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Original Research Article

MiR-33a-5p negatively regulates ovariectomy-induced osteoporosis in rats by decreasing WNT10B

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Abstract

***Purpose:** To investigate the role and potential mechanism of action of microribonucleic acid (miR)-33-5p in osteoporosis after ovariectomy.*
***Methods:** A postmenopausal osteoporosis model was established using an ovariectomized (OVX) rat. Primary bone marrow-derived mesenchymal stem cells (BMMSCs) were obtained from female rats, and were transfected with mimics or inhibitors. Reverse transcription-polymerase chain reaction (RT-PCR) and western blotting were preformed to determine the level of miR-33a-5p and target proteins, while luciferase reporter assay was used to verify whether WNT10B is a target of miR-33a-5p. Micro-computed tomography (micro-CT) analysis was employed to evaluate bone formation.*
***Results:** Bone mineral density (BMD), determined by micro-CT, was significantly decreased in OVX group. MiR-33a-5p was in negative correlation with WNT10B after surgery, and WNT10B was found to be a target of miR-33a-5p. Additionally, inhibition of miR-33a-5p facilitated osteogenic differentiation in vitro, and promoted bone formation in vivo.*
***Conclusion:** Inhibition of miR-33a-5p accelerates bone formation by increasing WNT10B levels. Thus, miR-33a-5p is a potential target for the treatment of osteoporosis.*

***Keywords:** Postmenopausal, Osteoporosis, Ovariectomy, Bone marrow-derived mesenchymal stem cells (BMMSCs), Bone mineral density*

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INTRODUCTION

Over 40 % of postmenopausal women suffer from osteoporosis, which is mainly manifested as damaged bone tissue microstructure, reduced bone mineral composition and bone matrix, lowered bone mineral density (BMD), increased bone fragility and elevated fracture risk [1,2]. According to a previous study, China has 120 million aged people, and the number will reach 457 million in 2050, posing serious threats to health and huge economic burdens to the society [3].

Postmenopausal osteoporosis occurs due to an imbalance between bone resorption and bone formation [4-6]. Although multiple efficient anti-bone resorption medications have been available clinically, there has not yet been ideal therapeutic schemes for promoting bone formation [7,8].

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Bone is in a dynamic balance, and this balance is mainly maintained by bone formation and bone resorption, which are realized through the osteogenesis of osteoblasts and the activity of osteoclasts respectively [9]. In the lifetime of humans, osteogenesis gradually decreases with age, but the activity of osteoclasts is relatively enhanced. Hence, osteoporosis, a bone disease, occurs in the majority of elderly people, especially postmenopausal women [10]. Osteoporosis has as its main symptoms, skeletal pain and susceptibility to fracture, and this endangers life in severe cases. Therefore, it is vital to study osteoporosis.

Existing literature reports have shown that micro ribonucleic acids (miRNAs) may be involved in the differentiation of mesenchymal stem cells [11]. Several miRNAs like miR-503, are capable of regulating postmenopausal osteoporosis [12], however, the main mechanism of action of miR-503 in postmenopausal osteoporosis remains to be clarified. Previous studies have shown that miRNAs participate in the development of osteoporosis. MiR-33a-5p is significantly raised in the blood of osteoporosis patients, but whether it takes part in postmenopausal osteoporosis is still unknown [13]. The purpose of this study was to examine the effects and potential mechanisms of action of miR-33a-5p in postmenopausal osteoporosis.

EXPERIMENTAL

Establishment of osteoporotic models

This study was approved by the Animal Ethics Committee of The First Hospital of Hangzhou Fuyang Animal Center (IACUC-002471). After 1 week of adaptive feeding, the rats were randomly divided into a sham group and an ovariectomized (OVX) group, respectively (n = 10). The surgical procedures applied were as follows: After anesthetization via intraperitoneal injection with 0.8 mL 10 % chloral hydrate, the rats were placed in a horizontal abdominal position, and the skin was routinely prepared in the middle and lower parts of the abdomen, and disinfected using 3 % iodine. About 0.5 cm-long longitudinal incision was made at 1 cm below the costal margin, at the junction about 1 cm away from both sides of the spine, so as to separate the skin, cut open the dorsal muscle, fully expose the ovary and bluntly separate the fat around the ovary. After both the oviduct and vessels were ligated, the whole ovary was removed, and the uterus was placed in the abdominal cavity. Then, the incision was sutured and closed layer by layer. The other ovary was excised using the same methods as above. In the sham group, no ovary was surgically removed, but other treatments were the same as those in the OVX group.

On day 3 post-operation, penicillin was administrated at 20,000 IU/kg, to routinely resist or prevent inflammation and infection. At 45 or 90 days after surgery, the rats were sacrificed by overdose anaesthesia (pentobarbital sodium 150 mg/kg intraperitoneal injection) and cervical dislocation. The right femur was used for BMD and biomechanical determination. For analysis of protein and RNA expressions, the right femur was frozen in liquid nitrogen and preserved at -80 °C.

Isolation and culture of bone marrow-derived mesenchymal stem cells (BMMSCs)

Primary BMMSCs were obtained from female rats weighing about 200 g. The cells were then cultured in 10 % DMEM at 37 °C with humidity and 5 % CO_2. After that, the cells were transfected with miR-33a-5p mimic/inhibitor/inhibitor mutant and non-specific control purchased from Gene Chem (Shanghai, China).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNAs were isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's manual. The expressions of miR-33a-5p and WNT10B were measured by quantitative RT-PCR (qRT-PCR) with SYBR Green Master Mix (ABI) kit (Invitrogen, Carlsbad, CA, USA). Glyceraldheyde 3-phosphate dehydrogenase (GAPDH) or U6 was used as a control. The primer sequences used are shown in Table 1.

Table 1: Primer sequences used

Gene		Primer sequences
GAPDH	Forward	5'-TGGCCTTCCGTGTTCCTAC-3'
	Reverse	5'-GAGTTGCTGTTGAAGTCGCA-3'
WNT10B	Forward	5'-TCTCCTGTTCCTGGCGTTG-3'
	Reverse	5'-CGACAGCGTCAAGCAGACTG-3'
MiR-33a-5p	Forward	5'- GUGCAUUGUAGUUGCAUUGCA-3'
	Reverse	5'-CAAUGCAACUACAAUGCACUU-3'

Western blotting

Radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) and various proteinase inhibitors *viz,* phenylmethylsulfonyl fluoride (PMSF) were added to the tissues and cells, and then put on ice for 10 min. During this process, the cells were constantly blown and washed to avoid air bubbles. Then the mixture

was transferred into a tube for centrifugation at 4 °C and 12,000 rpm for 15 min.

The procedures were performed in ice bath to prevent degradation. In accordance with the operation instructions of bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China), this study determined the concentration of the samples. Additionally, the loading volume of all samples was adjusted to equal levels. Then the samples were added with sodium dodecyl sulphate (SDS)-loading buffer in the same volume, mixed evenly and boiled at 100 °C for 10 min. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted. First, the solution was loaded separately. The marker was added into the first well, and the protein was separated via electrophoresis. Next, the obtained proteins were transferred onto sandwich-structured polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) on ice at 100 V for 1.5 h.

The membranes with proteins were placed in a box in an appropriate size, and then added to 6 % milk for 1 h on a shaking table. The resulting membranes were added to the primary antibody, washed using tris buffered saline-tween (TBST) 3 times, and then added with the horseradish peroxidase-coupled secondary antibody. When proper enzyme substrates were added, the compounds were oxidized by horseradish peroxidase into chemiluminescent substances, followed by determination. After washing 4 times using TBST (8 min/time), exposure was carried out as follows: the substrates were added, and then the signals were collected using the films, followed by development.

Validation of WNT10B as a direct target of miR-33a-5p

In the formation and differentiation of osteoblasts, WNT10B activates and initiates the differentiation of MSCs into osteoblasts, and regulates the maturity of osteoblasts. In addition, WNT10B is involved in the regulation of several signaling pathways in osteogenic differentiation. To gain further insights into the molecular mechanism of WNT10B in regulating postmenopausal osteoporosis, this study sought to determine its upstream miRNAs. The TargetScan was used to predict the targets of WNT10B. Among the candidate target miRNAs, it was determined that there was complementarity between miR-33a-5p and WNT10B 3'-untranslated region (3'UTR). Luciferase reporter assay was conducted as described in a previous study [14].

Micro-computed tomography (micro-CT) analysis

At 45 or 90 days after surgery, the rats were intraperitoneally injected with pentobarbital sodium for anesthesia, and then fixed on the micro-CT stage to scan the distal living femur under a voltage of 80 kV and a current of 500 μA. Then bone histomorphometric analysis was conducted using the built-in software, and the parameters, BMD (mg/cm^2), bone volume relative to tissue volume (BV/TV, %), trabecula number (Tb.N, 1/mm) and trabecula thickness (Tb.Th, μm) were determined.

Statistical analysis

Statistical analysis was performed using SPSS statistical analysis software (version 26.0) software (IBM, Armonk, NY, USA). Data are presented as mean ± standard deviation (SD). Student’s t-test was used for analyzing measurement data and to determine differences between two groups. Comparison between multiple groups was done using one-way ANOVA followed by post hoc test (least significant difference). $P < 0.05$ was considered statistically significant.

RESULTS

BMD was significantly reduced in OVX group

Postmenopausal osteoporosis was simulated by the estrogen deficient OVX rat model. To confirm the effect of surgery on osteoporosis, at 45 or 90 days after surgery, the rats were sacrificed for the determination of BMD. As opposed to sham rats, OVX rats showed decreased BMD score (Figure 1), suggesting that osteoporosis model induced by ovariectomy has been established.

MiR-33a-5p was elevated and WNT10B decreased after ovariectomy

Compared to sham group, miR-33a-5p level was significantly higher in OVX group at 45 and 90 days after surgery (Figure 2 A - C). In addition, WNT10B decreased significantly ($p < 0.05$) after ovariectomy (Figure 2 D - F).

WNT10B was a target of miR-33a-5p

MiR-33a-5p expression was negatively correlated with WNT10B (Figure 3A). To further examine the underlying molecular mechanism by which miR-33a-5p regulated osteoporosis, the TargetScan was employed to ascertain target genes that have clarified functions in regulating osteoporosis. It was found that there was

complementarity between miR-33a-5p and WNT10B 3'UTR (Figure 3B). Results of the luciferase assays performed above (Figure 3 C) confirmed these findings.

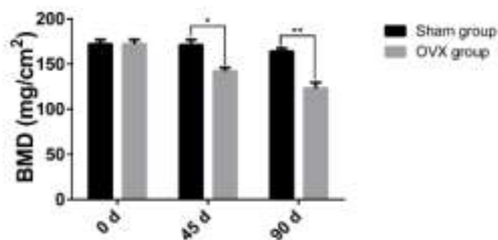

Figure 1: BMD of femur in control group was significantly higher than that in OVX group at 45 and 90 days after surgery (*$p < 0.05$)

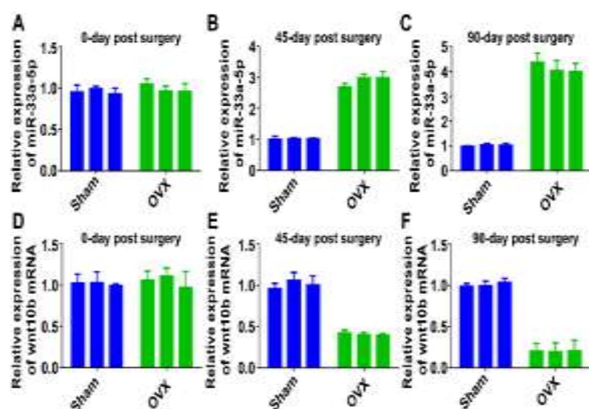

Figure 2: Expression of miR-33a-5p was significantly increased (A-C) whereas Wnt10b was significantly decreased (D - E) on days 45 and 90

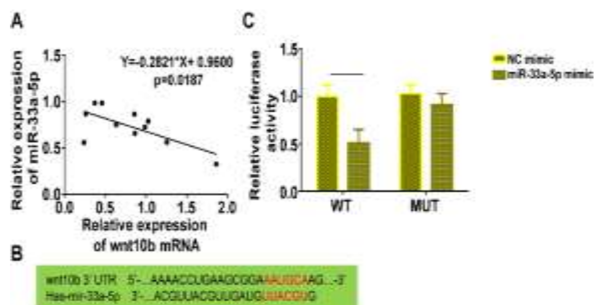

Figure 3: Correlation analysis of miR-33a-5p and Wnt10b expressions. (A) miR-33a-5p was negatively correlated with Wnt10b. (B) Schematic diagram of miR-33a-5p target site in the 3'UTR of Wnt10b. (C) Luciferase reporter showed Wnt10b as a target of miR-33a-5p

MiR-33a-5p inhibited osteogenic differentiation

RT-PCR was used to validate the impact of a mimic and an inhibitor on the expression levels of miR-33a-5p and WNT10B. The results showed that miR-33a-5p mimic increased miR-33a-5p level and decreased WNT10B level, whereas the inhibitor increased the mir-33a-5p level and increased WNT10B expression (Figure 4 A and B). The expressions of ALP and OCN in osteoblasts were determined via RT-PCR in MSCs transfected with miR-33a-5p mimic or miR-33a-5p inhibitor. Compared with the normal control, miR-33a-5p mimic significantly reduced the content of ALP and OCN ($p < 0.05$; Figure 4 C), whereas miR-33a-5p inhibitor significantly increased these osteoblast specific markers ($p < 0.05$; Figure 4 D).

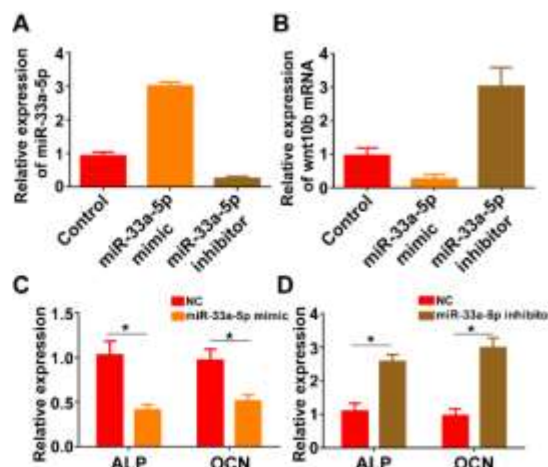

Figure 4: Role of miR-33a-5p in regulating MSC differentiation. (A, B) miR-33a-5p mimic increased miR-33a-5p while decreased Wnt10b level and miR-33a-5p inhibitor produced opposing results; (C) miR-33a-5p mimic significantly decreased the expressions of ALP and OCN; (D) miR-33a-5p inhibitor significantly increased the expression of ALP and OCN

MiR-33a-5p inhibitor promoted bone formation in rats

To determine the *in vivo* role of miR-33a-5p, the rats performed by sham surgery or OVX surgery were injected with miR-33a-5p inhibitor. The results showed that rats treated with miR-33a-5p showed increase in BMD as opposed to those in OVX group (Figure 5 A - C). Micro-CT was used to evaluate BV/TV, Tb.N, and Tb.Th and the results revealed that in OVX group, a significant decrease in BV/TV, Tb.N, and Tb.Th was occurred, while miR-33a-5p inhibitor up-regulated these bone formation indices (Figure 5 D - F).

DISCUSSION

Osteoporosis is a prevalent condition, especially among the elderly population, and finding effective treatments for this disease is of utmost importance. This study was aimed to investigate the role of miR-33a-5p in osteoporosis and its potential as a therapeutic target. The results

shed light on the underlying mechanisms and implications of miR-33a-5p in osteoporosis.

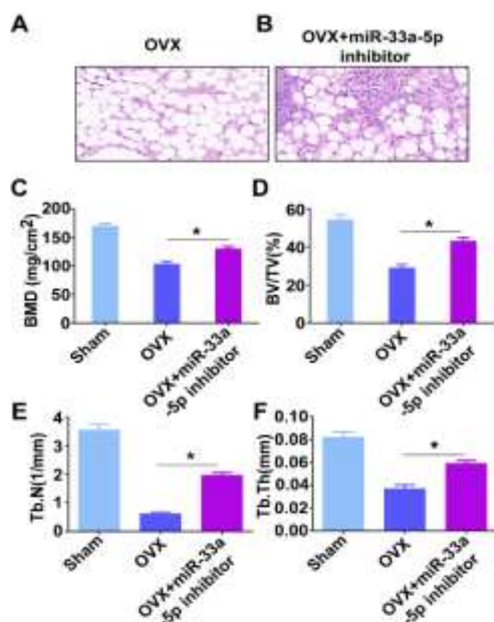

Figure 5: MiR-33a-5p inhibitor-treated rats showed increased bone formation. After 4 weeks, rats were injected miR-33a-5p inhibitor. (A - C) The miR-33a-5p improved the bone formation after ovariectomy compared to OVX group. (magnification: 400×). (D - F) miR-33a-5p inhibitor significantly up-regulated BV/TV, Tb.N. and Tb.Th ($p < 0.05$)

The findings demonstrate a significant reduction in bone mineral density (BMD) in the ovariectomized (OVX) group compared to the control group, confirming the successful establishment of an osteoporosis model. These results align with previous studies highlighting the detrimental effects of estrogen deficiency on bone health and the development of osteoporosis [15-17].

Dysregulation of miRNAs, including miR-33a-5p, has been implicated in various diseases, and their role in osteoporosis has gained increasing attention. We observed a significant elevation of miR-33a-5p expression in the OVX group compared to the control group, suggesting its involvement in the pathogenesis of osteoporosis. Moreover, the downregulation of WNT10B, a key player in bone metabolism, was observed after ovariectomy, indicating a potential link between miR-33a-5p and WNT10B in osteoporosis development [18].

Our study further elucidated the relationship between miR-33a-5p and WNT10B by demonstrating that miR-33a-5p directly targets WNT10B through complementary binding to its 3'UTR. This finding strengthens the notion that miR-33a-5p acts as a negative regulator of WNT10B expression, providing a molecular mechanism for the observed changes in bone metabolism.

To assess the functional consequences of miR-33a-5p dysregulation, *in vitro* experiments were conducted using primary bone marrow-derived mesenchymal stem cells (BMMSCs). The inhibition of miR-33a-5p led to enhanced osteogenic differentiation, as evidenced by the upregulation of osteoblast-specific markers ALP and OCN. These results suggest that miR-33a-5p exerts inhibitory effects on osteogenic differentiation, potentially contributing to the development of osteoporosis.

Furthermore, *in vivo* experiments utilizing miR-33a-5p inhibitor-treated rats provided valuable insight into the therapeutic potential of targeting miR-33a-5p in osteoporosis. Treatment with miR-33a-5p inhibitor resulted in increased bone formation, as indicated by improved BMD, and enhanced bone parameters, including BV/TV, Tb.N, and Tb.Th. These findings highlight the promising role of miR-33a-5p inhibition in promoting bone formation and mitigating bone loss associated with osteoporosis.

In the context of existing literature, this study contributes to the growing body of evidence implicating miRNAs in the regulation of osteoporosis. While previous studies explored the roles of various miRNAs in osteoporosis, limited research has focused on the specific involvement of miR-33a-5p in this context. The present study fills this knowledge gap by providing novel insights into the inhibitory role of miR-33a-5p in osteogenic differentiation and its direct targeting of WNT10B.

The implications of these findings extend beyond fundamental research and offer potential applications in the development of osteoporosis therapeutics. By identifying miR-33a-5p as a key regulator of bone formation, our study opens avenues for the design of targeted interventions that modulate miR-33a-5p activity or promote WNT10B.

CONCLUSION

The findings of this study reveal that miR-33a-5p plays a significant role in the pathogenesis of osteoporosis by downregulating WNT10B expression. Dysregulation of miR-33a-5p inhibits osteogenic differentiation and contributes to the bone loss observed in osteoporosis. Targeting miR-33a-5p may hold promise as a therapeutic strategy for promoting bone formation and mitigating osteoporosis. These findings expand

our understanding of the molecular mechanisms involved in osteoporosis, and provide a foundation for future research and the development of novel therapeutic interventions for this prevalent bone disease.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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