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Linc00963 up-regulation alleviates postmenopausal osteoporosis through suppression of miR-506-3p

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Abstract

Background This study aimed to investigate the regulatory effect of linc00963 on postmenopausal osteoporosis and the potential molecular mechanisms.

Methods Taking MC3T3-E1 cells as the study object, a cell cycle assay was used to evaluate the effect of linc00963 on cell proliferation. mRNA levels of Runx2, OCN, collagenia-1, OPG, RANKL and RANK were detected. Dual luciferase reporter assay verified the targeting relationship between linc00963 and miR-506-3p. A postmenopausal osteoporosis rat model was established after ovariectomy in 32 Sprague-Dawley rats. The rats were divided into sham group, OVX group, linc00963 overexpression group, and blank plasmid group. The bone mineral density (BMD) of the rat femur was measured by X-ray bone densitometer. Serum linc00963 expression in rat was detected by RT-qPCR. The protein expression of ALP, and BGP in the serum of rats was detected by ELISA.

Results Cell studies have shown that linc00963 alleviates postmenopausal osteoporosis by down-regulating the expression of miR-506-3p. Animal studies showed that compared with the sham group, the serum linc00963 level, BMD, serum Ca, P, LEP, SOD, and OPG levels in the OVX group were significantly decreased, while the levels of body weight, ALP, BGP, IL-6, IL-13, RANKL, and RANK were significantly increased. Compared with the OVX group, the use of linc00963 overexpression plasmid can significantly improve the above indexes and play a corresponding therapeutic effect on menopausal osteoporosis rats.

Conclusion Linc00963 is involved in the pathogenesis of postmenopausal osteoporosis by up-regulating miR-506-3p and activating the OPG/RANKL/RANK pathway. Linc00963 is expected to be a potential therapeutic target for postmenopausal osteoporosis.

Keywords Postmenopausal osteoporosis, Linc00963, miR-506-3p, OPG/RANKL/RANK signal pathway, Osteogenic differentiation

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Introduction

Osteoporosis is a systemic bone disease characterized by low bone mass, damaged bone microstructure, and increased bone brittleness [1–3]. The disease will get worse with age, and the risk of females is higher than that of males [4]. Postmenopausal osteoporosis belongs to primary osteoporosis, and its incidence not only accounts for more than half of osteoporosis but also has a trend of gradually getting younger [5]. Inflammation serves as a significant contributory factor in the development of postmenopausal osteoporosis, as evidenced by studies. Specifically, proinflammatory cytokines like interleukin-6 (IL-6) and IL-13 have been implicated in stimulating bone resorption processes in individuals with postmenopausal osteoporosis [6]. Concurrently, parathyroid hormone activity surges, activating osteoclasts and enhancing bone resorption beyond bone formation, ultimately resulting in osteoporosis [7]. With the intensification of populating aging, the proportion of individuals aged 60 and above suffering from osteoporosis has increased significantly, which brings a huge burden to the family and society [8, 9].

In recent years, studies on long non-coding RNA (lncRNA), as biomarkers and potential targets of diseases, have developed rapidly, and many studies have reported that lncRNA plays an important role in immune system diseases, cardiovascular diseases, and tumors [10–12]. In the process of osteogenic differentiation, lncRNA can promote osteoblast differentiation in Wnt/β-catenin and Notch signaling pathways, while inhibiting osteoclast differentiation in RANK, MAPK, and Akt pathways [13]. Yang et al. reported that when lncRNAp21 expression decreased, the Wnt/β-catenin signaling pathway was activated, resulting in increasing estrogen secretion. This, in turn, stimulated osteogenic differentiation and promoted bone formation [14]. Linc00963, also known as MetaLnc9, is located on human chromosome 9q34.11 [15, 16]. As one of many lncRNAs, linc00963 can play a carcinogenic role by regulating cell proliferation, migration, invasion, and apoptosis [17]. With the deepening of research, the role of linc00963 in non-cancerous diseases have gradually been discovered. Specifically, linc00963 is aberrantly expressed in rheumatoid arthritis and correlates with inflammatory markers [18]. Furthermore, up-regulation of linc00963 promotes osteogenic differentiation of bone marrow mesenchymal stem cells and contributes to bone repair in osteoporosis [19]. Notably, Fei et al. reported that 51 differentially expressed lncRNAs, including linc00963, were identified in blood samples of postmenopausal osteoporosis patients through RNA sequencing. This provided clues for mechanism research and biomarker development of postmenopausal osteoporosis [20]. However, the mechanism of linc00963 in postmenopausal osteoporosis remains unclear, prompting our study to address this knowledge gap.

MicroRNA (miRNA) are small endogenous non-coding RNAs (ncRNAs) that regulate gene expression post-transcription. As a regulatory factor, miRNA expression is strictly controlled and plays a role in numerous life processes, including cell proliferation, apoptosis, body development, and metabolism [21]. There have been numerous studies conducted on miR-506-3p in the context of osteoporosis. Zhao et al. reported that circulating miR-506-3p may serve as a potential noninvasive biomarker for osteoporosis in postmenopausal women [22]. The targeting relationship between linc00963 and miR-506-3p was predicted in the ENCORI database. Furthermore, the targeted binding of linc00963 to miR-506-3p has been demonstrated in gliomas. As the ncRNA molecules that regulate bone formation and bone absorption, there are few studies on the co-regulation of osteoporosis by linc00963 and miR-506-3p.

Therefore, we suspected that linc00963 might be involved in postmenopausal osteoporosis progression by targeting miR-506-3p to regulate the osteogenic differentiation of the OPG/RANKL/RANK system as well as MC3T3-E1. To this end, we assessed the role of linc00963 by in vivo animal models and in vitro cellular models. Our study highlights the role of linc00963/miR-506-3p in osteogenic differentiation, which can provide new ideas and methods for the treatment of postmenopausal osteoporosis.

Materials and methods

Cell culture and transfection

Mouse embryonic osteoblast progenitor cells (MC3T3-E1 cells, catalog number: SCSP-5218) were purchased from the Shanghai Institute of Biochemistry and Cell Biology. Passages 3–5 MC3T3-E1 cells were cultured in complete medium (89% α-MEM [HyClone; catalog number: 12571063], 10% fetal bovine serum [Wisent; catalog number: 080–450], and 1% penicillin/streptomycin [Gibco; catalog number: 15140122]) at 37℃ in a humid incubator with 5% CO_2 concentration. The culture medium is changed every two days until the cells have reached 80-90% fusion. Osteogenic differentiation was induced by adding 10mM β- glycerophosphate (Sigma-Aldrich; catalog number: G6501), 50 μg/mL ascorbic acid (Sigma-Aldrich; catalog number: A4544), and 100nM dexamethasone (Sigma-Aldrich; catalog number: A4544) into the medium.

Linc00963 small interfering RNA (si-linc00963) and its negative control (si-NC), linc00963 overexpressed plasmid (oe-linc00963), and blank plasmid (oe-NC), miR-506-3p mimics, miR-506-3p inhibitors and their corresponding negative controls (mimic-NC and inhibitor-NC) were provided by GenePharma (Shanghai,

China). The cells were inoculated into 6-well plates, and 2mL of cell suspension with a cell concentration of 1×10^6cells/mL was added to each well and cultured overnight. The siRNAs and/or miRNAs were transfected into the cells using Lipofectamine 2000 (Invitrogen; catalog number: 11668019) at 37℃ for 6 h. Serum-free medium was replaced with a complete medium and continued for 48 h.

RT-qPCR

RNA samples were extracted from MC3T3-E1 cells using TRIzol reagent (Invitrogen). The RNA was reverse transcribed into cDNA using PrimeScript RT Master Mix (Takara, Dalian, China; catalog number: RR036A) for mRNA or PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China; catalog number: 6210 A) for miRNA according to the manufacturer's scheme. The cDNA was then amplified on the ABI 7500 system (Applied Biosystems, USA) using the SYBR Premix Ex Taq kit (Takara, Dalian, China; catalog number: RR420A). GAPDH and U6 were used as reference genes, and the relative expression of the genes was calculated by the $2^{-\triangle\triangle CT}$ method. Thermal cycling conditions for RT-qPCR: 95℃ for 10 min; 40 cycles of 95℃ for 15 s, 60℃ for 1 min, and 95℃ for 15 s.

Cell cycle

The cell cycle was detected by flow cytometry.Cells in the logarithmic growth phase was inoculated into a 6-well plate at a density of 1×10^6 cells/mL, transfected according to the experimental procedure, and tested 48 h later. The cells were centrifuged at 800 rpm, washed with PBS, and then fixed with 70% ethanol. The cells were incubated at 4℃ for 2 h. After washing again, 25μL of RNase A solution (200 μg/mL; Promega; catalog number: A7973) was added at 37℃ for re-suspension, and then propidium iodide (PI; Sigma-Aldrich; catalog number: P-4170) was added and incubated at 4℃ for 1 h. Finally, flow cytometry was performed and the percentage of cells in the G1, S, and G2 phases were counted respectively.

Cell viability

Cell counting kit-8 (CCK-8) assay was utilized for the evaluation of cell viability. Briefly, MC3T3-E1 cells were inoculated into 96-well plates at a density of 1×10^3 cell/well. The cells were transfected as described in *Cell culture and transfection* item and treated with 100 μL CCK-8 solution (Dojindo; catalog number: CK04) for 1 h at 0 h, 24 h, 48 h, and 72 h, respectively. Subsequently, OD values of the cells were detected with a microplate reader at 450 nm.

Luciferase reporter gene

ENCORE (https://rnasysu.com/encori/index.php) was used to predict the target gene miR-506-3p of linc00963, and wild (WT) and mutant linc00963 fragments containing miR-506-3p binding sites were cloned into pmir reporter vectors. Lipofectamine 2000 was employed to co-transfect the constructed vectors with either miR-506-3p mimic/inhibitor or mimic-NC/inhibitor-NC into MC3T3-E1 cells. After 48 h, the cells were collected, and the luciferase activity of each group was assayed using the dual luciferase reporting system (Promega; catalog number E1960).

Grouping and modeling of experimental animals

Grouping: Thirty-two 6-month-old female SD rats (weighing between 220 and 250 g) were used as experimental subjects in this study. Ovariectomized (OVX) rats are used to simulate postmenopausal osteoporosis. The rats were kept in separate cages in a clean, pathogen-free environment, with a light-dark cycle set for 12 h. The feeding environment maintained a temperature of 25 ± 2℃, a relative saturation humidity of 50 ± 5%, the rats had free access to food and drink. The bedding material was changed every other day. This study was approved by the Ethics Committee of Yunnan Provincial Hospital of Traditional Chinese Medicine in accordance with the relevant provisions of the Regulations on the Administration of Experimental Animals of the People's Republic of China and met the requirements of animal ethics. The rats were randomly divided into 4 groups with 8 rats in each group: (1) Sham group: sham operation group; (2) OVX group (model group); (3) OVX + oe-NC group (after modeling, the blank plasmid was injected); (4) OVX + oe-linc00963 group (after modeling, linc00963 overexpression plasmid was injected).

Modeling: Fasting for 6 h before surgery. Each rat was weighed and anesthetized with 1% pentobarbital sodium (40 mg/kg; Sigma-Aldrich; catalog number: P3761) according to body weight. After fixation, the skin over the dorsal lumbar vertebrae and bilateral areas of the rats were prepared for sterilization, and a longitudinal incision of 2–3 cm was made along with the middle of the lumbar vertebrae to expose the abdominal cavity on both sides. According to previous studies [23], in the OVX group, the ovarian tissue was removed after ligation of both fallopian tubes, while in the Sham group, only an equal volume of adipose tissue around the ovarian tissue was removed. The abdominal cavity and skin were sutured after the corresponding tissues were removed. Intramuscular penicillin was injected for 5 consecutive days after the operation to prevent wound infection (80,000 units/animal), and the rats were kept in a single cage after the operation to avoid wound rupture caused by biting. In the Sham group and OVX group, 0.5 mL

normal saline was injected intraperitoneally on the first three days of 1, 3, and 5 weeks after surgery, respectively. OVX + oe-NC group and OVX + oe-linc00963 group were intraperitoneally injected with oe-NC and oe-linc00963 respectively at the dose of 7 mg/kg during the same period. All rats were kept in their original environment for another 6 weeks.

The tests of bone mineral density (BMD) and body weight of rats

After anesthesia, the rats were fixed and placed on LUNAR-PRODIGY dual-energy X-ray bone densitometry to detect the femoral density of the rats. The scanning speed was 10 mm/s, and the resolution was 0.5 × 0.5 mm. The experimental results were analyzed by using small animal step standard analysis software. The body weight of each rat was measured and recorded using an electronic scale at the same period on the weekend of weeks 0, 2, 4, and 6.

Detection of serum indicators

(1) Serum Ca and P levels were detected using Beckman CX9 Automatic biochemical analyzer; (2) ELISA: serum levels of alkaline phosphatase (ALP; Elabscience; catalog number: E-BC-K091-M), Bone Gla-Protein (BGP; Elabscience; catalog number: E-EL-R3070), leptin (LEP; Elabscience; catalog number: E-EL-R0582), superoxide dismutase (SOD; Elabscience; catalog number: E-BC-K020-M), interleukin-6 (IL-6; Elabscience; catalog number: E-EL-R0015), IL-13 (Elabscience; catalog number: E-EL-R0563), osteoprotegerin (OPG; Elabscience; catalog number: E-EL-R3005), nuclear factor kappa B receptor activator ligand (RANKL; Elabscience; catalog number: E-EL-R0841), nuclear factor kappa B receptor activator (RANK; USbiological; catalog number: 027808) in each group were determined according to ELISA kit instructions.

Data analysis

SPSS 22.0 (IBM Corp. Armonk, NY, USA) and GraphPad Prism 7.0 (GraphPad Software, CA, USA) were used for statistical data processing and graphing. Data were expressed as mean ± standard deviation (SD), and one-way ANOVA was used to compare multiple groups. $P < 0.05$ was considered a significant difference.

Results

Effect of linc00963 on proliferation, osteogenic differentiation, and OPG/RANKL/RANK system of MC3T3-E1 cells

Transfection with si-linc00963 could significantly down-regulate, while transfection with oe-linc00963 could significantly up-regulate the expression of linc00963 in MC3T3-E1 cells (Fig. 1A, $P < 0.001$). Cell cycle and CCK-8 experiments revealed that overexpression of linc00963 could significantly reduce the number of cells in the G1 phase and improve cell viability, which was conducive to cell proliferation (Fig. 1B-C, $P < 0.001$). As for osteogenic differentiation, the decrease of linc00963 inhibited it, as evidence by the decreased expression levels of Runx2, OCN, and Collagen 1. Conversely, the up-regulation of linc00963 promoted the osteogenic differentiation in MC3T3-E1 cells (Fig. 1D-F, $P < 0.001$). The effect of linc00963 on the OPG/RANKL/RANK signaling pathway was evaluated by RT-qPCR. The results showed that inhibition of linc00963 significantly down-regulated the expression of OPG, while up-regulated the expression levels of RANKL and RANK. The opposite result was observed when linc00963 was overexpressed (Fig. 1G-I, $P < 0.01$).

Linc00963 can directly target miR-506-3p

The online database predicted that linc00963 and miR-506-3p had targeted complementary sites, and the sequence was exhibited in Fig. 2A. The interaction between linc00963 and miR-506-3p was further verified using luciferase reporter genes assay. The results in Fig. 2B demonstrated that increasing or decreasing the level of miR-506-3p could correspondingly inhibit or promote luciferase activity in the WT group ($P < 0.001$), whereas no effect on the MUT group ($P > 0.05$). As shown in Fig. 2C, transfection with si-linc00963 was observed to up-regulated miR-506-3p expression, while transfection with oe-linc00963 inhibited miR-506-3p expression ($P < 0.001$). Transfection experiments involving miR-506-3p mimics or inhibitors illustrated that their addition could significantly upregulate or down-regulate the expression of miR-506-3p in MC3T3-E1 cells, respectively (Fig. 2D, $P < 0.001$). Co-regulation experiments showed that cells transfected with miR-506-3p mimics could reverse the inhibitory effect of overexpressed linc00963 on miR-506-3p expression (Fig. 2E, $P < 0.001$).

Linc00963 promotes the proliferation and osteogenic differentiation of MC3T3-E1 cells by targeting miR-506-3p

In cell viability experiments, it was observed that overexpression of linc00963 could significantly promote cell viability. On this basis, further upregulation of miR-506-3p inhibited cell viability (Fig. 3A, $P < 0.001$). As shown in Fig. 3B-D, the addition of miR-506-3p mimic reversed the promoting effects of linc00963 overexpression on Runx2, OCN, and Collagen-1 expression ($P < 0.001$). These results indicated that the increase in miR-506-3p inhibited the positive effect of linc00963 overexpression on osteoblastic differentiation of MC3T3-E1 cells. Furthermore, the increase of linc00963 enhanced the expression level of OPG in cells, but this effect could be inactivated after the addition of miR-506-3p mimic

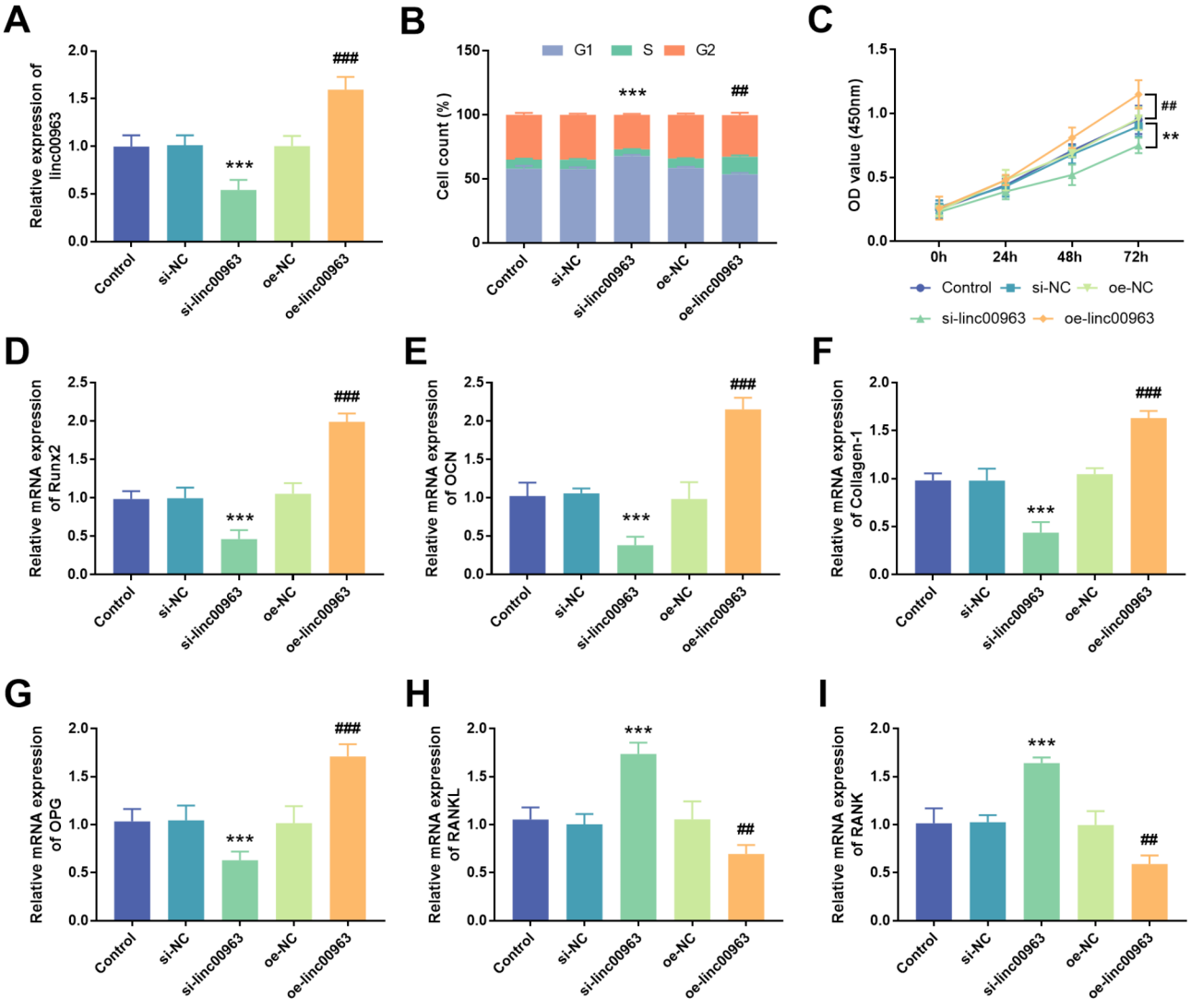

Fig. 1 Overexpression of linc00963 can promote the proliferation and osteogenic differentiation of MC3T3-E1 cells. **A**. Transfection of si-linc00963 or oe-linc00963 down-regulated or up-regulated the expression of intracellular linc00963. **B**. The effect of linc00963 on cell cycle was detected by flow cytometry. **C**. The role of overexpression of linc00963 on Cell viability was evaluated by cell counting kit-8 experiments. **D-F**. mRNA expression levels of osteogenic markers were detected by RT-qPCR. **G-I**. The effect of linc00963 on the expression level of OPG/RANKL/RANK was detected by RT-qPCR. $^{***}P<0.001$, $^{**}P<0.01$ vs. si-NC group. $^{###}P<0.001$, $^{##}P<0.01$ vs. oe-NC group

(Fig. 3E, $P<0.001$). For RANKL/RANK, the increase of miR-506-3p significantly promoted its expression level (Fig. 3F-G, $P<0.01$).

Expression and effect of linc00963 in OVX rat model

It was observed that the expression level of linc00963 in the serum of rats in the OVX group was significantly lower than that in the sham group, and the level of linc00963 in the serum of rats in OVX group was significantly increased after injection of oe-linc00963 (Fig. 4A, $P<0.001$). As illustrated in Fig. 4B-C, the BMD of OVX rats was lower than that of the sham group, while their body weight was higher than that of the sham group ($P<0.001$). Upregulation of linc00963 could improve BMD and body weight in OVX rats ($P<0.01$). As shown in Fig. 4D-F, compared with the sham group, the serum Ca and P levels of rats in the OVX group were significantly reduced, whereas the ALP levels were significantly increased ($P<0.001$). Compared with the OVX group, treatment with linc00963 could markedly increase the serum Ca and P levels and decrease the ALP levels ($P<0.01$). Subsequently, the osteogenic function-related indexes in the serum of rats from all groups were detected. The results indicated that, compared with the sham group, the levels of BGP in the OVX group were increased, while the levels of LEP and SOD were decreased, indicating that the osteogenic ability of rats in the OVX group was inhibited. Compared with the

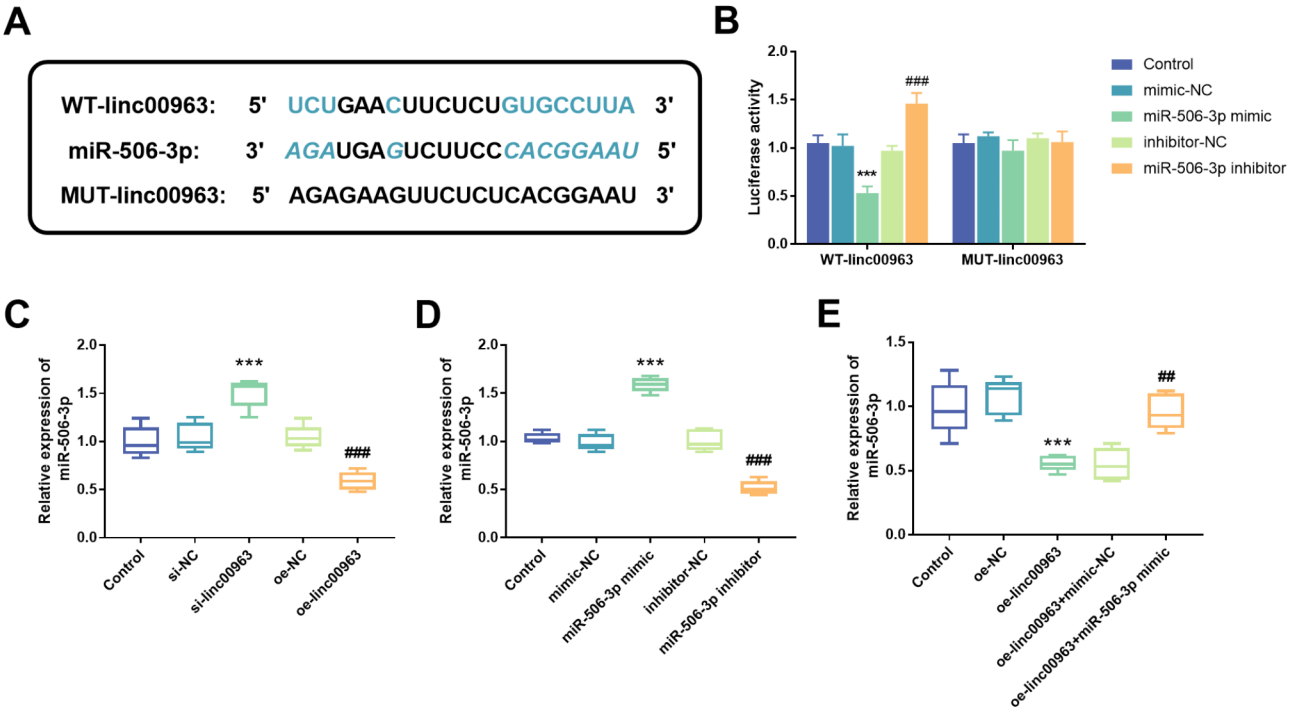

Fig. 2 miR-506-3p is the target gene of linc00963. **A**. Binding sites of linc00963 and miR-506-3p predicted by ENCORI. **B**. The luciferase reporter gene was used to verify the interaction between linc00963 and miR-506-3p. $^{***}P<0.001$ vs. mimic-NC group. $^{\#\#\#}P<0.001$ vs. inhibitor-NC group. **C**. The expression level of miR-506-3p after transfection with si-linc00963 or oe-linc00963 was detected by RT-qPCR. **D**. The expression level of miR-506-3p after transfection with miR-506-3p mimics or inhibitors was detected by RT-qPCR. $^{***}P<0.001$ vs. si-NC group. $^{\#\#\#}P<0.001$ vs. oe-NC group. **E**. The expression of miR-506-3p after co-transfection with oe-linc00963 and Mir-506-3p mimic was detected by RT-qPCR. $^{***}P<0.001$ vs. oe-NC group. $^{\#\#}P<0.01$ vs. oe-linc00963 + mimic-NC group

OVX group, overexpression of linc00963 significantly enhanced the osteogenic ability of rats in the OVX group (Fig. 5A-C, $P<0.001$). In the serum of OVX rats, it was found that the production of osteoclast function-related inflammatory factors IL-6 and IL-13 was increased compared with that of the sham group (Fig. 5D, $P<0.001$). Compared with the OVX group, up-regulation of linc00963 significantly inhibited the expression of osteoclast-related inflammatory factors (Fig. 5D, $P<0.01$).

Effect of linc00963 expression on OPG/RANKL/RANK signaling pathway in rat serum

As shown in Fig. 6A-C, compared with the sham group, the content of OPG in the serum of rats in the OVX group decreased, while the concentration of RANKL/RANK increased, suggesting that modeling process had an impact on the OPG/RANKL/RANK system ($P<0.001$). Further results showed that compared with the OVX group, injection of oe-linc00963 significantly increased the serum expression of OPG in model rats and inhibited the expression of RANKL/RANK, demonstrating that overexpression of linc00963 exhibited a therapeutic effect on OVX rats ($P<0.01$).

Discussion

As the most common type of osteoporosis, it is crucial to explore the molecular mechanisms and potential therapeutic targets of postmenopausal osteoporosis [24–29]. MECT3-E1 cells, derived from mouse cranial tissue, are pivotal in bone metabolism, exhibiting osteogenic and osteoclastic properties. Due to their ease of culture and reproducibility, these cells mimic the pathophysiological process of postmenopausal osteoporosis in vitro, offering valuable insights into its prevention and treatment. In vitro cell studies, it was found that upregulation of linc00963 enhanced the proliferation capacity of MC3T3-E1 cells by promoting the entry of cells into the S phase of mitosis. Linc00963 promotes osteogenic differentiation of MC3T3-E1 cells by enhancing the expression of Runx2 and OCN. In addition, the addition of linc00963 promoted the expression of OPG and inhibited the expression of the RANKL/RANK system. The ENCORI database predicted that linc00963 had binding sites with miR-506-3p. An increase in linc00963 can inhibit the expression of miR-506-3p. Animal studies have shown that BMD and body weight in OVX rat models are higher than those in sham groups, suggesting the successful construction of a postmenopausal osteoporosis model. In the OVX model, overexpression of linc00963 promotes

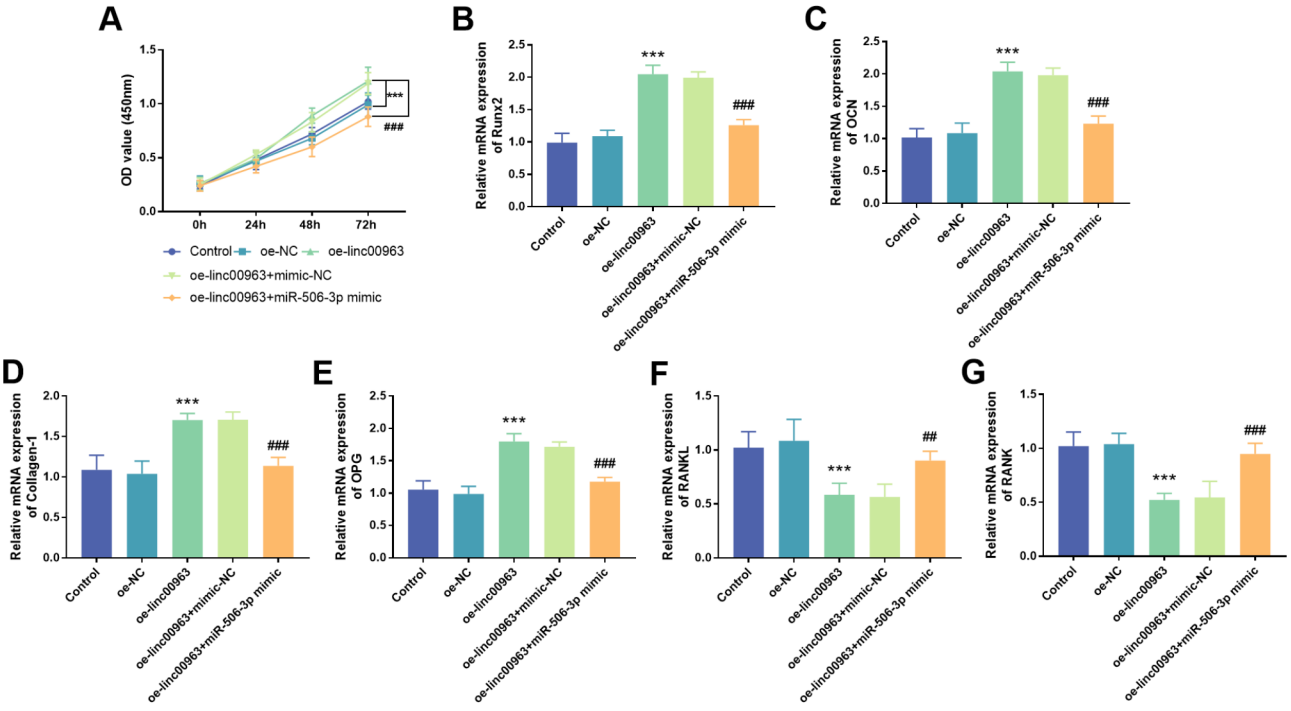

Fig. 3 Linc00963 promotes proliferation and osteogenic differentiation of MC3T3-E1 cells by down-regulating miR-506-3p. **A**. Upregulation of miR-506-3p can inhibit the promoting effect of overexpression of linc00963 on cell viability. **B-D**. The increase of miR-506-3p reversed the promoting effect of overexpression of linc00963 on markers of osteogenic differentiation, which showed decreased expressions of Runx2, OCN and collagen-1. **E-G**. The increase of miR-506-3p inhibited the expression of OPG but promoted the expression of RANKL and RANK. $^{***}P < 0.001$ vs. oe-NC group. $^{###}P < 0.001$, $^{##}P < 0.01$ vs. oe-linc00963 + mimic-NC group

the recovery of BMD and serum Ca and P levels, thereby alleviating osteoporosis in rats. Simultaneously, it was also proved that upregulation of linc00963 promoted serum OPG levels and inhibited RANKL/RANK in the model group.

Linc00963 has previously been extensively studied as an oncogene, associated with drug resistance in oral and gastric cancers, poor prognosis in liver cancer, and predictive value of taxane-anthracyclines in breast cancer [15]. In recent years, linc00963 has been extensively studied in osteoporosis, but the results have been inconsistent. Ren et al. reported that linc00963 promotes osteogenic differentiation of hBMSCs in vitro and weakens the progression of osteoporosis in vivo by directly targeting miR-760 [19]. Conversely, Wu et al. observed that reduced expression of linc00963 in osteogenic hBMSCs was associated with its overexpression inhibiting proliferation, differentiation, and promoting apoptosis of these cells [30]. Notably, the regulatory function of linc00963 in MC3T3-E1 cells, a prototypical cell line for osteoporosis research, along with its underlying mechanisms, remains unexplored. Innovatively, our study analyzed the stimulatory effect of linc00963 on MC3T3-E1 cell proliferation and osteogenic differentiation and further validated these findings in an animal model. Our observation revealed that linc00963 expression was reduced in OVX rats, and the upregulation of linc00963 played a certain therapeutic effect on osteoporosis in OVX rats, which was firstly reflected in the increase of BMD, serum Ca, and P. In addition, overexpression of linc00963 significantly promoted the expression of key osteogenic differentiation regulators such as OCN, collagen I, Runx2, and OPG in preosteoclasts (MC3T3-E1 cells), suggesting that linc00963 significantly enhances osteoblasts' differentiation.

The OPG/RANKL/RANK system plays an important role in regulating the homeostasis of osteoblasts and osteoclasts and in preventing osteoporosis [31]. Studies have found that RANK mainly binds to RANKL to promote osteoclast differentiation and maturation, while OPG inhibits the combination of RANK and RANKL through the activation of positive regulatory factors (such as estrogen and TGF-β) to achieve the purpose of inhibiting osteoclast differentiation [32]. In addition to regulating bone metabolism, this system is also involved in processes such as inflammation and immunity. Inflammatory factors (IL-6 and IL-13) have strong osteoclast activity, which can directly activate osteoclast precursors or stimulate RANKL expression to induce osteoclast production [33, 34]. A large increase in RANKL directly binds to normal levels of OPG, resulting in a compensatory decrease in OPG and a significant increase in

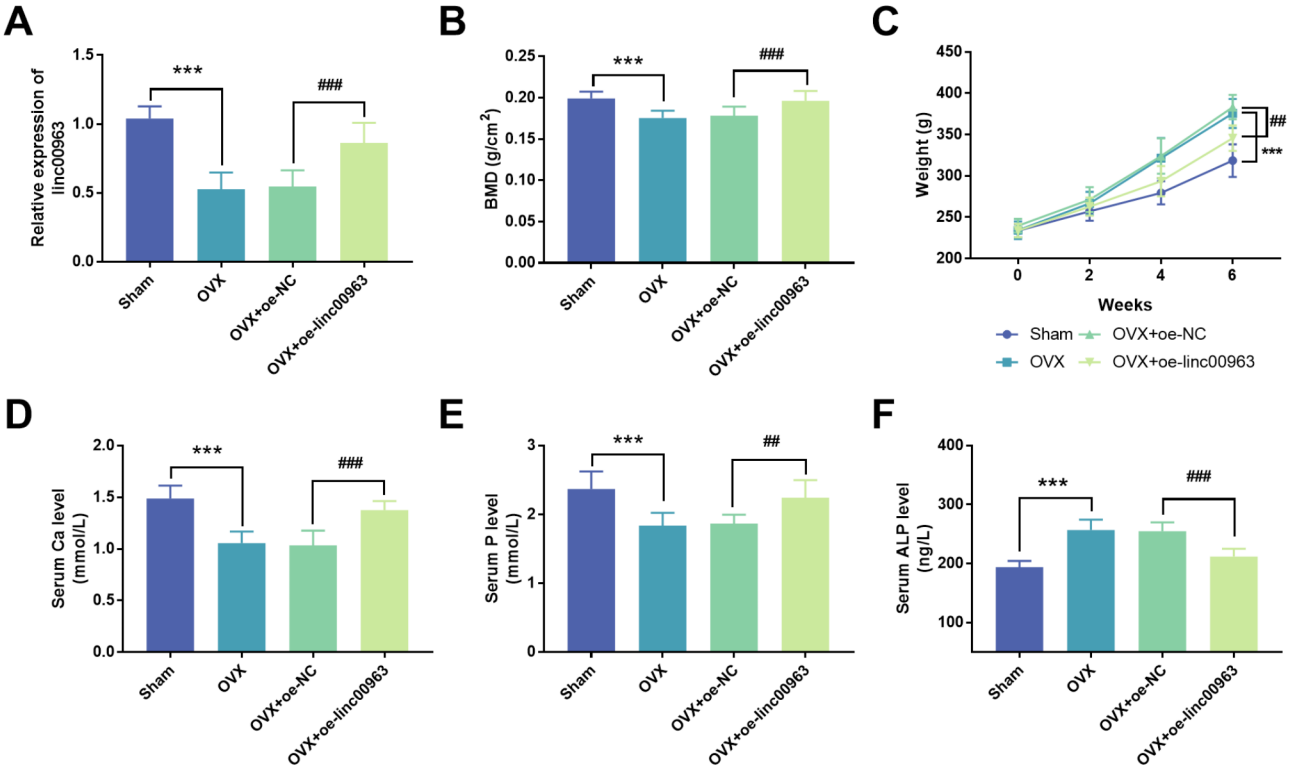

Fig. 4 Construction of menopausal osteoporosis rat model. **A**. The expression of linc00963 decreased in the OVX model group. **B**. Bone mineral density (BMD) of OVX model rats decreased, and BMD of OVX model rats increased after treatment with overexpression of linc00963. **C**. The body weight of the OVX group rats increased with the extension of time, and treatment with overexpression of linc00963 significantly reduced the body weight of the OVX group rats at the end of the sixth week. **D**. Overexpression of linc00963 can significantly increase the serum calcium level of OVX group rats. **E**. Up-regulation of linc00963 can increase the level of serum P in the model group. **F**. Overexpression of linc00963 can inhibit the expression of ALP in the serum of rats in the model group. $^{***}P < 0.001$ vs. Sham group. $^{###}P < 0.001$, $^{##}P < 0.01$ vs. OVX + oe-NC group

bone resorption [35]. In vitro studies, we found that the decrease of linc00963 reduced the expression level of OPG, while the level of RANKL/RANK increased, indicating that reduction of linc00963 promotes osteoclast activity and accelerates bone resorption. Similarly, this trend has been observed in animal studies. Overexpression of linc00963 can significantly up-regulate the expression of OPG in serum and decrease the level of RANKL/RANK in the OVX group, indicating that the addition of linc00963 can inhibit osteoclastic activity and the inflammatory response, thereby reducing bone resorption, protecting bone tissue, and alleviating osteoporosis. The regulation of miRNAs plays a crucial role in controlling bone homeostasis and metabolism, and the disturbance of miRNA expression may impact the function of osteoblasts and osteoclasts. In past studies, abnormal expression of certain miRNAs has been observed in the blood of patients with osteoporosis. For example, low levels of miR-29a-3p and miR-30b-5p are expected to be biomarkers for postmenopausal osteoporosis [36, 37]. In the context of disease, regulating miRNAs alone is often inadequate due to their multiple targets and potential interference with normal physiological processes. Thus, understanding the intricate interplay between miRNAs and lncRNAs within the regulatory network is crucial for unraveling disease mechanisms and advancing diagnostic and therapeutic strategies.

Upon analysis of the ENCORI database, miR-506-3p emerged as a promising target gene for linc00963. Despite not ranking highest in the target-binding algorithm scores, this miRNA can be harnessed to establish a ceRNA network and has garnered significant attention due to its association with bone metabolism. Studies have revealed that miR-506-3p regulates the RNAKL/NFATC1 signaling pathway, thereby attenuating osteoclast genesis and ultimately impacting bone erosion [38]. Furthermore, it has been shown to inhibit osteogenic differentiation in bone marrow mesenchymal stem cells [39]. Notably, elevated levels of miR-506-3p have been observed in the blood of postmenopausal women with osteoporosis [22]. Given the demonstrated target binding of linc00963 to miR-506-3p in gliomas [40] and its reported role in osteoporosis, we selected this miRNA for further studies on linc00963. We assessed the effect of miR-506-3p on osteogenic differentiation through cell experiments. In MC3T3-E1 cells, overexpression

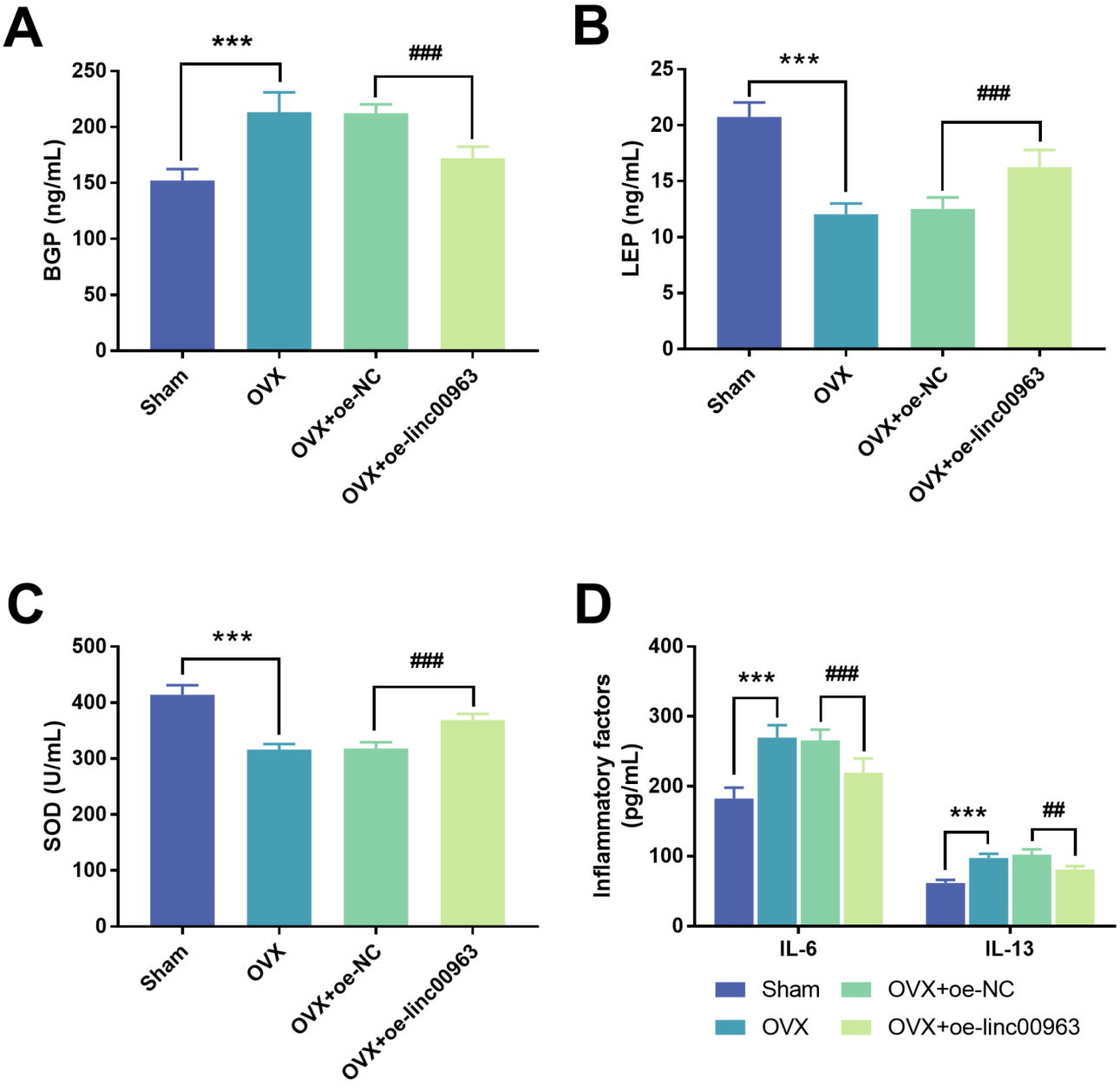

Fig. 5 Effect of overexpression of linc00963 on serum indexes in OVX rats. **A**. Up-regulation of linc00963 inhibited the expression of BGP in the serum of model rats. **B**. Up-regulation of linc00963 promoted the expression of LEP in the serum of model rats. **C**. Up-regulation of linc00963 improved the expression of SOD in the serum of model rats. **D**. Up-regulation of linc00963 inhibited the expression of IL-6 and IL-13 in the serum of model rats. $^{***}P < 0.001$ vs. Sham group. $^{###}P < 0.001$, $^{##}P < 0.01$ vs. OVX + oe-NC group

of linc00963 can correspondingly inhibit the expression level of intracellular miR-506-3p, suggesting the targeting relationship between linc00963 and miR-506-3p and the negative regulatory effect of linC00963 on the latter. Subsequent studies showed that overexpression of miR-506-3p overruled the promoting effect of up-regulation of linc00963 on cell proliferation and osteogenic differentiation, and the positive effect on OPG/RANKL/RANK system. In our study, we demonstrated the novel finding that overexpression of linc00963 through targeted inhibition of miR-506-3p, upregulates the expression of RUNX2, OCN, and collagen I in osteoblasts. This enhancement promotes osteogenic differentiation and accelerates the process of bone formation. Concurrently, the linc00963/miR-506-3p interaction also exerts a suppressive effect on osteoclast activity. By stimulating OPG expression and inhibiting RANKL and RANK levels, as well as dampening inflammatory responses, this interaction effectively reduces osteoclast activity, halts the progression of bone resorption, and facilitates bone repair.

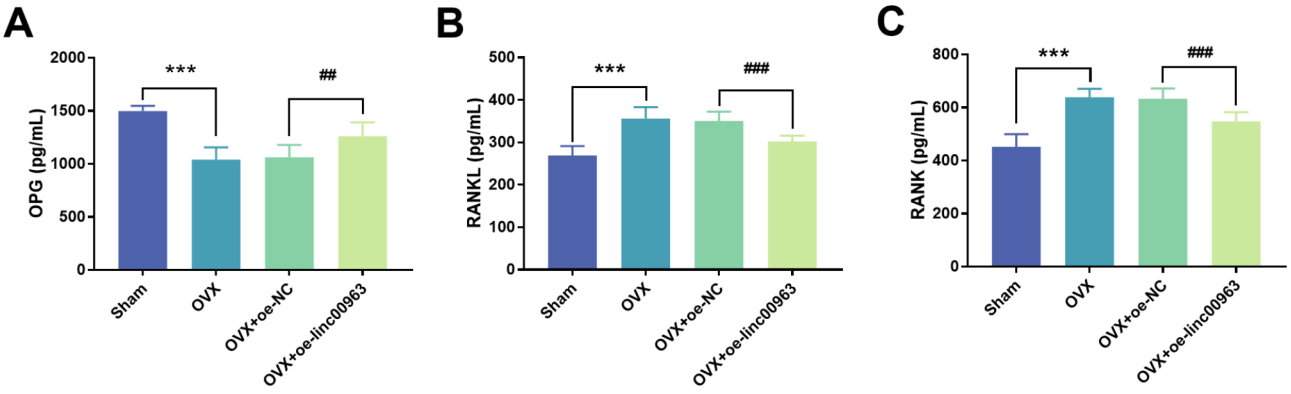

Fig. 6 Effect of linc00963 on OPG/RANKL/RANK signal pathway. A. Up-regulation of linc00963 can increase the expression of OPG in the OVX group. **B-C**. Overexpression of linc00963 inhibited the expression levels of RANKL and RANK in the serum of the OVX group. $^{***}P < 0.001$ vs. Sham group. $^{###}P < 0.001$, $^{##}P < 0.01$ vs. OVX + oe-NC group

Consequently, these findings suggest a protective role of linc00963 against postmenopausal osteoporosis. There are undeniable limitations to this study. First, although osteogenic differentiation indexes, inflammation, and the levels of OPG, RNAKL, and RNAK were significantly modulated, they were all carried out under the conditions of relatively mild transfection, and we have now planned to explore more effective transfection conditions in our subsequent studies to gain just a deeper understanding of the specific roles of linc00963 and miR-506-3p in postmenopausal osteoporosis. In addition, although the role of miR-506-3p in osteoporosis and postmenopausal osteoporosis has been widely reported and we confirmed that miR-506-3p attenuates the role of linc00963 in postmenopausal osteoporosis, we will separately verify the role of miR-506-3p in our later study. Finally, we found that linc00963 expression in mouse cell lines and rat models aligns with that in human osteoporosis patients reported by Fei et al., and both regulate osteogenic differentiation. However, a full sequence similarity analysis between human and mouse linc00963 has not been conducted; this will be addressed in a follow-up study. Although this study has some limitations as described above, this study still confirms that linc00963 as mentioned above, this study still confirms that linc00963 may regulate osteogenic differentiation and alleviate postmenopausal osteoporosis by targeting miR-506-3p.

In conclusion, this study has proven that linc00963, acting as a sponge for miR-506-3p, regulates the OPG/RANKL/RANK system and promotes the proliferation and osteogenic differentiation of MC3T3-E1 cells. Furthermore, we have verified the alleviating effect of upregulated linc00963 on osteoporosis in OVX rat models. This finding offers a potential theoretical basis for understanding the pathogenesis and developing targeted treatment for postmenopausal osteoporosis.

Abbreviations

ALP	Alkaline Phosphatase
BGP	Bone Gla-Protein
BMD	Bone Mineral Density
IL-6	Interleukin-6
LEP	Leptin
LncRNA	Long Non-Coding RNA
OPG	Osteoprotegerin
miRNA	MicroRNA
PI	Propyl Iodide
SD	Standard Deviation
SOD	Superoxide Dismutase

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Not Applicable.

Author contributions
All authors designed this study. Y X and LH G conducted the experiment and analyzed the data. Q L and J Z wrote the manuscript. LH G and XX Y revised the manuscript. All authors reviewed and approved for publication.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This experiment was conducted with the approval of the Animal Ethics Committee of Yunnan Provincial Hospital of Traditional Chinese Medicine. All institutional and national guidelines for the care and use of laboratory animals were followed. Appropriate measures were taken to minimize the number and suffering of animals.

Consent for publication
All patients provided written informed consent.

Competing interests
The authors declare no competing interests.

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