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Article

Icariin stimulates differentiation of bone marrow-derived mesenchymal stem cells (BM-MSCs) through activation of cAMP/PKA/CREB

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Icariin, a prenylated flavonol glycoside isolated from *Epimedium*, has been considered as a potential alternative therapy for osteoporosis. The present study aimed to clarify the detailed molecular mechanisms of action of icariin on osteoblast function, using bone marrow-derived mesenchymal stem cells (BM-MSCs). BM-MSCs were first stimulated by icariin. Then, gene and protein expression of cAMP/PKA/CREB signaling molecules were analyzed by RT-PCR and western blotting (WB), and alkaline phosphatase (ALP) was analyzed in cell lysates by ELISA. MTT assays indicated that icariin did not have significant effects on cell viability up to 1 μM. Icariin showed a dose-dependent effect on the alkaline phosphatase activity of BM-MSCs. WB analysis showed that icariin treatment of BM-MSCs significantly enhanced the protein expression of protein kinase A (PKA) and cAMP-responsive element binding protein (CREB), while RT-PCR results showed that icariin dose-dependently increased the mRNA levels of *PKA* and *CREB*. Icariin induced BM-MSC differentiation by BMP2, Smad1, and Runx2. RT-PCR and WB results indicated that icariin significantly increased the expression of BMP2, Smad1, and Runx2 in BM-MSCs. These results suggest that icariin is an agonist of the cAMP/PKA/CREB pathway in BM-MSC differentiation, raising the possibility that it could be used in the treatment of osteoporosis.

Keywords: Icariin/molecular mechanisms. BM-MSCs. cAMP. PKA. CREB.

INTRODUCTION

Osteoporosis is characterized by low bone mineral density (BMD) and loss of the structural and biomechanical properties that are required to maintain bone homeostasis (Ivanova *et al.*, 2015). Icariin is the main active flavonoid glucoside of *Epimedium pubescens*, which has been reported to enhance bone healing and reduce the incidence of osteoporosis (Hsieh *et al.,* 2010). Icariin restores the osteogenic differentiation and bone formation of bone marrow stromal cells in a rat model of estrogen deficiency-induced osteoporosis (Luo *et al.,* 2015). In the ovariectomized (OVX) calvarial defect model, icariin loaded on CPC scaffolds enhanced both osteogenesis and angiogenesis, while a system of local sustained release of icariin combined with systemic administration achieved a better effect on bone defect regeneration (Wu *et al.,* 2017).

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Osteoblasts, osteocytes and osteoclasts are the three types of bone cells that are in direct contact with all the cellular elements in the bone marrow (Carrington, 2005). Mesenchymal stem cells (MSCs) are a promising source of cells for use in cell-based therapeutics and regenerative medicine due to their ability to self-renew and differentiate into a number of functional cell types. To date, bone marrow-derived mesenchymal stem cells (BM-MSCs) have been the most widely-studied family of stem cells (Li *et al.,* 2016). BM-MSCs have been estimated to represent 0.01–0.0001% of the nucleated cells in adult human bone marrow (Dazzi *et al.,* 2006). BM-MSCs are progenitors of skeletal tissue components such as bone, cartilage, the hematopoiesis-supporting stroma, and adipocytes (Bianco, Riminucci, Gronthos, 2001). Bone morphogenesis protein-2 (BMP-2), a member of the BMP family, is one of the best-characterized inducers of osteochondrogenesis and can increase the expression of markers of both osteo- and chondrogenic lineage differentiation of progenitor cells, such as MSCs. It has recently been shown that protein kinase A (PKA) activation

also forms an important component of the mechanism by which BMP-2 mediates MSC differentiation. Once activated, PKA usually phosphorylates its prototypical downstream transcription factor, cAMP-responsive element binding protein (CREB), which is associated with a number of important physiological functions including osteochondrogenic differentiation (Zhao, Li, Zhou, 2009).

Icariin has been shown to have an anti-osteoporotic effect by regulating the bidirectional differentiation of BM-MSCs through the canonical Wnt signaling pathway (Huang *et al.,* 2017). Icariin is also a bone anabolic agent that may exert its osteogenic effects through the induction of BMP-2 and NO synthesis, subsequently regulating expression of Cbfa1/Runx2, OPG, and RANKL. This effect may contribute to its effect on the induction of osteoblast proliferation and differentiation, resulting in bone formation (Hsieh *et al.,* 2010). In this study, we clarified the detailed molecular mechanisms of action of icariin on the osteoblastic function of BM-MSCs. The results demonstrate that icariin induces osteoblast differentiation via BMP2, through activation of cAMP/PKA/CREB signaling in osteoblasts.

MATERIAL AND METHODS

Material and reagents

Icariin was obtained from Sigma-Aldrich (St. Louis, MO, USA) and the purity of the compound was more than 99%. Stock solutions of icariin were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich). In all studies, the final concentration of DMSO was 0.1% (v/v). The icariin stock solutions were stored at -20 ºC.

Cell culture

BM-MSCs were purchased from Saiye Biological Technology Co., Ltd. (Guangzhou, China). BM-MSCs were cultured in 25 cm^2 flasks in complete medium (DermaLife® K Medium Kit, Lifeline Cell Technology, Frederick, MD, USA) consisting of DMEM, 10% fetal bovine serum (FBS), and 100 U/mL penicillin and streptomycin. Cells were trypsinized and passaged at 80-90% confluence. The cells were reseeded into 96-well plates or 6-well plates at a density of 1×10^4 or 1×10^6 cells/well, respectively, and cultured in a humidified atmosphere of 5% CO_2 and 95% air, at 37ºC.

Cell viability assay

BM-MSCs were plated into 96-well plates at a density of 1×10^4 per well. After 2 days, cells were stimulated with icariin at concentrations of 0, 0.001, 0.01, 0.1, 1 or 10 μM for 24 h. Cell proliferation was investigated using the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. To perform the assay, 20 μL of MTT was added to every well, and the plates were placed into a 5% CO_2-humidified incubator at 37ºC for 4 h. After incubation, supernatants were removed from the plates and 100 μL of DMSO solution was added for 15 min. The plates were read on a micro-plate reader at a wavelength of 492 nm.

RNA preparation and RT-PCR analysis

BM-MSCs were seeded into 6-well plates at a density of 1×10^6 cells per well. Total RNA was extracted from the cells using TRIzol® reagent according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA) after stimulation. First strand cDNA was synthesized using a Reverse Transcription System Kit (Tiangen Biotech, Beijing, China). RNA was reverse transcribed into cDNA and analyzed by RT-PCR using an ABI PRISM® 7000 sequence detector (Applied Biosystems, Foster City, CA, USA). CREB, PKA, BMP2, Smad1, and Runx2 mRNA levels were detected by real-time PCR in a reaction mixture of 20 μL total volume containing 10 μL of 2 × SYBR® TransStar Green PCR Super Mix, 0.6 μL sense and antisense primers, and 50 ng RT product. The utilized primer sequences are shown in Table I.

TABLE I - Primer for real-time PCR assays

Genes	Forward primer	Reverse primer
CREB	5'-CTGAGGAGCTTGTACCACCG-3'	5'-TGGATACCTGGGCTAATGTGG-3'
PKA	5'-GAGCAGGAGAGCGTGAAAGAG-3'	5'-GGGCTGTATTCTGAGAAGGGG-3'
BMP2	5'-GGGACCCGCTGTCTTCTAGT	5'-TCAACTCAAATTCGCTGAGGAC-3'
Smad1	5'-ACCTGTGGCTTCCGTCTC-3'	5'-ATCGTGGCTCCTTCGTC-3'
RUNX2	5'-GGGAACCAAGAAGGCACAGA-3'	5'-GGTGGAATGGATGGATGGGG-3'
β-actin	5'-CGCGAGTACAACCTTCTTGC-3'	5'-CGTCATCCATGGCGAACTGG-3'

Western blot analysis

Cells were lysed with lysis buffer (20 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1% [v/v] Triton™ X-100, 10 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 0.05% [v/v] 2-mercaptoethanol, and 1 × protease inhibitor solution). Cell debris was removed by centrifugation at 14,000 × *g* for 15 min, and equal amounts of protein extracted from each sample were heat denatured and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% (v/v) polyacrylamide gel. After electrophoresis, proteins were electrotransferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% (w/v) skimmed milk at room temperature for 2 h and probed with primary antibody at 4°C overnight. The primary antibodies used were CREB, PKA, BMP2, Smad1, and RUNX2. After washing, membranes were incubated with the appropriate secondary antibody in 5% (w/v) skimmed milk/Tris-buffered saline containing 0.05% Tween® 20 for 1 h at 4°C. Proteins were detected using SignalFire™ ECL Reagent (Cell Signaling Technology, Danvers, MA, USA).

Enzyme-linked immunosorbent assay (ELISA) analysis

Supernatants from BM-MSC cell culture were harvested at 24 h after stimulation, and centrifuged at 1000 × *g* for 20 min. The supernatants were analyzed for ALP by ELISA following the manufacturers' instructions (Nanjing Jiancheng Bioengineering Institute).

Statistical analysis

Data are expressed as the mean ± SD of three independent determinations, and significance was analyzed using the *t*-test and one-way ANOVA. $^{*}P < 0.05$ was considered statistically significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) statistical software for Windows, Version 18.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Effect of icariin on cell viability

The effect of icariin on cell viability of BM-MSCs was determined by MTT assay. As shown in Figure 1, 0.001 μM icariin had no significant effect on the proliferation of BM-MSCs. However, icariin at 0.01, 0.1, or 1 μM promoted MSC proliferation ($P < 0.01$), and showed a quantitative relationship. Meanwhile, 10 μM of icariin showed a certain cytotoxicity, significantly inhibiting the proliferation of BM-MSCs ($P < 0.05$).

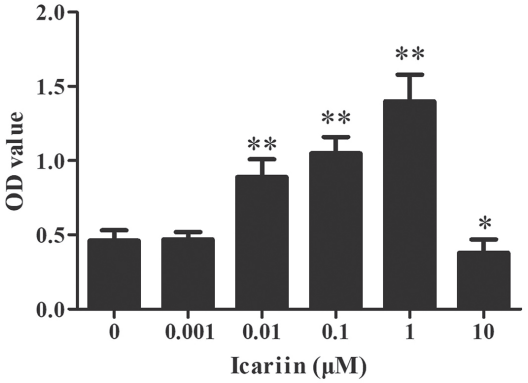

FIGURE 1 - Effects of different concentrations of icariin on the proliferation of BM-MSCs at 24 h. *vs.* treatment with 0 μM, $^{*}P < 0.05$, $^{**}P < 0.01$.

Assessment of ALP activity under different treatment conditions

BM-MSCs were treated with different concentrations of icariin for 24 h. the groups treated with 0.01, 0.1, or 1 μM icariin showed a significant increase in ALP activity compared with the control group ($P< 0.05$, Figure 2 A).

Icariin administration enhanced BM-MSC gene and protein expression *via* the cAMP/PKA/CREB signaling pathway

The cAMP/PKA/CREB signaling pathway has been shown to play an important role in differentiation of BM-MSCs. To determine the mechanism of icariin on BM-MSC differentiation, we examined the effect of icariin on the expression of components of the cAMP/PKA/CREB signaling pathway. Our qPCR data showed that icariin significantly enhanced mRNA expression of *CREB* and *PKA* in BM-MSCs. As shown in Figure 2, treatment with icariin at a concentration of 1 μM induced the most significant effect with a 1.9-fold increase in expression of *CREB* and a 1.6-fold increase in *PKA*, compared to the blank group (0 μM). The effect of icariin on BM-MSC mRNA expression showed a dose-response relationship (Figure 2. C, D). Finally we examined the effect of icariin on protein expression of PKA/CREB signaling molecules, and found that icariin stimulation of BM-MSCs showed a dose-response relationship. Expression of PKA/CREB was significant increased after treatment with icariin at the concentration of 1 μM, including both mRNA and protein levels (Figure 2 B). Taken together, these

results demonstrate that icariin may regulate BM-MSC differentiation through activation of the cAMP/PKA/CREB signaling pathway.

Icariin induced BMP2, Smad1, and RUNX2 mRNA and protein expression in BM-MSCs

The CREB receptors BMP2, Smad1, and Runx2 were constitutively expressed on BM-MSCs (Figure 3). The mRNA and protein levels of BMP2, Smad1, and Runx2 were analyzed after treatment of BM-MSCs with icariin to determine whether icariin increased BMP2, Smad1, and Runx2 expression. Analysis by RT-PCR showed that BM-MSCs treated with icariin exhibited a significant and dose-dependent increase in the mRNA expression of *BMP2*, *Smad1*, and *Runx2*, compared with untreated cells (Figure 3 A–C). The effect of icariin on BMP2, Smad1, and Runx2 protein expression was also dose-dependent. Meanwhile, the mRNA and protein expression of BMP2, Smad1, and RUNX2 showed a significantly greater sensitivity to icariin (1 μM) stimulation than untreated cells (all $P < 0.01$).

DISCUSSION

The herb *Epimedium* has long been used in traditional Chinese medicine to treat bone fractures and prevent osteoporosis. Researchers believe that the flavonoids contained in the herb are the effective component responsible for this activity. Icariin should be an effective agent for bone-strengthening activity, and one of its mechanisms is to stimulate the proliferation and enhance the osteogenic differentiation of MSCs (Chen *et al.,* 2005). Icariin has been shown to increase osteogenic differentiation and mineralization of BMSCs and osteoblasts, and to inhibit osteoclast formation and bone resorption activity (Zhang et al., 2011; Ma *et al.,* 2014; Zhang *et al.,* 2016). In this paper, we found that low concentrations (0.01, 0.1, 1 μM) of icariin stimulated BM-MSC proliferation ($P < 0.01$), but that 10 μM icariin had a certain cytotoxicity, and significantly inhibited the proliferation of BM-MSCs ($P < 0.05$).

Hormones that stimulate the synthesis of cAMP regulate many cell type-specific processes including gene transcription, cell proliferation, differentiation,

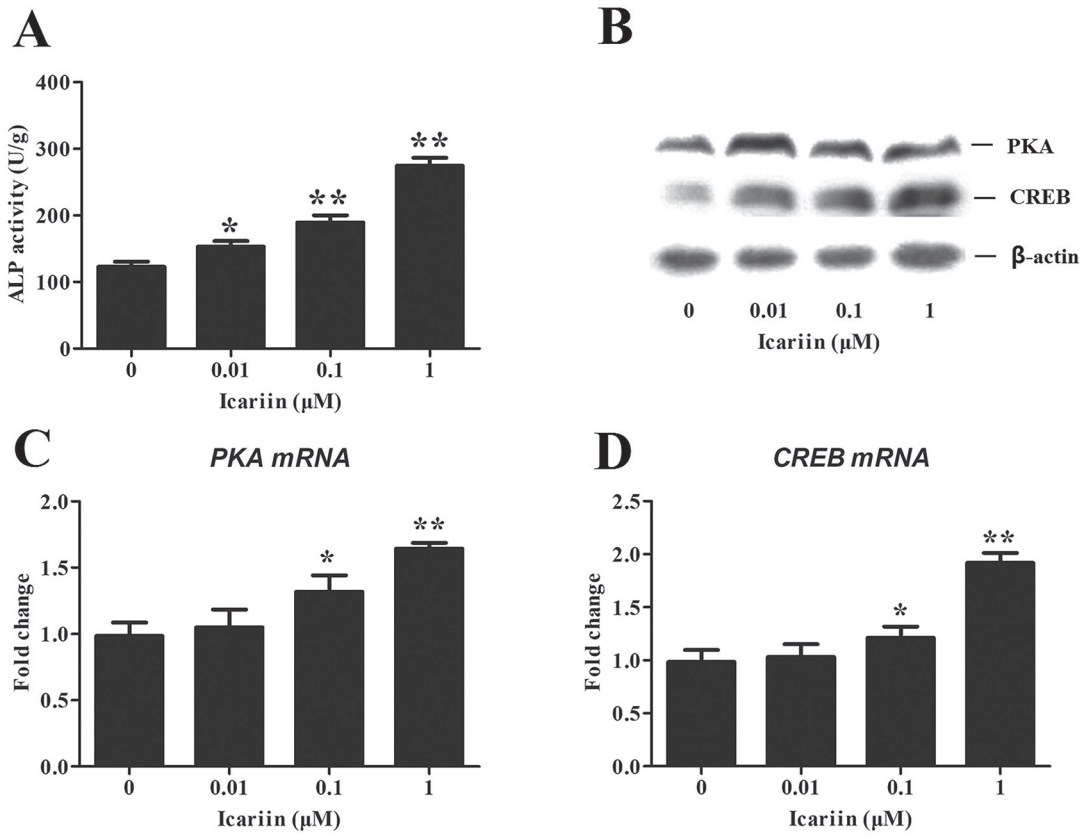

FIGURE 2 - Icariin actives the cAMP/PKA/CREB signaling pathway in BM-MSCs. (A) Icariin showed a dose-dependent effect on alkaline phosphatase (ALP) activity in BM-MSCs. (B) WB analysis showed that icariin treatment of BM-MSCs significantly enhanced protein expression of PKA and CREB. (C)(D) RT-PCR results showed that icariin dose-dependently increased the mRNA levels of *PKA* and *CREB* in BM-MSCs. *vs.* treatment with 0 μM, $^{*}P < 0.05$, $^{**}P < 0.01$.

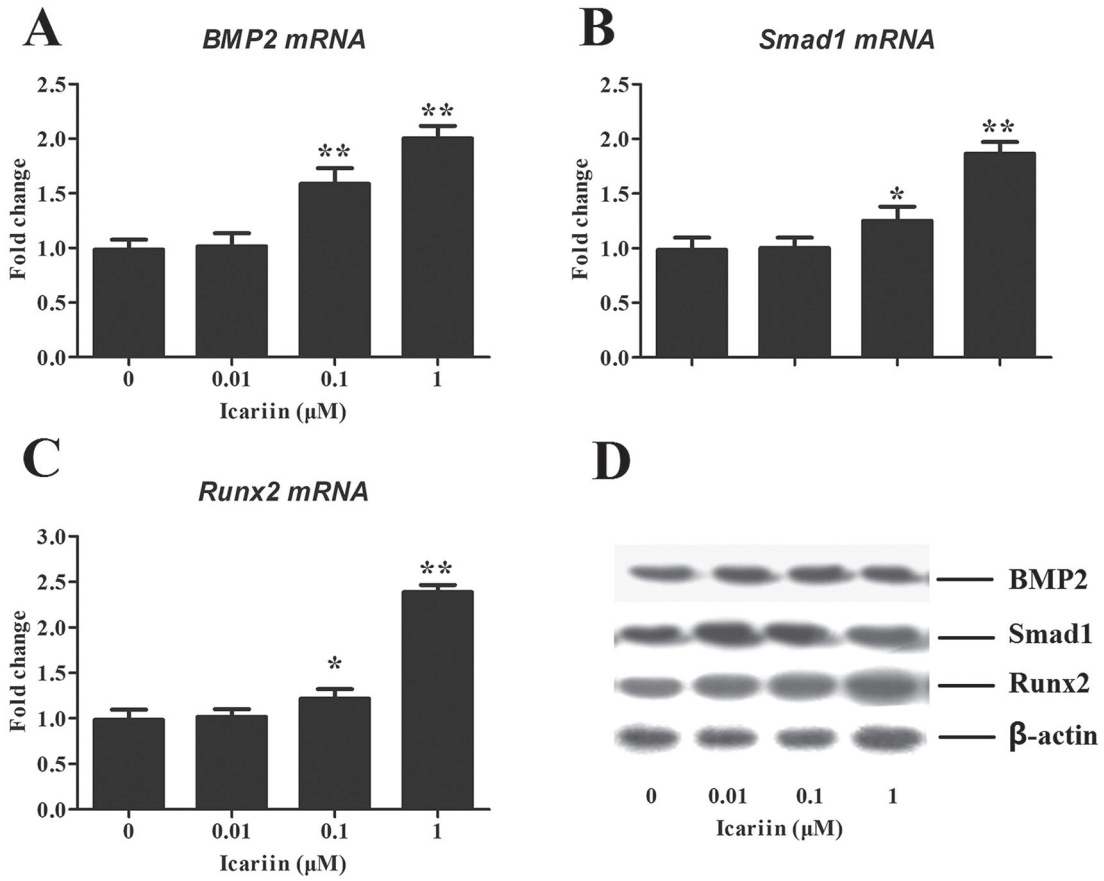

FIGURE 3 - Icariin induced BM-MSC differentiation by BMP2, Smad1, and Runx2. RT-PCR and WB results indicated that icariin significantly increased the expression of BMP2, Smad1, and Runx2 in BM-MSCs. *vs.* treatment with 0 μM; $^{*}P < 0.05$, $^{**}P < 0.01$

and survival. These processes are mediated, in part, by the MAP (mitogen-activated protein) kinase (or ERK; extracellular signal response kinase) cascade in a wide range of diverse cell types including pancreatic islet cells, bone cells, pituitary cells, thyroid cells, neuronal cells and others (Li *et al.,* 2016). In this study, we found that cAMP consistently enhanced the expression of the osteogenesis marker ALP. The results of cell proliferation assay demonstrated that icariin stimulated BM-MSC proliferation. These results indicated that icariin treatment at different concentrations promoted proliferation of BM-MSCs through the cAMP/PKA/CREB pathway. Further, we sought to examine the effect of icariin on expression of PKA/CREB mRNA and protein, by analyzing the mRNA and protein levels of PKA/CREB after treatment of BM-MSCs. Analysis using RT-PCR and WB showed that BM-MSCs treated with icariin exhibited a dose-dependent increase in the mRNA and protein expression of PKA/CREB, compared with untreated cells. Several previous studies have evaluated the role of the PKA pathway in osteogenic differentiation of hMSCs, and reported that pretreatment of human MSCs with a cAMP analog or forskolin enhanced bone formation. cAMP is a pivotal intracellular signaling molecule, the main function of which is to activate cAMP-dependent PKA (Yang *et al.,* 2008). Adenylate cyclase is activated through dissociation of G-proteins, causing the conversion of adenosine triphosphate into cAMP. Subsequently, cAMP activates PKA, which in turn phosphorylates CREB protein, and this translocates into the nucleus where it activates transcription of target genes (Kim et al., 2013; Chen *et al.,* 2016). However, precisely how the administration of icariin regulates osteogenic differentiation of BM-MSCs by the cAMP/PKA/CREB signaling pathway requires further elucidation. In the current study, cell proliferation assays, osteogenic gene expression testing, and ALP activity detection were used in order to determine the underlying molecular mechanism by which icariin administration modulates the cAMP/PKA/CREB signaling pathway.

Previous studies have shown that activation of the PKA pathway synergistically participates in BMP-2-induced osteoblastic differentiation, possibly by mediating the CREB and/or Ras/MAPK pathways, and activation of the PKA pathway may be one of the key BMP-2-activated signaling events that lead to osteogenic differentiation

(Zhao *et al.,* 2006). Based on these findings, we also tested the mRNA and protein expression of BMP2, Smad1, and Runx2 in BM-MSCs stimulated with icariin to investigate whether the administration of icariin regulates osteogenic differentiation of BM-MSCs via the cAMP/PKA/CREB signaling pathway. BMP/Smad signals are mediated by BMP receptors (type I and II). Smad1, 5, and 8 are phosphorylated by the BMP receptors and form a complex with Smad4, following which the complex is translocated into the nucleus. Within the nucleus, the phosphorylated Smads interact with other transcription factors such as Runx2 to initiate transcription of osteogenic-related genes (Xu *et al.,* 2015). We focused on signaling pathways involved in icariin-mediated BMP2, Smad1, and Runx2 expression in BM-MSCs according to previous reports (Guo *et al.,* 2016; Su *et al.,* 2015). Analysis by RT-PCR and WB showed that BMP2, Smad1, and Runx2 were activated by icariin in BM-MSCs (Figure 3). Taken together, our results indicate that icariin regulates the expression of BMP2 in BM-MSCs through the cAMP/PKA/CREB signaling pathway.

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