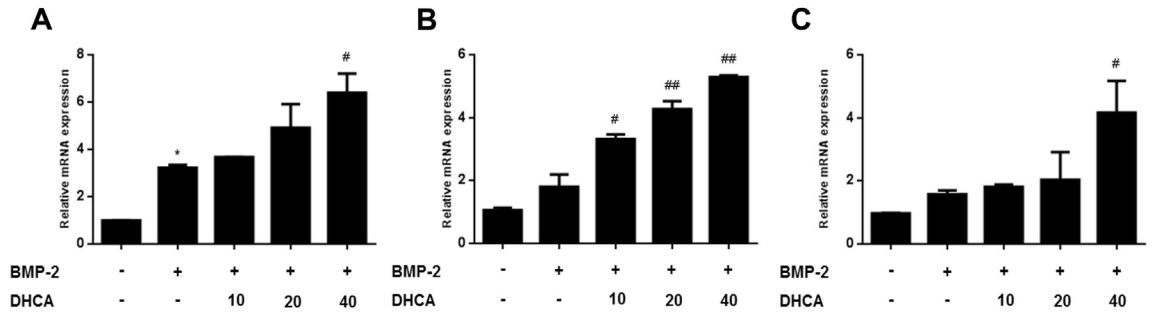

Fig. 2. Effects of DHCA on the expression of osteoblastogenic genes during BMP-2-induced osteoblastogenesis. MC3T3-E1 cells were treated with BMP-2 (25 ng/mL) and cultured in the presence of DHCA (10, 20 and 40 μM) for 24 h. Total RNAs were isolated and analyzed by quantitative RT-PCR for ALP (A), Osteocalcin (B) and OPG (C). Values represent the mean ± S.E.M. of three independent experiments. $^{*}p < .05$ compared with control; $^{\#}p < .05$, $^{\#\#}p < .01$ compared with those treated with BMP-2 alone.

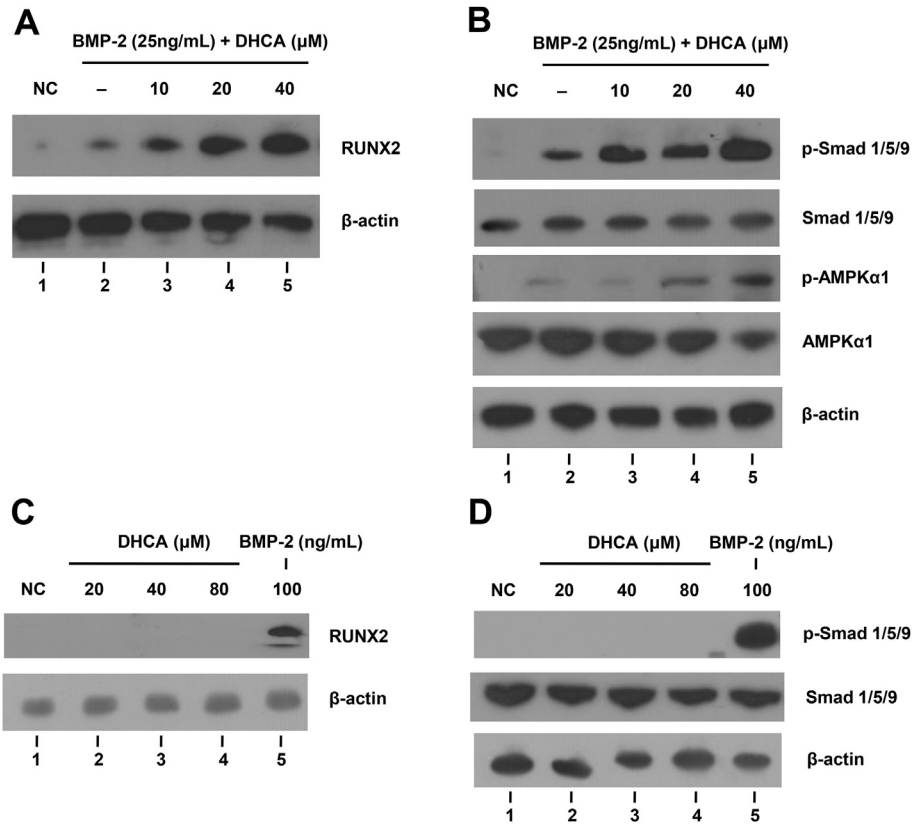

Fig. 3. Effects of DHCA on osteoblastogenesis-related signaling pathways. MC3T3-E1 cells were treated with or without BMP-2 (25 ng/mL), and cultured in the presence of DHCA (10, 20, 40 and 80 μM) for 2 h (for RUNX2) or 30 min (for Smad1/5/9 and AMPKα1), respectively. The positive control group (lane 5 in Fig. 3C and D) was treated with BMP-2 (100 ng/mL) alone. Total proteins were prepared following by Western blot using antibodies specific for respective proteins; (A) Effects of DHCA on the protein level of RUNX2; (B) Effects of DHCA on the phosphorylation status of Smad1/5/9 and AMPKα1; (C) Effects of DHCA on the protein level of RUNX2 in the absence of BMP-2; (D) Effects of DHCA on the phosphorylation status of Smad1/5/9 and AMPKα1 in the absence of BMP-2.

has been recently shown that Wnt/β-catenin signaling controls osteoblast differentiation by cross-talking with BMP-2 signaling [30]. Treatment with Wnt-ligand, Wnt3a, increased the level of luciferase activity from the BMP/Smad reporter plasmid, and also the expression of osteoblastogenic genes and the level of ALP activity in C2C12 cells [30]. Furthermore, co-treatment with Wnt3a and BMP-2 further increased the level of osteoblastogenic gene expression and ALP activity compared to when either activator was used [30]. Estrogen receptor signaling facilitates osteogenic differentiation by cross-talking with Wnt/β-catenin signaling [31], while in this study, DHCA was revealed as an agonist for estrogen receptor. Taken together, DHCA may up-regulate BMP-2 induced osteoblastogenesis by affecting the Wnt/β-catenin signaling pathway.

Our data indicate that DHCA promotes BMP-2-induced osteoblast differentiation by interacting with either ERα or ERβ as an

cells in the absence of BMP-2; (D) Effects of DHCA on the level of ALP activity in the absence of BMP-2. (E) Effects of DHCA on cell viability. MC3T3-E1 cells were co-treated with BMP-2 (25 ng/mL) and DHCA (10, 20 and 40 μM) for 72 h. Cells were then subjected to MTT assay as described in Materials and Methods. Values represent the mean ± S.E.M. of three independent experiments. $^{\#\#}p < .01$ compared with those treated with BMP-2 alone.

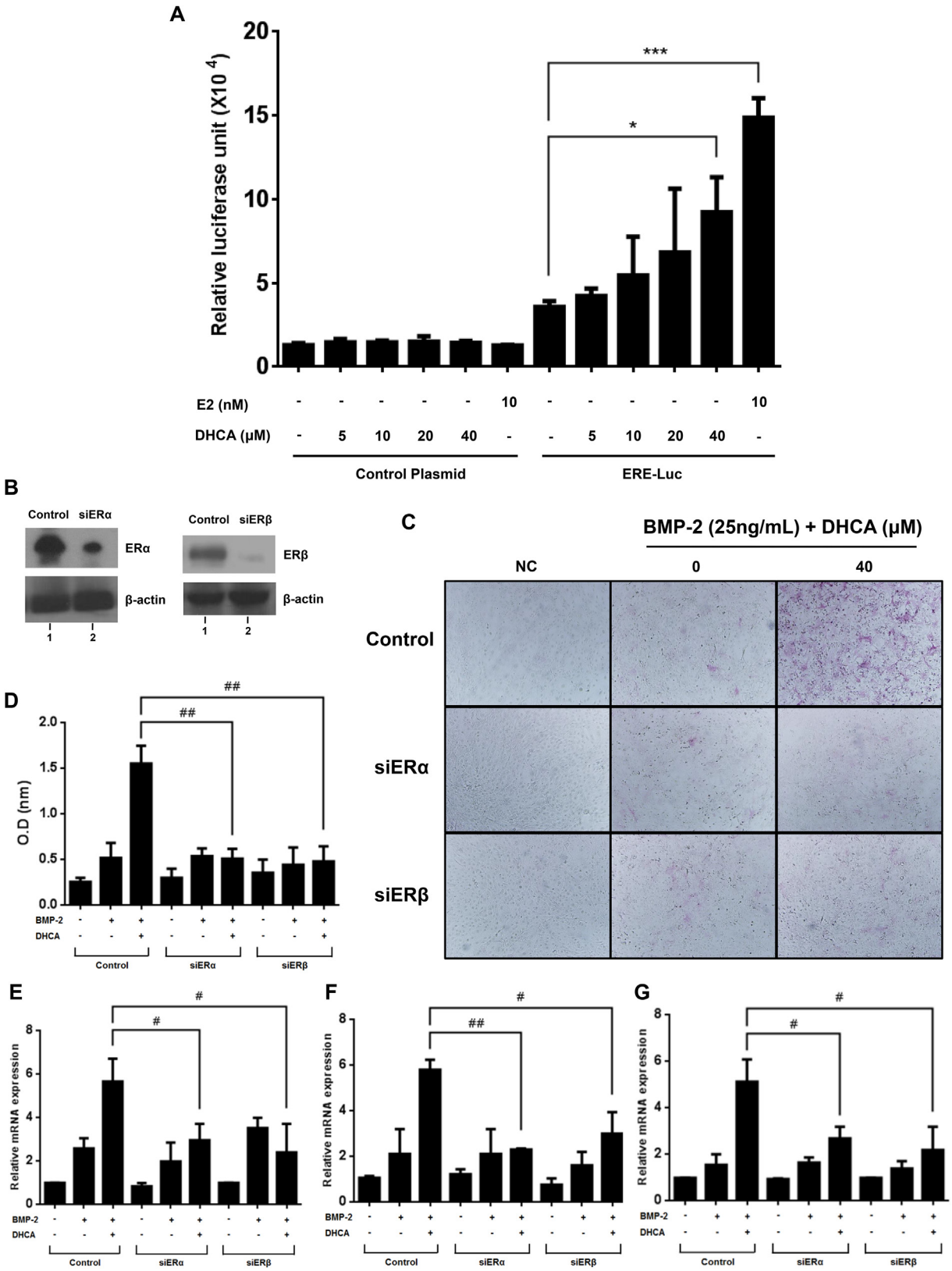

Fig. 4. Effects of DHCA on the estrogen receptors during BMP-2-induced osteoblastogenesis. MC3T3-E1 cells were transfected with a control or luciferase reporter plasmid containing sequences for ERE, and then treated with DHCA (5, 10, 20 and 40 μM) or E2 (10 nM) as a positive control for 6 h. (A) Effect of DHCA on luciferase activity. MC3T3-E1 cells were transfected with siRNAs for ERα, ERβ or scramble control, and then co-treated with BMP-2 (25 ng/mL) and DHCA (40μM) for 5 days. ALP-positive cells were visualized by ALP stain, while ALP activity was measured at 450 nm following ALP activity assay as described in Materials and Methods. (B) ERα and ERβ protein were knocked down by siRNA; (C) Effects of DHCA on the number of ALP-positive cells; (D) Effects of DHCA on the level of ALP activity; The RNA level of ALP (E), Osteocalcin (F) and OPG (G) was analyzed by quantitative RT-PCR after 24 h. Values represent the mean ± S.E.M. of three independent experiments. $^{*}p < .05$, $^{**}p < .01$ compared with control; $^{\#}p < .05$, $^{\#\#}p < .01$ compared with control siRNA group that was treated with BMP-2 and DHCA.

agonist for both receptors. We have recently shown that DHCA inhibits RANKL-induced osteoclast differentiation in vitro and ovariectomy-induced bone loss in vivo (submitted for publication). Taken together, our results indicate that DHCA may be developed as an efficient therapeutic for osteoporosis by controlling the osteoclast/osteoblast ratio through its estrogenic effects.

Author's contribution

W. Lee and S. Kim designed the research. W. Lee performed the whole experiments. K. R. Ko performed ALP staining and western blot. H. Kim performed ALP staining. S. Lim performed RT-qPCR. W. Lee and S. Kim wrote the main manuscript text.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

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