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

SHIJIN LU*, CHANGYING SUN*, CONGXIU MIAO and ZHONGFU ZHAO

Department of Orthopedics, The Affiliated Peace Hospital of Changzhi Medical College, Changzhi, Shanxi 046000, P.R. China

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 synergistic 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.

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 proliferation 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 remodeling 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% CO₂.

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: (CCGGTCAGTGCCGAGATTCCGAGTTATCT GAATTTCGACTGTATTTTTT) and ERβ shRNA (CCGGGCCAGTTAAGGGCATGGAATCGTTTCACTGC CCTTTTACTCGCATTTTTTTTT) sequences were designed and synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). A scrambled shRNA sequence (CCTAAGGTGATACGGCAAGGCAGGCAATCCTACGTAGG) 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'-TGCGCCGGACCTG CACTACAC-3' and reverse, 5'-CAGCCACCATCTGCAAGCGCCG AT-3'; Runx2 forward, 5'-AAACACCCGGCCCTCCCTGAC TCT-3' and reverse, 5'-ACTGCGCAGGGGGTAGTAAAG GTG-3'; OPG forward, 5'-GTCTCCGTACGCTTCAAAA3' and reverse, 5'-AAACACCCCATACGACATT-3'; GAPDH forward, 5'-CAGCGGAGAAGGGCGGGG-3' and reverse, 5'-GACGCGACATGTTGGAGT-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^ΔΔCq 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, 2x10⁴ 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 ~8x10⁴ and 1x10⁵ 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.
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...
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 down-regulation 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 ERs 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 ERs play 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 ERs 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.

**References**

1. Windahl SH, Norgård M, Kuiper GG, Gustafsson JA and Andersson G: Cellular distribution of estrogen receptor beta in neonatal rat bone. Bone 26: 117-121, 2000.
2. Centrella M and McCarthy TL: Estrogen receptor dependent gene expression by osteoblasts-direct, indirect, circumpect, and speculative effects. Steroids 77: 174-184, 2012.
3. Martin-Millan M, Almeida M, Ambrogini E, Han L, Zhao H, Weinstein RS, Jilka RL, O’Brien CA and Manolagas SC: The estrogen receptor-alpha in osteoclasts mediates the protective effects of estrogens on cancellous but not cortical bone. Mol Endocrinol 24: 323-334, 2010.
4. Zhao C, Matthews J, Tujaugue M, Wan J, Ström A, Toresson G, Lam EW, Cheng G, Gustafsson JA and Dahlman-Wright K: Estrogen receptor beta negatively regulates the transactivation of estrogen receptor alpha in human breast cancer cells. Cancer Res 67: 3955-3962, 2007.
5. Zhao C, Dahlman-Wright K and Gustafsson JA: Estrogen receptor beta: An overview and update. Nucl Recept Signal 6: e003, 2008.
6. Bhargavan B, Singh D, Gautam AK, Mishra JS, Kumar A, Goel A, Dixit M, Pandey R, Manickavasagam L, Dwivedi SD, et al: Medicarpin, a legume phytoalexin, stimulates osteoblast differentiation and promotes peak bone mass achievement in rats: Evidence for estrogen receptor β-mediated osteogenic action of medicarpin. J Nutr Biochem 23: 27-38, 2012.
7. Braidman IP, Hainey L, Batra G, Selby PL, Saunders PT and Hoyland JA: Localization of estrogen receptor beta protein expression in adult human bone. J Bone Miner Res 16: 214-220, 2001.
8. Stossi F, Barnett DH, Frasor J, Komm B, Lyttle CR and Katzenellenbogen BS: Transcriptional profiling of estrogen-regulated gene expression via estrogen receptor (ER) alpha or ERbeta in human osteosarcoma cells: Distinct and common target genes for these receptors. Endocrinology 145: 3473-3486, 2004.
9. Snickers YH, van Osch GJ, Ederveen AG, Inzunza J, Gustafsson JA, van Leeuwen JP and Weinsans H: Development of osteoarthritic features in estrogen receptor knockout mice. Osteoarthritis Cartilage 17: 1356-1361, 2009.
10. Komm BS and Chines AA: Bazedoxifene: The evolving role of third-generation selective estrogen-receptor modulators in the management of postmenopausal osteoporosis. Ther Adv Musculoskel Dis 4: 21-34, 2012.
11. Huang QY, Li GH and Kung AW: The -9247 T/C polymorphism in the SOST upstream regulatory region that potentially affects C/EBPalpha and FOXA1 binding is associated with osteoporosis. Bone 45: 289-294, 2009.
12. Morales-Santana S, Diez-Pérez A, Olmos JM, Nogués X, Sosa M, Díaz-Curiel M, Pérez-Castrillón JL, Pérez-Cano R, Torrijos A, Jodar E, et al: Circulating sclerostin and estradiol levels are associated with inadequate response to bisphosphonates in postmenopausal women with osteoporosis. Maturitas 82: 402-410, 2015.

13. Jami A, Gadi J, Lee MJ, Kim EJ, Lee MJ, Jung HS, Kim HH and Lim SK: Pax6 expressed in osteocytes inhibits canonical Wnt signaling. Mol Cells 35: 305-312, 2013.

14. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402-408, 2001.

15. Rochira V, Kara E and Carani C: The endocrine role of estrogens on human male skeleton. Int J Endocrinol 2015: 165215, 2015.

16. Heino TJ, Chagin AS and Sävendahl L: The novel estrogen receptor G-protein-coupled receptor 30 is expressed in human bone. J Endocrinol 197: R1-R6, 2008.

17. Roepke TA, Bosch MA, Rick EA, Lee B, Wagner EJ, Seidlova-Wuttke D, Wuttke W, Scanlan TS, Ronnekleiv OK and Kelly MJ: Contribution of a membrane estrogen receptor to the estrogenic regulation of body temperature and energy homeostasis. Endocrinology 151: 4926-4937, 2010.

18. Lindberg MK, Alatalo SL, Halleen JM, Mohan S, Gustafsson JA and Ohlsson C: Estrogen receptor specificity in the regulation of the skeleton in female mice. J Endocrinol 171: 229-236, 2001.

19. Roman-Blas JA, Castañeda S, Largo R and Herrero-Beaumont G: Osteoarthritis associated with estrogen deficiency. Arthritis Res Ther 11: 241, 2009.

20. Sims NA, Dupont S, Krust A, Clement-Lacroix P, Minet D, Resche-Rigon M, Gaillard-Kelly M and Baron R: Deletion of estrogen receptors reveals a regulatory role for estrogen receptors-beta in bone remodeling in females but not in males. Bone 30: 18-25, 2002.

21. Hara Y, Waters EM, McEwen BS and Morrison JH: Estrogen effects on cognitive and synaptic health over the lifecourse. Physiol Rev 95: 785-807, 2015.

22. Cai M, Ma YL, Qin P, Li Y, Zhang LX, Nie H, Peng Z, Dong H, Dong HL, Hou WG and Xiong LZ: The loss of estrogen efficacy against cerebral ischemia in aged postmenopausal female mice. Neurosci Lett 558: 115-119, 2014.

23. Sevetson B, Taylor S and Pan Y: Cbfal/RUNX2 directs specific expression of the sclerosteosis gene (SOST). J Biol Chem 279: 13849-13858, 2004.