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Original Article

TETRATHIOMOLYBDATE ALLEVIATES OSTEOPOROSIS BY ACTIVATING THE PI3K/AKT SIGNALING AXIS

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Abstract

To investigate the role and possible mechanisms of tetrathiomolybdate (TTM) in ovariectomy (OVX)-induced osteoporotic mice. Eighteen 12-week-old C57BL/6 female mice were randomized into three groups: sham-operated group (Sham group, n = 6), OVX-induced osteoporosis (OP) group (OP group, n = 6), and OVX-induced OP+TTM gavage-treated group (OP+TTM group, n = 6). The mice body weight results and abdominal fat macroscopic view showed significant improvement in obesity in the OP+TTM group of mice as compared to those in the OP group. HE staining and micro-CT scanning results demonstrated that mice in the OP+TTM group showed milder bone loss than those in the OP group. The results of TRAP staining also supported these findings. In addition, serum lipid profiling and immunohistochemical staining showed that compared with mice in the OP group, mice in the OP+TTM group had significantly lower serum levels of triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C), as well as PPARγ and FABP4 protein expression in the femur. Further, the results of transcriptome sequencing analysis indicated that the differential genes between OP+TTM and OP groups were mainly enriched in the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway. Immunofluorescence assay preliminarily confirmed that the expression of p-PI3K and p-Akt proteins was significantly elevated in the femur of mice in the OP+TTM group, especially in the bone marrow cavity. Taken together, we reported for the first time that TTM ameliorated obesity and inhibited bone loss in OP mice, which may be related to the activation of PI3K/Akt signaling.

Keywords: Tetrathiomolybdate, osteoporosis, bone formation, PI3K/Akt signaling, ovariectomy, cuproptosis.

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Introduction

Osteoporosis (OP) is an age-related bone metabolic disorder characterized by decreased bone mass, increased bone fragility, and increased fracture risk (Walker and Shane, 2023). Osteoporotic fractures usually heal poorly, and in severe cases may lead to disability or even death, which imposes a huge economic burden on the patient's family and society (Khosla and Hofbauer, 2017; Schwartz, 2015). Maintenance of bone homeostasis is dependent on coupled osteoclast-mediated bone resorption and osteoblast-mediated bone formation (Compston *et al.*, 2019). Disruption of bone homeostasis due to excessive bone resorption or inadequate bone formation is the underlying cause of the initiation and development of OP (Compston *et al.*, 2019). Studies have suggested that microelement abnormalities may be a risk factor for OP (Ceylan *et al.*, 2021; Hendrickx *et al.*, 2015; Zhang *et al.*, 2021).

Copper ions are essential trace elements for human health (Scheiber *et al.*, 2013). Previous studies have shown that copper ions are necessary for the growth and development of tissues and organs such as bone, brain and cardiovascular (Bost *et al.*, 2016). Copper ion deficiency or excess may lead to impaired bone metabolism, which in turn promotes the development of bone metabolism-related dis-

eases (Rondanelli *et al.*, 2021). Tetrathiomolybdate (TTM), consisting of a small inorganic tetrahedral molybdenum-sulfur anion ($MoS4^{2-}$), is an anti-copper drug that has been used in clinical trials primarily for the treatment of Wilson's disease, a copper-metabolizing autosomal recessive disorder (Pan *et al.*, 2002). Recent studies have demonstrated the ability of TTM to protect against a wide range of diseases and injuries through antioxidant damage (Hsu *et al.*, 2018), Alzheimer's disease (Voss *et al.*, 2014), cardiac ischemia-reperfusion injury (Dyson *et al.*, 2017), and pulmonary fibrosis (Ovet and Oztay, 2014). Since lowering copper ion levels inhibits a large number of copper-dependent angiogenic cytokines, TTM has shown excellent efficacy in the treatment of a variety of tumors, including breast cancer (Pan *et al.*, 2002). In addition, Morisawa *et al.* (2018) demonstrated that TTM not only inhibits tumor growth, but also inhibits bone resorption, resulting in a positive therapeutic effect on bone-invasive head and neck squamous carcinoma. The underlying mechanism may be that the copper chelating effect of TTM can down-regulate the expression of receptor activator of nuclear factor-κB ligand (RANKL) in osteoblasts through the suppression of lysyl oxidases, thus inhibiting osteoclast differentiation (Dahl *et al.*, 2005a; Morisawa *et al.*, 2018). However, currently, there is no literature on whether TTM can alleviate OP by modulating bone metabolism.

Phosphatidylinositol 3-kinase (PI3K) is a phosphatidylinositol kinase that, together with the downstream molecule protein kinase B (Akt), constitutes the PI3K/Akt signaling pathway, involving in the regulation of cell proliferation, differentiation and apoptosis (Wang *et al.*, 2022). Cumulative evidence confirms that the PI3K/Akt pathway may be closely related to the pathogenesis of OP (Hao *et al.*, 2022; Ma *et al.*, 2023). Activation of the PI3K/Akt pathway not only promotes osteogenic differentiation of bone marrow-derived stem/stromal cells (BMSCs) (Ye *et al.*, 2019), but also mediates the regulation of chondrogenic differentiation (Srivastava *et al.*, 2014), whereas inhibition of PI3K/Akt suppresses long bone growth (Ulici *et al.*, 2008). In addition, Weng *et al.* (2023) demonstrated that konjac glucomannan, a soluble dietary fiber used as an ingredient in weight-loss supplements, could prevent high-fat diet-induced atherosclerosis by activating the PI3K/Akt pathway to regulate blood lipids and attenuate inflammation in rabbits (Weng *et al.*, 2023; Yang *et al.*, 2017). Thus, PI3K/Akt signaling may be involved in the regulation of both bone metabolism and lipid metabolism.

Currently, there are limited clinical treatment strategies for OP (Lai *et al.*, 2022). Commonly used OP therapy medications in the clinic are ineffective and have side effects, including nausea, dizziness, leg cramps (Oryan and Sahvieh, 2021), and even an increased risk of cardiovascular disease, breast cancer, and osteonecrosis of the jaw (Cui *et al.*, 2022; Reid and Billington, 2022). The aim of this study was to explore potential strategies for the treatment of OP and to provide new ideas for targeted therapy of OP. In this study, we investigated for the first time the therapeutic efficacy and potential mechanisms of TTM in mice with ovariectomy (OVX)-induced OP, which provides new ideas for targeted treatment of OP.

Materials and Methods

Animal Experiments

All procedures of this study were reviewed and approved by the Ethics Committee of Exercise Science Experiment of Beijing Sport University (Approval No. 2023026A). Eighteen 12-week-old female C57BL/6 mice were purchased from Beijing Huafukang Bio-technology Co., Ltd. and housed in an SPF-class animal laboratory environment with temperature and relative humidity at (22 ± 2) °C and 55 %–75 %. Normal circadian rhythms were administered. All mice were allowed to move freely within the cage. Adaptive feeding of mice for one week was required before the formal experiment begins.

After the end of adaptive feeding, eighteen mice were randomly divided into three groups: sham-operated group (Sham group, n = 6), OVX-induced osteoporosis (OP) group (OP group, n = 6), and OVX-induced OP+TTM gavage-treated group (OP+TTM group, n = 6). The steps for OVX are as follows: Mice were anesthetized in the prone position by isoflurane. A small cut of about 0.5 cm was made in the depression on each side of the thigh root of the mice. Ophthalmic scissors were then used to cut a layer of muscle under the skin on the back. Ophthalmic forceps were used to pick out the mouse ovaries and later cut them out using ophthalmic scissors (OP group and OP+TTM group). Ophthalmic forceps were used to cut out the same volume of adipose tissue near the mouse ovaries (Sham group). The wounds were finally closed with absorbable sutures. All mice were injected intramuscularly with antibiotics postoperatively. Mice in the OP+TTM group were subjected to TTM gavage once a day from 1 w postoperatively (30 mg/kg for 11 weeks). Mice in the OP and Sham groups were subjected to equal volume solvent gavage once a day from 1 w postoperatively. All mice were sacrificed at 12 w postoperatively. Mice were anesthetized with isoflurane and executed by neck dissection after removal of the eyeballs for blood. No mice suffered accidental deaths during the experiment. Transcriptomics sequencing samples were transported using dry ice to Shanghai Sangong Biotechnology Co., Ltd. for RNA extraction, detection, and data analysis.

Micro-CT

The femur and tibia were isolated from the mouse knee joint. The intact femur was placed in 10 % PFA solution for 36–48 h for fixation before micro-CT scanning. The Skyscan1276 scanning device (Bruker, Kontich, Belgium) was used to scan the entire femur. The femur was placed in the Skyscan1276 *ex vivo* sample scanning bed.

The scanning parameters were as follows: Camera Pixel Size (μm) = 9.01; Source Voltage (kV) = 69; Source Current (μA) = 100; Image Pixel Size (μm) = 9.92. After scanning, NRecon (version 1.7.4.6, Bruker, Kontich, Belgium) and DataViewer software (version 1.5.2.4, Bruker, Kontich, Belgium) were used to adjust all femur samples to the same position in the 3D axes to facilitate subsequent more precise selection of the region of interest for reconstruction. CTan software (version 1.20, Bruker, Kontich, Belgium) was used for further analysis of bone microstructural parameters, including bone mineral density (BMD), bone volume fraction (BV/TV), bone surface/bone volume (BS/BV), trabecular thickness (Tb.Th), the number of trabeculae (Tb.N), and trabecular segregation (Tb.Sp). The place where the growth plate disappeared was chosen as the starting point of the region of interest, and 100 layers were selected upwards as the region of interest for 3D reconstruction. Finally, CTvox (version 2.0, Bruker, Kontich, Belgium) was used for 3D reconstruction of regions of interest. After the micro-CT scan was completed, the femur was placed in an EDTA decalcification solution for decalcification for subsequent paraffin embedding and pathological sectioning.

Hematoxylin-eosin Staining

Mouse femurs were fixed in PFA solution for 36–48 h before being decalcified in EDTA decalcification solution at room temperature. The EDTA decalcification solution was changed every other day until the bone tissue became soft enough to be easily pierced by a needle tip. Subsequently. The samples were then dehydrated, transparent, waxed, embedded in a tissue wax block, and finally sectioned (4 μm per section). Subsequently, routine HE staining was performed according to the instructions. After staining, the sections were observed and photographed under an inverted optical microscope.

Immunohistochemical and Immunofluorescence Staining

For immunohistochemical staining, the mouse femurs paraffin sections were placed in a 60 °C oven for 30 minutes and then subjected to treatment with xylene, xylene, xylene, 100 % ethanol, 95 % ethanol, and 80 % ethanol. After dewaxing, antigen repair, blocking, primary antibody incubation (anti-PPARγ, abcam, ab310323; anti-FABP4, abcam, ab92501), secondary antibody incubation, and DAB staining were performed. The staining process was terminated after positive expression was observed under the microscope. Subsequently, the nucleus was stained using hematoxylin staining. Conventional dehydration and xylene transparency were performed. Finally, neutral resin adhesive was used for sealing. The sections were observed and photographed under an inverted optical microscope. Similar to the immunohistochemical staining steps, the mouse femurs paraffin sections were sequentially subjected to dewaxing, antigen repair (microwave repair can reduce bone tissue loss), blocking, primary antibody incubation (anti-Runx2, abcam, ab192256; anti-p-PI3K, abmart, T40116), secondary antibody incubation, re-antigen repair, addition of primary antibody (anti-COL-I, abcam, ab270993; anti-p-Akt, abmart, T40067), secondary antibody incubation, DAPI re-staining of the cell nucleus, and blocking. Finally, images were taken under the SP8 Leica laser confocal microscope (Leica, Wetzlar, Germany).

Serum Lipid Profile

Mindray-BS240VET was used to detect the expression of Serum lipid profile in mice including triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) according to the instructions. TC and TG serum concentrations were determined by the oxidase method, and HDL-C and LDL-C serum concentrations were determined by the direct lipoprotein method.

Data Analysis

The data from each group were analyzed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA). GraphPad Prism 10 (GraphPad Software, Inc., San Diego, CA, USA) was used to graph statistical data. Results are expressed as mean ± standard deviation ($\bar{x} \pm S$). One-way ANOVA and the LSD method were used to compare the differences between multiple groups. $p < 0.05$ indicates a significant difference.

Results

TTM Ameliorates Obesity in OP Mice

As shown in Fig. 1**A**,**B**, a mouse model of OP was constructed by OVX. At 12 w postoperatively, the mice were sacrificed and the uteri were removed for observation and photographs. Compared with the Sham group, the uteri of mice in the OP group were significantly atrophied due to the surgical removal of both ovaries (Fig. 1**C**). However, the uteri of mice in the OP+TTM group were not significantly different from those in the OP group. Compared to the Sham group, mice in the OP group were significantly obese, while the body size of mice in the OP+TTM group did not differ from that of the Sham group. We speculated that TTM might be able to inhibit adipogenesis in mice. Therefore, the abdominal fat of mice in each group was removed and photographed (Fig. 1**D**). Obviously, the abdominal fat of mice in the OP group was significantly increased compared to the Sham group, whereas the abdominal fat of mice in the OP+TTM group was almost the same as that of the Sham group. In addition, the results of weekly weighing of mice showed that the weight of mice in the OP+TTM group was significantly lower than that in the OP group (Fig. 1**E**), which further demonstrated that TTM could hinder obesity in OP mice. Notably, TTM was administered by gavage. We questioned whether the loss of body weight in the OP+TTM group of mice might have been caused by

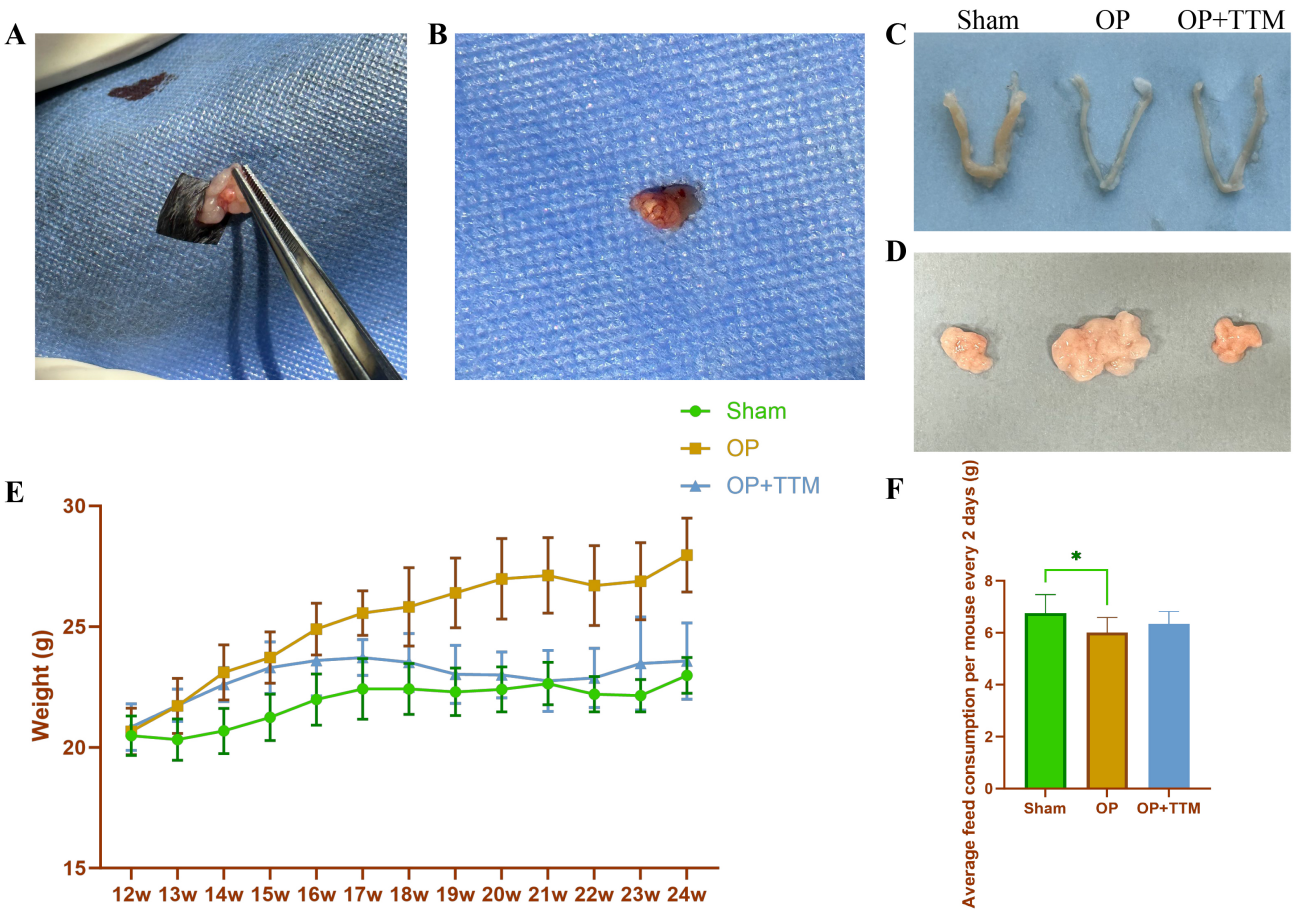

Fig. 1. Changes in body weight of mice in each group. (**A**,**B**) Images of OVX; (**C**) Gross observation of the uterus; (**D**) Gross observation of the abdominal fat; (**E**) Weight changes after OVX; (**F**) Average food intake per mouse per two days. TTM, tetrathiomolybdate; Sham, Sham group; OP, OP model group; OP+TTM, OP + TTM administration group; OVX, ovariectomy; n = 6, *$p < 0.05$.

anorexia after TTM gavage. Therefore, we continuously monitored the intake of each group of mice by weighing the feed every two days (Fig. 1**F**). It was found that the average food intake of mice in the OP group was lower than that of mice in the Sham group, but there was no significant difference in the average food intake between the mice in the OP group and the mice in the OP+TTM group, suggesting that the weight loss in the mice in the OP+TTM group was not caused by anorexia. Thus, TTM treatment can mitigate obesity in OP mice.

TTM Alleviates Bone Loss in OP Mice

Mouse femurs in each group were harvested for HE staining to evaluate histomorphometric changes. The growth of long bones is largely dependent on endochondral ossification. Growth plates with active osteogenesis have a typical four-layer structure: resting-zone, proliferative zone chondrocytes, hypertrophic zone, and ossification zone. As shown in Fig. 2**A**, the chondrocytes in the growth plate of mice in the OP group were disorganized and not clearly stratified, suggesting weak osteogenic function. Moreover, compared to the Sham group, the OP group of mice had a large number of distinct fat vacuoles in the bone marrow cavity. It suggests that mice in the OP group have a weakened osteogenic differentiation function and an active lipogenic differentiation function. Although the bone marrow cavity of mice in the OP+TTM group had more fat vacuoles than those in the Sham group, however, it was significantly lower than that in the OP group. In addition, micro-CT was used to evaluate bone formation function in mice by detecting bone morphometric indices (Fig. 2**B**,**C**). The results suggested that BMD, BV/TV and BS/TV were significantly lower and Tb.Sp was significantly higher in the OP group of mice compared to the Sham group. However, the BV/TV and BS/TV of mice in the OP+TTM group were significantly higher than those of mice in the OP group, although there was no significant difference in BMD. Further, immunofluorescence was used to detect the expression of Runx2 and COL-I (markers of osteogenic differentiation) in mouse femur. The results demonstrated that the expression of Runx2 and COL-I in the femur of mice in the OP group was significantly lower than that in the Sham group, whereas the expression of Runx2 and COL-I in mice in the OP+TTM group was significantly higher than that in the OP group (Fig. 3**A**,**B**). Since bone homeostasis depends on coupled bone formation and bone resorption, distribution of

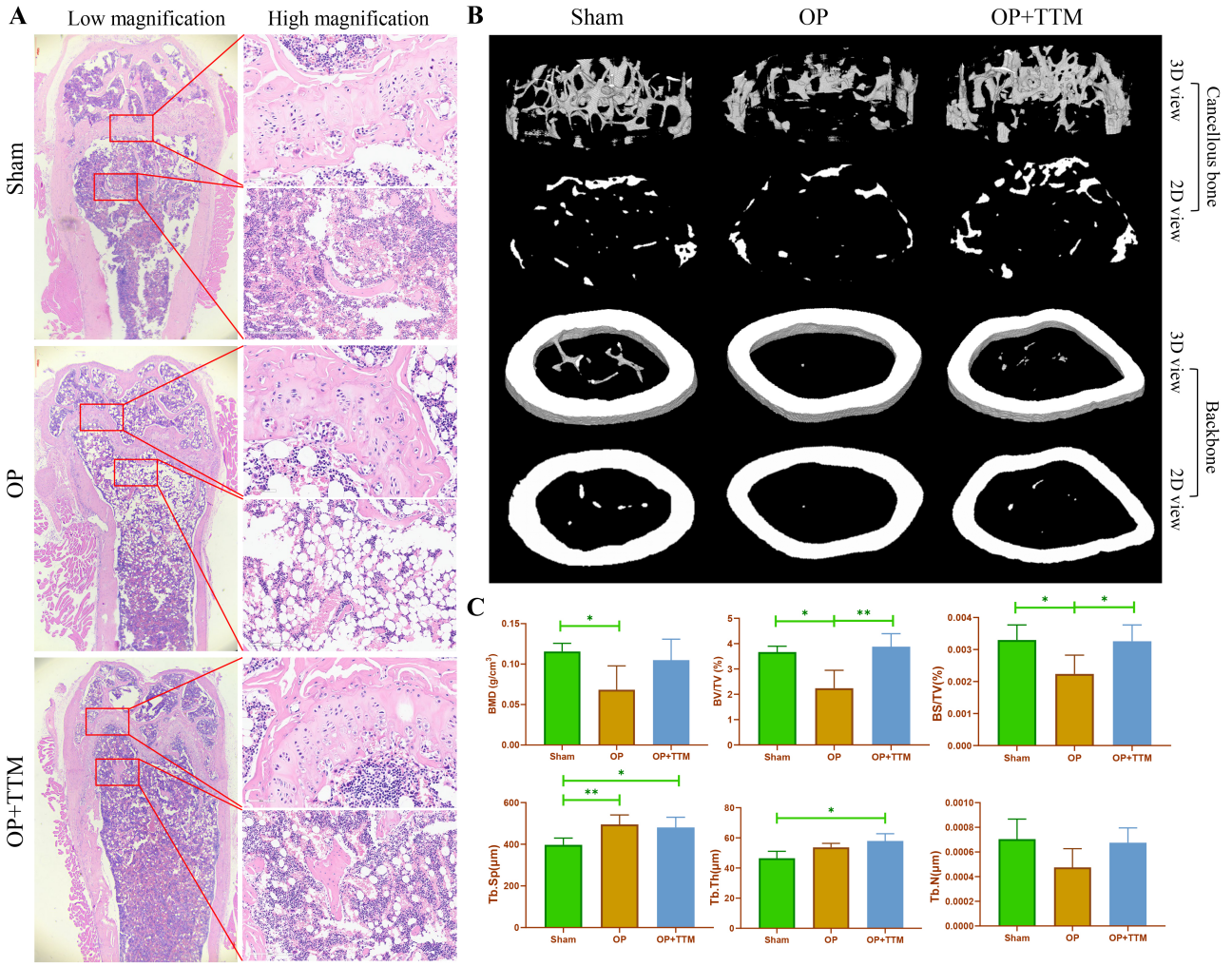

Fig. 2. Morphologic changes in bone tissue of mice in each group. (**A**) HE staining of the mouse femur; (**B**,**C**) Reconstruction images and bone morphometric indices of changes in the mouse femur by micro-CT scanning. BMD, bone mineral density; Tb.Sp, abecular segregation; Tb.Th, trabecular thickness; Tb.N, number of trabeculae; BS/BV, bone surface/bone volume; BV/TV, bone volume fraction; Sham, Sham group; OP, OP model group; OP+TTM, OP + TTM administration group; n = 6, *$p < 0.05$, **$p < 0.01$.

osteoclasts in bone tissue was detected by TRAP staining. Compared with the Sham group, osteoclasts were more active in the OP group, while the distribution of osteoclasts in the bone tissue of the OP+TTM group was sparser than that of the OP group (Fig. 3**C**,**D**). Thus, TTM treatment may alleviate OP by reducing bone loss.

TTM Inhibits Adipose Differentiation in OP Mice

Since the body weight of mice in the OP+TTM group was significantly lower than that in the OP group, we focused on the effects of TTM treatment on adipose tissue in mice. Mindray-BS240VET was used to detect lipid-related markers such as TG, TC, HDL-C, and LDL-C in serum (Fig. 4**A**). Both HDL-C and LDL-C in the serum of mice in the OP group were found to be significantly higher than those in the Sham group, whereas HDL-C and LDL-C in mice in the OP+TTM group were not significantly different from those in the Sham group. Interestingly, although there was no significant increase in serum TG levels in mice in the OP group compared to the Sham group, it was significantly lower in the OP+TTM group. In addition, there was a trend of decreasing TC levels in the serum of mice in the OP+TTM group, compared to the OP group, although the difference was not significant. Further, immunohistochemistry was used to analyze the protein expression of PPARγ and FABP4, markers of adipose differentiation, in mouse femurs (Fig. 4**B**,**C**). The results suggested that both PPARγ and FABP4 showed high expression in the femur of mice in the OP group, while the expression of PPARγ and FABP4 in the femur of mice in the OP+TTM group was significantly lower than that in the OP group. Thus, TTM treatment may alleviate OP by inhibiting adipose differentiation.

Adipose Tissue Transcriptome Sequencing

The above results preliminarily reveal the potential of TTM for the regulation of BMSC differentiation fate.

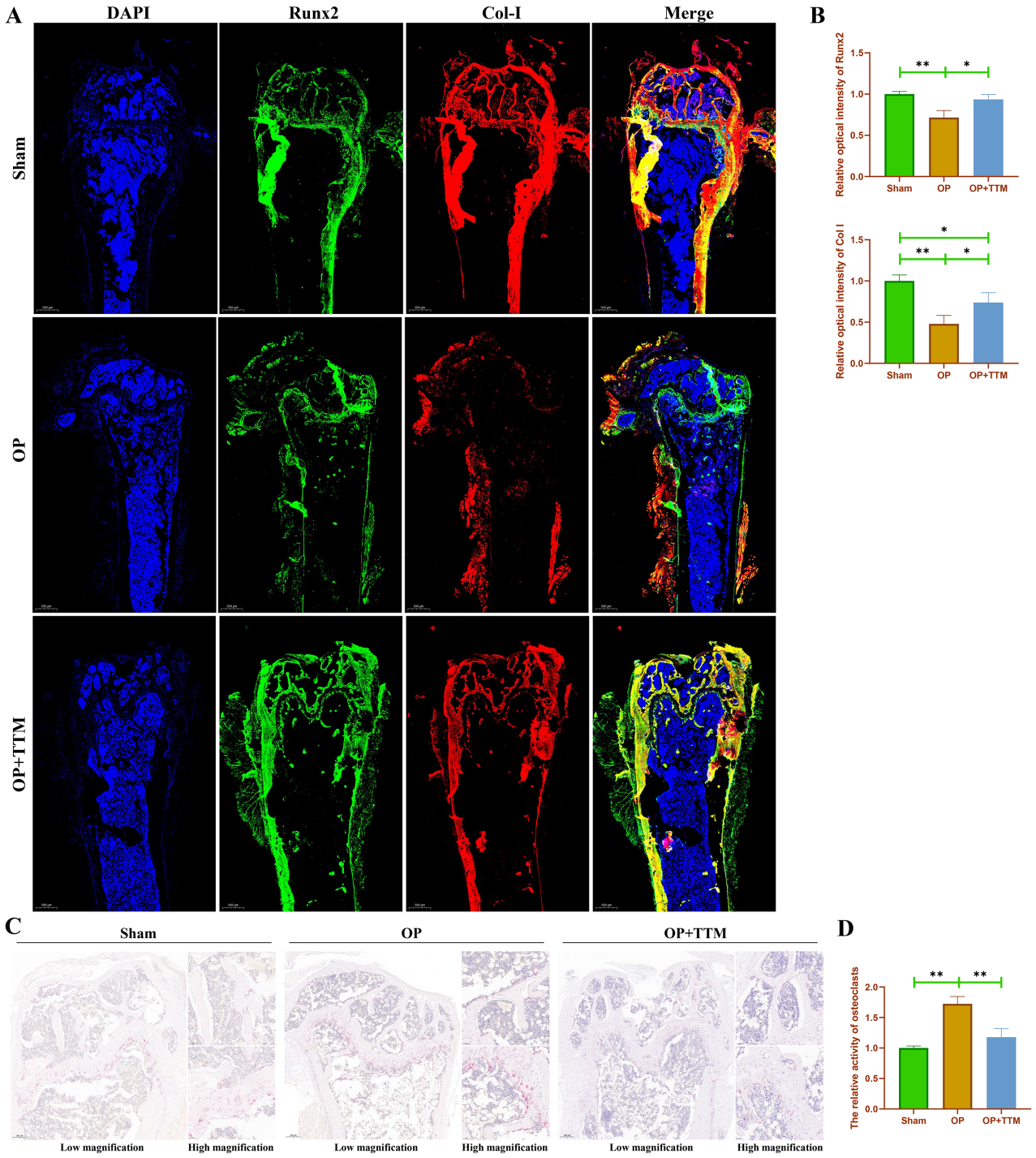

Fig. 3. Detection of bone formation and bone resorption in bone tissue of mice in each group. (**A**,**B**) Runx2 and COL-I protein expression in mouse femur by immunofluorescence; (**C**,**D**) Osteoclast staining by TRAP. Sham, Sham group; OP, OP model group; OP+TTM, OP + TTM administration group; n = 6. *$p < 0.05$, **$p < 0.01$.

We then explored the potential mechanisms by which TTM regulates fat accumulation and adipose differentiation by transcriptomic sequencing analysis of adipose tissue. The transcriptome sequencing analysis results of the OP group and the Sham group intuitively presented the overall distribution of differentially expressed genes between the two groups (Fig. 5**A**,**B**). The enrichment analysis circle plot (Fig. 5**C**) showed the enrichment results of the differentially expressed genes in Gene Ontology (GO) categories and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, revealing the close association of gene expression differences between the OP and Sham groups with biological functions and metabolic pathways from different perspectives. According to the KEGG pathway enrichment

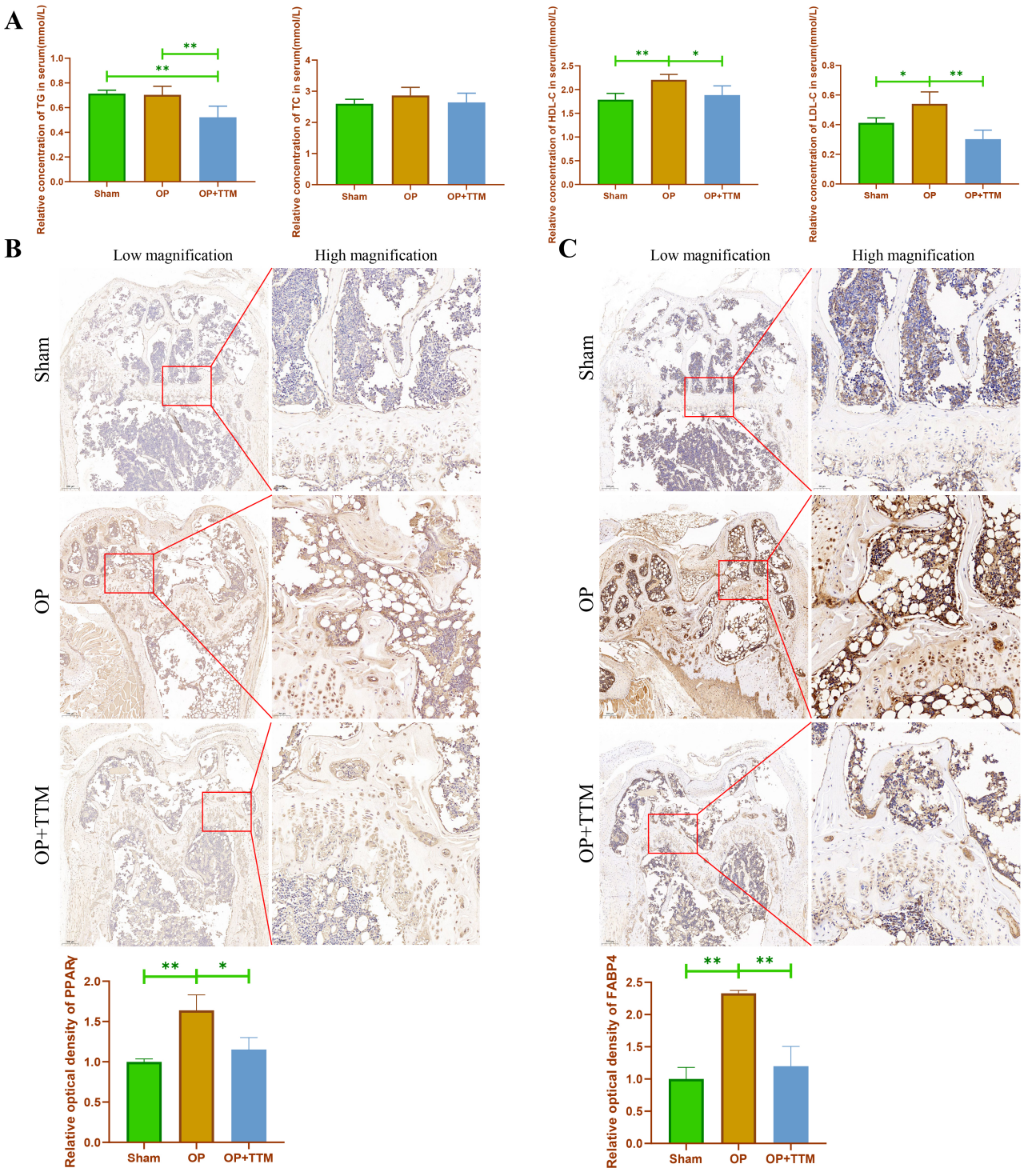

Fig. 4. Detection of adipose differentiation in each group of mice. (**A**) Serum lipid profile was detected by Mindray-BS240VET; (**B**) PPARγ protein expression in mouse femur by immunohistochemistry; (**C**) FABP4 protein expression in mouse femur by immunohistochemistry. TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Sham, Sham group; OP, OP model group; OP+TTM, OP + TTM administration group; n = 6, *$p < 0.05$, **$p < 0.01$.

analysis, the differentially expressed genes in the OP group compared to the Sham group were mainly concentrated in pathways such as complement and coagulation cascades, alcoholic liver disease, fat digestion and absorption, carbon metabolism, and fatty acid metabolism. This suggests that OP mice may have pathophysiological changes such as inflammatory responses and lipid metabolism disorders. GO functional enrichment analysis indicated that the differentially expressed genes in the OP group were mainly involved in the composition of cellular structures such as

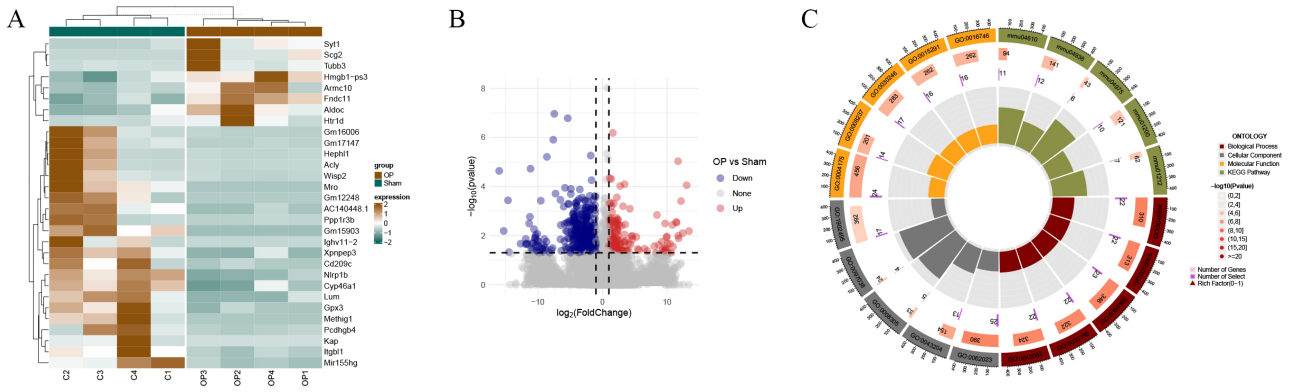

Fig. 5. Comparison of transcriptomics sequencing analysis between OP and Sham groups. (**A**) Heatmap of gene expression in OP vs. Sham Comparison; (**B**) Volcano plot of gene expression differences between OP and Sham groups; (**C**) Enrichment analysis circle plot of differentially expressed genes between OP and Sham Groups. OP, osteoporosis.

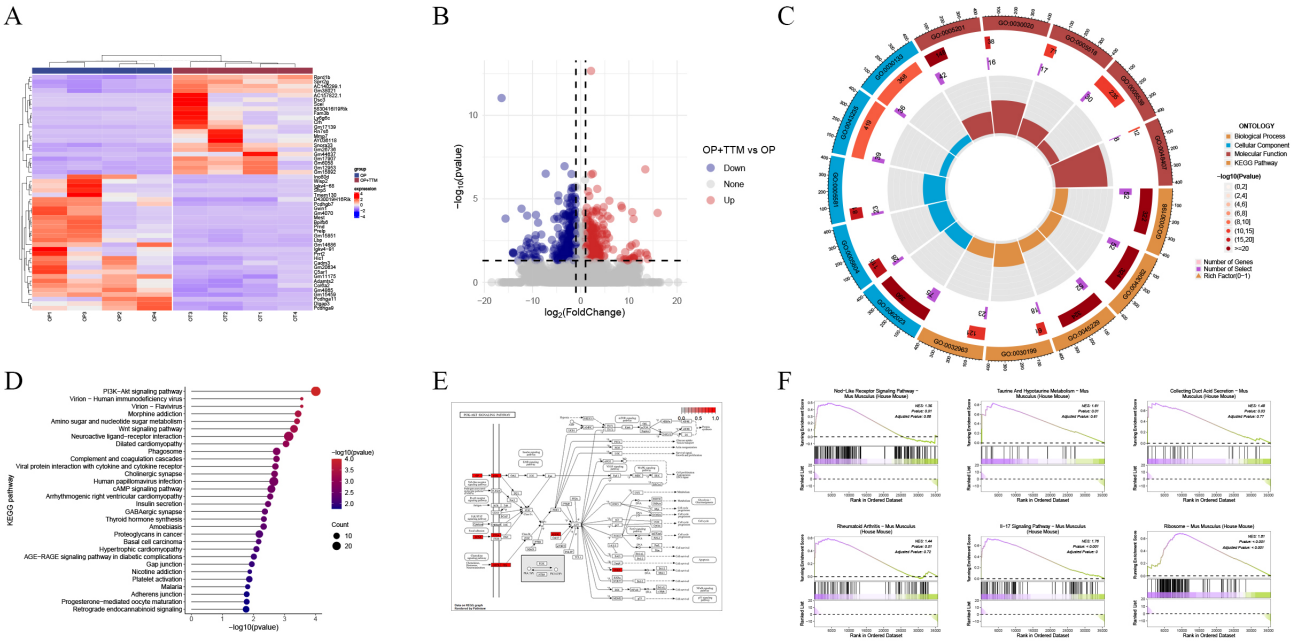

Fig. 6. Comparison of transcriptomics sequencing analysis between OP+TTM and OP groups. (**A**) Heatmap comparison of gene expression levels between OP+TTM and OP groups; (**B**) Volcano plot of gene expression differences between OP+TTM and OP groups; (**C**) GO enrichment analysis bubble chart for differential genes between OP+TTM and OP groups; (**D**) KEGG pathway enrichment analysis lollipop chart for differential genes between OP+OT and OP groups; (**E**) Enrichment of differentially expressed genes in the PI3K-Akt Signaling Pathway between OP+TTM and OP groups; (**F**) GSEA Enrichment analysis of differential genes between OP+TTM and OP groups. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B.

the collagen-containing extracellular matrix, perikaryon, and endoplasmic reticulum, as well as molecular functions like endopeptidase activity, metallopeptidase activity, and carbohydrate binding. This implies that OP may involve biological processes such as extracellular matrix remodeling, proteolysis, and carbohydrate metabolism changes. These results comprehensively demonstrated the gene expression profile changes in the OP group compared to the Sham group, providing important clues for understanding the pathogenesis of OP. Moreover, the transcriptome sequencing analysis results of the OP+TTM group and the OP group visually displayed the overall distribution of differentially expressed genes between the two groups (Fig. 6**A**,**B**), revealing changes in the gene expression profile following TTM treatment. The GO enrichment analysis circle plot (Fig. 6**C**) showed the enrichment of differentially expressed genes in the GO database, indicating that these genes are mainly related to biological processes such as extracellular matrix organization, collagen fibril organization, and collagen metabolic process. They are also related to

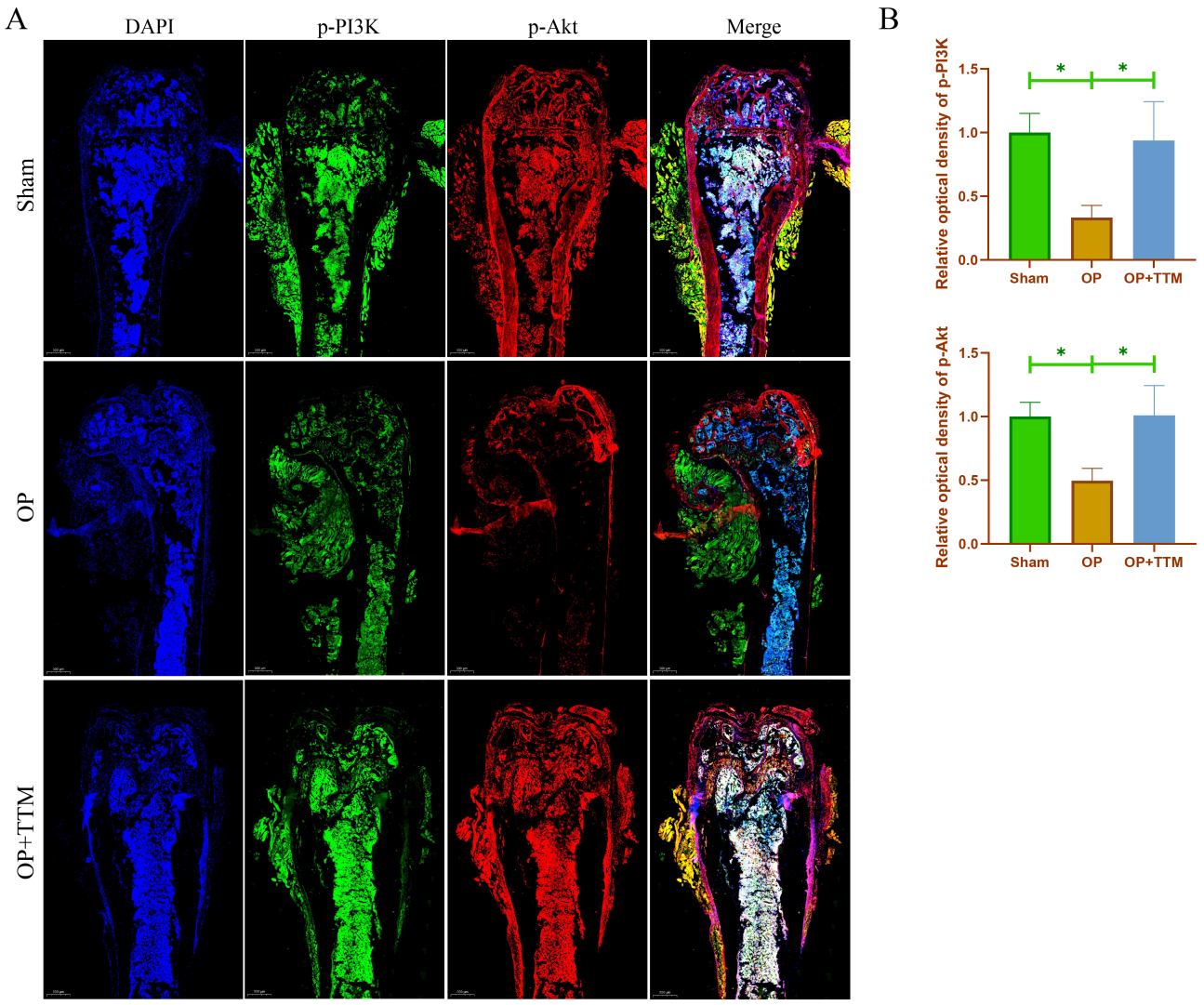

Fig. 7. Protein expression of p-PI3K and p-Akt in mouse femurs. (**A**) p-PI3K and p-Akt protein expression in mouse femur by immunofluorescence staining; (**B**) Statistical results of p-PI3K and p-Akt protein expression in mouse femur. Sham, Sham group; OP, OP model group; OP+TTM, OP + TTM administration group; n = 6, *$p < 0.05$.

cellular components such as collagen-containing extracellular matrix, basement membrane, and collagen trimer, as well as molecular functions like extracellular matrix structural constituent, extracellular matrix structural constituent conferring tensile strength, and collagen binding. This suggests that TTM may exert its effects by regulating extracellular matrix remodeling and collagen metabolism. The KEGG pathway enrichment lollipop chart (Fig. 6**D**) revealed that the differentially expressed genes are primarily enriched in metabolic pathways such as the PI3K-Akt signaling pathway. The significant enrichment of the PI3K-Akt signaling pathway (Fig. 6**E**) suggests that TTM may alleviate OP by regulating this pathway, affecting extracellular matrix remodeling and collagen metabolism. Subsequent functional experiments confirmed that TTM activates the PI3K/Akt signaling pathway, thereby influencing the balance between osteogenic and adipogenic differentiation and ultimately improving the OP phenotype. Additionally, GSEA enrichment analysis (Fig. 6**F**) further validated the enrichment of differentially expressed genes between the OP+TTM and OP groups in various metabolic pathways and biological processes, including the Nod-Like Receptor Signaling Pathway, suggesting that TTM may exert its effects by activating the NOD-like receptor signaling pathway. These results provide important clues for understanding the mechanisms of action of TTM.

TTM may Alleviate OP by Activating PI3K/Akt Signaling

Based on the results of transcriptomics analysis, we found that the differential genes were mainly enriched in the PI3K/Akt signaling pathway after TTM treatment. Therefore, immunofluorescence was used to detect the expression of p-PI3K and p-Akt proteins in mouse femurs (Fig. 7**A,B**). The results revealed that p-PI3K and p-Akt proteins were

significantly reduced in the femur of mice in the OP group compared with the Sham group, especially in the bone marrow cavity where p-PI3K and p-Akt proteins were hardly expressed. The expression of p-PI3K and p-Akt proteins was significantly elevated in the mouse femur after TTM treatment, particularly in the mouse bone marrow cavity. It suggests that TTM may affect BMSC osteogenic differentiation/lipogenic differentiation by activating PI3K/Akt signaling, which ultimately alleviates OP. It is worth mentioning that we observed that the protein expression difference of p-PI3K was smaller among the groups compared to that of p-Akt. We speculate that this may be due to the greater effect of OVX-induced mouse OP on p-Akt signal in mouse bone tissue. The possible reason for this is that the effect of de-ovulation on bone tissue may be related to other signaling pathways such as Akt/FOXO1 signal in addition to the PI3K/Akt pathway (Cai *et al.*, 2021). However, the protein expression of p-PI3K did not differ significantly between the TTM-treated group and the Sham group, suggesting that TTM has a potential therapeutic effect in OVX-induced OP mice.

Discussion

OP, a common bone metabolic disease of old age, affects about 200 million people worldwide (Sun *et al.*, 2015). Since the efficacy of clinical treatment strategies for OP is unsatisfactory, exploring novel targeted therapies for OP is necessary. In this study, OP mouse models were constructed by OVX. Subsequently, the copper ion chelator TTM was used to treat OP mice by gavage to explore the therapeutic effects and potential mechanisms. Surprisingly, we unexpectedly found that TTM treatment significantly reduced adipogenesis and improved obesity in OP mice. Specifically, body weight and abdominal fat volume were significantly reduced in mice after TTM treatment. The results of serum lipid profile further revealed that TTM administration significantly reduced the serum levels of TG, HDL-C and LDL-C, which are markers of lipid metabolism, in mice. Immunohistochemical staining showed similar results. TTM was able to significantly inhibit the protein expression of PPARγ and FABP4 in the femur of OVX-induced OP mice. PPARγ, a member of the PPAR subfamily, is a major regulator of adipogenesis and insulin sensitivity (Liu *et al.*, 2022). FABP4, a member of the intracellular lipid-binding protein superfamily (Garin-Shkolnik *et al.*, 2014), can to regulate adipogenesis together with PPARγ (Garin-Shkolnik *et al.*, 2014). Studies have reported a significant decrease in vertebral bone density along with an increase in fat density in postmenopausal women with OP (Kühn *et al.*, 2013; Li *et al.*, 2014). BMSCs are the common parent cell of origin for osteoblasts and adipocytes in the bone marrow. The differentiation of BMSCs toward osteogenic or lipogenic differentiation is balanced and competitive with each other (Li *et al.*, 2020; Muruganandan *et al.*, 2009). The absence of estrogen in postmenopausal OP patients causes an increase in adipogenesis and a decrease in the number of osteoblast precursors. This imbalance between osteogenesis and adipogenesis during bone remodeling results in partial replacement of bone tissue with adipose tissue (Cao *et al.*, 2019; Zhi *et al.*, 2021). It has been shown that BMSCs isolated from postmenopausal OP patients are more likely to differentiate toward adipocytes, while osteogenic differentiation potential is inhibited (Rodríguez *et al.*, 2000). Fat accumulation leads to compromised bone conversion and bone density, which is one of the risk factors for fractures in postmenopausal OP patients (Patsch *et al.*, 2013). Inhibition of adipogenesis and promotion of osteogenesis are essential targets for OP therapeutic strategies (Chen *et al.*, 2021). In our study, TTM treatment can inhibit fat accumulation in OP mice, which has positive implications for the treatment of OP.

The growth plate, composed of chondrocytes and extracellular matrix, is located in the cartilaginous tissue between the epiphysis and the diaphysis, which is the primary area of differentiation for long bone growth (Kronenberg, 2003; Stegen *et al.*, 2019). The cartilage growth plate with active osteogenic function has a typical four-layer structure: resting zone, proliferative zone, hypertrophic zone and ossification zone. HE staining of mouse femurs showed a significant reduction of fat vacuoles in the bone marrow cavity and a relatively neat arrangement of growth plate chondrocytes in OP mice after TTM treatment. Thus, TTM may be involved in the fate regulation of osteogenic differentiation and adipogenic differentiation. The results of micro-CT scanning and immunofluorescence of mouse femurs also support these findings. In addition, copper ions are cofactors for antioxidant enzymes that scavenge bone free radicals and promote osteoblast activity (Qu *et al.*, 2018). Previous studies have found that appropriate concentrations of copper ions can stimulate the differentiation of MSCs toward osteogenesis (Ding *et al.*, 2014). It suggests that the fate-regulating effect of the copper ion chelator TTM on adipogenic differentiation and osteogenic differentiation may be related to its modulating effect on tissue copper ion concentration. The main function of copper ions in tissues is to constitute enzymes that transfer electrons (oxidases) to reduce molecular oxygen (Rondanelli *et al.*, 2021). Among these enzymes, LOX is a copper-dependent monoamine oxidase that utilizes lysine and hydroxylysine (present in collagen and elastin) as substrates to produce cross-links, which are necessary for the development of connective tissues such as bone (Dahl *et al.*, 2005b). TTM was found to inhibit osteoclast differentiation by down-regulating the protein expression of LOX (Morisawa *et al.*, 2018). Moreover, a study of copper-deficient diets in chicks showed that copper deficiency prevents the formation of collagen and elastin cross-bonds in various tissues, leading to bone fragility associated with copper deficiency (Rucker *et al.*, 1975). In our study, TRAP staining showed that TTM treatment partially inhibited osteoclast activity in OP mice, sug-

gesting that TTM may exert a therapeutic effect on OP by reducing bone loss. The underlying mechanism may be related to the copper ion chelating effect of TTM. However, how TTM affects pathological changes in OP by modulating copper ions has not been reported.

Previous studies have revealed that TTM is able to exert anti-copper effects by forming a tripartite complex with copper and proteins in serum (Mills *et al.*, 1981), which are very stable and prevent copper from being taken up by cells in the blood circulation (Gromadzka *et al.*, 2023). In a clinical study, 728 postmenopausal women (aged 45–80 years) were divided into OP group and non-OP group. Evaluation of bone mineral density and blood copper levels in both groups showed that women in the OP group had significantly lower serum copper ion levels than that in non-OP group. Further, serum copper ion levels were positively correlated with bone mineral density (Okyay *et al.*, 2013). Similarly, some studies have also found that elevated serum copper concentrations are negatively associated with the risk of OP (Gür *et al.*, 2002; Okyay *et al.*, 2013). In our study, ICP-MS analysis was used for the determination of absolute copper ion concentration in mouse serum. The results showed that TTM treatment was able to significantly up-regulate serum copper ion levels in OP mice (**Supplementary Fig. 1**). We hypothesize that TTM may alleviate OP by preventing the uptake of copper ions from the blood into the cells, resulting in higher serum copper levels and lower intracellular (e.g., osteoblasts or BMSCs) copper ion concentrations. We speculate that this mechanism may also be related to the recently proposed concept of "cuproptosis". Cuproptosis is a novel copper-dependent mode of cell death associated with mitochondrial respiration proposed by Tsvetkov *et al*. (2022). Unlike other recognized mechanisms of cell death (e.g., apoptosis, pyroptosis, and ferroptosis), cuproptosis is a unique programmed death mechanism (Bao *et al.*, 2022). However, bone tissue or intracellular copper ion concentrations and the presence of cuproptosis were not further examined in our study. Therefore, more evidence is still needed to support whether the hypothesis is valid. Additionally, in our study, TTM was administered by gavage, which had no significant toxic effects on the viscera in our study (**Supplementary Fig. 2**). It has been demonstrated that pharmacokinetics depends on whether the drug is taken with food or not. If the drug is taken with food, very little of the drug may be absorbed. If the drug is administered directly through the vein, the drug may be absorbed relatively well and form a tripartite complex in the circulation (Brewer, 2009; Brewer *et al.*, 1991). Therefore, the mode of administration of TTM may also be an important factor affecting its efficacy. TTM via tail vein injection could be considered for OP treatment, which is yet to be explored in further experiments. It is worth mentioning that systemic administration involves the pharmacokinetics of TTM. The present study lacks the exploration of the metabolic pathways and clearance mechanisms of TTM, which needs to be further explored by more adequate and in-depth studies. In addition, further assessment of the half-life of TTM *in vivo* and whether it produces toxic metabolites to evaluate its long-term toxicity and potential side effects is essential.

Taken together, these results suggest that despite the lack of evidence that TTM alleviates OP by modulating copper death, the effect of TTM in inhibiting bone loss is significant. We attempted to use transcriptomic sequencing analyses of bone and adipose tissue to explore the potential mechanisms by which TTM affects fate regulation of lipogenic differentiation and osteogenic differentiation. Unfortunately, RNA extraction from bone tissue failed. Adipose tissue transcriptomics sequencing revealed that the differential genes after TTM treatment were mainly enriched in the PI3K/Akt signaling pathway, which tentatively indicates that TTM may affect adipose differentiation and ultimately alleviate OP by modulating PI3K/Akt signaling. Subsequently, we detected the protein expression of p-PI3K and p-Akt by immunofluorescence in mouse femoral tissues. The results revealed that TTM treatment was able to partially reverse the protein expression of p-PI3K and p-Akt in the femoral tissues of OP mice, especially in the bone marrow cavity, indicating that TTM may influence the fate regulation of BMSCs by activating p-PI3K and p-Akt protein expression, thereby ultimately inhibiting bone loss in OP mice. The PI3K/Akt signaling pathway has been reported to play a key role in regulating cellular functions, including cell proliferation and survival as well as gene transcription and protein synthesis (Ye *et al.*, 2019). Many studies have reported that the PI3K/Akt pathway can mediate the treatment of OP with herbal formulas. For example, Ma *et al*. (2023) demonstrated that Erxian Tang ameliorated ovariectomy-induced bone loss by modulating lipid metabolism, fatty acid metabolism, and the IGF1/PI3K/Akt pathway. Moreover, Dai *et al*. (2007) demonstrated that biomechanical forces can activate nuclear factor erythroid 2-related factor 2 (Nrf2) signaling through the PI3K/Akt pathway, effectively regulating endothelial cell redox homeostasis and attenuating high-fat diet-induced atherosclerosis. Nrf2 is a major antioxidant response regulator that exerts critical antioxidant effects through transcriptional regulation of several antioxidant proteins (Yang and Zhang, 2021). Recent studies revealed that TTM can act as a novel Nrf2 agonist to up-regulate the expression of Nrf2 downstream antioxidant genes HMOX1, GCLM, and SLC7A11, thus exerting a protective effect against NaAsO2-induced oxidative stress (Zhang *et al.*, 2022). Although it has been shown that bone tissue is inherently naturally hypoxic, which is essential to protect it from reactive oxygen species (ROS)-mediated damage (Stegen *et al.*, 2018; Yellowley and Genetos, 2019), sustained exposure to hypoxic conditions leads to alterations in the bone marrow microenvironment, increasing RANKL protein expression and promoting osteo-

clast formation, which in turn leads to OP (Zhu *et al.*, 2019). In addition, TTM has been reported to act as a superoxide dismutase inhibitor effectively reducing copper ion levels and inhibiting lipid peroxidation by suppressing SOD1 enzyme activity, thereby delaying disease progression and prolonging survival time (Członkowska *et al.*, 2018). Therefore, TTM may also exert a therapeutic effect on OP by affecting osteogenic differentiation/adipogenic differentiation of BMSCs through inhibition of cellular copper toxicity and lipid peroxidation responses, although this hypothesis needs to be supported by further evidence. However, the investigation of the mechanism of action of TTM in this study is still relatively preliminary. The distribution and targeting of TTM *in vivo* after administration are not yet clear. Future studies are needed to determine whether TTM has specific targeting effects on bone tissue and BMSCs to better validate the specific mechanism of action of TTM.

Limitations and Perspectives

This study provides preliminary evidence that TTM may alleviate OP by activating PI3K/Akt signaling. However, the specific molecular mechanisms underlying these effects remain unclear. The potential mechanisms by which TTM regulates PI3K/Akt signaling by binding to cell membrane surface receptors or affecting intracellular signaling need to be further explored. In addition, previous studies found that TTM was able to inhibit osteoclast differentiation by inhibiting LOX to downregulate RANKL expression in osteoblasts and osteocytes. Our results of TRAP staining of bone tissue were similar to the results of this study, indicating that TTM treatment was able to inhibit osteoclast activity in mice, which in turn inhibited bone resorption. However, the potential mechanism by which TTM inhibits bone resorption needs to be further explored.

It is noteworthy that we preliminarily examined copper ion alterations in blood and bone tissues of mice. We found that copper ion concentrations in blood decreased in OP mice (**Supplementary Fig. 1**) and increased in bone tissue (**Supplementary Fig. 3**), whereas TTM supplementation reversed this trend. We speculate that it is possible that TTM supplementation chelated the copper ions in the bone tissue, which allowed the copper ions to exist in the blood as a complex. Therefore, it is possible that the underlying mechanism by which TTM alleviates OP is by inhibiting copper ion-related pathological changes in bone tissue, such as cuproptosis. However, further investigation is required to elucidate the precise molecular targets and pathways involved. For example, detecting the expression levels of cuproptosis protein markers such as FDX1 in bone tissue would further support or refute the speculation that cuproptosis is involved in the alleviation of OP by TTM.

Conclusions

In this study, the copper ion chelator TTM was used for the first time in the treatment of an OVX-induced OP mouse model. Unexpectedly, our results showed that TTM treatment significantly ameliorated obesity and reduced bone loss in OP mice. Transcriptomics sequencing implied that TTM may alleviate OP by modulating PI3K/Akt signaling to affect the differentiation fate of BMSCs.

List of Abbreviations

TTM, tetrathiomolybdate; OVX, ovariectomy; OP, osteoporosis; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; $MoS4^{2-}$, molybdenum-sulfur anion; RANKL, receptor activator of nuclear factor-κB ligand; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; BMSCs, bone marrow-derived stem/stromal cells; BMD, bone mineral density; BV/TV, bone volume fraction; BS/BV, bone surface/bone volume; bone surface/bone volume, trabecular thickness; Tb.N, number of trabeculae; Tb.Sp, trabecular segregation; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species.

Availability of Data and Materials

The data presented in this study are available from the corresponding author and first author upon request.

Author Contributions

GXN designed and coordinated the study; XCZ, CHC, DXW, HLD, and YJY performed the experiments, acquired and analyzed data; CHC, HLD, SYL and YJY interpreted the data; GXN and SYL acquired the Fund; XCZ and DXW wrote the manuscript. All authors contributed to editorial changes in the manuscript, read and approved the final manuscript, and have participated sufficiently in the work to take public responsibility for appropriate portions of the content.

Ethics Approval and Consent to Participate

Approval has been given by the Ethical Committee of Beijing Sport University on the Care and Use of Animal Subjects in Research (Approval Number: 2023026A). All mice were anesthetized and humanely euthanized after the procedures were completed.

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Conflict of Interest

The work presented in this article is the subject of a pending patent (Application number: 202410462218.2) filed by Guoxin Ni (the First Affiliated Hospital of Xiamen University).

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.22203/eCM.v048a02.

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