Icariin induces the growth, migration and osteoblastic differentiation of human periodontal ligament fibroblasts by inhibiting Toll-like receptor 4 and NF-κB p65 phosphorylation

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Abstract. The proliferation, migration and differentiation capacities of human periodontal ligament fibroblasts (HPDLCs) are important for the treatment of periodontal diseases. The aim of the present study was to investigate whether icariin could promote these abilities in HPDLCs, and explore the cellular mechanisms therein. The results indicated that icarrin markedly blocked apoptosis, and increased the viability and migration of HPDLCs, particularly at the concentrations of 20 and 50 μM. In addition, icariin significantly promoted HPDLCs to synthesize extracellular matrix, which was reflected by the decreased expression of matrix matalloproteinase-1 and increased expression of tissue inhibitor of metalloproteinase-1. Furthermore, the levels of bone morphogenetic protein 2, collagen I, osteoprotegerin and alkaline phosphatase were markedly elevated by icariin, indicating that icariin was able to promote the osteogenic differentiation capability of HPDLCs. Icariin also inactivated the Toll-like receptor 4 (TLR)-4/nuclear factor (NF)-κB signaling pathway by suppressing the expression levels of TLR-4 and phosphorylated p65, and by blocking p65 nuclear translocation. These results suggested that icarrin increased the survival, migration and osteoblastic differentiation of HPDLCs by inhibiting the TLR-4/NF-κB signaling pathway.

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Abbreviations: ECM, extracellular matrix; MMP-1, matrix matalloproteinase-1; TIMP-1, tissue inhibitor of metalloproteinase-1; BMP2, bone morphogenetic protein 2; Col I, collagen I; OPG, osteoprotegerin; TLR-4, Toll-like receptor 4; NF-κB, nuclear factor-κB

Key words: growth, migration, osteoblastic differentiation, TLR-4, NF-κB

Introduction

Human periodontal ligament, a band of fibrous connective tissue, has shock absorption ability and prevents tooth and alveolar bone injury during chewing (1). As the most abundant cells in human periodontal ligament, human periodontal ligament fibroblasts (HPDLCs) are involved in pathological changes of the human periodontal ligament and periodontal tissue regeneration (2). Besides that, HPDLCs have been demonstrated to have the capacity to generate extracellular matrix and regulate osteoclastic differentiation within periodontal tissue (3). For these reasons, HPDLCs was considered to play a vital role in the damage repair of periodontal diseases.

Icariin (2-(4'-methoxyphenyl)-3-rhamnosido-5-hydroxyl-7-glucosido-8-(3'-methyl-2-butylenyl)-4-chromanone, the major active ingredient of epimedii, possesses a broad spectrum of pharmacological applications, such as the protection of bone, cartilage, neuro and cardiovascular system (4). In addition, it has been suggested and supported by studies that icarrin could induce rat adipose-derived stem cells differentiation into osteoblasts (5) and regulate extracellular matrix (ECM) synthesis (6). However, little is known that whether icariin could affect ECM synthesis and osteoblastic differentiation in HPDLCs.

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that degrade components of ECM (7). According to published reports, numerous pathways such as Toll-like receptors (TLRs) (8), NF-κB (9) play important roles in MMPs expressions. However, activation of NF-κB and TLRs inhibit osteogenic differentiation in various cells, including pre-osteoblasts (10), human bone marrow mesenchymal stem cells (11). Therefore, TLR/NF-κB pathway maybe a major target for the prevention and treatment of damaged HPDLCs-induced periodontal diseases.

In the present study, HPDLCs were isolated and cultured in the absence or presence of icariin at different concentrations to explore the molecular mechanisms of icarrin in HPDLCs. These results strongly demonstrated that icariin promote HPDLCs to differentiate into osteoblasts and stimulate ECM synthesis via suppressing TLR-4 and phosphorylation of NF-κB.

Materials and methods

Primary culture of HPDLCs. HPDLCs were isolated from the molar of a female patient (25 years old) at Shanghai Stomatological Hospital. Briefly, periodontal ligament tissue dissected from patients were chopped into pieces and digested with 0.25% Trypsin/EDTA (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C for 1 h. Then, cells were centrifugated at 1,000 x g for 10 min and subsequently cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS) at 37˚C with 5% CO_2. A complete clinical examination was performed in all participants, clinical data were recorded and written consent was obtained from each subject. The experimental protocols were approved by the Ethics Committee of Shanghai Stomatological Hospital (Shanghai, China).

CCK-8 assay. Icariin was obtained from the Chinese National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Cell viability was measured using Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Briefly, HPDLCs were planted into a 96-well plate at the concentration of $5x10^3$/well. After adherence, cells were treated with icariin at different concentrations for different times. Then, CCK-8 solution was added into cells, and cells were incubated for another 4 h. The average value of optic density was detected using a Microplate Spectrophotometer (Thermo Fisher Scientific, Inc.) at a wavelength of 450 nm.

Detection of cell apoptosis. HPDLCs were cultured in the in the absence and presence of various concentrations of icariin for 24 h. Then, cells packed by centrifugation at 1,000 x g. After washing with PBS for 3 times, cells were stained with Hoechst 33258 at 37˚C for 5 min in the dark. Images (magnificaion, x400) were captured under a confocal laser scanning microscope (Carl Zeiss AG, Oberkochen, Germany).

Transwell assay. $2x10^4$ cells in each group were seeded into serum-free medium in the insert coated with Matrigel (BD Biosciences, San Jose, CA, USA), and the lower chamber was filled with DMEM containing 10% FBS. After incubation for 24 h, the invaded cells were stained with crystal violet. Cells were counted in at least 6 randomly selected fields under a light microscope (Olympus, Tokyo, Japan).

Western blot analysis. Proteins were isolated from HPDLCs using a Total Protein Extraction kit (Applygen Technologies, Inc., Beijing, China). Then, proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore , Billerica, MA, USA). After blocking with 5% BSA, membranes were incubated with primary antibodies specific for matrix matalloproteinase-1 (MMP-1), bone morphogenetic protein 2 (BMP2), collagen I (Col I), osteoprotegerin (OPG), toll-like receptor 4 (TLR-4), nuclear factor-κB (NF-κB), GAPDH (Abcam, Cambridge, MA, USA) at 4˚C overnight, followed by incubation with horseradish peroxidase-conjugated IgG at room temperature for 1.5 h. Blots were observed using enhanced chemiluminescence.

ELISA assay. HPDLCs were planted into a 96-well plate at the concentration of $2x10^5$ cells/ml. After different treatment, the cells were incubated for another 24 h. Then, culture supernatants were collected and ALP level was assessed using ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA). The experiment performed strictly according to the manufacturer's instructions.

Detection of p65 nuclear translocation. HPDLCs were cultured in chamber slides, followed by different treatment. Then, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Trion and blocked with 5% BSA in PBS for 1 h at room temperature. Then, cells were incubated with NF-κB p65 (Abcam) at 4˚C overnight, followed by incubation with second antibody for 30 min. After washing, cells were counterstained with DAPI. Cells were observed under a confocal laser scanning microscope (Carl Zeiss Group, Germany).

Flow cytometry. Cell apoptosis was measured using Annexin V Apoptosis Detection kit (BD Biosciences). Briefly, HPDLCs were treated with Icariin at different concentrations for 24 h. Then, cells were stained with Annexin V-FITC and PI for 15 min. Cell apoptosis was detected using FACS Calibur flow cytometer (BD Biosciences).

Statistical analysis. All statistical analysis was carried out using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation. Analysis was performed using one-way analysis of variance followed by a Bonferroni post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

The effect of icariin on HPDLCs viability. The structure of icariin was shown in Fig. 1A. To select the proper concentration of icariin for subsequent experiments, HPDLCs were treated with icariin at different concentration (0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, 50, 100, 200, 300 and 400 μM) for 24 h. As illustrated in Fig. 1B, cell viability was remarkably suppressed in HPDLCs cells treated with icariin at the concentration of 50 μM above. To exclude cell toxicity, the concentrations of 10, 20 and 50 μM were chosen for the next experiments. CCK-8 assay results indicated that cell viability was increased in a dose-dependent manner when treated by icariin at day 2, 3 and 4 (Fig. 1C).

Icariin suppresses the apoptosis of HPDLCs. To investigate whether icariin was related to cell apoptosis, the morphological changes of icariin-treated HPDLCs were measured by Hoechst 33258 staining and flow cytometry. Results suggested that the icariin treatment decreased the cell apoptosis in a dose-dependent manner (Fig. 2).

Icariin induces HPDLCs motility and fibrosis. To explore the effect of icariin on the motility of HPDLCs, cell motility was measured using Transwell. As illustrated in Fig. 3A, cell

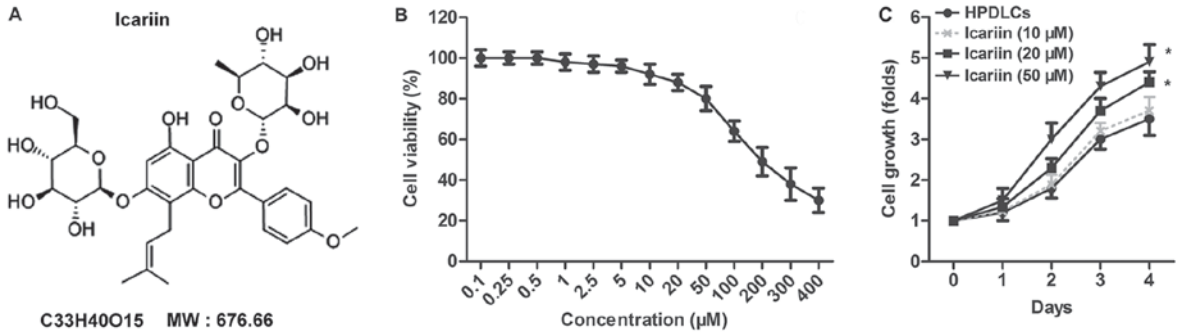

Figure 1. Effect of icariin on HPDLC proliferation. (A) The structure of icariin. (B) HPDLC viability was detected by CCK-8 assay. Cells were treated with icariin at different concentrations (0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, 50, 100, 200, 300 and 400 μM) for 24 h. (C) Cell viability was measured using CCK-8 in HPDLC cells treated with icariin at different concentrations (0, 10, 20 and 50 μM) and different times (1, 2, 3 and 4 days). The experiments were repeated at least 3 times with similar results, and data are presented as the mean ± standard deviation. *P<0.05 vs. HPDLC. CCK, Cell Counting Kit; HPDLC, human periodontal ligament fibroblast.

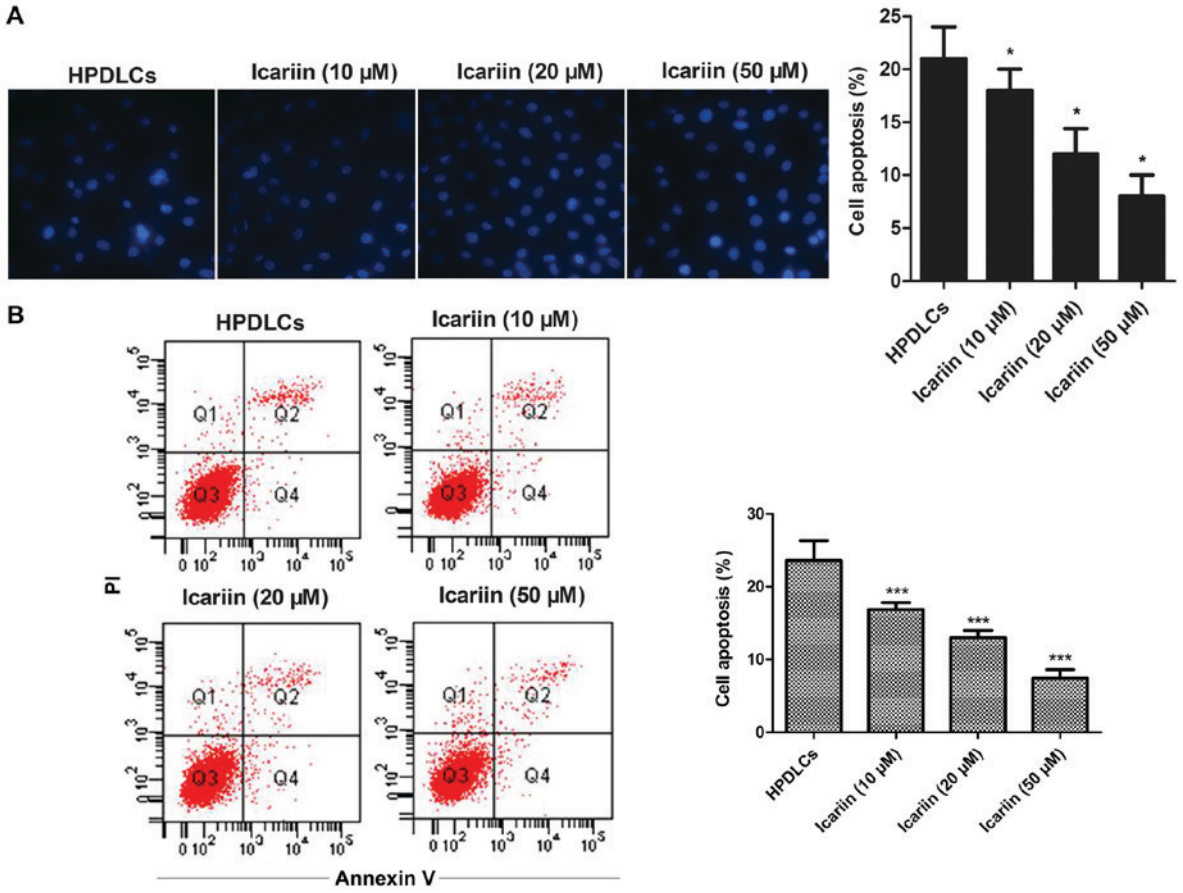

Figure 2. Icariin suppresses the apoptosis of HPDLCs. HPDLCs were administered icariin at different concentrations (0, 10, 20 and 50 μM) for 24 h. (A) Cell apoptosis was determined using immunofluorescence assay (magnification, x400). (B) Histogram representing the statistical analysis of the rate of cell apoptosis. The experiments were repeated at least 3 times with similar results, and data are presented as the mean ± standard deviation. *P<0.05 and ***P<0.001 vs. HPDLCs. HPDLC, human periodontal ligament fibroblast; PI, propidium iodide.

motility increased in a dose-dependent manner when treated with icariin. Besides that, MMP-1 expression was reduced and TIMP-1 expression was elevated with the increasing concentration of icariin (Fig. 3B), indicating that icariin play a vital role in inducing HPDLCs fibrosis.

Icariin enhances HPDLCs osteogenic differentiation ability. To determine whether icariin could affect the osteogenic differentiation of HPDLCs, the level of related proteins were measured using western blotting. Results suggested that, BMP2, Col I and OPG expressions were slightly elevated in icariin (20 μM) group and remarkably increased in icariin (50 μM) group compared with control group (Fig. 4A). In addition, ELISA assay indicated that icariin treatment enhanced ALP level in a dose-dependent manner in the supernatant of HPDLCs (Fig. 4B). These results indicated that HPDLCs

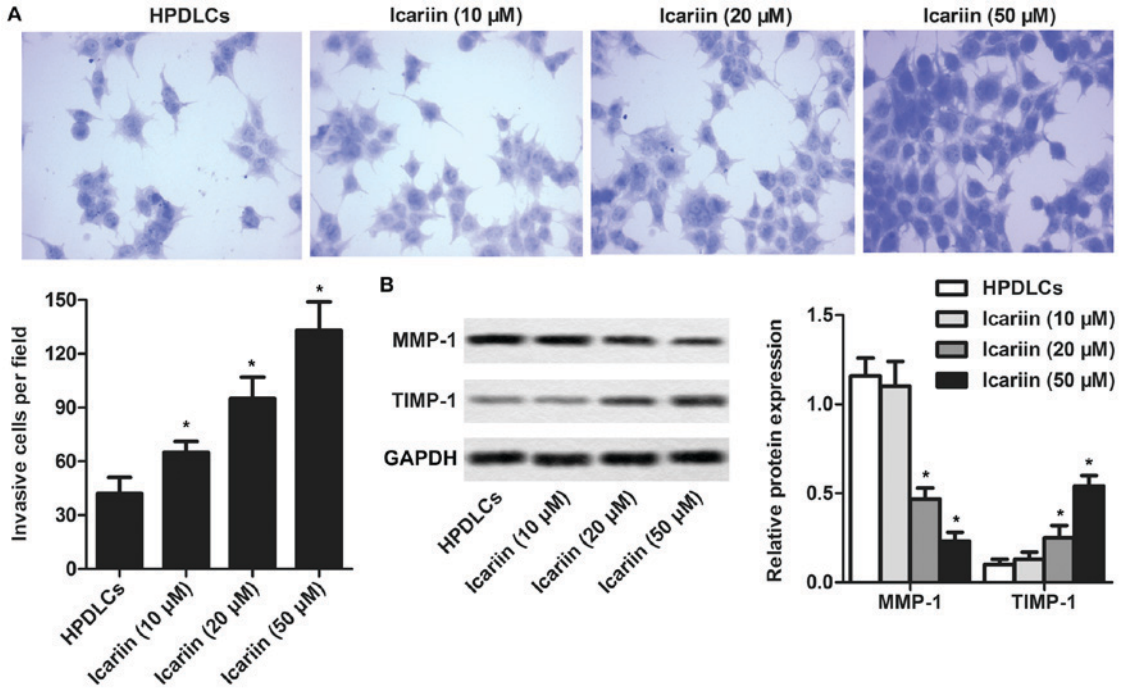

Figure 3. Icariin induces HPDLC motility and fibrosis. HPDLCs were administered icariin at different concentrations (0, 10, 20 and 50 μM) for 24 h. (A) A Transwell assay was performed to assess the migration ability of HPDLCs (magnification, x400). (B) The expression levels of MMP-1 and TIMP-1 were evaluated by western blotting. The experiments were repeated at least 3 times with similar results, and data are presented as the mean ± standard deviation. *P<0.05 vs. HPDLCs. HPDLC, human periodontal ligament fibroblast; MMP-1, matrix matalloproteinase-1; TIMP-1, tissue inhibitor of metalloproteinase-1.

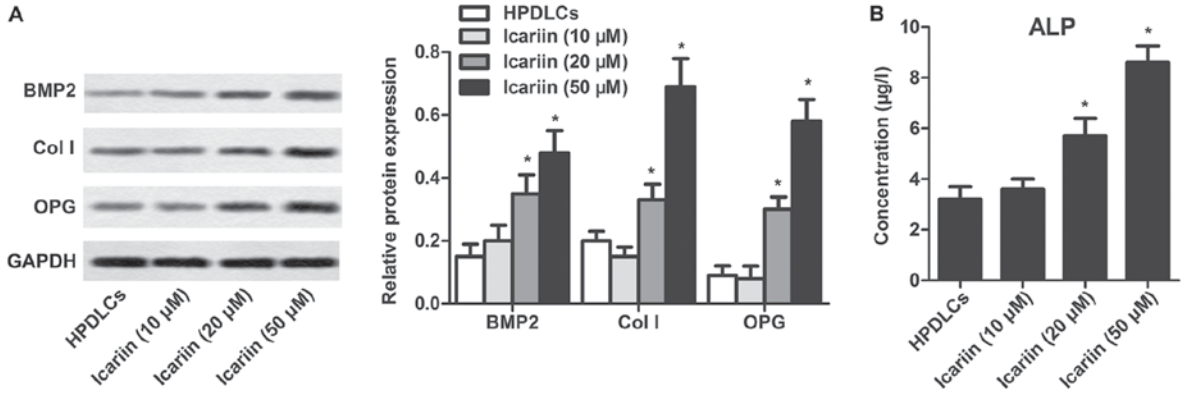

Figure 4. Icariin enhances HPDLC osteogenic differentiation ability. HPDLCs were administered icariin at different concentrations (0, 10, 20 and 50 μM) for 24 h. (A) The levels of BMP2, Col I and OPG were detected by western blotting. (B) ELISA was employed to evaluate the ALP levels. The experiments were repeated at least 3 times with similar results, and data are presented as the mean ± standard deviation. *P<0.05 vs. HPDLCs. HPDLC, human periodontal ligament fibroblast; BMP2, bone morphogenetic protein 2; Col I, collagen I; OPG, osteoprotegerin; ALP, alkaline phosphatase.

osteogenic differentiation ability could be enhanced by icariin treatment.

Icariin inactivates TLR-4/NF-κB pathway. Recently, various researches have suggested that the activation of TLR-4/NF-κB pathway could inhibit osteogenic differentiation, cell viability and motility. Thus, TLR-4/NF-κB pathway was examined. It is observed that icarrin decreased TLR-4 expression and p65 phosphorylation in a dose-dependent manner (Fig. 5A). Moreover, p65 nuclear translocation was remarkably suppressed by icariin treatment (Fig. 5B). These results indicated that TLR-4/NF-κB pathway could be remarkably suppressed by icariin treatment.

Discussion

HPDLCs is one of the most important cells in periodontal ligament. Due to the stem cell-like property, HPDLCs have potent proliferation, differentiation and migration abilities (12). According to published reports, HPDLCs could differentiate into osteoblastic cells and collagen-forming cells (13). Accumulated studies have indicated that HPDLCs play a vital role in maintaining the integrity and homeostasis of the periodontal ligament during alveolar bone remodeling (14). Thus, improving the proliferation, differentiation and migration capacity is one of the beneficial strategies to suppress periodontal ligament degradation.

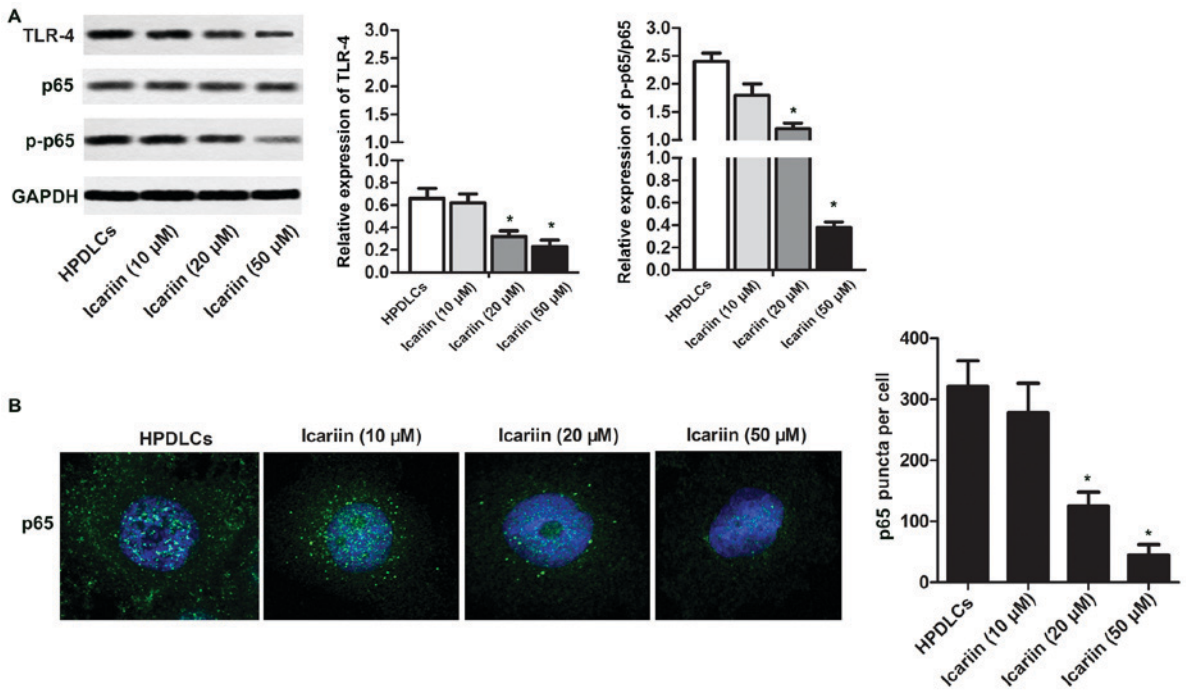

Figure 5. Icariin inactivates the TLR-4/NF-κB signaling pathway. HPDLCs were administered icariin at different concentrations (0, 10, 20 and 50 μM) for 24 h. (A) Western blot analysis was performed to detect the expression of TLR-4, p65 and p-p65. (B) The nuclear translocation of p65 was observed using an immunofluorescence assay (magnification, x400). The experiments were repeated at least 3 times with similar results, and data are presented as the mean ± standard deviation. *P<0.05 vs. HPDLCs. HPDLC, human periodontal ligament fibroblast; TLR-4, Toll-like receptor 4; NF-κB, nuclear factor-κB; p-, phosphorylated.

Both proliferation and migration of HPDLCs are essential for repair and regeneration. The results from current studies have demonstrated that icariin could promote the proliferation and migration of various cells. For example, icariin was found to tremendously enhance the osteoblast proliferation and Col I level using membrane chromatography coupled with liquid chromatography and time-of-flight mass spectrometry (15). Icariin treatment increased the proliferation of human neural stem cells and the formation of neurospheres (16). Moreover, human umbilical cord mesenchymal stem cells treated with icariin (100 μg/ml) exhibit remarkable cell migration to kidney tissue in mice with acute kidney (17). Endothelial cell migration could be promoted by icariin treatment (18). Similarly, in our research, the proliferation and migration of HPDLCs were tremendously elevated when the concentration of icariin was 20 and 50 μM.

Previous studies have demonstrated that ECM degradation is closely related to MMPs and TIMPs, for TIMPs help to regenerate ECM via binding to MMPs to decrease their activity (19). Icariin was reported to be involved in the expression of MMPs and TIMPs. According to Wang *et al* (20), icariin suppressed the MMP-1, MMP-3 and MMP-13 expression via MAPK pathways in IL-1β-induced SW1353 chondrosarcoma cells. Icariin treatment inhibits MMP-9 expression and carbonic anhydrase II in the tibia of glucocorticoid-induced hypocalcemia and hypercalciuria mice (21). Lipopolysaccharide-induced the increase in the level of MMP-1, MMP-3, MMP-13, cyclooxygenase-2 and iNOS could be remarkably suppressed by the pretreatment of icariin in neonatal mice chondrocytes (22). Among the MMPs, MMP-1 is the major enzyme to participate in degrading collagen types I and III, which are the most abundant components of the periodontal tissue matrix (23). In our research, icariin treatment decreased MMP-1 expression and elevated TIMP-1 expression in a dose-dependent manner, indicating ECM degradation could be restrained by icariin in HPDLCs. According to published stidies, MMP-1 expression is always associated with enhanced cell mobility, which is not consistent to what they found. Thus, we speculated that icariin may be involved in some other mechanism that has a moderation effect on MMP-1 expression and cell mobility, which need further exploration.

Apoptosis, a common cellular behavior, has irreplaceable functions in multicellular organisms. Accumulated studies have indicated that HPDLCs apoptosis is closely associated with the development of periodontitis (24). Icariin was reported to possess the anti-apoptosis ability. According to published researches, H_2O_2-induced apoptosis was inhibited by icariin via the PI3K/Akt Pathway in rat nucleus pulposus intervertebral disc cells (25). Besides that, oral administration of icariin remarkably suppressed cardiomyocyte apoptosis and attenuated left heart ventricle remodeling and abnormal mitochondria (26). Similarly, our research indicated that the apoptosis of HPDLCs was reduced by icariin in a dose-dependent manner.

BMP2, Col I, OPG and ALP have potent capacity of promoting osteoblast differentiation and inducing osteogenesis (27,28). Arising studies have demonstrated that icarrin play a vital role in regulating these genes. Wang *et al* used ALP activity assay to observe that icariin stimulate BMP2 osteogenesis in a concentration-dependent manner (29). Besides, the expression of osteogenic genes (Col I, Runx2, osteopotin and DLX5) were significantly elevated by icariin in

rat bone marrow stromal cells (30). Moreover, Mok *et al* (31) demonstrated that icariin inhibited the loss of bone mass and strength in the distal pat of the femur and elevated the mRNA level of OPG in the ovariectomized mouse. A similar result was drawn in our research, the expression levels of BMP2, Col I, OPG and ALP were tremendously enhanced by icariin at a dose-dependent manner.

It has been suggested and supported by studies that the inactivation of TLR-4 and NF-κB was involved in ECM synthesis and osteogenic differentiation (11,32). The results obtained from the current studies have demonstrated that icarrin was closely related to TLR-4/NF-κB pathway. According to Zhang *et al*, icariin treatment could remarkably suppress NF-κB nuclear translocation and the activation of Nlrp3 inflammasome in IgAN rats (33). In addition, icariin can elevate the osteogenic differentiation activity, decrease the NF-κB gene and protein expression, increase the OPG expression, enhance of ALP gene expression level in MC3T3-E1 cells (34). A similar result was drawn in our research, the expression of TLR-4 and P-65 was dramatically blocked by icarrin treatment. Moreover, icarrin further suppressed NF-κB p65 nuclear translocation in HPDLCs.

In conclusion, the current study has illustrated that icarrin treatment decreased the apoptosis and increased the viability and migration of HPDLCs. However, HPDLCs have stem cell-like characteristics and the osteogenic differentiation and ECM synthesis abilities could be remarkably enhanced by icariin treatment via inactivating of TLR-4/NF-κB pathway. Our study is valuable for unraveling the underlying mechanism of icarrin as a candidate drug for periodontal diseases.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HJL analyzed and interpreted the main data regarding the cell function study and immunofluorescence. XYL was responsible for study design and the drafting of the manuscript. DBJ conducted the statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experimental protocols were approved by the Institute Research Medical Ethics Committee of Shanghai Stomatological Hospital (Shanghai, China); written informed consent was obtained from all participants.

Patient consent for publication

Written informed consent was obtained from all participants.

Competing interests

The authors declare that they have no competing interests.

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