Icaritin promotes the osteogenesis of bone marrow mesenchymal stem cells via the regulation of sclerostin expression

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Abstract. Icaritin, a metabolite of icariin, is a potent promoter of bone marrow-derived mesenchymal stem cells (BMSCs) osteogenesis, but the underlying mechanisms remain unclear. To examine the effects of icaritin on osteogenic differentiation, BMSCs were exposed to osteogenic induction medium with or without icaritin pretreatment in the present study. It was identified that icaritin (0.01-1 μM) exhibited no cytotoxicity on the proliferative abilities of the BMSCs. Icaritin at 1 μM increased alkaline phosphatase activity, mineral deposition and osteoblast-specific gene expression. Treatment with 1 μM Icaritin upregulated osteocalcin, RUNX family transcription factor 2, tissue-nonspecific alkaline phosphatase and β-catenin, and suppressed sclerostin (*SOST*) gene expression in different stages of osteogenic differentiation. It was also demonstrated that SOST overexpression inhibited icaritin-induced osteogenesis. The western blot analysis data suggested that ICI 182780, which causes estrogen receptor α (ERα) degradation, reversed the icaritin-induced decrease in SOST expression, which was inconsistent with the results of immunofluorescence analysis. In conclusion, icaritin was demonstrated to promote the osteogenesis of hBMSCs by downregulating SOST expression, and icaritin-induced suppression of *SOST* was regulated in part via the Wnt/β-catenin/ERα axis.

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Introduction

Due to their self-renewal capabilities, multi-directional differentiation potential and the fact that they are easily accessible, bone marrow-derived mesenchymal stem cells (BMSCs) have been applied in tissue engineering, especially in the construction of bone defects models (1). As bone defects such as osteoporosis or osteonecrosis of femoral head are primarily due to the decreased osteogenic differentiation of BMSCs (2), it is crucial to develop strategies to promote the osteogenic differentiation of BMSCs in a clinical setting (3).

Sclerostin (SOST), the protein product of the *SOST* gene, serves a key role in inhibiting osteoblast activity (4). It is also well-known that *SOST* negatively regulates bone formation through antagonizing the Wnt/β-catenin pathway (5). In addition, knockdown of the *SOST* gene in mice results in a high bone mass phenotype and resistance to bone loss. In addition, antibodies directed against SOST stimulate bone formation and represent a novel therapeutic option in the anabolic treatment of osteoporotic conditions (6,7). Mirza *et al* (8) demonstrated that serum SOST levels were inversely associated with estrogen levels (8). Kim *et al* (9) demonstrated that estrogen signaling functions as an negative regulator of *SOST* expression involving the Wnt/β-catenin/estrogen receptor α (ERα) pathway in human osteoblasts.

Icariin, extracted from *Herba epimedii*, has been suggested to significantly improve osteogenesis to decrease bone loss in ovariectomized rats and steroid-induced osteonecrosis of the femoral head (10). However, recent studies have demonstrated that icariin is enzymatically hydrolyzed, and then subsequently metabolized to desmethyl icaritin and icaritin; desmethyl icaritin is suggested to have more potent pharmacological effects compared with icariin (11,12). Icaritin is able to promote the proliferation and differentiation of osteoblasts and enhance matrix calcification due to its estrogen-like activity (13). In addition, icaritin may decrease the activity of osteoclasts *in vitro* and increase the expression of osteogenic-associated mRNA levels in human BMSCs (hBMSCs) (14). The osteogenic effects of icaritin have been clearly described, but the underlying mechanisms

remain unclear. As icaritin has been suggested to regulate the Wnt/β-catenin pathway (10) and ERα (9), which are closely associated with *SOST*, we hypothesized that icaritin may promote osteogenesis through inhibiting *SOST*.

The present study assessed the effects of icaritin on osteogenesis of hBMSCs and explored the role of SOST in icaritin-induced osteogenesis of hBMSCs.

Materials and methods

Reagents. Icaritin (>98% purity) was purchased from Shanghai U-sea Biotech Co. Ltd. modified Eagle's medium of alpha (α-MEM) and fetal bovine serum (FBS) were both obtained from Hyclone (Hyclone; GE Healthcare Life Sciences); The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. Dimethyl sulfoxide (DMSO), trypsin and TRIzol® reagent were purchased from Gibco; Thermo Fisher Scientific, Inc.; the alkaline phosphatase (ALP) activity kit, enhanced chemiluminescent detection reagent and micro-BCA assay kit were obtained from Beyotime Institute of Biotechnology. DKK-1 and ICI182780 were purchased from R&D Systems, Inc. The RNA extraction kit was purchased from Takara Bio, Inc. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) primers were obtained from Invitrogen; Thermo Fisher Scientific, Inc. The antibodies used were obtained from Cell Signaling Technology, Inc., unless otherwise indicated. Other reagents used in the experiment were purchased from Sigma-Aldrich; Merck KGaA. Icaritin was dissolved in DMSO and the final concentration of DMSO was 0.05% (v/v). ICI 182780 was dissolved in DMSO and stored at 4˚C. Cells were pretreated with ICI 182780 (1 μM) for 30 min at 37˚C, followed by icaritin treatment.

Cell culture of hBMSCs and SOST transfection. hBMSCs were purchased from Cyagen Biosciences, Inc. (cat. no., HUXMA-01001). The hBMSCs were cultured in α-MEM containing 10% FBS and 1% penicillin-streptomycin in a humidified incubator with 5% CO_2 at 37˚C. After reaching 80-90% confluence, the adherent cells were harvested with trypsin for 1 min and sub-cultured at a ratio of 1:2. Cells from passages 3-6 were used in the following experiments.

The *SOST* gene overexpression lentivirus was constructed and purchased from Shanghai GeneChem Co., Ltd. Briefly, the *SOST* gene was amplified using PCR with the following primers: *SOST* forward, CACCGCTGCACTTCACCC GCTACGTTTCAAGAGAACGTAGCGGGTGAAGTGCAG CTTTTTTG; and *SOST* reverse, GATCCAAAAAAGCTG CACTTCACCCGCTACGTTCTCTTGAAACGTAGCGGG TGAAGTGCAGC. The gene was then cloned into plenti-U bcP-IKZF2-V2-3xHA-pGK-Pur plasmid (Addgene; plasmid no. 107393) by *in vitro* recombination. Lentiviruses were generated by transient transfection of 293FT packaging cells (Invitrogen; Thermo Fisher Scientific, Inc.) using the calcium phosphate method. After 72 h of transfection, the supernatant was collected. The supernatant was filtered with a 0.45 μm syringe filter (Sigma-Aldrich; cat. no. CLS431220), centrifuged for 90 min in at 4˚C at 42,000 x g, and resuspended in 200 μl α-MEM. For lentivirus transduction, hBMSCs from the third passage were seeded in a 6-well plate ($5x10^6$ cells/well). After 24 h, the lentivirus solution was added to each well in serum-free Opti-MEM® (Invitrogen; Thermo Fisher Scientific, Inc.). The medium was replaced with fresh complete medium after 8 h. The cells were selected with puromycin (10 μg/ml) following transduction for 48 h. Transduction efficiency was examined by PCR and western blot analysis. The transduced cells were termed BMSCs-vector (lentiviral vector only) and BMSCs-SOST (lentiviral vector containing the *SOST* gene).

Identification of hBMSCs. An hBMSC suspension of $1x10^6$ cells/ml was prepared. The cells were washed twice with cold PBS, centrifuged at 1,000 x g for 5 min at 4˚C and resuspended in 100 ml stain buffer (BD Biosciences). The resuspended cells were incubated with phycoerythrin (PE)-labeled primary antibodies against surface markers integrin-β1 (CD29; cat. no. 34971T; 1:400) and hematopoietic progenitor cell antigen CD34 (CD34; cat. no. 3569S; 1:400), as well as a corresponding isotype control antibody, at room temperature according to the manufacturers' protocol. The positively stained cells were immediately analyzed by flow cytometry using FlowJo software 8.7.1 (FlowJo, LLC). hBMSCs from passages 3-6 were used in the experiments.

Cell proliferation assay. To examine the effects of icaritin on hBMSC proliferation, the cells were seeded into a 96-well plate (5000 cells/well). The medium was removed after 24 h, then the cells were treated with complete medium with or without different concentrations of icaritin (0.01, 0.1, 1 and 10 μM) accordingly for 4 days. The cells were treated with 10% CCK-8 in 100 μl complete medium for 40 min at 37˚C each day. The absorbance was determined at 450 nm using a microplate reader (ELX808; BioTek Instruments, Inc.). Cell proliferative rate (%) was calculated as: Optical density (OD) treatment/OD control x 100.

Osteogenic differentiation and ALP/Alizarin Red S staining. hBMSCs were seeded onto a 24-well plate (10^5 cells/well) and cultured in osteogenic induction medium (OIM; Cyagen Biosciences, Inc.), consisting of 1 nM dexamethasone, 50 mM L-ascorbic acid-2-phosphate and 20 mM β-glycerolphosphate in complete medium, with various doses of icaritin (0.01, 0.1 and 1 μM) for 21 days. The OIM was changed every 3 days. ALP staining and Alizarin Red S staining were conducted 7 and 14 days following the induction, respectively, and were performed separately to evaluate the positive rates of ALP and calcium deposit formation. For ALP staining, cells were fixed with 10% formaldehyde for 15 min, rinsed three times with deionized water, and treated with the BCIP®/NBT solution (Sigma-Aldrich; Merck KGaA) for 20 min. After an additional washing, the stained cultures were photographed (magnification, x10). To measure ALP activity, cell lysates were tested using a commercial ALP assay kit. For Alizarin red S staining, hBMSCs were stained with pH 4.2, 0.1% Alizarin red S (Aladdin Industries Corporation) for 5 min and the images were captured using a scanner. The calcium deposition was dissolved in 10 cetylpyridinium chloride (Sigma-Aldrich; Merck KGaA), and the absorbance of the extracts was determined at 570 nm.

RT-qPCR. Cellular RNA was extracted from the hBMSCs cultured in OIM with or without icaritin using the RNA extraction kit, and cDNA was synthesized with the M-MLV

Table I. Primer sequences used in PCR analysis.

Genes	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
β-actin	GTCATCCATGGCGAACTGGT	CGTCATCCATGGCGAACTGG
ALP	CAAGGATGCTGGGAAGTCCG	CTCTGGGCGCATCTCATTGT
Runx2	GCTTCATTCGCCTCACAAAC	GTAGTGACCTGCGGAGATTAAC
OCN	AAATAGCCCTGGCAGATTCC	CAGCCTCCAGCACTGTTTAT
β-catenin	CTTCACCTGACAGATCCAAGTC	CCTTCCATCCCTTCCTGTTTAG
SOST	GGGCAACTGTAGATGTGGTT	GTCCCGAAGGAGAATTGTGTAG

ALP, alkaline phosphatase; Runx2, RUNX family transcription factor 2; OCN, osteocalcin; SOST, sclerostin.

reverse transcriptase according to the manufacturer's protocols. qPCR was conducted using Power SYBR® Green PCR Master Mix on the ABI StepOnePlus System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycler conditions were as follows: Initial denaturation at 95˚C for 5 min; 40 cycles at 95˚C for 15 sec and 60˚C for 1 min; and a final extension at 72˚C for 5 min. The primers are listed in Table I. The target gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (15), and were normalized using the expression level of *β-actin*.

Protein extraction and western blot analysis. Cell samples were rinsed twice with cold PBS and harvested in the extraction buffer (cat. no. P0013; Beyotime Institute of Biotechnology). The lysate was centrifuged at 4˚C with 16,000 x g for 30 min and the suspension was collected. Then, the protein content was examined using a BCA kit. Total proteins (20 μg) were separated by 12% (w/v) SDS-PAGE. The proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was washed and blocked with freshly prepared TBST containing 5% (w/v) non-fat dry milk for 90 min at room temperature. The membrane was incubated with antibodies targeting β-actin (1:1,000; cat. no. 4970S; Cell Signaling Technology, Inc.), osteocalcin (OCN; 1:1,000; cat. no. MAB1419; R&D Systems, Inc.), RUNX family transcription factor 2 (Runx2; 1:1,000; cat. no. 12556; Cell Signaling Technology, Inc.), ALP (1:1,000; cat. no. AF2910; R&D Systems, Inc.), β-catenin (1:1,000; cat. no. 8480S; Cell Signaling Technology, Inc.) and SOST (1:1,000; cat. no. MAB1406; R&D Systems, Inc.) overnight at 4˚C. Following three washes, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody (1:1,000; cat. nos. 93702 and 7076S, respectively; Cell Signaling Technology, Inc.) for 1 h at room temperature. The membrane was washed three times and then the protein-antibody complexes were examined using an enhanced chemiluminescent detection reagent. Antibody signals were developed using a Bio-Rad XRS chemiluminescence detection system (Bio-Rad Laboratories, Inc.). Band densities were analyzed using Quantity One Software (Bio-Rad Laboratories, Inc.; v4.52). The mean expression levels of the proteins relative to β-actin were presented.

Immunofluorescence staining. Following fixing with 4% paraformaldehyde at 4˚C for 15 min, the hBMSCs were treated with 0.25% Triton X-100 and 2% bovine serum albumin at 4˚C for 15 and 30 min, respectively. hBMSCs were washed and incubated overnight with primary anti-SOST antibody (1:100; cat. no. MAB1406; R&D Systems, Inc.) at 4˚C. After washing three times with PBS, the samples were incubated with a green fluorescence-labeled rabbit anti-mouse secondary antibody (1:100; cat. no. ab150113; Abcam) for 2 h in the dark at 4˚C. After the nuclei were stained with DAPI (5 μg/ml) for 5 min, the cells were observed using a fluorescence microscope (magnification, x100; Leica Microsystems GbmH).

Statistical analysis. All data acquisitions were repeated 3 times and were analyzed using SPSS v.18.0 (SPSS, Inc.). A one-way analysis of variance followed by Bonferroni's post-hoc test was used for multi-group comparison, and differences between two groups were determined by Student's t-test. The data are presented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of human MSCs. hBMSCs at passage 3 were harvested and the surface phenotypes were assessed by flow cytometry (Fig. 1). The results showed that 94.7% of the cells expressed CD29 and only 1.3% of the cells expressed CD34. These results suggested that the isolated hBMSCs expressed standard markers and were suitable for use in the following experiments.

Icaritin did not affect proliferation of hBMSCs. To determine whether icaritin would promote cell proliferation of hBMSCs, a CCK-8 assay was used. hBMSCs were treated with various concentrations of icaritin (0, 0.01, 0.1, 1 and 10 μM) for 1-4 days. The results indicated that icaritin at 0.01-1 μM did not cause any significant toxicity to the cells; however, icaritin started to exert toxic effects when the dose reached 10 μM (Fig. 2). Therefore, concentrations of icaritin from 0.01 to 1 μM were chosen for further experimentation.

Icaritin enhanced osteogenesis of hBMSCs. The data indicated that 0.01-1 μM icaritin could induce the formation of calcified nodules of hBMSCs, particularly at the concentration of 1 μM (Fig. 3A), which was confirmed by the results from Alizarin red S quantitative assay (Fig. 3B). ALP activity can

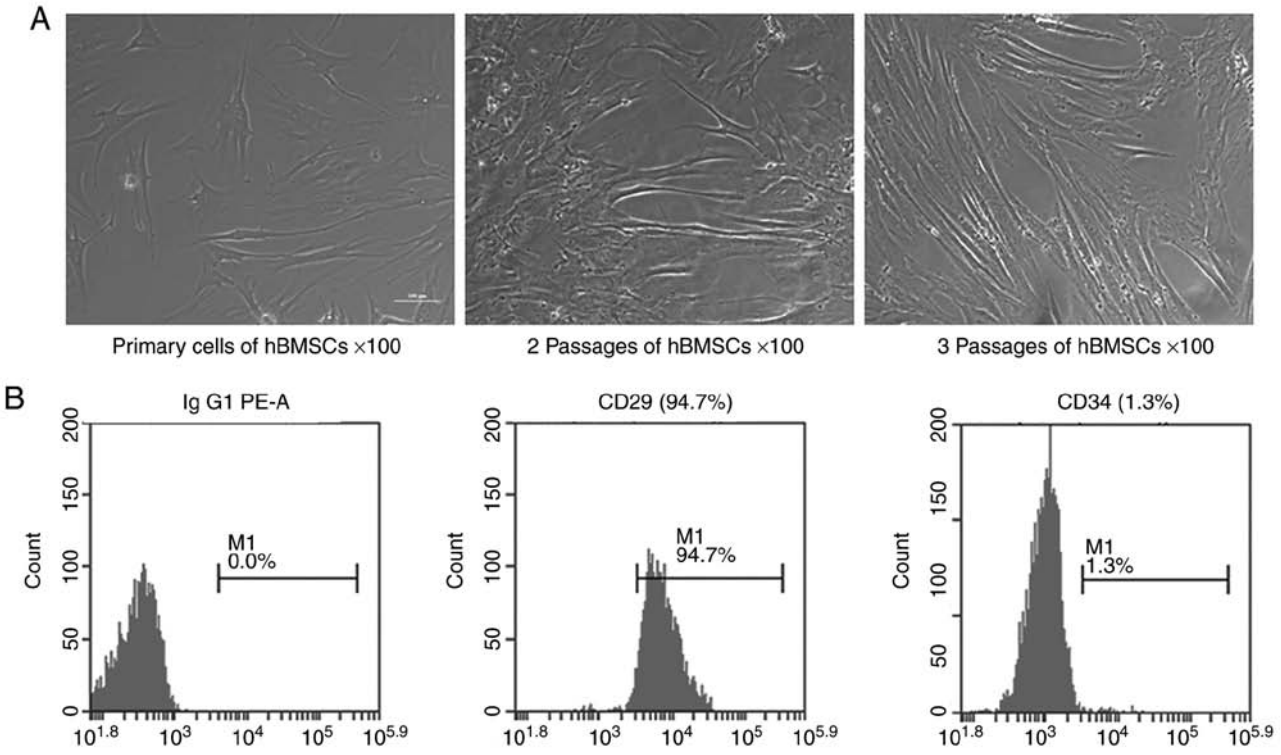

Figure 1. Characterization of hBMSCs. (A) Morphology and (B) flow cytometry analysis of hBMSCs. hBMSCs expressed CD29, but not CD34. hBMSCs, human bone marrow-derived mesenchymal stem cells; PE, phycoerythrin; CD29, integrin-β1; CD34, hematopoietic progenitor cell antigen CD34.

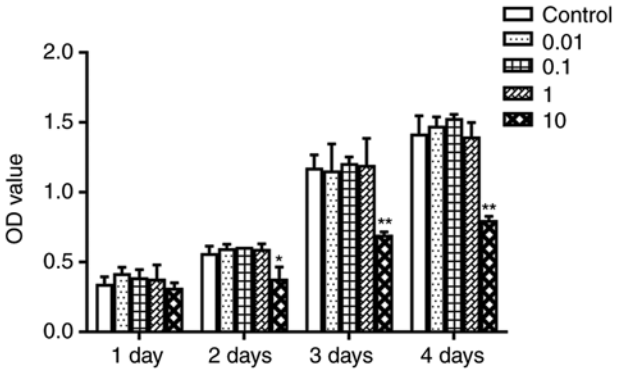

Figure 2. Effects of icaritin on hBMSC proliferation measured by CCK-8 assay. The cells were incubated with icaritin (0.01-10 μM) for 1, 2, 3 and 4 days. At each time point, CCK-8 was added to each group to detect the proliferation ability. All data are presented as mean ± standard deviation from 3 independent experiments. *P<0.05 and **P<0.01 vs. control group. hBMSCs, human bone marrow-derived mesenchymal stem cells; CCK-8, Cell Counting Kit-8; OD, optical density.

be used to detect the early stage of osteogenesis. Therefore, the ALP activity of BMSCs treated with or without icaritin was examined at 7 days after osteogenic induction. The results demonstrated that ALP activity in the icaritin group was increased almost 1.8-fold compared with that in the control group (Fig. 3C). RT-qPCR was also used to detect the osteogenic gene expression at 7 days. The results revealed that the levels of osteogenic genes (*OCN*, *Runx2* and *ALP*) in the icaritin group were significantly increased compared with those in the control group (Fig. 3D-F). The results suggested that icaritin significantly enhanced osteogenesis of hBMSCs.

Effects of icaritin on mRNA and protein levels of osteogenic genes. The effects of icaritin on the mRNA levels of *OCN*, *Runx2*, *ALP* and *β-catenin* were further determined at days 3, 7 and 14. The RT-qPCR results suggested that icaritin upregulated *OCN* at days 7 and 14, and *Runx2* at day 7, and increased ALP and β-catenin transcript levels at days 3 and 7 (Fig. 4A-D). However, icaritin decreased the expression level of *SOST* at days 7 and 14 (Fig. 4E). In addition, the data from the western blot analysis indicated that icaritin exhibit similar effects on the expression levels of osteogenic proteins as with the mRNA levels (Fig. 5).

Lentivirus-mediated SOST overexpression reverses the effects of icaritin on osteogenic differentiation. To investigate *SOST* function, hBMSCs were transfected with lentiviruses encoded with the *SOST* gene to overexpress *SOST*. The mRNA and protein expression levels of SOST were significantly increased in the *SOST* overexpression group compared with in the vector control group, as determined by RT-qPCR and western blot analysis. Furthermore, overexpression of *SOST* partly inhibited the icaritin-induced increase of ARS level, ALP activity and osteogenic gene expression. As demonstrated in Fig. 6, the ARS level and ALP activity in the BMSCs-vector + icaritin group were significantly increased compared with that in the BMSCs-SOST + icaritin group. In addition, the BMSCs-SOST + icaritin group also exhibited decreased levels of *β-catenin* and osteogenic genes including *OCN*, *Runx2* and *ALP*.

Icaritin suppresses SOST protein expression mediated by the Wnt/ERα signaling pathway in hBMSCs. To examine whether the Wnt/ERα signaling pathway was involved in icaritin-induced suppression of SOST, the protein level of

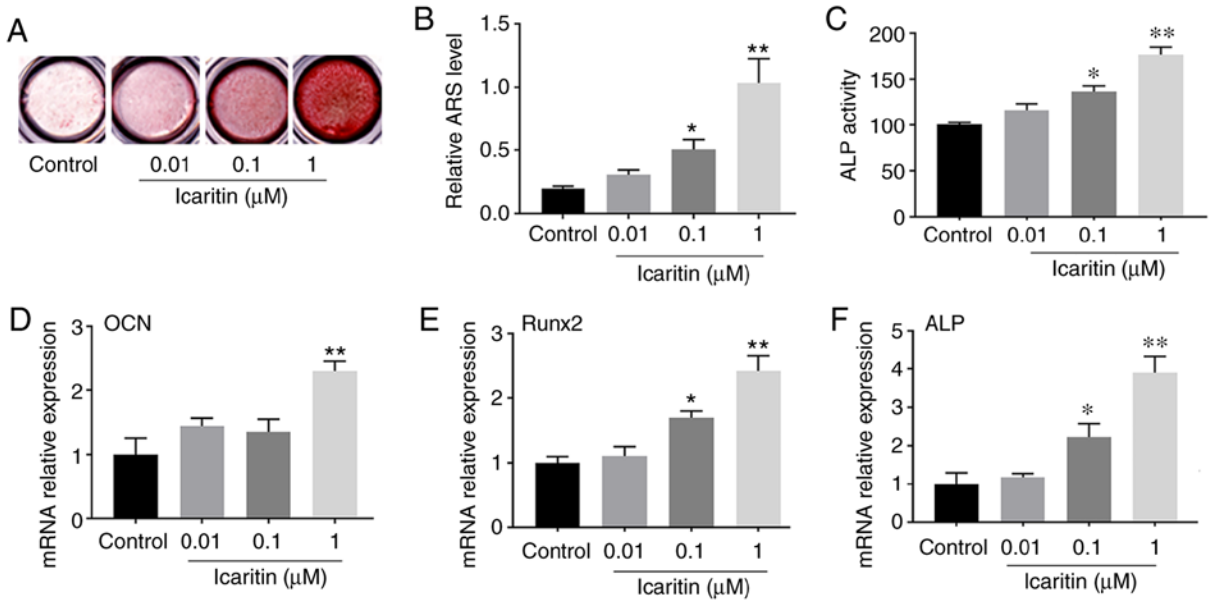

Figure 3. Icaritin promotes osteogenesis on hBMSCs. (A) Alizarin Red S stainingto visualize mineralization was applied following treatment of the hBMSCs with icaritin at various concentrations (0.01-1 μM) for 21 days. Magnification, x10. (B) The mineralization of hBMSCs of Alizarin red S was quantified. (C) ALP activity of hBMSCs treated with icaritin for 7 days. (D-F) hBMSCs were treated with Icaritin for 3 days, and the mRNA levels of (D) OCN, (E) Runx2 and (F) Alp were determined by reverse transcription quantitative polymerase chain reaction. Data are presented as mean ± standard deviation (n=3). *P<0.05 and **P<0.01 vs. control group. hBMSCs, human bone marrow-derived mesenchymal stem cells; ALP; OCN, osteocalcin; Runx2, RUNX family transcription factor 2; Alp, alkaline phosphatase.

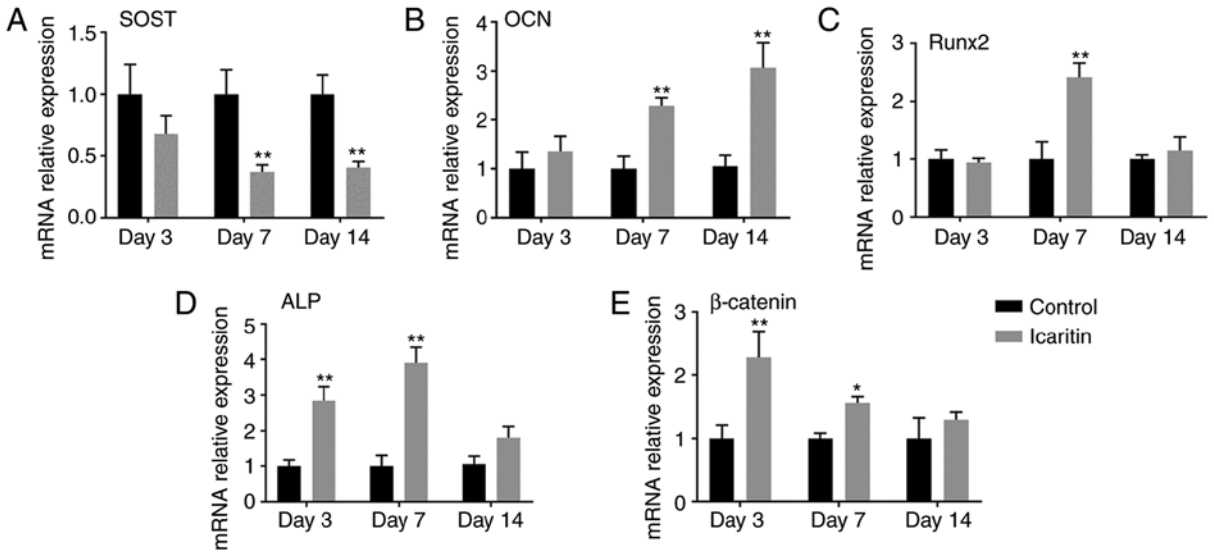

Figure 4. Icaritin (1 μM) increases mRNA levels of osteogenic marker genes. (A) *SOST*, (B) *OCN*, (C) *Runx2*, (D) *Alp* and (E) *β-catenin* at the different time points of osteogenesis of human bone marrow-derived mesenchymal stem cells. DMSO was used as the control group. Data are presented as the mean ± standard deviation (n=3). *P<0.05 and **P<0.01 vs. control group at the same stage. SOST, sclerostin; OCN, osteocalcin; Runx2, RUNX family transcription factor 2; Alp, alkaline phosphatase.

SOST in hBMSCs treated with icaritin and the Wnt inhibitor, ICI 182,780, which causes ERα degradation, were detected by western blot analysis at days 3, 7 and 14. Icaritin decreased the protein expression of SOST significantly at day 3, while the decrease was not that marked at days 7 and 14. ICI 182,780 treatment partially reversed the suppression of SOST caused by icaritin, to levels close to those of the control group. The results of the present study indicated that icaritin decreased SOST expression and was regulated via the Wnt/ERα signaling pathway (Fig. 7A and B). To confirm these data, a immunofluorescence assay was conducted; a decreased SOST (green) fluorescence signal in the cells treated with icaritin was observed, and pretreatment with ICI 182780 was demonstrated to reverse the process (Fig. 8).

Discussion

The results of the present study demonstrated that the small phytomolecule icaritin enhanced osteogenic differentiation of hBMSCs *in vitro*, with the greatest effect demonstrated

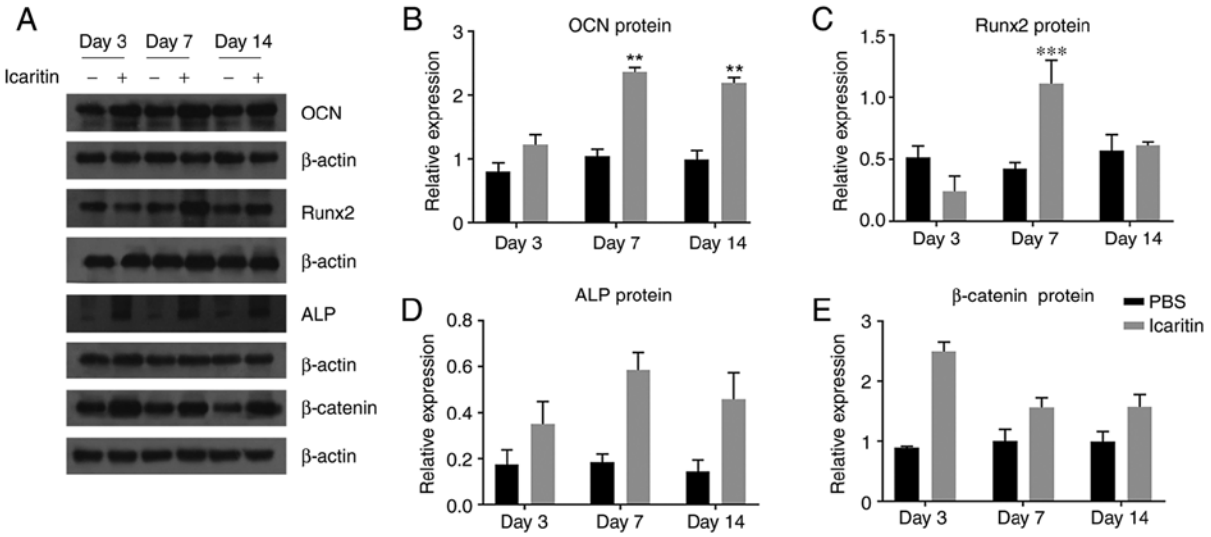

Figure 5. Icaritin (1 μM) increases protein levels of osteogenic genes. (A) Human bone marrow-derived mesenchymal stem cells were cultured in OIM for 3, 7, 14 days, and OCN, Runx2, ALP, β-catenin protein levels were measured. DMSO was used as control group. (B-E) Band density of (B) OCN, (C) Runx2, (D) ALP and (E) β-catenin were quantified by densitometry. Data are presented as mean ± standard deviation (n=3). **P<0.01, ***P<0.001 vs. control group at the same day. OCN, osteocalcin; Runx2, RUNX family transcription factor 2; Alp, alkaline phosphatase.

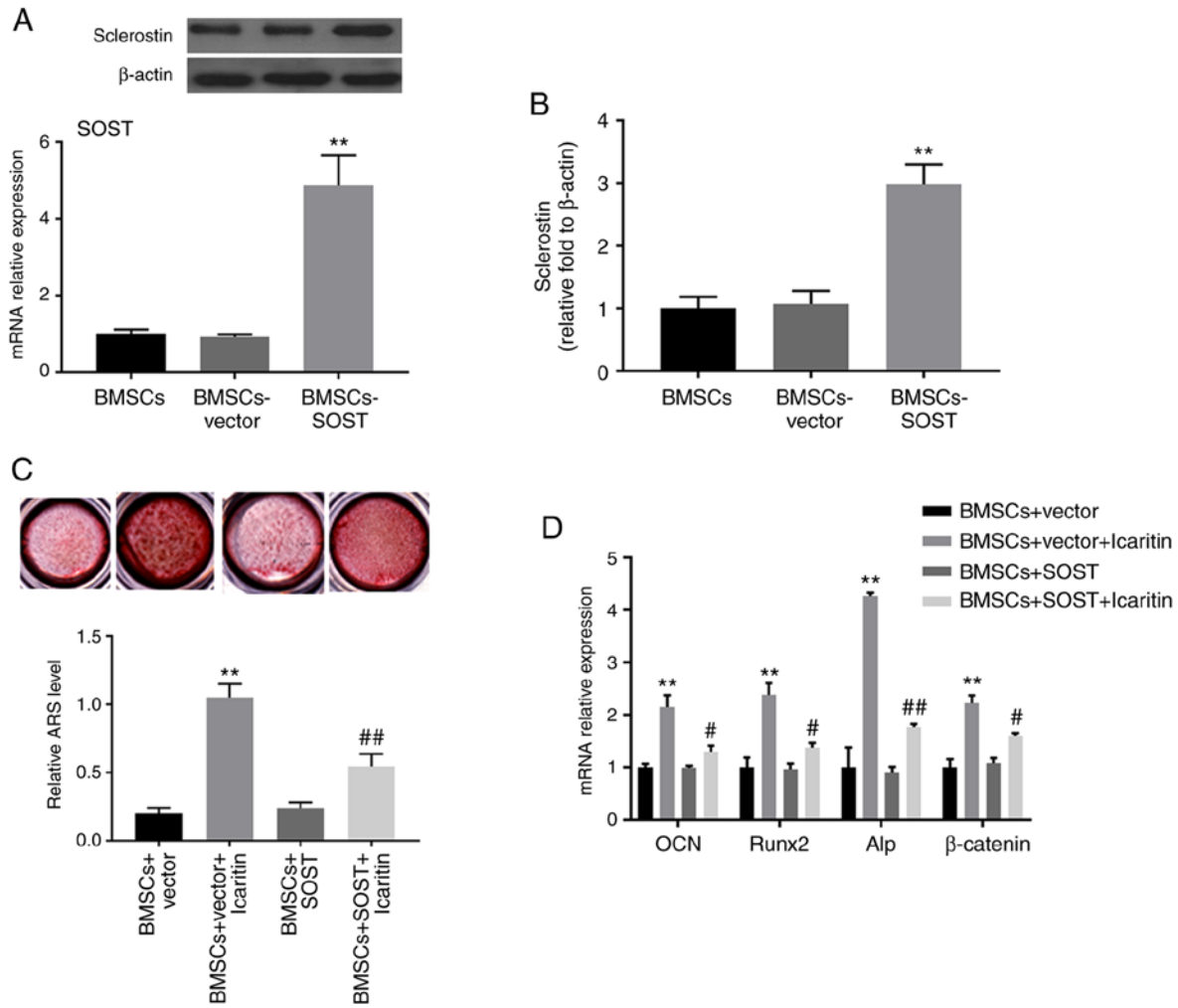

Figure 6. SOST overexpression reverses icaritin-induced osteogenesis of hBMSCs. (A) Western blot analysis for the protein level of SOST. (B) The mRNA expression of *SOST* in BMSCs, BMSCs-vector, and BMSCs-SOST. (C) Mineralization in cultured hBMSCs in BMSCs-vector and BMSCs-SOST groups with or without icaritin were detected at day 14. Magnification, x10. (D) The mRNA levels of OCN, Runx2, Alp and β-actin were determined by reverse transcription quantitative polymerase chain reaction. Data are presented as mean ± standard deviation (n=3). **P<0.01 vs. BMSCs group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. BMSCs-vector group. SOST, sclerostin; BMSCs, bone marrow-derived mesenchymal stem cells; OCN, osteocalcin; Runx2, RUNX family transcription factor 2; Alp, alkaline phosphatase.

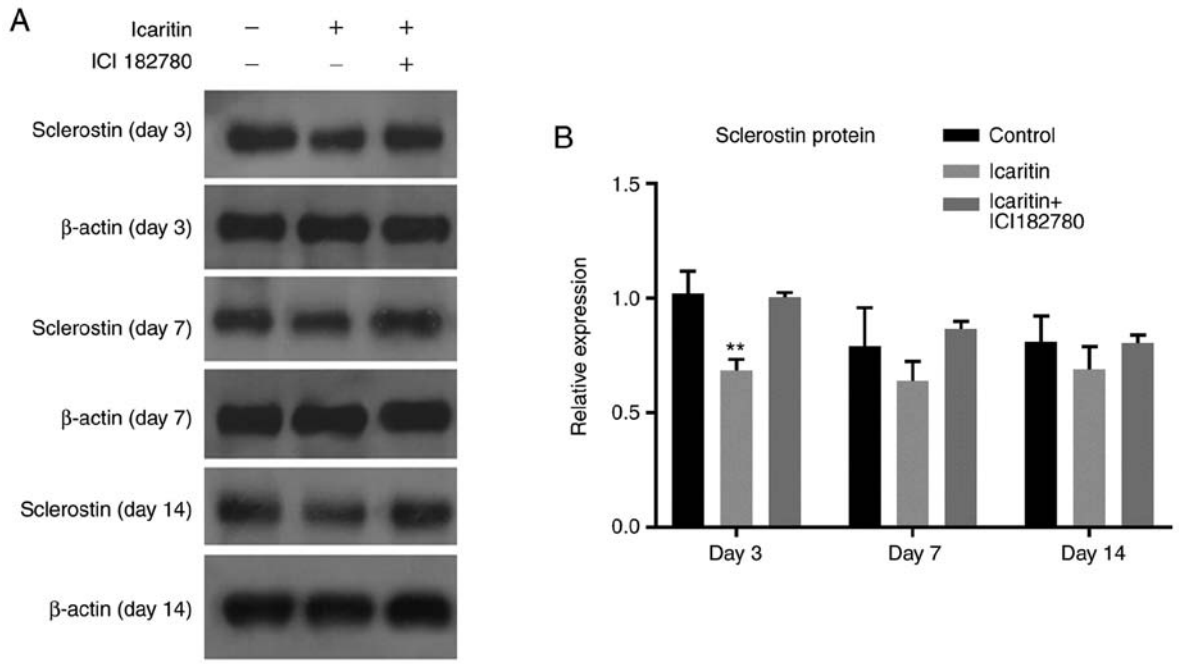

Figure 7. Icaritin downregulates SOST, which is reversed by ICI 182780. hBMSCs were cultured in osteogenic induction medium for 3, 7 and 14 days, and SOST protein levels were measured. DMSO was served as the control group. (A) SOST protein expression was measured by western blot analysis. (B) Band density of SOST was quantified by densitometry. Data are presented as mean ± standard deviation (n=3). **P<0.01 vs. control group at the same day. SOST, sclerostin; BMSCs, bone marrow-derived mesenchymal stem cells; OCN, osteocalcin; Runx2, RUNX family transcription factor 2; Alp, alkaline phosphatase.

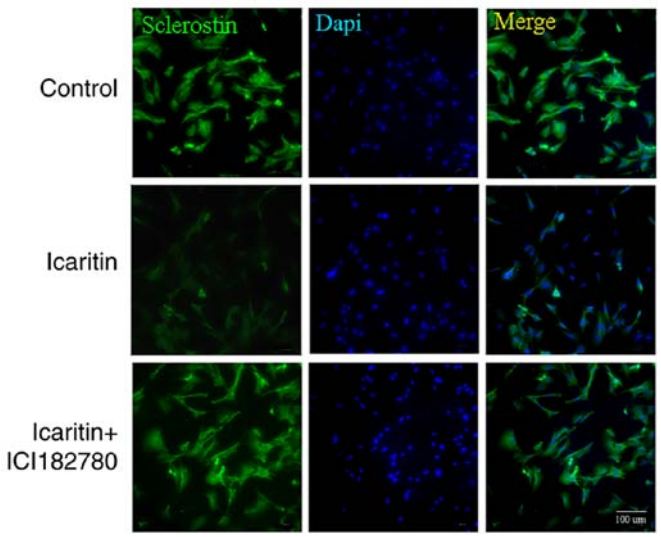

Figure 8. Effects of icaritin + ICI 182780 on the expression of sclerostin detected by immunofluorescence staining. hBMSCs were divided into three groups: Icaritin; icaritin + ICI 182780; and DMSO. Following culture in osteogenic induction medium for 14 days, all the groups were collected for immunofluorescence staining. Sclerostin was labeled with green fluorescence and the nuclei were stained with DAPI.

at 1 μM concentration. The results also demonstrated that SOST served a crucial role in icaritin-induced osteogenesis of hBMSCs. Icaritin downregulated SOST expression at mRNA and protein level, as demonstrated by the RT-qPCR and western blot analysis. Overexpression of SOST inhibited icaritin-induced osteogenic differentiation. It was also identified that SOST expression was mediated through icaritin-induced activation of the Wnt/β-catenin and ERα signaling pathways. These results suggest that SOST is a key factor in the icaritin-mediated osteogenesis of hBMSCs.

Herba epimedii is used for curing bone-associated disorders in China (10). Icariin is considered to be the major pharmacologically active component and it promotes osteogenesis of hBMSCs (10). Recent studies have revealed that the pharmacological effects of icaritin, which is an intestinal metabolism of icariin, were more potent than icariin *in vitro* (11,12). In the present study, it was first demonstrated icaritin had no effects on the proliferative ability or viability of hBMSCs at a wide range of concentrations (0.01-1 μM). The dose would be much lower if used *in vivo*, suggesting that icaritin may achieve a bio-safety level for clinical use. Whether icaritin enhanced osteogenesis of BMSCs was further explored through the detection of ALP activity and calcium nodule formation. The results demonstrated that icaritin promoted both of these osteoblast markers. Next, the effects of icaritin on osteogenic marker genes of hBMSCs were examined. It was identified that the levels of ALP, Runx2 and OCN were elevated following icaritin treatment. ALP, an early marker of osteogenesis, is a key regulator in bone formation (16), and is able to produce phosphate, which reacts with calcium to form hydroxyapatite, further promoting mineralization. Runx2, a pivotal transcription regulator, is the runt family transcription factor, which serves an important role in osteogenesis (17,18). *OCN* is a marker gene of late stage osteoblast differentiation (17). Icaritin significantly increased the mRNA and protein levels of these osteogenic marker genes, suggesting that icaritin promoted osteogenesis of hBMSCs.

The Wnt/β-catenin pathway is well-known as a key axis for modulating bone mass (13). *SOST*, the Wnt/β-catenin regulator, inhibits bone formation by exerting antagonistic effects

on Wnt pathway. Antagonism or loss of the SOST will result in elevated bone formation and a high bone mass (19,20). The SOST antibody (SclAb) has attracted attention as an anabolic strategy for treating post-menopausal osteoporosis (21). Furthermore, in phase II (ClinicalTrials.gov identifier: NCT01059435) (22) and III clinical trials (ClinicalTrials.gov identifier: NCT01575834) (23), SclAb increased bone mass and strength in osteoporotic women. The results of the present study indicated that icaritin downregulated the mRNA and protein levels of *SOST* and upregulated the mRNA level of *β-catenin*. When *SOST* was overexpressed in hBMSCs, the mRNA level of *β-catenin* decreased, which is consistent with previous data indicating that SOST antagonizes the Wnt signaling pathway (9). To the best of our knowledge, the present study demonstrated for the first time that overexpression of SOST diminishes the icaritin-induced increases in ALP activity, ARS level and osteogenic genes.

To determine whether the Wnt/ERα signaling pathway was involved in icaritin-induced suppression of SOST, hBMSCs were treated with icaritin and the Wnt inhibitor, ICI 182,780, which causes ERα degradation (24). The results demonstrated that icaritin treatment decreased the levels of SOST protein expression, while treatment with ICI 182,780 partly reversed the icaritin-mediated suppression of SOST, indicating that the Wnt/β-catenin and ERα signaling pathways were involved in icaritin-induced *SOST* suppression. These results were consistent with previous data suggesting that estrogen-mediated suppression of *SOST* expression was associated with the Wnt/β-catenin/ERα axis in human osteoblasts (24).

Stem cell transplantation has been applied in clinical settings for the treatment of multiple conditions, such as diabetic neuropathy (25) and leukemia (26). However, stem cell transplantation alone has exhibited limited satisfactory results Previously, the combination of stem cells with other compounds with osteogenic effects may provide improved therapeutic effects. Icariin, which has been suggested to exhibit weaker pharmacological effects (12) was demonstrated to result in significantly decreased levels of bone loss in postmenopausal women (27). Based on these data, we hypothesized that icaritin, which had significant osteogenic effects, may also be used for decreasing the bone loss in postmenopausal women.

In conclusion, icaritin induced osteogenesis in hBMSCs by suppressing SOST expression, and icaritin-induced suppression of *SOST* was regulated in part via the Wnt/β-catenin and ERα pathways. The results of the present study suggested that icaritin may be a promising agent for diseases caused by decreased levels of osteogenic differentiation of BMSCs, such as osteoporosis or osteonecrosis of the femoral head.

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Availability of data and materials

All the materials included in the manuscript, including all relevant raw data, may be made freely available to any researchers who wish to use them for non-commercial purposes, while preserving any necessary confidentiality and anonymity.

Authors' contributions

YC conceived and supervised the project. BW and HH were in charge of the PCR, western blotting, flow cytometry and immunofluorescence staining. QW was responsible for the experimental design, data analysis, drafting and revision of the article. CX analyzed data. LL and JG were responsible for the culture of the hBMSCs, ALP/Alizarin Red S staining, CCK-8 assay and lentiviral transduction. All authors read and approved the final manuscript.

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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