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MiR-137 affects bone mineral density in osteoporosis rats through regulating RUNX2

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Abstract. – OBJECTIVE: To study the influence of micro ribonucleic acid (miR)-137 on osteoporosis rats by regulating runt-related transcription factor 2 (RUNX2).

MATERIALS AND METHODS: A total of 36 Sprague-Dawley rats were randomly assigned to the normal group (n=12), model group (n=12), and inhibitor group (n=12). No treatment was performed in the normal group. The osteoporosis model in rats was prepared in the model group, and miR-137 inhibitor was administered in osteoporosis rats of inhibitor group. Following 12 weeks of intervention, sampling was conducted. The expression of RUNX2 was detected *via* immunohistochemistry, and its protein expression level was determined *via* Western blotting. Quantitative Polymerase Chain Reaction (qPCR) was carried out to detect the mRNA level of miR-137. The contents of serum bone Gla protein (BGP) and total alkaline phosphatase (TALP) were measured using enzyme-linked immunosorbent assay (ELISA). Finally, bone mineral density was determined with a dual-energy X-ray absorptiometry instrument.

RESULTS: According to the immunohistochemistry detection, the rats in model group and inhibitor group had a notably lower positive expression level of RUNX2 than normal group ($p<0.05$), and its expression level in the inhibitor group was substantially higher than that in the model group ($p<0.05$). Western blotting results showed that compared with that in the normal group, the protein expression level of RUNX2 was notably lowered in the model and inhibitor group ($p<0.05$), which was markedly higher in the inhibitor group than that in the model group ($p<0.05$). It was found through the qPCR that the expression level of miR-137 was remarkably raised in both model group and inhibitor group compared with that in the normal group, showing statistically significant differences ($p<0.05$). The rats in the inhibitor group had a remarkably lower expression level of miR-137 than the model group ($p<0.05$). ELISA results revealed that the model group and inhibitor group had substantially lower contents of serum BGP and TALP than the normal group ($p<0.05$), and that their contents rose dramatically in the inhibitor group compared with that in the model group ($p<0.05$). Additionally, based on the measurement of bone mineral density, compared with that in the normal group, bone mineral density declined considerably in the model group and inhibitor group ($p<0.05$). It was markedly elevated in inhibitor group in comparison with that in the model group ($p<0.05$).

CONCLUSIONS: MiR-137 regulates RUNX2 to affect the bone mineral density of osteoporosis model rats.

Key Words
Osteoporosis, RUNX2, MiR-137.

Introduction

With the increase in the aging population, and changes in people's lifestyles, the morbidity rate of osteoporosis, a clinically common bone metabolism abnormality disease, is growing year by year[1,2]. According to the definition by the World Health Organization, osteoporosis is characterized by bone mass loss and destruction of bone microstructure, thus increasing bone fragility and incidence rate of fracture[3]. Osteoporosis-induced fracture seriously affects the elderly, and it tends to cause long-term immobilization and various complications to old people, affecting the quality of life of patients and even their life health. Therefore, osteoporosis has gradually become a research hotspot worldwide.

As studies of osteoporosis progress, it has gradually been recognized that imbalanced bone destruction and bone formation make the function of osteoclasts more potent than that of osteoblasts and ultimately results in bone mass loss and decrease in bone mineral density. Runt-related transcription factor 2 (RUNX2) is one of the most important transcription factors involved in the differentiation of bone marrow mesenchymal stem cells into osteoblasts. RUNX2 regulates the gene transcription of relevant crucial proteins in the differentiation of bone marrow mesenchy-

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mal stem cells, contributing to their differentiation into osteoblasts[4,5]. RUNX2 is, therefore, considered as a novel target for the treatment of osteoporosis. Additionally, as a class of non-coding ribonucleic acids (RNAs) containing about 18-25 nucleotides, microRNAs (miRNAs) bind to the 3' non-coding region of the target RNAs to inhibit the translation of the target RNAs[6-9]. In the miRNA family, miR-137 can modulate osteogenesis and plays a vital role in the post-fracture bone morphogenesis and bone formation[10], but its regulatory mechanism in osteoporosis is still not very clear.

Thus, the present study aims to investigate the influence of miR-137 on osteoporosis rats *via* the regulation of RUNX2 and further elucidate the important role of miR-137 in osteoporosis and its underlying mechanism.

Materials and Methods

Laboratory Animals and Grouping

A total of 36 female Sprague-Dawley rats weighing (220±20) g [Shanghai SLAC Laboratory Animal Co., Ltd., License No.: SCXK (Shanghai) 2014-0003, Shanghai, China] were randomly divided into normal group (n=12), model group (n=12), and inhibitor group (n=12). This study was approved by the Animal Ethics Committee of Central South University Animal Center.

Experimental Reagents and Instruments

The main reagents and instruments were: dexamethasone injection (CSPC, Shijiazhuang, China), miR-137 inhibitor agomir-137 (MCE, Monmouth Junction, NJ, USA), anti-RUNX2 primary and secondary antibodies (Abcam, Cambridge, MA, USA), bone Gla protein (BGP), and total alkaline phosphatase (TALP) enzyme-linked immunosorbent assay (ELISA) kits (BOSTER Biological Technology Co., Ltd., Wuhan, China), AceQ quantitative polymerase chain reaction (qPCR) SYBR Green Master Mix kit and HiScript II Q Reverse Transcriptase SuperMix for qPCR [+genomic deoxyribonucleic acid (gDNA) wiper] kit (Vazyme Biotechnology, Nanjing, China), optical microscope (Leica DMI 4000B/DFC425C, Wetzlar, Germany), fluorescence qPCR instrument (ABI 7500, Applied Biosystems, Foster City, CA, USA), and Image-Pro image analysis system (Bio-Rad, Hercules, CA, USA).

Modeling

The rats were intraperitoneally injected with dexamethasone injection at a dose of 0.6 mg/kg/3 d for 12 consecutive weeks to establish the osteoporosis model.

Rat Treatment in Each Group

The rats in the normal group were given an equal volume of normal saline through intraperitoneal injection and normally fed without any treatment. The rats in the model group underwent the above procedures for preparation of the osteoporosis model, and those in the inhibitor group were intraperitoneally injected with dexamethasone injection and 10 nmol agomir-137. At 12 weeks after treatment, sampling was performed for detection.

Sampling

Upon successful anesthesia, blood was first drawn from the abdominal aorta of all the rats. Then, 6 rats in each group were fixed through paraformaldehyde perfusion, and lumbar and femoral tissues were obtained. The tissues were fixed in 4% paraformaldehyde at 4°C for 48 h and prepared into paraffin-embedded tissue sections for immunohistochemistry detection. Besides, lumbar and femoral tissues were directly sampled from the remaining 6 rats in each group and placed in Eppendorf (EP) tubes for Western blotting and qPCR.

Immunohistochemistry Detection

Paraffin-embedded tissues were sliced into 5 μm-thick sections, placed in warm water at 42°C for extending, mounting, and baking. Then, the tissue sections were soaked in the xylene solution and gradient ethanol successively for de-paraffinization and hydration. Subsequently, the sections were immersed in citrate buffer and heated repeatedly in a microwave oven for 3 times (heating for 3 min and braising for 5 min per time) for complete antigen retrieval. After rinsing, the tissue sections were added dropwise with endogenous peroxidase blocker for 10 min of reaction, rinsed, and sealed with goat serum for 20 min. With the goat serum blocking solution discarded, the tissue sections were incubated with the anti-RUNX2 primary antibody (1:200) in a refrigerator at 4°C overnight. On the next day, the sections were rinsed, incubated with secondary antibody for 10 min, and fully rinsed. Subsequently, they were reacted with streptomycin avidin-peroxidase solution for 10 min and color development using diaminobenzidine (DAB; Solarbio, Beijing,

China). Finally, cell nuclei were counterstained using hematoxylin, and the sections were sealed and observed.

Western Blotting

The lumbar and femoral tissues stored at an ultralow temperature were added with lysis buffer, bathed on ice for 1 h and then centrifuged at 14,000 g for 10 min. Protein concentration was determined using BCA. The proteins were denaturalized and isolated *via* sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) that was terminated when the marker protein stayed at the bottom of the glass plate in a straight line. Subsequently, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and blocked with sealing solution for 1.5 h. The membranes were incubated successively with anti-RUNX2 primary antibody (1:1,000) and secondary antibody (1:1,000). After rinsing, images were fully developed in the dark by reacting with a chemiluminescent reagent for 1 min.

QPCR

The total RNA was first extracted and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using a reverse transcription kit. Then, qPCR was conducted in a reaction system (20 μL) under the following conditions: reaction at 51°C for 2 min, pre-degeneration at 96°C for 10 min, degeneration at 96°C for 10 s, and annealing at 60°C for 30 s, for 40 cycles. The relative expression levels of the related messenger RNAs (mRNAs) were calculated, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal reference. The primer sequences were shown in Table I.

Measurement of Bone Mineral Density

The femoral bone mineral density was measured using the dual-energy X-ray absorptiometry instrument. Femurs fixed using paraformaldehyde was placed in the instrument with the variable coefficient set as <1.0% to measure the femoral bone mineral density.

ELISA

The abdominal aortic blood was centrifuged at 14,000 g in a high-speed centrifuge for 10 min, and the supernatant was obtained. Then, according to the instructions of the ELISA kit, the samples and standard were separately loaded into a plate and added with biotinylated antibody working solution and enzyme conjugate working solution, and the plate was washed. Finally, the products were detected at 450 nm using a microplate reader.

Statistical Analysis

In this study, the Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA) was employed for statistical analysis. Enumeration data were expressed as mean ± standard deviation. The *t*-tests were performed for data conforming to normal distribution and homogeneity of variance. The comparison between groups was done using One-way ANOVA test, followed by the post-hoc test (Least Significant Difference). Ranked data and enumeration data were subjected to the rank sum test and chi-square test, respectively. p-values < 0.05 were considered statistically significant.

Results

Immunohistochemistry Detection Results

Tan represents the positive expression, and the normal group exhibited higher positive expression of RUNX2 than the model and inhibitor groups (Figure 1). According to the statistical results (Figure 2), compared with that in the normal group, the average optical density of positively expressed RUNX2 declined substantially in the model and inhibitor groups, showing statistically significant differences (p<0.05). Its average optical density in the inhibitor group was higher than that in the model group, with a statistically significant difference (p<0.05).

Table I. Primer sequence.

Name	Primer sequence
MiR-137	Forward: 5' TCCACCAAGAAGCTGAGCGAG 3' Reverse: 5' GTCCAGCCCATGATGGTTCT 3'
GAPDH	Forward: 5' ACGGCAAGTTCAACGGCACAG 3' Reverse: 5' GAAGACGCCAGTAGACTCCACGAC 3'

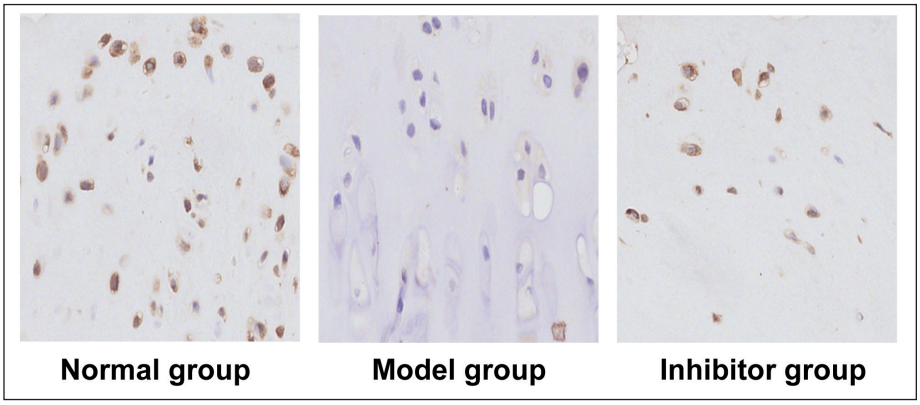

Figure 1. RUNX2 expression detected *via* immunohistochemistry (×200).

Expressions of Related Proteins Detected Via Western Blotting

As shown in Figure 3, the protein level of RUNX2 was higher in the normal group than the model and inhibitor groups. The statistical results revealed that the relative protein expression of RUNX2 was substantially lowered in the model and inhibitor groups compared with that in the normal group, with statistically significant differences (p<0.05). Protein expression of RUNX2 in the inhibitor group was remarkably higher than that in the model group, showing a statistically significant difference (p<0.05) (Figure 4).

Expression Level of Relevant mRNA Determined Via qPCR

Compared with that in the normal group, the relative expression of miR-137 markedly rose in the model and inhibitor groups, with statistically significant differences (p<0.05). Its relative expression level in the inhibitor group was remarkably higher than that in the model group, displaying a statistically significant difference (p<0.05) (Figure 5).

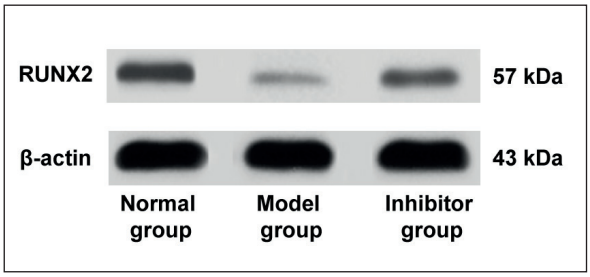

Figure 3. Protein expression of RUNX2 detected *via* Western blotting.

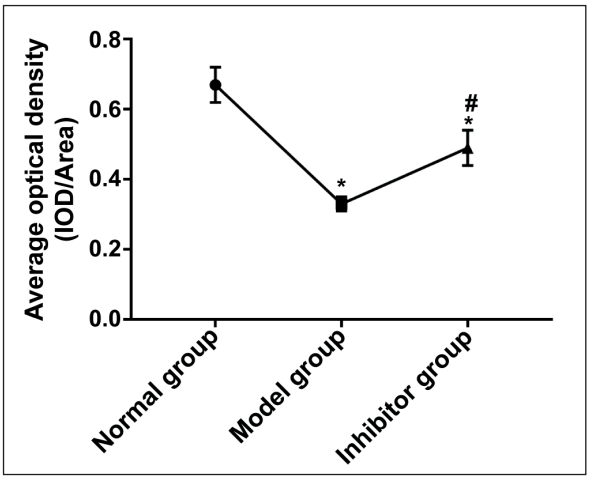

Figure 2. Average optical density of positively expressed RUNX2 in each group. Note: *p<0.05 *vs.* normal group, and #p<0.05 *vs.* model group.

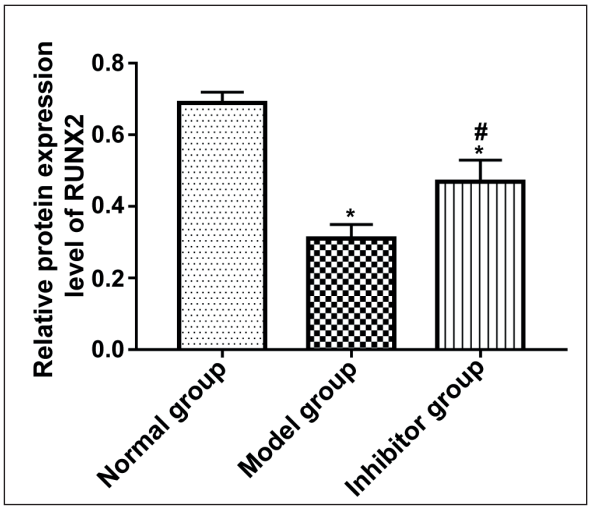

Figure 4. Relative protein expression level of RUNX2 in each group. Note: *p<0.05 *vs.* normal group, and #p<0.05 *vs.* model group.

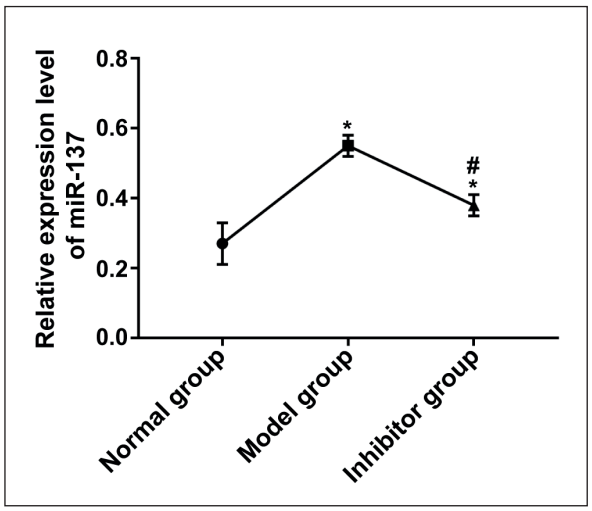

Figure 5. Relative expression level of miR-137. Note: *p<0.05 *vs.* normal group, and #p<0.05 *vs.* model group.

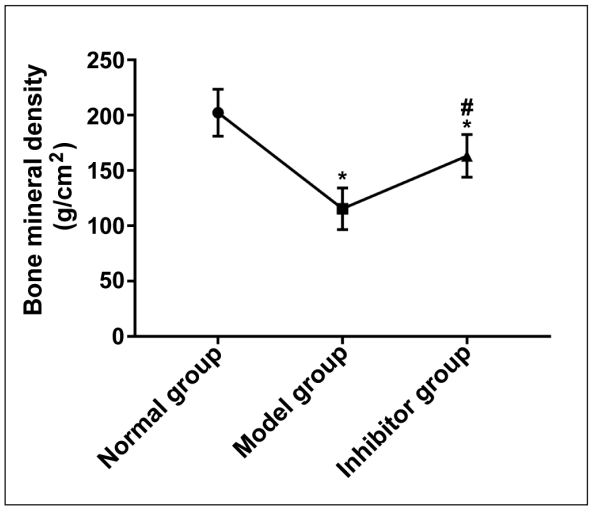

Figure 7. Bone mineral density in each group. Note: *p<0.05 vs. normal group, and #p<0.05 *vs.* model group.

ELISA Results

The rats in the model group and inhibitor group had notably lower contents of serum BGP and TALP than the normal group, with statistically significant differences (p<0.05). Their contents in the inhibitor group were markedly higher than that in the model group, showing statistically significant differences (p<0.05) (Figure 6).

Bone Mineral Density

Compared with that in the normal group, bone mineral density was notably lowered in both model group and inhibitor group, displaying statistically significant differences (p<0.05). Bone mineral density in the inhibitor group was substantially higher than that in the model group, with a statistically significant difference (p<0.05) (Figure 7).

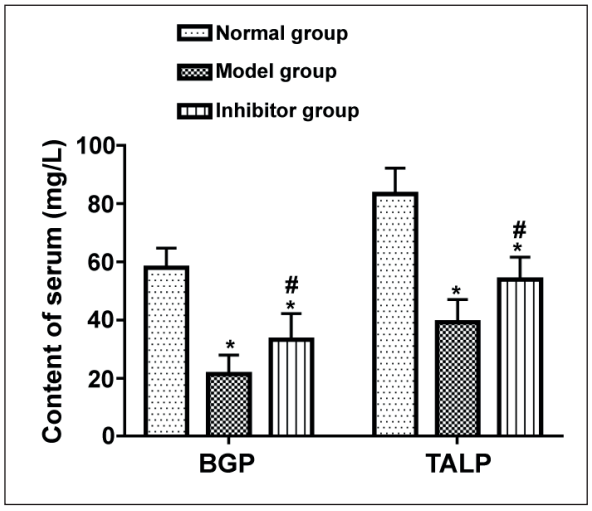

Figure 6. Contents of serum BGP and TALP. Note: *p<0.05 *vs.* normal group, and #p<0.05 *vs.* model group.

Discussion

Osteoporosis is now considered as a common disease in the elderly, especially postmenopausal elderly women. The endocrine disorder caused by the degradation of ovarian function affects bone metabolism, leading to increased bone fragility and damage the internal structure of bone tissues over time[11,12]. Besides, osteoporosis caused by the use of hormone drugs has received growing special attention from medical staff. The increase in bone tissue fragility causes a fracture in the damaged bone, which further induces malformation and function loss, and affects heart and lung functions in severe cases. In particular, due to osteoporosis-induced fracture and malformation, the length of stay is prolonged, which seriously affects the living quality of them and even enhances mortality. Further research has demonstrated that[13,14] RUNX2, an important member in the RUNX family, is able to bind to the target gene containing an osteoblast-specific cis-acting element (OSE) to trigger transcription. It exerts a crucial regulatory role in the maturity and differentiation of bone marrow mesenchymal stem cells and osteoblast proliferation. Based on the results of some studies[15,16], RUNX2 can bind to OSE under normal physiological conditions to further regulate the expressions of the substances that

are closely related to osteoblast proliferation and differentiation, such as osteopontin, bone morphogenetic protein, and osteocalcin. Eventually, RUNX2 mediates the differentiation of the bone marrow mesenchymal stem cells into osteoblasts, and accelerates osteoblast differentiation and osteogenesis, as well as increases bone mass. In this study, RUNX2 was abnormally lowly expressed, and bone mineral density was dramatically lowered in the hormone-induced osteoporosis model rats. It is suggested that RUNX2 was involved in the onset of hormone-induced osteoporosis, and its aberrant low expression was associated with the decline in bone mineral density.

The latest studies have manifested that miRNAs are a class of non-coding RNAs that can bind to their target genes to degrade the target genes or inhibit their translation and modulate transcription, which have become research hotspots in the fields of cancer, cardiovascular disease, bone disease, etc.[17-19]. Amongst them, miR-137 plays an important role in bone morphogenesis and fracture healing[20]. MiR-137 can regulate several downstream cellular signaling pathways to modulate the transcription and expressions of the downstream genes, thereby acting as a crucial player. According to the findings in the present study, the relative expression level of miR-137 was substantially elevated in the bone tissues of the hormone-induced osteoporosis model rats. The silence of miR-137 increased bone mineral density and BGP and TALP contents in osteoporosis rats. The above results indicated that miR-137 is implicated in the onset of osteoporosis and closely related to the decline in the bone mineral density in the osteoporosis model rats. Meanwhile, aberrant low expression of RUNX2 was detected in the bone tissues of the osteoporosis model rats, which could reduce osteoblasts and lower bone mineral density. As the downstream effector molecule, RUNX2 can be regulated by miR-137. It was discovered in this study that the high expression of miR-137 in the bone tissues of the hormone-induced osteoporosis model rats lowered the expression of RUNX2, while the expression of RUNX2 was raised after the knockdown of miR-137.

Conclusions

In summary, it can be concluded that miR-137 affects the bone mineral density of osteoporosis model rats by modulating RUNX2.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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