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Original Article

Foxf1 gene increases the risk of osteoporosis in rats by inhibiting osteoblast formation and promoting osteoclast differentiation through the upregulation of NF-κB pathway

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Abstract

Objectives: a) To explore the expression of Foxf1 and NF-κB in bone tissue of ovariectomized rats with osteoporosis and b) to investigate the role and mechanism of NF-κB pathway regulated by Foxf1 gene in the differentiation and formation of rat osteoclasts and osteoblasts with cell experiments. **Methods**: Ovariectomized rat model of osteoporosis was established with 3-month-old female SD rats. The rats were divided into sham group (n=10) and osteoporosis group (n=10). Real time fluorescent quantitative PCR and Western blot were used to detect the expression levels of Foxf1 and NF-κB genes and proteins in the femur tissues of rats and analyze their correlation. **Results**: Both Foxf1 and NF- κB were highly expressed in the femur tissues. Upon the overexpression of Foxf1 gene in osteoblasts and osteoclasts *in vitro*, the gene and protein expression of NF-κB were also upregulated, significantly reducing the gene and protein expression levels of osteogenic factors, including ATF4, OCN, ALP and Runx2. **Conclusions**: Foxf1 gene could inhibit osteoblast formation and promote osteoclast differentiation by NF-κB pathway, which may increase the risk of osteoporosis in rats.

Keywords: Foxf1 Gene, NF-κB Pathway, Osteoblasts, Osteoclasts, Osteoporosis

Introduction

Osteoporosis (OP) is a systemic bone metabolic disease, which is mainly characterized by the decrease of bone mineral density and bone quality, destruction of bone microstructure, increase of bone fragility, and increasing possibility of fracture. The fracture can be mostly found in hip joint, wrist joint and spine[1]. Over 200 million people worldwide have osteoporosis, and the incidence rate increases with age[2]. Every year, about 9 million people suffer from fractures due to osteoporosis. One third of females and one fifth of males over the age of 50 suffer from osteoporotic fracture[3]. Postmenopausal osteoporosis is caused by estrogen deficiency and represents the most common type of primary osteoporosis[4]. Although achievements have been made in the prevention and treatment of osteoporosis, it is still of great significance to continue with the development of new methods and drugs due to the concern of the rare side effects of drugs and to obtain convincing evidence of long-term efficacy of the drugs[5].

Osteoporosis is mainly caused by the imbalance between bone resorption and bone formation. At present, the specific pathogenesis is still unclear[6]. Bone homeostasis and remodeling is a process where bone formation and bone resorption are balanced to remodel and maintain the bone constantly. And bone loss will occur if this balance is destroyed. The cells playing an important role in bone remodeling mainly include osteoclasts that stimulate bone resorption, osteoblasts responsible for new bone formation, osteoblasts, macrophages, etc[7]. The interaction between osteoblasts and osteoclasts has always been frequently studied. Osteoclasts are derived from mononuclear macrophage. The formation of osteoclasts requires macrophage colony-stimulating

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factor (M-CSF) and receptor activator of nuclear factor-κB ([NF-κB], RANKL)[8,9]. With RANKL stimulation, osteoclast precursor cells differentiate into osteoclasts through the recruitment of tumor necrosis factor receptor-related factor 6 (TRAF6) and activate NF-κB and mitogen activated protein kinase (MAPK) signaling pathways, inducing the expression of osteoclast marker genes, such as tartrate resistant acid phosphatase (TRAP), Cathepsin-K and matrix metalloproteinase 9 (MMP9)[8-10]. Osteoblasts originate from bone marrow mesenchymal stem cells, and their differentiation is controlled by several transcription factors and signal cascades[11]. NF-κB can inhibit the activity of bone morphogenetic protein 2 (BMP2) or transforming growth factor-β (TGF-β) downstream Smad protein, inhibiting the mineralization of osteoblasts[12]. BMP2 activates MAPK signaling pathway to promote the expression and activation of osteogenic specific transcription factor, Runt-related transcription factor 2 (Runx2)[13]. Runx2 can regulate the expression of several osteogenic genes, including alkaline phosphatase (ALP) and osteocalcin (OCN), which are also key molecules and markers of osteoblast differentiation and function[14]. NF-κB plays an important role in bone formation by regulating osteoclasts and osteoblasts[11,15-17]. Therefore, recently, NF-κB is considered to be the key factor in the inhibition of bone formation in osteoporosis. NF-κB inhibition can lead to increased bone formation and decreased bone resorption.

Recent studies have shown that Foxf1 (Forkhead box F1) gene knockout could significantly promote the osteogenesis of bone marrow mesenchymal stem cells[18] and prevent bone loss, which may play an important regulatory role in osteoporosis. Forkhead protein F1 (Foxf1), a member of forkhead box transcription factor family, has a common pterygoid helix or forkhead domain[19]. Members of the forkhead box transcription factor family play a role in cell differentiation, development, metabolism and senescence. It has been reported that Foxf1 is related to a variety of bone metabolism related factors, such as BMP4, Wnt2, Wnt11, Wnt5a, β-catenin, and NF-κB[20-22], regulating the differentiation and formation of osteoclasts and osteoblasts. In addition, other Fox transcription factor families also play a key role in regulating bone metabolism. Foxa2 knockout promotes bone marrow-derived mesenchymal stem cells to differentiate into bone and repairs bone defects in rats by regulating Wnt/β-catenin signaling pathway[23]. Foxc2 gene can induce osteogenesis by promoting Wnt signal and BMP4 expression[24]. In addition, studies have found that FoxF1 can protect the lung injury induced by paraquat in rats through the related IκB/NF-κB axis, indicating that FoxF1 and NF-κB had a regulatory relationship[25]. Therefore, we believe that Foxf1 may be a new potential factor in regulating the stability of bone homeostasis. However, the interaction between FoxF1 gene and classical NF-κB pathway regulating bone homeostasis remains unclear.

In this study, the expression of Foxf1 and NF-κB in bone tissue of ovariectomized rats with osteoporosis was determined, and the role and mechanism of NF-κB pathway regulated by Foxf1 gene in the differentiation and formation of rat osteoclasts and osteoblasts was explored with cell experiments *in vitro*, providing a scientific basis of considering Foxf1 gene as a target for prevention and treatment of osteoporosis.

Materials and methods

Animal experiment

Animal source and grouping: This study was approved by the ethics committee of Xuzhou Third People's Hospital experimental animal center. Twenty 3-month-old female SPF Grade Sprague Dawley (SD) rats were purchased from Guangdong Medical Laboratory Animal Center. Five rats occupied one cage and were given food and water available ad libitum in an SPE Grade environment. The rats were numbered and were randomly divided into sham group and ovariectomized rats with osteoporosis (OVX) group.

Animal operation: 1% pentobarbital sodium (40 mg/kg) was used to anesthetize the rats abdominally, and then the abdominal hair was removed, and the abdominal skin was disinfected after the rats were fixed in supine position. In OVX group, the skin was cut about 2 cm in the middle of the abdomen, and the muscles were separated bluntly to conduct operation in the abdominal cavity. After ligation, bilateral oophorectomy was performed, and the incision was sutured layer by layer. In sham group, the same incision was made to remove the same volume of adipose tissue around the bilateral ovaries, and the ovaries and fallopian tubes were kept intact[26].

Animal sacrifice and tissue sampling: after all the above treatments for 3 months, the rats were anesthetized with 1% pentobarbital sodium (40 mg/kg), and the end of the tail vein was cut to collect the peripheral blood for measurement. The rats were sacrificed by rapid cardiac acute massive blood loss method, and the bilateral femurs and L5 lumbar vertebrae were taken and wrapped with 0.9% sodium chloride wet gauze and tin foil, and stored at -20°C for test.

Bone mineral density (BMD) measurement: the right femur of the rats to be tested was taken out and allowed to equilibrate to room temperature for 30 minutes. The dual energy X-ray absorption scanner was used to scan all femurs and the upper and lower areas of the femoral end and the BMD value was analyzed and calculated with software[27].

Biomechanical measurement of bone tissue: according to the literature[27], the right femur was taken out, and allowed to equilibrate to room temperature for 30 minutes. The length of the right femur was measured by vernier caliper. The mechanical test was carried out on MTS-858 electronic universal biomechanical material testing machine, and the maximum load (N) and positioning elongation displacement (mPa) were recorded by computer recorder.

Enzyme linked immunosorbent assay (ELISA) was used to determine serological indicators: the concentrations of BGP (Abcam, ab270202, UK), Ca (J&L Biological, KB18306, Shanghai, China) and P (Feiya Biotechnology, F8258-A,

Table 1. Reference Primer Sequences for qRT-PCR.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Foxf1	ACGCCGTTTACTCCAGCTC	CGTTGTGACTGTTTTGGTGAAG
NF-κB	ATGGCAGACGATGATCCCTAC	TGTTGACAGTGGTATTTCTGGTG
ATF4	ATGGCGCTCTTCACGAAATC	ACTGGTCGAAGGGGTCATCAA
OCN	CTGACCTCACAGATCCCAAGC	TGGTCTGATAGCTCGTCACAAG
ALP	GCCCTCTCCAAGACATATA	CCATGATCACGTCGATATCC
Runx2	ATGCTTCATTCGCCTCACAAA	GCACTCACTGACTCGGTTGG
TRAP	AAATCACTCTTTAAGACCAG	TTATTGAATAGCAGTGACAG
MMP9	CTGGACAGCCAGACACTAAAG	CTCGCGGCAAGTCTTCAGAG
CLC7	CGCCAGTCTCATTCTGCACT	GCTTCTCGTTGTGTGGAATCT
Cathepsin-k	CTTCCAATACGTGCAGCAGA	TCTTCAGGGCTTTCTCGTTC

Jiangsu, China) in peripheral blood were determined with ELISA kit according to the instructions. The collected serum samples were added into a 96-well plate of the corresponding specific primary antibody, and incubated in water bath at 37°C for 2 h. After washing, the corresponding secondary antibody was added and the samples were incubated at room temperature for 1 hour. Finally, 100 μl sulfuric acid was added to stop the reaction and the mixture was oscillated. And then dual wavelength determination (determination wavelength 450 nm, correction wavelength 650 nm) was performed with the microplate analyzer (Tecan, Sunrise) and result was analyzed.

Real-time quantitative polymerase chain reaction (qRT-PCR) was used to detect gene expression: the collected femur tissue was fully ground to powder in liquid nitrogen, and total RNA was extracted with Trizol Kit (Tiangen Biotech, Beijing, China) to detect RNA concentration and purity (NanoDrop 2000 ultra micro spectrophotometer, Thermo Fisher, USA). Then, reverse transcription kit (No.RR036A, Takara, Japan) was used to obtain cDNA. The corresponding PCR reaction system was prepared with PCR reaction kit (No.RR820A, Takara, Japan) according to the instructions. CFX96 system (Bio-Rad) was used for amplification detection, and 2-ΔΔCT was used to calculate the relative expression of each gene. The primer sequence is shown in the Table 1.

Western blotting (WB) was used to detect protein expression: the femur tissue was lysed and lysed and then extracted. Femur tissues were lysed in the lytic solution and proteins were extracted. The SDS buffer was added to boil for 5 minutes. After 10% SDS-PAGE gel electrophoresis (35 mA, 90 min), the samples were transferred to nitrocellulose membrane. 5% skim milk powder was added to seal the sample at room temperature for 60 minutes. Primary antibodies included ATF4 (ab186284, Abcam, 1:1000), OCN (AB10911, Sigma-Aldrich, 1:1000), ALP (ab95462, Abcam, 1:1000), Runx2 (ab192256, Abcam, 1:1000), TRAP (ab133238, Abcam, 1:1000), MMP9 (ab76003, Abcam, 1:1000), CLC7 (ab136016, Abcam, 1:1000), and Cathepsin-k (ab187647, Abcam, 1:1000). After incubating for 16 hours at 4°C, the samples were washed with PBST three times for 10 minutes each time, and incubated with the corresponding secondary antibody (ab205718, Abcam, 1:3000) for 2 hours at room temperature. After being washed with PBST for three times, ECL chemiluminescence solution was used for development, and Imag J software was used for analysis.

Cell experiment

Cell culture: after 8-week-old SD rats were sacrificed, femur and tibia were separated with sterilized forceps and scissors under aseptic conditions, and marrow cavity was cleaned with phosphate buffered saline (PBS) containing heparin (2500 U/mL). Bone marrow mesenchymal stem cells were isolated and cultured[28]. About 1.073 g/mL cell separation solution was added for centrifugation (3000 g, 30 min), and the intermediate mononuclear cells were collected and washed with PBS for three times. The cells were resuscitated in the medium containing 10% fetal bovine serum, 1% L-glutamine, 1% penicillin and streptomycin (Sigma-Aldrich). The samples were inoculated in a 75 cm^2 flask at a density of $2\times10^6/cm^2$ and cultured in the 5%CO_2 incubator at 37°C. Bone marrow macrophages were isolated and cultured[29]. The bone marrow was collected by centrifugation (5000 g, 5 min), and the cells were separated with a 40 μm cell filter, and the RBC was dissolved in sterile distilled water. After centrifugation (245 g, 5 min), the remaining bone marrow cells were suspended in the macrophage medium composed of 10% fetal bovine serum, 2% glutamic acid, 1% penicillin and streptomycin and 20 ng/mL recombinant macrophage colony-stimulating factor. The macrophage medium was inoculated in the culture dish, and cultured in the 5% CO_2 cell incubator at 37°C. After 2 days of pre-culture, the culture dish was washed with PBS and all non-adherent cells in the culture supernatant were removed. The adherent cells were identified as macrophages. The adherent cells were isolated with 0.02% EDTA in PBS buffer, cultured on ice for

Table 2. Body weight, BMD, and biomechanical index values of rats in each group.

	Sham (n=10)	OVX (n=10)	*P* value
Pre-test body weight of the rats (g)	246.2 ± 25.7	228.9 ± 28.6	0.1719
After-test body weight of the rats (g)	308.7 ± 36.7	358.7 ± 26.3	0.0025
BMD (g/cm^2)	0.234 ± 0.022	0.201 ± 0.031	0.0133
BMD of L5 lumbar (g/cm^2)	0.189 ± 0.026	0.135 ± 0.032	0.0006
Maximum load (N)	147.58 ± 0.608	85.37 ± 0.283	< 0.0001
Positioning elongation displacement (mPa)	1.45 ± 0.021	0.17 ± 0.0121	< 0.0001

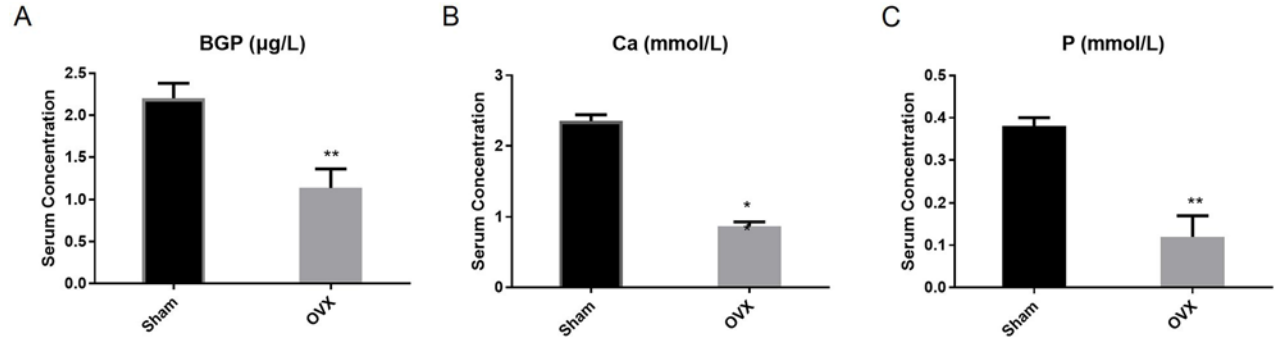

Figure 1. Concentration values of bone metabolism indexes in peripheral blood of rats in each group were detected using ELISA. A: BGP. B: Ca. C: P.

5 minutes, and then cultured at -20°C for 1 minute. Then the adherent cells were separated by cell scraper and collected with centrifugation. The collected cells were inoculated in a 75 cm^2 flask at a density of 2×10^6/cm^2 and cultured in the 5% CO_2 incubator at 37°C. The above cells were cultured to the fifth to ninth formations and used for subsequent *in vitro* cell experiments.

Overexpression and transfection of Foxf1 gene: the Foxf1 gene overexpression plasmid vector pcDNA3.1-EGFP-eFoxf1 (RiboBio, Guangzhou, China) and negative control (RiboBio, Guangzhou, China) were constructed *in vitro*. According to the instructions, the Foxf1 gene overexpression plasmid vector pcDNA3.1-EGFP-eFoxf1 constructed *in vitro* was transfected into rat bone marrow mesenchymal stem cells and macrophages respectively for 48 hours.

Osteoblast induction: Osteogenic induction medium (α-MEM containing 10% FBS, 0.2 mM ascorbic acid, 10 mM β-glycerol phosphate, 1% penicillin/streptomycin, and 10^{-7} mM dexamethasone) was used to induce bone marrow mesenchymal stem cells to differentiate into osteoblasts[18]. The osteogenic induction medium was changed every 3 days. After 21 days of osteogenic induction, the expression of Foxf1 and osteogenic related genes and proteins were detected by qRT-PCR and WB.

Osteoclast induction: Bone marrow macrophages were treated with M-CSF (50ng/mL) and RANKL (100 ng/mL, R & D, USA)[17] to induce them to differentiate into osteoclasts. The solution was changed every day. After 5 days of induction, the expressions of Foxf1 and osteoclast related genes and proteins were detected by QRT PCR and WB.

Detection of gene expression indexes of Foxf1, NF-κB, osteoclasts and osteoblasts with qRT-PCR: after cell culture, the supernatant was discarded, and Trizol was used to collect cells and extract RNA. The rest steps were the same as those in method 1.1.7. The primer sequence of detection indicators was shown in Table 1.

Detection of expression indexes of Foxf1, NF-κB, osteoclasts and osteoblasts with WB: after cell culture, the supernatant was discarded, the cells were collected by operation on ice. The cells were lysed in lysate and cell protein was extracted, and the rest steps were the same as those in method 2.1.8. Specific proteins were detected, including Foxf1 and NF-κB, osteoblast-related proteins, ATF4, OCN, ALP, Runx2, and osteoclast related proteins, TRAP, MMP9, CLC7, and Cathepsin-K.

Statistical analysis

Data were obtained from at least three independent repeated experiments and were expressed as mean ±

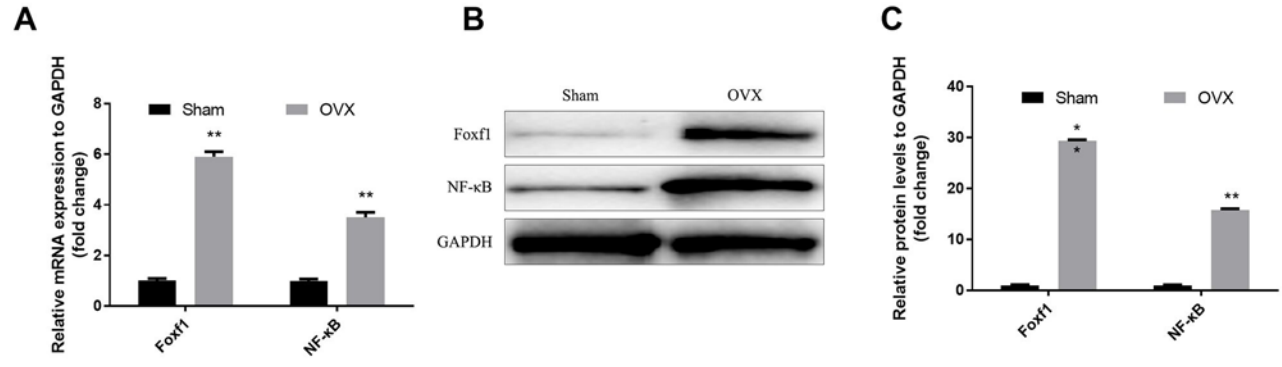

Figure 2. Expressions of Foxf1 and NF-κB genes and proteins in femur tissues of rats in each group were detected using qRT-PCR and WB, respectively. A shows the detection of Foxf1 and NF-κB gene expression in femur tissues of rats in each group using qRT-PCR; B and C show the expression bands of Foxf1 and NF-κB proteins in the femur tissues of rats in each group detected by WB and the statistical chart of quantitative analysis.

standard deviation. SPSS 20.0 software was used for statistical analysis. The data were consistent with continuous variables and normal distribution. The differences between groups were compared by one-way analysis of variance or independent sample t test. Compared with the control group, if $P<0.05$ or $P<0.01$, it indicated that the difference was statistically significant.

Result

Establishment of ovariectomized rat model of osteoporosis

In order to investigate the expression of Foxf1 and NF-κB in animal model of osteoporosis, an ovariectomized rat model of osteoporosis was established and related indexes were detected. The results showed that 3 months after operation, compared with those in sham group (sham), the body weight, femur BMD and L5 lumbar BMD of OVX group were significantly decreased (Table 2) with statistical significance. In terms of biomechanical indexes, compared with those in sham group, the maximum load and location elongation displacement of right femur of rats in OVX group were decreased (Table 2) with statistical significance. In terms of bone metabolism indexes in rats' peripheral blood, ELASA results showed that compared with those in sham group, the concentration values of BGP, Ca and P of rats in OVX group were significantly decreased (Figure 1) with statistical significance. These results indicate that the ovariectomized rat model of osteoporosis was established successfully.

High expression of Foxf1 and NF- κB in the femur tissues of the ovariectomized rat model of osteoporosis

Quantitative analysis of the femur tissues of rats was performed with qRT-PCR and WB, and the results showed that compared with sham group, the genes and proteins of Foxf1 and NF-κB were highly expressed in the femoral bone tissues of rats in OVX group (Figure 2) with statistical significance.

Foxf1 inhibits osteoblast formation and promote osteoclast differentiation by promoting NF-κB pathway in vitro

In order to investigate whether Foxf1 gene regulates the expression of NF-κB gene and its role in the development of osteoporosis, a plasmid vector of Foxf1 gene was constructed and transfected into rat bone marrow mesenchymal stem cells and macrophages, respectively, to induce osteoblasts and osteoclasts, respectively. Then qRT-PCR and WB were used to detect the expression of osteoblast and osteoclast-related genes and proteins, as well as the levels of Foxf1 and NF-κB genes and proteins in osteoblasts and osteoclasts, respectively. The results in osteoblasts showed that compared with those in the control group, the gene and protein expressions of ATF4, OCN, ALP and Runx2 in OeFoxf1 group were decreased (Figure 3A-C) with statistical significance. Furthermore, the results in osteoclasts showed that compared with those in the control group, the gene and protein expressions of ATF4, OCN, ALP and Runx2 in OeFoxf1 group were increased (Figure 3A-C) with statistical significance. The results of Foxf1 and NF-κB gene and protein detection in the transfected osteoblasts and osteoclasts showed that compared with those in the control group (Ctrl), the expression levels of Foxf1, and NF-κB genes and proteins in osteoblasts and osteoclasts in the Foxf1 overexpression group (OeFoxf1) were increased (Figures 4 A-C, D-F-C) with statistical significance. These results indicated that the osteoblast and osteoclast models with Foxf1 overexpression were successfully constructed, and that Foxf1 overexpression also promoted the expression of NF-κB gene. These results showed that Foxf1 inhibited osteoblast formation and promoted osteoclast differentiation by promoting NF-κB pathway *in vitro*.

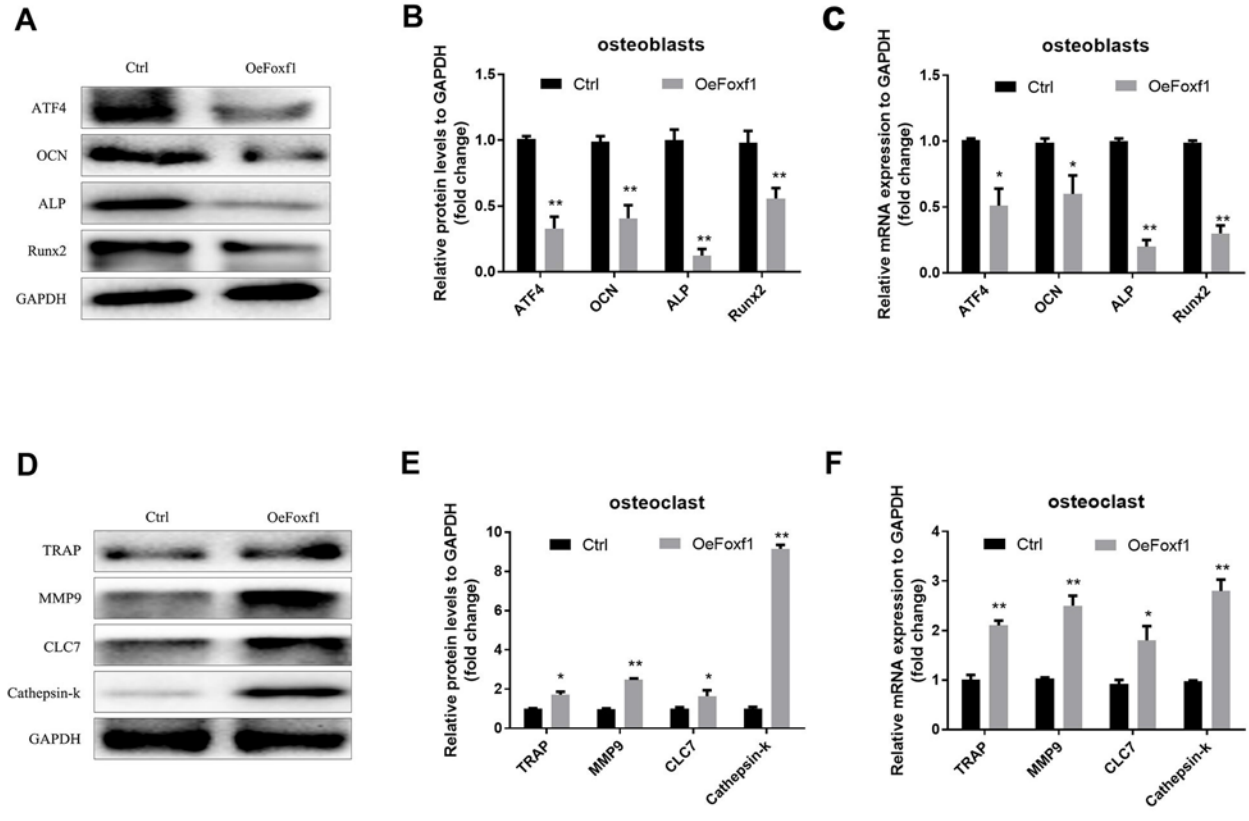

Figure 3. Expression of related genes and proteins of osteoblasts and osteoclasts derived from the rat bone marrow mesenchymal stem cell and macrophages transfected with Foxf1 gene overexpressed plasmid were detected by qRT-PCR and WB. A and B show the expression bands of ATF4, OCN, ALP and Runx2 proteins induced osteogenesis after the transfection of rat bone marrow mesenchymal stem cells with Foxf1 overexpressed plasmid by WB, and the statistical analysis of the expression levels. C shows the expression levels of ATF4, OCN, ALP and Runx2 genes induced osteogenesis after the transfection of rat bone marrow mesenchymal stem cells with Foxf1 overexpressed plasmid by qRT-PCR. D and E show the expression bands of TRAP, MMP9, CLC7 and Cathepsin-K proteins related to the function of osteoclasts after osteoclasts were induced by the transfection of macrophages with rat-deprived Foxf1 overexpressed plasmid by WB, and the statistical analysis of the expression levels. F shows the expression levels of TRAP, MMP9, CLC7 and cathepsin-K genes related to the function of osteoclasts after osteoclasts were induced by the transfection of macrophages with rat-deprived Foxf1 overexpressed plasmid by qRT-PCR.

Discussion

Osteoporosis is mainly caused by the imbalance between bone resorption and bone formation. The specific pathogenesis is still unclear[6]. It has been widely documented that the NF-κB pathway plays an important role in the differentiation of osteoclasts and osteoblasts[11,15,16]. Recent studies have shown that Foxf1 gene knockout could significantly promote the osteogenesis of bone marrow mesenchymal stem cells[18] and improve bone loss. In addition, studies have found that FoxF1 can protect the lung injury induced by paraquat in rats by regulating IκB/NF-κB pathway[25]. However, the regulatory role of Foxf1 gene and NF-κB pathway in bone metabolism remains unclear. In this study, a classic ovariectomized rat model of osteoporosis was established. Subsequently, it was found that Foxf1 and NF-κB genes and proteins were highly expressed in the femur tissues of the rats with osteoporosis. Then, the regulatory relationship between Foxf1 and NF-κB gene was investigated *in vitro* in osteoblasts and osteoclasts deprived from rats. Their effect in the development of osteoporosis was also explored. The results showed that compared with those in the control group, Foxf1 overexpression promoted the expression of NF-κB gene in osteoblasts and osteoclasts. Meanwhile, in the Foxf1 overexpression group, the gene and protein expressions of ATF4, OCN, ALP, and Runx2, indexes of osteogenesis function, were decreased. The gene and protein expressions of TRAP, MMP9, CLC7 and Cathepsin-K, indicators of osteoclast function, were significantly increased. These results showed that Foxf1 inhibited osteoblast formation and promoted osteoclast differentiation by promoting NF-κB pathway *in vitro*. Foxf1 gene knockdown may be a potential choice for

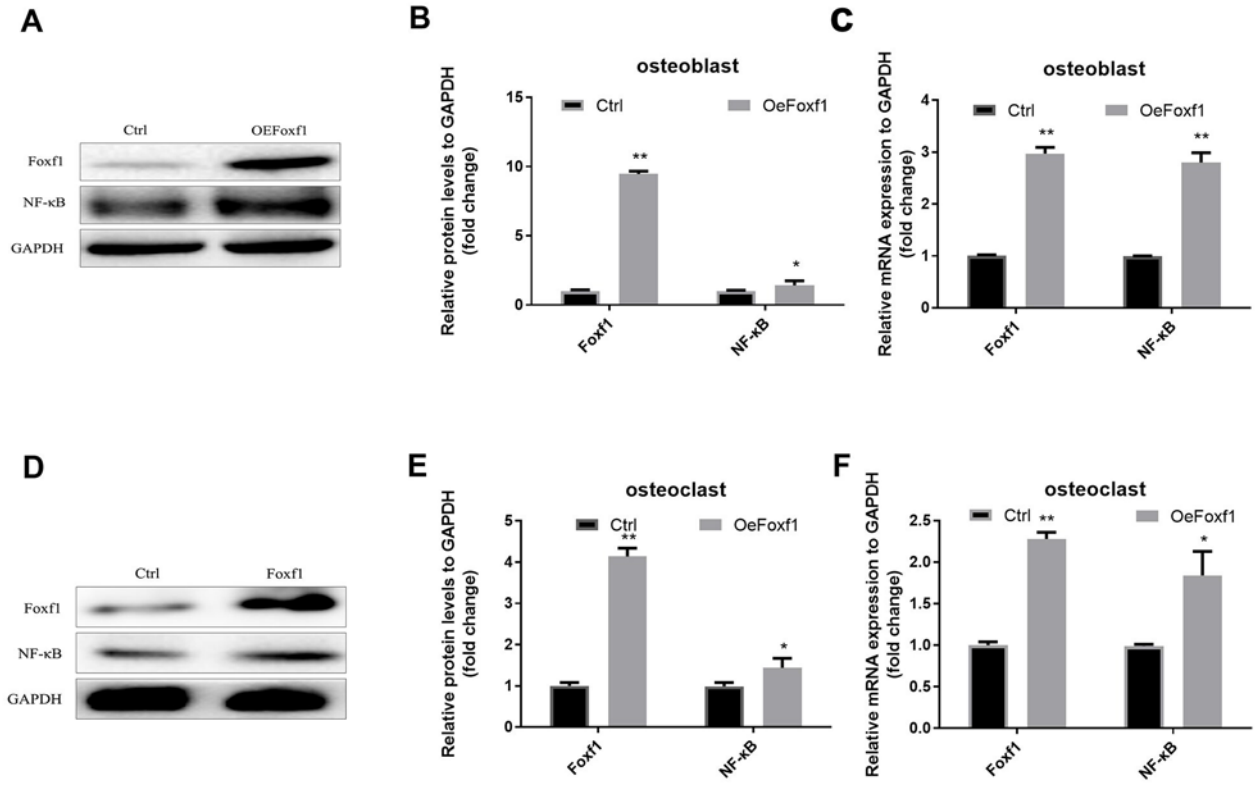

Figure 4. Expression of the Foxf1, NF-κB genes and proteins of osteoblasts and osteoclasts derived from the rat bone marrow mesenchymal stem cell and macrophages transfected with Foxf1 gene overexpressed plasmid were detected by qRT-PCR and WB. A and B show the expression bands of Foxf1x and NF-κB proteins induced osteogenesis after the transfection of rat bone marrow mesenchymal stem cells with Foxf1 overexpressed plasmid by WB, and the statistical analysis of the expression levels. C shows the expression of Foxf1x and NF-κB proteins induced osteogenesis after the transfection of rat bone marrow mesenchymal stem cells with rat-deprived Foxf1 overexpressed plasmid by qRT-PCR. D and E show the expression bands of Foxf1x and NF-κB proteins after osteoclasts were induced by the transfection of macrophages with rat-deprived Foxf1 overexpressed plasmid by WB, and the statistical analysis of the expression levels. F shows the expression levels of Foxf1x and NF-κB proteins after osteoclasts were induced by the transfection of macrophages with rat-deprived Foxf1 overexpressed plasmid by qRT-PCR.

the prevention and treatment of osteoporosis.

In order to further explore the regulatory relationship between Foxf1 and NF-κB and their roles in the development of osteoporosis, a classic ovariectomized rat model of osteoporosis was established and identified as postmenopausal osteoporosis is the most common type of osteoporosis. The reproductive organs and body weight of three-month-old female rats were mature, and bone metabolism was in a relatively balanced state. The ovariectomized rat model of osteoporosis could replicate the clinical characteristics of osteoporosis in postmenopausal women[30]. Our results showed that the body weight, femur BMD, BMD of L5 lumbar vertebrae, biomechanical parameters of right femur (maximum load and positional elongation displacement values), and concentration of bone metabolic indexes BGP, Ca, and P of theovariectomized rats were significantly decreased. These results were consistent with those in previous studies on ovariectomized rats with osteoporosis[28,31], indicating that our animal model was successfully established.

NF-κB pathway is closely related to bone metabolism and is a transcription factor regulating the inflammatory response of bone forming cells and bone resorptive cells and the process of bone remodeling. The study showed that the decrease of NF-κB activity in osteoblasts can enhance the differentiation and mineralization of osteocytes and promote bone formation[32]. Activation of NF-κB depends on phosphorylation of NF-κB/p65[33]. The NF-κB pathway in osteoclasts has been extensively studied[34]. RANKL, TNF-α or IL-1-activated NF-κB signaling pathway can induce the expression of osteoclast differentiation genes, prolong the life of osteoclasts and increase bone resorption[35]. The FOX transcription factor family plays an important regulatory role in cell growth and metabolism, and studies have also

confirmed that Foxa2, Foxc2 and other genes also play a key role in the regulation of bone metabolism[23,24]. Foxf1 gene, a member of the Fox family, is a newly discovered regulatory factor of bone metabolism. Studies have shown that Foxf1 gene knockout can significantly promote the osteogenesis of bone marrow mesenchymal stem cells and prevent bone loss. However, the specific regulatory mechanism remains unclear, and there is no more evidence to support its potential as an option for the prevention and treatment of osteoporosis[18]. After the establishment of the rat model of osteoporosis, Foxf1 gene and its NF-κB pathway, which is closely related to bone metabolism, were detected. The results showed that both Foxf1 and NF-κB were highly expressed in the femur tissue of ovariectomized rats with osteoporosis, suggesting that the two may have a synergistic effect. The above results are consistent with our prediction and similar to the results reported in the previous studies[18,27].

The rat bone marrow mesenchymal stem cells and macrophages were transfected with Foxf1 gene overexpressed plasmid vector to induce osteoblasts and osteoclasts, respectively. The expression of NF-κB and its effect on osteoblast and osteoclast function were detected by qRT-PCR and WB. The results showed that upon the overexpression of Foxf1 gene, the expression of NF-κB were also up regulated, significantly reducing the gene and protein expression levels of osteogenic factors, including ATF4, OCN, ALP and Runx2. However, the expression levels of osteoclast related factors, including TRAP, MMP9, CLC7 and Cathepsin-k, were significantly increased. Therefore, Foxf1 can promote the gene and protein expression of NF-κB *in vitro*, decrease the function of osteoblasts, increase the activity of osteoclasts, and increase the risk of osteoporosis. In addition, the Wnt/β-catenin signaling pathway has been reported to be associated with the promoted osteogenic differentiation of bone marrow mesenchymal stem cells induced by Foxf1 gene knockout[18], which provides further evidence for our conclusion that Foxf1 gene may be a potential new target for the effective prevention and treatment of osteoporosis.

Currently, although a variety of drugs are used to prevent osteoporosis and its associated fractures, there are still huge challenges[5]. For example, bisphosphonates were previously considered a first-line treatment for postmenopausal osteoporosis, but unexpected adverse events such as osteonecrosis of the jaw and atypical femoral fractures have been reported in some patients, restricting the application of bisphosphonates[36]. Therefore, it is of great importance to continue to develop new treatment methods and drugs for osteoporosis. To our knowledge, the interaction between Foxf1 and NF-κB in osteoblasts and osteoclasts has been reported for the first time. Foxf1can promote the expression of NF-κB gene and protein *in vitro*, which may increase the risk of osteoporosis, suggesting that Foxf1 knockdown may be a potential choice for the prevention and treatment of osteoporosis. However, there are some limitations to the study. For example, the molecular mechanisms of bone formation are complex, and further studies are needed to confirm the role of Foxf1 knockdown in rat osteoporosis and further explore the molecular mechanisms of the potential effects. In addition, we did not evaluate the effect of Foxf1 on other important bone metabolic processes, such as angiogenesis and inflammatory response.

In conclusion, our study shows that Foxf1 gene increases the risk of osteoporosis in rats by inhibiting osteoblast formation and promoting osteoclast differentiation through the promotion of the NF-κB pathway. This study provides a theoretical basis and experimental evidence for the future application of Foxf1 gene knockdown in the prevention and treatment of osteoporosis and other diseases associated with bone loss.

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