Cell Proliferation

Cell Prolif., 2015, **48**, 375–384 doi: 10.1111/cpr.12185

Icariin induces osteogenic differentiation of bone mesenchymal stem cells in a MAPK-dependent manner

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Received 30 August 2014; revision accepted 14 December 2014

Abstract

Objectives: Icariin, a flavonoid isolated from *Epimedium pubescens*, has previously been identified to exert beneficial effects on preventing bone loss and promoting bone regeneration. However, molecular mechanisms for its anabolic action have, up to now, remained largely unknown.

Materials and methods: Effects of icariin on cell proliferation and osteogenic differentiation of rat bone mesenchymal stem cells (BMSCs) were systematically evaluated. To characterize underlying mechanisms, its effects on mitogen-activated protein kinase (MAPK) signalling pathways were determined.

Results: Results showed that icariin might not have enhanced effects on cell proliferation. However, it seemed to significantly enhance osteogenic differentiation of BMSCs, demonstrated by increasing alkaline phosphatase (ALP) activity and gene expression of collagen type I (*Col I*), osteocalcin (*OCN*) and osteopotin (*OPN*). It was demonstrated that icariin rapidly phosphorylated extracellular signal-regulated kinase (ERK), p38 kinase and c-Jun N terminal kinase (JNK). Furthermore, icariin-stimulated osteogenic effects on BMSCs were dramatically attenuated by treatment with either specific ERK inhibitor of PD98059, p38 inhibitor of SB202190 or JNK inhibitor SP600125.

Conclusions: These results provide a potential mechanism of anabolic activity of icariin on BMSCs involving ERK, p38 and JNK MAPK pathways.

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Introduction

Bone fractures or defects resulting from, for example, ageing, traffic accidents or bone cancer, have become increasingly prevalent and remained a challenge for surgeons. As growth factors play an important role in the process of bone formation, a range of exogenous osteoinductive growth factors has already been successfully applied in surgery to promote bone repair. However, with consideration to high costs of production, high quality preservation conditions as well as limited active periods, clinical application of exogenous growth factors has been limited. Over recent years, traditional Chinese medicine, an empirical system of multicomponent therapeutics (1), can potentially meet the demands of treating bone fractures and bone defects, and provide a remedy with exogenous growth factors.

The herb *Epimedium pubescens*, recorded in the Chinese pharmacopoeia as Chinese medicine "yinyanghuo", has been used in Chinese traditional medicine for treatment of osteoporosis, kidney, joint and liver disorders. Icariin, the main active flavonoid glucoside isolated from the herb, has been identified to exert beneficial effects on preventing postmenopausal bone loss (2). Studies on rat models have indicated that icariin could prevent ovariectomy (OVX)-induced bone loss, and reduction in femoral and tibial strength (3,4). Recent studies have demonstrated that icariin can improve maturation and mineralization of osteoblasts, and enhance osteoblastic differentiation of bone

mesenchymal stem cells (BMSCs) *in vitro* (5–7). However, systematic effects and underlying mechanisms of icariin on BMSCs have, up to now, largely been unknown.

Mitogen-activated protein kinases (MAPKs) compose the family of messengers that convey signals from the cell surface to nucleus, in response to a wide range of stimuli, including hormones, chemicals and stress (8). MAPK signalling cascades modulate gene expression to regulate proliferation, differentiation and apoptosis (9). Extracellular signal-regulated kinase (ERK), p38 kinase and c-Jun N terminal kinase (JNK) form the three major families of MAPKs and each of these has its own subfamilies. The ERK signalling pathway has been intensively investigated in the regulation of osteogenic differentiation of BMSCs. Previous study has shown that commitment of hBMSCs to osteogenic or adipogenic lineages is governed by activation or inhibition, respectively, of ERK (10). Meanwhile, inhibiting or activating p38 in turn inhibits or promotes osteogenic differentiation *in vitro*, making it plausible that the p38 signalling pathway is a mediator in the process of osteogenic differentiation (11). Previous study has also revealed that the p38 signalling pathway contributes to osteoblast functions *in vitro* and corresponding bone mineralization *in vivo* (12). Different MAP kinases are activated by phosphorylation, stimulated by upstream kinases activated by further extracellular stimuli. Also, it has been shown that numerous bone-active agents, including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and insulin-like growth factor-I (IGF-I) can induce osteogenic differentiation *via* the ERK signalling pathway (13,14). Other studies demonstrated that a number of cytokines can activate the p38 signalling pathway in osteoblasts, and subsequently promote osteogenic proliferation and differentiation (15,16). Additionally, JNK signalling has been reported to play an important role in effects of osteoblast differentiation of human periosteal-derived cells (17).

In the present study, our hypothesis was that icariin could induce osteogenic differentiation of BMSCs in a dose-dependent manner, related to MAPK signalling pathways. To verify this, MTT assay, cell cycle analysis, assay of apoptosis, alkaline phosphatase (ALP) activity and real-time PCR analysis for osteogenesis-related markers, were performed to evaluate cell proliferation and osteogenic differentiation of rat BMSCs, treated with different concentrations of icariin. Furthermore, ERK, p38 and JNK signalling pathways were explored to discover whether the MAPK signalling pathway could be activated by icariin.

Methods and materials

Animal and cell culture

Tibiae and femurs were dissected from euthanised 160 ± 10 g, male Sprague–Dawley rats (Shanghai SLAC Experimental Animal Center, Shanghai, China) and bone marrow was flushed out using Dulbecco's modified Eagle's medium (DMEM; Hyclone, Waltham, MA, USA) supplemented with 100 unit/ml penicillin and 100 μg/ml streptomycin (Hyclone). To remove blood cells, washouts were collected and centrifuged at 500 ***g*** for 10 min. The precipitate was then mixed with complete DMEM supplemented with 10% foetal bovine serum (FBS; Hyclone), and plated into culture flasks maintained at 37 °C in 5% CO_2. Non-adherent cells were removed by changing medium every 3 days. When large colonies formed and became confluent, primary rat BMSCs were trypsinized with 10% trypsin–ethylene diamine tetraacetic acid (EDTA; Hyclone) and passaged. BMSCs of passage 2–3 were used for the experiments.

Cytotoxicity evaluation (LD50)

BMSCs were seeded in 96-well plates at 5×10^3cells/well. After 24-h incubation, cells were treated with 0, 10, 20, 40, 80 and 160 μM icariin respectively. At 24 h, cytotoxicity evaluation was carried out using the MTT assay. According to the manufacturer's instructions, 20 μl 5 mg/ml MTT (Amresco, Solon, OH, USA) solution was added and incubated at 37 °C for 4 h to form MTT formazan. Then, medium was replaced with 200 μl dimethyl sulphoxide (DMSO; Sigma, St. Louis, MO, USA) to dissolve the formazan, and absorbance was measured at 590 nm, using an ELX Ultra Microplate Reader (Bio-tek, Burleigh, QLD, Australia). Percentage of viable cells was obtained by comparing absorbances of samples with and without icariin.

Cell proliferation

For the cell proliferation assay, BMSCs were seeded in 96-well plates at 5×10^3 cells/well. After 24-h incubation, they were treated with icariin at concentrations of 5, 10, 20 and 40 μM for 1, 3, 5 and 7 days respectively. Equal volume of vehicle alone (1 μL DMSO/ml medium) was added to form the control group (0 μM). According to the manufacturer's instructions, cell proliferation was assessed using the MTT assay as described above. All experiments were performed in triplicate.

Cell cycle analysis

BMSCs were plated into 6-well plates at 2×10^5 cells/well and incubated for 24 h, followed by being incubated with icariin at concentrations of 0, 10, 20 and 40 μM respectively. After incubation for 3 days, both adherent and floating cells were harvested and then fixed overnight in cold 70% ethanol in phosphate-buffered saline (PBS). Cells were then treated with RNase A (Beyotime, Suzhou, China) for 30 min prior to nucleic acid staining with propidium iodide (PI, Sigma) for 5 min. Cell cycles were then analysed using a BD Calibur flow cytometer (Becton Dickinson, Franklin Lake, NJ, USA).

Assay of apoptosis

An annexin V-FITC kit (Beyotime) was used to quantify apoptosis. After being treated with the range of chosen concentrations of icariin, for 3 days, BMSCs cultured in 6-well plates were harvested, washed in PBS at 4 °C, then resuspended in 300 μl binding buffer containing 5 μl annexin V-FITC and 10 μl PI. After 15 min incubation at room temperature, stained cells were analysed by flow cytometry (Becton Dickinson).

Real-time PCR assay

BMSCs were plated on 12-well plates at 1×10^5 cells/well, and incubated for 24 h, followed by incubation with icariin at concentrations of 0, 10, 20 and 40 μM respectively. Total RNA was isolated after icariin treatment for 3, 6, 12 and 24 h, using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommended protocol. RNA concentrations were determined using a NanoDrop spectrophotometer (Thermo, Logan, UT, USA). Complimentary DNA (cDNA) was synthesized by means of cDNA Synthesis Reverse Transcription Kit (Fermentas; Thermo). Real-time PCR assay for runt-related transcription factor 2 (Runx2), Collagen I (Col I), osteopontin (OPN) and osteocalcin (OCN) was performed using a Light-Cycler system with SYBR Premix Ex Taq™ (Takara, Japan), according to the manufacturer's instructions. Conditions of real-time PCR were as follows: denaturation at 95 °C for 10 s; 50 cycles at 95 °C for 10 s and 60 °C for 30 s; and a final dissociation stage (95 °C for 5 min) was added at the end of the amplification procedure. β-actin was used as internal control. Data were analysed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method and expressed as fold change respective to control. Each sample was analysed in triplicate. Primer sequences used are listed in Table 1.

Table 1. List of primers used and respective forward and reverse sequences

Gene	Forward sequence Reverse sequence
β-actin	5′-GTAAAGACCTCTATGCCAACA-3′ 5′-GGACTCATCGTACTCCTGCT-3′
Runx2	5′-ATCCAGCCACCTTCACTTACACC-3′ 5′-GGGACCATTGGGAACTGATAGG-3′
Collagen type I(COL I)	5′-CTGCCCAGAAGAATATGTATCACC-3′ 5′-GAAGCAAAGTTTCCTCCAAGACC-3′
Osteopontin(OPN)	5′-CCAAGCGTGGAAACACACAGCC-3′ 5′-GGCTTTGGAACTCGCCTGACTG-3′
Osteocalcin(OCN)	5′-GCCCTGACTGCATTCTGCCTCT-3′ 5′-TCACCACCTTACTGCCCTCCTG-3′

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity, quantitation and staining assays were performed at day 7 after BMSC treatment with icariin at concentrations of 0, 10, 20 and 40 μM respectively. Samples of all groups were incubated in p-nitrophenyl phosphate (pNPP) (Sigma) at 37 °C for 30 min. Absorbance values (OD) were recorded at 405 nm to determine ALP activity. Total protein contents were assessed using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA), and OD values were normalized to bovine serum albumin (BSA; Sigma) standard curve, at 590 nm. ALP activity was accessed as OD value at 405 nm per milligram of total protein. Meanwhile, ALP staining was also performed according to the manufacturer's instructions (Beyotime). Each sample was rinsed three times in PBS and fixed in 4% paraformaldehyde for 15 min. Samples were soaked in 0.1% naphthol AS-MX phosphate and 0.1% fast red violet LB salt, in 56 mM 2-amino-2-methyl-1,3-propanediol, for 45 min at 37 °C, then observed using a digital camera (ECLIPSETS 100, NIKON, Tokyo, Japan). All experiments were performed in triplicate.

Western blotting

For the MAPK signalling pathway, BMSCs were cultured in medium of 20 μM icariin for 0, 15, 30, 60 and 120 min. For protein expression of Runx2 and OPN, BMSCs were cultured in 20 μM icariin medium for 0, 3, 6, 12 and 24 h. Cells were lysed on ice for 30 min in RIPA lysis buffer (Thermo, DE) supplemented with protease inhibitor cocktail, phosphatase inhibitor cocktail and phenylmethanesulphonyl fluoride (PMSF) (Kangchen, Shanghai, China). Protein concentration was measured using a BSA protein assay kit. Of the sample, 20 μg was resolved on 10% SDS-PAGE gel and electro-transferred to polyvinylidene difluoride membrane

(PVDF, Pall, New York, USA). Membranes were blocked and incubated with appropriate primary antibodies including rabbit anti-rat ERK, p38, JNK, phosphorylated-ERK (p-ERK), phosphorylated-p38 (p-p38), phosphorylated-JNK (p-JNK) (CST, USA), Runx2 (ablcam, USA) and OPN (CST, USA) at dilution of 1:1000. For normalization of protein loading, mouse anti-rat β-actin (Sigma) antibody was used at 1:10000 dilution. Finally, membrane reactions were visualized using horseradish peroxidase (HRP)-conjugated secondary antibodies (Beyotime, dilution, 1:1000) with ECL plus reagents (Amersham Pharmacia Biotech, Buckinghamshire, England) by UVItec ALLIANCE 4.7 gel imaging system. Protein band intensities on scanned films were compared to their respective controls using Quantity One Image software. Bands were first rounded up using a volume rect tool, then target area intensity was calculated. Density of β-actin was used as control for protein expression of Runx2 and OPN. Densities of ERK, p38 and JNK were quantified as control groups for protein expression of p-ERK, p-p38 and p-JNK respectively.

ERK, p38 and JNK inhibitor treatment analysis

BMSCs treated with icariin at concentrations of 0 and 20 μM were cultured in medium supplemented with ERK signalling pathway inhibitor PD98059 (CST), p38 signalling pathway inhibitor SB202190 (CST) or JNK signalling pathway inhibitor SP600125 (CST), final concentration 20 μM, for 7 days respectively. Then, total RNA was isolated and cDNA was synthesized, and real-time PCR was performed on Collagen I, OCN, OPN as specified above. In addition, ALP activity was measured by ALP staining assay as described above. Meanwhile, BMSCs treated with icariin at concentrations of 0 and 20 μM, were cultured in medium without ERK, p38 or JNK inhibitor, identified as control groups respectively.

Statistical analysis

All experiments were performed a minimum of three times. All measurements are expressed as mean ± SD. Significant differences between groups were determined using ANOVA (SPSS, v.17.5; Chicago, IL, USA), while $P < 0.05$ denotes statistical significance.

Results

Icariin exerted no enhanced effect on proliferation of BMSCs

LD50 assay was applied to explore appropriate concentration of icariin for this *in vitro* study and concentrations of 80 and 160 μM led to more than half BMSCs succumbing to apoptosis (Fig. 1a). MTT assay had negative effect on BMSC proliferation in icariin-treated groups from day 1 to day 7 (Fig. 1b), particularly at 40 μM concentration. There were no signs of cell proliferation of BMSCs treated with icariin at 5–40 μM with cell cycle detection (Fig. 1c). As shown in Fig. 1d and 1e, icariin at 40 μM concentration induced significantly higher levels BMSC apoptosis. Levels of BMSC apoptosis treated with icariin at 40 μM were 9.87 ± 0.18%, while the control group was 6.935 ± 0.94% ($P < 0.05$). However, icariin at other concentrations had no significant effect on level of apoptosis compared to the control group. These results indicate that icariin had no effect on enhancing cell proliferation of BMSCs, and little effect on their apoptosis. Proliferation-inhibition effect of icariin was in a concentration-dependent manner, identified by cytotoxicity of BMSCs induced by icariin at concentration of more than 40 μM.

Icariin enhanced osteogenic differentiation of BMSCs

Osteogenic differentiation of BMSCs was evaluated by real-time PCR and ALP activity. Over the range of 5–40 μM, icariin enhanced mRNA expression of *Runx2, Col I*, *OCN* and *OPN* of BMSCs, in a dose-dependent manner, at concentrations of 20 and 40 μM it achieved higher values (Fig. 2a–d). mRNA expression of Runx2 was promoted by almost eight times over early stages of icariin treatment at concentrations of 20 and 40 μM; then this increasing tendency slowed down as treatment time extended. In contrast, mRNA expression of *OCN* of icariin-treated groups increased over the whole culture time, and icariin of 20 μM group reached 6.36 ± 0.78 times compared to the control group after 24-h treatment ($P < 0.01$). Similar to *Runx2,* mRNA expression of *Col I* was promoted significantly by icariin at concentrations from 10 to 40 μM after 3-h treatment. This was specially obvious for 20 μM concentration, at which *Col I* mRNA expression was induced more than 10 times compared to the control group. Then, *Col I* mRNA expression fell back to normal levels after 12-h treatment. *OPN* mRNA expression was also significantly promoted by icariin at concentrations of 20 and 40 μM. In contrast to the others, increase in *OPN* mRNA expression at concentrations of 20 and 40 μM remained stable from 3 to 12 h, whereas they all fell back to normal levels after 24-h treatment. Similar to mRNA expression, Runx2 protein was up-regulated at 6 h after treatment of 20 μM icariin, and then fell back to normal levels at 24 h. OPN protein increased over the first 3 h, and remained at similar levels from 6 to 24 h.

As shown in Fig. 3a, ALP staining at 7 days indicated that icariin significantly increased ALP activity,

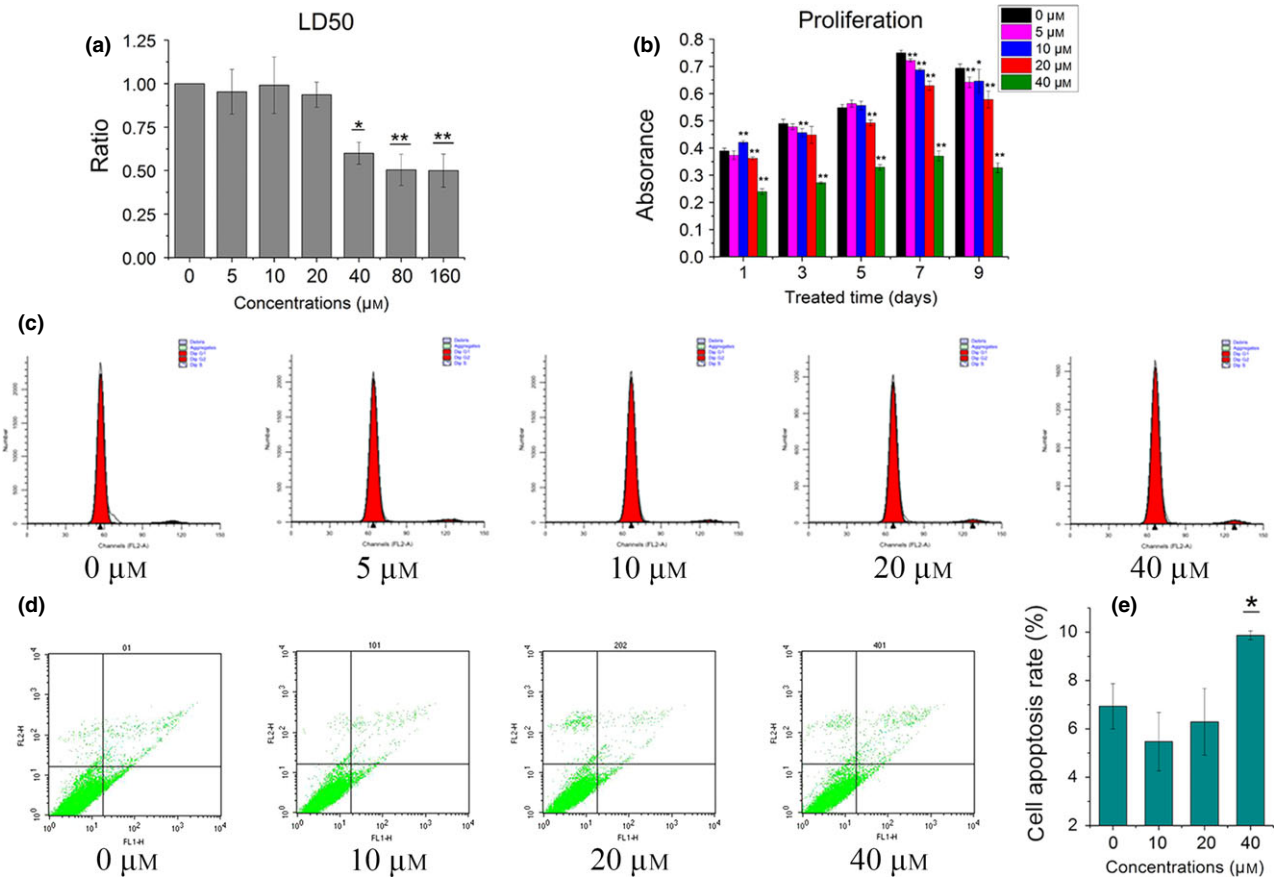

Figure 1. Proliferation and apoptosis of BMSCs treated with icariin. (a) Cytotoxicity evaluation with different concentrations of icariin (μM). (b) Proliferation of BMSCs after treatment of icariin by MTT assay. (c) Cell cycle assay of BMSCs after treatment of icariin. (d, e) Apoptosis assay of BMSCs (*as compared to 0 μM group at each time point, $*P < 0.05$, $** P < 0.01$; $n = 3$).

with its peak level at 20 μM concentration. However, staining showed ALP activity seemed to be depressed by icariin at 40 μM, while its activity quantitative assay did not demonstrate any reduction (Fig. 3b). In combination with MTT assay, it may be inferred that light staining of the 40 μM group was given rise to by lower numbers of BMSCs.

Icariin affected phosphorylation in MAPK signalling pathways

To address the role of the MAPK cascade in induction of osteogenic differentiation by icariin, we investigated protein levels of p-ERK, ERK, p-p38, p38, p-JNK and JNK under icariin-stimulated conditions at 0, 15, 30, 60 and 120 min. Dose-dependent studies revealed that optimal concentration of icariin was 20 μM, and hence this concentration was adopted in the following studies. Results of western blotting showed that icariin (20 μM) phosphorylated ERK (p-ERK) during the first 15 and 30 min ($P < 0.01$, Fig. 4a, b), while ERK level itself, seemed to be down-regulated at 15 and 30 min (Fig. 4a). p38 was phosphorylated by icariin from 15 to 120 min ($P < 0.05$, Fig. 4a, c), while p38 level was also promoted over the first 15 and 30 min (Fig. 4a). Though JNK expression had no significant change, its phosphorylated level (p-JNK) was promoted over the first 15 and 30 min (Fig. 4a, d).

ERK, p38 and JNK pathways involved in icariin-stimulated osteogenic differentiation

To further investigate the role of MAPK pathways in icariin-stimulated osteogenesis, BMSCs treated with icariin were cultured in medium supplemented with ERK signalling pathway inhibitor PD98059, p38 signalling pathway inhibitor SB202190 and JNK signalling pathway inhibitor SP600125, for 7 days, separately. Results of real-time PCR showed that except *OCN* mRNA, ERK inhibitor PD98059 did not show high inhibition of expression of osteogenic genes for BMSCs cultured without icariin. Similar to PD98059, p38 inhibitor

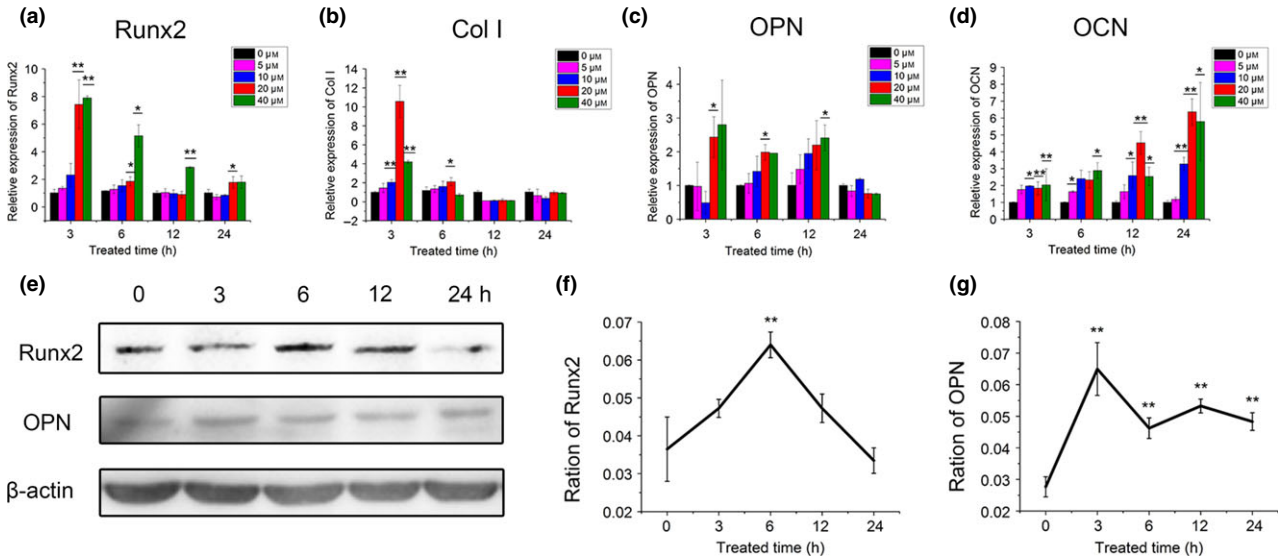

Figure 2. Protein and gene expression of osteoblastic markers in BMSCs treated with icariin. (a–d) Real-time PCR analysis of Runx2, Collagen I, OCN and OPN mRNA in BMSCs treated with icariin (*compared to 0 μM at each time point). (e) Protein expression of Runx2 and OPN at concentration of 20 μM by western blotting assay. (f, g) Densitometric analysis of Runx2 and OPN expression; β-actin density was used as control (*compared to ratio at 0 min group) (*$P < 0.05$, **$P < 0.01$; $n = 3$).

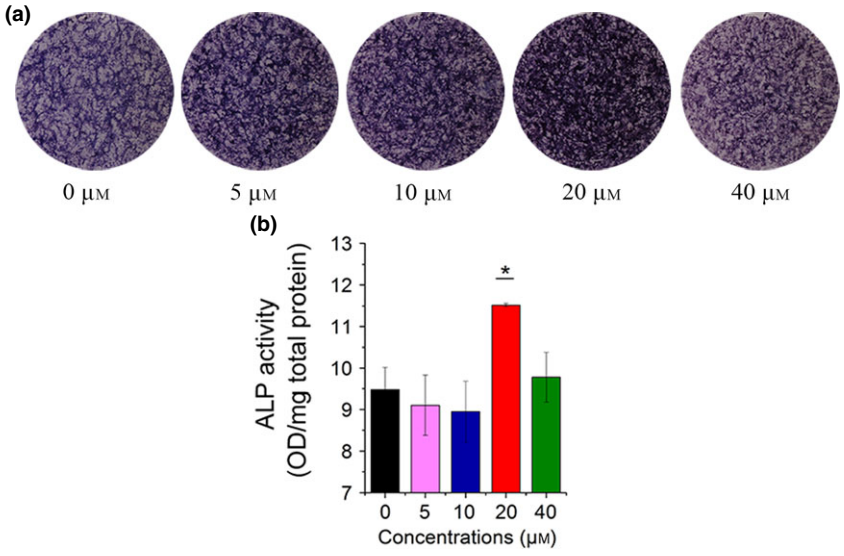

Figure 3. ALP activity of icariin-treated BMSCs on day 7. (a) ALP staining of BMSCs after treatment with icariin. (b) ALP activity quantitative assay of icariin-treated BMSCs measured by pNPP assay (*compared to 0 μM group, *$P < 0.05$, $n = 3$).

SB202190 did not show high inhibition of expression of osteogenic genes, except *OPN* mRNA of BMSCs cultured without icariin. However, enhanced mRNA expression of osteogenic genes *Collagen I*, *OPN* and *OCN* induced by icariin, was significantly inhibited by PD98059 and SB202190 respectively. JNK inhibitor SP600125 down-regulated expression of *OPN* and *OCN* mRNA cultured without icariin, and also inhibited enhanced mRNA expression of osteogenic genes *Collagen I*, *OPN* and *OCN* induced by icariin (Fig. 5a–c). ALP staining showed that ALP activity in BMSCs treated with or without icariin was inhibited after PD98059, SB202190 and SP600125 treatment (Fig. 5d). These results strongly suggest that icariin promoted osteogenic differentiation of rat BMSCs, at least in part, *via* activation of ERK, p38 and JNK signalling pathways.

Discussion

Exogenous cytokines or growth factors, essential for bone development, can effect different cell responses, such as promoting proliferation, migration and osteo-

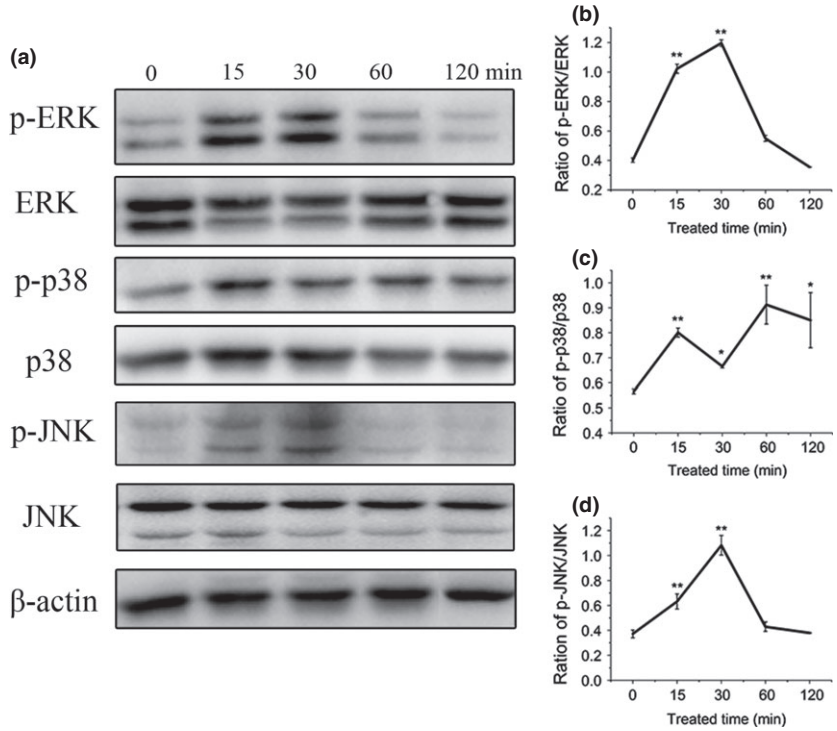

Figure 4. Effect of icariin on MAPK signalling pathways. (a) Western blotting of total and phosphorylated ERK, p38 and JNK following icariin treatment at 0, 15, 30, 60 and 120 min. (b–d) Densitometric analysis of p-ERK/ERK, p-p38/p38 and p-JNK/JNK expression by western blot analysis (*$P < 0.05$, **$P < 0.01$; *compared to phosphorylated protein expression at 0 min).

genic differentiation. Some growth factors have been used in bone regeneration, such as bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs) and platelet-derived growth factor (PDGF) (18–20). However, due to the high quality conditions for preservation and production costs, it is difficult to further promote clinical application of growth factors. Recently, specific traditional Chinese medicine, that can promote osteogenesis (with a high levels of production and low cost), has been explored extensively.

Icariin, a major active constituent in *Epimedium*, has been shown to prevent osteoporosis, and promote bone formation during mandibular distraction osteogenesis (21,22). Effects of icariin on osteoblasts are similar to its anabolic action, including induction and promotion of osteoblastic differentiation (7,23). Appropriate drug concentration *in vitro* plays a highly important role in research on its effects and mechanisms. The present study was first designed to investigate optimal concentration of icariin, to evaluate osteogenesis and its underlying mechanisms. Though there was cytotoxicity when concentration of icariin was higher than 10^{-5} M (10 μM), as reported in some studies (24), other investigations have demonstrated that icariin at 5, 10 and 50 μM did not cause any significant microglial cell viability change compared to control groups (25,26). To screen optimized concentration of icariin for inducing proliferation or osteogenic differentiation of rat BMSCs, concentrations from 5 to 160 μM were selected to test its median lethal dose in the present study. It was found that concentration higher than 80 μM induced more than half BMSC apoptosis. Moreover, both MTT assay and cell cycle analysis showed that icariin had no positive effect on proliferation of rat BMSCs, in contrast to results on osteoblasts reported by Song *et al.* (27). This may be related to the different cell type tested in the present study.

Here, icariin was found to have excellent ability to improve osteogenic differentiation and osteogenic function, as demonstrated by ALP activity and mRNA expression of *Runx2, Collagen I, OCN* and *OPN* at concentrations of 20 and 40 μM, as well as Runx2 and OPN protein levels. ALP staining and quantitative analysis demonstrated ALP activity was induced by icariin, particularly at 20 μM (Fig. 3). Runx2, an osteoblast transcriptional activator, has been reported to play an important role in regulating expression of osteoblast genes at early stages (28). Collagen I, which provides the structural framework for inorganic molecule deposition, has an apparent effect on biomechanical strength of bone tissue. In the present study, mRNA expression of *Runx2* and *Col I* in 20 and 40 μM icariin-treated groups peaked at 3 h, of the time points examined, then fell back from 6 to 24 h (Fig. 2a, b). Similar to mRNA expression, protein expression of Runx2 was shown to be up-regulated at early stages of icariin treatment.

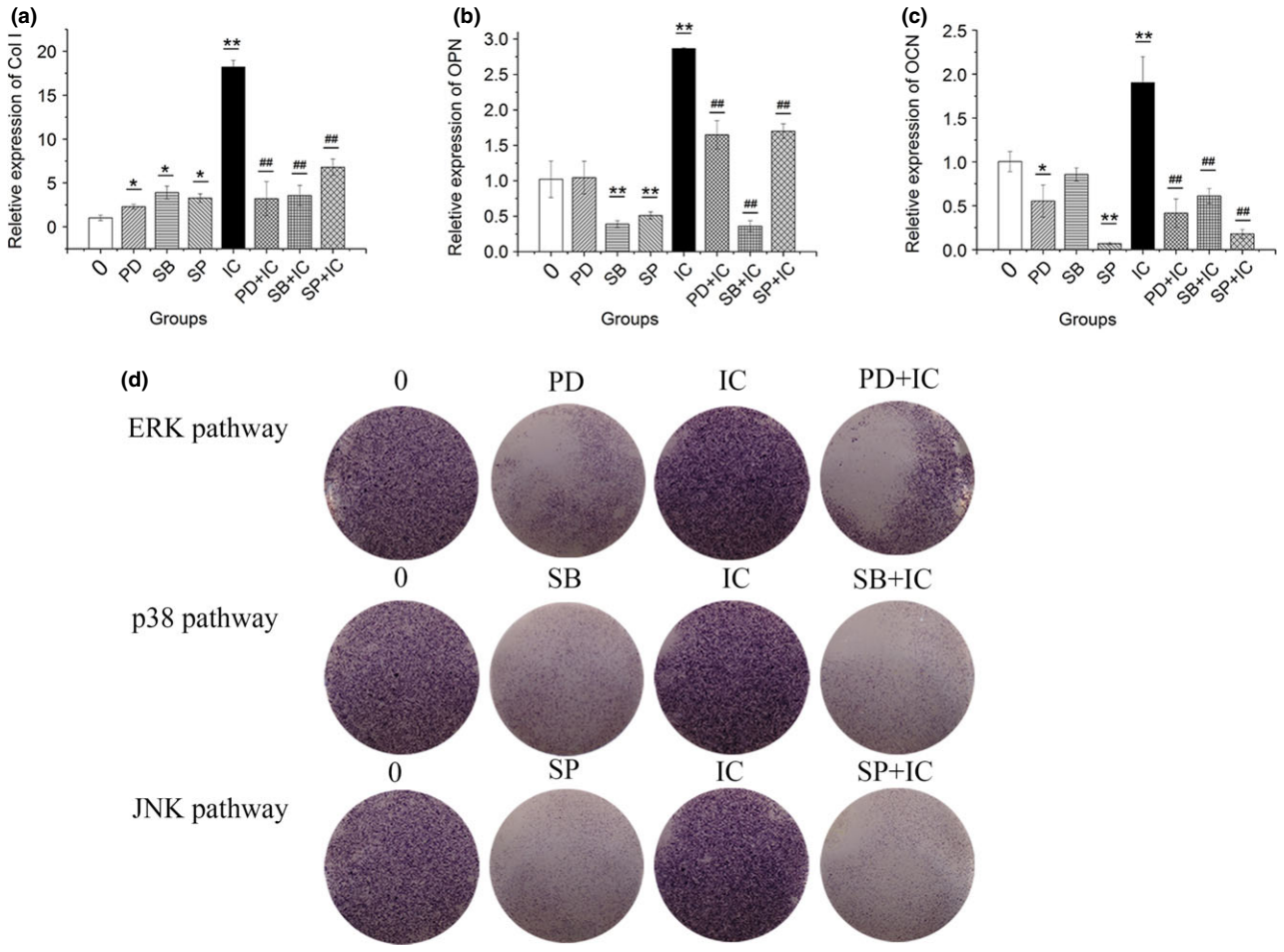

Figure 5. Effect of ERK, p38 and JNK signalling pathways on osteogenic differentiation of BMSCs treated with or without icariin. (a–c) Effect of PD98059 (20 μM, PD in short), SB202190 (20 μM, SB in short) and SP600125 (20 μM, SP in short) on osteogenic gene expression of BMSCs without or with icariin (0, IC in short respectively) treatment. (d) ALP staining assay. (*$P < 0.05$, **.$^{\#\#}P < 0.01$; *compared to 0 μM group, $^{\#}$compared to icariin group).

OPN, as an intermediate or relatively earlier marker of osteogenic differentiation, is associated with the maturation stage of osteoblasts during attachment, and matrix synthesis before mineralization. The present study showed that *OPN* mRNA expression in icariin-treated groups at 20 and 40 μM was promoted from 3 to 12 h, while it almost maintained at the same level for the control group at 24 h (Fig. 2c). OPN protein expression was also up-regulated in the 20 μM group. As late marker of osteogenic differentiation, OCN is related to matrix deposition and mineralization. In the present study, icariin caused increase in *OCN* mRNA expression as extension of icariin-treatment time, and peaked at 24 h (Fig. 2d). Its time course expression seemed to closely follow that of Runx2, which was similar to the result previously reported by Ma *et al.* (25). Though *Runx2* mRNA expression at 40 μM was higher than that at 20 μM at 6 and 12 h, there was no significant difference for mRNA expression of *OPN* and *OCN* between 20 and 40 μM icariin-treated groups. Considering cytotoxicity at concentration of 40 μM, as shown by apoptosis assay, optimal concentration of icariin in the present study was 20 μM (Fig. 1, 2).

MAPK kinases, including ERK, p38 and JNK pathways, are secondary messengers that convey signals from the cell surface to the nucleus in response to a wide range of stimuli, and then regulate multiple cellular activities in osteoblasts. ERK has been reported to regulate osteoblast proliferation, apoptosis and differentiation by regulating expression of cell cycle regulators as well as activity of the skeletal-specific transcription factor Runx2 (29–32). Previous studies have shown that the ERK signalling pathway is involved in osteogenesis, by enhanced ALP activity, in osteoblast progenitor cells (10,33–35). The p38 signalling pathway has been reported to involve phosphorylation of smad-1, thus

resulting in ALP expression and activation of osteoblasts (36). Activation of the JNK pathway is commonly linked to promoting apoptosis and cell death (37–39). For instance, activation of the JNK signalling pathway leads to permeabilization of outer mitochondrial membranes resulting in mitochondrial cytochrome release into the cytosol allowing apoptosis to progress (40). Recently, the JNK pathway was found to be involved in osteogenic differentiation of BMSCs or periodontal ligament stem cells (41,42). In the present study, we examined the effects of icariin on ERK, p38 and JNK MAPK signalling pathways. In contrast to the Song *et al.* report that icariin induces osteoblast proliferation, differentiation and mineralization through ERK and JNK signal activation (27), the present study showed that ERK, p38 and JNK signalling pathways were all phosphorylated in BMSCs treated with icariin, while total JNK expression did not show any significant changes (Fig. 4a, b). Furthermore, we blocked ERK, p38 and JNK signalling pathways for BMSCs treated with icariin, and then ALP activity and expression of *Collagen I, OPN* and *OCN* were significantly inhibited respectively. These results suggest that ERK, p38 and JNK signalling pathways are involved in the process of icariin-induced osteogenesis. Moreover, icariin has also been reported to stimulate osteogenic differentiation of BMSCs *via* activating the PI3K–AKT–eNOS–NO–sGC–cGMP–PKG signalling pathway (43). Previous studies have also shown that there is crosstalk between MAPK and AKT signalling pathways (44). However, whether this may occur for enhanced effects of icariin on BMSCs needs to be explored in the future.

In conclusion, in the present study, it has been demonstrated that the optimal concentration of icariin to promote osteogenic differentiation of BMSCs, was 20 μM. Furthermore, the ERK, p38 and JNK MAPK signalling pathways were involved in this process. This investigation has provided scientific evidence and support for the belief that icariin as traditional Chinese medicine, could be applied for bone regeneration.

Acknowledgements

This project was supported by National Research Program of China (973 Program, 2012CB933604), National Outstanding Youth Foundation (81225006), National Natural Science Foundation of China (81170939), the Shanghai Science and Technology Development Fund (12nm0501600), Specialized Research Fund for the Doctoral Program of Higher Education (20110073110076); Biomedical Engineering Cross Research Foundation of Shanghai Jiao Tong University School (YG2012MS29) and Shanghai Jiao Tong University Doctor Innovation Program (BXJ201226).

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