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Nobiletin Improves Bone Loss due to Natural Aging by Regulating RORα*

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Abstract With the acceleration of aging population in the world, the incidence of age-related bone loss has shown an obvious rising trend. It is of great significance to find effective approaches to relieve senile osteoporosis. Nobiletin is one of the most abundant flavonoids in citrus genus with many important biological properties. Herein, 20-month natural aging mice were used as senile osteoporosis model and were treated with nobiletin by consecutive intraperitoneal injection for 15 days. Micro-CT results showed that nobiletin significantly improved the bone microstructure featured by increased bone volume fraction and decreased trabecular separation. HE staining results indicated that nobiletin increased the number of osteoblasts of trabecular bone surface. Serum osteocalcin (gene *Bglap*) level was also found to be significantly increased in mice by nobiletin treatment. We then tested the effect of nobiletin in mouse pre-osteoblast (MC3T3-E1 cells) model and found that nobiletin significantly up-regulated *Mki67* expression, increased *Bglap* expression and alkaline phosphatase enzyme activity, and markedly enhanced the alizarin red S staining, suggesting that nobiletin can promote osteoblast proliferation, differentiation and mineralization. Further study of the underlying mechanism showed that nobiletin increased the retinoic acid receptor-related orphan receptor α (RORα, gene *Rora*), down-regulated sclerostin (SOST, gene *Sost*) and up-regulated osteocalcin, while knocking down of *Rora* significantly compromised the regulation of nobiletin on *Sost* and *Bglap*, indicating that the improving effect of nobiletin on age-related bone loss depends on RORα. To our knowledge this is the first report that nobiletin shows improvement effect on bone loss in natural aging mice and the first report that nobiletin down-regulates *Sost* through *Rora*. These results provide a new mechanism of nobiletin in age-related bone loss and a new potential strategy to improve the senile osteoporosis.

Key words nobiletin, aging, osteoporosis, bone formation, bone loss, RORα/*Rora*, sclerostin/*Sost*, osteocalcin/*Bglap*
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Senile osteoporosis is a common disease that mainly affects the elderly in clinical and may result in substantial bone-associated diseases, especially for post-osteoporotic fractures, which increases the mortality of the elderly and the burden of social medical seriously[1-2]. Seeking drugs to treat or relieve osteoporosis in the elderly has always been important in basic and translational research. Currently, osteoporosis treatment options are mainly divided into the following strategies: resisting bone resorption (Denosumab, Odanacatib, Saracatinib, *etc*.), enhancing bone formation [Parathyroid hormones (PTH 1-84) or its N-terminal fragment (teriparatide)] and promoting bone remodeling (Calcilytic agents, inhibitors of Wnt antagonists, sclerostin antibody, *etc*.) [2]. Therapy of senile osteoporosis based on supplement of calcium, vitamin D and treatment with

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bisphosphonates (bone resorption inhibitor) such as Alendronate, was generally linked to increase bone density and reduce the risk of fracture. Full-length parathyroid hormone (PTH 1−84) was a common drug for senile osteoporosis by daily subcutaneous injections with appropriate dosage. However, side effect and the chronic application are still submerged in uncertainty.

Natural products present a better alternative and safety. Nobiletin, a polymethoxylflavone in citrus fruits, has effects of improving cognitive impairment[3-6], anti-osteoporosis[7-9], preventing obesity[8], cardiovascular protection[10-12], anti-cancer[13-14], anti-metabolic disorders[15-16], and anti-oxidation[17-18]. Polymethoxyflavonoids mainly composed of nobiletin showed the effect of inhibiting osteoclast differentiation and bone resorption[19]. In terms of inhibiting bone mineral density, nobiletin was reported to suppress bone resorption by inhibiting NFκB-dependent prostaglandin E (PGE) synthesis in osteoblasts and rescue postmenopausal bone loss due to estrogen deficiency[7-9]. However, whether nobiletin benefits in natural aging bone loss lacks direct evidence.

The retinoic acid receptor-related orphan receptors (RORs) are an important subfamily of transcriptional regulators in the nuclear receptor superfamily. Nobiletin has been studied as an agonist of RORα (a subtype of RORs) in the regulation of circadian rhythm[15]. RORα plays an important role in regulating bone metabolism and bone development[20]. RORα activates mouse bone sialoprotein up to 7-fold[20-21]. We therefore deduce that ROR agonists may have utility in the treatment of osteoporosis. Another important player sclerostin (SOST), a secreted glycoprotein encoded by *Sost* gene participates in regulating bone formation and resorption[22-23]. As a negative regulator in bone formation, sclerostin inhibits the Wnt signaling pathway when binding to a co-receptor in the Wnt signaling pathway[23-24]. In terms of clinical disease, sclerostin shows major impact on bone health[25-29]. The regulation of nobiletin on sclerostin also remains unknown.

In the present study, we investigated whether nobiletin could improve age-related bone loss and the involving mechanism. Using cultured pre-osteoblasts and natural aging mice as models, we detected the effect of nobiletin on bone formation. Our results showed that nobiletin can improve bone loss in natural aging mice through regulation of RORα-SOST signaling pathway.

1 Materials and methods

1.1 Animals

20-month natural aging C57BL/6J male mice housed in SPF conditions were randomly divided into two groups, 5 mice per group, the vehicle group and nobiletin group. Nobiletin (30 mg/kg) or vehicle (DMSO) were administered daily by consecutive intraperitoneal injection for 15 days. The dosage of nobiletin and administration procedures used were based on descriptions in the published literature[30]. All experimental procedures involving animals were approved by the Institute of Biophysics, Chinese Academy of Science Animal Centre.

1.2 Cell culture

The mouse pre-osteoblasts (MC3T3-E1) was purchased from the cell bank of Chinese academy of sciences. The growth culture medium was minimum essential medium (α-MEM; HyClone, SH30265.01) supplemented with 10% fetal bovine serum (FBS; Gibco, 10099-1633101) and 1% penicillin-streptomycin (HyClone, SH40003-12). The differentiation culture medium was MEM supplemented with 10% FBS, l-ascorbic acid (50 g/ml; Sigma, A4034), 0.1 mol/L dexamethasone (Sigma, St. Louis, MO; D4902), 10 mmol/L glycerophosphate (Sigma, G6376), and 1% penicillin-streptomycin (HyClone, SH40003-12), and the medium was refreshed every 2 days. MC3T3-E1 cells were seeded at 1×10^5 cells per well in 6-well plates, and cell cultures were incubated in a humidified environment containing 5% CO_2 at 37℃.

1.3 HE staining

HE staining was conducted according to the published literature. Briefly, mouse femurs from 20-month old mice were excised and fixed in 4% PFA for 24 h and decalcified in a 10% EDTA for 4 weeks at 4℃. The samples were embedded in paraffin and sectioned at 5 μm[31], stained with H and E (hematoxylin and eosin) [32], then examined and imaged using a Leica Aperio Versa 200 whole slide scanner.

1.4 Alizarin red S staining

We plated MC3T3-E1 cells at a density of 1×10^5 cells per well in 6-well plates and expanded them in growth medium for 3 days. We induced osteoblast

differentiation by culturing in differentiation culture medium. At day 21 after inducing osteoblast differentiation, cells were fixed with 4% paraformaldehyde for 15 min and then stained with 1% alizarin red S (Sigma, A5533-25G), using standard protocols.

1.5 ALP（alkaline phosphatase）activity assay

After removing the medium, 24-well plates were washed with phosphate-buffered saline (PBS), and then 0.1% Triton X-100 buffer was added. Cells were frozen and thawed three times, and then incubated at 37℃ for 30 min in substrate buffer (Sigma, N7653-100 ML). The reaction was then terminated with 3 mol/L NaOH. The optical density of the solution was determined at 405 nm. The ALP activity was adjusted by the total protein in each well.

1.6 Evaluation of serum markers of bone formation

Blood samples were taken from the fundus of mice after anaesthesia and the serum was obtained after centrifugation (2 000 *g*, 20 min, 4℃). Markers of bone formation including bone alkaline phosphatase (BALP) and osteocalcin were measured using BALP ELISA Kit and osteocalcin ELISA Kit, respectively according to the manufacturers' protocols.

1.7 Analysis of bone by micro−computed tomography（Micro−CT）

Micro-CT was undertaken with Inveon MM system (Siemens, Munich, Germany) as previously described with some modification[33] and trabecular morphometric analysis was according to the manufacturer's manual of operation. Measured trabecular metric parameters included BMD (Bone Mineral Density), bone volume/total volume (BV/TV) and trabecular separation (Tb. Sp). Briefly, the right femur samples were scanned at an effective pixel size of 8.89 μm, a voltage of 60 kV, a current of 220 μA, and an exposure time of 1 500 ms in each of the 360 rotational steps, *in vivo* and *ex vivo*. The images consisted of 1 536 slices and had a voxel size of 8.89 μm in all three axes. Three-dimensional (3D) visualization images were reconstructed by 2D images and the parameters were calculated using Inveon Research Workplace (Siemens).

1.8 Western blot analysis

MC3T3-E1 cells were collected after differentiation and femur tissues were harvested to extract proteins. Proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Millipore). Membranes were blocked with 5% skimmed milk in TBS containing 0.05% Tween-20 for 2 h, and incubated with the indicated antibody for 2 h at room temperature or overnight at 4°C, followed by incubation with peroxidase-conjugated anti-rabbit or mouse IgG (Santa Cruz) for 2 h. Protein bands were analysed quantitatively using imaging software (BioRad).

1.9 Quantitative real−time PCR assay

The expression of *Bglap* (gene of osteocalcin), *Rora* (gene of RORα), *Sost* (gene of sclerostin), *Mki67* (gene of Ki67), and *Gapdh* in the femur samples and cells were measured. RNA extraction was done using Trizol reagent (87803 Invitrogen, USA) and the concentration was determined using a NanoDrop ™ 2000 Spectrophotometer (ThermoFisher Scientific, UK). The reverse transcription reaction was performed with 2 μg of total RNA that was reverse transcribed into cDNA using a cDNA Synthesis SuperMix kit (Transgene, China). Quantitative real-time PCR analysis was carried out in triplicate on SYBR green reagents (1725121, Bio-Rad, USA) using an iQTM5 system (Bio-Rad, USA). The qPCR set up consisted of a 100 ng total RNA in a 25 μl reaction medium with sequence-specific primers (InvitrogenTM). *Gapdh* was used as an endogenous control. The change in the expression of mRNA of mice in each treatment group was assessed by the 2-ΔΔCq method. The primers used in this study are listed in Table 1.

Table 1 Primers used for Real−time qPCR（SYBR Green）

Gene	Sense primer (5'→3')	Antisense primer (5'→3')
Rora	GGAAGAGCTCCAGCAGATAACG	GCTGACATCAGTACGAATGCAG
Bglap	AGGGAGGATCAAGTCCCG	GAACAGACTCCGGCGCTA
Sost	CCTGAGAACAACCAGACCATGA	TGGGCCGTCTGTCAGGAA
Mki67	GCTGTCCTCAAGACAATCATCA	GGCGTTATCCCAGGAGACT
Gapdh	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG

1.10 Data analysis

Two-tailed Student's *t*-tests were used for two-group comparisons. ANOVA and appropriate post hoc analyses were used for comparisons of more than two groups, $P<0.05$ was considered statistically significant.

2 Results

2.1 Nobiletin improves bone microstructure and bone formation in natural aging mice

The molecular structure of nobiletin is shown as in Figure 1a. The bone microstructure in the femur of natural aging mice was detected by Micro CT scanning and HE staining. Figure 1b showed the representative CT images of femurs from 20-month old mice and the quantitative analysis showed that the trabecular bone volume per total volume (BV/TV) was increased and the trabecular separation (Tb. Sp) was decreased in the nobiletin group compared to the vehicle group (Figure 1c). To examine whether the better bone microstructure phenotype was due to increased bone formation, we next performed histomorphometric analysis of osteoblast surfaces in the femur. It can be seen that Ob. S/BS, osteoblast surface per bone surface, was significantly higher in nobiletin treatment group (Figure 1d) although nobiletin treatment did not increase bone mineral

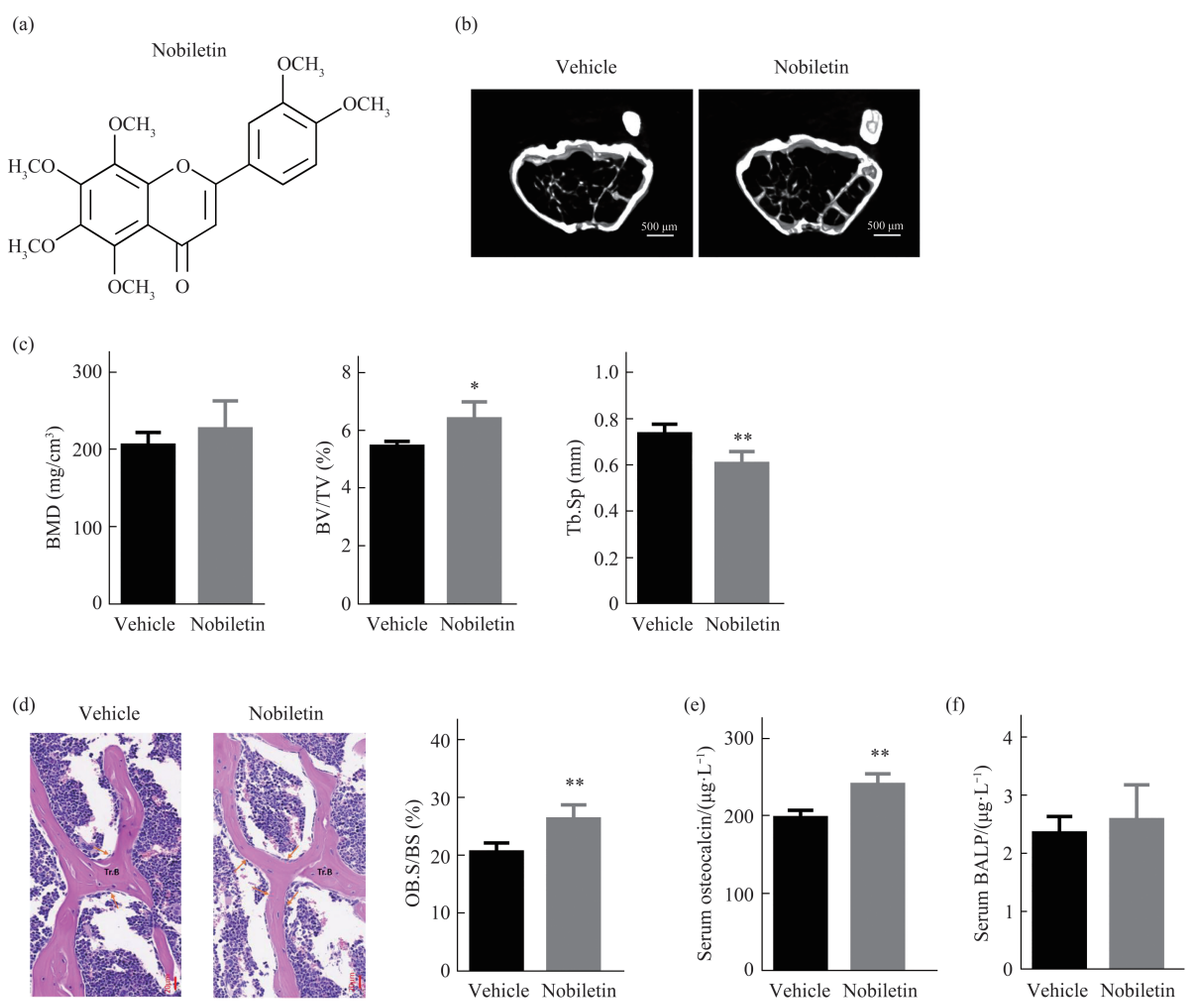

Fig. 1　Nobiletin treatment promoted the bone formation in natural aging mice

(a) The chemical structure of nobiletin. (b) Representative CT images of femurs from 20-month old mice of vehicle and nobiletin treated group. (c) Quantitative analysis of CT images with trabecular bone parameters of femurs from 20-month old mice of vehicle and nobiletin treated group. BMD, bone mineral density; BV/TV, bone-volume/tissue-volume ratio; Tb.Sp, trabecular separation; **P* < 0.05, ***P* < 0.01, *n*=5. (d) Representative images of HE staining of the femur trabecular bone from 20-month old mice of vehicle and nobiletin treated group and the analysis of histomorphometric parameters. Ob. S/BS, osteoblast surface per bone surface; ***P* < 0.01, *n*=5. (e) Serum level of osteocalcin. ***P* < 0.01, *n*=5. (f) Serum level of BALP. *n*=5.

density (BMD) (Figure 1c). In consistent with these results, osteocalcin, a bone formation marker, was remarkably increased in the serum of nobiletin treated mice (Figure 1e). The BALP result indicated that the bone turnover was not changed by nobiletin (Figure 1f). Together, all results indicated that nobiletin significantly improved the bone microstructure and bone formation in natural aging mice.

2.2 Nobiletin promotes osteoblast proliferation, differentiation and mineralization

In order to explore how nobiletin affects bone formation, we analyzed the effect of nobiletin on osteoblast proliferation, differentiation and mineralization in MC3T3-E1 cells. *Mki67* mRNA level and protein level both were markedly increased by nobiletin in the osteoblast proliferation test (Figure 2a). Osteoblast differentiation was significantly promoted indicated by the up-regulation of two osteoblast differentiation markers, ALP enzyme activity and *Bglap* expression (Figure 2b). The alizarin red S staining result showed that the osteoblast mineralization was markedly promoted by nobiletin (Figure 2c). We thus concluded that nobiletin treatment enhances osteoblast proliferation, differentiation and mineralization.

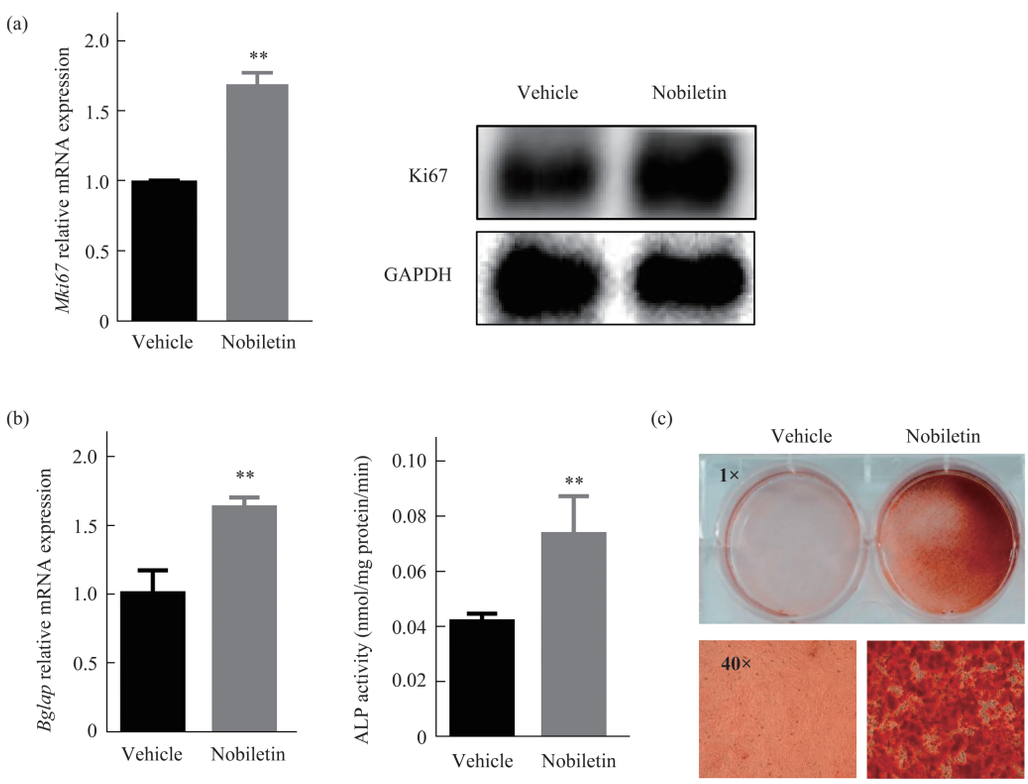

Fig. 2　Nobiletin treatment enhances osteoblast proliferation，differentiation and mineralization

(a) mRNA level of *Mki67* and protein level of Ki67 in vehicle and nobiletin treated cells. $^{**}P < 0.01$. (b) Quantitative real-time PCR of *Bglap* expression and measurement of ALP enzyme activity in vehicle and nobiletin treated cells. $^{**}P < 0.01$, n=5. (c) Mineralization of MC3T3-E1 cells with and without nobiletin treatment was detected with alizarin red S staining (21st day) assay.

2.3　Nobiletin regulates RORα and SOST in natural aging mouse

To explore the mechanism by which nobiletin promoted bone microstructure in natural aging mouse, we examined RORα signaling pathway regulated by nobiletin in the femur. RT-PCR results showed that *Rora* gene expression was increased and *Sost* gene expression was decreased after nobiletin treatment in 20-month natural aging mice (Figure 3a). Western blotting results showed that RORα protein level was significantly upregulated in natural aging mice after treatment with nobiletin (Figure 3b). While SOST protein level was significantly decreased in natural

aging mice by nobiletin treatment (Figure 3b). These results demonstrated that nobiletin rescued age-related bone loss may be related to the regulation of RORα and SOST.

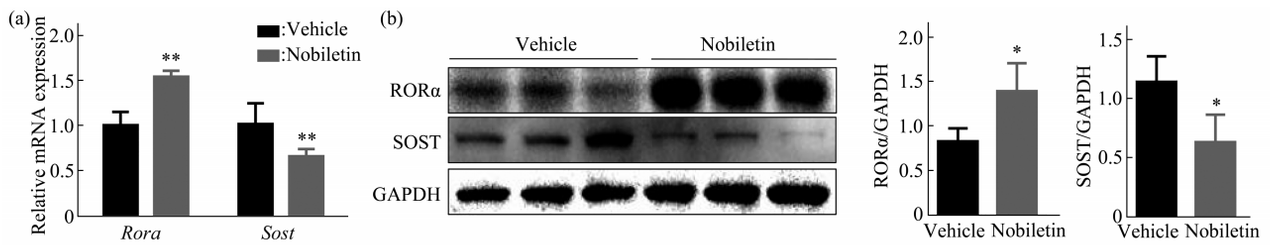

Fig. 3　Regulation of nobiletin on RORα and SOST in the femur of natural aging mice

(a) Gene expression of *Rora* and *Sost* in the femur of 20-month natural aging mice of vehicle or nobiletin group. $^{**}P < 0.01$, n=5. (b) Protein levels of RORα and SOST in the femur of 20-month natural aging mice of vehicle or nobiletin group measured by Western blot and the quantification analysis. $^{*}P < 0.05$, n=5.

2.4　The effect of nobiletin on bone formation depends on RORα

To validate whether nobiletin enhances bone formation depending on RORα, we choose knocking down *Rora* with siRNA (si*Rora*) to see its effect on the regulation of *Sost* and *Bglap*. As illustrated in Figure 4a, *Rora* gene was successfully knocked down. In accordance with the previous results, *Rora* silencing led to the up-regulation of *Sost* and the down-regulation of *Bglap* (Mock *vs*. si*Rora*) and nobiletin led to the down-regulation of *Sost* and up-regulation of *Bglap* (Mock *vs*. Mock + nobiletin) shown as in Figure 4b. While all the effects that the down-regulation of *Sost* and the up-regulation of *Bglap* by nobiletin were significantly inhibited by *Rora* silencing (Figure 4b, (Mock + Nobiletin) *vs*. (si*Rora* + Nobiletin)). Accordingly, the effect of improvement of bone mineralization by nobiletin was also markedly compromised as shown in Figure 4c ((Mock + Nobiletin) *vs*. (si*Rora* + Nobiletin)). Taken together, the results indicate that nobiletin down-regulates *Sost* and up-regulates *Bglap* depending on

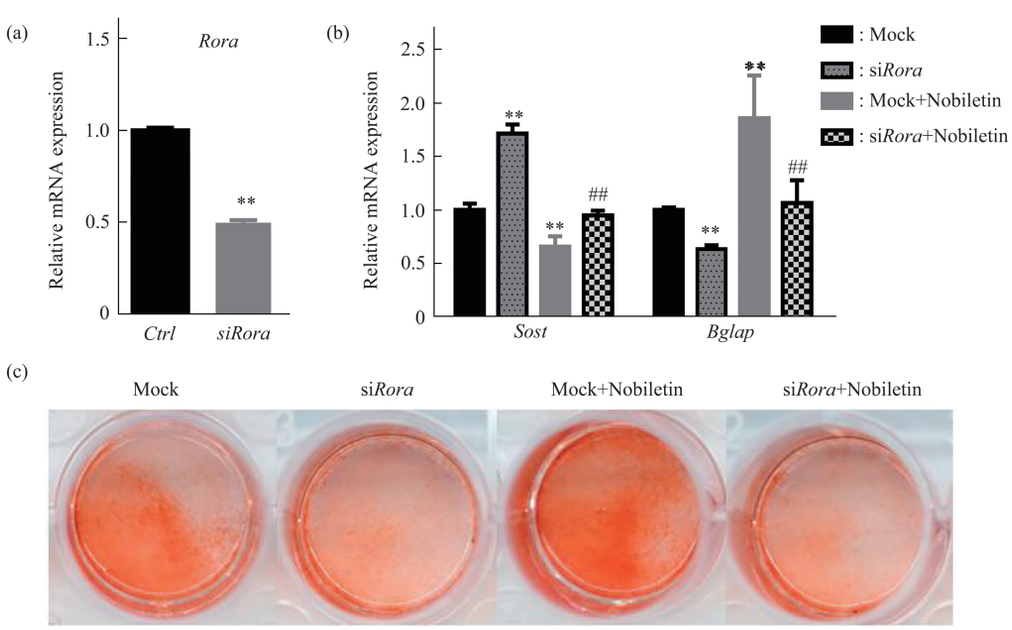

Fig. 4　Nobiletin enhances bone formation depending on RORα

(a) *Rora* was knocked down by siRNA. (b) Quantitative real-time PCR analysis of the effect of si*Rora* on *Sost* and *Bglap*. $^{**}P < 0.01$ (Compare to the Mock group). $^{\#\#}P < 0.01$ (Compare to the Mock + nobiletin group). (c) Effect of nobiletin and si*Rora* on the mineralization of MC3T3-E1 cells with alizarin red S staining (21st day) assay.

Rora.

3 Discussion

In this study, our research firstly demonstrated the effect of nobiletin on the bone loss in natural aging mouse and the involved mechanism. Nobiletin improved the bone microstructure of natural aging mice and enhanced osteoblast proliferation, differentiation and mineralization. The mechanism study showed that nobiletin can activate RORα and then down-regulate *Sost* and up-regulate *Bglap* in *Rora*-dependent pathway to improve bone formation. These results provide a new mechanism of nobiletin in improving age-related bone loss and a new potential strategy to improve the senile osteoporosis.

Osteoporosis is a prevailing skeletal disease and aging is one of the most risk factors. Natural aging model is the most appropriate model to best mimic senile bone loss although requiring a long time. Although some studies have investigated the effect of nobiletin on osteoporosis[7-9], they are in adult mice with estrogen deficiency or ovariectomized model, rather than real natural aging mice. Whether nobiletin improves aging induced bone loss and the involved mechanism have not been determined. In this point, our study of nobiletin in natural aging mouse model owns its advantage. Apart from this, the mechanism of nobiletin on senile osteoporosis in our model is also different from its mechanism in other models. Our research firstly uncovered that nobiletin can up-regulate RORα in the femur of natural aging mice and down-regulate osteocalcin level in the serum. Both *Sost* and *Bglap* were regulated by nobiletin dependent on RORα.

Currently although there are some drugs available to treat age-related osteoporosis, such as Alendronate and PTH 1-84, there are certain limitation and chronic effect is still uncertain. Nobiletin possesses many important biological properties as one most prevalent flavone. Moreover, it has been reported that nobiletin-rich Citrus reticulata peel extract was used in clinical trials by one year oral-administration with no side effects[34]. Therefore, nobiletin may be a potentially effective and safe compound for drug development.

In conclusion, this study demonstrates that nobiletin showed good potential to improve age-related bone loss involving regulation of RORα and SOST. Further research on the optimized dosage and best effect of nobiletin on bone formation is required. The mechanism beyond the nobiletin regulation of SOST *via* RORα is of great interesting. Nobiletin may be worth clinical studying as natural product supplement to treat the age-related bone loss in the future.

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川陈皮素通过调控RORα改善自然衰老小鼠骨流失*

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摘要　随着世界人口老龄化的加速，老年性骨质疏松症的发病率呈现出明显的上升趋势. 衰老相关的骨质流失的机制研究对寻找有效的治疗骨质疏松药物具有重要意义. 天然产物为治疗骨质疏松症药物的发现提供了丰富的来源. 川陈皮素是柑橘属植物果实中含量最丰富的黄酮类成分之一，具有多种生物活性. 在本研究中以20个月自然衰老小鼠为模型，连续15天腹腔注射给予川陈皮素，Micro-CT结果表明川陈皮素可以增加自然衰老小鼠骨体积分数和降低骨小梁分离度，改善自然衰老小鼠的骨微观结构，HE染色结果表明川陈皮素可增加骨小梁表面成骨细胞数量，川陈皮素处理小鼠血清中骨钙蛋白（Osteocalcin）水平显著增加. 在细胞水平，川陈皮素处理小鼠前成骨细胞（MC3T3-E1），*Mki67*水平显著增加，骨碱性磷酸酶水平和骨钙蛋白水平增加，茜素红染色明显增强，表明川陈皮素能促进MC3T3-E1细胞增殖、分化和矿化. 进一步机制研究发现川陈皮素可直接激活视黄酸受体相关孤儿受体α（retinoic acid receptor-related orphan receptors，RORα），并以RORα依赖的方式下调骨硬化蛋白（Sclerostin，SOST）水平、上调骨钙蛋白水平，从而调节成骨细胞功能. 本研究揭示了川陈皮素改善自然衰老相关骨流失的新功能和新机制，为川陈皮素这一天然产物在自然衰老骨质疏松方面应用提供了新的依据.

关键词　川陈皮素，衰老，骨质疏松，骨形成，骨流失，视黄酸受体相关孤儿受体α（RORα），骨硬化蛋白，骨钙蛋白

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