

Icariin may benefit the mesenchymal stem cells of patients with steroid-associated osteonecrosis by ABCB1-promoter demethylation: a preliminary study

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Abstract

Summary In this study, we found out a previously undefined function of icariin which restored the dynamic balance between osteogenic and adipogenic differentiation of mesenchymal stem cells (MSCs) in patients with osteonecrosis of femoral head (ONFH) via ABCB1-promoter demethylation. These findings provided important information regarding potential implication of icariin targeting epigenetic changes for the treatment of steroid-associated ONFH.

Introduction Here, we investigated whether icariin can also exert a beneficial role in the reactivation of MSCs in the patients with steroid-associated ONFH via ABCB1-promoter demethylation.

Methods Bone marrow was collected from the proximal femur in patients with steroid-associated ONFH ($n=20$) and patients with new femoral neck fractures ($n=22$), and then MSCs were isolated. We investigated cell viability, intracellular reactive oxygen species (ROS) level, mitochondrial membrane potential (MMP), P-glycoprotein (P-gp) activity, the transcript levels of ABCB1 and oxidative stress-related genes, methylation extent at CpG islands of ABCB1 promoter, and osteogenic and adipogenic differentiation ability of MSCs from the femoral neck fractures group and from the steroid-associated ONFH group treated with or without icariin.

Results We observed that MSCs from the steroid-associated ONFH group showed reduced proliferation ability, elevated ROS level, depressed MMP, weakened osteogenesis, and enhanced adipogenesis while low P-gp activity, transcription level of ABCB1, and oxidative stress-related genes as well as aberrant CpG islands hypermethylation of ABCB1 were also noted in steroid-associated ONFH group. Treatment with icariin obviously induced de novo P-gp expression, decreased oxidative stress, and promoted osteogenesis.

Conclusion Icariin may be a potential drug targeting epigenetic changes for the treatment of steroid-associated ONFH.

Keywords ABCB1 · Hypermethylation · Icariin · Steroid-associated osteonecrosis

Introduction

Nowadays, there are several alternative mechanisms responsible for glucocorticoid (GC)-associated osteonecrosis of femoral head (ONFH) such as fat embolisation [1], intramedullary pressure changes [2], modified artery constriction [3, 4], circulatory impairment [5], coagulation disorders [6], and cell apoptosis and dysfunction [7–9]. Among them, bone marrow mesenchymal stem cell (MSC) adipogenesis and apoptosis is a principal mechanism involved in the onset and progression of this disease. When the population of adipocytes becomes more numerous than the osteoblasts, the intraosseous pressure may rise, ultimately causing ONFH. Hence, it is quite essential for the treatment of GC-associated ONFH to inhibit MSCs from adipogenesis and apoptosis and promote their osteogenesis.

P-glycoprotein (P-gp), encoded by ABCB1, plays an important role in absorption and distribution of drugs and can protect normal cells or tissues from hazards [10–12]. As a member of the ATP-binding cassette transporter super-family,

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P-gp can transport substrates from the inside to the outside of cells using adenosine triphosphate as an energy source. GC, orally administered to patients, was demonstrated to be a substrate for P-gp [13–15]. Recent studies have found out that P-gp is closely related to the development of GC-associated ONFH and able to prevent GC-induced adipogenesis or apoptosis [16]. In particular, increased P-gp activity was regarded as an ideal predictor for low risk of developing GC-associated ONFH [17].

Epigenetics, as an effective method to study the interplay between environmental signals and the genome, have received a great deal of recent attention. Epigenetic mechanisms play crucial roles in the control of gene activity and nuclear architecture [18]. Emerging evidence shows that epigenetics may be a candidate mechanism for individual differences in GC sensitivity [19]. Our previous study has demonstrated that aberrant epigenetic modification of ABCB1 gene existed in MSCs of patients with GC-associated ONFH [20]. It is beneficial for the dysfunctional MSCs to reverse the aberrant methylation status of ABCB1 promoter.

Herba Epimedii, an important traditional Chinese herbal medicine, is widely used for “tonifying kidney and strengthening bone” for thousands of years in China, Korea, and Japan. Icariin ($C_{33}H_{40}O_{15}$; molecular weight 676.67) is a flavonoid extracted from Epimedii Herba and considered as the main pharmacological active constituent. It was reported that icariin could improve bone mineral density and tensile force in ovariectomized rats [21]. An *in vivo* study showed that osteoprogenitor was the key target of icariin in regulation of bone metabolism [22]. An *in vitro* study demonstrated that icariin regulated the anabolism of osteoblasts in a BMP- and Runx2-dependent manner [23]. However, to date, no investigation has yet focused on the relevance of icariin and epigenetic modification in the treatment of bone metabolism-related diseases.

In this study, we investigate a previously undefined function of icariin which induces *de novo* P-gp expression in MSCs of patients with GC-associated ONFH via ABCB1-promoter demethylation. These findings could provide important information regarding the application of therapeutic agents targeting epigenetic changes in ONFH and potential implication of icariin as a new drug for the treatment of GC-associated ONFH.

Methods

Patients

This study was approved by the Ethics Committee of Wuhan Union Hospital. Between Jan 2012 and May, 2013, 20 patients (10 men, 10 women; mean age 53.5, ranging from 39 to 67 years) with GC-associated ONFH were selected at the

authors' institution (Union Hospital, Wuhan, China) and 22 subjects with femoral neck fractures (12 men and 10 women; mean age 56.2, ranging from 40 to 70 years) were enrolled as controls. Clinical characteristics for all participants were summarized in Table 1. For GC-associated ONFH, the steroid exposure threshold is 1,800 mg GC or its equivalent over 4 weeks [24]. After written informed consent was obtained from patients, bone marrow aspirates (5 mL) were procured from the proximal end of femur or near the site of osteonecrosis while inserting the tapered awl into the femoral canal during hip replacement surgery.

Bone marrow separation for primary cell cultures

Human mesenchymal stem cells (hMSCs) were isolated and cultured as described in our previous study [25]. Adherent cells were cultured for 12 to 14 days until they attained a confluence of greater than 80 %. The cells were then digested with a solution of 0.25 % trypsin and 0.02 % EDTA (Invitrogen) and replated at a 1:2 dilution for the initial subculture. hMSCs were passaged three times after the initial 12–14-day culture period.

Cell viability measurement

hMSCs were plated in the 96-well plate at a density of 2×10^3 cells/well. After adherence to the plate, the initial defining media were aspirated away and replaced with complete medium supplemented with different concentrations of icariin (National Institutes for Food and Drug Control, Beijing, China). At 24, 48, and 72 h, cell viability was assayed by MTT as the manufacturer's instruction.

Reactive oxygen species

The intracellular ROS level was measured by reactive oxygen species (ROS)-specific fluorescent probe, 2-,7-dichlorofluorescein diacetate (DCFH-DA; Beyotime, China) [26]. The collected cells at a density of 2×10^5 cells/mL were resuspended in DCFH-DA and incubated in the dark at 37 °C for 20 min. They were then washed with serum-free medium three times to remove the excessive probes. The mean fluorescence intensity (MFI) of DCF in different samples was analyzed by flow cytometry with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was determined by 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolycarbocyanine iodide (JC-1, Beyotime, China) staining following the manufacturer's instructions. Images were collected and analyzed with a Zeiss LSM

Table 1 Clinical characteristics of the subjects

	ONFH (<i>n</i> =20)	Controls (<i>n</i> =22)	<i>P</i> value
Age (years)	51.9±8.5	54.8±9.1	0.270
Gender (male/female)	10/10	12/10	0.768
Body mass index (kg/m ²)	25.8±2.4	24.5±3.0	0.143
Hypertension	3 (15.0 %)	2 (9.1 %)	0.903
Smoker	7 (35.0 %)	6 (27.3 %)	0.588
Alcoholism	3 (15.0 %)	4 (18.2 %)	0.782
Glucocorticoid medication	20 (100 %)	2 (9.1 %)	
Total cholesterol (mmol/L)	5.48±0.63	4.71±0.74	0.001
LDL cholesterol (mmol/L)	3.26±0.37	3.16±0.36	0.384
HDL cholesterol (mmol/L)	1.19±0.36	1.32±0.38	0.272
Triglycerides (mmol/L)	1.76±0.40	1.57±0.33	0.099

Data are mean±standard derivation. Statistical significances of differences (*p* values) between groups were determined by paired Student's *t* test (continuous values) and chi-square test (categorical values)

GC glucocorticoid, LDL low-density lipoprotein, HDL high-density lipoprotein

510 laser scanning confocal microscope (Germany). The value of MMP staining from each sample was expressed as ratio of red fluorescence intensity over green fluorescence intensity.

Assay of P-gp activity of MSCs

MSCs were incubated in DMEM containing 10 % FBS and 2 mg/mL rhodamine 123 (Sigma, USA) at 37 °C for 10 min and centrifuged at 200*g* for 10 min, washed twice with PBS containing 10 % FBS, maintained in DMEM containing 10 % FBS at 37 °C for 60 min, and finally assessed by flow cytometry (Cytomics FC-500, Beckman Coulter, Fullerton, CA, USA). The excitation and emission wave lengths were 488/525 nm.

Adipogenic and osteogenic differentiation

Adipogenic and osteogenic differentiation of human MSCs were, respectively, performed as previously described [27]. For oil red O staining, the cells were washed twice with PBS and fixed with 4 % formaldehyde in PBS for 30 min at room temperature. They were next stained for 1 h at room temperature with filtered oil red O solution, washed twice with PBS, visualized under light microscopy, and photographed. To extract the incorporated oil red O, 1 mL isopropanol was added to each well followed by 15 min of shaking at room temperature. After appropriate dilution, the absorbance of triplicate samples was read at 490 nm.

For alizarin red S (ARS) staining, the cells were washed twice with PBS and fixed with 4 % formaldehyde in PBS for 30 min at room temperature. After a brief wash with PBS, they were stained for 20 min with 40 mM ARS solution (pH 4.2).

Next, they were rinsed five times with PBS to reduce nonspecific staining. Osteogenic differentiation was quantified by measurement of area stained with alizarin red S by using Meta Morph imaging software (Universal Imaging, Downingtown, PA). Measurements were done in duplicate at each experiment and experiments were repeated three times.

Real-time PCR

Total RNA was extracted via standard protocols using standard commercial kits (TRIZOL[®] Reagent, Invitrogen, USA). Real-time PCRs were performed using SYBR Green Master mix according to the protocols of the supplier (Invitrogen). The primer sequences are shown in Table 2. The SYBR Green signal was detected by a StepOne[™] real-time PCR machine (ABI, USA). The relative levels of transcript expression were quantified using the $\Delta\Delta C_t$ method. All real-time PCRs were run in triplicate and gene expression was analyzed using an ABI PRISM 7900HT Sequence detection system (Applied Biosystems, USA).

Bisulfite sequencing

Bisulfite conversion was performed as previously described [28]. Briefly, total genomic DNA was isolated from MSCs using a DNeasy Tissue Kit (Cwbiotech, Beijing, China). Two micrograms of genomic DNA were denatured in a volume of 50 μ L by freshly prepared 0.3 M NaOH for 30 min at 42 °C. After denaturation, 30 μ L freshly prepared hydroquinone (10 mM) and 510 μ L sodium bisulfite (3.6 M, pH 5.0) were added and incubated at 50 °C for 16 h. Modified DNA was purified using the DNeasy spin column (Qiagen) and eluted in 50 μ L. This was followed by desulfonation by adding 5.5 μ L 3 M NaOH for 15 min at 37 °C. Samples were neutralized by adding 33 μ L ammonium acetate (10 M, pH 7.0), followed by ethanol precipitation and resuspension in water.

PCR was performed at 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min with a final extension at 72 °C for 7 min. The primers used for PCR analysis were shown in Table 2. The PCR products were tested in 2 % agarose gel and then cloned into the pEASY-T1 vector (TransGen Biotech, Beijing, China). The colony PCR was undertaken to screen the positive colonies. The clones with the right sizes of PCR products were sequenced on an ABI sequencer with dye terminators (Applied Biosystems, Foster City, CA, USA). With sequencing results of ten clones, the methylation frequency was determined for each CpG site.

Statistical analysis

The statistical analysis was carried out via SPSS version 12.0 for Windows (SPSS, Chicago, IL, USA). Significance of

Table 2 Primers used for real-time PCR and bisulfite sequencing

Genes	Sequence (5'→3')	Product size
Real-time PCR		
Runx2-F	AGATGATGACACTGCCACCTCTG	125 bp
Runx2-R	GGGATGAAATGCTTGGGAAGTGC	
Osteocalcin-F	CACTCCTCGCCCTATTGGC	112 bp
Osteocalcin-R	CCCTCTGCTTGGACACAAAG	
PPAR γ -F	CGAGAAGGAGAAGCTGTGG	122 bp
PPAR γ -R	TCAGCGGAAGGACTTTATGTATG	
SOD-F	GGTGGGCCAAAGGATGAAGAG	227 bp
SOD-R	CCACAAGCCAAACGACTTCC	
Catalase -F	TGGGATCTCGTTGGAAATAACAC	146 bp
Catalase -R	TCAGGACGTAGGCTCCAGAAG	
ABCB1-F	TTGCTGCTTACATTCAGGTTTCA	105 bp
ABCB1-R	AGCCTATCTCCTGTGCGATTA	
GAPDH-F	GGCACAGTCAAGGCTGAGAATG	143 bp
GAPDH-R	ATGGTGGTGAAGACGCCAGTA	
Bisulfite sequencing		
ABCB1-region1-F	AACCTACTCTCTAAACCCRC	273 nt
ABCB1-region1-R	GTTGGAGGTGAGATTAATTTTATGTT	
ABCB1-region2-F	TTATTTGTGGTGAGGTTGATT	353 nt
ABCB1-region2-R	ACCAAAAACTTCACACTATCC	

Runx2 runt-related transcription factor 2, *PPAR γ* : peroxisome proliferator-activated receptor gamma, *SOD* superoxide dismutase

difference was determined using a one-way analysis of variance (ANOVA). Data are presented as mean \pm standard deviation. Probabilities lower than 5 % ($P<0.05$) were considered statistically significant.

Results

Measurement of hMSCs' viability

We can learn from Fig. 1a that the cellular viability reached the peak at the concentration of 10^{-9} M and an obvious decrease in cell viability emerged when treated with icariin at concentrations above 10^{-6} M. Hence, the concentration of 10^{-9} M was considered moderate and chosen for use in subsequent experiments.

As shown in Fig. 1b, the cellular viability in the control group was remarkably higher than the GC-associated ONFH group at three different time points ($P<0.05$). Treated with 10^{-9} M icariin, the cellular viability in the GC-associated ONFH group increased by more than one fifth, but still a little lower than the control group at $t=72$ h ($P<0.05$).

Icariin decreased ROS in hMSCs of patients with ONFH

As shown in Fig. 2a, c, d, ROS level was obviously higher in the GC-associated ONFH group than the control group ($P<0.01$). After 72-h treatment of 10^{-9} M icariin, the expected increased ROS showed an evident decrease in the GC-associated ONFH group ($P<0.01$, Fig. 2a, b, d).

Icariin increased MMP in hMSCs of patients with ONFH

To further illustrate the mitochondria dysfunction in hMSCs of GC-associated ONFH groups, we measured MMP in the isolated hMSCs by using JC-1 staining in situ. The decline of MMP is considered as a symbolic event of early cellular apoptosis. The results from Fig. 3a–j showed that the MMP was obviously lower in the GC-associated ONFH group than the control group and the treatment of 10^{-9} M icariin can restore the polarization state of the mitochondria, as indicated by an obvious increase in red (JC-1 aggregates)/green (JC-1 monomers) ratio.

Icariin enhanced P-gp activity in hMSCs of patients with ONFH

Enhanced P-gp activity could decrease the overall intracellular rhodamine 123 fluorescence by the efflux function. Thus, P-gp activity increased as MFI decreased. P-gp activity was quantified by the analysis of MFI in different samples using flow cytometry. The MFI of the GC-associated ONFH group was obviously higher than that of the control group ($P<0.01$, Fig. 4a, c, d). The treatment of icariin can increase P-gp activity with a lower MFI ($P<0.01$, Fig. 4b, d).

Real-time PCR

Our results showed that 10^{-9} M icariin markedly promoted ABCB1, oxidative stress-related genes (SOD, catalase), and osteogenesis-related genes (Runx2, osteocalcin) transcription while inhibiting adipogenesis-related gene (PPAR γ) transcription. As shown in Fig. 5h, the amount of ABCB1 transcripts in the GC-associated ONFH group was the lowest for all the groups ($P<0.01$). It reached nearly 28-fold level at 72 h after treatment with icariin, almost the same level as the control group ($P>0.05$). Meantime, SOD and catalase transcription elevated to varying degrees at the treatment of icariin (Fig. 5e–g). Pretreatment of icariin could also help to promote Runx2 transcription and decrease PPAR γ transcription (Fig. 5a–d).

Bisulfite sequencing

To determine whether the increased expression of ABCB1 in MSCs had an underlying epigenetic basis, the DNA methylation status in ABCB1 promoter was examined by bisulfite

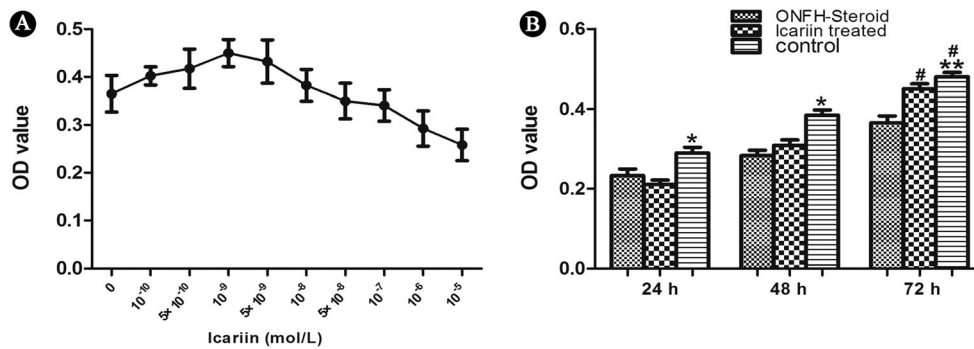


Fig. 1 Dose response curves for icariin-treated cultures and cell proliferation assay. **a** Cellular viability reached the peak at the dosage of 10⁻⁹ M. **b** The proliferation capacity of MSCs at *t*=24, 48, and 72 h.

Data are represented as mean values±standard deviation. **P*<0.05 vs. ONFH group, icariin group at *t*=24 and 48 h; ***P*<0.05 vs. icariin group at *t*=72 h; #*P*<0.01 vs. ONFH group at *t*=72 h

sequencing. A schematic overview of the promoter structure is shown in Fig. 6. Two regions in the promoter were selected, region 1 -760 to -407 and region 2 -328 to -55. CpG hypermethylation was detected within the CpG island in the GC-associated ONFH group. After treatment with 10⁻⁹ M

icariin for 72 h, the methylation ratio of region 1 was decreased from 40.0 % to 0 while the methylation level of region 2 was clearly reduced from 32.2 to 4.3 %. The methylation ratio of the control group is 2.3 % (region 1) and 2.2 % (region 2), respectively (Fig. 6).

Fig. 2 Decrease in ROS level by the treatment of icariin. **a** The ROS level in ONFH group. **b** The ROS level in icariin group. **c** The ROS level in control group. **d** The MFI of DCF was measured by flow cytometry. Data are represented as mean values±standard deviation. **P*<0.01 vs. control group, icariin group

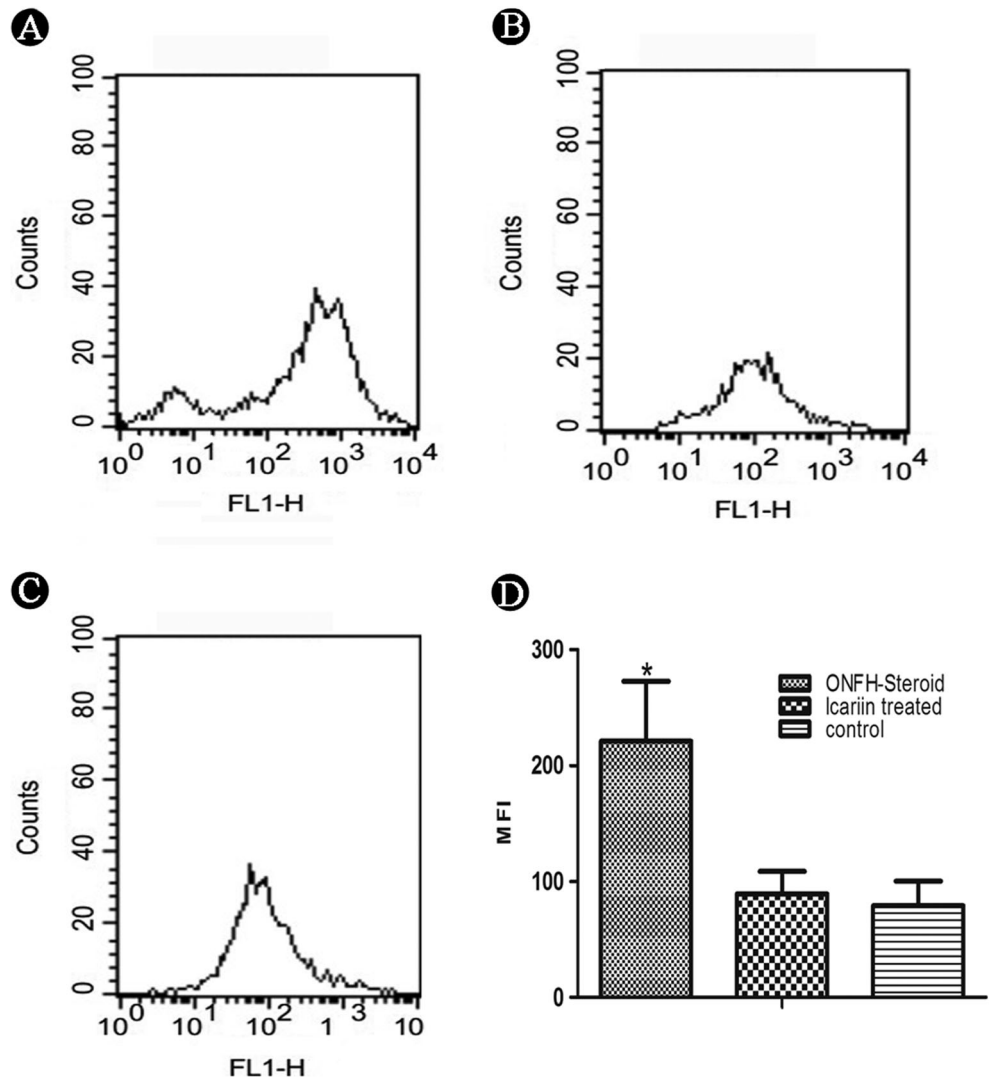
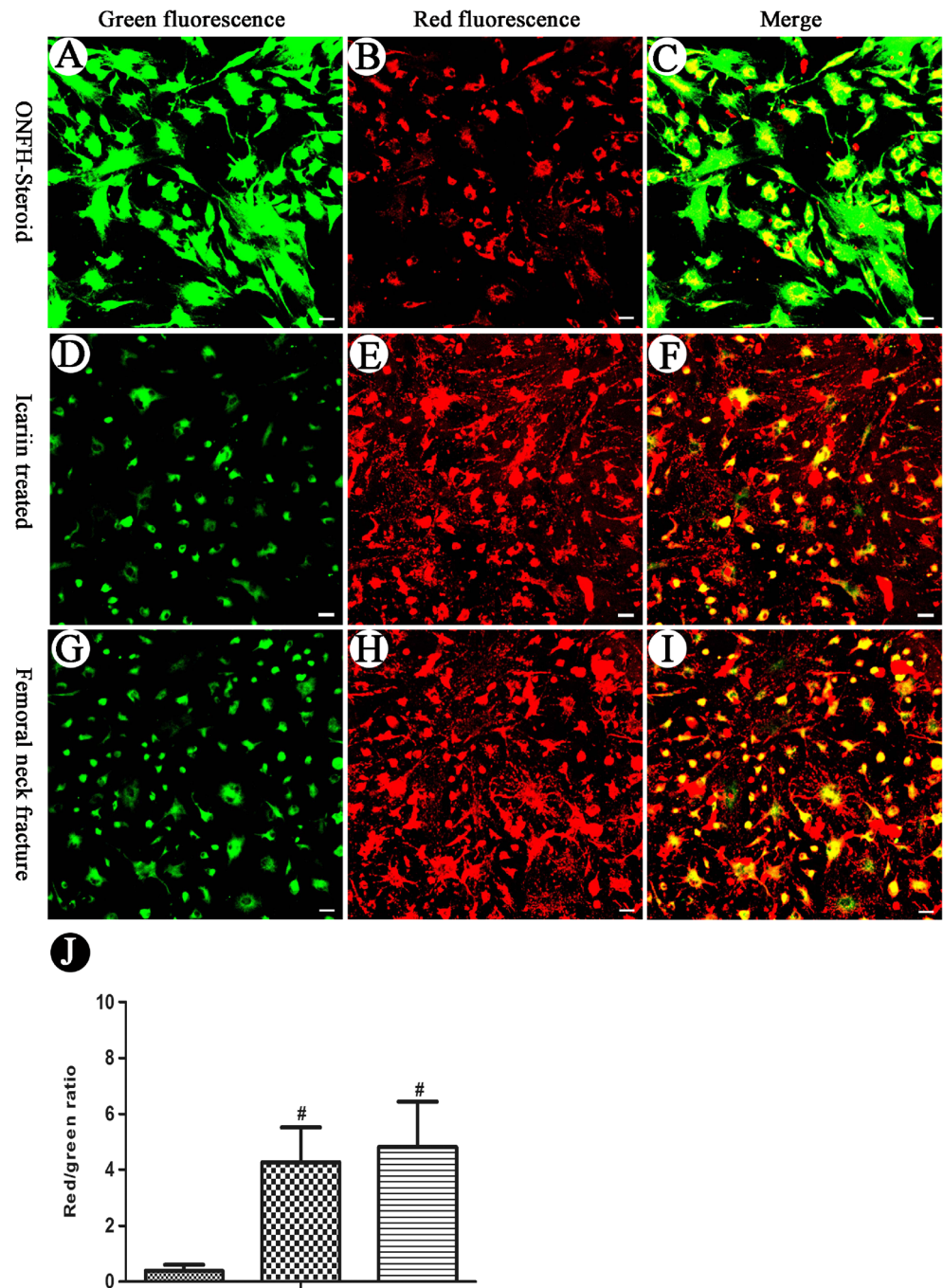


Fig. 3 An increase in MMP level by the treatment of icariin. **a–c** JC-1 staining in ONFH group. **d–f** JC-1 staining in icariin group. **g–i** JC-1 staining in control group. **j** The ratio of red fluorescence intensity over green fluorescence intensity was analyzed with a Zeiss LSM 510 laser scanning confocal microscope. $^{\#}P<0.01$ vs. ONFH group (bars 50 μm)



Adipogenic and osteogenic differentiation

As shown in Fig. 7a–c, oil red O has a strong deposit in the GC-associated ONFH group upon 3-week induction, compared to control group ($P<0.05$). The treatment of 10^{-9} M icariin resulted in a weakened staining, almost as the same level as the control group ($P>0.05$).

As shown in Fig. 7d–f, the ARS staining of the GC-associated ONFH group is weakest among all the groups. The pretreatment of icariin resulted in a deeper intensity of

alizarin red S staining, which indicated enhanced calcium mineralization, when compared with the GC-associated ONFH group ($P<0.05$).

Discussion

P-gp plays an important role in the development of GC-associated ONFH. An in vivo study showed that increased

Fig. 4 Assay of P-gp activity of MSCs. Low intracellular fluorescence means enhanced P-gp activity. **a** Rhodamine 123 level in ONFH group. **b** Rhodamine 123 level in icariin group. **c** Rhodamine 123 level in control group. **d** The MFI of Rhodamine 123 was measured by flow cytometry. Data are represented as mean values \pm standard deviation. * $P > 0.05$ vs. control group; # $P < 0.01$ vs. icariin group, control group

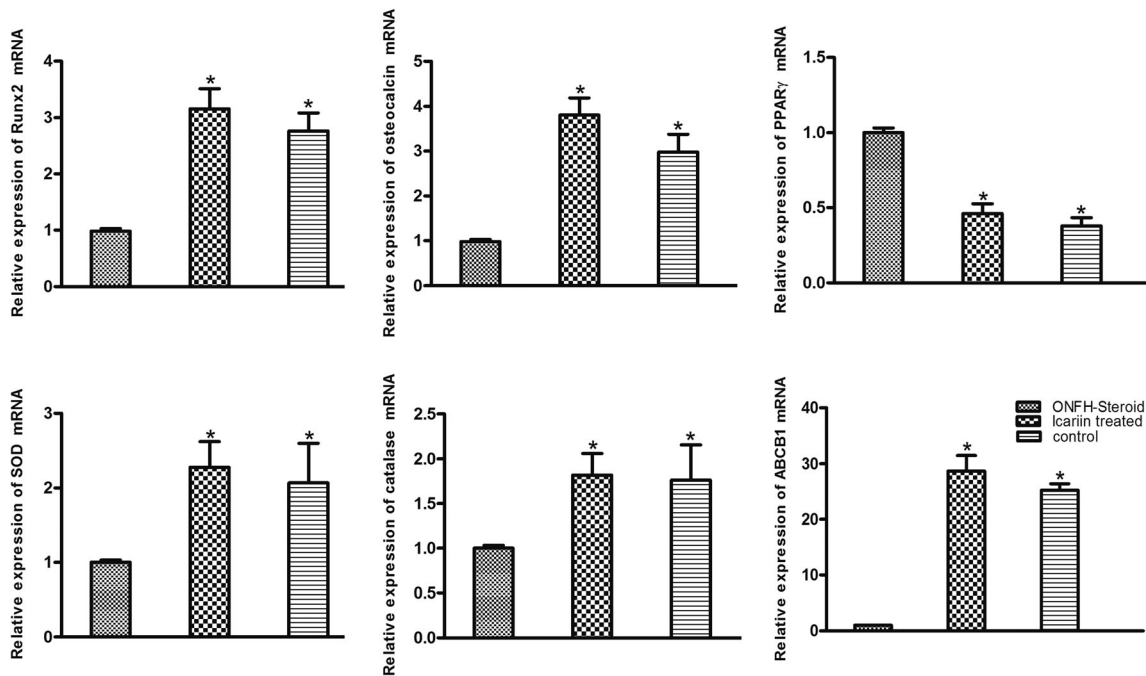
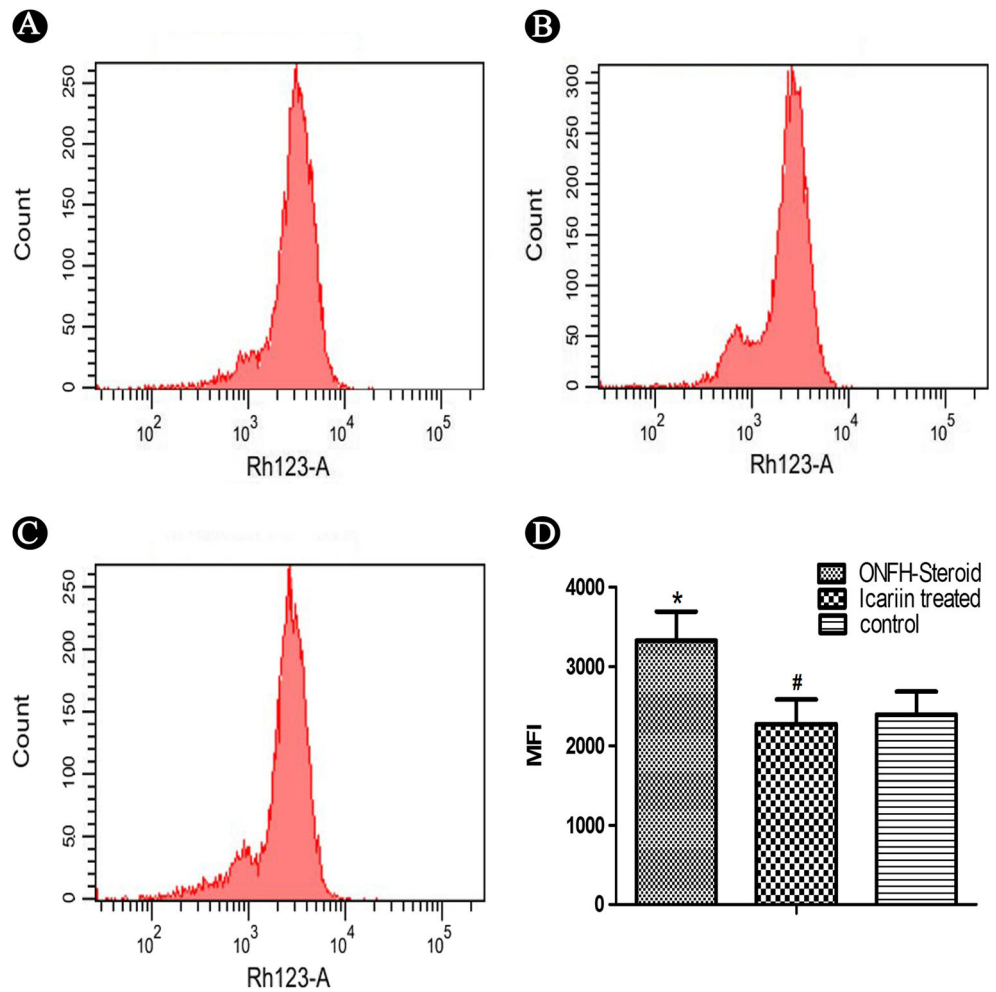
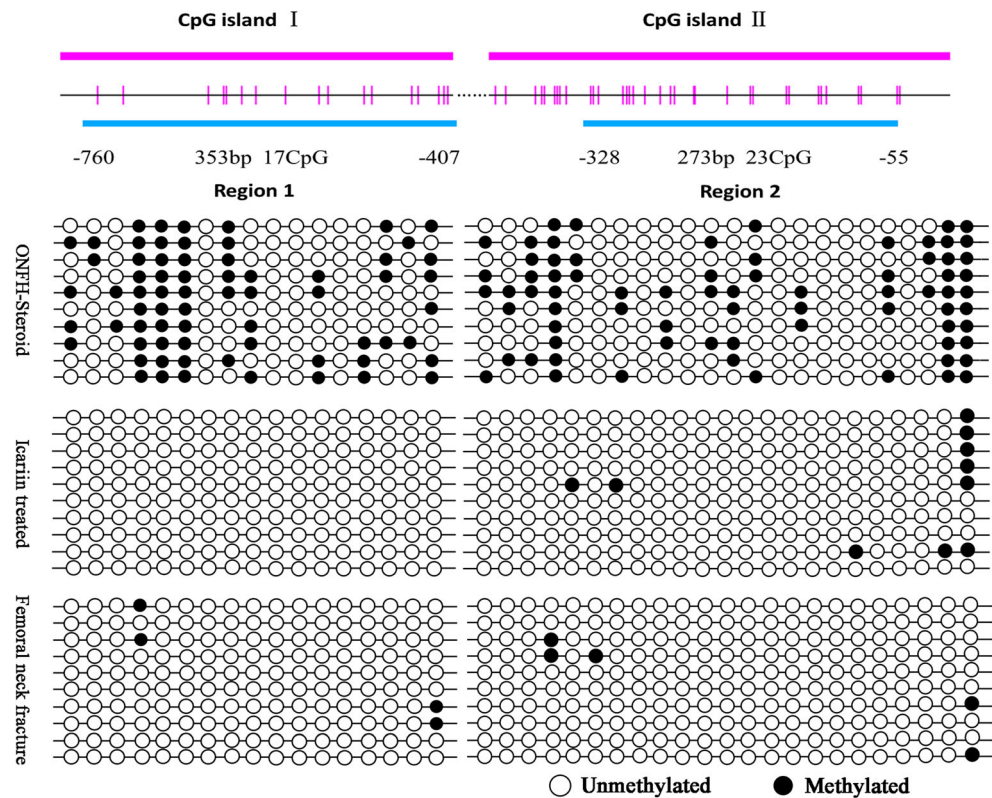


Fig. 5 Osteogenesis, adipogenesis, oxidative stress-related genes, and ABCB1 transcript levels in the three groups. Data are represented as mean values \pm standard deviation. * $P < 0.01$ vs. ONFH group

Fig. 6 Bisulfite sequencing. Region 1 (−760 to −407 bp): The methylation ratio of ONFH group was 40.0 % in ONFH group, 0 in the icariin group, and 2.3 % in the control group. Region 2 (−328 to −55 bp): The methylation ratio was 32.2 % in ONFH group, 4.3 % in the icariin group, and 2.2 % in the control group



P-gp activity could decrease the risk of GC-associated ONFH [16]. Our previous study demonstrated that aberrant CpG island hypermethylation of ABCB1 gene may be responsible for individual differences in the development of GC-associated ONFH and it was beneficial for the retardation of progressive ONFH to reverse the aberrant epigenetic modification and restore ABCB1 expression [20]. Hence, it is the key for the treatment of GC-associated ONFH to enhance P-gp activity and expression. New strategies for reactivation of P-gp and resistance of P-gp-negative MSCs to GC administration are required. The present study provides evidence that the exposure of P-gp-low MSCs in the patients with GC-associated ONFH to icariin leads to re-expression of ABCB1 mRNA and the P-gp protein.

Icariin, extracted from *Herba Epimedii*, has been proved to play an important role in bone anabolism [22, 23, 29–31]. Although some of the underlying molecular mechanisms have been partly clarified by previous studies, the relevance of icariin and epigenetic modification remains to be elucidated. Here, we observed that icariin could also improve cellular viability and osteogenic differentiation of MSCs in addition to upregulating ABCB1 expression. The state of CpG islands in MSCs was examined by BSP. Our results demonstrated that the CpG islands of the control group were hypomethylated in contrast with the ONFH group, and the exposure of MSCs in ONFH group to icariin led to a significant reduction in the extent of CpG dinucleotide methylation in comparison with untreated

cells. This suggests that icariin induces the demethylation of ABCB1 promoter in MSCs. Our results confirm that icariin can act as a demethylation agent, and the demethylation of ABCB1 promoter induced by icariin is not site specific.

MSCs, a kind of multipotent stem cells, were originally identified in adult bone marrow [32]. They can proliferate and differentiate into multiple mesodermal lineages, such as osteoblasts [33], cardiocytes [34], neurocytes [35], chondrocytes [36], and adipocytes [37]. In the physiological state, there is a balance between osteogenic and adipogenic differentiation of MSCs, but this balance may be disrupted by extraneous factors, such as steroids, alcohol, and so on. The imbalance between adipogenesis and osteogenesis of MSCs is considered to be a principle mechanism in the development of ONFH [38]. Since adipocytes and osteoblasts share a common pool of stem cells and steroids stimulate the differentiation of MSCs into adipocytes and suppress their differentiation into osteoblasts, it is insufficient to provide enough osteoblasts to meet the needs of bone remodelling; this, in turn, gives rise to bone necrosis. Accordingly, how to promote osteogenic differentiation of MSCs and inhibit their adipogenic differentiation is a focused issue in the control of ONFH progression.

Recently, the importance of oxidative stress in the regulation of adipogenesis and osteogenesis has received much attention [39, 40]. Edaravone, a novel free radical scavenger,

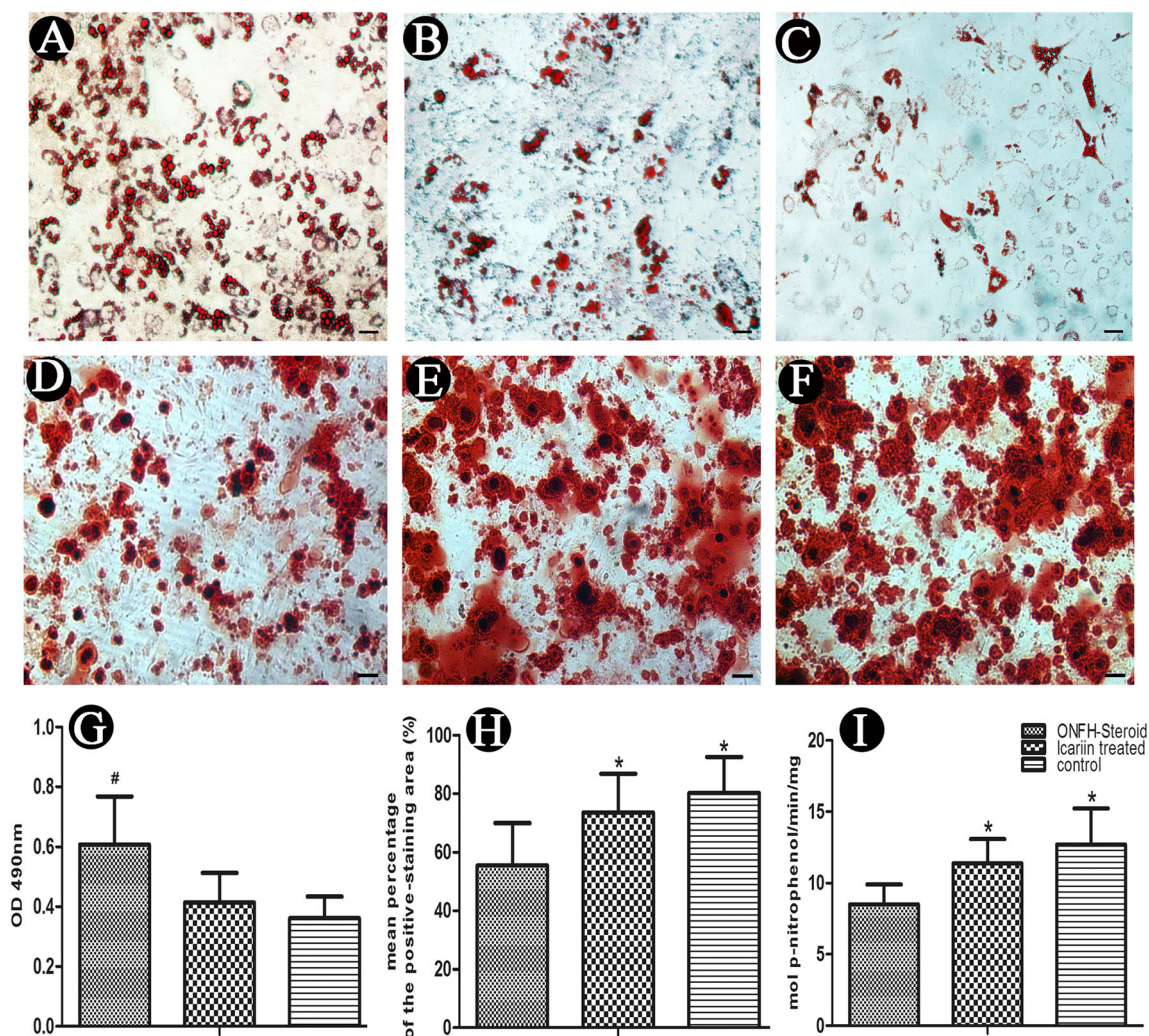


Fig. 7 Adipogenic and osteogenic differentiation. MSCs from all the groups were, respectively, induced to adipocytes and osteoblasts for 21 days. **a** Oil red O staining of ONFH group. **b** Oil red O staining of icariin group. **c** Oil red O staining of control group. **d** Alizarin red S staining of ONFH group. **e** Alizarin red S staining of icariin group. **f** Alizarin red S staining of control group. **g** The incorporated oil red O was

extracted with isopropanol and the quantification was performed as described in “Methods”. **h** The area stained with alizarin red S was measured as described in “Methods”. Data are represented as mean values±standard deviation. [#] $P<0.05$ vs. icariin group, control group; ^{*} $P<0.05$ vs. ONFH group (bars 100 μm)

is demonstrated to prevent steroid-induced osteonecrosis by suppressing the accumulation of lipid peroxidative products and oxidative DNA damage [41]. For MSCs, oxidative stress is regarded as a crucial mediator for adipogenic differentiation under GC’s administration [42]. Our previous study has demonstrated that oxidative stress happened in the MSCs of GC-associated ONFH could be relieved by de novo P-gp expression [20]. Here, we observed that treatment with icariin restored ABCB1 expression to a certain degree and concomitantly, the intracellular ROS level, MMP and other important indices of oxidative stress, reached almost the same levels as the control group. Correspondingly, the proliferation of MSCs in the GC-associated ONFH group was also improved. Hence, we speculate that icariin may alleviate the oxidative stress by de novo P-gp expression as well. Moreover, it is observed that MSCs in the ONFH group, treated with icariin, exhibited a

weakened adipogenesis and an enhanced osteogenesis. Taking together, there is a good possibility that the therapeutical effect of icariin on GC-associated ONFH is attributed to ABCB1-promoter demethylation of MSCs. However, the underlying mechanism remains to be studied further.

In this study, we found that icariin may be used as a potential drug targeting epigenetic changes for the treatment of GC-associated ONFH. By comparative observation, we confirmed that icariin, at a suitable concentration, protected MSCs of the patients with GC-associated ONFH from oxidative stress injury and adipogenesis while inducing de novo P-gp expression through ABCB1-promoter demethylation. This study has provided new information regarding the application of icariin targeting epigenetic changes in the treatment of ONFH. However, a better understanding of the regulatory mechanisms will benefit the conception of combined therapies

and innovative techniques for drug delivery. In the future, *in vivo* studies will be performed to further evaluate the effect of icariin on the treatment of ONFH animal models.

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Conflicts of interest None.

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