

Estrogen-induced *Tgfb1* and *Bmpr1a* Expression Repressed via Estrogen Receptor Beta in MC3T3-E1 Cells

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Abstract

Background: Estrogen, as an important hormone in human physiological process, is closely related to bone metabolism. The aim of this study was to investigate the mechanism of estrogen on osteoblasts metabolism in MC3T3-E1 cells.

Methods: We treated the MC3T3-E1 cells with different concentrations of β -estradiol (0.01, 0.1, 1, and 10 nmol/L), observed the morphological changes of the cells, and detected the cell's proliferation and apoptosis of MC3T3-E1 cells. Two transcriptome libraries were constructed and sequenced. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to confirm the differentially expressed genes (DEGs), and then treated the MC3T3-E1 cells with estrogen receptor (ER) inhibitors α and β , respectively, and then examined the expression of *Tgfb1* and *Bmpr1a* genes. The promoter of *Tgfb1* and *Bmpr1a* gene was analyzed, and the ER response elements were identified. Finally, ChIP was used to verify the binding of ER to *Tgfb1* and *Bmpr1a* promoter.

Results: In the high-concentration β -estradiol treatment group (1 nmol/L and 10 nmol/L), there was no significant difference in the morphology of the cells under the microscope, 1 nmol/L and 10 nmol/L treated group appeared statistically significant difference in cell apoptosis and proliferation ($P < 0.05$ and $P < 0.01$, respectively). We found 460 DEGs compared with the control group. Among the DEGs, there were 66 upregulated genes and 394 downregulated genes. Gene ontology classification and Kyoto Encyclopedia of Genes and Genomes pathway analysis revealed that many bone metabolism-related biological processes and cell signaling pathways were disordered. The qRT-PCR verification showed that the expressions of *Tgfb1*- and *Bmpr1a*-related genes in bone metabolism pathway in the 10 nmol/L treatment group were significantly decreased ($P < 0.05$). ER β was involved in the inhibitory effect of *Tgfb1* and *Bmpr1a* genes. The bioinformatics of the promoter found that there were three ER response elements in the promoter of *Tgfb1*, and there were two ER response elements in *Bmpr1a* promoter regions. ChIP experiments showed that estrogen could enhance the binding of ERs to *Tgfb1* and *Bmpr1a* genes.

Conclusions: Estrogen can promote the apoptosis and proliferation of osteoblasts simultaneously, and the mechanism may be the joint action of transforming growth factor-beta, Wnt, mitogen-activated protein kinase, and nuclear factor-kappaB bone metabolism-related signaling pathway. Estrogen inhibits the expression of *Tgfb1* and *Bmpr1a* genes through ER β and affects the metabolism of MC3T3-E1 osteoblasts.

Key words: *Bmpr1a*; Estrogen Receptor Beta; Estrogen; MC3T3-E1 Cells; *Tgfb1*

INTRODUCTION

Estrogen, a steroid hormone in the human body, plays an important role in many physiological processes,^[1] such as the maintenance of secondary sexual characteristics, the development of neurons, and bone homeostasis.^[2] Estrogen is critical to maintain bone and bone density.^[3] Estrogen deficiency often leads to the emergence of osteoporosis. In postmenopausal women, estrogen secretion is markedly

reduced, as is bone mineral density, whereas the incidence of bone fractures is increased. This form of osteoporosis is

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called postmenopausal osteoporosis (PO).^[4] An imbalance of osteoblasts and osteoclasts in the bone causes osteoporosis.^[5] PO has become one of the most important diseases that affect elder women, as it entails huge financial burdens for individuals and society, and reduces the quality of life.

In 1988, Komm *et al.*^[6] found that estrogen receptors (ERs) are expressed in osteoblasts, suggesting that estrogen may have a regulatory role for osteoblasts. After the discovery of ERs on osteogenic precursors and bone cells, an increasing number of studies has shown that estrogen has a direct effect on osteogenesis, as well as an indirect effect that does not depend on ERs. Kousteni *et al.*^[7] have found that estrogen can inhibit osteoblast apoptosis and prolong osteoblast life, thereby increasing the osteogenic capacity of each osteoblast. The effect of estrogen is achieved by rapid activation of ER-mediated regulatory kinases.^[8] The lack of estrogen causes a marked increase in nuclear factor-kappaB (NF-κB) activity in osteoblasts.^[9] Estrogen deficiency leads to an imbalance in bone metabolism between bone formation and resorption, eventually leading to osteoporosis. As the mechanism is not yet clear, further studies on estrogen regulation of bone metabolism and its signaling pathways are required to achieve a better understanding.

MC3T3-E1 cells are a clonal preosteoblastic cell line derived from newborn mouse calvarias, which is utilized in many osteoblast studies.^[10]

METHODS

Cell culture

The clonal murine preosteoblastic cell line MC3T3-E1 was used in this study. The cells were thawed from frozen stocks and cultured in 100-mm culture dishes in α -minimum essential medium supplemented with 10% (v/v) fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 1% (w/v) antibiotics/antimycotics (the stock solution contained 100 U/ml penicillin G sodium, 100 μ g/ml streptomycin sulfate, and 0.25 μ g/ml amphotericin B, in saline), 1.0 mmol/L sodium pyruvate, 0.1 mmol/L nonessential amino acids, and 1.5 g/L sodium bicarbonate (Thermo Fisher Scientific) at 37°C in a humidified atmosphere containing 5% (v/v) CO₂. After establishing cultures from frozen cells, we subcultured the cells several times. Confluent cells were detached using 0.25% trypsin in Mg²⁺- and Ca²⁺-free phosphate-buffered saline (PBS, Thermo Fisher Scientific) before use. For the treatment of MC3T3-E1 with E2, the cells were seeded in each well of a 24- or 6-well plate with 0.5 or 2.0 ml of culture medium. The cells were incubated in the presence of E2 at different concentrations (0.01, 0.1, 1, and 10 nmol/L), and each concentration of E2 was maintained for three days. A group that received dimethyl sulfoxide (DMSO) served as the control.

Cell proliferation assay

Cell proliferation was analyzed using the Cell Counting kit 8 (Dojindo, Tokyo, Japan). MC3T3-E1 cells were detached and seeded into 96-well plates at a density of

3×10^3 cells/well. After 24 h, cells were treated with E2 at various concentrations (0, 0.01, 0.1, 1, and 10 nmol/L) and they were then incubated for another 24 h. Cell proliferation was analyzed according to the manufacturer's instructions. The optical density of each group was measured at 450 nm using a BioTek Microplate reader.

Cell apoptosis assay

Cell apoptosis of MC3T3-E1 cells was analyzed with the TUNEL Apoptosis Detection Kit (Beyotime, Shanghai, China). Briefly, the cells were collected two days after treatment with E2, washed with PBS, and suspended in 500 μ l binding buffer. Then, the cells were stained for the detection of apoptosis, according to the manufacturer's instructions. The ratio of apoptotic cells was analyzed under an optical microscope by counting terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, Beyotime) positive cells in six randomly selected areas.

Total mRNA extraction

Total cell mRNA was extracted using TRIzol® (Thermo Fisher Scientific), according to manufacturer instruction (Invitrogen, USA). Smart Spec Plus (Bio-Rad, Hercules, CA, USA) was used to measure the absorption at 260/280 nm to assess the quality and quantity of the collected RNA. Finally, the integrity of the extracted RNA was further assessed using 1.5% agarose gel electrophoresis. Subsequently, the RNA was transcribed to first strand cDNA using the First Strand cDNA Synthesis Kit (TAKARA, SHIGA-KEN, Japan) for gene expression analysis.

RNA sequencing library construction and sequencing

We used 20 μ g of total RNA from the control group and the 10 nmol/L E2-treated group for the RNA sequencing (RNA-seq) library preparation. The collected mRNA was purified and concentrated using oligo (dT)-conjugated magnetic beads (Invitrogen) before library preparation. The purified mRNA was randomly cut into fragments with the fragmentation buffer. We used the mRNA as a template, with 6-base random primers (Random hexamers, Thermo Fisher Scientific), to synthesize the first strand of cDNA. We then added dNTPs, RNase H, and DNA polymerase I to the buffer, to synthesize the second strand of cDNA. Finally, we used AMPure® XP beads (Beckman, CA, USA) to purify the synthesized cDNA. The purification of double-stranded cDNA was performed with end repair and A-tailing. The AMPure® XP (Beckman) beads were used to select the size of the fragments. Finally, the cDNA library was obtained by PCR. Using "sequencing by synthesis" technology, we sequenced the cDNA libraries with the HiSeq2500 (Illumina, San Diego, CA 92122, USA) high-throughput sequencing platform and obtained high-quality reads. The reads and bases sequenced by the platform were usually considered raw data, and most of them got a Q30 score for base quality.

Analysis of differentially expressed genes

Gene expression has time- and space-specific characteristics, and both external stimulation and the internal environment can affect gene expression. Under two different

conditions (such as the control and treatment, wild-type and mutant, different times, different organizations, *etc.*), genes that have significantly different expression are considered differentially expressed genes (DEGs). Similarly, transcripts with significantly different expression are called differentially expressed transcripts. In bioinformatics, the process of finding differentially expressed transcripts or DEGs is known as differential expression analysis.

Annotation and analysis of differentially expressed genes

The gene ontology (GO) database was built in 2000 and is a standard, structured biological annotation system aimed at establishing a system of standard vocabulary and knowledge of genes and their products. The GO annotation system contains three main branches: biological process, molecular function, and cellular component. We used the GO database to predict the functions of the DEGs identified in this study.^[11] Enrichment analysis of the differences between samples was carried out using Top GO. Top GO directly displays the GO node and the hierarchical relationship of the DEGs. The clusters of orthologous groups (COG) of proteins database is constructed on the basis of the phylogeny of bacteria, algae, and eukaryotes to classify the orthologs of gene products. To annotate the pathways of the DEGs, it is necessary to analyze the functions of the genes. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database is a major metabolic pathways database.^[12] The analysis of the overrepresentation of the DEGs in a pathway is important to determine the functions of the genes. In our study, COG and KEGG were used to analyze the DEGs.

Quantitative real-time polymerase chain reaction validation

Total cell mRNA was extracted using TRIzol[®], according to manufacturer instruction (Invitrogen). We subjected 10 µg of total RNA to DNaseI treatment with 1 U DNaseI (NEB, MA, USA). The reaction was carried out at 37°C for 10 min followed by heat inactivation at 65°C for 10 min. We then used 2.5 µg of DNase-treated RNA for cDNA synthesis with reverse transcriptase (Bio-Rad), in accordance with the manufacturer's protocol. The expression of the *Gapdh* gene, which the transcriptome database indicated was stable, was used as the control for quantitative real-time polymerase chain reaction (qRT-PCR) experiments. Primers were designed for selected transcripts from the transcriptome database, and real-time PCR was performed with SYBR[®] Green I master mix (TAKARA) on a CFX Connect[™] Real-Time PCR Detection System (Bio-Rad). The relative expression of the transcripts was calculated using the 2^{ΔΔCT} method.

Estrogen receptor α and estrogen receptor β antagonist treatment of MC3T3-E1 cells

For the ERα and ERβ antagonist experiments, the ERα antagonist 1,3-bis (4 hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyloxy)phenol]-1H-pyrazole dihydrochloride (MPP) and the ERβ antagonist 4-(2-phenyl-5,7-bis[trifluo-

romethyl] pyrazolo[1,5-a]pyrimidin-3-yl) (PTHPP) were added to the MC3T3-E1 cultures. After 72 h of treatment with the antagonists, the cells were harvested to quantify the target gene expression.

ChIP assays

ChIP assays were performed according to the manufacturer's protocol (ChIP Assay Kit, Millipore, MA, USA). Briefly, MC3T3-E1 cells were collected after culture with or without E2, cross-linked in 2% formaldehyde at 28°C for 30 min, then treated with a 1 / 10 volume of 1.25 mol/L glycine to stop cross-linking, followed by PBS washes (three washes for 10 min each). We used purified rabbit or mouse IgG (Invitrogen) as a negative control and an antibody against ERβ to pull down the DNA. We performed ChIP PCR using primers flanking the estrogen response element (ERE) sites, as well as primers not flanking the ERE sites in the promoter regions of *Tgfb1* and *Bmpr1a* as controls.

Statistical analysis

The statistical analyses were performed with JMP13.0 (SAS Institute Inc. Cary, NC, USA), and a *P* < 0.05 was considered statistically significant. Data were presented as mean ± standard deviation (SD). Statistical differences between two groups were determined with Student's *t*-test. Statistical differences among groups were analyzed with a one-way analysis of variance (ANOVA) followed by Student's test. All experiments were repeated at least three times, and representative experiments were shown.

RESULTS

E2 increased MC3T3-E1 cell proliferation and apoptosis

To check whether the estrogen effects the MC3T3-E1 cell's proliferation and apoptosis, different concentrations of estrogen (0, 0.01, 0.1, 1, and 10 nmol/L) were used to treat the MC3T3-E1 cells. CCK-8 and TUNEL experiments were performed. The results show MC3T3-E1 cell proliferation and apoptosis was not changed in the low concentration-treated groups (0.01 and 0.1 nmol/L estrogen-treated groups) compared with DMSO control groups. However, in the high-concentration estrogen groups (1 and 10 nmol/L estrogen-treated groups) the MC3T3-E1 cell apoptosis significantly increased compared with the DMSO control groups [Figure 1]. The 10 nmol/L concentration is higher than the physiological concentration, for obtain the robust data, so we sampled the 10 nmol/L treated group to perform RNA-seq and explore the mechanisms of the estrogen effects on MC3T3-E1 cells.

RNA sequencing and identification of differentially expressed genes

To assess the effects of E2 on gene transcription, we used the Cuffdiff analysis module Cufflinks to analyze the differential gene expression in the samples.^[13] The screening criteria for significant differences in the expression of genes are $|\log_2\text{Ratio}| \geq 1$ and $q \leq 0.05$. Using these criteria, we identified 460 DEGs. Among

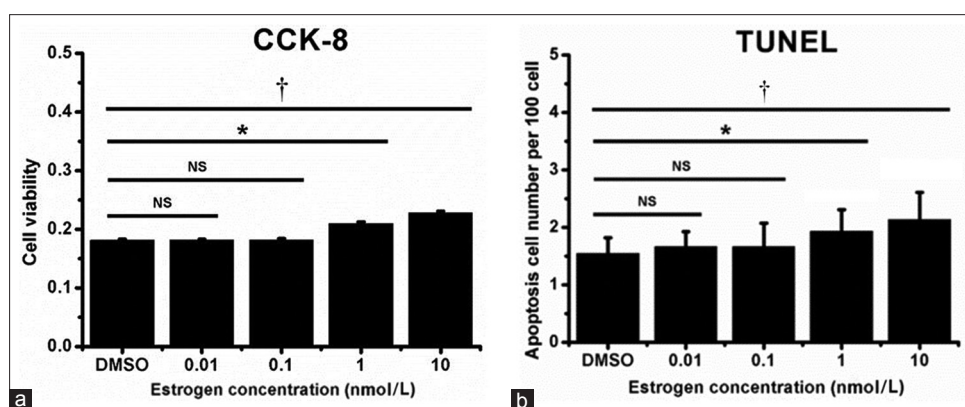


Figure 1: Effects of estrogen on MC3T3-E1 proliferation (a) and apoptosis (b). MC3T3-E1 cells were treated with different concentrations of E2, then subjected to analysis with a CCK-8 assay (a) and the TUNEL Apoptosis Detection Kit (b). Student's *t*-test. **P* < 0.05; †*P* < 0.01.

those DEGs, 66 genes were upregulated and 394 genes were downregulated in the cells treated with 10 nmol/L E2-treated group [Figure 2].

Gene ontology analysis and Kyoto encyclopedia of genes and genomes pathway functional analysis of enriched differentially expressed genes

GO is an internationally standardized gene functional classification system that provides a set of dynamically updated vocabularies to comprehensively describe the properties of genes and gene products in organisms. The most enriched GO results for the three ontologies are shown in Table 1. We found that genes that affect biological regulation, metabolic processes, development, anatomical structure development, responses to stimulation, systems development, cell differentiation, cell communication, regulation of gene expression, and signal transduction were enriched in the DEGs [Table 2].

Multiple genes coordinate their biological functions *in vivo*. Pathway-based analysis enables further understanding of the biological functions of the genes. KEGG is the main public pathway database. Because we focus on the bone metabolism process, so bone metabolism pathway was come into our view. We found that the DEGs were enriched for genes involved in the pathways for cancer, mitogen-activated protein kinase (MAPK) signaling, transforming growth factor-beta (TGF- β) signaling, focal adhesion, Wnt signaling, NF- κ B signaling, and osteoclast signaling [Table 2]. The TGF- β and Wnt signaling pathways are important in osteoblasts. Through the pathway maps, we identified alterations in the expression of *Bmpr1a*, *Tgfbr1*, *Rock1*, and *sp1*, which are involved in the TGF- β signaling pathway [Table 2]. We also noted alterations in the expression of *Lrp5*, *Vangl2*, and *Rock1*, which are involved in Wnt signaling [Table 2]. In the cell cycle signaling pathway, we noted alteration in the expression *Ccna2* *Stag2*, *Atr*, *Smc1a* and *Rb1l*. These data show that the effects of estrogen maybe mediated via the TGF- β and Wnt signaling pathways in MC3T3-E1 cells.

Confirmation of differentially expressed genes expression by quantitative real-time polymerase chain reaction

We chose the candidate target genes from the bone metabolic signaling pathway. The candidate genes and their relative gene functions are listed in Figure 3a. Among those genes, *Sp1*, *Tgfbr1*, and *Rock1* belong to the TGF- β signaling pathway; *Bmpr1a* belongs to the BMP signaling pathway; *Fos* is from the osteoclast differentiation pathway; *Lrp6* is from the Wnt signaling pathway; and *Adralb* belongs to the calcium signaling pathway. The qRT-PCR results indicated that *Bmpr1a*, *Tgfbr1*, *Rock1*, *Lrp6*, and *Sp1* were downregulated by E2 treatment whereas *Fos* and *Adralb* were upregulated [Figure 3b-3h]. These results confirmed the differential expression after E2 treatment of the genes identified by RNA-seq.

Estrogen negatively regulated gene expression via estrogen receptor β

Estrogen is an important endocrine regulator of bone metabolism, which mediates its effects via ERs.^[14] ERs are transcription factors that bind ERs to activate or repress target gene expression.^[15] Two mammalian ERs have been identified, ER α and ER β .^[16] Previous studies revealed that estrogen represses *SOST* expression through ER β .^[17] To investigate if the differential gene expression in the E2-treated MC3T3-E1 cells was mediated through ER β , we analyzed the *Bmpr1a* and *Tgfbr1* promoters using online bioinformatics software (<http://jaspar.binf.ku.dk/>). The results identified three EREs and two EREs in the *Bmpr1a* and *Tgfbr1* gene promoters, respectively [Figure 4a]. To determine if E2 repressed the candidate genes via a particular ER, we treated MC3T3-E1 cells with 10 nmol/L E2, then added the ER α antagonist MPP or the ER β antagonist PTHPP. We then performed qRT-PCR to investigate candidate gene expression. We found that E2 treatment significantly downregulated *Bmpr1a* and *Tgfbr1*, compared to their expression in the DMSO control groups, in the MC3T3-E1 cells. MPP did

not rescue the E2-mediated downregulation of *Bmpr1a* and *Tgfbr1*. However, PTHPP treatment rescued the downregulation of *Bmpr1a* and *Tgfbr1* [Figure 4b and 4c]. Rescue of the candidate target gene expression by PTHPP, but not MPP, revealed that estrogen negatively regulates gene expression via ER β .

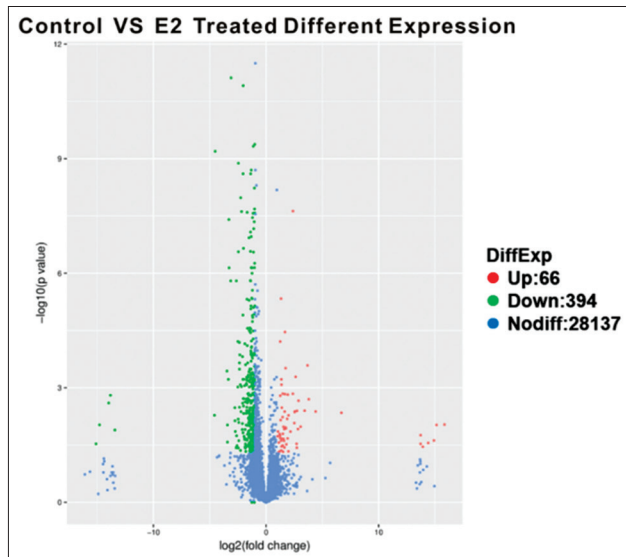


Figure 2: The RNA-seq identified DEGs. The screening criteria to identify significant differences in the expression of genes are $|\log_2\text{Ratio}| \geq 1$ and $q \leq 0.05$. RNA-seq: RNA sequencing; DEGs: Differentially expressed genes.

E2 increased estrogen receptor β binding to the EREs of the *Bmpr1a* and *Tgfbr1* promoters in MC3T3-E1 cells

We next addressed the potential functionality of the EREs located in the *Bmpr1a* and *Tgfbr1* promoters. We performed ChIP experiments using an ER β antibody to determine if ER β bound to the EREs in the *Bmpr1a* and *Tgfbr1* promoters. We found that ER β bound to ERE1 and ERE3, but not ERE2, in the *Bmpr1a* promoter [Figure 5a]. Both of the EREs located in the *Tgfbr1* promoter were bound by ER β [Figure 5b]. To investigate if E2 treatment affected the binding of ER β in the *Bmpr1a* and *Tgfbr1* promoters, we treated the MC3T3-E1 cells with E2 for 72 h, then performed ChIP, followed by qRT-PCR. E2 treatment increased ER β binding to ERE1 and ERE3 in the *Bmpr1a* promoter [Figure 5c] and ERE1 and ERE2 in the *Tgfbr1* promoter [Figure 5d]. These data demonstrated that ER β can bind to the *Bmpr1a* and *Tgfbr1* promoters and that E2 increased ER β binding to the EREs of the *Bmpr1a* and *Tgfbr1* promoters in MC3T3-E1 cells.

DISCUSSION

E2, a synthetic, exogenous estrogen that is functionally similar to endogenous estrogen, is commonly used to study the function of estrogen. MC3T3-E1 cells are a newborn mouse calvarias cell line, which is commonly used in the study of osteoblasts. ER α and ER β have also been identified in this cell line, so MC3T3-E1 can be used to study the role of estrogen in the regulation of osteoblast function.

Table 1: GO enriched by differentially expression genes

GO ID	GO-term	Hits	t	P
GO:0065007	Biological regulation	216	2.787	0.00455
GO:0008152	Metabolic process	180	3.098	0.00234
GO:0032502	Developmental process	118	3.135	0.00265
GO:0048856	Anatomical structure development	103	3.191	0.00012
GO:0048731	System development	83	3.113	0.00265
GO:0050896	Response to stimulus	109	2.812	0.00326
GO:0030154	Cell differentiation	73	3.206	0.00174
GO:0007154	Cell communication	73	2.744	0.00354
GO:0010468	Regulation of gene expression	98	3.175	0.00104
GO:0007165	Signal transduction	62	3.231	0.00278

GO: Gene ontology.

Table 2: KEGG pathway enriched by differentially expression genes

Pathway ID	Pathway	Hits genes	P	t
ko05200	Pathways in cancer	<i>Kras, Cul2, App11, Chuk, Hif1a, Msh2, Mecom, Birc2, Ccna2, Fgf5, Fos</i>	0.00176	4.025
ko04350	TGF-beta signaling pathway	<i>Sp1, Inhba, Tgfbr1a, Rbl1, Rock1, Sp1, Bmpr1a</i>	0.00480	4.325
ko04010	MAPK signaling pathway	<i>Kras, Chuk, Rasa1, Tgfbr1a, Mecom, Rasa2, Fgf5, Fos</i>	0.01030	3.169
ko04510	Focal adhesion	<i>Pdpk1, Vav3, Arhgap5, Birc2, Rock1, Actn2</i>	0.01145	3.148
ko04310	Wnt signaling pathway	<i>Lrp6, Vangl2, Rock1</i>	0.01264	4.751
ko04064	NF- κ B signaling pathway	<i>Chuk, Birc2</i>	0.01721	5.462
ko04380	Osteoclast differentiation	<i>Chuk, Fos, Tgfbr1a</i>	0.03135	3.198
ko04810	Cell cycle	<i>Atr, Smc1a, Rbl1, Ccna2, Stag1</i>	0.04867	2.765

KEGG: Kyoto encyclopedia of genes and genomes; TGF: Transforming growth factor; MAPK: Mitogen-activated protein kinase; NF- κ B: Nuclear factor-kappaB.

Gene ID	Gene name	Regulated	Relative Function	Pathway
ENSMUST0000001326	<i>Sp1</i>	Down	Transcription factor binding	TGF-beta signaling pathway
ENSMUST00000049005	<i>Bmpr1a</i>	Down	BMP receptor activity	BMP signaling pathway
ENSMUSG00000007613	<i>Tgfb1</i>	Down	Component of the TGF-beta signaling pathway	TGF-beta signaling pathway
ENSMUST00000067947	<i>Rock1</i>	Down	Rho GTPase binding	TGF-beta signaling pathway
ENSMUSG00000021250	<i>Fos</i>	Up	osteosarcoma oncogene	Osteoclast differentiation
ENSMUSG00000030201	<i>Lrp6</i>	Down	low density lipoprotein receptor gene family	Wnt signaling pathway
ENSMUSG00000050541	<i>Adra1b</i>	Up	G protein-coupled receptor superfamily	Calcium signaling pathway

a

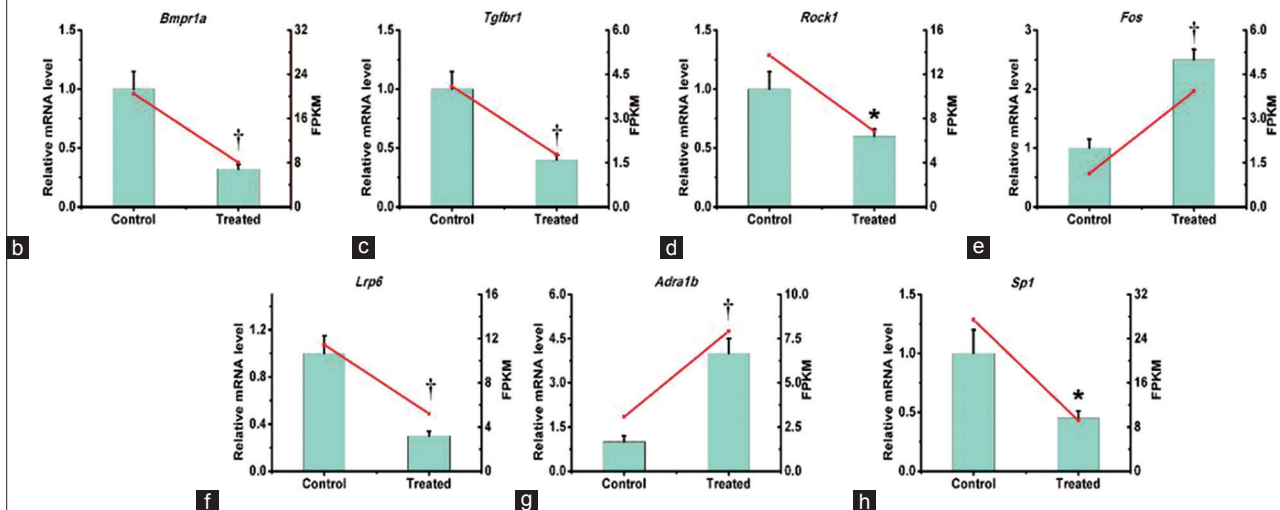


Figure 3: Confirmation of the mRNA levels of the DEGs by qRT-PCR. We treated MC3T3-E1 cells with 10 nmol/L E2, then extracted total RNA for qRT-PCR. Three independent experiments were conducted. The candidate genes and their relative gene functions are listed (a). The qRT-PCR results indicated that *Bmpr1a* (b), *Tgfb1* (c), *Rock1* (d), *Lrp6* (f), and *Sp1* (h) were downregulated by E2 treatment, whereas *Fos* (e) and *Adra1b* (g) were upregulated. Student's *t*-test was performed. * $P < 0.05$; † $P < 0.01$. DEGs: Differentially expressed genes; qRT-PCR: Quantitative real-time polymerase chain reaction.

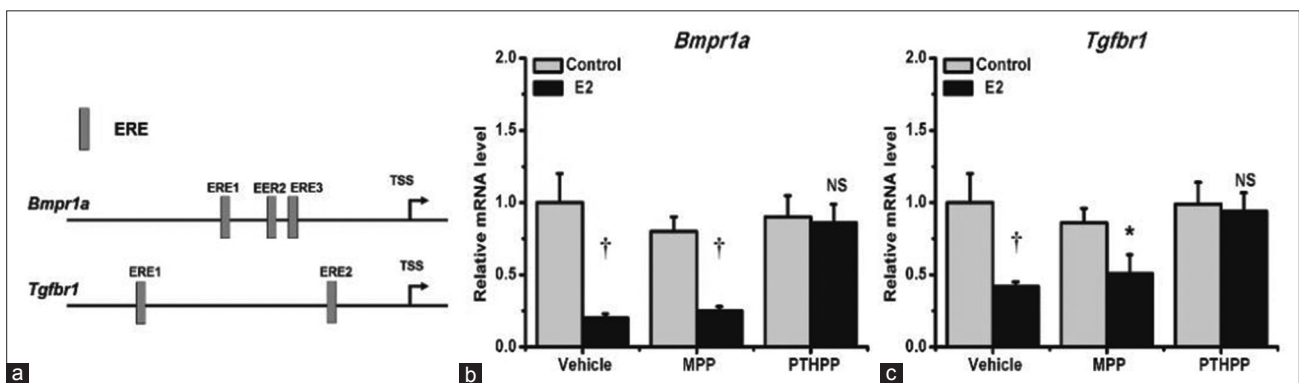


Figure 4: Estrogen negatively regulated gene expression via ER β . The promoter analysis of *Bmpr1a* and *Tgfb1* (a). MC3T3-E1 cells were treated with 10 nmol/L E2, and 0.1 μ mol/L MPP or 0.1 μ mol/L 4-(2-phenyl-5,7-bis[trifluoromethyl] pyrazolo[1,5-a]pyrimidin-3-yl), the candidate target mRNA level was quantified via qRT-PCR. The expression level of *Bmpr1a* under different treated (b). The expression level of *Tgfb1* under different treated (c). Student's *t*-test was performed. * $P < 0.05$; † $P < 0.01$. NS: No significant; qRT-PCR: Quantitative real-time-polymerase chain reaction; ER: Estrogen receptor.

We found that estrogen increases the rates of proliferation and apoptosis in MC3T3-E1 cells. We used CCK-8 and TUNEL assays to investigate the proliferation and apoptosis of MC3T3-E1 cells after E2 treatment. We found no significant differences in the groups treated

with low concentrations of E2 after three days. However, high concentrations of E2 promoted the proliferation and apoptosis of MC3T3-E1 cells. E2-mediated increases in the proliferation of MC3T3-E1 cells have been reported in the previous studies,^[18] but we also found a slight but statistically

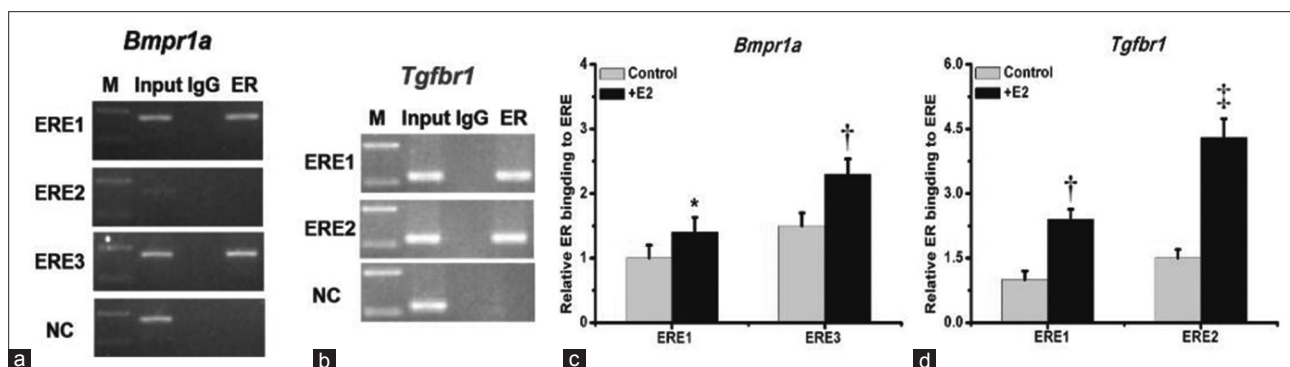


Figure 5: E2 increased ER β binding to the EREs located in the *Bmpr1a* and *Tgfr1* promoters in MC3T3-E1 cells. Ch-IP experiments were performed in the MC3T3-E1 cells use ER β antibody. ER β binding to ERE1 and ERE3 not ERE2 located in *Bmpr1a* promoter (a). Both of the EREs located in the *Tgfr1* promoter were bound by ER β (b). MC3T3-E1 cells were treated with 10 nmol/L E2 for 72 h, then performed ChIP, followed by qRT-PCR. E2 treatment increased ER β binding to ERE1 and ERE3 in the *Bmpr1a* promoter (c), and increased ER β binding to ERE1 and ERE2 in the *Tgfr1* promoter (d). Student's *t*-test was performed. * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$. qRT-PCR: Quantitative real-time polymerase chain reaction; ER: Estrogen receptor; ERE: Estrogen response element.

significant increase in the number of apoptotic cells in our high-concentration group, which indicated that treatment with E2 for three days was slightly toxic to MC3T3-E1 cells.

Estrogen affects many signaling pathways that are associated with bone metabolism. In both transcriptome libraries, these pathways can be identified by GO enrichment and KEGG pathway analysis of the DEGs. After E2 treatment of the MC3T3-E1 cells, cell differentiation- and cell cycle-related genes were significantly enriched among the DEGs. The involvement of these pathways can explain why E2-treated MC3T3-E1 cells undergo increased cell proliferation and apoptosis. KEGG signaling pathway analysis indicated that genes in the Wnt, MAPK, and calcium signaling pathways, and osteoclast differentiation genes were significantly enriched among the DEGs. The enrichment of genes in these bone metabolic pathways among the DEGs demonstrated osteoblast-like differentiation of the MC3T3-E1 cells.

However, in our sequencing results, in addition to these signaling pathways, we found that the TGF- β signaling pathway and cancer-related signaling pathways were also enriched, in contrast with the research of Zhen *et al.* The TGF- β signaling pathway plays an important role in bone differentiation, especially in osteogenesis.^[19] The TGF- β superfamily of ligands includes BMPs, growth and differentiation factors, anti-müllerian hormone, activin, nodal, and the TGF- β proteins.^[20] Signaling begins with the binding of a TGF- β superfamily ligand to a TGF- β type II receptor.^[21] The type II receptor is a serine/threonine receptor kinase, which catalyzes the phosphorylation of the type I receptor.^[20] Each class of ligand binds to a specific type II receptor. In mammals, there are seven known type I receptors and five type II receptors.^[20] In our sequencing results, we found that *Tgfr1* and *Bmpr1a* levels were significantly reduced in MC3T3-E1 cells after E2 treatment, and qRT-PCR confirmed this result. *Tgfr1* is also called activin receptor-like kinase receptor (*Alk5*),^[22] ALK5 protein is strongly expressed in perichondrial progenitor cells for osteoblasts and in a thin chondrocyte layer located

adjacent to the perichondrium in the peripheral cartilage.^[23] Conditional knockout of the TGF- β type I receptor *Alk5* in skeletal progenitor cells resulted in growth plates that had an abnormally thin perichondrial cell layer, as well as reduced proliferation and differentiation of osteoblasts.^[24]

The BMP receptors are a family of transmembrane serine/threonine kinases that include the type I receptors *Bmpr1a* and *Bmpr1b*, and the type II receptor *Bmpr2*.^[20] Deletion of the receptor *Bmpr1a* in the osteoblast lineage cells with a *Dmp1-Cre* caused a dramatic increase in trabecular bone mass in postnatal mice, which was due to a marked increase in osteoblast numbers. Another study revealed that conditional deletion of *Bmpr1a* in differentiated osteoclasts negatively regulates osteoclast differentiation.^[25] However, osteoblast-specific deletion of *Bmpr1a* resulted in increased bone volume with marked decreases in bone formation rate (BFR) in tibias at 8 weeks of age.^[26] Thus, physiological *Bmpr1a* signaling in bone exerts a dual function in both restricting preosteoblast proliferation and promoting osteoblast activity. The impact of decreased *Tgfr1* and *Bmpr1a* expression on MC3T3-E1 cell differentiation or increasing osteoclast activity must be further explored.

Estrogen inhibits the expression of *Tgfr1* and *Bmpr1a* through ER β . Previous studies have also shown that estrogen can inhibit the expression of *SOST* through ER β .^[17] To confirm that estrogen inhibits the expression of *Tgfr1* and *Bmpr1a* through ER β , we used ER α - and ER β -specific inhibitors and found that only the ER β inhibitor rescued the expression of *Tgfr1* and *Bmpr1a*. In addition, our ChIP experiment also demonstrated that ER β binds to the *Tgfr1* and *Bmpr1a* promoters. In mouse lung cancer tissue, estrogen was found to inhibit the expression of *Bmpr2* through ER α .^[27] Estrogen also inhibits the activity of the TGF- β signaling pathway by promoting Smad2/3 degradation.^[28] In another study, estrogen replacement decreases the accumulation of TGF- β 1 on vascular smooth muscle cell (VSMC).^[29] In this study, we found that the

ER signaling pathway and the TGF- β signaling pathway participate in crosstalk, and ER β can inhibit *Tgfb1*. Therefore, estrogen can downregulate the expression of *Tgfb1* and *Bmpr1a* in MC3T3-E1 cells.

In conclusion, Estrogen can affect bone metabolism through several bone metabolism-related signaling pathways. In MC3T3-E1 cells, estrogen appears to affect bone by negatively regulating *Tgfb1* and *Bmpr1a* expression. Our research provides a new understanding of the mechanism by which estrogen acts on osteoblasts.

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Conflicts of interest

There are no conflicts of interest.

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雌激素通过雌激素受体 β 抑制MC3T3-E1细胞Tgfbr1和Bmpr1a基因的表达

摘要

背景: 雌激素作为人体生理过程中重要的激素,与骨代谢密切相关。本研究利用MC3T3-E1作为细胞模型,进一步研究雌激素对骨代谢的影响。

方法: 分别用0.01、0.1、1和10 nmol/L浓度的 β -雌二醇处理成骨细胞MC3T3-E1,并设对照组;然后利用CCK-8试剂盒、TUNEL试剂盒检测不同浓度下MC3T3-E1细胞的增值和凋亡状况;选用10 nmol/L β -雌二醇的处理组,与对照组构建两个转录组库,进行RNA深度测序分析;利用qRT-PCR验证差异表达的基因,使用雌激素受体抑制剂分别处理MC3T3-E1细胞,检测Tgfbr1和Bmpr1a两个基因的表达。启动子分析软件分析Tgfbr1和Bmpr1a基因,ChIP实验验证雌激素受体 β 对Tgfbr1和Bmpr1a启动子的结合作用。

结果: 在低浓度(0.01 nmol/L和0.1 nmol/L)的处理组中,在细胞的形态、细胞的增值和细胞的凋亡等细胞行为方面相对于DMSO对照组都没有产生明显的差异($P>0.05$);在1 nmol/L和10 nmol/L高浓度 β -雌二醇处理组中,细胞的凋亡和细胞的增值活性增强,1 nmol/L处理组有明显差异($P<0.05$),并且在10 nmol/L处理组出现了极其显著的差异($P<0.01$)。深度测序发现10 nmol/L处理组中有460个差异表达基因,在这些差异表达基因中,上调的基因有66个,下调的基因有394个。利用这些差异表达基因,本研究进行GO分类和pathway分析,发现许多骨代谢相关的生物过程和细胞信号通路。在这些被富集的信号通路中,包括经典的TGF- β 信号通路,Wnt信号通路,MAPK信号通路。差异表达基因qRT-PCR验证显示,10 nmol/L处理组中骨代谢通路相关基因Tgfbr1和Bmpr1a表达明显下降($P<0.05$)。雌激素受体 β 参与了雌激素对Tgfbr1和Bmpr1a基因的抑制性作用,生物信息学分析发现,在Tgfbr1的启动子区域存在3个雌激素受体应答原件,在Bmpr1a的启动子区域有2个雌激素受体应答原件。ChIP实验证明,雌激素能够增强雌激素受体 β 对Tgfbr1和Bmpr1a基因的绑定性作用。

结论: 雌激素能同时促进成骨细胞凋亡、增值,其机制可能是TGF- β 、Wnt、MAPK及NF-kappaB骨代谢相关信号通路共同作用结果;雌激素通过雌激素受体 β 抑制Tgfbr1和Bmpr1a基因的表达,影响MC3T3-E1成骨细胞代谢。