Icariin promotes osteogenic differentiation of BMSCs by upregulating BMAL1 expression via BMP signaling

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Abstract. Increasing research has demonstrated that expression of brain and muscle ARNT-like 1 (BMAL1) and other circadian clock genes can be regulated by drugs and toxicants. We previously demonstrated that icariin, extracted from Herba Epimedii, sromotes osteogenic differentiation. However, the mechanism underlying the association between icariin and BMAL1 in osteogenic differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) remains unclear. The present study was designed with an aim to clarify the association between icariin and BMAL1 in osteogenic differentiation of BMSCs. The Cell Counting Kit-8 assay was used to evaluate cell proliferation. The expression of bone morphogenetic protein 2 (BMP2), RUNX family transcription factor 2 (RUNX2), alkaline phosphatase (ALP), osteocalcin (OC) and BMAL1 in BMSCs was evaluated by reverse transcription-quantitative PCR and western blotting. ALP and Alizarin red S (ARS) staining were also performed. Icariin promoted BMSC proliferation, and upregulated expression of osteogenic genes and BMAL1. In addition, expression of the osteogenic genes BMP2, RUNX2, ALP and OC were upregulated by BMAL1 overexpression. Furthermore, we confirmed that BMAL1 deficiency suppressed osteogenic differentiation in BMSCs. Finally, ARS staining of BMAL1$^{-/-}$ BMSCs revealed that BMAL1 was an essential intermediary in matrix mineralization during osteogenic differentiation. In conclusion, these results demonstrated that icariin promoted osteogenic differentiation through BMAL1-BMP2 signaling in BMSCs. The present study thus described a novel target of icariin that has potential applications in the treatment of osteogenic disorders.

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Introduction

Osteonecrosis of the femoral head (ONFH) is a debilitating and progressive disease which can lead to femoral head collapse and subsequent osteoarthritis. This disease is the major cause of total hip arthroplasty in young adults (1). It is reported that more than 1 million new patients are affected with this disease annually and the annual incidence is 15 to 20 million according to a nationwide survey (2). Accumulating evidence has alluded to several etiologic and pathogenic mechanisms for ONFH (3). The principal mechanism involves interruption of osteogenic differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) (4). Thus, investigation of the disordered mechanism of osteogenic differentiation in BMSCs and development of effective methods of prevention and early therapy are crucial.

A number of previous studies have reported that osteogenic differentiation-related genes are expressed periodically (5,6). Brain and muscle ARNT-like 1 (BMAL1) is the most important component of the molecular biological clock, expression of which has been found to have 24-h periodicity in bone. The circadian system rhythmically regulates cellular proliferation, physiology, and behavior (7,8). Moreover, it is implicated in drug efficacy, metabolism, detoxification and toxicity (9-11). The core transcriptional activator of the clock network is BMAL1 which is regulated by delicate systems in mammals (7). Increasing numbers of studies have demonstrated that BMAL1 and other circadian clock genes could be regulated by drugs and toxicants. Miranda *et al* (12) showed that resveratrol could reverse circadian disruption induced by a high-fat diet in rats. Chen *et al* (13) demonstrated that carbon tetrachloride altered circadian rhythms of liver clock genes in a mouse model of hepatic fibrosis, while Gabás-Rivera *et al* (14) reported that dietary oleanolic acid supplementation mediated circadian clock gene expression in an animal model.

Icariin is extracted from Herba Epimedii which is widely used as a major active ingredient to prevent osteonecrosis

induced by glucocorticoids in patients with severe acute respiratory syndrome. Our previous study revealed that icariin regulated cell proliferation and osteogenic differentiation in MC3T3-E1 cells (15) and acted as an effective ingredient for preventing the progression of ONFH induced by glucocorticoids in a rat model (16). However, the mechanism underlying the association between icariin and BMAL1 in osteogenic differentiation of BMSCs remains unclear.

In the present study, it was demonstrated that icariin could alter BMAL1 and induce osteogenic differentiation of BMSCs *in vivo*. Furthermore, it was also shown that icariin enhanced the bone morphogenetic protein 2 (BMP2)/RUNX family transcription factor 2 (RUNX2) signaling pathway through upregulation of BMAL1 expression.

Materials and methods

Cell culture and treatment. According to the method described by Gong *et al* (17), a total of 20 3-week-old female Sprague-Dawley rats (Laboratory Animal Center of Huazhong University of Science and Technology) were sacrificed by the intraperitoneal injection of 100 mg/kg sodium pentobarbital and BMSCs were extracted from femurs and tibias by aseptic manipulation. Cells were expanded in minimum essential medium (α-MEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin G-streptomycin and cultured in 5% CO2 at 37˚C. BMSCs at a density of 80-90% were passaged and cells at passage three to six were used in the following experiments. For osteogenic induction, basic medium was supplemented with 10^{-7} mol/l dexamethasone, 10^{-2} mol/l sodium β-glycerophosphate and 50 μg/ml L-ascorbic acid. The medium was changed every 3 days for BMSC differentiation. Each experiment was performed in triplicate. All the experimental protocols involving animals were approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology (IACUU no. S496; date of permission, October, 31, 2017).

Icariin (purity >98%; Abcam) was dissolved in dimethyl sulfoxide (DMSO) and then stored at -20˚C without light. Subsequent experiments used a DMSO concentration of ≤0.1%. All experiments were performed in triplicate.

Cell Counting Kit-8 (CCK-8) assay. The effect of icariin on cell proliferation was evaluated using the CCK-8 assay. Briefly, BMSCs ($5x10^3$ cells/well) were plated in 96-well plates and treated with icariin (10^{-10}-10^{-3} mol/l) for 48 h at 37˚C. Subsequently, cell proliferation was assessed using the CCK-8 reagent (Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's instructions, at a wavelength of 450 nm using an ELx800 multifunctional microplate reader (BioTek Instruments, Inc.). The concentration of icariin used to treat the cells that were analyzed by RT-qPCR and western blotting was based on the results of the CCK-8 assay.

Flow cytometry. After reaching confluence, BMSCs at passage 3-4 were used for flow cytometric analysis. First, the cells were incubated with trypsin for 1 min at 37˚C, and then the cell pellet was collected by transient centrifugation at 400 x g at room temperature for 5 min. Next, the cell pellet was washed with ice-cold PBS and then centrifuged at 400 x g at room temperature for 5 min. Subsequently, the cell pellet was resuspended in 100 μl ice-cold PBS supplemented with 0.5% BSA (Invitrogen; Thermo Fisher Scientific, Inc.) for 20 min at 4˚C. Additionally, the cells were treated with Rat Fc block (cat. no. 550271; BioLegend, Inc.) for 20 min at 4˚C. Finally, the cells were identified by negative expression of CD34 [phycoerythrin (PE)-labeled; cat. no. 128611; BioLegend, Inc.] and CD45 (FITC-labeled; cat. no. 202205; BioLegend, Inc.) and positive expression of CD73 (FITC-labeled; cat. no. 127219; BioLegend, Inc.), CD90 (PE-labeled; cat. no. 205903; BioLegend, Inc.) and CD105 (PE-labeled; cat. no. 120407; BioLegend, Inc.) using a FACSscan flow cytometer (Beckton Dickinson) and analyzed by FlowJo software (version X; FlowJo LLC).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted with TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) from BMSCs and the reverse transcription RT reaction (starting with 1 μg of total RNA) was performed using the EasyScript One-Step gDNA Removal and cDNA Synthesis Supermix (Beijing TransGen Biotech Co., Ltd.) according to the manufacturer's instructions. The SYBR Green/ROX RT-qPCR mix (Takara Bio, Inc.) and the StepOnePlus™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) were used for mRNA quantification, according to the manufacturer's protocol. The primer sequences used for PCR were as follows: BMAL1 forward, 5'-AACCTTCCCGCAGCTAACAG-3' and reverse, 5'-AGTCCTCTTTGGGCCACCTT-3'; $BMAL1^{-/-}$, forward 5'-CCACCAAGCCCAGCAACTCA-3' and reverse, 5'-TCGCCTTCTATGGCCTTCTTGACG-3'; BMAL1 (overexpression) forward, 5'-AGGTCGACTCTAGAGGATCCCGCCACCATGGCGGACCAGAGAATGGACATTTC-3' and reverse, 5'-TCCTTGTAGTCCATACCCAGCGGCCATGGCAAGTCACTAAAG-3'; BMP2 forward, 5'-AGGATGCTGGGAAGTCCG-3' and reverse, 5'-AGGTGCCACGATCCAGTCAT-3'; RUNX2, forward, 5'-AGCGGACGAGGCAAGAGTTT-3' and reverse, 5'-AGGCGGGACACCTACTCTCATA-3'; alkaline phosphatase (ALP) forward, 5'-CAAGGATGCTGGGAAGTCCG-3' and reverse, 5'-CTCTGGGCGCATCTCATTGT-3'; osteocalcin (OC) forward, 5'-TCAACAATGGACTTGGAGCCC-3' and reverse, 5'-AGCTCGTCACAATTGGGGTT-3'; and β-actin forward, 5'-GAGACCTTCAACACCCCAGC-3' and reverse, 5'-ATGTCACGCACGATTTCCC-3'. The reaction conditions were as follows: 95˚C for 15 sec, 60˚C for 20 sec and 75˚C for 10 sec after denaturing DNA templates at 95˚C for 10 min; 40 cycles. The $2^{-\Delta\Delta Cq}$ method (18) was used to determine the relative expression levels.

Western blotting. Total protein was extracted with RIPA buffer (Sigma-Aldrich; Thermo Fisher Scientific, Inc.) according to standard protocols. Cell lysates with 50 μg of total protein (quantified using the bicinchoninic acid assay) were separated by 10% SDS-PAGE, transferred onto PVDF membranes, and then blocked in TBST with 5% non-fat dried milk for 1 h at room temperature. The membranes were incubated with primary monoclonal mouse antibodies targeted against: BMAL1 (1:1,000; cat. no. ab93806; Abcam), BMP2 (1:1,000; cat. no. ab14933; Abcam), RUNX2 (1:1,000; cat. no. ab23981; Abcam), ALP (1:1,000; cat. no. ab95462; Abcam), OC (1:1,000;

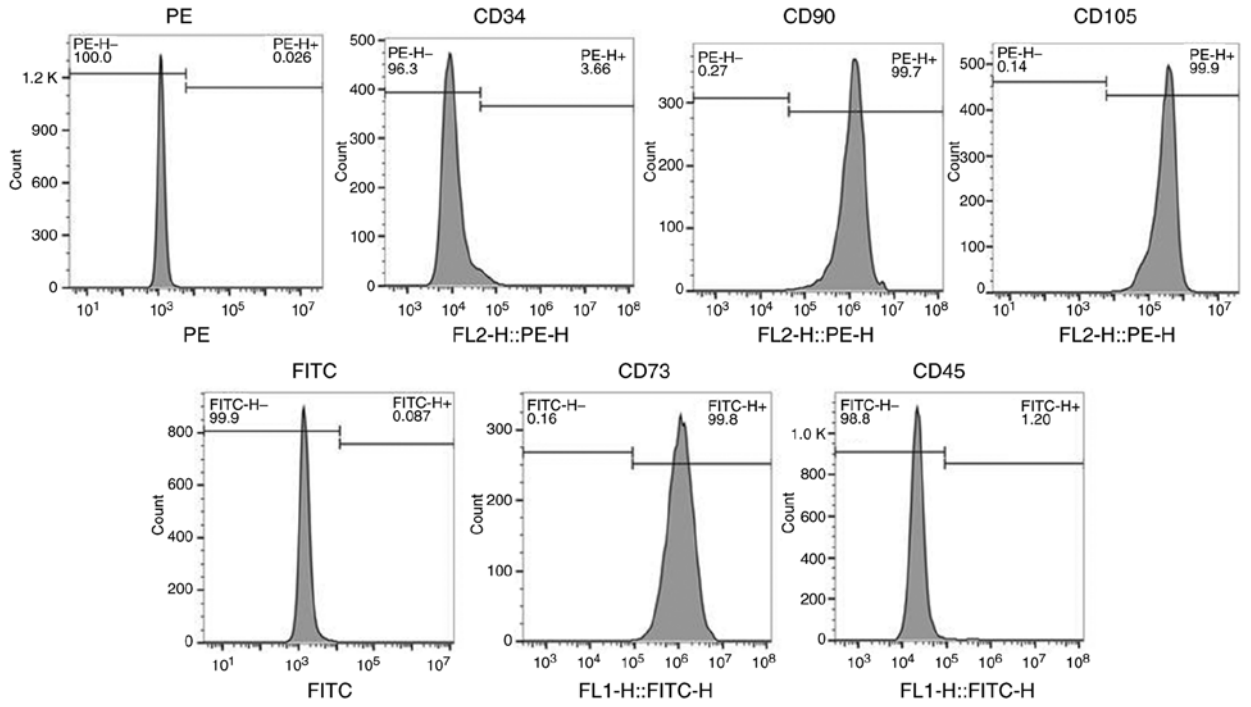

Figure 1. Flow cytometric analysis of cell surface markers of BMSCs. BMSCs were stained for cell surface markers. BMSCs were largely positive for CD73, CD90, CD105 and negative for CD34, CD45. BMSCs, bone marrow-derived mesenchymal stem cells; CD, cluster of differentiation.

cat. no. ab34710; Abcam) and β-actin (1:1,000; cat. no. ab8227; Abcam) at 4˚C overnight, and then followed by secondary antibodies conjugated with horseradish peroxidase (1:10,000; cat. no. ASP00001; Dako; Agilent Technologies, Inc.) for 1 h at room temperature after washing. The immunoblots were visualized and detected using the ECL system (Pierce; Thermo Fisher Scientific, Inc.). Protein expression was quantified using Fusion Solo software (version 4; Vilber Lourmat Deutschland GmbH).

Transient transfection and luciferase assay. BMAL1 short hairpin RNA (shRNA) interference lentiviral vector was constructed and synthesized by Shanghai Genechem Co., Ltd. The BMAL1 shRNA interference target sequence was 5'-ACA CGCAATAGATGGGAAA-3', and a scramble sequence 5'-TTCAAGATCCTCAATTATA-3' was used as a negative control. BMSCs were cultured in 6-well plates with specific media to appropriate confluence and then transfected with the virus assisted with 6 μg/ml polybrene for 12 h at 37˚C. The viral supernatants were removed and the culture was continued in complete medium. BMSCs were grown to 30-70% confluence and then transfected with BMAL1, BMAL1-shRNAs or empty vector overnight. The following day, the cells were co-transfected with pRL-SV40 *Renilla* luciferase reporter plasmids (10 ng; Promega Corporation) and firefly luciferase reporter vectors (50 ng), using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as the delivery system and a control. Cells were lysed after a 48-h transfection and firefly and *Renilla* luciferase activities were measured using the Dual Luciferase Reporter Assay system (Promega Corporation).

ALP and Alizarin red S (ARS) staining. BMSCs were seeded at a density of $1x10^5$ cells/well in 6-well plates and incubated with osteogenic medium. According to the manufacturer's instructions, ALP staining was assessed using an ALP staining kit (Nanjing Jiancheng Bioengineering Institute) on day 7.

After osteogenic induction for 14 days, the cells were washed three times with PBS and fixed in 4% paraformaldehyde for 30 min at room temperature. Then paraformaldehyde was removed and the cells were washed three times with ddH_2O. Finally, the cells were stained with 0.04 M ARS for 30 min at room temperature, and then rinsed twice with ddH_2O and visualized under a light microscope (magnification, x40). For quantitative analysis of ARS staining, the absorbance at 540 nm was measured using a UV spectrophotometer. Results of ARS are expressed as μg/mg protein with average and standard deviation.

Statistical analysis. Data are presented as the mean ± SD. Statistical analyses were performed using SPSS software (version 17.0; SPSS, Inc.). Differences between groups were analyzed using an unpaired Student's t-test or one-way ANOVA followed by Dunnett's post hoc test. $P<0.05$ was considered to indicate statistical significance.

Results

Identification of BMSCs using stem cell markers. Flow cytometry was performed to identify BMSCs by expression of CD34, CD45, CD73, CD90 and CD105 in the isolated cells. The percentages of CD73-positive cells, CD90-positive cells and CD105-positive cells were 99.8, 99.7 and 99.9%, respectively; while the percentages of CD34-positive cells and CD45-positive cells were 3.66 and 1.2%, respectively, indicating that the isolated cells were BMSCs (Fig. 1).

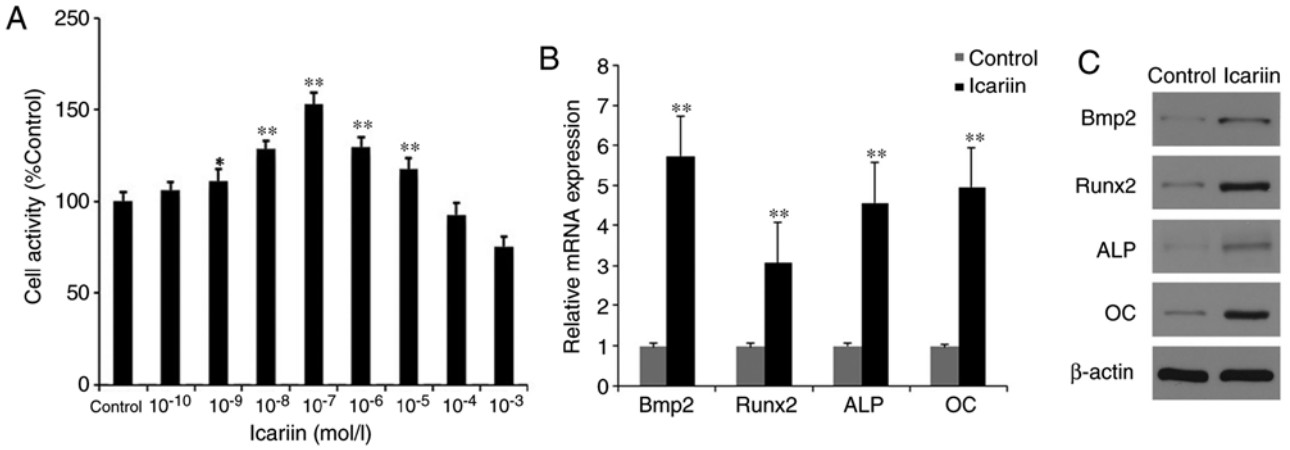

Figure 2. Icariin promotes cell proliferation activity and expression of osteogenic genes in BMSCs. (A) BMSCs were treated with icariin (10^{-10}-10^{-3} M) for 48 h and assessed by the Cell Counting Kit-8 assay. (B) mRNA expression and (C) protein level of osteogenic genes in control and 10^{-7} M icariin-treated groups. Data are expressed as mean ± SD. *P<0.05 and **P<0.01 (compared with the control); by one-way ANOVA followed by Dunnett's post hoc test or the Student's t-test. BMSCs, bone marrow-derived mesenchymal stem cells; BMP2, bone morphogenetic protein 2; RUNX2, RUNX family transcription factor 2; ALP, alkaline phosphatase; OC, osteocalcin.

Icariin induces expression of osteoblastic genes in BMSCs. The proliferative activity of BMSCs in the presence of different concentrations of icariin (10^{-10}-10^{-3} M) was determined using the CCK-8 assay (Fig. 2A). The results showed that cell proliferation was significantly increased by lower concentrations of icariin (10^{-9}-10^{-5} M; P<0.05), with 10^{-7} M being the most effective.

RT-qPCR and western blotting demonstrated that icariin significantly increased the expression of BMP2, RUNX2, ALP and OC (Fig. 2B and C).

Icariin regulates BMAL1 in BMSCs. To investigate whether icariin induces BMAL1 expression in BMSCs, BMAL1 expression of 10^{-7} M icariin-treated cells was evaluated by RT-qPCR and western blotting. Icariin significantly increased BMAL1 mRNA and protein levels (Fig. 3). These results suggested that icariin induced BMAL1 expression in BMSCs.

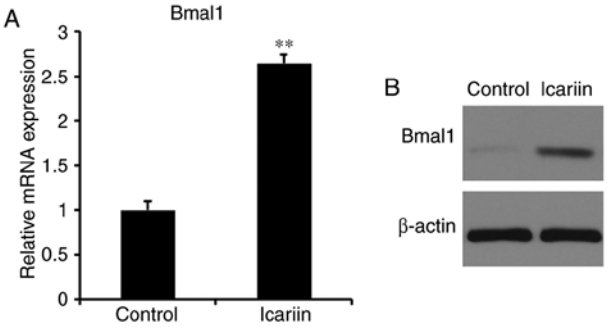

Figure 3. Icariin increases BMAL1 expression in BMSCs. (A) mRNA expression of BMAL1. (B) Protein level of BMAL1 as assessed by western blot analysis. **P<0.01 (compared with control). BMSCs, bone marrow-derived mesenchymal stem cells; BMAL1, brain and muscle ARNT-like 1.

BMAL1 induces osteogenic differentiation via BMP2 in BMSCs. To evaluate whether BMAL1 regulates osteogenic differentiation via BMP2, BMSCs were transduced with BMAL1. BMAL1 overexpression increased expression of osteogenic differentiation-associated genes. Moreover, BMAL1 overexpression enhanced both mRNA expression and the promoter activity of BMP2 (Fig. 4E and F).

BMAL1 deficiency suppresses osteogenic differentiation in BMSCs. In order to determine whether BMAL1 is essential for osteogenic differentiation induced by icariin, the osteogenic differentiation-inducing effects of 10^{-7} M icariin treatment were further investigated in BMAL1-knockdown ($BMAL1^{-/-}$) BMSCs. Compared with the effects of icariin in wild-type (WT) BMSCs, expression of osteogenic genes was reduced in the $BMAL1^{-/-}$ BMSCs evaluated by PCR analysis (Fig. 4E). In accordance with the PCR results, western blotting also showed that levels of BMP2, RUNX2, ALP and OC were lower in the $BMAL1^{-/-}$ BMSCs compared to the WT BMSCs (Fig. 4F). Furthermore, ARS of $BMAL1^{-/-}$ BMSCs indicated that BMAL1 was required for mineralized nodule formation during osteogenic differentiation (Fig. 4B and D). In addition, less positive staining was detected in $BMAL1^{-/-}$ BMSCs (Fig. 4A and C).

Taken together, icariin increased the BMAL1, BMP2 and RUNX2 expression in BMSCs. BMAL1 overexpression also upregulated BMP2 and RUNX2 expression compared with the control group. However, these levels of expression were significantly downregulated after BMAL1 knockdown whether icariin treatment was administered or not. The results suggested the icariin could enhance the BMP2 signaling pathway by upregulating BMAL1 expression *in vitro*.

Discussion

In the present study, it was demonstrated that icariin promoted osteogenic differentiation by upregulating BMAL1 expression through BMP signaling in BMSCs. These findings suggested that BMAL1 plays a critical role in icariin-mediated osteogenic differentiation.

In fact, a circadian clock exists in every cell of the human body. The central players in these are BMAL1 encoded by the gene ARNTL and clock circadian regulator (CLOCK)

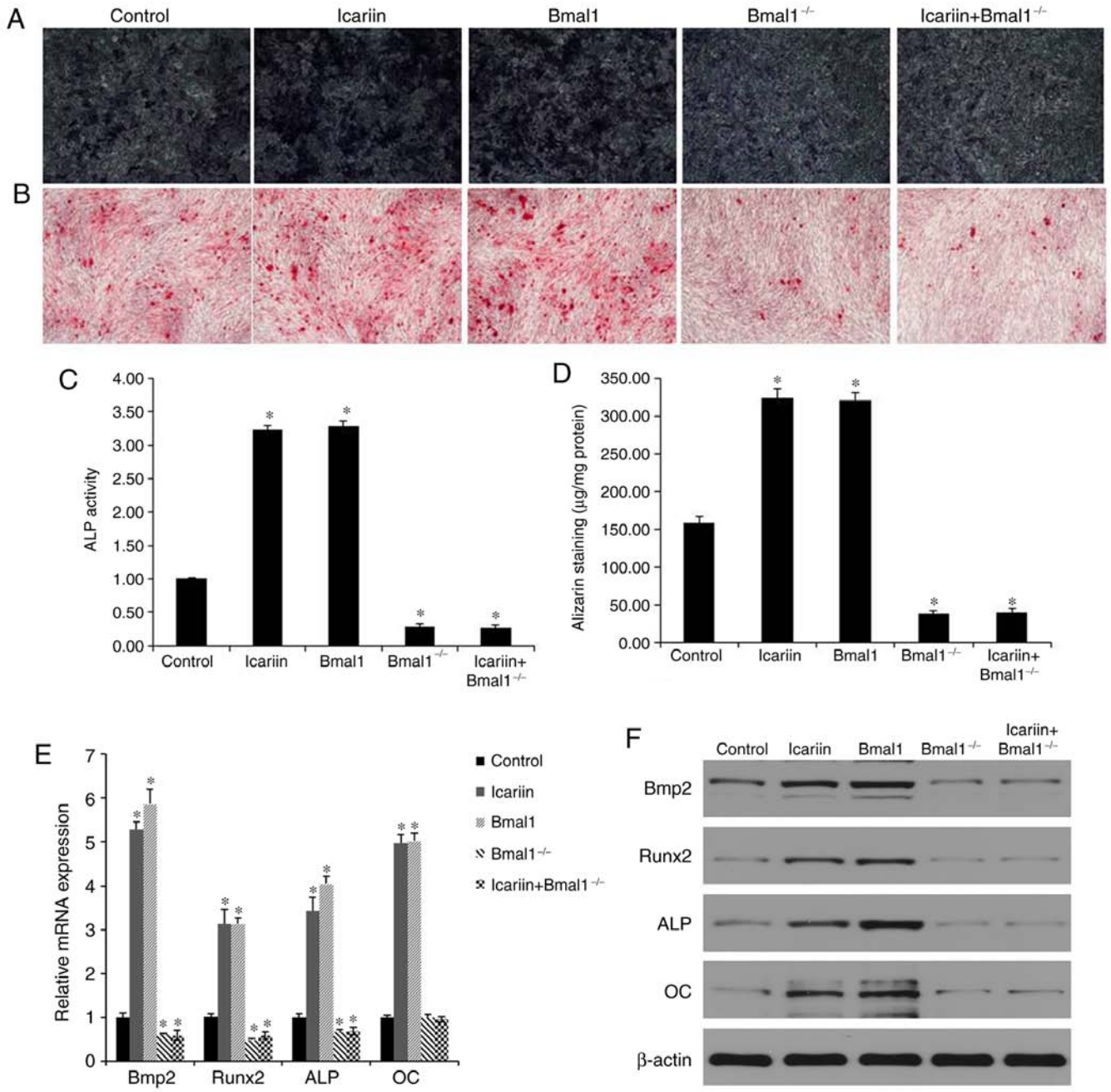

Figure 4. Osteogenic differentiation of BMSCs with BMAL1 overexpression (BMAL1) and BMAL1 deficiency (BMAL1$^{-/-}$). (A) ALP staining. (B) ARS staining performed to examine extracellular mineralization. (C) Quantitative ALP analysis. (D) Quantitative ARS staining analysis. (E) Reverse transcription-quantitative PCR and (F) western blot analysis of osteogenic gene expression in BMSCs. *P<0.05 (compared with the control) by one-way ANOVA followed by Dunnett's post hoc test. BMSCs, bone marrow-derived mesenchymal stem cells; BMAL1, brain and muscle ARNT-like 1; BMP2, bone morphogenetic protein 2; RUNX2, RUNX family transcription factor 2; ALP, alkaline phosphatase; OC, osteocalcin; ARS, alizarin red S.

by the gene CLOCK (19). BMSCs are stem cells with the ability to differentiate *in vitro* into adipocytes, osteoblasts and cartilage-forming chondroblasts (20). Regardless of its exact developmental origin, the function of the circadian clock gene is related to the behavior of various stem cells involved in homeostasis and repair of bone and adipose tissue (21). A recent report revealed impairment of osteogenic differentiation of BMSCs from BMAL1$^{-/-}$ mice (5). This finding was in line with our present study as well as an observational study that showed that the numbers of active osteocytes and osteoblasts were reduced in BMAL1$^{-/-}$ mice *in vivo* (22). However, a previous study showed that the amount of osteoblastic activity was increased in BMAL1$^{-/-}$ mice (21). These differences may due to the different ages of the mice. In this study, we clearly evaluated the positive regulatory mechanism of BMAL1 on osteogenic differentiation. Our results indicated that BMAL1 could accelerate osteogenic differentiation through BMP2 signaling. BMP2, a member of the transforming growth factor (TGF)-β superfamily, is considered to be a key regulator of cell growth and differentiation (23-25). A number of studies have suggested that the activity of clock genes is closely correlated with BMPs. One study reported that regulation of clock genes controlled BMP2 expression in osteoblasts (26). Meanwhile, BMAL1 regulation of TGF-β and BMP signaling in the control

of fat adipogenesis has also been reported (27). A recent study showed that BMAL1 silencing-induced BMP2 was found in rat uterus endometrium stromal cells (28). Samsa *et al* (5) recently reported that BMAL1$^{-/-}$ mice had reduced osteoblast differentiation *in vitro* and they proposed some potential molecular mechanism of BMAL1-dependent control of osteogenic differentiation including oxidative stress response, the mTOR signaling pathway and translation. However, the precise mechanism via which BMAL1 induces osteogenic differentiation of BMSCs remains unclear. In the present study, it was demonstrated that BMAL1 deficiency decreased BMP2 expression during osteogenic differentiation of BMSCs.

The disorder and downregulation of clock genes may be the cause of disease, and in some cases could also be used as a target of drug action to improve disease symptoms and treat disease. Recently, in order to alleviate circadian clock-related disease, there has been significant progress in the development of chemical compounds which are capable of manipulating clock genes as novel preventive and therapeutic agents (29). For example, resveratrol, which is considered a natural antioxidant polyphenol compound, has also been demonstrated to ameliorate circadian disorders of lipid metabolism (30). Moreover, a recent study revealed that glucocorticoids altered the circadian clock in the iris-ciliary body in a mouse model (31). However, few studies have yet demonstrated the potential mechanism by which osteogenic differentiation is mediated between icariin and BMAL1.

The results of the present study suggested that icariin at a concentration range between 10^{-9} and 10^{-5} mol/l significantly promoted the proliferation of BMSCs, especially at 10^{-7} mol/l. A number of previous studies have reported that icariin increased cell proliferation at different concentrations. Yang *et al* (32) showed enhanced proliferation of human neural stem cells *in vitro* at a concentration of 10^{-5} mol/l. In addition, Mok *et al* (33) demonstrated that icariin stimulated the proliferation of UMR 106 cells at concentrations ranging from 10^{-14} to 10^{-6} mol/l. These differences may be attributable to the variety of cells used in the experiments.

Icariin has been demonstrated to have therapeutic properties, with reports of antitumor, anti-hepatotoxic, immune-enhancing, anti-inflammatory, neurite outgrowth activity and erection dysfunction-improving effects over the past decades among Chinese researchers (34-36). Furthermore, the bone-strengthening activity of icariin has attracted much recent attention. However, there have been few reports on the effect of icariin on the circadian clock in BMSCs. The present study suggested that icariin increased BMAL1 expression. Furthermore, reduced expression of BMP2/RUNX2 was observed in the present study during osteogenic differentiation of BMAL1-deficient cells. In addition, mineralized nodule formation was suppressed both in BMAL1$^{-/-}$ BMSCs and in icariin-mediated BMAL1$^{-/-}$ BMSCs. Taken together; these findings demonstrated that icariin promoted osteogenic differentiation by upregulating BMAL1 expression through BMP signaling in BMSCs. However, as the effects of icariin on BMSCs in the present study were mainly observed *in vitro*, further studies will be needed to confirm these results in animal models.

In conclusion, to the best of our knowledge, the present study is the first to provide evidence that icariin promoted osteogenic differentiation through BMAL1-BMP2/RUNX2 signaling.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

ZH and SZ conceived and designed the study. HW, ZL, JX and ZW performed the experiments. ZH and HW wrote the paper. XW, YH, YX and XL analyzed the data, and reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research.

Ethics approval and consent to participate

All the experimental protocols involving animals were approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology (IACUU no. S496; date of permission, October, 31, 2017).

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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