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Icariin Reduces Cartilage Degeneration in a Mouse Model of Osteoarthritis and is Associated with the Changes in Expression of Indian Hedgehog and Parathyroid Hormone-Related Protein

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Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The aim of this study was to determine the role of icariin, a Chinese traditional herbal medicine extracted from *Epimedium*, in osteoarthritis (OA), using the murine anterior cruciate ligament transection (ACLT)-induced model of OA and micromass culture of murine chondrocytes.

Material/Methods: Twenty-four three-month-old C57/6J mice were randomly divided into three groups: the sham group (no surgery and joint injection with normal saline) (N=8); the ACLT + ICA group (ACLT surgery and icariin treatment) (N=8); and the ACLT group (ACLT surgery and joint injection with normal saline) (N=8). At 12 weeks after ACLT surgery, murine articular cartilage was harvested from all mice for histological evaluation of any differences in cartilage degeneration. *In vitro* micromass culture of mouse chondrocytes was used to study the effects of icariin on chondrocyte differentiation and growth from the three mouse groups.

Results: Icariin treatment (mice in the ACLT + ICA group) significantly reduced degeneration of cartilage in OA with increased cartilage thickness, associated with increased expression of collagen type II alpha 1 (COL2A1), decreased chondrocyte hypertrophy, and decreased expression of collagen type X (ColX) and matrix metalloproteinase 13 (MMP13). *In vitro*, icariin promoted chondrocyte differentiation by upregulating the expression of agrrecan, Sox9 and parathyroid hormone-related protein (PHrP) and down-regulation of Indian hedgehog (Ihh) and genes regulated by Ihh.

Conclusions: In a mouse model of OA icariin treatment reduced destruction of cartilage, promoted chondrocyte differentiation, upregulated expression of PHrP and down-regulated the expression of Ihh.

MeSH Keywords: Chondrocytes • Hedgehog Proteins • Osteoarthritis, Knee

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Background

Osteoarthritis (OA) is a chronic and progressive joint disease characterized by the destruction of cartilage, sclerosis of subchondral bone, and synovial inflammation [1]. OA may cause severe joint pain, joint stiffness, and dysfunction of the joint, resulting in disability, particularly in older people. Articular cartilage is composed mainly (99%) of extracellular matrix (ECM) and comprises approximately 1% of chondrocytes. Few previous studies on chondrocytes in OA have been undertaken. Treatments for OA include the relief of joint pain and inflammation using nonsteroidal anti-inflammatory drugs (NSAIDs) including cyclooxygenase inhibitors [2,3]. However, due to the side effects of drug treatments for OA, new prevention and treatment strategies are needed.

Chondrocytes in the articular cartilage are multifunctional cells that synthesize, secrete, and regulate the distribution of the ECM. Recent studies have shown that the proliferation, differentiation, and hypertrophy of chondrocytes have a significant role in the pathogenesis of OA [4,5]. Hypertrophic chondrocytes are associated with the onset and progression of OA and with the mineralization of ECM, with increased vascularity, and apoptosis of chondrocytes [6,7]. Therefore, the control of chondrocyte hypertrophy might be a potential approach for the treatment of OA.

Icariin (molecular formula: $C_{33}H_{40}O_{15}$; molecular weight: 676.65) is a bioactive flavonoid and phytoestrogen compound extracted from *Epimedium* and is widely used in traditional Chinese herbal medicine Asian countries to treat osteoporosis [8]. Icariin has also been studied for its anti-inflammation, anti-cancer, and antioxidant activities [9,10]. *In vitro* studies have shown that icariin may have a protective function in OA as it promoted the differentiation of chondrocytes, reduced chondrocyte apoptosis, and accelerated the secretion of ECM by chondrocytes [11,12]. Although icariin has been shown to have beneficial effects on chondrocyte differentiation and cartilage regeneration *in vitro*, its role *in vivo* during the process of OA and the underlying mechanisms of its action remain to be investigated.

Multiple signaling pathways have been showed to regulate the differentiation of chondrocytes, including the WNT/β and catenin pathway, the bone morphogenetic protein (BMP) and transforming growth factor-β (TGF-β) pathways, and the parathyroid hormone-related peptide (PTHrP), Indian hedgehog (Ihh), and fibroblast growth factor (FGF) signaling pathways [13]. Among these signaling pathways, Ihh plays a regulatory role in both chondrocyte and osteoblast differentiation, and its expression is increased in hypertrophic chondrocytes [14]. Also, Ihh stimulates proliferating chondrocytes to produce PTHrP, which inhibits Ihh expression and maintains chondrocytes in the proliferative state, preventing the onset of hypertrophy, which means that Ihh and PTHrP together form a negative feedback loop to maintain homeostasis of articular cartilage [15].

The aim of this study was to determine the role of icariin on chondrocytes in OA using the murine anterior cruciate ligament transection (ACLT)-induced model of OA and micromass culture of murine chondrocytes. The mechanisms of any effects of icariin on the differentiation of chondrocytes were evaluated by study of the expression of genes and proteins associated with chondrogenesis, including those involved in the Ihh and PTHrP negative feedback loop.

Material and Methods

Study design of the experimental mouse groups

The study was approved by the local Ethics Committee and performed in compliance with the Guidelines for the Care and Handling of Experimental Animals of the Laboratory Animal Center, Shanghai Tongji University. The study used the murine anterior cruciate ligament transection (ACLT)-induced model of osteoarthritis (OA) [16].

Twenty-four C57/6J mice, 3-months-of-age, weighing between 19–21 gm, were randomly divided into three groups: the sham group (no surgery and joint injection with normal saline) (N=8); the ACLT + ICA group (ACLT surgery and icariin treatment) (N=8); and the ACLT group (ACLT surgery and joint injection with normal saline) (N=8). Mice in the ACLT + ICA group and ACLT group underwent ACLT surgery to induce osteoarthritis. The experiments were repeated in triplicate, resulting in a total of 72 mice being used throughout the study. ACLT surgery was performed as previously described [16]. Briefly, the anterior cruciate ligament of the right knee was transected to generate a destabilized OA animal model. Mice in the sham group underwent surgical opening of the joint capsule, injection with normal saline, followed by suturing of the incision in the right knee.

Icariin treatment of mice *in vivo* following surgery

On the second day after the operation, the ACLT + ICA group of mice were treated with oral doses of icariin (25 mg/kg/day) (>99% purity) Taotu Biotech, China). Mice in the other two groups, the sham group, and the ACLT group were treated with the same amount of normal saline, once into the joint. The icariin treatment doses were chosen according to the findings from previous pharmacological studies [17,18]. *In vitro*, icariin was administered into the chondrogenic medium at concentrations of 10^{-7} M, 10^{-6} M, and 10^{-5} M.

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Mice joint sample collection and tissue preparation

Twelve weeks following ACLT surgery, the murine right knee joints were harvested and fixed in 4% paraformaldehyde (PFA) overnight. Tissue samples were dehydrated by using the graded concentrations of ethanol, and fixed in 70% ethanol. For the histological analysis, the murine joints were decalcified for two weeks using the 10% ethylenediaminetetraacetic acid (EDTA). For paraffin sections, samples were embedded in paraffin wax and sectioned at 6 μm using a microtome (Leica, Germany).

Light microscopy and histochemical staining with hematoxylin and eosin (H&E), safranin O, and Weigert's iron hematoxylin

Tissue sections of 6 μm thickness were mounted on glass slides (Fisher Scientific, USA). Sections were deparaffinized and hydrated through xylene and graded ethanol. For routine light microscopy, tissue sections were stained with hematoxylin and eosin (H&E). For safranin O staining for chondrocytes, slides were stained with Weigert's iron hematoxylin, then rinsed in water, and counterstained with fast green before staining with 0.1% safranin O solution. Tissue sections on the glass slides were mounted and observed under light microscopy and photographed (Leica, Germany). The morphology of cartilage and subchondral bone formation were evaluated by using 2010 Osteoarthritis Research Society International (OARSI) recommended semi-quantitative scoring system [19].

Immunohistochemical (IHC) staining and immunofluorescence (IF) staining

Immunohistochemical (IHC) staining was performed to analyze the proteins expressed by chondrocyte-related genes. Tissue sections were processed for antigen retrieval by digestion with 0.05% trypsin at 37°C for 15 minutes. Tissue sections were incubated with primary antibodies to Sox9 (sc-166505) (Santa Cruz, USA), collagen type II alpha 1 (col2a1) (sc-517571) (Santa Cruz, USA), collagen type X (ColX) (ab58632) (Abcam, Cambridge, MA, USA), and matrix metalloproteinase 13 (MMP13) (sc-101564) (Santa Cruz, USA) overnight at 4°C. Then, sections were incubated with the secondary antibodies for 1 hour and developed in 3,3'-diaminobenzidine (DAB) chromogen (brown) (Invitrogen, USA). The sections were counterstained with hematoxylin, and observed by light microscopy (Leica, Germany).

For the immunofluorescence (IF) staining, the sections were stained with primary antibodies against runt-related transcription factor (RUNX2) (ab23981) (Abcam, Cambridge, MA, USA), Indian hedgehog (Ihh) (ab39634) (Abcam, Cambridge, MA, USA) and parathyroid hormone-related protein (PHrP) (sc-12722) (Santa Cruz, USA). Imaging was performed using a Leica fluorescence microscope. Quantification of the positively-stained cells was performed by microscopy.

Primary cell culture of murine chondrocytes

Chondrocytes from of the new murine growth plates were harvested and micromass culture was performed, as previously described [20]. Briefly, chondrocytes were seeded in 6-well culture plates at a density of 1.0×10^5 per well and cultured in chondrogenic medium to induce chondrocyte differentiation. The chondrogenic medium consisted of Dulbecco's modified Eagle's medium (DMEM) (high glucose), 10% fetal bovine serum (FBS) (Gibco Biocult), penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively), 100 mM L-glutamine (Sigma, USA), 50 μg/ml ascorbic acid (pH7.1) and 10 mM β-glycerolphosphate (Sigma, USA). There were four groups of cells: the control group (no icariin treatment); chondrocytes treated with 10^{-7} M icariin; chondrocytes treated with 10^{-6} M icariin; and chondrocytes treated with 10^{-5} M icariin. After 7 days and 14 days of chondrogenic differentiation, the cells were harvested and stained with 1% alcian blue (Sigma, USA) in 3% glacial acetic acid to compared the degree of proteoglycan synthesis. To quantify the intensity of the staining, the plates were rinsed with PBS, and cell lysates were prepared with 6 M guanidine hydrochloride (Beyotime, China).

Enzyme-linked immunosorbent assay (ELISA)

The levels of Col2a1 protein in cell culture supernatants was measured by using a Col2a1 enzyme-linked immunosorbent assay (ELISA) kit (CSB-EL005739MO) (Cusabio, China), according to the manufacturer's instructions. The optical density (OD) of the extracted lysate supernatants was measured at 630 nm with a microplate reader. All the experiments were performed in triplicate.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) analysis

RNA samples were harvested from the cultured cells at day 7 and day 14. Isolated RNA was dissolved in RNase-free water and quantified by measuring the absorbance at 260 and 280 nm with a spectrophotometer. The RNA samples were then treated with DNAse I, and cDNA was prepared for each sample, using 0.5 mg of total RNA and the cDNA synthesis reagent in a final volume of 20 μl.

The primers of the target genes were as follows:
Sox9, AGGAAGCTGGCAGACCAGTA (F),
TCCACGAAGGGTCTCTTCTC (R);
Aggrecan, CTGAAGTTCTTGGAGGAGCG (F),
CGCTCAGTGAGTTGTCATGG (R);
MMP13, CTGGACCAAACTATGGTGGG (F),

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GGTCCTTGGAGTGATCCAGA (R);
Clo10α1, CCTGGTTCATGGGATGTTTT (F),
ACCAGGAATGCCTTGTTCTC (R);
β-*actin,* GTTGTCGACGACGAGCG (F),
GCACAGAGCCTCGCCTT (R);
To evaluate the gene expression levels, qRT-PCR was performed with SYBR Green PCR Kit using iCycler (Bio-Rad). All experiments were performed in triplicate. The copy numbers of each gene were determined with cycle threshold (ΔΔCT) method. The mean values of the copy numbers of β actin were used as internal controls. Standard curves for all primers were prepared from total normal cDNA, amplified by semi-quantitative PCR, and cloned using the TOPO II TA Cloning Kit, following the manufacturer's instructions.

Statistical analysis

Data were presented as the mean ± standard deviation (SD). Statistical significance was assessed using the Student's t-test. Multiple comparisons within and between groups were analyzed by one-way analysis of variance (ANOVA) for multiple comparisons. P-values <0.05 were considered significant (* P<0.05, ** P<0.01, *** P<0.005).

Results

Icariin treatment reduced degeneration of articular cartilage in the murine anterior cruciate ligament transection (ACLT)-induced osteoarthritis (OA) phenotype

The mice used in this study showed good tolerance for the experimental treatment. The murine anterior cruciate ligament transection (ACLT)-induced model of osteoarthritis (OA) was chosen in this study. The mice were divided into three groups: the sham group (no surgery and joint injection with normal saline); the ACLT + ICA group (ACLT surgery and icariin treatment); and the ACLT group (ACLT surgery and joint injection with normal saline).

The effects of icariin treatment during the process of OA were compared between the sham group and ACLT model group. The integrity of cartilage at 12-weeks following ACLT was examined by using the histological analysis and 2010 OARSI recommended semi-quantitative scoring system [19]. Light microscopy using routine hematoxylin and eosin (H&E) staining showed severe structural degeneration in the ACLT group as indicated by the decreased thickness of cartilage and increased volume of subchondral bone formation when compared with sham group (Figure 1A). Icariin treatment restored the cartilage in the ACLT mice, shown by the decreased subchondral bone (SB) formation and increased the thickness of cartilage (Figure 1A, 1D). Safranin O staining for cartilage supported these results and showed increased staining of glycosaminoglycan in murine cartilage in the ACLT + ICA group when compared with the ACLT group (Figure 1B). Consistent with the histological findings, the OASRI scoring demonstrated that mice in the ACLT group had increased calcified cartilage (CC) and decreased the thickness of hyaline cartilage (HC) when compared with the sham group (Figure 1D). Therefore, icariin treatment could reduce the degeneration of ACLT-induced OA in the ACLT + ICA group when compared with the ACLT group.

Icariin promoted the expression of chondrogenic-associated genes expression while inhibiting chondrocyte hypertrophic gene expression *in vivo*

To further investigate the chondroprotective function of icariin during the process of OA, immunohistochemical (IHC) staining was performed on the paraffin sections of murine cartilage tissue with antibodies to Sox9 and collagen type II alpha 1 (COL2A1). Sox9 is a key transcription factor that regulates chondrocyte differentiation and is a typical chondrocyte marker; Col2a1 is expressed by the gene for the synthesis of collagen II in cartilage. Icariin treatment upregulated the expression of Sox9 and Col2a1 in the murine cartilage of the ACLT + ICA group when compared with the ACLT group (Figure 2A–2C).

The proteins expressed by hypertrophic chondrocyte marker genes, collagen type X (ColX) and matrix metalloproteinase 13 (MMP13) were studied in the murine cartilage. Mice in the ACLT group showed significantly increased expression levels of ColX and MMP13 in cartilage when compared with the sham group. Mice in the ACLT + ICA group showed reduced expression of ColX and MMP13 when compared with ACLT group (Figure 2A, 2C, 2D). Therefore, consistent with routine light microscopy, IHC staining demonstrated that treatment with icariin could promote chondrocyte differentiation while inhibiting its chondrocyte hypertrophy during the process of OA.

Icariin treatment increased proteoglycan synthesis during chondrogenic differentiation *in vitro*

The *in vivo* data above showed that icariin treatment reduced the degeneration of chondrocytes during the process of ACLT-induced OA. To determine the function of icariin in the differentiation of chondrocytes, micromass culture of chondrocyte from the newly formed murine growth plates, as previously described [20]. Increasing concentrations of icariin (10^{-7} M, 10^{-6} M, and 10^{-5} M) were added to the culture medium. The levels of proteoglycan were detected using alcian blue staining on day 7 and day 14 cells. The results showed that the groups of cells treated with 10^{-5} M and 10^{-6} M of icariin had a higher density of staining compared with the sham (control) group and the group treated with 10^{-7} M of icariin. The absorbance values in the groups treated with 10^{-5} M and 10^{-6} M of icariin

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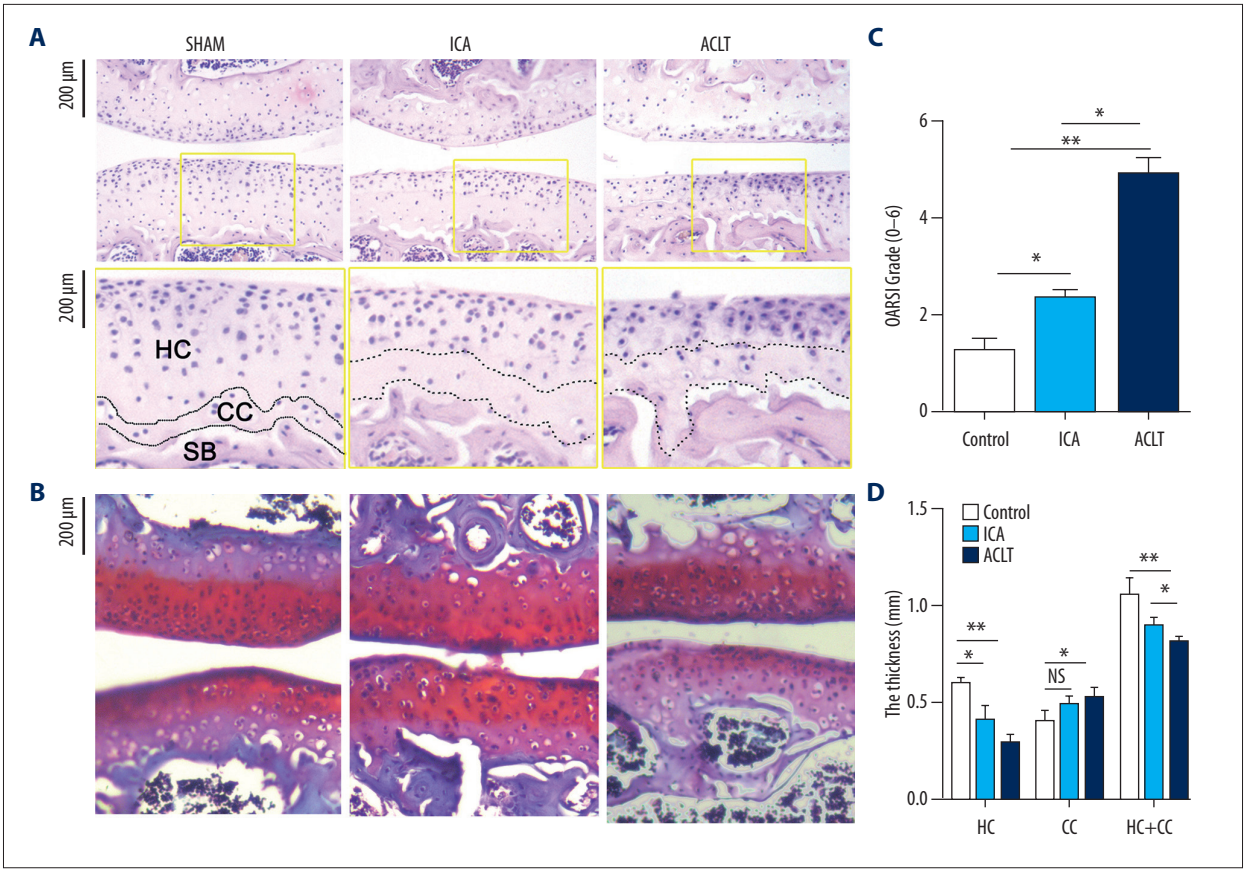

Figure 1. Icariin treatment reduced the degeneration of articular cartilage in the murine anterior cruciate ligament transection (ACLT)-induced model of osteoarthritis (OA). (**A**) Photomicrographs of the histology of the murine articular cartilage of the three mouse groups: the sham group (no surgery and joint injection with normal saline), the ACLT + ICA group (ACLT surgery and icariin treatment) and the ACLT group (ACLT surgery and joint injection with normal saline). The yellow squares are the higher magnification of the upper images, black dots imply the separation lines among hyaline cartilage (HC), calcified cartilage (CC), and subchondral bone (SB). Hematoxylin and eosin (H&E). (**B**) Photomicrographs of the histology of the Safranin O staining of murine cartilage in the sham, ACLT + ICA, and ACLT mouse groups. (**C**) The degeneration of cartilage was assessed by using Osteoarthritis Research Society International (OARSI) scoring system. (**D**) The thickness of cartilage in three groups of mice, 12 weeks after surgery: the sham group, where the incision was made and sutured immediately; the ACLT + ICA group of mice underwent ACLT surgery and icariin treatment; the ACLT group underwent ACLT surgery and joint injection with normal saline. Data are presented as the mean ±SD. N=5. NS – nonsignificant. * P<0.05; ** P<0.01.

were significantly increased when compared with the other two groups. However, there were no differences in the absorbance values between the groups of cells treated with 10^{-5} M and 10^{-6} M of icariin (Figure 3A, 3B).

Enzyme-linked immunosorbent assay (ELISA) was used to detect the protein expression level of Col2a1 in the micromass culture of chondrocytes. The results showed that the expression of Col2a1 in the groups of cells treated with 10^{-5} M and 10^{-6} M of icariin were significantly upregulated by 2.7-fold and 2.5-fold when compared with those in control group (Figure 3C). These results indicated that icariin treatment with the concentration of 10^{-6} M and 10^{-5} M could increase the chondrogenesis during the chondrocyte differentiation. Since the concentration of 10^{-6} M icariin resulted in higher expression levels of Col2a1 than 10^{-5} M, the dose of 10^{-6} M as the optimal concentration of icariin in the further *in vitro* studies.

Icariin treatment promoted the expression of chondrogenic-related genes and inhibited the expression of chondrocyte hypertrophic-related genes in micromass culture of mouse chondrocytes *in vitro*

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was used to detect the chondrocyte-related gene expression *in vitro* during the chondrocyte differentiation. Treatment with icariin promoted the expression of *Sox9* and *Aggrecan* on day 7 and day 14. The mRNA expression level of chondrocyte hypertrophy-related genes, runt-related transcription factor (*RUNX2*), *MMP13*, and *ColX* showed that icariin

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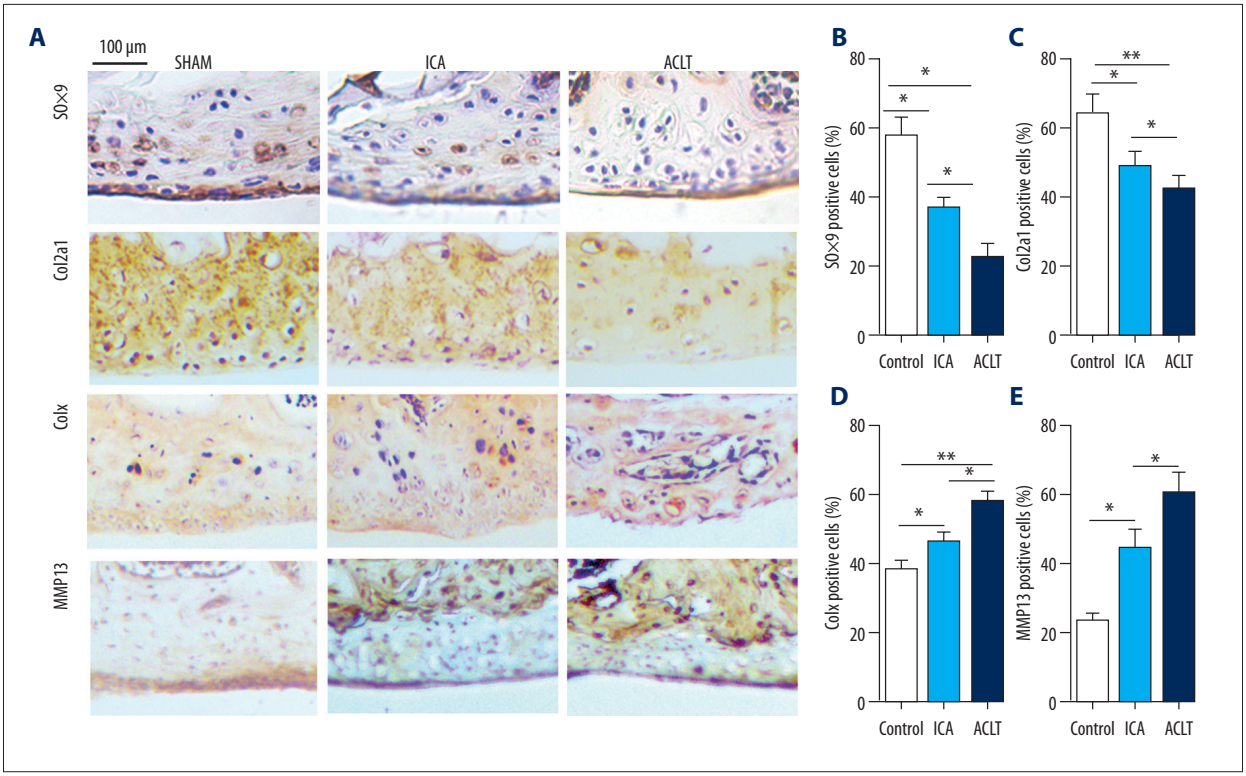

Figure 2. Icariin treatment promoted the expression of chondrogenic marker genes and inhibited the expression of chondrocyte hypertrophic genes *in vivo*. (**A**) The expression of Sox9, collagen type II alpha 1 (COL2A1), collagen type X (ColX), and matrix metalloproteinase 13 (MMP13) in articular cartilage, 12 weeks after murine anterior cruciate ligament transection (ACLT), were detected by immunohistochemical (IHC) staining. (**B–E**) The quantification of IHC staining of Sox9, Col2a1, ColX, and MMP13 in (**A**). The three mouse groups include: the sham group (no surgery and joint injection with normal saline), the ACLT + ICA group (ACLT surgery and icariin treatment) and the ACLT group (ACLT surgery and joint injection with normal saline). Data are presented as the mean ±SD. N=5. NS – nonsignificant. * P<0.05; ** P<0.01.

treatment down-regulated the expression of *MMP13* and *ColX* on day 7, and this effect was further enhanced on day 14 cells (Figure 4). These results indicated that icariin promoted the expression of chondrogenic-related genes while inhibiting the expression of chondrocyte hypertrophic-related genes.

Icariin may affect Ihh and PTHrP signaling in promoting chondrogenesis *in vitro* and *in vivo*

Ihh and PTHrP signaling have been previously shown to be an important signaling pathway in the regulation of homeostasis of chondrocyte differentiation both in the growth plate and in cartilage by forming a negative regulatory loop [21]. In this study, the protein expression level of Ihh and PTHrP was studied by using immunofluorescence (IF) staining on day 14 cells. The results showed icariin significantly enhanced the expression of PTHrP on day 14 cells when compared with the control cells; icariin treatment in day 14 cells significantly inhibited the expression level of Ihh when compared with controls (Figure 5).

Investigation of the expression of genes regulated by Ihh, including *CyclinD1*, *Gli1*, and *Ptch1* in day 7 and day 14 cells was undertaken using quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). The results showed the icariin treatment inhibited the mRNA expression levels of *CyclinD1*, *Gli1*, and *Ptch1* in a time-dependent manner. The mRNA expression levels of *CyclinD1*, *Gli1*, and *Ptch1* in icariin-treated cells decreased by 60%, 75%, and 80%, respectively when compared with control cells (Figure 6A).

Immunofluorescence (IF) staining with the antibodies to Ihh, PTHrP, and runt-related transcription factor (RUNX2) on the cartilage tissue sections also confirmed the *in vitro* results, which showed the number of PTHrP-positively-stained cells in the ACLT + ICA group in cartilage were significantly increased when compared with the ACLT group (Figure 6B, 6D). However, the number of Ihh positively-stained cells in the ACLT + ICA group decreased by 50% when compared with the ACLT group (Figure 6C, 6E). Therefore, these findings indicated that icariin treatment might exert its effects by Ihh and PTHrP signaling to prevent cartilage degeneration in the process of OA.

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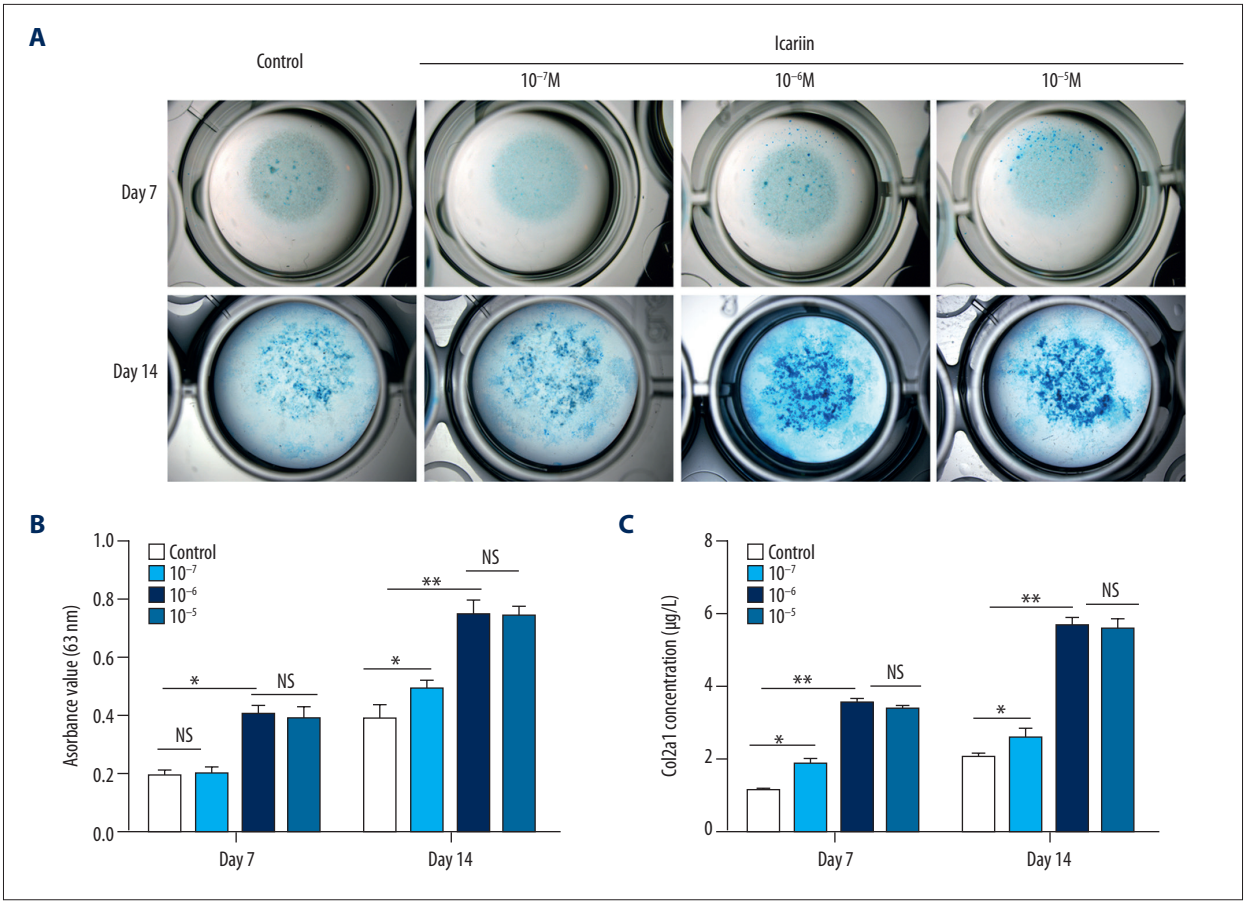

Figure 3. Icariin treatment of chondrocytes increased proteoglycan synthesis during chondrogenic differentiation. (**A**) Micromass culture of chondrocytes in the chondrogenic medium without or with different concentration of icariin: 10^{-7} M, 10^{-6} M, and 10^{-5} M. Alcian blue staining was used to detect the proteoglycans synthesis in day 7 and day 14 cells. (**B**) Quantification of the Alcian blue staining in (**A**). (**C**) Enzyme-linked immunosorbent assay (ELISA) results of the expression of collagen type II alpha 1 (COL2A1) of cells in culture on Day 7 and Day 14 treated with or without icariin. Data are presented as the mean ±SD. N=4. NS – nonsignificant * P<0.05; ** P<0.01.

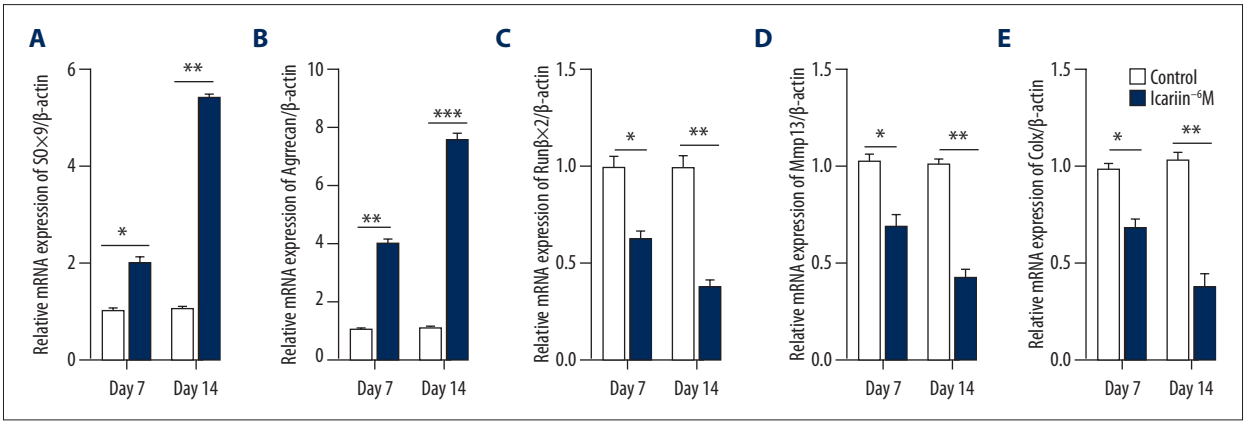

Figure 4. Icariin treatment increased the expression of chondrogenic-related genes and inhibited the expression of chondrogen hypertrophy-related genes in micromass culture of chondrocytes *in vitro*. Chondrocytes were cultured in chondrogenic medium in the absence or presence of icariin (10^{-6} M). Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis was used to detect the mRNA expression level of *Sox9* (**A**), *Agrrecan* (**B**), runt-related transcription factor (*RUNX2*) (**C**), *MMP13* (**D**), and *ColX* (**E**). Data are presented as the mean ±SD. N=4. NS, nonsignificant * P<0.05; ** P<0.01; *** P<0.005.

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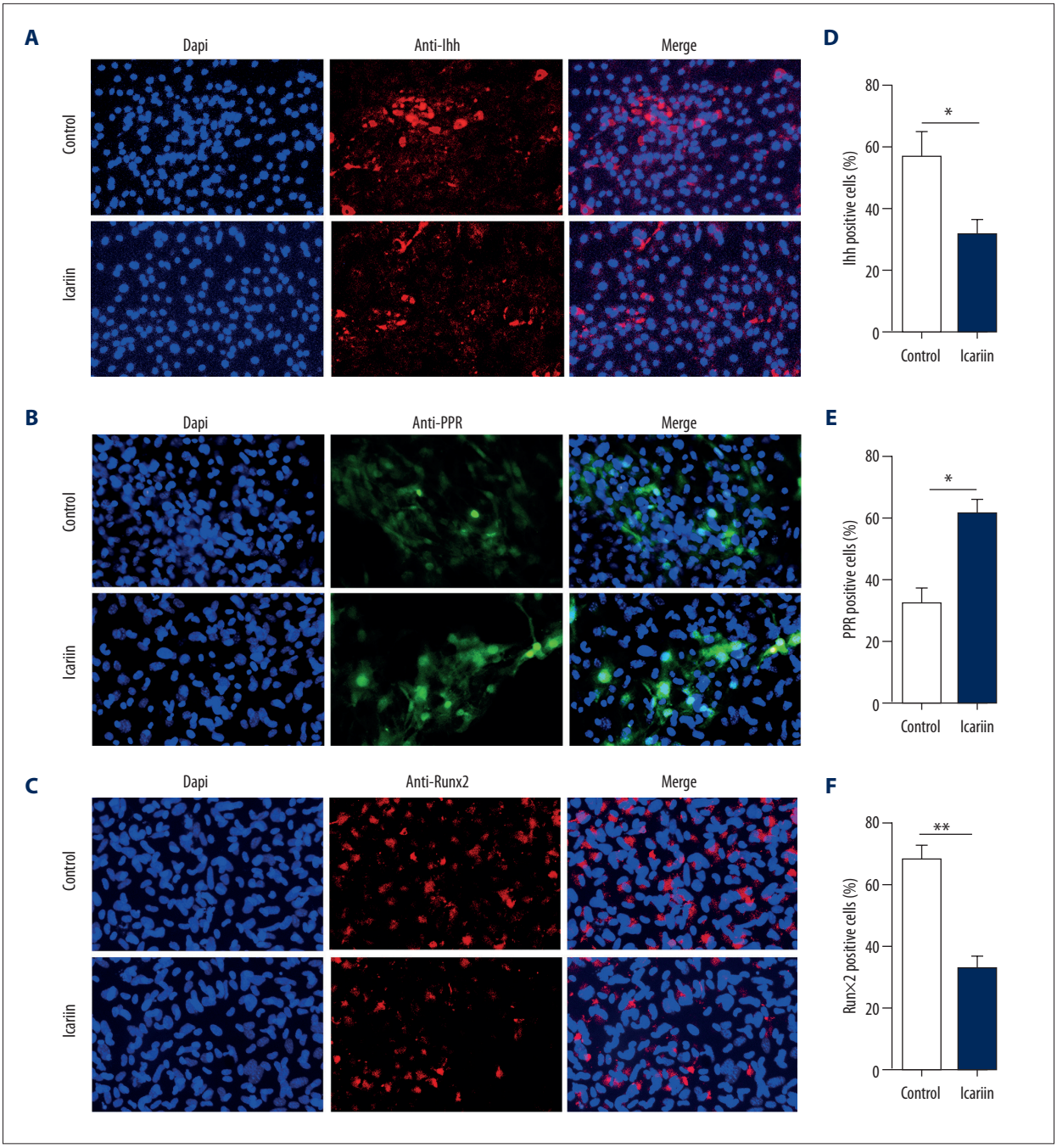

Figure 5. The effects of icariin treatment on Indian hedgehog (Ihh) and parathyroid hormone-related protein (PHrP) signaling in promoting chondrogenesis *in vitro*. Mouse chondrocytes were cultured and induced to differentiation in the chondrogenic medium in the absence or presence of icariin (10^{-6} M). Immunofluorescence (IF) staining was used to detect the protein expression level of Indian hedgehog (Ihh) (**A**), parathyroid hormone-related protein (PHrP) (**B**), and runt-related transcription factor (RUNX2) (**C**). (**D–F**) The quantification of IF staining in **A–C**. Data are presented as the mean ±SD. N=4. NS – nonsignificant. * P<0.05.

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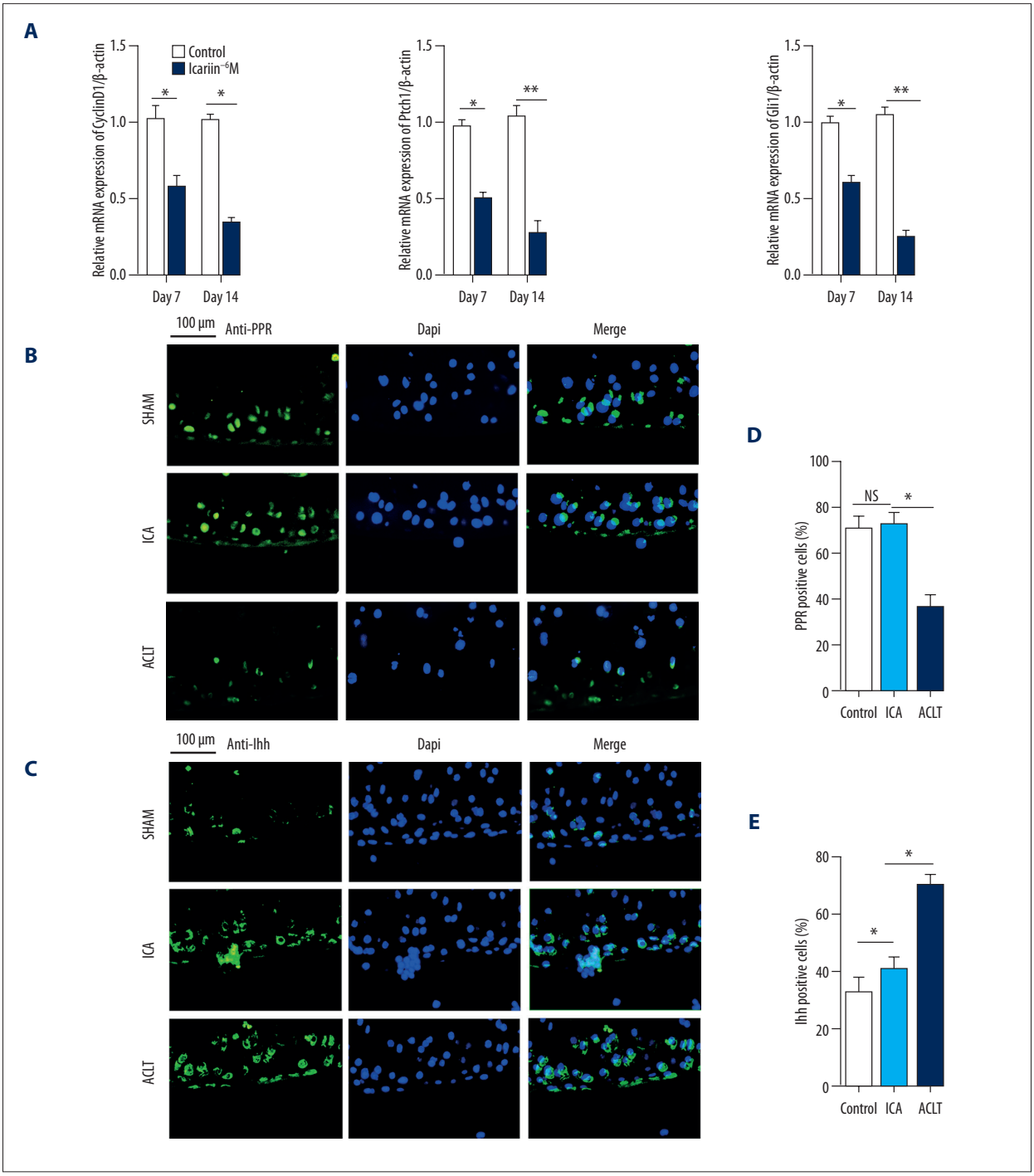

Figure 6. Icariin treatment and the effects on Indian hedgehog (Ihh) and parathyroid hormone-related protein (PHrP) signaling in promoting chondrogenesis *in vitro* and *in vivo* in the murine anterior cruciate ligament transection (ACLT)-induced model of osteoarthritis (OA). (**A**) Chondrocytes in culture show differentiation in the chondrogenic medium in the absence or presence of icariin (10^{-6} M). Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis detects the mRNA expression levels of genes regulated by Indian hedgehog (Ihh): *Cyclin D1*, *Ptch1*, and *Gli1*. Data are presented as the mean ±SD. N=4. (**B, C**) Immunofluorescence (IF) staining detects the protein expression level of parathyroid hormone-related protein (PHrP) (**B**) and Indian hedgehog (Ihh) (**C**) in murine articular cartilage of in the three mouse groups: the sham group (no surgery and joint injection with normal saline), the ACLT + ICA group (ACLT surgery and icariin treatment) and the ACLT group (ACLT surgery and joint injection with normal saline). (**D, E**) The quantification of IF staining in **A, B.** Data are presented as the mean ±SD. N=5. NS – nonsignificant. * P<0.05; ** P<0.01.

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Discussion

The murine anterior cruciate ligament transection (ACLT)-induced model of osteoarthritis (OA) was chosen in this study because of its reproducibility and its relevance to human post-traumatic arthritis. The mice were divided into three groups: the sham group (no surgery and joint injection with normal saline); the ACLT + ICA group (ACLT surgery and icariin treatment); and the ACLT group (ACLT surgery and joint injection with normal saline). The aim of the study was to investigate the role of parathyroid hormone-related protein (PHrP) and Indian hedgehog (Ihh) and genes related to chondrocyte degeneration and hypertrophy.

Due to the absence of vascular supply and low cell density, the prevention of the degeneration of cartilage during OA present a challenge for the treatment of this progressive condition. Icariin, isolated from Chinese traditional medicine *Herba epimedii*, has been widely used in Asian countries for the treatment of osteoporosis and chronic inflammation [22,23]. However, its role in the process of OA remains poorly studied *in vivo,* although the chondroprotective properties of icariin have been demonstrated *in vitro* [12]. In this study, the effects of icariin on the degeneration of articular cartilage were investigated using the ACLT-induced OA mouse model and micromass culture of chondrocytes. The results of this study provided histological and molecular support for the role of icariin in the prevention of the degeneration of articular cartilage during the progress of OA by its effects on Ihh and PTHrP signaling.

The murine ACLT-induced OA model was used in this study because of its reproducibility and reliability and its clinical similarity to the features of OA found clinically from trauma and aging [24]. Histological analysis of this model has shown that the severity of OA occurs in a time-dependent manner, from mild (4 weeks after surgery), to severe (12 weeks after surgery) [25]. In the present study, in order to identify the role of icariin during the process of OA, treatment began from the second day after surgery and cartilage samples were harvested 12 weeks after surgery when severe structural degeneration of the cartilage had occurred. Histological analysis of the OA model in this study showed thinning of the cartilage layer, disorganized chondrocytes, and increased subchondral bone formation. These histological changes are consistent with previous reports [26], indicating the relevance of the mouse model (Figure 1).

In the present study, treatment with icariin, in the ACLT + ICA group, increased the thickness of cartilage layers, prevented the degeneration of extracellular matrix (ECM), and decreased the subchondral bone mass when compared with the ACLT group without icariin treatment. These histological changes were demonstrated by the increased safranin O staining and decreased OARSI scale scores in the ACLT + ICA group when compared with ACLT group (Figure 1B, 1C).

The homeostasis of healthy cartilage largely depends on the balance between the chondrocyte differentiation and hypertrophy. In the progression of OA, chondrocyte hypertrophy is accelerated, accompanied by impaired chondrocyte differentiation, which results in the increased expression of collagen type X (ColX) and matrix metalloproteinase 13 (MMP13), and the decreased expression of collagen type II alpha 1 (COL2A1) and Sox9 in the cartilage [27,28]. In the present study, immunohistochemistry (IHC) staining demonstrated that icariin treatment upregulated the expression of COL2A1 and Sox9, and down-regulated the expression of ColX and MMP13 in the ACLT + ICA group when compared with ACLT group (Figure 2A). Therefore, the effects of icariin treatment in OA might occur by the promotion of chondrogenesis and the inhibition of chondrocyte hypertrophy and the reduction of cartilage destruction that occurs during the progression of OA.

The chondroprotective functions of icariin treatment in the ACLT-induced OA model were also supported by the findings of the *in vitro* studies. By using the micromass culture of chondrocyte from newly formed mouse bone growth plates, the study results showed that cells treated with icariin had more intense histochemical staining for proteoglycans and higher expression levels of chondrocyte related marker genes, including collagen type II alpha 1 (*COL2A1*) (Figure 3A), which were consistent with the findings from previous *in vitro* studies [11,29]. Also, the present study showed that icariin treatment inhibited the expression of chondrocyte hypertrophy-related genes, including *ColX*, *MMP13*, and *Ihh* (Figure 4).

There have been previous studies that have shown that Ihh signaling has a role in maintaining the homeostasis of cartilage, as chondrocytes in Ihh knockout mice, using *Col2a1*-cre showed decreased cartilage thickness and proteoglycan content [30,31]. Because Ihh signaling might have a chondroprotective function following icariin treatment during the process of OA, in this study, the genes regulated by Ihh, including *Gli1*, *Ptch1*, and *Sox9* were studied on day 7 and day 14 cells *in vitro*. The results showed that icariin down-regulated the expression of these genes at the mRNA level, when compared with the control cells (Figure 6A), indicating that icariin treatment inhibited Ihh expression during chondrocyte differentiation. Also, it has previously been demonstrated that Ihh can positively regulate the transcription and expression of *ColX* and *MMP13* through the downstream transcription factors of the Gli-Kruppel family members that include Gli1, which may partly explain the decreased expression levels of MMP13 and ColX following icariin treatment in this study [32].

Previously published studies have shown that Ihh can promote the expression of PTHrP in proliferating chondrocytes,

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preventing both the expression of Ihh and chondrocyte hypertrophy through a negative feedback loop [14,33]. As an important growth factor, PTHrP regulates chondrocyte differentiation by promoting the expression of Sox9 while inhibiting the expression of runt-related transcription factor (RUNX2) [34]. The *in vitro* results from the present study showed that icariin treatment upregulated the expression of Sox9 while down-regulating the expression of RUNX2 (Figure 4; Figure 5C).

In support of the findings icariin treatment may affect the expression of PTHrP in chondrocytes in OA, the present study examined the function of icariin treatment on the expression of PTHrP by using the immunofluorescence staining for the receptor of PTHrP *in vivo* and *in vitro*. The findings confirmed that expression of the receptor of PTHrP was increased about by 2-fold, both *in vivo* and *in vitro*, by icariin treatment, supporting the role of icariin in promoting the expression of PTHrP to prevent hypertrophy of chondrocytes in OA (Figure 5B, Figure 6B).

This study had several limitations. In this study, the anti-inflammatory and anti-apoptotic effects of icariin in cartilage on the mouse model of OA were not studied. As both inflammation and apoptosis are involved in the degeneration of articular cartilage in OA, these factors remain to be studied. Also, although primary micromass culture of chondrocytes in this study demonstrated the function of icariin during the chondrocyte differentiation, the use of *in vitro* methods cannot be assumed to reflect an *in vivo* disease process, such as OA. Furthermore, the present study only investigated the function of icariin in the mouse OA model, and further controlled clinical studies are required to examine whether and how icariin might improve cartilage regeneration and joint function in patients with OA.

Conclusions

The findings of the study, in a mouse model of osteoarthritis (OA), showed that icariin treatment reduced the destruction of cartilage, promoted chondrocyte differentiation, inhibited chondrocyte hypertrophy, upregulated the expression of parathyroid hormone-related protein (PHrP) and down-regulated the expression of Indian hedgehog (Ihh). These findings support that icariin may have a beneficial role in OA by its effects on Ihh and PTHrP signaling to regulate chondrocyte differentiation.

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Conflict of interest

None.

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