Myricitrin promotes osteogenesis and prevents ovariectomy bone loss through PI3K/AKT signaling pathway

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Research Article
Abstract

Background: Osteoporosis is characterized by reduced bone mass and destruction of trabecular bone microstructure, increasing fracture risk. Newly marketed drugs have certain advantages in osteoporosis, but high health care expenditures and side effects limit their application. Therefore, it is urgent to explore new approaches to break the therapeutic deadlock in the current clinical condition. Myricitrin has many biological activities. However, the effect on antiosteoporosis has not fully been elucidated yet.

Methods: The effect of myricitrin on the proliferation of immortalized bone marrow mesenchymal stem cells was evaluated using Cell Counting Kit-8 assays. Alizarin Red staining, Alkaline phosphatase staining, and activity were performed to elucidate osteogenesis. qRT-PCR and Western blot were performed to evaluate the expression of osteo-specific genes and proteins. To screen for candidate targets, sequencing of mRNA transcriptome genes was used through bioinformatics analysis. Western blot and Molecular docking analysis were used to examine target signaling markers. Rescue experiments were used to confirm myricitrin can enhance osteogenic differentiation of imBMSCs reversely. Moreover, ovariectomy mice were used to assess the effect of myricitrin on bone loss in vivo by Western blot, Micro-CT, Hematoxylin and Eosin staining, and immunochemistry.

Results: Myricitrin can significantly enhance osteo-specific genes and proteins expression, alkaline phosphatase activity, and calcium deposition in imBMSCs in vitro. These phenomena were accompanied by an upregulation of the PI3K/AKT signaling pathway via bioinformatics analysis and Western blot. In addition, the improvement of osteogenesis due to myricitrin intervention was partially reversed by copanlisib, which is a PI3K inhibitor. Osteopontin (OPN), osteoprotegerin (OPG), p-PI3K, and p-AKT were significantly increased in the low dose and high dose myricitrin groups compared with the OVX group. Micro-CT, H&E staining, and immunochemistry showed that bone mass was significantly increased in low dose and high dose groups compared with the OVX group. Furthermore, myricitrin can attenuate bone loss in ovariectomy mice.

Conclusion: These data suggest that myricitrin enhances the osteogenic differentiation of imBMSCs, partly via activation of the PI3K/AKT signaling pathway. Myricitrin may be a potential drug development for the treatment of postmenopausal osteoporosis.

Introduction

Osteoporosis is characterized by reduced bone mass and destruction of trabecular bone microstructure, increasing fracture risk. Osteoporosis is becoming a public health issue and an estimated 5 million new osteoporotic fractures occur every year in China[1]. In mechanism, osteoporosis is caused by increased bone resorption and decreased bone formation. The destruction of the coupling balance between osteoblast and osteoclast is the direct cause of osteoporosis. With the increase of age, the elderly's absorption of calcium and vitamin D and PTH secretion decreased, which causes a decrease in calcium salt deposition. However, osteoclasts are still active, leading to the fracture of trabecular microstructure,
eventually lower bone strength. In clinical, anti-osteoporosis drug therapy can be divided into promoting bone formation and anti-bone resorption according to the function. The representative drugs promoting bone formation are parathyroid analogs, such as teriparatide and abaloparatide\[2, 3\]. Anti-bone resorption drugs are calcitonin, bisphosphonates, selective estrogen receptor modulators (SERMs), specific RANKL monoclonal antibodies, and Cathepsin K (CTSK) inhibitor. The representative drugs respectively are salmon calcitonin\[4\], zoledronate sodium\[5\], raloxifene\[6\], denosumab\[7\], and odanacatib\[8\]. Currently, effective prevention of osteoporosis mainly relies on the combined intervention of calcium, vitamin D, and anti-osteoporosis drugs for the treatment of the improvement of symptoms\[9\]. What is more, there are increasing numbers of osteoporosis patients’ intolerant of adverse effects of newly marketed drugs and/or those who cannot afford expensive health care expenditures. Therefore, it is urgent to explore new approaches to break the therapeutic deadlock in the current clinical condition.

Traditional Chinese medicine (TCM) is becoming an important source for the development of antiosteoporosis drugs. Especially in recent years, TCM has made remarkable progress toward the prevention of osteoporosis. What is more, many studies have demonstrated that TCM can effectively improve the symptoms of osteoporosis\[10–12\].

*Chinese Waxmyrtle Bark*, traditional herbal medicine in East Asia, has been widely used to treat patients against bone-resorbing and osteoarthritis\[13, 14\]. But its active ingredients and mechanism are not elucidated. Myricitrin is widely derived from *polygonum aviculare*, myrica bark, and myrica leaves. Myricitrin is the primary active principle component of *Chinese Waxmyrtle Bark*, which has many biological activities, including antioxidative and anti-inflammatory effects. However, the effect of myricitrin on antiosteoporosis has not fully been elucidated yet.

Bone-forming cells build mineralized microstructure and couple with bone-resorbing cells, harmonizing bone mineral acquisition and remodeling to maintain bone mass homeostasis. Mesenchymal stem cells have attracted much attention due to their multiple differentiation pathways that can differentiate into multiple cells under different conditions, seed cells suitable for tissue engineering, and their role as immunomodulators in autoimmune diseases\[15, 16\]. Marrow mesenchymal stem cells (MSCs) can differentiate into mesoderm and neuroectodermal tissue cells, including osteoblasts, chondrocytes, myoblasts, fibroblasts, and glial cells\[17, 18\]. Bone mesenchymal stem cells (BMSCs) isolated from the bone marrow, a subset of marrow stromal cells, are one of the parts with rich sources and convenient samples. Osteoblasts construct mineralized microstructures and couple with osteoclasts to coordinate the acquisition of bone minerals and remodeling to maintain bone mass homeostasis. Osteogenic precursor cells have a variety of sources. BMSCs are one of the ideal cells for bone and cartilage tissue engineering seed cells\[19\]. Even though BMSCs are one of the most widespread applications of MSCs, their isolation and maintenance culture are time-consuming. Thus, immortalized bone marrow stromal stem cells (imBMSCs) are used in this study.

To explore the therapeutic potential and molecular mechanism of myricitrin in antiosteoporosis, sequencing of mRNA transcriptome genes combined with a bioinformatics approach was used to explore
the potential targets of myricitrin in treating osteoporosis. In this study, we observed that myricitrin promoted osteogenic differentiation of imBMSCs in vitro and was partially dependent on PI3K/AKT signaling pathway. In vivo, we used the ovariectomy model in female mice to evaluate the ability of myricitrin to reduce bone loss.

Materials And Methods

Reagents and antibodies

Myricitrin (> 98%) was purchased from Feiyubio (Jiangsu, China). Copanlisib was purchased from Topscience (Shanghai, China). Primary antibodies against Collagen alpha-1 type I (Collagen-I/Col1a1), Sp7/Osterix, osteopontin (OPN), osteocalcin (OCN), osteoprotegerin (OPG), PI-3 Kinase p85 alpha (phospho Y607) and PI-3 Kinase p85 alpha were from Abcam (Cambridge, UK). Primary antibodies against runt-related transcription factor 2 (Runx2), Phospho-Akt, and AKT1 were from Cell Signaling Technology (Danver, USA). Minimum essential medium-alpha modification (MEM-α), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco BRL (Thermo Fisher Scientific, USA). Amphotericin-B was purchased from Viva Cell (Shanghai, China). The complete medium consisted of MEM-α and 10% FBS, 1% penicillin/streptomycin, and amphotericin-B. The osteogenic differentiation medium was supplemented with 10 mM/L β-sodium glycerophosphate, 50 µg/mL Vitamin C, 100 nM/L Dexamethasone (Sigma-Aldrich, USA) based on a complete medium.

Culture of immortalized mouse bone marrow stromal cells (imBMSCs)

Reversibly imBMSCs were donated by the T-C HE Research Group at the University of Chicago. imBMSCs were previously characterized by MSC markers and osteogenic, chondrogenic, and adipogenic differentiation in vitro and in vivo[20]. The imBMSCs were cultured in a complete medium and incubated at 37 °C with 5% CO₂ in a carbon dioxide cell incubator. The cells were passaged every 3 days at a ratio of 1:3 with 0.25% trypsin-EDTA (Gibco, USA). The cells from passages 3–8 were used in experiments.

Cell viability assay

The Cell Counting Kit-8 (CCK-8) assay kit (Bimake, USA) was used for the determination of cell viability in cell proliferation and cytotoxicity. The effect of myricitrin on the proliferation of imBMSCs cells was assessed using a CCK-8 kit (Bimake, USA). Cells (3×10³/well) were incubated in 96-well plates and cultured with different concentrations of myricitrin (0, 1, 5, 10, 25, 50, and 100 µM) for 24, 48, 72, and 96 h. Subsequently, 10 µL CCK-8 solution was added to each well at the respective time point and then incubated for 2 h at 37 °C. The absorbance of the samples was assessed at 450 nm with a Microplate Spectrophotometer (Omega, USA).

Alkaline phosphatase (ALP) and Alizarin Red staining (ARS) and activity measurement
ALP staining and ARS were performed to determine the osteogenesis in imBMSCs cells. After 8 days of osteogenic differentiation, cells were fixed with 4% polyoxymethylene for 30 min at room temperature. The ALP staining was detected using a BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, Shanghai) according to the manufacturer's instructions. For measurements of ALP activity, cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai). The ALP activity in the cellular fraction was measured using BCIP/NBT Alkaline Phosphatase Activity Assay Kit (Beyotime, Shanghai) following the manufacturer's instructions. The absorbance of the samples was assessed at 405 nm with Microplate Spectrophotometer (Omega, USA).

As for ARS, after 16 days of osteogenic induction, cells were subjected to Alizarin Red (pH 4.2, Cyagen, USA) for 5 min at room temperature. Subsequently, they were washed two times with ultrapure water (Mi Milli-Q® IQ 7000) to reduce nonspecific staining. Images were scanned under a microscope (Nikon, Japan). To quantify mineralization, calcium deposition was desorbed with 10% cetylpyridinium chloride (Sigma-Aldrich) for 30min in the dark. We collected and diluted the solution 5 times and the absorbance of the samples was assessed at 562 nm with Microplate Spectrophotometer (Omega, USA).

**RNA extraction and real-time PCR**

Gene expression was detected by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). The total RNA was extracted by Trizol™ reagent (Yeasen, Shanghai). 2 µg of RNA from each group was reverse transcribed to cDNA using Hifair® Strand cDNA Synthesis SuperMix for qPCR (Yeasen, Shanghai) according to the provided protocol. Primers used for the qRT-PCR assay were obtained from (Tsingke Biotechnology Co.Ltd, Beijing). The primer sequences were shown in Table 1. GAPDH was used as an internal control for normalizing mRNA expression and the gene expression levels were calculated employing the $2^{-\Delta\Delta C_t}$ method.

**Western blot (WB)**

Proteins were extracted from imBMSCs and the fourth vertebrae of mice with RIPA (Beyotime, Shanghai) supplied with a protease inhibitor cocktail (Bimake, USA) and phosphatase inhibitor cocktail (Bimake, USA). 30 µg total protein was subjected to SDS-PAGE separation and then the proteins were transferred to PVDF membranes (Millipore, Germany). Next, the membranes were blocked with QuickBlock™ Blocking Buffer (Beyotime, Shanghai) and incubated with primary antibodies overnight at 4°C. Then the membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology, USA) for 1 h and finally, the proteins were visualized using a Chemiluminescent ECL reagent (Tanon, Shanghai). The following antibodies were used: Anti-Collagen-antibody, anti-Runx2 antibody, anti-Osterix antibody, anti-PI3K antibody, anti-p-PI3K antibody, anti-AKT1 antibody, anti-p-AKT antibody, anti-OPN antibody, anti-OPG antibody, anti-GAPDH antibody.

**RNA sequencing analysis**

Total RNA was extracted from the cultured in different concentrations of myricitrin (0, 10, 25 µM) imBMSCs for 8 days by using Trizol™ reagent. The quality and integrity of total RNA samples were
assessed using a 2200 TapeStation (Beijing Genomics Institution) (BGI) (Wuhan, China). The preparation of whole transcriptome libraries and deep sequencing were performed by BGI. We used the Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway and Gene Ontology (GO) enrichment analysis for the results by RNA-seq. Dr. Tom bioinformatics tools were used for functional annotation enrichment and clustering.

**Molecular docking analysis**

Molecular docking tool BIOVIA Discovery Studio 2019 (Pipeline Pilot, USA) and Auto Dock Vina (Scripps, USA) software were used to analyze myricitrin ligand docking and PI3 Kinase p110δ receptor binding pocket. The PDB format structure of PI3 Kinase p110δ was downloaded from the Protein Data Bank (https://www.rcsb.org/structure/5ngb). The molecular structure of myricitrin was downloaded from the PubChem dataset and saved in pdbqt format. Subsequently, it was evaluated to the binding affinity of the compound-target relationship and docked into a grid generated from the protein structure using standard precision docking mode by Auto Dock Vina 1.5.7. The binding models were visualized by Discovery Studio 2019 software.

**Immunofluorescence (IF)**

The expression of Collagen-I and Runx2 were detected in different concentrations of myricitrin (0, 25 µM) and copanlisib 10 nM imBMSCs for 8 days. We seeded the cell at a density of 4×10^4 in a Confocal (Corning, USA) dish. Then imBMSCs were fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.3% Triton X-100 for 15 min, and blocked with goat serum albumin for 1 h at room temperature. After washing 3 times with PBS, cells were incubated overnight at 4  ºC with primary antibodies specific to Collagen-I(1:400, Abcam, UK)and Runx2(1:400, Cell Signaling Technology, USA). Cells were incubated with 488 Alexa Fluor conjugated secondary antibodies (1:200, Cell Signaling Technology, USA) for 1 h at room temperature. The nuclei were stained with Hoechst 33358 for 10 min at room temperature. Fluorescence images were captured on a Nikon fluorescence microscope (Nikon Life Science, Japan). The level of expression was determined using integrated optical density through the ImageJ software (Media Cybernetics, Inc, USA).

**Dual-luciferase reporter assay**

The Dual-luciferase reporter assay was used to examine the effect of myricitrin on PI3K/AKT downstream transcriptional activity. imBMSCs were seeded in 96-well plates at 5×10^3 cells/well and then transiently transfected with pMyc-TA-luc (Beyotime, Shanghai), Renilla luciferase reporter plasmid, and pFR-luc for 24 h. Then cells were treated with 25 µM Myricitrin for 24 h, followed by treatment with 25 µM myricitrin and copanlisib for another 24 h. Cells were then lysed with lysis buffer (Beyotime, Shanghai). The luciferase activities were measured with the dual-luciferase reporter assay system (Promega, USA) using a luminometer. The luciferase activity was normalized to Renilla Luciferase activity as an internal control and expressed as the fold change.

**Ovariectomy (OVX) osteoporosis mice model experiments**
All animal experimental procedures were approved by the Animal Welfare Ethics Committee of First Affiliated Hospital of Guangzhou University of Chinese Medicine (2021006). The mice were carried out concerning the criteria outlined in the Guide for the Care and Use of Laboratory Animals (NIH, USA). A total of 8-week-old female mice were used to construct the osteoporosis model by removing bilateral ovaries. The mice were housed in ventilated cages in groups of 5 under the SPF conditions of 22–25 °C and 12-hour daylight. The mice were randomly divided into sham operation group (only cutting the skin of mice without damaging the peritoneal structure), model group (ovariectomy followed by PBS vehicle treatment), low-dose group (ovariectomy followed by 5 mg/kg myricitrin once every two days) and high-dose group (ovariectomy followed by 25 mg/kg myricitrin once every two days). After 8 weeks of intervention, the mice were sacrificed. The fourth vertebrae were taken for WB. The bones of the lower limbs were decalcified for 24 h. Micro-CT instrument was used to analyze the relevant physical parameters of the distal femur. Hematoxylin and Eosin (HE) staining and immunohistochemistry (IHC) were performed to analyze the expression of OPN, OPG, OCN, and signal pathway marker p-PI3K.

**Micro-CT scanning (µCT)**

After intraperitoneal injection for eight weeks, femurs harvested from the mice were fixed in 4% paraformaldehyde for 24 h and scanned using Micro-CT Skyscan 1276 system (Kontich, Belgium). Scan settings are as follows: voxel size 6.5 µm, medium resolution, 85 kV, 200 mA, 1 mm Al filter, and integration time 384 ms. Density measurements were calibrated to the manufacturer's calcium hydroxyapatite (CaHA) phantom. Analysis was performed using the manufacturer's evaluation software. Reconstruction was accomplished by NRecon (version 1.7.4.2). 3D images were obtained from contoured 2D images by methods based on distance transformation of the grayscale original images (CTvox; version 3.3.0). 3D and 2D analyses were performed using the software CT Analyser (version 1.18.8.0). Analyses of the bone microarchitecture were carried out in a region of interest (ROI). We chose the cancellous bone of the distal femur 1.3 mm above the growth plate as ROI. The parameters for evaluating the trabecular bone, include mean volumetric bone mineral density (BMD, mg/cm³), bone volume/tissue volume (BV/TV, %), trabecular thickness (Tb. Th, mm), trabecular spacing (Tb. Sp, mm), trabecular number (Tb. N, 1/mm), Structure model index (SMI), and Degree of Anisotropy were analyzed using software CT Analyzer program (Bruker micro-CT, Kontich, Belgium).

**Histological observation(HE·IHC)**

The dissected femurs were washed in PBS, fixed in 4% polyoxymethylene for 24 h, and decalcified in 0.5 M EDTA for 4 weeks. Next, all the samples were dehydrated and embedded in paraffin before sectioning (5 µm). The sections were then processed with H&E staining after deparaffination. As for IHC analysis, slides were subjected to sodium citrate buffer for antigen retrieval and then incubated with primary antibodies against OPN(1:200, Abcam), OPG (1:200, Abcam), OCN (1:200, Abcam), and p-PI3K (1:200, Abcam) for detecting their expression changes. Images were acquired with the automatic digital slide scanner (Pannoramic MIDI, 3DHISTECH Ltd, Hungary.) and histopathological lesions were evaluated with Case Viewer 2.4(3DHISTECH Ltd.). Images of mean integrated optical density were analyzed by Image-Pro Plus 6.0(Media Cybernetics, Inc, USA).
Table 1
Sequences of primers for quantitative RT-PCR analyses

<table>
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<tr>
<th>Gene ID</th>
<th>Sequence (5’ to 3’)</th>
<th>Length of product (bp)</th>
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<tbody>
<tr>
<td>Runx2</td>
<td>F: CCGGTCTCCCTCCAGGAT</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>R: GGGACTGCTGCTGCTTC</td>
<td></td>
</tr>
<tr>
<td>Osterix</td>
<td>F: GAAGTCAATGGGGATCTGA</td>
<td>189</td>
</tr>
<tr>
<td></td>
<td>R: AGAATCCTTTGCCCTCTCCA</td>
<td></td>
</tr>
<tr>
<td>Col1a1</td>
<td>F: GAGCAGAGGTACTGGATCG</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>R: GCTTCTTTCTTTGGGTTC</td>
<td></td>
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<tr>
<td>OPN</td>
<td>F: CCTCCGGTGAAATGAC</td>
<td>127</td>
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<tr>
<td></td>
<td>R: CTGTGGCGCAAGGAGATT</td>
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<tr>
<td>OCN</td>
<td>F: CTTTCATGTCCAAGGAGA</td>
<td>161</td>
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<tr>
<td></td>
<td>R: GGCCTCTTTCAAGCCATA</td>
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<tr>
<td>ALP</td>
<td>F: CCCCATGTGATGGCGTAT</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>R: CGGTAGGGAGAGCACGAC</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: CATCACTGCCACCCAGAAGACTG</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td>R: ATGCCAGTGAGCTTCCTCCGTTCAG</td>
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</table>

Statistical analysis

Experiments were repeated at least three times. The data are presented as mean ± standard error of the mean (SEM). Statistical comparisons were performed using the one-way ANOVA followed by Newman-Keuls multiple comparison tests. Then the results were visualized by GraphPad Prism (8.0.2). Differences with a p-value of less than 0.05 were considered to be statistically significant.

Results

Myricitrin enhanced the proliferation of imBMSCs

The 2D and 3D structures of myricitrin (pentahydroxyflavonoid-3-rhamnoside) were shown, which is a natural polyphenolic hydroxyl flavonoid glycoside (Fig. 1A, B). We used CCK-8 assays to determine the effect of myricitrin on cells viability. As shown in Fig. 1C, myricitrin can significantly increase cell proliferation at 10 µM and 25 µM compared with the control group (p < 0.0001).

Myricitrin promoted osteogenesis of imBMSCs
ALP staining, ALP activity, and ARS were performed to elucidate the effect of myricitrin on osteogenesis in vitro. We measured the ALP activity of imBMSCs after incubating with different concentrations of myricitrin. As shown in ALP staining, myricitrin increased the ALP activity of imBMSCs in a dose-dependent manner (Fig. 1D). Similar results were observed by the ALP activity detection (Fig. 1E) ($p < 0.0001$). We detected ARS of imBMSCs after incubating with different concentrations of myricitrin for 16 days. Moreover, myricitrin enhanced the number of calcium deposits, which was examined by ARS (Fig. 1F). In addition, semiquantitative analysis of ARS further confirmed the previous results (Fig. 1G) ($p < 0.0001$).

**Myricitrin increased the expression of osteogenic differentiation-related marker genes and proteins**

The mRNA expression levels of osteogenic differentiation-related marker genes were measured by qRT-PCR. The results showed that the mRNA expression levels of Col1a1, Runx2, Osterix, OPN, OCN, and ALP were significantly increased by myricitrin at the concentration of 25 µM compared to the OIM group (Fig. 2A-F). Furthermore, the results by WB demonstrated that the protein levels of Col1a1, Runx2, Osterix, OPN, and OCN were significantly increased by myricitrin at 25 µM compared to the OIM group ($p < 0.001$) (Fig. 2H-K). In addition, we tested OPG by WB and found that myricitrin also enhanced its protein expression ($p < 0.01$) (Fig. 2M). Taken together, these results suggest that myricitrin can promote osteogenic differentiation of imBMSCs in vitro.

**Identification of myricitrin-responsive mRNA in imBMSCs**

To explore the mechanism of myricitrin to regulate osteogenic differentiation, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) analysis. According to the criteria of $|\log2FC|\geq 1$ and Q value $\leq 0.05$, a total of 77 genes was up-regulated and 169 genes was down-regulated (Fig. 3A). Further, we performed a clustering heat map analysis of the top 50 differential genes. The results demonstrated that Myc was the thirteenth up-regulated gene in the myricitrin intervention group compared to the OIM (Myciritrin 0) group. Based on the heatmap, Myc level was significantly inhibited in the OIM group, while the expression of Myc was significantly increased after the myricitrin intervention group, especially in the myricitrin 25 µM group (Fig. 3B). KEGG pathway analysis revealed that PI3K/AKT, HIF-1, and Rap1 signaling pathways may be involved in the treatment of osteoporosis by myricitrin. The top 20 of the most significant KEGG pathways were shown in Fig. 3C. The GO terms included cell components, biological process, and molecular function. The results suggested that these differentially expressed genes were highly related to the extracellular complex, collagen-containing extracellular matrix, angiogenesis, growth factor activity, and collagen protein binding. The top 20 most significant GO terms were shown in Fig. 3D-3F.

**Myricitrin activated the PI3K/AKT signaling pathway to promote osteogenesis of imBMSCs**
To determine the mechanism through which myricitrin affects imBMSCs osteogenic differentiation. We verified the expression of proteins associated with the PI3K/AKT signaling pathway after different concentrations of myricitrin intervention. The results revealed fold changes of p-PI3K ($p < 0.001$) (Fig. 4B) and p-AKT ($p < 0.0001$) (Fig. 4C) were increased after myricitrin intervention.

Based on this study (Fig. 3C,4A-B,4D), myricitrin and its corresponding key target mouse phosphatidylinositol 3-kinase p110delta (PIK3CG) (PDB ID:5ngb) appeared higher degree of connectivity according to the corresponding relationship between the active ingredients and their targets. The greater the absolute value of the docking affinity, the stronger the binding ability appeared between the compounds and the active site of the targets. The Affinity of myricitrin and 5ngb was −8.5 kcal/mol, which means they can interact well indeed. (Fig. 4D)

Copanlisib can partially inhibit the increased osteogenesis of imBMSCs proliferation by myricitrin

To further investigate the involvement of the PI3K/AKT signaling pathway, the inhibitory effect of this pathway on osteogenesis in the myricitrin intervention group was evaluated. Copanlisib, an ATP competitive selective PI3 kinase inhibitor, acting on PI3Kα, PI3Kδ, PI3Kβ, and PI3Kγ, which was used in the rescue experiment (Fig. 5A, B). We first verified CCK-8 to detect the effect of copanlisib on the cell proliferation of imBMSCs. The results demonstrated that 7.8125 nM and 15.625 nM copanlisib group can suppress cell proliferation more than the control group in 72 h and 96 h. (Fig. 5D). Therefore, we selected 10 nM copanlisib to further detect the effect on osteogenesis. With copanlisib intervention, ALP staining and activity assay results revealed higher ALP expression in the 25 µM myricitrin group than in the 25 µM myricitrin + copanlisib 10 nM group ($p < 0.0001$) (Fig. 5D, E), and similar calcium deposition was examined by Alizarin Red staining and semi-quantitative analysis ($p < 0.01$) (Fig. 5F, G).

Moreover, inhibition of the PI3K/AKT signaling pathway partially reversed the positive effects of myricitrin on the expression of osteo-specific genes in Runx2, Collagen-1, OPN, OCN ($p < 0.0001$) and Osterix, ALP ($p < 0.001$) as demonstrated by the qRT-PCR (Fig. 6A–F). The results were similar to proteins by WB. (Fig. 6G). Copanlisib also can prevent osteo-specific proteins in Col1a1 ($p < 0.001$), OPN ($p < 0.0001$), Runx2 and Osterix ($p < 0.01$) (Fig. 6H–L). In addition, copanlisib also reduced the cellular immunofluorescence intensity levels of Runx2 and Col1a1 ($p < 0.01$). (Fig. 7A-D).

Furthermore, we detected whether copanlisib affected the expression of downstream transcription factor Myc in PI3K/AKT signaling pathway by dual-luciferase reporter gene assay. The results demonstrate that copanlisib can partially reverse the positive effects of Myc induced by myricitrin ($p < 0.01$) (Fig. 7E). It indicated that copanlisib can not only directly inhibit PI3K function, but affect PI3K/AKT signaling pathway.

Myricitrin attenuated ovariectomy-induced bone loss

We performed WB, Micro-CT analysis, HE staining, and IHC to evaluate the effect of myricitrin on OVX-induced osteoporosis in vivo. The results showed that the expression levels of p-PI3K and p-AKT were
significantly increased in a dose-depend manner when compared to the OVX group (Fig. 8A-C). Compared to the OVX group, treatment with myricitrin was significantly up-regulated the levels of OPN and OPG, but there was no significant statistical difference between the low dose and high dose groups. (Fig. 8D-E).

As shown in Fig. 8F, myricitrin significantly improved bone microarchitecture compared to the OVX group. Meanwhile, BV/TV, Tb. Th, and Tb. N were also remarkably increased in the low dose and high dose groups (Fig. 8G-I). Consistently, Micro-CT analysis showed that BMD was increased in the myricitrin-treated group, while treatment with myricitrin could decrease Tb. Sp, the degree of anisotropy, and SMI indicating that myricitrin can partially rescue bone loss in ovariectomy mice (Fig. 8K-M).

The representative images of HE staining revealed that bone microarchitecture gradually deteriorated in the OVX group, but myricitrin can partially rescue the tendency (Fig. 9A-B). The representative images of IHC demonstrated that the expression of p-PI3K, OPN, OPG, and OCN were down-regulated in the OVX group, treatment with myricitrin can rescue their expression (Fig. 9C-J). These data revealed that myricitrin can partially prevent ovariectomy-induced bone loss and plays a positive role in the treatment of osteoporosis.

**Discussion**

Bone formation includes two main pathways, namely endochondral ossification and intramembranous ossification. This balance can be accomplished by regulating the activity of osteoblasts and osteoclasts. Bone marrow mesenchymal stem cells play a central role in the osteogenesis pathway. In the process of osteogenic differentiation, the basic biological characteristics are the development and maturation of trabecular bone. Runx2 and Osterix are considered to be two key transcription factors regulating the osteogenic differentiation of MSCs.[21] Osterix, also known as SP7, belongs to the Sp family transcription factors and is another important transcription factor regulating MSCs osteogenic differentiation. Osterix is a downstream gene of Runx2 that can be activated by Runx2.[22] Cells first synthesize extracellular matrix such as collagen-I, osteocalcin, osteopontin, and release calcium ions and alkaline phosphatases through matrix vesicles. Calcium ions precipitate on collagen fibers under the action of alkaline phosphatase, complete the matrix mineralization process, and finally form bone tissue.[23] Bone marrow macrophages play an important role in the osteoclast differentiation pathway. Osteoclast differentiation is mainly affected by RANKL/ RANK/OPG signaling axis. RANKL activates osteoclasts to participate in the regulation of bone resorption by binding to the receptor activator of nuclear factor kappa B(NF-κB) receptor activator (RNAK)[24]. The process is affected by NF-κB[25], MAPK signaling pathway[26], reactive oxygen species (ROS)[27], and calcium[28]. Subsequently, these processes can activate NFATc1, which is the most critical regulator of osteoclast differentiation by directly regulating the transcriptional expression of osteoclast-related specific genes, such as c-Fos, Ctsk, MMP9, Acp5. Therefore, once abnormal osteoblast differentiation occurs, it will have adverse effects on bone metabolism balance. Therefore, promoting the osteogenic differentiation of MSCs and inhibiting osteoclasts differentiation to correct the imbalance of bone metabolism are principles for the treatment of osteoporosis.
In recent years, Traditional Chinese Medicine (TCM) is attracting more attention to deal with osteoporosis. The role of traditional Chinese medicine in regulating the osteogenic differentiation of MSCs is mainly reflected in the key signaling pathways and transcription factors in osteogenic differentiation. The key signaling pathways and transcription factors, post-transcriptional level, and non-coding RNA were modified to promote the expression of osteogenic genes, inhibit bone resorption and promote bone formation. Flavonoids have been widely demonstrated to have favorable osteogenic effects\[29\]. Quercetin enhanced the phosphorylation of AMPK and SIRT1 protein, thus activating the AMPK/SIRT1 signaling pathway in mice BMSCs. Quercetin promoted osteogenic differentiation and inhibited antioxidant responses of mice BMSCs by activating the AMPK/SIRT1 signaling pathway\[30\]. Panax Notoginseng Saponins promoted angiogenesis and prevented bone loss in ovariectomy mice\[31\]. Total flavonoid extract of Epimedium herb increases bone mass. The expression levels of bone-formation regulatory genes were upregulation by the serum turnover\[32\]. Myricitrin (pentahydroxyflavonoid-3-rhamnoside) is a natural polyphenolic hydroxy flavonoid glycoside, which has been found in the fruit, bark, and leaves of the Myrica rubra abundantly. Myricitrin was originally used as an antioxidant to inhibit cell apoptosis by reducing redox reactions\[33\]. Myricitrin inhibits vascular endothelial growth factor-induced angiogenesis in immune-deficient nude mice via downregulation matrix metalloproteinase (MMP) activity\[34\]. Myricitrin has great potential to treat atherosclerosis by inhibiting excess endothelial cell apoptosis, which is the first step of atherosclerosis\[35\]. However, the specific mechanisms by which myricitrin promotes osteogenic differentiation remain unclear.

In vivo, we observed that myricitrin can stimulate osteogenic differentiation of imBMSCs by evaluating the deposition of calcium salt and the expression level of osteogenic related genes and proteins. Based on the above results, we concluded that 10µM and 25µM are suitable doses to promote the osteogenesis of imBMSCs. Therefore, RNA-sequencing analysis was used for the exploration of the mechanisms underlying the osteogenesis effects of myricitrin. Accordingly, we observed the increased expression of PI3K/AKT signaling pathway-related genes by KEGG Pathway Analysis. PI3K/AKT is an important pathway in osteogenic differentiation\[41\]. To further confirm the role of the PI3K/AKT signaling pathway, we further verified it through Auto molecular docking and WB. The results showed myricitrin increased the expression of p-PI3K and p-AKT, suggesting that myricitrin might promote osteogenic differentiation of imBMSCs through PI3K/AKT signaling pathway. Based on previous experiments, we found that the expression of osteogenic related
proteins, p-PI3K, and p-AKT proteins in the 25 µM myricitrin group are higher than those in the 10 µM myricitrin group. Therefore, we chose 25 µM myricitrin to continue exploring rescue experiments.

Subsequently, we conducted the rescue experiment, using copanlisib, an ATP competitive selective PI3 kinase inhibitor, acting on PI3Kα, PI3Kδ, PI3Kβ, and PI3Kγ, which can partially inhibit the osteogenic differentiation of imBMSCs promoted by myricitrin. To verify that, we detected luciferase activity downstream factor Myc. It was activated significantly by 25µM myricitrin and inhibited by copanlisib 10 nM, which meant the PI3K/AKT signaling pathway might be indicative of a key role of myricitrin in promoting osteogenesis in imBMSCs.

In vitro, we used the ovariectomy model to evaluate the ability of myricitrin to delay osteoporosis. We observed that bone loss in mice in low-dose and high-dose myricitrin groups was alleviated than that in the model group by Micro-CT. Myricitrin attenuated histomorphology damage in low dose and high dose myricitrin groups compared to the OVX group. We found that myricitrin can promote the expression of OPG, OPN, OCN, and p-PI3K in bone tissue of mice in low dose and high dose myricitrin groups through WB and immunohistochemistry. Our results suggest that myricitrin reduces bone loss in ovariectomy mice via PI3K/AKT signaling pathway.

**Conclusions**

In our study, we used sequencing of mRNA transcriptome genes to elucidate multiple targets of myricitrin. These data suggest that myricitrin enhances the osteogenic differentiation of imBMSCs, partly via activation of the PI3K/AKT signaling pathway. Myricitrin can reduce bone loss in the ovariectomy mice model. Myricitrin may be a potential drug development for the treatment of postmenopausal osteoporosis.

**Abbreviations**

CCK-8
Cell Counting Kit-8
ALP: Alkaline phosphatase
ARS
Alizarin Red Staining
imBMSCs: immortalized mouse bone marrow stromal cells
BV/TV
Bone volume/Total volume
FBS
Foetal bovine serum
HE
Hematoxylin-eosin
IF
Immunofluorescence
OIM
Osteogenic induction medium
OVX
Ovariectomy
PBS
Phosphate-buffered saline
TBST
tris-buffered saline containing 0.1% Tween-20
qRT-PCR
Quantitative reverse transcription-polymerase chain reaction
WB
Western Blot
RIPA
Radioimmunoprecipitation assay
SD
Standard deviation
Tb. N
Trabecular number
Tb.Th
Trabecular thickness
Tb.Sp
Trabecular separation
BMD
Bone mineral density
MEM-α
Modified Eagle’s medium alpha modification.

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

We thank Pro.f Tong-Chuan He from Molecular Oncology Laboratory at the University of Chicago for providing immortalized mouse bone marrow stromal cells for this study.

Author’s contributions

Haibin Wang designed the study. Jianliang Li and Jiale Mai composed the manuscript. Meng Zhang designed the cell experiment. Jianliang Li and Jiale Mai designed the animal experiment. Qi He, Yanhuai Ma, Jiacong Xiao, Miao Li, WeiJian Chen, and Zhen Li participated in animal experiments. Jianliang Li and Jiale Mai accomplished micro-CT, H&E staining, and IHC. Jianliang Li accomplished the analysis of bioinformatics. Jianliang Li accomplished CCK-8, qRT-PCR, ALP, and ARS. Jianliang Li and Jiale Mai
participated in Western blot and Molecular Docking. Zhaofeng Pan and Shaocong Li performed the statistical analysis. Meng Zhang, Dawei Gong, Shuai Chen, and Haibin Wang revised the manuscript. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

**Funding**

The work was sponsored by grants from the National Natural Science Foundation of China (82074462) and the Major Research Project of Guangzhou University of Chinese Medicine (2021xk53).

**Ethics approval and consent to participate**

All animal experimental procedures were approved by the Animal Research Committee of First Affiliated Hospital of Guangzhou University of Chinese Medicine and were performed by the guidelines of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**References**


**Figures**

**Figure 1**

Myricitrin enhanced osteogenic differentiation of imBMSCs in vitro. A 2D chemical structure of myricitrin. B 3D chemical structure of myricitrin. C Cell proliferation after myricitrin intervention was determined via CCK-8 assay. D, F Osteogenic differentiation was assessed by ALP staining and ARS. E Diethanolamine (DEA) value of Alkaline phosphatase activity. G ARS semiquantitative analysis was determined by measuring optical density. The data are presented as the means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns:not significant.
Figure 2

Myricitrin increased the expression of osteogenic-specific genes and proteins. A-F The expression of osteogenic-specific genes was measured by qRT-PCR. G-L The expression of osteogenic-specific proteins and semi-quantitative analysis were assessed by WB. The data are presented as the means ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns:not significant.
Figure 3

Differential genes were expressed by volcano map and cluster analysis heat map. A-B KEGG signaling pathway and GO enrichment analysis was used for results by RNA-seq. C-G A volcanic plot was used to express the expression of differential genes between myricitrin and osteogenic groups. 77 genes were up-regulated and 169 genes were down-regulated among them. B We applied a cluster analysis of the top 50 differential genes and showed them by heat map. C We applied KEGG signaling pathway enrichment analysis and showed the top 20 signaling pathways. D We expressed the top 20 different components on differential genes by GO Cellular component. E We performed the top 20 biological functions on differential genes by GO Biological Process. F We selected the top 20 molecular functions on differential genes by GO Molecular Function.

Figure 4

Myricitrin activated PI3K/AKT signaling pathway through WB. A Fold change of p-PI3K/PI3K. B Fold change of p-AKT/AKT. D Schematic diagram of 3D and 2D simulation of the docking results of myricitrin and 5nbg. The data are presented as the means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns: not significant.
Figure 5

Copanlisib prevented the proliferation of imBMSCs in vitro. A 2D chemical structure of copanlisib. B 3D chemical structure of copanlisib. C Cell proliferation after copanlisib intervention was determined via CCK-8 assay. D, F Osteogenic differentiation was assessed by ALP staining and ARS. E Diethanolamine (DEA) value of Alkaline phosphatase activity. G ARS semiquantitative analysis was determined by
measuring optical density. The data are presented as the means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns: not significant.

Figure 6

Copanlisib prevented the expression of osteogenic-specific genes and proteins. A-F The expression of osteogenic-specific genes was measured by qRT-PCR. G-L The expression of osteogenic-specific proteins...
and semi-quantitative analysis were assessed by WB. The data are presented as the means ± SD. 
*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns:not significant.

Figure 7

Copanlisib prevented the mean optical density value of Runx2 and Col1a1 by immunofluorescence. 
Copanlisib prevented Myc which was a downstream transcription factor of the PI3K/AKT signaling pathway. 
A, B The Runx2 level of fluorescence intensity was measured by semiquantitative analysis. 
C, D The Col1a1 level of fluorescence intensity was measured by semiquantitative analysis. 
E Myc fluorescence was assessed by dual-luciferase reporter assay. The data are presented as the means ± SD. 
*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns:not significant.
Figure 8

Myricitrin prevented bone loss in ovariectomy mice. A-C Myricitrin protected the expression of phosphorylated PI3K and AKT. A, D, E Myricitrin protected the expression of OPN and OPG. F 3D reconstruction of the coronal and transverse images of the distal femur 2 months after ovariectomy in different groups. G-L Micro-CT analysis of bone included volume/total volume (BV/TV), trabecular thickness (Tb.Th), trabecular number(Tb.N), trabecular separation(Tb.Sp), bone mineral density(BMD),
Structure model index (SMI), and Degree of Anisotropy. (n=5) The data are presented as the means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns: not significant.

Figure 9

Myricitrin attenuated histomorphology damage and bone loss induced by ovariectomy in vivo. A-B Compared to the OVX group, thickened and coarse trabeculae were observed in the low and high dose group in the representative HE staining images of the distal femur. C-J Myricitrin improved the trabecular structure and stimulated the expression of the p-PI3K, OPN, OPG, and OCN in low dose and high dose groups. The mean density of the antibody-positive cells was calculated as integrated optical density per area of positive cells. (n=5). The data are presented as the means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns: not significant.

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