CBP-mediated FOXO4 acetylation facilitates postmenopausal osteoporosis (PMO) progression through the inhibition of the Wnt/β-catenin signaling pathway

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Abstract
FOXO4 was previously identified as a potential biomarker and therapeutic target for postmenopausal osteoporosis (PMO) using bioinformatic analysis, but its specific function and molecular mechanism in the progression of osteoporosis was not reported. The current study was designed to investigate the biological function and underlying mechanism of FOXO4 in PMO. Our results showed that FOXO4 expression was significantly upregulated in the serum samples of PMO patients, which was also negatively correlated with the expression of osteogenesis genes (OCN and ALP). In addition, FOXO4 depletion alleviated osteoporosis by facilitating osteogenic differentiation and inhibiting adipogenic differentiation in human bone marrow mesenchymal stem cells (hBMSCs). Overexpression of FOXO4 exerted the opposite effects on the osteogenic/adipogenic differentiation in hBMSCs. Moreover, FOXO4 knockdown activated the Wnt/β-catenin signaling whereas the inhibition of Wnt/β-catenin signaling overturned the effects of FOXO4 deficiency on osteoporosis. Furthermore, FOXO4 upregulation in PMO was caused by CBP-induced acetylation.

In summary, our data demonstrated that FOXO4 was a potent biomarker for PMO and mediated the balance between osteogenesis and adipogenesis in hBMSCs by regulating Wnt/β-catenin signaling.

Keywords: postmenopausal osteoporosis, FOXO4, osteogenesis, adipogenesis

Introduction
Postmenopausal osteoporosis (PMO), a metabolic disorder characterized by reduced bone quality and deteriorated bone microstructure, ultimately causes an increased risk of fracture (Li et al., 2020). It is reported that the incidence of fractures in postmenopausal women is as high as 40% (Chen et al., 2021). Bone homeostasis is maintained by bone remodeling, a dynamic process between osteoblast-mediated bone formation and osteoclast-mediated bone resorption (Zhang et al., 2019). Bone marrow mesenchymal stem cells (BMSCs) can undergo multidirectional differentiation, including osteogenic and adipogenic differentiation (Wang et al., 2021). The imbalance...
of the decreased osteogenic and increased adipogenic differentiation tendency in hBMSCs causes more fat to deposit in the bone marrow cavity, which inhibits the bone formation process and ultimately leads to osteoporosis (Gao et al., 2015). However, the mechanism underlying the decreased bone formation capacity in hBMSCs in osteoporosis remains poorly understood.

Forkhead box O4 (FOXO4) is one of the key members in the forkhead transcription factor O (FOXO) family of proteins which participate in various biological processes, including cell proliferation, apoptosis, oxidative stress response, and metabolism (Liu et al., 2019). Studies have demonstrated that the FOXO family proteins are involved in the pathogenesis of bone diseases, including osteoporosis. For example, FOXO1 deficiency inhibited bone formation in young mice but alleviated age-related bone loss in aged mice (Xiong et al., 2022). FOXO3 facilitated osteogenic differentiation in BMSCs by enhancing autophagy (Long et al., 2021). Particularly, FOXO3a was highly expressed in bones and osteoblasts, and played protective role against oxidative stress-induced apoptosis of osteoblasts during osteoporosis progression (Ambrogini et al., 2010; Zou et al., 2021). A published article identified that FOXO4 was upregulated and might be a key biomarker in PMO using bioinformatic analysis (Yang et al., 2022). Nevertheless, it is unknown whether FOXO4 mediates the osteogenic and adipogenic differentiation potential of BMSCs.

The present study revealed that FOXO4 was abundantly expressed in the serum plasma of PMO patients and negatively correlated with the expression of osteogenesis genes, suggesting that FOXO4 is a potential biomarker for PMO. Besides, FOXO4 mediated the balance between osteogenesis and adipogenesis in hBMSCs through the regulation of Wnt/β-catenin signaling. Furthermore, it was identified that FOXO4 upregulation was caused by CBP-induced acetylation.

Materials and methods

Clinical specimens
Postmenopausal patients with osteoporosis (n=36) and without osteoporosis (n=36) were identified at the Liyang People’s Hospital between May 2016 and May 2019 and were involved in this study. Peripheral blood was collected and total RNA was isolated. The study protocol was approved by the Ethics Committee of the hospital and conducted in compliance with the Helsinki Declaration. All participants provided written consent.

Cell culture
Human bone marrow mesenchymal stem cells (hBMSCs, HUXMA-01001) were purchased from Cyagen Biosciences (Soochow, China) and cultured in Alpha Modified Eagle’s Medium (α-MEM) containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin (All from Thermo Fisher, USA) in the incubator with 5% CO₂ and 95% humidity at 37 °C. Upon arriving at 85% confluence, the cells were trypsinized and passaged using 0.25% trypsin-EDTA (Thermo Fisher, USA). Osteogenic or adipogenic differentiation was induced following previous steps (Zhi et al., 2021).
Cell transfection
Short hairpin RNA (shRNA) against FOXO4 (shFOXO4), shNC, pcDNA3.1 vector containing FOXO4 (oeFOXO4), and empty pcDNA3.1 vector as control were designed and synthesized by GenePharma (Shanghai, China). Briefly, hBMSCs were seeded into a 6-well plate (1x10^6 cells/well) and were cultured to 85% confluence. Cell transfection was performed using Lipofectamine 2000 reagent (Thermo Fisher, USA).

Antibodies and reagents
The antibodies utilized in this study were: FOXO4, RUNX2, OCN, ALP, C/EBPα, PPARγ, GSK-3β, p-GSK-3β, total β-catenin, nuclear β-catenin, LEF1, TCF1/7, Acetylated-Lysine, FLAG, HA, CBP, Actin, GAPDH. Transfection procedures were accomplished by using Lipofectamine 2000 (Invitrogen, 11668019).

RT- qPCR
Total RNA was extracted from serum plasma or cells using TRIzol reagent (Thermo Fisher). cDNA was transcribed using the First Strand cDNA Synthesis Kit (Beyotime, China). PCR was performed on an ABI 7500 Real-Time PCR system (Thermo Fisher) using the TB Green Premix Ex Taq II (Taraka, Japan). GAPDH was used as the reference gene.

Alkaline phosphatase (ALP) activity assay
ALP activity was measured using the Alkaline Phosphatase Assay Kit (Jiancheng, China) as per the manufacturer’s instructions. Absorbance was determined at a wavelength of 520 nm by BioTek Synergy 2 (BioTek).

Alizarin red staining (ARS) assay
After fixation in standard solution (PBS+4% paraformaldehyde), the cells were stained with 0.1% Alizarin red (pH 4.2, Solarbio) and imaged under the IX71 microscope (Olympus, Japan). To quantify mineralization, the bound dye was dissolved in the eluent containing 10% cetylpyridinium chloride and then transferred to a 96-well plate. Absorbance was measured at 570 nm using a BioTek Synergy 2 (BioTek, USA).

Co-immunoprecipitation (Co-IP) and western blot
Cells were washed by phosphate buffer saline (PBS) twice and lysed by protein lysis buffer containing protease inhibitors on ice for 0.5h. Cell supernatant was collected after centrifugation and the protein concentration was measured using a BCA assay kit. For immunoprecipitation, the protein solution was incubated with corresponding antibodies followed by the addition of protein A/G agarose Beads (Santa Cruz). Subsequently, the beads-protein complex was washed 3 times using lysis buffer (without protease inhibitors). The protein was eluted by boiling SDS-PAGE loading buffer. An appropriate amount of protein was loaded into SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked with 5% skim milk for at least 1h and then incubated with indicated primary antibodies overnight at 4°C. The
membrane was rinsed three times with TBST and then incubated with an appropriate secondary antibody for 1h at room temperature. The membrane was washed, incubated with an enhanced chemiluminescence reagent (Thermo Fisher, USA), and observed on the ChemiDoc Touch Imaging System (Bio-Rad, USA).

**Statistical analysis**
Each experiment was repeated no less than three times and data are presented as the mean ± SEM. Statistics were analyzed using GraphPad Prism 7.0. Student’s t test was used to compare the difference between two groups. The one-way analysis of variance (ANOVA) was used to compare the difference between no less than three groups. P<0.05 was considered statistically significant.

**Results**
**FOXO4 was upregulated in PMO patients and negatively correlated with osteogenesis genes**
To determine whether FOXO4 is involved in the development of PMO, FOXO4 expression in plasma was detected. RT-qPCR indicated that FOXO4 expression levels were significantly higher in the plasma of PMO patients compared to healthy controls (Fig. 1A). Besides, FOXO4 expression was negatively correlated with the expression of osteogenesis genes (OCN and ALP) (Fig. 1B). These results implied that FOXO4 was upregulated in PMO patients and might play critical roles in the pathophysiology of osteoporosis.

**FOXO4 suppressed osteoblastic differentiation**
To determine the role of FOXO4 in postmenopausal osteoporosis, FOXO4 expression during osteoblastic differentiation was assessed using RT-qPCR. As presented in Fig. 2A, FOXO4 expression showed a time-dependent decrease in OIM-induced cells. Next, FOXO4 was overexpressed or silenced in hBMSCs (Fig. 2B and F). The levels of osteoblast differentiation markers (RUNX2, OCN, and ALP) were assessed following the transfection. RT-qPCR showed that FOXO4 supplementation suppressed while FOXO4 depletion augmented the expression of RUNX2, OCN and ALP (Fig. 2C-E). Analogously, western blot detected that the protein expression of RUNX2, OCN and ALP was decreased and increased by oeFOXO4 and shFOXO4, respectively (Fig. 2F). In addition, the ALP activity of hBMSCs was significantly reduced by oeFOXO4 and enhanced by shFOXO4 (Fig. 2G). Calcified nodules were markedly increased following FOXO4 knockdown and were significantly decreased after FOXO4 overexpression (Fig. 2H). These data imply that FOXO4 inhibited the osteoblastic differentiation of hBMSCs.

**FOXO4 facilitated adipogenic differentiation**
Next, the function of FOXO4 in the adipogenic differentiation in hBMSCs was focused on. Fig. 3A illustrates that the mRNA level of FOXO4 gradually increased during the adipogenic differentiation. A significant increase of FOXO4 expression was detected 12 days after induction of adipogenic differentiation. Moreover, the mRNA level of
adipogenic markers (C/EBPα and PPARγ) was remarkably elevated by FOXO4 overexpression whereas FOXO4 knockdown drastically repressed C/EBPα and PPARγ mRNA expression (Fig. 3B and C). In agreement, the protein level of C/EBPα and PPARγ was enhanced by oeFOXO4 and reduced by shFOXO4 (Fig. 3D). Taken together, FOXO4 promoted the adipogenic differentiation of hBMSCs.

**FOXO4 depletion stimulated the activation of Wnt/β-catenin signaling pathways**

To determine whether FOXO4 mediated Wnt/β-catenin signaling, the expression of Wnt-associated genes and proteins was assessed in FOXO4-silenced cells. RT-qPCR indicated that FOXO4 knockdown markedly increased β-catenin expression, but had no significant influence on Gsk-3β expression (Fig. 4A). Western blot results demonstrated that protein level of p-GSK-3β, total β-catenin and nuclear β-catenin was dramatically enhanced by silencing FOXO4. However, GSK-3β protein expression was barely affected by shFOXO4 (Fig. 4B). In addition, the expression of LEF1 and TCF1/7, two downstream molecules of Wnt/β-catenin signaling, was remarkably increased following FOXO4 knockdown (Fig. 4B). In sum, FOXO4 knockdown activated Wnt/β-catenin signaling in hBMSCs.

**DKK1 abated the effect of FOXO4 knockdown on the adipogenesis and osteogenesis of hBMSCs**

Subsequently, the participation of Wnt/β-catenin signaling in FOXO4-mediated behaviors of hBMSCs was explored. With DKK1 treatment, the levels of p-GSK-3β and β-catenin expression in FOXO4-silenced hBMSCs were significantly decreased (Fig. 5A). Inhibition of Wnt/β-catenin signaling partially reversed the effects of shFOXO4 on the expression of osteo-specific and adipo-specific genes and proteins, as demonstrated by qRT-PCR and WB results (Fig. 5B and C). In addition, ALP activity assay results revealed higher ALP expression in the shFOXO4 group than in the shFOXO4 + DKK1 group (Fig. 5D). These results demonstrated that FOXO4 regulated the osteoblastic and adipogenic differentiation through the inhibition of Wnt/β-catenin signaling.

**CBP-induced acetylation stabilized FOXO4 expression in hBMSCs**

Finally, we investigated whether acetylation regulated the function of FOXO4 in hBMSCs. First, immunoprecipitation was performed using an acetylated-lysine (AcK) antibody, followed by western blot detection using a FOXO4 antibody, which demonstrated that FOXO4 can be acetylated (Fig. 6A). We speculated that CBP might be the acetyltransferase that mediates the acetylation of FOXO4. It was observed that CBP overexpression markedly increased the acetylation of FOXO4 (Fig. 6B). Co-immunoprecipitation indicated that FOXO4 interacted with CBP in hBMSCs (Fig. 6C). In addition, CBP overexpression or depletion in hBMSCs caused remarkable upregulation or down-regulation in FOXO4 expression (Fig. 6D). To summarize, CBP-induced acetylation of FOXO4 caused the upregulation of FOXO4 in hBMSCs.
Discussion

Increasing studies have shown that FOXO4 could play key roles in the pathogenesis and progression of human diseases. For example, FOXO4 was identified as a key stimulator for soluble guanylyl cyclase β protein which produces cGMP and relaxes vascular smooth muscle cells (SMCs) needed for vasodilation (Galley et al., 2022). FOXO4 supplementation enhanced HT-22 cell viability and alleviated hippocampal neuronal damage in the mouse model of epilepsy (Jin et al., 2022). FOXO4 inhibited the transcription of TRAF3IP2 to suppress the growth, migration, and extracellular matrix deposition of keloid fibroblasts through the TGF-β1/Smad pathway (Yan and Li, 2022). In this study, it was uncovered that FOXO4 was significantly upregulated in the serum plasma of PMO patients and negatively correlated with osteogenesis genes.

Previous studies have indicated that the imbalance between fat and bone is a critical factor in the pathology of osteoporosis (Du et al., 2021). The imbalance between bone formation and bone loss happens with aging and osteogenic differentiation is inhibited and bone formation is reduced, leading to fat accumulation, and thereby inducing osteoporosis (Lai et al., 2016). Mounting evidence has proved that genetic factors can affect the osteo-/adipogenic differentiation capabilities of BMSCs. For instance, miR-130a was drastically down-regulated in BMSCs of aged mice and the overexpression of miR-130a facilitated the osteoblastic differentiation but suppressed the adipogenic differentiation of BMSCs (Lin et al., 2019). CircRNA CDR1as was upregulated and promoted adipogenic differentiation while inhibited osteogenic differentiation of BMSCs in steroid-induced osteonecrosis of the femoral head (Chen et al., 2020). FOXC2 overexpression in BMSCs stimulated osteogenesis and suppressed adipogenesis (You et al., 2014). Herein, we discovered that FOXO4 depletion caused increased osteogenic differentiation and decreased adipogenic differentiation whereas the supplementation of FOXO4 exerted the opposite effects.

Wnt signaling has been demonstrated to play crucial roles in organogenesis during embryonic development (Huybrechts et al., 2020). Noticeably, the canonical Wnt/β-catenin pathway is reported to be of importance in adult skeletal homeostasis and bone remodeling (Aditya and Rattan, 2021). Existing studies have indicated that the activation of Wnt/β-catenin signaling is of critical importance in the progression of osteogenesis. For example, GPR35 abundance alleviated osteogenesis through enhancing the activity of Wnt/GSK3β/β-catenin signaling (Zhang et al., 2021). FOXF1 knockdown activated the Wnt/β-catenin signaling to promote BMSCs osteogenesis and prevent ovariectomy-induced bone loss (Shen et al., 2020). MiR-23b-3p promoted PMO by inhibiting MRC2 and Wnt/β-catenin signaling (Li et al., 2021). Through our investigation, it was discovered that Wnt/β-catenin signaling could be activated by silencing FOXO4 in hBMSCs. Moreover, the inhibition of Wnt/β-catenin signaling by DDK1 effectively overturned the effects of FOXO4 knockdown on the osteogenic/adipogenic differentiation tendencies of BMSCs.

It has been proven that acetylation, a post-transcriptional modification, mediates the expression and activity of FOXO4 in many cases. For example, Sirt1-induced deacetylation of FOXO4 elevated SOX9 expression and maintained the ECM stability of cartilage. (Ma et al., 2021). CBP-induced acetylation negatively regulated the
transcriptional activity of FOXO4 (Fukuoka et al., 2003). FOXO4 down-regulation caused by Sirt1-mediated deacetylation alleviated the apoptosis of podocytes in diabetes mellitus (Chuang et al., 2011). Our results showed that FOXO4 was acetylated by CBP, which led to the upregulation of FOXO4 in hBMSCs.

In conclusion, our investigation revealed the function and molecular mechanism of FOXO4 in PMO for the first time. It was demonstrated that CBP-induced acetylation upregulated FOXO4 in hBMSCs, which further inhibited osteoblastic differentiation and facilitated adipogenic differentiation, leading to exacerbated osteogenesis. Our findings suggest a role of FOXO4 as a novel biomarker for osteoporosis, and the mechanism of FOXO4 induces imbalance between osteogenesis and adipogenesis, representing a novel target for osteoporosis treatment.

Reference:


**Figure legends**

**Figure 1** FOXO4 was abundantly expressed and negatively correlated with the expression of osteogenesis genes in PMO patients. (A) Relative FOXO4 expression was detected in the serum plasma of PMO patients and healthy controls by RT-qPCR. (B) The correlation between the levels of FOXO4 and osteogenesis genes (OCN and ALP) was analyzed by Pearson’s Correlation Analysis. **P<0.01.

**Figure 2** FOXO4 suppressed osteoblastic differentiation. (A) Relative FOXO4 expression in hBMSCs during osteogenic differentiation. (B-E) RT-qPCR detected the mRNA expression of FOXO4 (B), RUNX2 (C), OCN (D), and ALP (E) in hBMSCs transfected with oeFOXO4 or shFOXO4. (F) Western blot assessed the protein expression of FOXO4, RUNX2, OCN, and ALP in FOXO4 overexpressed or silenced hBMSCs. (G) ALP activity was detected by ALP assay in oeFOXO4- or shFOXO4-transfected hBMSCs. (H) Alizarin red staining was used to detect the mineralization ability of hBMSCs after transfection with oeFOXO4 or shFOXO4. *P<0.05; **P<0.01; ***P<0.001.

**Figure 3** FOXO4 promoted adipogenic differentiation. (A) Relative FOXO4 expression in hBMSCs during adipogenic differentiation. (B, C) Relative mRNA expression of C/EBPα and PPARγ in hBMSC after transfection with oeFOXO4 or shFOXO4. (D) Protein expression of C/EBPα and PPARγ expression in hBMSCs after transfection with oeFOXO4 or shFOXO4. *P<0.05; **P<0.01; ***P<0.001.

**Figure 4** FOXO4 mediated the activation of Wnt/β-catenin signaling. (A) The expression of β-catenin and Gsk-3β were assessed by qRT-PCR. (B) Western blot detected the expression of Wnt/β-catenin signaling-related proteins (GSK-3β, p-GSK-3β, total β-catenin, nuclear β-catenin, LEF1, and TCF1/7) at day 7 of osteogenic differentiation. **P<0.01.
Figure 5 FOXO4 mediated the osteoblastic/adipogenic differentiation of hBMSCs via Wnt/β-catenin signaling. hBMSCs were divided into shNC, shFOXO4, and shFOXO4+DKK1. (A-C) The protein expression of Wnt signaling proteins (p-GSK-3β and β-catenin), osteo-specific proteins (OCN and ALP), adipo-specific proteins (C/EBPα and PPARγ) were assessed. (D) Osteogenic differentiation was evaluated using ALP activity assay. *P<0.05; **P<0.01.

Figure 6 CBP-induced acetylation stabilized FOXO4 expression in hBMSCs. (A) Lysates of hBMSCs were immunoprecipitated with antibody (Ab) to acetylated-lysine (AcK) or control IgG. Western blot analysis was performed with anti-FOXO4 Ab and anti-Actin Ab. (B) FLAG-FOXO4 and CBP overexpression plasmid were co-transfected in hBMSCs, which were subjected to immunoprecipitation with M2-Beads (anti-FLAG), and then immunoblotted with the indicated antibodies. (C) hBMSCs were lysed and performed coimmunoprecipitation with antibody to CBP or IgG, and then detected by western blot analysis with FOXO4 Ab. (D) Protein expression of FOXO4 in hBMSCs transfected with shCBP or oeCBP was detected by western blot. **P<0.01.
Figure A: Relative FOXO4 expression in Control and PMO groups.

• Control group shows lower FOXO4 expression compared to PMO group.

Figure B: Correlation between FOXO4 expression and OCN, ALP levels.

- **B1**: Correlation of FOXO4 with OCN
  - Correlation coefficient: $r = -0.3776$
  - Significance: $p = 0.0232$
  - Sample size: $n = 36$

- **B2**: Correlation of FOXO4 with ALP
  - Correlation coefficient: $r = -0.6604$
  - Significance: $p < 0.0001$
  - Sample size: $n = 36$
A

B

GSK-3β

p-GSK-3β

β-catenin (T)

β-catenin (N)

LEF1 TCF1/7

GAPDH

shNC shFOXO4

Relative mRNA expression

Relative protein levels

β-catenin

Gsk-3β

GSK-3β p-GSK-β-catenin β-catenin LEF1 TCF1/7

shNC shFOXO4
A

p-GSK-3β
β-catenin
GAPDH

B

shNC
shFOXO4
shFOXO4+DKK1

p-GSK-3β
β-catenin

C

shNC
shFOXO4
shFOXO4+DKK1

OCN
ALP

D

shNC
shFOXO4
shFOXO4+DKK1

C/EBPα
PPARγ
GAPDH

ALP activity (unit/gprot)