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Icariin promotes osteoblast differentiation and inhibits adipogenesis of bone marrow stem cells through activation of Wnt/β-catenin signaling *in vivo* and *in vitro*

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Icariin has been proved to promote the bone regeneration and thus have a therapeutic the potential against osteoporosis. However, the role of icariin in the regulation of osteogenesis and aidpogenesis in osteoporosis remains unclear. The present study was designed to investigate the role of in the icariin in osteoblast and adipocyte commitment and differentiation in a osteoporosis mouse model and a primary culture of murine bone marrow stem cells (BMSCs). *In vivo*, a total of 72 mice were randomly divided into 3 groups: SHAM group (mice without ovariectomy + normal saline), ICA group (ovariectomy + icariin treatment, 25 mg/kg/day), OVX group (ovariectomy + normal saline). At 12 weeks after surgery, murine long bones were harvest for radiographic evaluation and histomorphological analyses. *In vitro*, BMSCs were harvested and cultured in osteogenic medium. ALP staining and Oil Red O staining were used to detect the differences of osteoblast and adipocyte differentiation between the icariin treated BMSCs (10^{-6}mol/L) and non treated BMSCs. Western blot and quantitative real time PCR (qPCR) were used to examine the mRNA and protein expression levels of osteogenic and adipogenic related genes and the underlying mechanisms. In mice, oral administration of icariin increased the trabecular bone formation and decreased the adipocyte numbers in bone. *In vitro*, icariin promoted the expression levels of mRNA and protein of osteogenic related genes and inhibits adipogenic related genes during the culture of BMSCs. Icariin also accelerated the accumulation of active β-catenin in nucleus and upregulated genes regulated by β-catenin during the osteogenesis. Our results indicated that icariin promotes osteogenesis while inhibiting adipogenesis of BMSCs through the Wnt/β-catenin signaling pathway.

1. Introduction

Osteoporosis is a progressive disease of the skeletal system which features in the low bone mass and micro-architectural deterioration of bone tissue (Busenlechner et al. 2014). As a result, it increases the incidence of bone fractures and declines the quality of life. Commonly, the underlying mechanism of osteoporosis is thought to be with the decreased osteogenesis accompanied by increased activity of osteoclasts in the bone tissues (Makitie 2013). However, studies have shown that the balance between osteogenesis and aidpogenesis in the bone marrow plays a fundamental role in the maintenance of skeletal mechanical integrity (Rosen and Bouxsein 2006). Ageing and menopause tend to cause the increasing accumulation of adipocytes in the bone marrow, which were initially assumed to be the results of bone resorption (Horowitz and Lorenzo 2004). Thus, the adipocytes in the bone marrow may play a direct role in maintaining bone health: First, adipocytes and osteoblasts are from the same progenitor cells, ageing and other pathological changes could shift the cell fate of BMSCs from osteoblasts to adipocytes (Chen et al. 2016; Casado-Diaz et al. 2017). Second, the superfluous adipocytes accumulating in the bone marrow serve as an endocrine organ releasing of cytokines, adipokines and fatty acids, which enhances the activities of osteoclasts (Duque 2008). Therefore, osteoporosis can be considered as a lipotoxic bone disease, and it is urgent to find a new agent which can promote osteoblasts while inhibiting adipogenesis.

Icariin (molecular formula: $C_{33}H_{40}O_{15}$, molecular weight: 676.65; Fig. 1A) is a bioactive compound derived from traditional Chinese herbal remedy *Epimedium* (Zhai et al. 2013). This compound exhibits anti-inflammatory, anti-cancer and anti-oxidant activities, which have been widely studied (Liu et al. 2012; Song et al. 2013; Zhai et al. 2013). As a phytoestrogen, icariin was reported to protect against bone loss and improve bone mineral density *in vivo*, and these anti-osteoporosis activities partly derive from the fact that icariin promotes osteoblast differentiation and mineralization (Mok et al. 2010; Shi et al. 2017). However, the role of icariin in the lineage direction of BMSCs remains unclear: Is this anti-osteoporosis function of icariin partly derived from shifting cell fates of BMSCs from adipocytes to osteoblasts? What is the mechanism underlying its osteogenesis function? A further understanding of the role of icariin in the development of osteoblast and adipocyte could provide new insights into the treatment role of icariin in the course of osteoporosis.

In this study, we used the ovariectomy-induced osteoporosis mouse model and a culture of murine BMSCs to examine the function of icariin in lineage commitments of osteoblast and adipocyte *in vivo* and *in vitro*. Our study demonstrated that icariin promotes osteogenesis while inhibiting adipogenesis of BMSCs. Icariin treatment reduced ovariectomy-induced trabecular and cortical bone loss and marrow fat accumulation. Moreover, icariin enhances the expression of osteoblast related genes while inhibiting adipogenesis related genes through canonical Wnt/β-catenin pathway. Thus, our study provides new insights into the function of icariin in the course of osteoporosis.

2. Investigations and results

2.1. Icariin attenuates ovariectomy-induced bone loss and weight gain

To study the function of icariin in osteoporosis, ovariectomy was performed on 3-month old mice. One week after the operation, mice in the ICA group were orally treated with icariin (Taotu Biotech, China), at a concentration of 25 mg/kg/day. The dose of icariin was calculated according to pharmacological experiments and has been tested in previous studies (Ma et al. 2011; Shi et al. 2017). The other two groups were treated with the same amount of normal saline. To investigate the effect of oral administration of icariin on the changes of murine body weight during osteoporosis, we recorded the murine body weight every week after the operation. There were no differences of body weights among the three groups of mice on the first week after the operation. All the mice in the three groups went through an increased body weight over time. In week 5, the murine body weights of the OVX group were significantly increased compared to the other two groups ($P<0.05$). Interestingly, there was no significant difference between the ICA group and the SHAM group. However, in week 10, the body weights of ICA group were 15% higher than the SHAM group ($P<0.05$), but still less than in the OVX group (Fig. 1A). The results indicated that icariin could attenuate the increasing body weight induced by postmenopause, but could not reverse this trend. Since ovariectomy could result in the deterioration of bone microarchitecture, we then examined the effects of ICA on the changes of bone mass by using micro-CT. The results showed the OVX group had a reduction in bone volume (BV/TV), trabecular bone number (Tb.N), and an increase in trabecular bone separation (Tb.Sp) compared with the other two groups. However, there were no significant changes of bone microarchitecture between mice of the SHAM group and those in the ICA group (Fig. 1C, 1D). To further confirm whether treatment with icariin could improve the bone mass of cortical bone in ovariectomized mice, we performed a H&E staining on the murine long bones. The results showed that the volume of cortical bone in ICA group was remarkable increased compared with the OVX group, but still less than in the SHAM group (Fig. 1E). Taken together, the results showed that icariin treatment could attenuate ovariectomy-induced bone loss and weight gain.

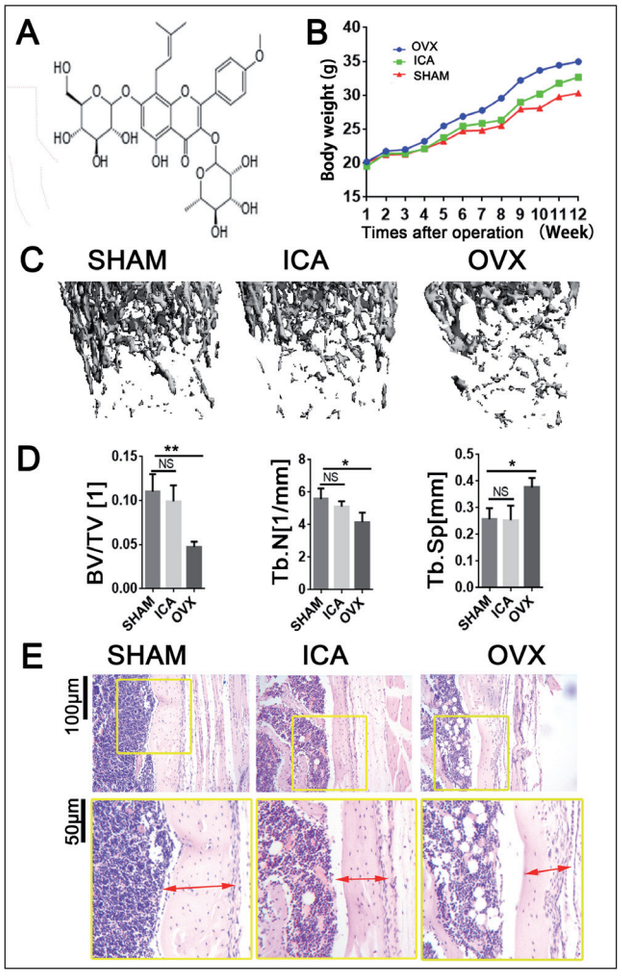

Fig. 1: Icariin attenuates bone loss and weight gain in murine osteoporosis model. (A) The chemical structure of icariin. (B) The body weights of every group of mice were recorded each week after operation for 12 weeks. (C) Micro-CT analysis of mice femurs. (D) Quantification data of Micro-CT indicates bone volume/tissue volume (BV/TV), trabecular number (Tb.N), and trabecular separation (Tb.Sp). (E) H&E staining of corticol bone in mice tibiae. Yellow squares are the higher magnification figures. Red arrows indicate the width of the cortical bone. Data were presented as mean±SD, n=5. NS denotes non-significant, *$P<0.05$; **$P<0.01$, ***$P<0.005$.

2.2. Icarin stimulates trabeculare bone formation and suppresses adipogenesis in murine bone marrow

To investigate the role of icariin in the lineage direction of BMSCs *in vivo*, we performed the H&E staining on murine femur sections. The OVX group showed tremendously decreased trabecular bone areas accompanied by increasing accumulation of adipocytes in the bone marrow as compared to the other two groups. Interestingly, ICA groups had fewer numbers of adipocytes in the bone marrow and higher trabecular bone areas compared with OVX groups (Fig. 2A, 2B, 2C). Although the trabecular bone areas of ICA group were decreased compared to the SHAM group, there were no differences of adipocyte formation in the bone marrow between these two groups. These results indicate that icariin treatment can reduce postmenopause related bone loss and adipocytes accumulation in the bone marrow.

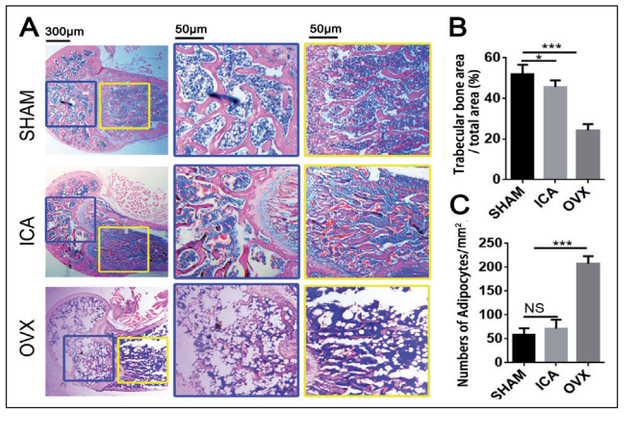

Fig. 2: Icarrin stimulates trabeculare bone formation and suppresses adipogenesis in murine bone marrow. (A) H&E staining of mouse femurs of SHAM, ICA, OVX. The blue and yellow squares are the higher magnification of left column. (B) Quantification of trabecular bone area of (A). (C) Quantification of the adipocyte numbers of (A). Data were presented as mean±SD, n =5. NS denotes nonsignificant, *$P<0.05$; ***$P<0.005$.

2.3. Icariin promotes osteoblast activity in vivo

The results above suggest that icariin can reduce bone loss and the numbers of bone marrow adipocytes in osteoporosis. To reveal if a reduction of bone loss after ICA treatment results from the increased activities of osteoblasts, we examined the osteoblast marker genes *in vivo* by using immunohistochemical (IHC) staining on the murine femur sections. Runt-related transcription factor 2 (Runx2) is the first transcription factor required for the determination of osteoblast commitment which is mostly expressed in the preosteoblast and immature osteoblasts. IHC staining results demonstrated that Runx2 expression level was similar between the ICA group and SHAM group (Fig. 3A, 3B). However, the expression level of oseocalcin (OCN), which is mostly expressed in mature osteoblasts, was lower in the ICA group than that in the SHAM group, but still upregulated

as compared to that in the OVX group (Fig. 3A, 3C). These results indicate that icariin treatment may promote osteoblast differentiation and mineralization in bone tissues.

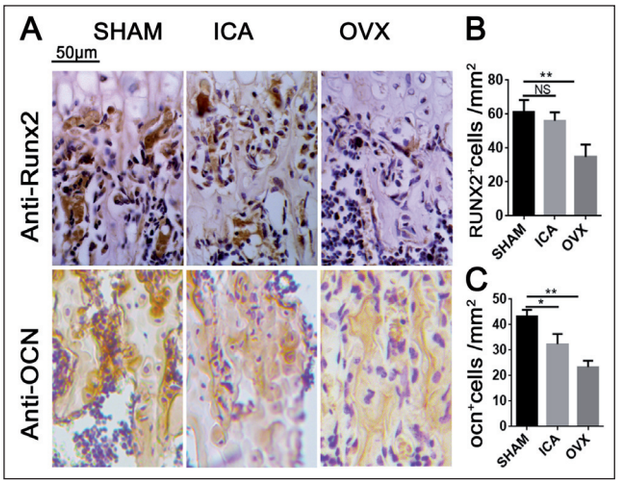

Fig. 3: Icarrin promotes osteoblast activity in vivo. (A) Immunohistochemical (IHC) staining with anti-Runx2 and anti-OCN antibodies of paraffin sections from murine femurs. (B) Quantification of Runx2 positive stained cells in (A). (C) Quantification of OCN positive stained cells in (A). Data were presented as mean±SD, n =5. NS denotes nonsignificant, **P*<0.05; ***P*<0.01, ****P*<0.005.

2.4. Icariin promotes BMSCs commitment to osteoblasts instead of adipocytes.

The *in vivo* data above show that icariin treatment prevented both bone loss and adipocytes accumulation in bone marrow under the conditions of osteoporosis. To determine the role of icariin in the regulation of the osteoblast and adipocyte lineage commitment, BMSCs from murine long bones were isolated and cultured in osteogenic medium. Cells were divided into two groups: Control group (no icariin) and ICA group (with 10^{-6}mol/L icariin). This concentration of icariin in the osteogenic medium has previously been examined to have better osteogenic function than other concentrations ranging from 10^{-8}mol/L to 10^{-4} mol/L (Liu et al. 2016). ALP staining and Oil Red O staining were performed on day14 cells to detect the differences of osteogenesis and adipogenesis between the control group and the ICA group. In comparison with the control cells, icariin treatment significantly facilitated osteoblast differentiation with higher intensity of ALP staining (Fig. 4A, 4B). Meanwhile, icariin conditional medium suppressed the adipogenesis of BMSCs as shown in the Oil Red O staining (Fig. 4C, 4D).

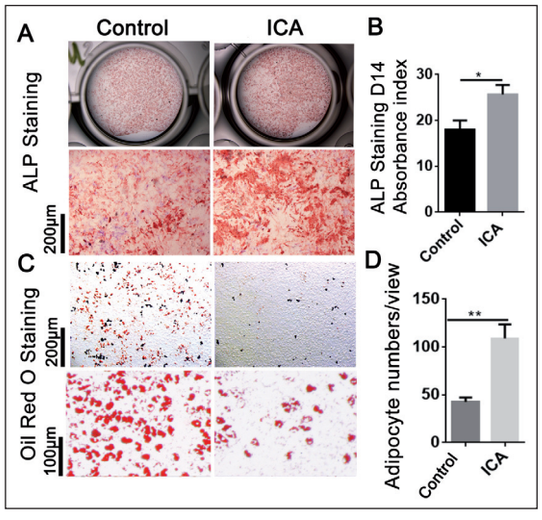

Fig. 4 Icariin promotes BMSCs commitment to osteoblast instead of adipocyte. (A) ALP staining of Day 14 BMSCs in osteogenic medium. (B) Quantification of ALP staining of (A). (C) Oil Red O staining of Day 14 BMSCs in osteogenic medium. (D) Quantification the adipocytes numbers of (C). Data were presented as mean±SD, n =7. NS denotes nonsignificant, **P*<0.05; ***P*<0.01.

2.5. Icariin activates osteogenesis related genes expression while suppresses adipogenesis related genes expression in vitro

To investigate the effects of icariin on the expression of osteogenesis and adipogenesis related genes expression, we harvested RNA and protein from day 14 BMSCs in osteogenic medium of two groups. The results of qRT-PCR showed: mRNA expression level of Runx2, Osterix (OSX), and OCN in the ICA group were increased by 5-fold, 3-fold, and 2.5-fold, respectively, compared to the control group, while the expression level of adipogenesis related genes in the ICA group were significantly decreased compared to control (Fig. 5A). As expected, Western blotting results confirmed that icariin effectively promoted the expression of Runx2 and OSX while suppressing the expression of CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor gamma (PPARγ) (Fig. 5B, 5C). Consistent with histological staining data, the results of qPCR and Western blot demonstrated that icariin promoted the expression of osteogenesis related genes while inhibiting the expression of adipogenesis related genes.

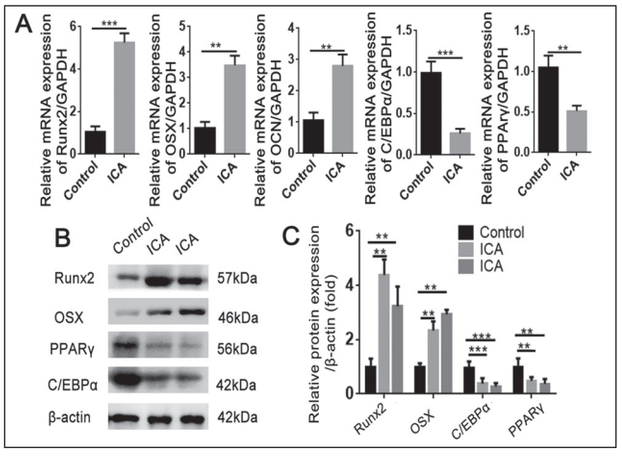

Fig. 5: Icariin activates osteogenesis related genes expression and suppresses adipogenesis related genes expression in vitro. (A) Expression of Runx2, OSX, OCN, C/EBPα and PPARγ in Day 14 BMSCs was detected by qRT-PCR and normalized to GAPDH. (B) Western Blot was used to detect the protein levels of Runx2, OSX, C/EBPα and PPARγ in D14 BMSCs. (C) Quantification the protein levels of (B) by normalizing to β-actin. Results were presented as mean±SD, n=7. NS denotes nonsignificant, **P*<0.05; ***P*<0.01, ****P*<0.005.

2.6. Icariin activates canonical Wnt/βcatenin pathway in vivo and in vitro

To further investigate the mechanism of how icariin regulates BMSCs commitment to osteoblasts instead of adipocytes, we performed *in vivo and in vitro* studies. Since Wnt signaling plays an important role in regulating BMSCs lineage commitmen (Kennell and MacDougald 2005), we detected the effects of icariin on the mRNA expression of Wnt ligands in Day14 BMSCs. The results showed that icariin significantly upregulated the expression of wnt5a and wnt10b (Fig. 6A). Then we investigated the β-catenin protein expression in the trabecular bone of murine femurs by using immunofluorescence (IF) staining, the results showed that icariin treatment increased the protein expression of β-catenin in trabecular bone by 2-fold as compared with that in the OVX group (Fig. 6B, 6C). We also examined the mRNA expression of Tcf1 and Lef1 which were regulated by β-catenin in D14 BMSCs. The results show that icariin promoted expression of Tcf1, Lef1

with 10-fold and 6-fold increases, respectively, compared to the control group (Fig. 6A). Taken together, these results demonstrate that icariin enhanced osteogenesis while inhibiting adipogenesis in BMSCs through the canonical Wnt/β-catenin pathway.

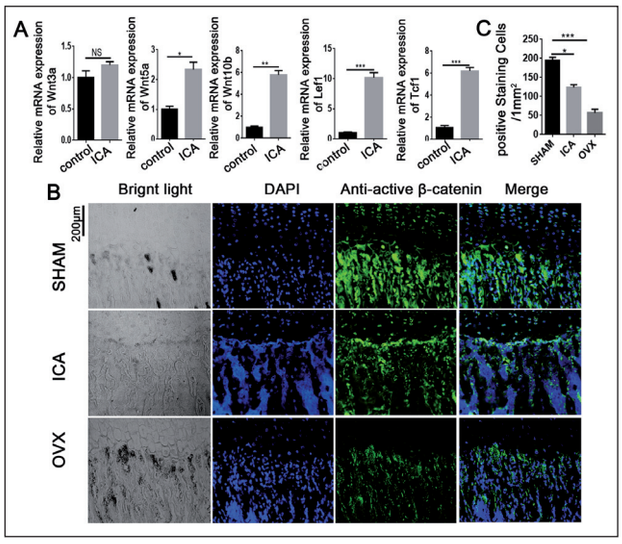

Fig. 6: Icarrin activates canonical Wnt/βcatenin pathway in *vivo* and in *vitro*. (A) Expression of Wnt3a, Wnt5a, Wnt10b, Lef1 and Tcf1 was detected by qRT-PCR and normalized to GAPDH. (B) Immunoflourence (IF) staining of active β-catenin on the 3 groups of murine femurs. (C) Quantification of positive staining cells of (B). Results were presented as mean±SD, n=5. NS denotes nonsignificant, **P*<0.05; ***P*<0.01, ****P*<0.005.

3. Discussion

Icariin, isolated from the Chinese traditional medicine Herba epimedii, has been widely proved to act against osteoporosis (Xu et al. 2016; Liu et al. 2017). Our previous studies also have demonstrated that icariin and its derivatives including icarisid I & II have osteoinduction and osteoconduction functions in bone regeneration (Liu et al. 2016; Luo et al. 2017). However, there is still doubt about the preventive role of icariin in the process of osteoporosis, especially its function in osteogenesis and adipogenesis in bone marrow (Ma et al. 2011; Li et al. 2014). In this study, we used the ovariectomized mouse model to examine the functions of icariin in the regulation of lineage commitments of osteoblasts and adipocytes *in vivo* and *in vitro*. Our results demonstrated that icariin played a protective role in maintaining the bone mass of ovariectomized mice by shifting lineage commitment of BMSCs from adipocytes to osteoblasts. Importantly, we found icariin to encourage osteogenesis while inhibiting adipogenesis by regulating the canonical Wnt/β-catenin pathway. This study provides direct evidence for the therapeutic potential of icariin in preventing bone loss and accumulating of marrow fat in the physiological and the pathological processes of osteoporosis.

In ovariectomized mice, we found that ovariectomy results in both trabecular and cortical bone loss, accompanied by the accumulation of bone fat 12-week after the surgery, which resembled the pathological features of osteoporosis (Fig. 1, Fig. 2). Body weight change in different groups of mice was used as another indicator to compare the functions of icariin treatment on bone formation, since it has been verified to be directly associated with bone mineral density (Gunn et al. 2014; Lecka-Czernik and Stechschulte 2014). Mice in the ICA group experienced less weight gain as compared with the OVX group, which partly might be explained by the changes of bone mass in ICA group lagged behind OVX group (Fig. 1).

To detect the function of icariin in osteoblast differentiation and mineralization during the process of osteoporosis, trabecular and cortical bone formation were checked by Micro-CT examination and H&E staining. The results demonstrated that icariin treatment not only improved trabecular bone volume and numbers, but also increased the thickness of cortical bones in murine femurs. These results were also confirmed by ALP staining *in vitro*, which showed increased osteoblast activities in the icariin treated BMSCs. As expected, the expression level of osteogenesis related genes in ICA group were significantly higher than those in the OVX group as shown in the IHC staining (Fig. 4), indicating a higher osteoblast differentiation and mineralization. These findings were in accordance with the in *vitro* studies, which demonstrated that icariin promoted BMSCs commitment to osteoblast and motivated osteoblast differentiation, correspondingly leads to upregulation of Runx2, OSX and OCN.

Bone marrow stem cells can differentiate into both osteoblasts and adipocytes. However, under some pathological conditions such as estrogen deficiency and ageing, BMSCs tend to commit to adipocytes instead of osteoblasts, which may cause osteoporosis characterized by reduced bone formation and increased marrow fat accumulation (Sui et al. 2016). Therefore, bone marrow adipocytes function as a negative factor in the deterioration of microstructure in bone diseases (Lecka-Czernik and Stechschulte 2014). Surprisingly, we observed that icariin antagonized adipogenesis in estrogen deficient bone marrow, which was confirmed by in *vitro* Oil Red O staining of BMSCs (Fig. 4C, 4D). To further understand the functions of icariin on the adipocyte commitment, we tested mRNA and protein expression levels of C/EBPα and PPARγ, which were two crucial transcription factors in adipogenesis (Kawai and Rosen 2010). The results demonstrated that icariin could significantly suppress the expression of C/EBPα and PPARγ. Taken together, our findings show that the preventive bone loss function of icariin comes not only from the promoting of osteogenesis but also from inhibition of BMSC adipogenesis.

The canonical Wnt/β-catenin pathway is an important regulator of lineage direction of BMSCs. It not only promotes osteogenic differentiation of BMSCs, but also strongly inhibits adipogenesis (Ross et al. 2000; Bennett et al. 2002). Loss of Wnt signaling results in a lineage shift of BMSCs from osteoblasts to adipocytes (Yuan et al. 2016). Accumulation of β-catenin was observed to cause a dose-dependent increase in osteogenesis and a dose-dependent decrease in adipocyte differentiation (Kokabu et al. 2016; Valenti et al. 2016). Interestingly, a higher expression of active β-catenin was observed in femurs of the ICA group compared to the OVX group (Fig. 7b). Furthermore, Lef1 and Tcf1, which are regulated by β-catenin, were also upregulated in the ICA group of cells. In addition to this, we examined the mRNA levels of Wnt ligands in D14 BMSCs, and it turned out that icariin could activate the expression of Wnt5a and Wnt10b. From our data, it is likely that icariin interacts with the canonical Wnt/β-catenin pathway to regulate the lineage direction of BMSCs. However, the mechanism by which icariin regulates the Wnt/β-catenin pathway still needs further investigation. Besides, our results showed icariin to strongly inhibit the expression of C/EBPα. Since C/EBPa can also prevent osteogenesis (Jules et al. 2016), inhibition of C/EBPα might be another mechanism for icariin to promote osteogenesis.

Our findings show, along with reduced bone mineral density in estrogen deficiency mice, that adipocytes were dramatically increased in murine bone marrow (Fig. 2), indicating shifting the lineage direction of BMSCs could be a new target for the treatment of osteoporosis. Moreover, our studies suggest that icariin effectively promotes the osteoblast commitment and differentiation while inhibiting adipogenesis, and the underlying mechanism might involve canonical Wnt/β-catenin signaling. Icariin comes from the traditional Chinese medicine Herba epimedii, which has been commonly used for thousands of years. As a result, icariin might be an effective candidate for the treatment of postmenopausal osteoporosis. Further investigation will be needed to elucidate the mechanisms by which icariin activates Wnt signaling, as well as other Wnt-independent pathways.

4. Experimental

4.1. Design of the experimental groups

The experiment was approved by the ethics committee and performed in compliance with the guidelines for the care and handling of experimental animals of laboratory animal center, Shanghai Tongji University. C57/6J female mice of 3-month-old weighing 19-21 g were used in the study. Mice were randomly divided into 3 groups

(8/group): Sham-operated group (SHAM), ovariectomized group (OVX) and oral administration of icariin combined with ovariectomy group (ICA). The experiments were repeated three times leasing to a total number of 72 mice. Mice in the groups of OVX and ICA underwent surgical removal of ovaries, while mice in the SHAM group were treated with the removal of the adipocyte tissues around the ovaries. The SHAM group acted as the positive control, while the OVX group as the negative control.

4.2. Icariin treatment

In the *in vivo* studies, one week after the operation, mice in the ICA group were orally treated with icariin (>99% purity, Taotu Biotech, China), at a concentration of 25 mg/kg/day. The other two groups were treated with the same amount of normal saline. Murine body weights were measured every week. Twelve weeks after the surgery, mice samples were harvested. For *in vitro* studies, BMSCs from murine long bones were isolated according to previously described protocols (Keating 2012). Cells were plated on six-well plates at a density of 10^5cells/well and cultured in osteogenic medium to induce osteoblast formation. The osteogenic medium is made of the growth medium added with 50 μg/ml l-ascorbic acid (Sigma, USA) and 5 mM β-glycerolphosphate (Sigma, USA). There were two groups of cells: the vontrol group (no icariin added to the medium) and ICA group with icariin added to the medium at a concentration of 10^{-6}mol/L,

4.3. Mice samples collection and tissue preparation

Murine femurs and tibiae were harvested and fixed in 4% paraformaldehyde (PFA) overnight. Samples were dehydrated by using the different concentration ethanol, they were then fixed in ethanol. Left femurs were used for the micro-CT examination, the right femurs were used for the histological analysis. For the histological analysis, the femurs and tibiae were decalcified for 2 weeks with 10% EDTA. For paraffin sections, samples were embedded in paraffin and sectioned at 6 μm using a microtome (Leica, Germany).

4.4. Radiographic evaluation

Micro-CT examination was performed as described before (Liu et al. 2016). Briefly, the specimens were scanned along the sagittal direction by micro-CT (Inveon, Siemens) with a resolution of 18 μm followed by an off-line reconstruction. The area of interest was chosen and the thresholding of mineralized bone was set at 350. The morphometric parameters were used to determine the bone formation by using the Siemens Inveon software: BV/TV: Relative bone volume; Tb.N: Trabecular number; Tb.Sp: Trabecular separation.

4.5. Histopathological examinations

For H&E staining, sections were cut at a thickness of 5 μm and mounted on slides (Fisher, USA). Sections were deparaffinized and hydrated through a xylene and graded ethanol series, stained with hematoxylin and then in eosin. After dehydration, the slides were mounted and observed under an optical microscope (Leica, Germany). Quantification of trabecular bone areas were analyzed using NIH Image J software, the adipocyte numbers in bone marrow were counted under the microscope. IHC staining was performed to analyze the osteoblast related genes. First, bone sections were incubated with primary antibodies against oseocalcin (OCN) (ab93876, Abcam, USA), Runt-related transcription factor 2 (Runx2) (ab23981, Abcam, USA) overnight at 4 °C. Then sections were incubated with the secondary antibodies for 1 h, and developed in DAB chromogen (Invitrogen). For IF staining, the sections were stained with primary antibody against β-catenin (Abcam, ab32572), images was taken by a Leica fluorescent microscope. Quantification of the positive stained cells was performed under the microscope.

4.6. In vitro osteoblastogenesis and adipogenesis assays

After 14 days cultured in the osteogenic medium, BMSCs were stained with ALP by according to manufacturers' manual (A2356, Sigma, USA). For the quantification of ALP activity, the cells were rinsed with PBS and lysed by 1% TritionX-100, then centrifuged for 30 min at 12000 x g. Total protein contents were determined by Pierce protein assay kit (Thermo Scientific, Rockford, USA). ALP activity was determined at 405 nm using *p*-nitrophenyl phosphate (Sigma, USA) as substrate. Adipogenesis was determined by Oil Red O staining, Cells were stained with solution of 0.5% Oil Red O (Sigma, USA) and 60% isopropanol with ratio of 4 to 6 at room temperature. Finally, cells were washed twice with distilled water and air dried. Adipocytes stained red were recorded by light microscopy (Leica, German).

4.7. RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

RNA samples from day 14 BMSCs were isolated with a genomic DNA eliminator. 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 kit in a final volume of 20 μl. The primers of the target genes are listed in the Table. To evaluate the gene expression levels, qPCR was performed with SYBR Green PCR Kit using iCycler (Bio-Rad). PCR reactions were performed at 0.5 μM for each primer in a 25 μl volume containing 1 μl cDNA sample. The reaction was initiated by activating the polymerase with 5 min pre-incubation at 95 ºC. Amplification was achieved with 45 cycles of 15 s denaturation at 94 ºC, 20 s annealing at 65 ºC and 10 s extension at 72 ºC. The program was concluded by a melting curve analysis. All experiments were performed in triplicate. The copy numbers of each gene were determined with cycle threshold (ΔΔCT) methods. The means of the copy numbers of GAPDH were used as internal controls. Standard curves of all primers were prepared from total normal cDNA, amplified by semi-quantitative PCR, and cloned using TOPO II TA Cloning Kit following the manufacturer's recommendations.

4.8. Western blot analyses

Cells were seeded into six-well plates and cultured in osteogenic medium. Total protein was harvested on day 14. Cells were washed twice in PBS and the cells were lysed in mammalian protein extraction reagent (Pierce Biotechnology, USA) to extract total cellular proteins. Equal amounts of cell lysates were separated on duplicate 8–10% SDS–PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membrane was blocked with 5% nonfat dry milk in TBST, and incubated with the first antibodies: peroxisome proliferator-activated receptor gamma (PPARγ) (ab59256, Abcam); OCN (ab93876, Abcam); Runx2 (ab23981, Abcam); Osterix (OSX) (SC22538, Santa Cruz); CCAAT/enhancer binding protein α (C/EBPα) (SC166258, Santa Cruz) respectively. The mean expression level of the target protein relative to β-actin was presented.

Talbe: Primer sequences utilized for qRT-PCR

Gene	Primer sequences
Rux2	F5′-GGGACCGACACAGCCATATA -3′
	R5′- TCTTAGGGTCTCGGAGGGAA -3′
OSX	F 5′- TCAAGATGGTGGCCGTTACT -3′
	R 5′- CATCTTGAGGTCACGGCATG -3′
OCN	F 5′- GACAAGTCCCACACAGCAACT -3′
	R 5′- GGACATGAAGGCTTTGTCAGA -3′
PPARγ	F 5′- GGAATCAGCTCTGTGGACCT -3′
	R 5′- TCAGCTCTTGTGAACGGGAT -3′
C/EBPα	F 5′- ATGTGCAGAAGTGGGATGGA -3′
	R 5′- TGCAAATTTCAGTCCAGGGC -3′
Wnt3a	F 5′-AACTGCACCACCGTCCAC -3′
	R 5′-AAGGCCGACTCCCTGGTA -3′
Wnt5a	F 5′-CTGCAGCACAGTGGACAATAC-3′
	R 5′- TAGCGTGGATTCGTTCCC-3′
Wnt10b	F 5′- GGCTGTAACCACGACATGGAC -3′
	R 5′- ACGTTCCATGGCATTTGCAC -3′
Lef1	F 5′- TGAGTGCACGCTAAAGGAGA -3′
	R 5′- GCTGTCATTCTGGGACCTGT -3′
Tcf1	F 5′- ACATGAAGGAGATGAGAGCCA -3′
	R 5′- CTTCTTCTTTCCGTAGTTATC -3′
GAPDH	F 5′- CAAGTTCAACGGCACAGTCA -3′
	R 5′- CCCCATTTGATGTTAGCGGG -3′

4.9. Statistical analysis

Data are presented as mean±SD (n>3). Statistical significance was assessed by using student's t-test. Multiple comparisons within and between groups were analyzed by one-way ANOVA for multiple comparison. P values less than 0.05 were considered significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$).

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