Original Article
Rutin suppresses FNDC1 expression in bone marrow mesenchymal stem cells to inhibit postmenopausal osteoporosis

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Abstract: A previous study revealed that rutin is the main component of Eucommia flavonoids that exerts a protective effect against osteopenia. The bone density and trabecular bone number of osteoporosis model rats can be significantly improved after treatment with rutin. Further study using whole gene expression profiling revealed that FNDC1, a fibronectin type III domain-containing protein, may be a novel bone metabolism-related factor that is decreased in rutin-treated rats. The mechanism underlying the effects of rutin treatment on osteoporosis is important to explore. Micro-CT, western blotting, quantitative PCR, transmission electron microscopy, and Alizarin Red mineralization staining assays were performed to evaluate bone density, FNDC1 expression and autophagy to determine whether FNDC1 might play a significant role in rutin-inhibited trabecular bone loss in rats. FNDC1 expression was high in the osteoporosis group, whereas rutin treatment facilitated FNDC1 downregulation. In addition, rutin promoted bone marrow mesenchymal stem cell autophagy by inhibiting phosphorylated Akt in osteoporosis. In summary, our study shows that rutin could regulate FNCD1 level and autophagy through the Akt/mTOR signaling pathway to provide a novel therapeutic strategy for osteoporosis.

Keywords: Rutin, FNDC1, autophagy, postmenopausal osteoporosis

Introduction

Postmenopausal osteoporosis, which is one of the most common bone metabolic diseases affecting more than 200 million people, may increase the risk of fracture when bone resorption surpasses bone formation and results in imbalance [1, 2]. Despite hormone replacement therapy (HRT) as well as antiresorptive or anabolic drugs that have been widely used in clinical practice to prevent and treat postmenopausal osteoporosis, serious side effects are inevitable, such as normal bone metabolism impairment and atypical fracture [3]. Therefore, new antiosteoporosis therapeutic candidates with fewer side effects are urgently needed.

Rutin, a natural compound flavonoid from Eucommia ulmoides Oliv, might play an important role in osteoporosis [4, 5]. Some studies have been conducted to determine the potential molecular changes involved in postmeno-pausal osteoporosis in rutin-treated and control groups. Therefore, a gene microarray profile was used to find differentially expressed genes and detect the mechanism that may be involved. The FNDC1 gene was highly expressed in the osteoporosis group, while the rutin treatment group exhibited low FNDC1 expression.

FNDC1, which encodes a fibronectin type III domain-containing protein, was first discovered and reported by Vereb Z [6]. This protein coding and disease-related gene is located on chromosome 6q25.3 [7]. As reported, FNDC1 exists ubiquitously in the cell matrix, and the associated membrane receptors and enzymes could mediate Cx43 phosphorylation and G protein signaling transduction to regulate cell permeability and apoptosis [8]. Ingen G V and Das D K's study also showed that FNDC1 expression might affect the occurrence and development of prostate cancer and acute otitis [9-11].

Autophagy can quickly supply energy or materials to update cellular components, and this process thus plays an irreplaceable role in cells confronted with starvation and other types of stress. When autophagy is absent, DNA damage can accumulate, which affects cell proliferation and leads to abnormal gene expression; ultimately, these cells may suffer death due to genotoxic stress [12]. In recent years, some evidence has shown that autophagy plays a significant role in stem cells by regulating remodeling and differentiation, as well as self-renewal, and the correlation between autophagy and bone metabolic disease pathogenesis has gained much attention [13]. Indeed, the autophagy-related regulatory signaling pathways are complex and include PI3K/Akt, AMPK and MAPK [14]. Based on other studies, we postulated that phosphorylated Akt can activate mTOR, thus regulating cell autophagy [15].

The results of our previous study showed that rutin could reverse trabecular bone reduction by promoting bone marrow mesenchymal stem cell (BMSC) differentiation into osteoblasts in vivo, whereas the effects of low FNDC1 expression facilitated autophagosome formation to promote autophagy in osteoporosis in vitro, revealing the potential mechanism of the functional pathways. Based on our previous research, we propose that FNDC1 might possess a function similar to that of fibronectin (FN) and that rutin could downregulate FNDC1 to decrease Akt phosphorylation and induce autophagy, thus inhibiting the Akt/mTOR/p70S6K signaling pathway.

Materials and methods

Animals and specimen collection

For our study, 21 female Sprague-Dawley (SD) rats aged 4 to 5 weeks were obtained from the Department of Animal Science of Nanchang University. Then, the rats were housed at 24°C (air conditioned) under a 12-h light/dark cycle and provided adequate food and water. To establish the osteoporosis model, the rats were divided randomly into three groups. Following oophorectomy, 14 rats were distributed equally to the oophorectomy group treated with a physiological saline solution (OVX, n=7) and the oophorectomy group treated with rutin by gavage (OVX + Rutin, n=7). The remaining rats were subjected to bilateral laparotomy as a

control group (Sham, n=7). We administered 10 mg rutin per kilogram every day for 10 weeks to the rats in the experimental group. The weights of the animals were recorded weekly during the experimental period. Rutin was purchased from Macklin Biotechnology Company and has been validated. All animal experiments adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were performed according to the protocols approved by the Medical Research Ethics Committee of the Second Affiliated Hospital of Nanchang University.

BMSC acquisition and cultivation

BMSCs were obtained from rat femurs and tibias by iteratively injecting complete medium into the marrow cavity. Then, the cells were cultivated in 6-well plates with DMEM/F12 medium (DMEM/F12, HyClone, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 2% penicillin/streptomycin (HyClone, USA) and 2% L-glutamine. In addition, the cells were incubated under standard conditions at 37°C in an atmosphere containing 5% CO_2. When the cells reached approximately 80% to 90% confluence, they were passaged by trypsinization. In addition, CD33 and CD44 expression was measured in the cells, suggesting that we succeed in purifying BMSCs. We collected a population of BMSCs by subculture with P3 for further experiments.

Transmission electron microscopy (TEM)

Firstly, cells were exposed to RF-EMFs (1, 2, or 4 W/kg) for 24 h, harvested, washed with PBS, and fixed with 2.5% glutaraldehyde. After washing three times with PBS, 1% osmic acid was added to fix the cells for approximately 3 h. Then, the cells were washed, dried, and embedded in paraffin, and 70-nm-thick sections were generated with an ultrathin slicing machine (Leica EM UC6; Leica Microsystems, Wetzlar and Mannheim, Germany) and stained with uranyl acetate-lead citrate. Finally, autophagic vacuoles were detected using a transmission electron microscope (JEM1230, JEOL Ltd, Tokyo, Japan) (1500×).

RNA sequencing and gene expression profiling

To identify altered gene expression in BMSCs isolated from diverse groups, we randomly

sequenced BMSC transcriptomes of 3 Sham specimens, 3 OVX specimens and 3 OVX specimens with rutin treatment. Differential gene expression was determined both by including all the pools in the analysis and by excluding some of the pools based on phenotypic characteristics. The BMSC transcriptomes were sequenced by Shanghai Biotechnology Corporation (Shanghai, People's Republic of China) with an Illumina sequencing platform. Total RNA was isolated using an RNeasy mini kit (Qiagen, Germany). Library constructs were prepared using a TruSeq Stranded Total RNA Sample Preparation Kit (Illumina, USA), and RNA sequencing was conducted using an Illumina HiSeq 2500 (Illumina, USA). The insert size confirmation of purified libraries was tested with an Agilent 2100 bioanalyzer (Agilent Technologies, USA). In this manner, we identified 9 differentially expressed genes, a subset of which was validated by qPCR.

Quantitative PCR

Total RNA was isolated from BMSCs using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. For quantitative PCR, total RNA was reverse-transcribed with a Transcriptor First-Strand cDNA Synthesis Kit (Roche Diagnostics). Then, the reaction system was loaded in 96-well plates and analyzed with a 7500 Real-Time PCR System and 7500 software. All PCR reactions were replicated three times. Gene expression differences was evaluated by fold change ($2^{-\Delta\Delta Ct}$), and significant differences were indicated by a *P* value <0.05(*).

Western blotting

Briefly, total protein was isolated from BMSCs, and the protein concentrations were measured using a BCA protein assay kit (Pierce, IL, USA). Protein extracts were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, Massachusetts, USA). The membranes were blocked overnight with 3% BSA Tris-buffered saline with Tween (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20). Subsequently, the membranes were incubated with rat anti-FNDC1 polyclonal antibody (orb1884, Biocompare, USA) at a 1:1000 dilution, LC3, P62, Akt, and p-Akt primary antibodies at a 1:2000 dilution (Proteintech, Hubei, China), while β-actin and

GAPDH (Proteintech, Hubei, China) polyclonal antibodies at a 1:5000 dilution at room temperature were used as loading controls. The appropriate rat secondary antibodies were then incubated with the membranes. The protein bands were visualized using an ECL detection reagent (Proteintech, Hubei, China), and the band densities were measured using ImageJ software.

Alizarin red staining for mineralization

After 3 weeks of appropriate BMSC stimulation, the stimulation was terminated according to the manufacturer's protocol. First, the cells were washed with PBS and then immobilized with a 4% neutral formaldehyde solution for 30 minutes. Then, the remaining liquid was removed, and the cells were washed twice with PBS and stained with an Alizarin Red solution (Cyagen, RASMX-90021) for 5 minutes. Finally, the Alizarin Red solution was discarded, and the cells were washed with PBS three times. Afterward, the mineralized nodes were investigated under a microscope.

Statistical analysis

The data were analyzed with Prism 5 (GraphPad Software, San Diego, CA, USA), and the values are presented as the mean ± SEM or SD. Student's t test and Fisher's exact test were used to compare the groups. SPSS 17.0 software (SPSS Inc., Cary, NC, USA) was also used for data analyses. *P* values less than 0.05 indicate a statistically significant difference and are denoted by (*).

Results

Rutin treatment could partially reverse osteoporotic trabecular microstructural degradation according to micro-CT analysis

In our preliminary study, rats were administered the appropriate dosage of rutin by gastric perfusion, but not treated in the Sham and OVX groups. The rats were then ethically sacrificed to collect their tibias, which were analyzed via micro-CT. The results revealed that oophorectomy led to significant trabecular reduction, whereas rutin treatment had the opposite effect in rats (**Figure 1**). The bone trabecular selected was approximately 0.2 mm below the proximal humerus growth plate and measured 0.5 mm length. In addition, the bone index

Figure 1. Rutin could partially reverse bone trabecula structural degeneration in vivo. A. Micro-CT images showing representative bone trabecular microarchitecture of the tibia for each treatment group (Sham group, OVX group and OVX + Rutin group). In comparison with the Sham group, the OVX group showed significantly decreased trabecular number and thickness in the tibia metaphysis, while in the presence of rutin, the trabecular quantity and thickness of the rat tibia metaphy-

sis were increased. B. The BMD, BV/TV, ConnD, Tb.N, and Tb.Th values in the trabecular bone of the OVX group were significantly decreased compared with those of the Sham group, but the SMI value in the proximal tibia was increased compared to that of the Sham group. In addition, treatment with rutin partially reversed the BMD, BV/TV, ConnD, Tb.N and Tb.Th values compared with no treatment in the OVX group; however, the SMI value was decreased. $P<0.05*$, the values are presented as the mean ± standard deviation.
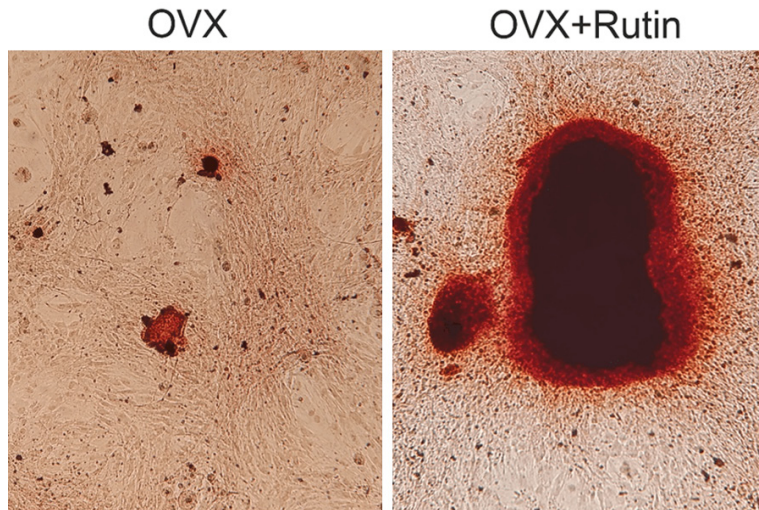
Figure 2. Rutin induces BMSC mineralization in vitro. After 21 days of cultivation, deep red and aggregated calcium nodules were observed under the microscope, and standard pictures are displayed. There were more mineralized calcium nodules with a long diameter and deep staining in the rutin group than in the control group. These results indicated that rutin induced cell mineralization and promoted BMSC differentiation into osteoblasts.

parameter was determined, and the results indicated that the OVX group had decreased BMD, Conn, BV/Tv, Tb.N and Tb.Th values, but an increased SMI value, thus indicating trabecular bone transition from a plate to rod structure (**Figure 1**). Treatment with rutin increased BMD, Conn, BV/Tv, Tb.N and Tb.Th values but decreased SMI values, revealing that the bone trabecular was more likely to transition from a rod to plate structure (**Figure 1**). Taken together, the images indicated that osteoporosis induced trabecular microstructural degradation, while rutin partially reversed these trabecular microstructural changes in vivo.

Rutin promotes BMSC mineralization in osteoporosis

To confirm rutin anti-osteoporosis function in vitro, BMSCs were extracted from the osteoporosis model group, and BMSCs treated with rutin were cultured for 3 weeks and compared with untreated BMSCs. The cells were stained with Alizarin Red (pH=4.2), air-dried and observed under an inverted phase-contrast microscope to detect mineralized nodules. The

rutin-treated group showed large amounts of crimson mineralized nodules with long diameters and dark colors compared with the untreated group (**Figure 2**). Therefore, the results indicated that rutin might play a significant role in BMSC conversion to osteoblasts in vitro.

Gene expression differences in BMSCs detected with a microarray profile were verified by quantitative PCR

Based on the images showing that rutin may affect bone remodeling, we hypothesized that rutin might have beneficial osteoporosis treatment effects. To further explore the underlying mechanism of rutin, we evaluated the gene expression of BMSCs with a microarray profile. Fifteen gene expression levels were significantly different when comparing the rutin group with the control groups using the following cutoff values: gene expression levels were changed by equal to or greater than 2-fold, at the same time the *P* value was less than 0.05 (**Figure 3**). Quantitative PCR was conducted for 9 genes, and the outcomes supported the microarray profile results. Otherwise, there displayed representative 4 genes Cd163, FNDC1, Prg4 and Krt79 (**Figure 4**). To further investigate the underlying molecular mechanism through which rutin affects osteoporosis, we examined the gene named FNDC1. FNDC1 was upregulated in BMSCs in osteoporosis, but rutin treatment significantly downregulated FNDC1; thus, it may be closely related to cell function in osteoporosis.

FNDC1 expression is deregulated in rutin-treated BMSCs

To confirm the previous finding, the FNDC1 expression level was examined in BMSC protein samples via western blotting. The results indi-
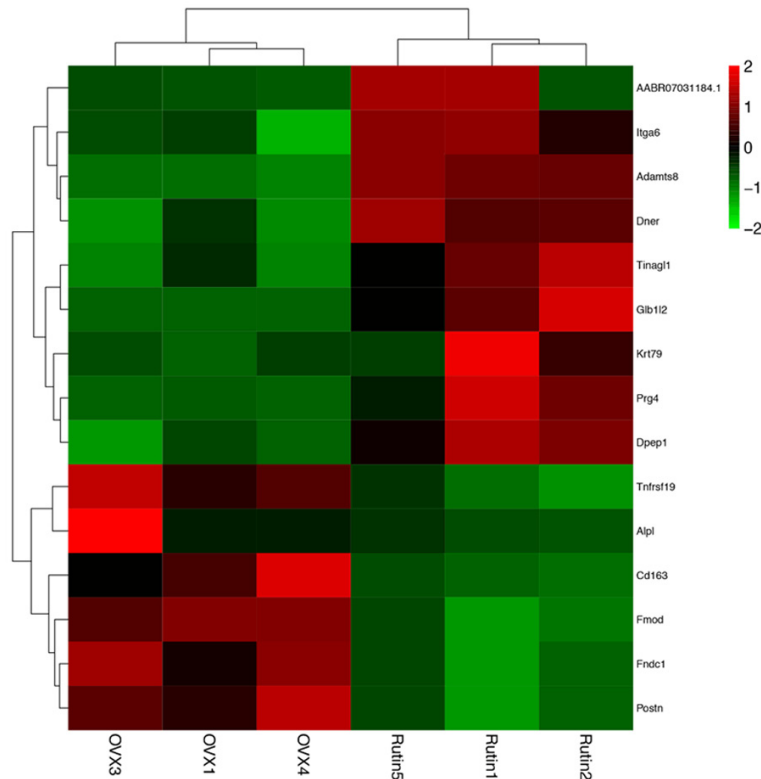
Figure 3. Heat map of the differential expression of genes in BMSC specimens from the OVX and OVX + Rutin groups. Based on the microarray profile results, 15 genes were successfully identified according to fold changes ≥2.0 or ≤0.5 when the OVX + Rutin group was compared with the control group. Notably, the heat map showed that rutin stimulated the expression of different genes, which revealed an interesting connection between rutin and gene expression.

cated that FNDC1 expression was lower in BMSCs treated with rutin than in OVX control cells (**Figure 5**). In addition, the mRNA expression level of FNDC1 was lower in BMSCs treated with rutin than in OVX control cells (**Figure 5**). The results showed that rutin may be implicated in inhibiting FNDC1 expression, thus prompting us to examine the molecular mechanism underlying rutin and FNDC1 gene expression suppression.

Rutin might affect osteoporosis by inducing autophagy in BMSCs

BMSCs were extracted from the oophorectomy model group, and the experimental group cells were treated with rutin. We evaluated LC3 and P62 expression by western blotting and found that LC3 expression was notably higher in rutin-treated osteoporosis BMSCs than in untreated osteoporosis BMSCs, whereas P62 expression was reduced following rutin treatment (**Figure**

6A). Additionally, as observed by transmission electron microscopy, autophagosome formation was significantly induced in rutin-treated BMSCs, which might indicate the mechanism of rutin in osteoporosis (**Figure 6B**). In conclusion, this finding established a link between autophagy and the effects of rutin in osteoporosis.

The regulatory effects of rutin on BMSC autophagy are partially due to decreased Akt phosphorylation in osteoporosis

Akt and Akt phosphorylation levels were detected by western blotting to further investigate the mechanism of rutin treatment in osteoporosis (**Figure 7**). Preliminary results showed that rutin might reduce Akt phosphorylation in BMSCs in osteoporosis, thereby promoting autophagy.

Discussion

Postmenopausal osteoporosis is a chronic human metabolic bone disease, and an in-depth understanding of its molecular mechanisms is urgently needed. Several recent studies have suggested that the Chinese herb Du zhong may play a significant role in inhibiting osteoporosis development and progression [16-18]. Our team previously demonstrated that chlorogenic acid (CGA), the most abundant polyphenol of Du zhong, could effectively counteract osteoporosis in OVX rat models [19]. This finding has attracted considerable attention to the functions of other compositions of the Chinese herb Du zhong in osteoporosis. Recently, several studies have focused on the antioxidant effects of flavonoids because it has been established that antioxidants are involved in the prevention of various diseases and could be used to treat postmenopausal osteoporosis [16].

Indeed, BMSCs are important sources of osteogenic precursor cells, which have a strong proliferation capacity and can easily differentiate
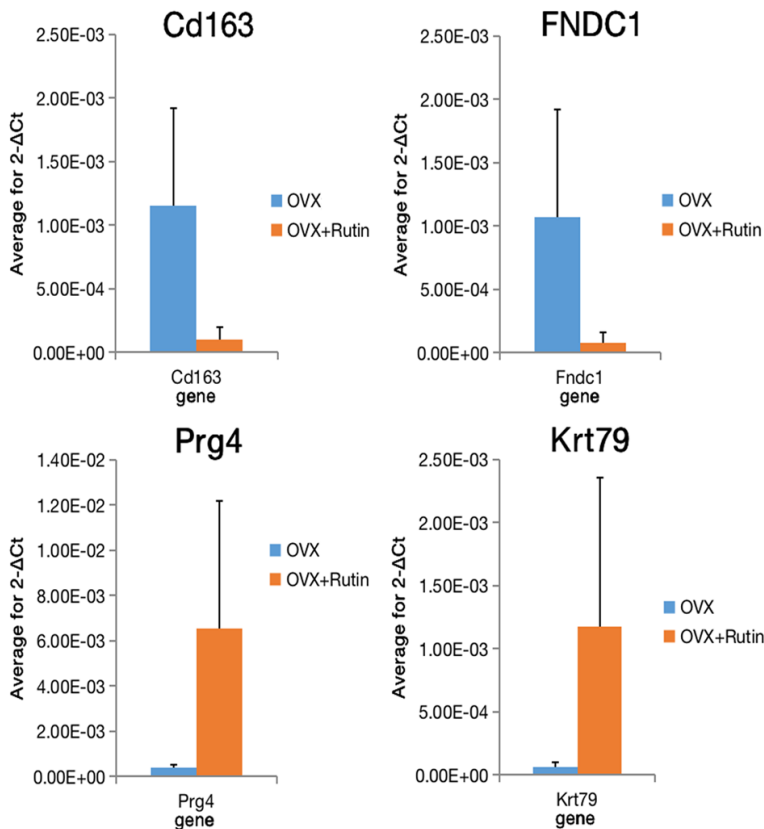
Figure 4. Nine genes were detected by quantitative PCR analysis. Nine gene expression levels were measured by quantitative PCR, and the results were similar to those of the microarray profile. There were 4 standard gene expression changes: Cd163, FNDC1, Prg4 and Krt79. Analysis of Cd163 and FNDC1 showed relatively low expression in the rutin-treated group; in contrast, the levels of Prg4 and Krt79 expression were increased after rutin treatment. $P < 0.05$*, the values are presented as the mean ± standard deviation.
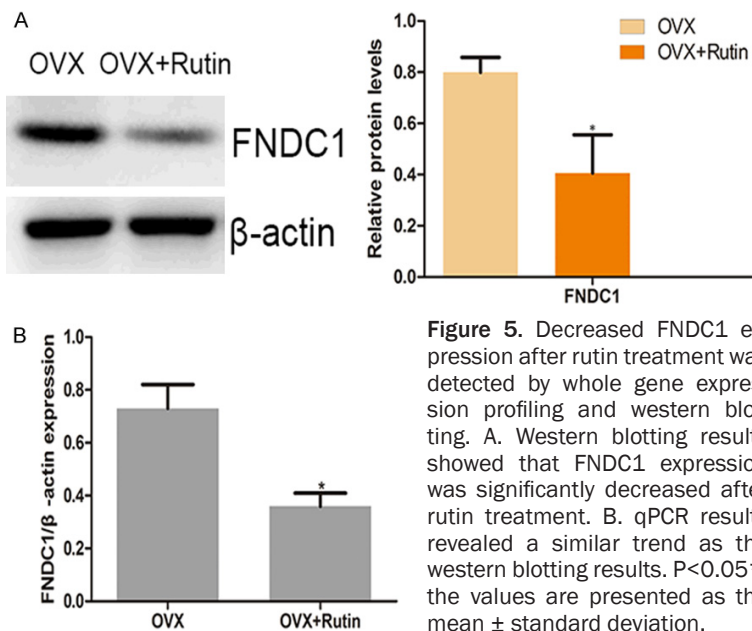
Figure 5. Decreased FNDC1 expression after rutin treatment was detected by whole gene expression profiling and western blotting. A. Western blotting results showed that FNDC1 expression was significantly decreased after rutin treatment. B. qPCR results revealed a similar trend as the western blotting results. $P < 0.05$*, the values are presented as the mean ± standard deviation.

into various cell lines, such as osteoblasts, chondrocytes and adipocytes, under specific microenvironment conditions [20]. Osteoblasts are cells that promote bone formation [21]. Osteoporosis occurs when bone resorption exceeds bone formation [2]. The ability of rutin, a flavonoid extract, to reverse this imbalance by promoting BMSCs that are more likely to translate to osteoblasts was explored in our current study. As shown, the OVX group manifested bone trabeculae sparsely, consistent with BMD loss, while rutin changed this imbalance to reestablish bone trabeculae and increase BMD. In this study, we aimed to carefully determine the role of rutin in osteoporosis and elucidate the associated signaling mechanisms.

Autophagy is a lysosomal degradation process that clears away protein aggregates and damaged cells, thus providing extra energy [22]. Interestingly, the role of autophagy in cells remains controversial [23, 24]. Studies have shown that autophagy could be used in the reconstruction of damaged cell membranes. In addition, autophagy may affect cell energy metabolism by regulating cellular mitochondrial function [23, 25]. It can also modulate the metabolism of intracellular reactive oxygen species (ROS) by blocking ROS accumulation; significant ROS accumulation can disturb cell proliferation and differentiation [26]. In the process of autophagy, p62 binds to the polyubiquitinated protein and aggregates; then, p62 binds to Atg8/LC3 on the autophagosome membrane to degrade the aggregate [27, 28]. Furthermore, it
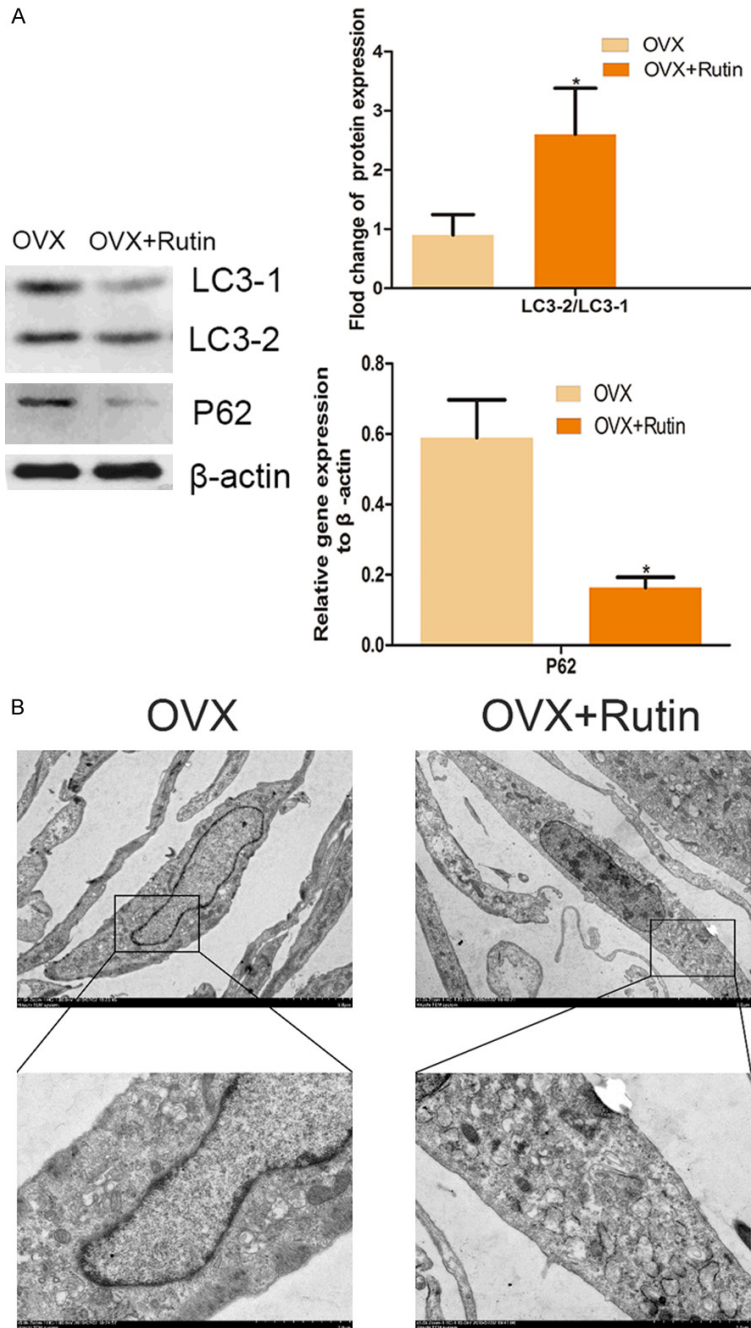
A

B

Figure 6. Rutin promotes BMSC autophagy. A. After treatment with rutin, LC3-2/LC3-1 expression was upregulated, while P62 expression was downregulated in BMSCs. Otherwise, the control group exhibited adverse outcomes. B. Transmission electron microscopy showed an increase in the number of autophagosomes in rutin-treated BMSCs. However, the untreated osteoporosis group demonstrated fewer autophagosomes, which further confirmed the promoted effects of rutin on autophagy. P<0.05*, the values are presented as the mean ± standard deviation.

the association of autophagy with bone metabolic disease pathogenesis [13]. Compared with no treatment, rutin treatment of the OVX group resulted in high LC3 expression and decreased P62 expression via western blotting. These results indicate that rutin may induce autophagy in osteoporosis. Transmission electron microscopy results were similar, indicating that rutin treatment promotes the formation of autophagosomes. It is thus important to explore the mechanism by which rutin plays a significant role in autophagy in the prevention of postmenopausal osteoporosis.

Through correlative functional structure analysis of FNDC1, W S's study confirmed that FNDC1 contains the main functional components of an FN domain called the type III conserved FN domain [29-31], which led us to the hypothesis that FNDC1 may possess a function similar to that of FN. FN could affect autophagy and differentiation by regulating key kinases in the TOR signaling pathway, including FAK, Akt, mTOR and 4E-BP1m [32]. In fact, FN acts as an important scaffold protein that supports the composition of tissues and extracellular matrix (ECM). Therefore, we investigated the functional mechanism of FNDC1 and found that FNDC1 can alter Akt and then regulate autophagy, which ultimately affects postmenopausal osteoporosis.

Furthermore, phosphorylated Akt could inhibit TSC2 and TSC, thus activating mTOR [33]. mTOR could induce p70S6k phosphorylation to promote mRNA translation via phosphorylated S6K1.

is worth noting that autophagy induces the capacity for stem cell remodeling and cell differentiation and self-renewal, which underlies
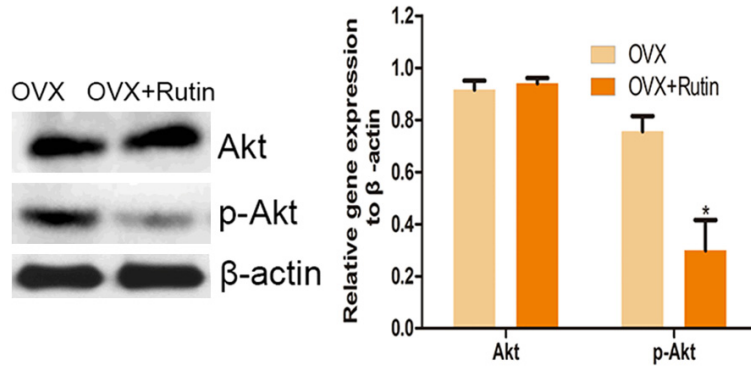
Figure 7. Comparison of Akt and phosphorylated Akt levels between the OVX + Rutin group and the control group. The levels of Akt and p-Akt expression were detected via western blotting. Akt showed no significant differences between the two groups. Interestingly, rutin-treated BMSCs showed lower p-Akt expression than nontreated osteoporosis model rat BMSCs. P<0.05*, the values are presented as the mean ± standard deviation.

When the ribosome and endoplasmic reticulum are closely adhered, it is difficult for the endoplasmic reticulum membrane to form autophagosome membranes, which inhibits autophagy activity [34]. At the same time, mTOR can phosphorylate 4EBP1 and inhibit its activity, relieving the inhibition of the eukaryotic translation initiation factor eIF4E and leading to decreased autophagy activity [35]. Based on the latest research and previous experiments, we believe that FNDC1 may play a role similar to that of FN in postmenopausal osteoporosis, where cell autophagy and BMSC differentiation into osteoblasts are affected by rutin treatment via this molecule.

Above all, our data revealed that ovariectomy in rats reduced estrogen levels, which led to a loss of bone density, decreased trabecular bone number and degraded bone microstructure, whereas rutin acted as an ossific promoter in osteoporosis. Moreover, rutin might play a crucial role in stimulating BMSC differentiation into osteoblasts, as osteoblast aggregation is considered to be an indicator of ossification; this series of results showed beneficial effects of enhanced bone density and improved trabecular bone number and bone microstructure. However, further investigations of the molecular mechanisms underlying the beneficial behavior of rutin in osteoporosis therapy are necessary to determine whether changes in BMSC gene expression could directly regulate antiosteoporosis functions.

Conclusion

In summary, we found that rutin could effectively counteract postmenopausal osteoporosis in OVX rat models. We anticipate that this study will provide a basis for establishing new strategic approaches for treating postmenopausal osteoporosis.

Acknowledgements

Disclosure of conflict of interest

None.

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