Icariin stimulates osteogenic differentiation and suppresses adipogenic differentiation of rBMSCs via estrogen receptor signaling

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Abstract. Icariin (ICA) is a major active ingredient in *Herba epimedii*, which is commonly used as a Chinese herbal medicine for the treatment of osteoporosis. Previous studies have revealed that ICA exerted a protective effect against bone loss and increased bone regeneration; however, the association between ICA and estrogen receptor (ER) signaling remains unclear. The aim of the present study was to determine the effect of ICA on rat bone marrow stromal cells (rBMSCs). Cell Counting Kit-8 assays were conducted to measure proliferation, alkaline phosphatase (ALP) activity was evaluated to assess osteoblast differentiation, and reverse transcription-quantitative polymerase chain reaction as well as western blotting were performed to detect the expression of cellular and molecular markers of osteogenic or adipogenic differentiation. The results demonstrated that treatment of rBMSCs with 10^{-6} M ICA stimulated rBMSC proliferation and ALP activity. Furthermore, ICA treatment increased the expression of the osteogenic markers runt-related transcription factor 2, collagen type 1 and bone morphogenetic Protein 2; however, it also decreased the expression of the adipogenic differentiation markers peroxisome proliferator-activated receptor gamma and CCAAT/enhancer-binding protein α. Treatment of rBMSCs with ICI182780, an ER antagonist, blocked the effects of ICA. Taken together, these findings indicated that ICA may stimulate osteoblast differentiation and inhibit adipogenic differentiation via the activation of the ER signaling pathway. Therefore, ICA has the potential to serve as a therapeutic alternative for the prevention and treatment of osteoporosis.

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Introduction

There is a dynamic balance between osteoblast and adipocyte differentiation of bone marrow stromal cells (BMSCs). Under the action of signaling pathways, BMSCs have the potential to differentiate into osteoblasts or adipocytes (1). Postmenopausal osteoporosis (PMOP) is a skeletal disorder that results from osteoclastic bone resorption outpacing osteoblastic bone formation, is induced by estrogen deficiency, and characterized by decreased bone mass with an increased risk of fragility fracture (2). Current drugs available to treat PMOP aim to decrease osteoclastic activity or increase osteoblastic activity. Although estrogen replacement therapy is commonly used for PMOP prevention and treatment, it increases the risk of breast cancer and venous thromboembolism, and therefore, the risks outweigh the beneficial anti-resorptive effects of estrogen therapy (3). Denosumab, an androgen deprivation therapy, has reported side effects including sexual dysfunction, fatigue and metabolic syndrome (4). Bisphosphonates, the first-line anti-resorptive drug, have also been used to treat PMOP (5). However, due to the high incidence of osteonecrosis of the jaw and atypical femoral fractures in patients administered oral bisphosphonates for PMOP, such drugs cannot be routinely administered to patients in clinic (6). Therefore, there is a great need to seek novel therapeutic strategies and/or natural therapies that stimulate an anabolic effect on osteoporotic bone in PMOP patients.

The process of rat BMSC (rBMSC) differentiation involves various signaling pathways including MAPK and Notch pathway. Recently, plant-derived phytochemicals have received attention for their ability to induce osteoblast and osteoclast activity. Phytochemicals with promising therapeutic potential for osteoporosis include isoflavones, stilbenes and flavonoids (7). Icariin (ICA) is a major active flavonoid

glycoside extracted from the Chinese herbal medicinal plant Epimedii, and it acts as a phytoestrogen. A study has shown that ICA protected against bone loss induced by ovariectomy or glucocorticoids (8). ICA treatment could increase osteogenic differentiation and bone formation of BMSCs in ovariectomized rat model of osteoporosis (9). In addition, ICA has recently been shown to play a vital role in the proliferation and osteogenic differentiation of rBMSCs and human BMSCs (10). Song *et al* (11) showed that ICA induced osteoblast differentiation through estrogen receptor (ER)-mediated activation of ERK and JNK signaling. Furthermore, this study showed that 17β-estradiol affects osteogenic and adipogenic differentiation through ER signaling (12). Therefore, we hypothesize that ICA may promote osteogenic differentiation of rBMSCs through an ER-mediated signaling pathway. ICI182780 (ICI) is a high affinity ER antagonist, which also acts as an agonist of the membrane-bound G-protein coupled ER (GPER).

In the experiments presented here, we used ICI as an ER antagonist. We investigate the effect of ICA on both osteogenic and adipogenic differentiation in rBMSCs, to further explore whether ICA regulates differentiation of rBMSCs via ER signaling.

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Gene	Sequence (5'-3')
ERα	Forward: CATCGATAAGAACCGGAGGA
	Reserve: TCTGACGCTTGTGCTTCAAC
ERβ	Forward: GAAGCTGAACCACCCAATGT
	Reserve: CCAATCATGTGCACCAGTTC
RUNX2	Forward: CCACCACTCACTACCACACG
	Reserve: GGACGCTGACGAAGTACTAT
BMP-2	Forward: GCCATCGAGGAACTTTCAGA
	Reverse: TGTTCCCGAAAAATCTGGAG
PPARγ	Forward: GGAATGCGTCATGAAAGGCG
	Reserve: GCGAACTTCAGTCCAGGTCA
C/EBPα	Forward: TTACAACAGGCCAGGTTTCC
	Reserve: CTCTGGGATGGATCGATTGT

ER, estrogen receptor; RUNX2, runt-related transcription factor 2; BMP-2, bone morphogenetic Protein 2; FABP, fatty acid-binding protein; C/EBPα, CCAAT/enhancer-binding protein α; PPARγ, peroxisome proliferator-activated receptor γ.

Materials and methods

Cell culture. rBMSCs were purchased from Cyagen Biological Sciences (RASMX-01001; Santa Clara, CA, USA), and cultured in α-MEM (Hyclone, Pittsburg, PA, USA) containing 10% fetal bovine serum (Tbd, China) in a humidified chamber with 5% CO_2 at 37˚C. The medium was replaced every three days.

Cell-counting kit-8 (CCK8) assay. rBMSCs were seeded into 96-well plates at a density of $5x10^3$ cells/well, then treated with different concentrations of ICA (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M). After culturing for 24 or 48 h, cell proliferation was measured using a CCK8 according to the manufacturer's instructions (Dojindo Laboratories, Kumamoto, Japan). Briefly, the culture medium was replaced with 100 μl α-MEM containing 10 μl CCK8, and the plates were incubated for 1 h at 37˚C. Absorbance was measured at 450 nm using a multi-well spectrophotometer (BioTek, Synergy H4).

Alkaline phosphatase (ALP) activity. rBMSCs were seeded into 6-well plates at a density of $2x10^3$ cells/well, and treated with different concentrations of ICA, as described in 2.2. Cells were treated with ICA for 3 and 7 days, and ALP activity assays were completed using ALP kits according to the manufacturer's instructions (Nanjing Jiancheng, Nanjing, China). Absorbance was measured at 520 nm using a multi-well spectrophotometer (BioTek, Synergy H4).

Alizarin red S staining. To determine calcium deposition, alizarin red staining was performed on day 7 of ICA treatment to evaluate the mineralized matrix. Cells were divided into control, 10^{-6} M ICA and β-estradiol (E2) treatment groups. After culturing for 7 days, the medium was removed, cells were washed three times with PBS and fixed in 95% ethanol for 10 min, then washed twice with PBS, and stained with 0.1% alizarin red at a pH of 7.2 (Sigma). After incubating for 5-10 min at room temperature, cells were again washed three times with PBS and examined via light microscopy.

Oil red O staining. rBMSCs were seeded into 6-well plates at a density of $2x10^3$ cells/well, and divided into control, 10^{-6} M ICA and adipocyte-induced treatment groups. After rBMSCs were cultured for 14 days, oil red O staining was used to evaluate adipocyte differentiation of rBMSCs. Cells were rinsed twice with PBS, fixed with 4% paraformaldehyde for 15 min, then rinsed twice more with PBS. treated with 60% isopropanol for 1 min, and infusion with oil red O (Sigma-Aldrich) that was dissolved in 60% isopropanol for 20 min. This process was followed by three rinses with PBS. and then photographed.

Western blot analysis. rBMSCs were seeded into 10-cm culture dishes and divided into control, ICA, ICI and ICI+ICA treatment groups. Total protein was extracted with RIPA lysis buffer (P0013B, Beyotime, Haimen, China). Total protein was separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were washed in TBST, blocked with 5% skim milk and incubated overnight with anti-ERα (1:1,000, ab133467, Abcam, USA) and anti-ERβ (1:1,000, ab32063, Abcam), anti-RUNX2 (1:1,000, 12556S, Cell Signaling Technology, Danvers, MA, USA), anti-BMP-2 (1:1,000, ab14933, Abcam), anti-PPARγ (1:1,000, C26H12, Cell Signaling Technology), anti-CEBP/α (1:1,000, 2295, Cell Signaling Technology). Anti-GAPDH (1:1,000, D16H11, Cell Signaling Technology) was used as a loading control. The following day, membranes were incubated with anti-rabbit IgG secondary antibody (1:4,000, 7074P2, Cell Signaling Technology). Immunoreactive bands were visualized with an electrochemiluminescence reagent (5312-1; DOCLAB, Guangzhou, China) and quantified using Image J (NIH).

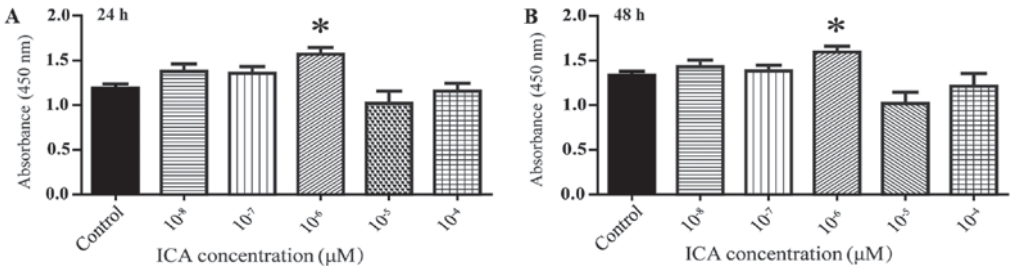

Figure 1. Effects of different concentrations of ICA on rBMSCs activity. rBMSCs were treated with different concentrations of ICA for (A) 24 or (B) 48 h. Data are presented as the mean ± standard deviation (n=3). *P<0.05 vs. control. ICA, icariin; rBMSCs, rat bone marrow stromal cells.

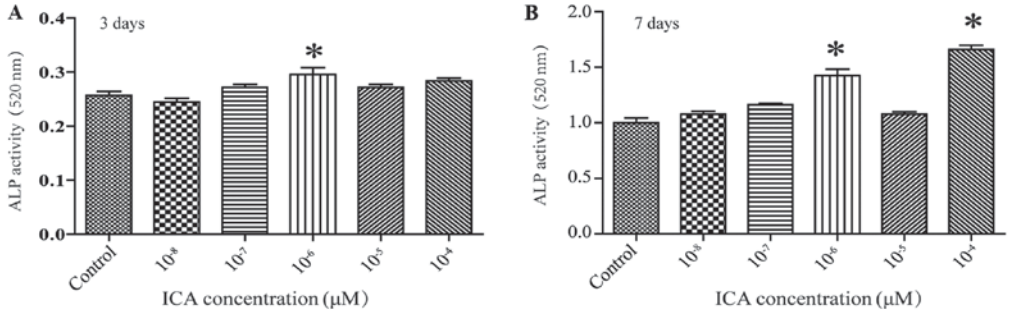

Figure 2. Effects of different concentration of ICA on ALP activity. Rat bone marrow stromal cells were treated with different concentrations of ICA for (A) 3 or (B) 7 days. Data are presented as the mean ± standard deviation (n=3). *P<0.05 vs. control. ICA, icariin; ALP, alkaline phosphatase.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). rBMSCs were seeded into 60-mm culture dishes, and divided into four groups, as aforementioned. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from mRNA using reverse transcriptase (AK4001; Takara, Shiga, Japan). After predenaturation at 95˚C for 2 min, 40 RT-qPCR cycles were performed (95˚C for 10 sec; and 60˚C for 30 sec), followed by a final extension at 72˚C for 10 min. Target gene expression was calculated using the formula $2^{-\Delta\Delta Cq}$ (13). Gene expression was normalized to *GAPDH*. Gene primer sequences are shown in Table I.

Statistical analysis. Data were collected from three separate experiments and expressed as the mean ± standard deviation. The statistical differences were analyzed by one-way analysis of variance with Least Significant Difference and Student-Newman-Keuls post hoc tests using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of different ICA concentrations on rBMSCs activity. CCK8 kits were used to measure cell activity. compared to control, treatment of rBMSCs with 10^{-6} M ICA resulted in significantly increased cell activity at 24 and 48 h (Fig. 1; *P<0.05).

ICA promotes osteogenic differentiation of rBMSCs. ALP is a glycoprotein associated with the formation of calcified tissue, and it is the most widely recognized marker of the osteoblast phenotype. ALP activity is partially indicative of osteogenic differentiation. Treatment of cells with 10^{-6} M ICA resulted in an increase in ALP activity by day of 3 of treatment, and this increase persisted through day 7 of treatment. (Fig. 2; *P<0.05). ALP activity was also increased by 10^{-4} M ICA on the seventh day (Fig. 2; *P<0.05). These results, together with those detailed in section 3.1, led us to choose a concentration of 10^{-6} ICA for the following experiments.

ICA promotes osteogenic differentiation. The number of mineralized nodules can serve as measure of osteogenic differentiation. Compare to control, the number of mineralized nodules was increased in cells treated with ICA, and was also increased cells treated with E2 (Fig. 3A-C). Fat droplets were identified by oil red O staining, and there was a reduction in the number and size of fat droplets in cells treated with ICA. (Fig. 3D-F; *P<0.05).

ICA promotes the expression of osteogenic differentiation marker proteins and inhibits the expression of adipose differentiation marker proteins. Runt-related transcription factor 2 (RUNX2) and collagen type 1 (COL1) are markers of osteogenic differentiation, whereas peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding protein alpha (C/EBPα) are important indicators of adipose differentiation. Compare with control group, the expression levels of ERα and ERβ proteins were significantly decreased with ICI treatment (Fig. 4). Treatment of cells with ICA, resulted in a significant decrease in PPARγ and C/EBPα protein expression, and a significant increase in ERα, ERβ, RUNX2 protein expression. However, compare to ICA treatment alone, when combined treatment of ICI+ICA, resulted in a significant increase in PPARγ protein expression and a significant decrease in RUNX2 and COL1 protein expression.

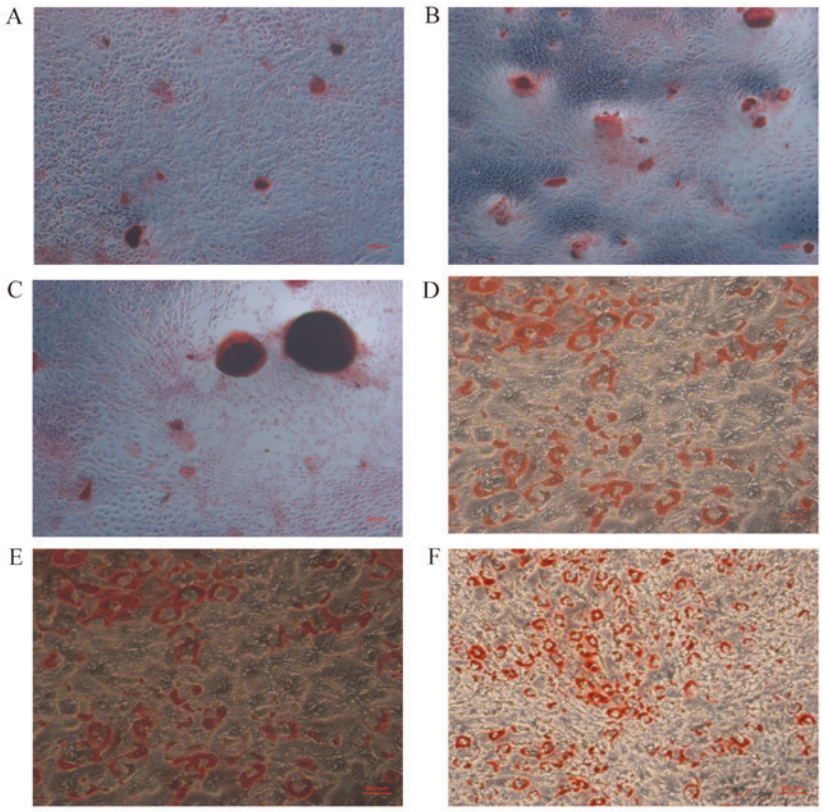

Figure 3. Alizarin red and Oil red O staining. Alizarin red staining of (A) control, (B) 10^{-6} M ICA and (C) E2 treated cells. Oil red O staining of (D) control, (E) 10^{-6} M ICA, and (F) adipocyte-induced cells (magnification, x200). ICA, icariin

While C/EBPα protein expression was not significantly different (Fig. 4; *P>0.05).

ICA promotes the expression of osteoblast-specific genes and inhibits the expression of adipose-specific genes. Treatment of cells with ICI resulted in a significant decrease in *ERα* and *ERβ* gene expression, (Fig. 5); treating cells with ICA resulted in a significant increase in the gene expression of bone morphogenic protein-2 (*BMP-2*) and *runx2* compared to control (Fig. 5). These genes expression changes are in accordance with the changes of protein expression detected with Western-blot, suggesting that ICA stimulates osteogenic differentiation of rBMSCs by upregulating runx2 and bmp-2 expression. In addition, *pparγ* and *c/ebpα* mRNA expression were decreased following treatment with ICA, suggesting that ICA acts to inhibit adipogenic differentiation. However, compared with ICA treatment, *pparγ* and *c/ebpα* were significantly increased, whereas *bmp-2* and *runx2* expression were significantly decreased with combined treatment of ICI+ICA. (Fig. 5; *P<0.05).

Discussion

ER regulates physiological functions in almost all tissues in both males and females. Recent studies have shown that ER signaling plays an important role in many bone metabolism diseases (14). The classic ERs include ERα and ERβ. ERα is predominantly expressed in cortical bone, whereas ERβ shows higher levels of expression in cancellous bone (15). ERα signaling activation regulates matrix mineralization *in vitro*, and its deficiency may lead to osteoporosis (16), ERβ signaling activation induces osteogenic differentiation of MC3T3-E1 cells and upregulates the expression of the osteogenesis-related factors including bone GLa protein and osteopontin. Previous study has indicated that ER signaling could regulate osteogenic and adipogenic differentiation (12). Therefore, in this study, we used ICI as an ER antagonist to determine whether ICA promotes osteogenic differentiation and inhibits adipogenic differentiation in an ER-dependent fashion. We demonstrated that ICI significantly downregulated ERα and ERβ protein and gene expression, suggesting that ICI successfully blocks ER-mediated signaling.

Bone metabolism is a balance between osteoblast-driven bone formation and osteoclast-mediated bone resorption. Osteogenic differentiation and adipogenic differentiation of rBMSCs are mutual inhibitory processes (11). Previous studies have demonstrated that ICA promotes osteogenic differentiation of MC3T3-E1 cells (17) and rBMSCs (18). Treatment with 10^{-6} M ICA significantly increased rBMSCs activity, demonstrating that ICA promotes rBMSCs proliferation. Alizarin red S staining was used to visualize mineral deposition, and adipocyte accumulation was assessed with oil red O staining. The number of mineralized nodules was significantly increased with ICA treatment; while the number of fat droplets was significantly decreased, suggesting that ICA induces osteogenic differentiation but inhibits adipogenic differentiation. ALP is an early marker of osteoblast differentiation, and a peak in ALP activity is indicative of osteogenic differentiation (19). Treatment of 10^{-6} M ICA significantly increased ALP activity by the third day of treatment, and ALP activity remained elevated on the seventh day of treatment. These

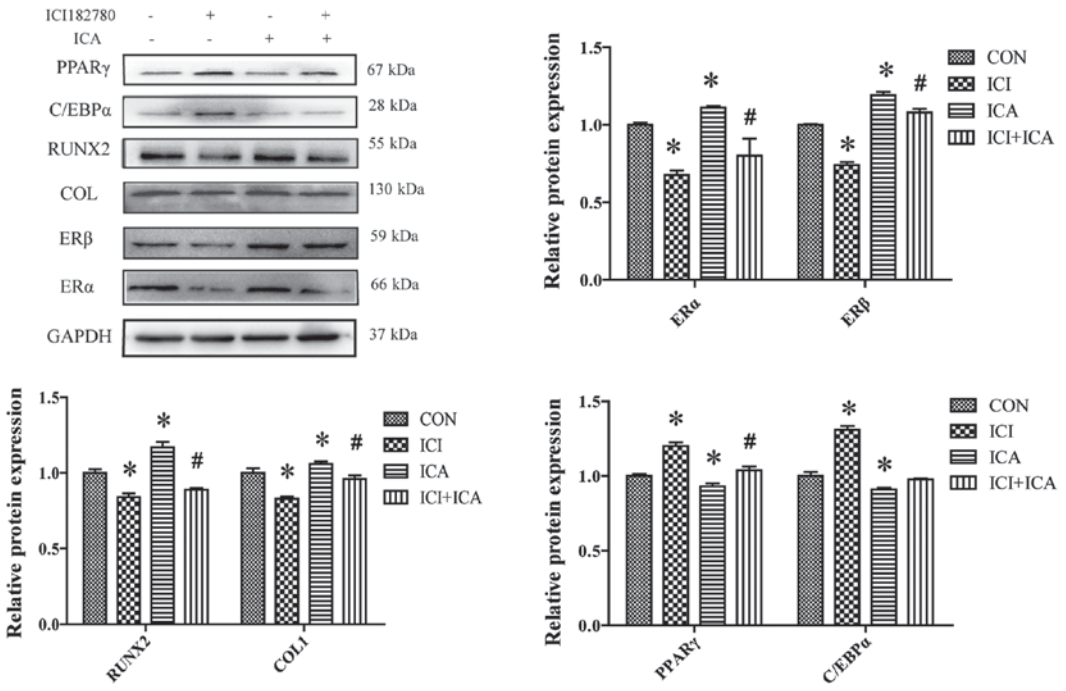

Figure 4. Western blot analysis of ERα, ERβ, RUNX2, COL1, C/EBPα and PPARγ protein expression in rBMSCs treated with ICA and/or ICI. ICA treatment resulted in the downregulation of C/EBPα and PPARγ expression, and upregulation RUNX2 and COL1 expression. All of the observed effects were blocked by ICI treatment, except for C/EBPα expression. Data are presented as the mean ± standard deviation (n=3). *P<0.05 vs. control; #P<0.05 vs. ICA. ICA, icariin; ICI, ICI182780; ER, estrogen receptor; RUNX2, runt-related transcription factor 2; COL1, collagen type 1; PPARγ, peroxisome proliferator-activated receptor-γ; C/EBPα, CCAAT/enhancer-binding protein α; rBMSCs, rat bone marrow stromal cells; CON, control.

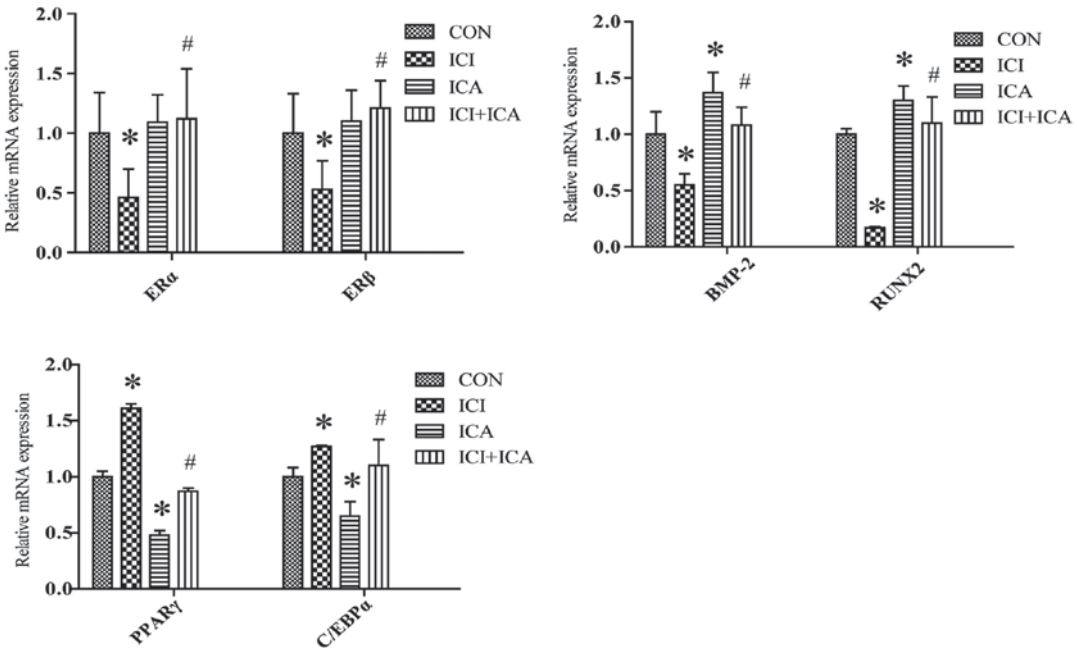

Figure 5. Reverse transcription-quantitative polymerase chain reaction analysis of *ERα, ERβ, RUNX2, BMP-2, C/EBPα* and *PPARγ* gene relative expression in rat bone marrow stromal cells treated with ICA or ICI. Data are presented as the mean ± standard deviation (n=3). *P<0.05 vs. control; #P<0.05 vs. ICA. ICA, icariin; ICI, ICI182780; ER, estrogen receptor; RUNX2, runt-related transcription factor 2; BMP-2, bone morphogenetic Protein 2; PPARγ, peroxisome proliferator-activated receptor-γ; C/EBPα, CCAAT/enhancer-binding protein α.

findings correspond to those from a previous study, which showed that ICA stimulated osteogenic differentiation (20) and inhibited adipocyte differentiation of rBMSCs (21).

RUNX2 acts as a scaffold for nucleic acids and regulatory factor involved in skeletal gene expression, and shows significantly increased expression in osteogenic differentiation (22); RUNX2 along with COL1, another important molecular marker of bone formation and bone remodeling, represent the foundation for matrix mineralization and have been shown to have increased expression or synthesis during

osteogenic differentiation (23). BMP-2 plays a pivotal role in growth and differentiation. Huang *et al* (24) demonstrated that BMP-2 overexpression significantly stimulated osteocalcin and ALP gene expression. ICA treatment significantly upregulated the protein expression of RUNX2 and COL1 and the gene expression of *runx2* and *bmp-2*. Together, these data suggest that ICA can promotes osteogenic differentiation. C/EBPα and PPARγ are the two molecules mostly likely to influence the regulation of adipocytes (25). In this study, the protein expression of PPARγ was significantly downregulated, as well as the genes expression of *pparγ* and *c/ebpα*, suggesting that treatment with ICA can inhibit the adipogenic differentiation of rBMSCs.

Our previous study has shown that ICA can improve osteoporosis via the Notch signaling pathway (21), as well as the MAPK signaling pathway has also been shown to be involved in ICA-mediated osteogenic differentiation (26). In the present study, we use ICI as an ER signaling pathway antagonist, to investigate the correlation between ICA and the ER signaling pathway. The results showed that ICA treatment significantly upregulate the expression of ERα and ERβ, suggesting that ICA may function by regulating ER signaling. Compared with ICA treatment alone, combined treatment with ICI+ICA resulted in a significant decrease in RUNX2 and COL1 protein expression, and a significant increase in PPARγ, but there is no change in C/EBPα protein expression. Furthermore, genes expression of runx2 and bmp-2 were also significantly decreased, while pparγ and c/ebpα were significantly with combined treatment increased. Together, these data suggest that the effects of ICA are blocked by ICI. Tao *et al* (27) have shown that prenylated flavonols can act as ER modulators. Taken together, these data demonstrate that ICA stimulates osteogenic differentiation and inhibits adipogenic differentiation via activation of ER signaling.

In conclusion, ICA promotes proliferation of rBMSCs, stimulates the osteogenic differentiation and mineralization of rBMSCs by regulating RUNX2, COL1 and BMP-2 expression, and inhibits adipogenic differentiation of rBMSCs by decreasing the expression of PPARγ and C/EBPα. These effects can be blocked by ICI, which suggests that ICA stimulates osteogenic differentiation and inhibits adipocyte differentiation via activation of ER signaling.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RZ and LY conceived and designed the present study. XyL and BP performed western blotting, reverse transcription-quantitative polymerase chain reaction and Alizarin S staining, and wrote the manuscript. YP completed CCK8 counting and data analysis. PW provided the cell line and performed cell culture. XtL and KS analyzed the western blotting data. LO and ZW performed the ALP activity experiments. XgL and HW conducted complete Oil red staining. HH, SM, YT, XP and XZ designed the structure of the article, performed the literature review, and critically revised the manuscript for important intellectual content.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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