ERβ compensates for the absence of ERα function to promote osteoblast viability by inhibition of SOST signaling

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Received January 27, 2016; Accepted January 26, 2017

DOI: 10.3892/etm.2017.5014

Abstract. Estrogen receptors α and β (ERα and ERβ) serve key functions in bone development and maintenance, and in the metabolism of bone mineral. ERβ and ERα form heterodimers, and ERβ negatively regulates the transactivation of ERα. ERβ also inhibits recruitment of ERα to the estrogen-responsive promoters. However, the relationship of ERα and ERβ in the regulation of osteoblast viability and differentiation remains unclear. The present study aimed to investigate whether ERβ plays a role in balancing ERα activity in osteoblast cells. Downregulation of ERα by short hairpin RNA (shRNA) was found to significantly increase cell cycle arrest at G1 phase ($P<0.01$). In addition, this effect was found to be significantly enhanced by downregulation of ERβ ($P<0.05$). Inversely, ERα-knocked down osteoblasts were treated with ERβ agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) to activate ERβ. It was found that activation of ERβ significantly rescued the arrest of cell cycle induced by the downregulation of ERα ($P<0.05$). Furthermore, downregulation of ERα was found to significantly inhibit cell viability ($P<0.01$), and knockdown of ERβ was found to have a significant synergic effect with ERα downregulation on the inhibition of cell viability ($P<0.01$). Treatment with ERβ agonist DPN significantly rescued the effects of downregulation of ERα on cell viability ($P<0.01$). It was also demonstrated that the synergic effects of ERα and ERβ deletion was via upregulation of SOST gene expression, and the subsequent inhibition of OPG and Runx2 gene expression. Thus, ERβ may serve a function in balancing osteoblast viability and differentiation induced by ERα.

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Key words: osteoblast, estrogen receptor, SOST

Introduction

Estrogen receptor α and β (ERα and ERβ) are expressed in osteoblast cells and their precursors (1). They play a key role in bone remodeling (2). Previous studies have suggested that ERα serves a key function in bone development and maintenance, and in the metabolism of bone mineral, by regulating osteoblast activity (2,3). ERβ and ERα form heterodimers, and ERβ negatively regulates the transactivation of ERα. ERβ also inhibits recruitment of ERα to estrogen-responsive promoters (4,5). In addition, recent studies have shown that ERβ is critical in the regulation of osteoblast prolifERαtion and differentiation via regulation of osteogenesis related genes (6). Braidman *et al* found that ERβ was expressed in osteoblasts derived from areas of active bone formation or bone remodeling (7). Stossi *et al* showed that estradiol upregulated several genes associated with cell motility selectively via ERβ (8). Sniekers *et al* observed an increase in number and size of osteophytes and thinning of the lateral subchondral plate in ERβ- and ERα-knockout ($ERβ^{-/-}$ and $ERα^{-/-}$) mice (9). However, no significant differences were found in cartilage damage score, osteophyte formation, or subchondral plate thickness between $ERβ^{-/-}$ or $ERα^{-/-}$ mice. Compared with wild-type mice, the bone volume fraction of the epiphyseal trabecular bone was unchanged in $ERα^{-/-}$ mice, while it was increased in $ERβ^{-/-}$ mice, and decreased in $ERβ^{-/-}ERα^{-/-}$ mice, indicating that ERβ and ERα may retain a compensatory function for each other. However, a previous study suggested that activation of ERβ had a similar effect on bone remolding with or without ERα (10). ERβ promotes expression of a subset of genes when ERα is deleted (9). However, the relationship of ERα and ERβ in the regulation of osteoblast viability and differentiation is yet to be elucidated, and the mechanism by which ERβ exerts its function is also unclear.

Huang *et al* found that the SOST gene binds to two cooperating transcription factors, CCAAT-enhancer-binding protein α (C/EBPα) and forkhead box protein A1 (FOXA1), which modulate estrogen receptor function at the core consensus recognition site, suggesting that SOST may be one of the target genes of estrogen (11). It was reported that serum levels of the SOST protein were negatively correlated with estradiol levels in postmenopausal osteoporosis women (12). SOST is able to suppress the canonical Wnt signaling pathway by binding to LRP5/6, and subsequently inhibits osteoblast

differentiation and proliferation (13). Low levels of estrogen may result in overexpression of SOST, which may be one of the pathogenic mechanisms of osteoporosis. However, whether ERβ mediates the osteoblastic context by regulating the expression of SOST under the condition of ERα expression inhibition is unclear.

The present study aimed to investigate whether ERβ serves a function in balancing ERα activity in osteoblastic cells. It was demonstrated that knockdown of ERβ promotes osteoblast viability, mediated by downregulation of ERα, via regulation of a subset of genes, including SOST, OPG and Runx2.

Materials and methods

Cell culture and treatment. The mouse osteoblastic cell line MC3T3-E1 (MCE) was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured (70-80% confluence) in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) at 37˚C in a humidified incubator containing 5% CO_2.

In order to activate ERβ, MCE cells were treated with 0.1 μM ERβ agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN; Tocris Bioscience, Bristol, UK) dissolved in dimethyl sulfoxide (DMSO) for 24 h.

Transfection. ERα short hairpin RNA (shRNA) sequences: (CCGGTACAGGCCAAATTCAGATAACTCGAGTTATCT GAATTTGGCCTGTAGTTTTT) and ERβ shRNA (CCG GGCGAGTAACAAGGGCATGGAACTCGAGTTCCATGC CCTTGTTACTCGCTTTTT) sequences were designed and synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). A scrambled shRNA sequence (CCTAAGGTTAAG TCGCCCTCGCTCGAGCGAGGGCGACTTAACCTT AGG) was used as a negative control (NC). To knockdown the expression of ERα or ERβ, MCE cells were transfected with ERα shRNA or ERβ shRNA using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.). NC cells were transfected with scrambled shRNA. Untreated cells were used as a mock control.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. Total RNA of cells after indicated treatment was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. RNA was converted into cDNA using a Reverse Transcription kit (Thermo Fisher Scientific, Inc.). To analyze mRNA expression, SYBR Green qPCR Master mix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to perform RT-qPCR. The primers used were: SOST forward, 5'-TGCCGCGAGCTG CACTACAC-3' and reverse, 5'-CACCACTTCACGCGCCCG AT-3'; Runx2 forward, 5'-AACCCACGGCCCTCCCTGAAC TCT-3' and reverse, 5'-ACTGGCGGGGTGTAGGTAAAG GTG-3'; OPG forward, 5'-GTTCCTGCACAGCTTCACAA-3' and reverse, 5'-AAACAGCCCAGTGACCATTC-3'; GAPDH forward, 5'-CACCATGGAGAAGGCCGGGG-3' and reverse, 5'-GACGGACACATTGGGGGTAG-3'. The conditions of the RT-qPCR reaction were as follows: 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing/elongation at 60˚C for 30 sec. GAPDH was used as an internal reference. The relative expression was analyzed by the $2^{-\Delta\Delta Cq}$ method (14).

Western blot analysis. Cells were solubilized in cold radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc.). Proteins were separated with 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was incubated with PBS containing 5% milk overnight at 4˚C, which was then incubated with rabbit monoclonal anti-ERα antibody (1:1,000, cat. no. ab32063), rabbit polyclonal anti-ERβ antibody (1:1,000, cat. no. ab5784; Abcam), and mouse monoclonal anti-GAPDH antibody (1:5,000, cat. no. 60004-1-Ig; Wuhan Sanying Biotechnology, Wuhan, China) overnight at 4˚C. The membrane was washed with PBS 3 times and incubated with goat anti-mouse secondary antibody or goat anti-rabbit secondary antibody (1:5,000, cat. no. SA00001-1; Wuhan Boster Biological Technology, Ltd., Wuhan, China) at room temperature for 1 h. Chemiluminescent detection was conducted using an enhanced chemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The relative protein expression was analyzed by Image-Pro Plus software 6.0, represented as the density relative to GAPDH.

MTT assay. In order to examine cell viability, $2x10^3$ MCE cells in each group were cultured in a 96-well plate. MTT (0.5 g/l; Thermo Fisher Scientific, Inc.) dissolved in 100 μl DMEM was added to each well, then the cells were cultured at 37˚C for 0, 12, 24, 48, 72 or 96 h. The medium was then removed and 150 μl DMSO was added. After incubation at 37˚C for 15 min, the optical density of each sample at 570 nm was measured using a Tecan Infinite M200 plate reader (Tecan Group Ltd., Männedorf, Switzerland).

ELISA determination of Runx2 and OPG levels. Human Runx2 and OPG immunoassay kits (Cedarlane, Burlington, ON, Canada) were used to determine the Runx2 and OPG levels in the cells according the manufacturer's instructions. Briefly, the samples were incubated with Runx2 and OPG antibodies overnight at 4˚C, then incubated with horseradish peroxidase-labeled anti-rabbit antibody for 30 min at room temperature. Wells were then developed with tetramethylbenzidine reagent in a dark environment and the absorbance was measured at 450 nm on an ELISA Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Cell cycle analysis. Cell cycle analysis was determined by flow cytometry. Briefly, between ~$8x10^4$ and $1x10^5$ cells were seeded in each well of a 6-well plate. After culture for 12 h, cells were treated with shRNA-ERα alone, or co-treated with shRNA-ERα and shRNA-ERβ, or co-treated with shRNA-ERα and ERβ agonist DPN. At 48 h, the cells were harvested and fixed in 70% ice-cold ethanol for 12 h, followed by staining with propidium iodide. The different phases of the cell cycle were analyzed using a BD FACSCalibur instrument (BD Biosciences, San Jose, CA, USA).

Statistical analysis. All experiments were performed in triplicate. Data are presented as the mean ± standard error.

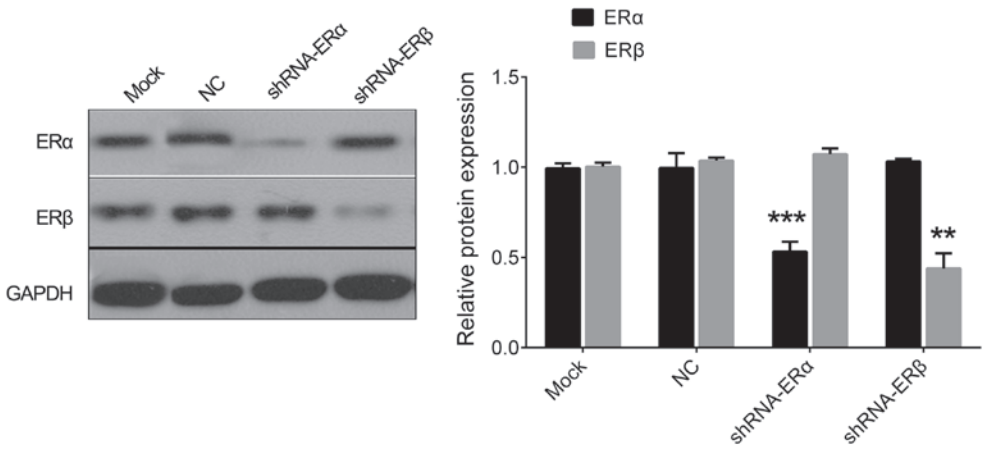

Figure 1. ERα and ERβ knockdown in MCE cells. Western blot analysis was performed for ERα and ERβ after shRNA-ERα or shRNA-ERβ treatment. **P<0.01, ***P<0.001 vs. NC. ER, estrogen receptor; shRNA, short hairpin RNA; NC, normal control.

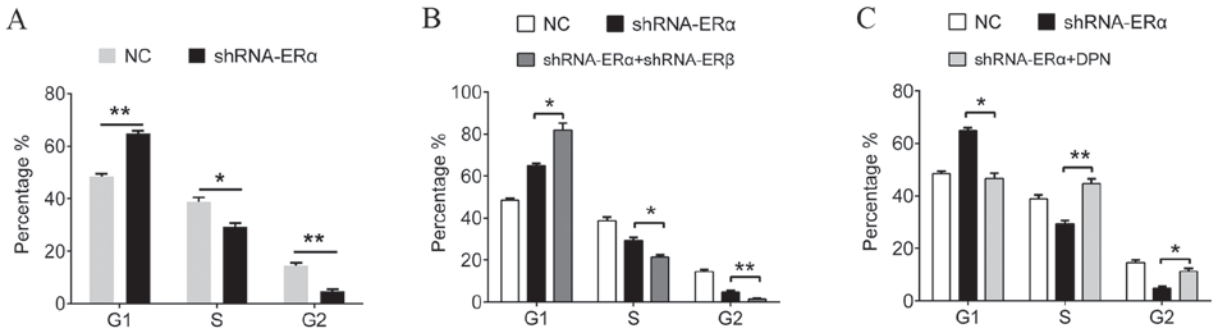

Figure 2. Effect of ERα and ERβ on MCE cell cycle. Cell cycle analysis in MCE cells treated with (A) shRNA-ERα alone, (B) co-treated with shRNA-ERα and shRNA-ERβ or (C) co-treated with shRNA-ERα and ERβ agonist DPN. *P<0.05, **P<0.01 as indicated. ER, estrogen receptor; shRNA, short hairpin RNA; NC, normal control; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile.

Statistical analysis was performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Differences between two groups were analyzed using an unpaired t-test. Differences among more than two groups were analyzed using analysis of variance with the Bonferroni post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

ERα and ERβ shRNA knockdown. To investigate the role of ERα and ERβ on the cell cycle of osteoblast cells, their expression was knocked down in MCE cells. Western blot analysis indicated that the expression of ERα was significantly decreased in shRNA-ERα cells (P<0.001), and the expression of ERβ was significantly decreased in shRNA-ERβ cells (P<0.01), compared with the NC group (Fig. 1).

Effect of ERα and ERβ on MCE cell cycle. Downregulation of ERα by shRNA significantly increased the percentage of cells in G1 phase (P<0.01), and significantly decreased the percentage of cells in S (P<0.05) and G2 (P<0.01) phases, indicating an increase in cell cycle arrest at G1 phase compared with the NC group (Fig. 2A). In addition, it was observed that knockdown of ERβ and ERα by shRNA significantly increased the percentage of cells in G1 phase (P<0.05) and significantly decreased the percentage of cells in S (P<0.05) or G2 (P<0.01) phase compared with ERα knockdown alone (Fig. 2B). Inversely, in osteoblasts treated

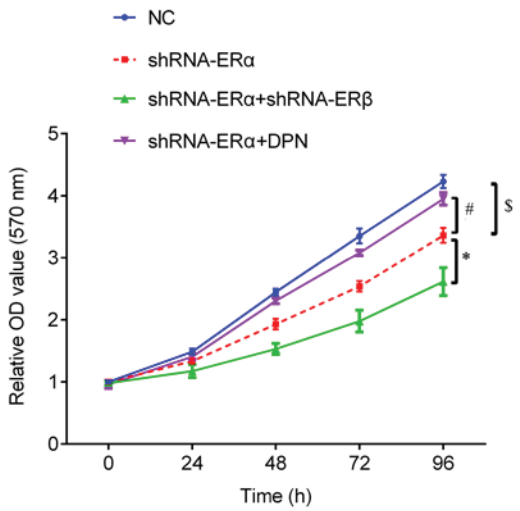

Figure 3. Effect of ERα and ERβ on MCE cell viability. MTT assay performed in cells treated with shRNA-ERα alone or co-treated with shRNA-ERα and shRNA-ERβ or co-treated with shRNA-ERα and ERβ agonist DPN. *P<0.05, shRNA-ERα vs. shRNA-ERα + shRNA-ERβ; #P<0.05, shRNA-ERα vs. shRNA-ERα+DPN; $P<0.05, shRNA-ERα vs. NC. OD, optical density; ER, estrogen receptor; shRNA, short hairpin RNA; NC, normal control; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile.

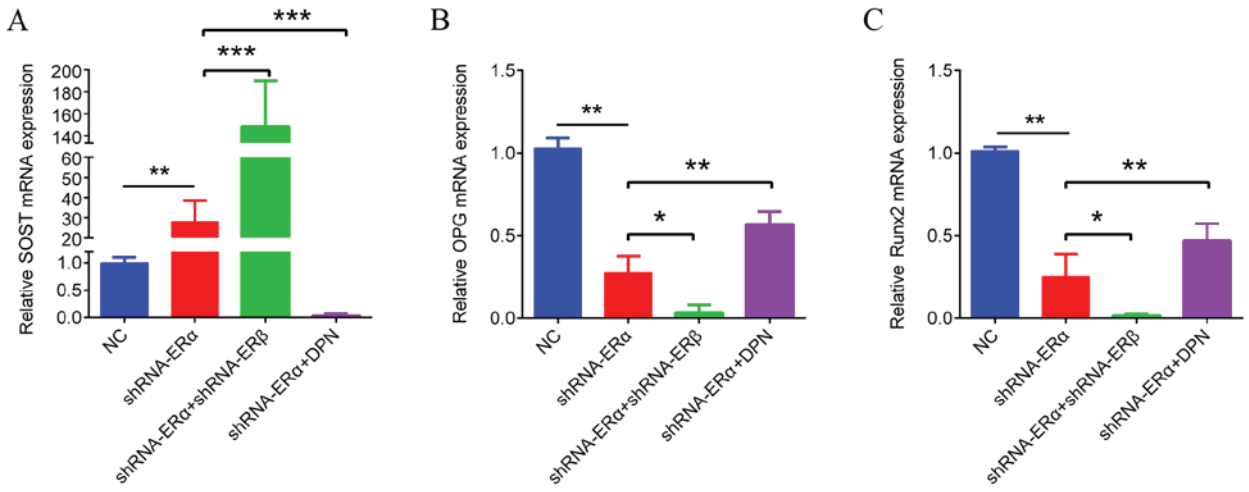

Figure 4. Effect of ERα and ERβ on SOST, Runx2 and OPG expression. Reverse transcription-quantitative polymerase chain reaction analysis for (A) SOST, (B) Runx2 and (C) OPG in cells with shRNA-ERα alone or co-treated with shRNA-ERα and shRNA-ERβ or co-treated with shRNA-ERα and ERβ agonist DPN. *P<0.05, **P<0.01, ***P<0.001 as indicated. ER, estrogen receptor; shRNA, short hairpin RNA; NC, normal control; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile.

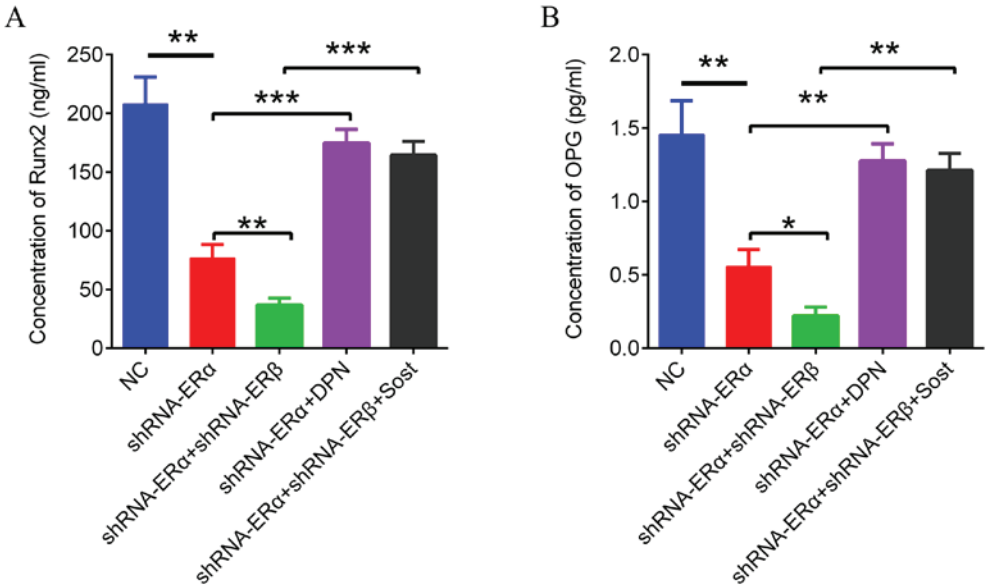

Figure 5. Effect of ERα and ERβ on Runx2 and OPG protein levels. ELISA was conducted for protein concentrations of (A) Runx2 and (B) OPG in cells with shRNA-ERα alone or co-treated with shRNA-ERα and shRNA-ERβ or co-treated with shRNA-ERα and DPN or co-treated with shRNA-ERα, shRNA-ERβ and Sost antibody. *P<0.05, **P<0.01, ***P<0.001 as indicated. ER, estrogen receptor; shRNA, short hairpin RNA; NC, normal control; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile.

with shRNA-ERα and ERβ agonist DPN to activate ERβ, it was found that activation of ERβ rescued the arrest of cell cycle induced by downregulation of ERα. The percentage of cells in G1 phase significantly decreased (P<0.05), and the percentage of cells in S or G2 phase significantly increased, compared with ERα knockdown alone (P<0.01 and P<0.05, respectively; Fig. 2C).

Effect of ERα and ERβ on MCE cell viability. An MTT assay was performed to investigate the role of ERα and ERβ in osteoblast cell viability. It was observed that downregulation of ERα by shRNA significantly decreased cell viability compared with the NC group (P<0.05; Fig. 3). Knockdown of ERβ had a synergic effect with knockdown of ERα, significantly decreasing cell viability compared with ERα knockdown alone (P<0.05). However, treatment of ERα knockdown cells with ERβ agonist DPN significantly rescued the effect of ERα downregulation on cell viability (P<0.05).

Effect of ERα and ERβ on the expression of SOST, OPG and Runx2. In order to investigate the potential mechanism underlying the regulation of cell cycle and cell viability by ERα and ERβ, the expression of SOST, OPG and Runx2 were analyzed by RT-qPCR after knockdown of ERα and ERβ. The results showed that knockdown of ERα significantly increased the expression of SOST (P<0.01; Fig. 4A), while it significantly decreased the expression of OPG and Runx2 compared with

the NC group (P<0.01; Fig. 4B and C). Knockdown of ERβ significantly enhanced the effects of ERα knockdown on the expression of SOST (P<0.001), OPG (P<0.05) and Runx2 (P<0.05). Activation of ERβ by DPN significantly reversed the increase in SOST expression (P<0.001) and the decrease in OPG and Runx2 expression (P<0.01 for both) induced by knockdown of ERα.

Effect of ERα and ERβ on the OPG and Runx2 protein concentration. The protein concentration of OPG and Runx2 after knockdown of ERα and ERβ was evaluated using ELISA. Consistent with the mRNA expression results in Fig. 4, knockdown of ERα significantly decreased the protein concentration of OPG and Runx2 (P<0.01; Fig. 5). Knockdown of ERβ and ERα significantly enhanced the decrease in protein concentration compared with knockdown of ERα alone (P<0.01 for Runx2, P<0.05 for OPG). Activation of ERβ by DPN significantly reversed these decreases in Runx2 and OPG expression (P<0.001 and P<0.01, respectively). Notably, inhibition of SOST by an exogenous antibody significantly rescued the effects of ERα and ERβ knockdown on Runx2 and OPG protein levels (P<0.001 and P<0.01, respectively).

Discussion

Estradiol (E2) is the principal human circulating sex steroid to act on bone tissue. A decline in circulating E2 is directly correlated with bone loss from adulthood onwards (15). Both ERα and ERβ are expressed in osteoblasts and their precursors. They mediate the stimuli responsiveness of E2 for bone remodeling. Previous evidence has indicated that ERα plays an important role in bone development and maintenance processes (16,17). ERβ prevents the stimulation of ERα in bone formation by regulating the activity of ERα (18,19). However, other evidence has suggested that ERβ and ERα have similar effects on bone metabolism, the expression of osteogenic cytokines and osteoblast function (6). Sims *et al* suggested that both ERα and ERβ influence bone remodeling in females, and could compensate for each other at least under basal knockout conditions (20). The results of the present study indicated that silencing of ERα resulted in decreased osteoblast viability, which was enhanced by silencing of ERβ. However, activation of ERβ by the selective ERβ agonist DPN was able to rescue the decrease in osteoblast viability induced by the deletion of ERα. These results indicate that the activity of ERβ can partly compensate for the regulatory role of ERα in osteoblast viability.

Osteogenic cytokines, such as SOST, OPG, Runx2, are closely related to the effects of estrogen. Huang *et al* found that two cooperating transcription factors, C/EBP and FOXA1 were located 10 kb upstream of the SOST transcription start site, and could modulate estrogen receptor function, suggesting that SOST may be one of the target genes of estrogen (11). It was reported that serum SOST levels were negatively correlated with circulating estradiol levels in postmenopausal females with osteoporosis (12). In addition, the distribution and protein expression of ER subtypes (ERα and ERβ) are altered with aging and estrogen loss (21,22). The results of the present study suggested that knockdown of ERα significantly increased the expression of SOST, which was enhanced by the silencing of ERβ. The decrease in SOST expression due to ERα deletion was abolished by the selective ERβ agonist DPN. These data suggest that ERβ is required for inhibition of SOST expression by ERα.

It has previously been reported that the juxtaposition of Runx2, E-box and C/EBP binding sites in the SOST promoter is notably similar to the structure of the osteocalcin promoter (23), suggesting that a regulatory feedback loop is present between SOST and Runx2, which may be regulated by estrogen signaling. In the present study, it was investigated whether the expression of osteogenic cytokines (OPG and Runx2) may be regulated by ERα through Sost, mediated by ERβ activity. The results indicated a synergic effect between downregulation of ERα and ERβ on the inhibition of OPG and Runx2 expression. However, the inhibition of OPG and Runx2 induced by downregulation of ERα and ERβ was abolished by inhibition of Sost, indicating that the synergic effects of ERα and ERβ deletion were via upregulation of Sost expression, and the subsequent inhibition of OPG and Runx2 expression. Thus, ERβ may serve a function in balancing the osteoblast viability and differentiation induced by ERα.

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