Mir-152 Regulates Osteoblast Apoptosis, Proliferation and Differentiation through Targeted Inhibition of Runx2 in Osteoporosis Running Title: Role and Potential Mechanism of Mir-152 in Osteoporosis

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Objective: To explore the role and potential mechanism of miR-152 in osteoporosis. Methods: Fifty-four osteoporotic patients and 54 healthy subjects were recruited from August 2017 to January 2019. Serum samples of the two groups were obtained, and the miR-152 expression in serum was detected and compared. The human osteoblast cell line hFOB1.19 was obtained and miR-152 in cells was increased. The biological behavior changes such as cell proliferation, apoptosis and differentiation were observed by MTT, flow cytometry and detection of osteoblast differentiation markers (ALP, OCN). Results: miR-152 was elevated in osteoporosis patients, and AUC value of serum miR-152 in diagnosing osteoporosis was 0.939. After miR-152 in osteoblasts was elevated, cell proliferation was inhibited, cell apoptosis rate increased, and ALP and OCN content in cells reduced, while increasing cell RUNX2 simultaneously was totally different. Dual luciferase report showed that RUNX2 could be targeted and regulated by miR-152. Conclusion: miR-152 is elevated in serum of osteoporosis patients and can be used as a biological indicator for diagnosing osteoporosis. In addition, miR-152 can inhibit osteoblast proliferation, differentiation and induce apoptosis through negative regulation of RUNX2.

Keywords: miR-152, osteoporosis, RUNX2, osteoblast
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Osteoporosis is a progressive bone disease characterized by low bone mass and poor microstructure of bone tissue [1]. Around the world, about 200 million patients suffer from osteoporosis. Unbearable pain and various complications seriously affect the quality of life of patients [2]. According to reports, with the aging population getting more and more serious, the number of osteoporosis patients will also continue to grow [3]. Compared with normal people, osteoporosis patients have a higher incidence of fractures, which will undoubtedly have negative effects on public and human health [4]. Therefore, it is necessary to clarify the pathogenesis of osteoporosis and find potential therapeutic targets.

miR is a kind of highly conservative endogenous short-chain non-coding RNA, which can save one third of human genes [5]. With the continuous understanding of miR function, it has been found that miR has obvious abnormal expression in various orthopedic diseases and can participate in the development of these diseases. For example, miR-495 is up-regulated in osteoarthritis and can promote disease progression by regulating AKT1 [6]. miR-106b can inhibit osteoblast differentiation and bone formation by negatively regulating BMP2 expression [7]. miR-142-5p can promote fracture healing in aged mice by stimulating osteoblast

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activity [8]. miR-152 is a miR widely reported recently, because it acts as an anti-cancer in ovarian cancer, breast cancer, endometrial cancer and other tumors, and is considered as a vital target for treatment [9]. In osteoporosis related reports, some studies have found that miR-152 is elevated in postmenopausal osteoporosis and fragile fracture patients [10]. This suggests that miR-152 is also very likely involved in the development of osteoporosis. Osteoblast is the main cell type of bone formation and plays an important role in metabolic balance, growth and injury repair of bone tissue [11]. Previous studies have found that miR can interfere with the biological behavior of osteoblasts by regulating the expression of its target genes, thus participating in the pathogenesis of osteoporosis [12, 13]. We suspect that miR-152 can also participate in the pathogenesis of osteoporosis in the same way.

In this study, we will explore the role and potential mechanism of miR-152 in osteoporosis through a series of experiments. Our results may be helpful to further understand the pathogenesis of osteoporosis.

DATA AND METHODS

General Information

Fifty-four patients with osteoporosis admitted to our hospital from August 2017 to January 2019 were selected as the research participants. Fifty-four patients were recruited for physical examination in our hospital during the same period. There was no statistical difference in age, gender, BMI and other general data between patients and health subjects. Venous blood of both groups was obtained 8 h after fasting and sent to the examination room for centrifugation to collect serum. Next, miR-152 content was tested. Inclusion criteria: patients met the diagnostic criteria for osteoporosis [14], i.e. T score of femoral neck and/or lumbar vertebra ≤ -2.5; patients had complete clinical data; patients signed an informed consent form. Exclusion criteria: patients took hormones and other drugs that affect bone metabolism in the near future; patients suffered from endocrine metabolic diseases such as diabetes, renal insufficiency and hyperparathyroidism; patients had tumors. This study was approved and implemented by the Ethics Committee of our hospital.

Hfob1.19 Cells

The human osteoblast cell line hFOB1.19 was obtained from the American ATCC cell bank and placed in DMEM medium (Gibco, USA) containing 10% fetal bovine serum (PBS, Gibco, USA). The medium was placed in a 37℃, 5% CO_2 incubator and logarithmic growth cells were taken for subsequent experiments. Cell transfection: miR-152 and RUNX2 overexpression or inhibition plasmids were respectively established using pcDNA 3.1 plasmids as vectors, and different plasmids were respectively transfected into cells through Lipofectamine™ 2000 kit (Invitrogen, USA) and cultured right along.

Qrt-Pcr

The content of miR-152 in the collected serum and cells was detected by qRT-PCR, and the total RNA was extracted based on the instructions of Trizol kit (Invitrogen, USA), and its purity, concentration and integrity were detected by ultraviolet spectrophotometer and agarose gel electrophoresis. Altogether 2 μg of total RNA was taken and reverse transcribed into cDNA using reverse transcription kit (Invitrogen, USA). PrimeScript RT Master Mix kit was used for amplification (Takara Bio, Japan). The amplification system was as follows: 10 μL SYBR qPCR Mix, upstream and downstream primers 0.8 μL each, 2 μL cDNA product, 0.4 μL 50×ROX reference dye, RNase-free water made up to 20 μL. PCR reaction conditions were as follows: pre-denaturation at 95℃ for 60 s, denaturation at 95℃ for 30 s, annealing extension at 60℃ for 40 s, a total of 40 cycles. The miR-152 expression was calculated via $2^{-\Delta\Delta ct}$ [15].

Western Blot (Wb)

RIPA was used to lyse cells to be tested and BCA method was used to detect protein concentration (Thermo Fisher, USA). Protein concentration was adjusted to 4 μg/μL, and protein was separated by 10% SDS-PAGE protein (V100). Then, it was transferred to PVDF membrane, soaked 5 min by TBST, washed, and sealed 2 h with 5% defatted milk powder. RUNX2, ALP, OCN and β-catenin (Abcam, USA) primary antibody were added, and the dilution ratio was 1: 1000. It was incubated overnight at 4℃ and then rinsed 3 times with TBST. Goat anti-mouse secondary antibody (Abcam, USA) with dilution ratio of 1: 4000 was added, and it was incubated 2 h at 37℃ and rinsed 3 times with TBST. It developed in dark room using chemiluminescence (ECL) method, and the relative expression of each protein was analyzed by Quantity One.

Cell Proliferation Detection

Cell proliferation was measured by MTT assay (Beyotime Biotechnology Co., Ltd., China). Twenty-four hours after transfection, target cells were taken and cultured in 96-well plates at a cell density of 2.5×10^3 per well. After cells were cultured for 24, 48, 72 and 96 h respectively, 50 μL MTT and 150 μL of culture medium were added to each well of the cell culture plate, and the supernatant was discarded after culturing at 37℃ for 4 h. Next, 200 μL of DMSO solution was added dropwise to each well of the cell culture plate to dissolve MTT 30 min. The absorbance at

570nm was detected with an automatic enzyme reader (Bio-Rad, Hercules, CA, USA).

Apoptosis Detection

Transfected cells were collected and digested with 0.25% trypsin. After digestion, they were prepared into 1×10^6 /mL suspension, followed by 10μL AnnexinV-FITC/PI (Shanghai Yisheng Biotechnology Co., Ltd.), incubated 5min at indoor temperature in the dark, and analyzed by flow cytometry, and the apoptosis rate was calculated.

Dual Luciferase Report

The potential target gene of miR-152 was predicted using the biological prediction website miRDB (http://www.mirdb.org/). The RUNX2-3'UTR wild type (Wt) and RUNX2-3'UTR mutant (Mut) were respectively established usingLipofectamine™ 2000 kit, and then RUNx2-3' UTR mutant, miR-152-mimics and miR-NC were transferred into HEK293 cells (ATCC). The change of luciferase activity was detected by dual-Lucy Assay Kit (Solarbio, CA, China).

Statistical Methods

The research data were statistically processed via SPSS18.0, and the required pictures were drawn via GraphPad 7. The comparison between the two groups was assessed via independent-samples t test, comparison between multiple groups was assessed via one-way analysis of variance (ANOVA), post hoc pairwise comparison was analyzed via LSD-t test, multi-time point expression was assessed via repeated measures ANOVA, and back testing was under Bonferroni. There was statistical difference when $P < 0.05$.

RESULTS

Mir-152 Elevates in Serum of Osteoporosis Patients

miR-152 in serum of patients and health subjects was detected through qRT-PCR, and its expression in serum of the former was higher than that of the latter. After drawing ROC curve of serum miR-152 for diagnosing osteoporosis, we found that AUC value was 0.939.

Figure 1: expression and diagnostic value analysis of miR-152 in osteoporosis

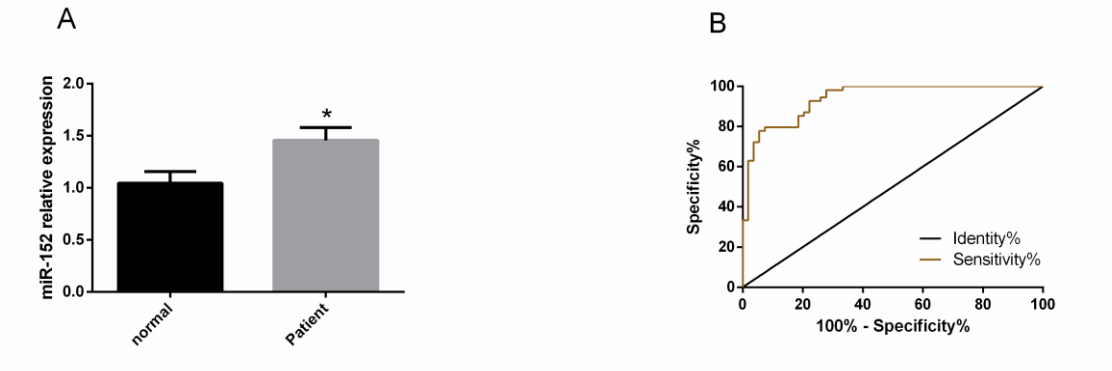

Figure A: miR-152 expression in serum of patients is higher than that of physical examinees. Figure B: ROC curve of serum miR-152 in diagnosing osteoporosis. Note: * represents $P < 0.05$ compared with physical examinees.

Mir-152 Can Inhibit Proliferation and Differentiation of Hfob1.19 Cells

hFOB1.19 successfully increased miR-152 in cells after transfecting miR-152-mimics. MTT assay revealed that cell proliferation was inhibited after transfecting miR-152-mimics. Flow cytometry showed that the apoptosis rate of cells transfected with miR-152-mimics increased. WB detection of osteoblast differentiation markers showed that hFOB1.19 cells were transfected with miR-152-mimics, and the contents of ALP and OCN in the cells decreased. (Figure 2)

Figure 2: effect of increasing miR-152 on proliferation, apoptosis and differentiation of hFOB1.19

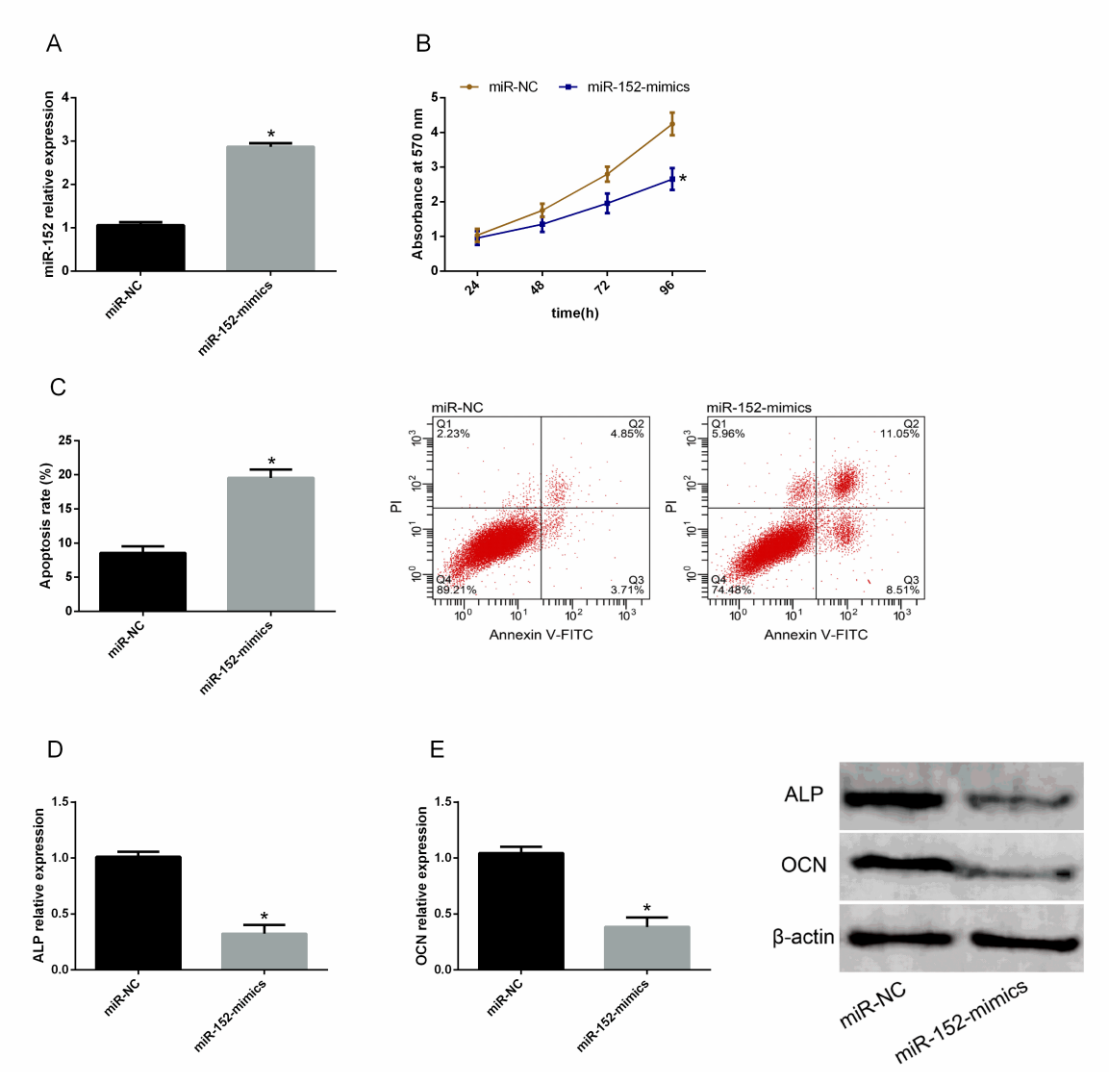

Figure A: After hFOB 1.19 was transfected with miR-152-mimics, miR-152 in cells increased. Figure B: After hFOB 1.19 was transfected with miR-152-mimics, cell proliferation was inhibited. Figure C: After hFOB 1.19 was transfected with miR-152-mimics, the apoptosis rate increased. Figure D/E: After hFOB 1.19 was transfected with miR-152-mimics, the contents of ALP and OCN in cells decreased. Note: compared with miR-NC group, * represents $P < 0.05$.

Mir-152 Can Regulate Runx2 Targetedly

miR can affect cell biological behavior through regulation of target genes. In order to further explore the mechanism of miR-152 regulating hFOB1.19 cell biological behavior, we found that RUNX2 and miR-152 have targeted binding sites through miR target gene prediction. Therefore, we detected the changes of RUNX2 in transfected miR-NC and miR-152-mimics cells and found that RUNX2 decreased after the cells were transfected with miR-152-mimics. Moreover, dual luciferase report experiment found that miR-152-mimics could inhibit the fluorescent activity of RUNX2-3'UTR Wt and had no obvious influence on the activity of RUNX2-3'UTR Mut. (Figure 3)

Figure 3: miR-152 can regulate RUNX2 targetedly

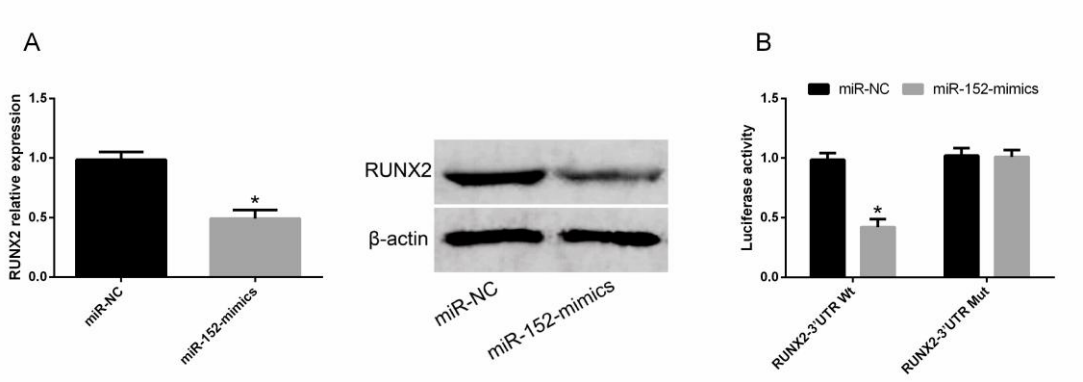

Figure A: After hFOB 1.19 was transfected with miR-152-mimics, RUNX2 in cells decreased. Figure B: miR-152-mimics can inhibit RUNX2-3'UTR Wt fluorescence activity and has no obvious effect on RUNX2-3'UTR Mut activity. Note: compared with miR-NC group, * represents $P < 0.05$.

Increasing Runx2 Can Weaken Mir-152'S Effect on Proliferation and Differentiation of Hfob1.19 Cells

In order to find out whether miR-152 can affect the biological behavior of hFOB1.19 cells by regulating RUNX2, we transfected hFOB1.19 cells with miR-152-mimics while carrying out sh-RUNX2 treatment to observe whether such treatment can reverse or weaken proliferation, apoptosis and differentiation changes of hFOB1.19 cells caused by transfection of miR-152-mimics. Our results displayed that compared with cells transfected with miR-152-mimics, those transfected with miR-152-mimics+sh-RUNX2 had higher RUNX2, higher cell proliferation, lower apoptosis rate, and lower ALP and OCN contents. (Figure 4)

Figure 4: effect of simultaneous increase of RUNX2 and miR-152 on proliferation, apoptosis and differentiation of hFOB1.19 cells

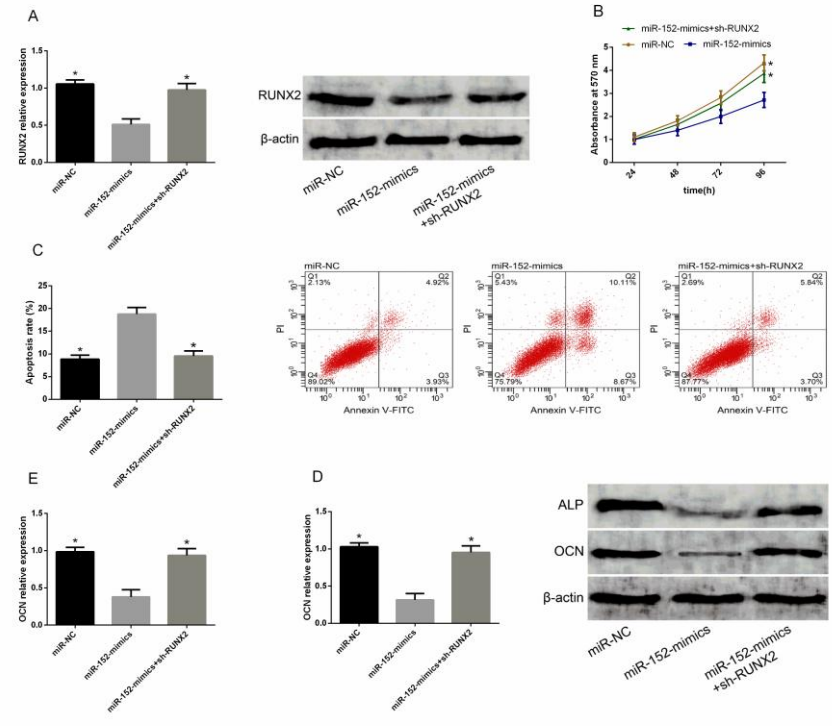

Figure A: RUNX2 in transfected miR-152-mimics+sh-RUNX2 cells was higher than that in transfected miR-152-mimics cells. Figure B: Compared with cells transfected with miR-152-mimics, those transfected with miR-152-mimics+sh-RUNX2 had enhanced proliferation. Figure C: Compared with cells transfected with miR-152-mimics, the apoptosis rate of those transfected with miR-152-mimics+sh-RUNX2

increased. Figure D: Compared with cells transfected with miR-152-mimics, ALP and OCN contents in those transfected with miR-152-mimics+sh-RUNX2 reduced. Note: compared with miR-152-mimics group, * represents $P < 0.05$.

DISCUSSION

Osteoporosis is a disease related to aging. As the population aging becomes more and more serious, the number of osteoporosis patients is also increasing, which has caused serious burden to individual families and society [16]. Hence, studying the pathological process of osteoporosis has important social and economic value. In this study, we have partially revealed the role and potential mechanism of miR-152 in osteoporosis. The results showed that miR-152 was higher in serum of osteoporosis patients than that of healthy subjects, and the AUC value of serum miR-152 in diagnosing osteoporosis was 0.939. Increasing miR-152 in osteoblasts can inhibit cell proliferation, differentiation and induce apoptosis, which was tied to its negative regulation of RUNX2.

More and more evidences show that the abnormal expression of miR in human body is one of the vital factors inducing diseases or affecting the development of diseases [17, 18]. The pathogenesis of osteoporosis is still not fully known, but studies have found that various miR play a vital role in disease development and progression [19, 20]. miR-152 is a crucial member of miR. Previous studies have found that it is elevated in patients with osteoporosis [10], so does this study. The two results can be mutually demonstrated. This suggests that miR-152 may be involved in the pathogenesis of osteoporosis. Osteoporosis patients have no obvious clinical manifestations before easy fracture without obvious inducement, therefore, they are easy to miss the best treatment period [21]. Studies have found that miR abnormally expressed in serum can be used as a potential marker for diagnosing various diseases including osteoporosis [22-24]. Thus, we analyzed the diagnostic effect of serum miR-152 on osteoporosis, and found that the AUC value of miR-152 for osteoporosis diagnosis was 0.939, which indicated that miR-152 had potential as a biological indicator for osteoporosis diagnosis.

Decrease of osteoblast proliferation, increase of apoptosis and abnormal differentiation are one of the important reasons for the development and progression of osteoporosis [25]. It is well known that miR can regulate many cell biological processes, such as cell proliferation, migration, apoptosis, differentiation and metabolism [26]. This study analyzes miR-152's role in osteoporosis by observing the changes of cell proliferation, apoptosis, differentiation and other biological behaviors after increasing miR-152 in osteoblasts. Through MTT and flow cytometry, we found that proliferation of osteoblasts was inhibited and the apoptosis rate increased after miR-152 in osteoblasts increased. In the process of osteoblast differentiation, it involves the regulation of various related proteins [27]. ALP and OCN are vital indicators of osteoblast differentiation. The former is a calcium binding transporter whose expression gradually increases with the degree of cell differentiation [28]; the latter is a non-collagen protein, which is essential for bone mineralization [29]. In our experiment, we detected ALP and OCN in osteoblasts and found that miR-152 inhibitor could inhibit the expression of both ALP and OCN. This indicated that miR-152 could affect osteoblast proliferation, apoptosis and differentiation to participate in the progress of osteoporosis.

miR could participate in biological processes by regulating downstream target genes. In order to understand the mechanism through which miR-152 regulates osteoblast biological behavior, we predicted the potential target genes of miR-152 through the biological prediction website miRDB, and found that there was a targeted binding site between RUNX2 and miR-152. RUNX2 gene, located on chromosome 6 p21.1, belongs to RUNT-related transcription factor family and is an essential regulator of osteoblast differentiation and bone formation [30, 31]. In this article, we first analyzed the relationship between RUNX2 and miR-152, and the results showed that RUNX2 decreased after knocking down miR-152 in osteoblasts. In addition, miR-152-mimics could inhibit the fluorescent activity of RUNX2-3'UTR Wt and had no obvious effect on the activity of RUNX2-3'UTR Mut. This indicated that the influence of miR-152 on proliferation, apoptosis and differentiation of osteoblasts might be related to its negative regulation of RUNX2. Next, we treated osteoblasts with simultaneously increasing miR-152 and RUNX2, and found that increasing RUNX2 could reverse miR-152's effect on biological behavior of osteoblasts. This indicated that miR-152 could affect osteoblast proliferation, apoptosis and differentiation through negative regulation of RUNX2.

There are some limitations in this study. First, we did not design an in vivo experiment and know whether miR-152/RUNX2 axis could improve osteoporosis symptoms. Second, we only explored the role of miR-152 and RUNX2 in osteoporosis but failed to know whether miR-152 could affect osteoblast biological behavior by regulating other target genes. These were expected to be supplemented in subsequent experiments.

To summarize, miR-152 is elevated in serum of osteoporosis patients and can be used as a

biological indicator for diagnosis. Furthermore, miR-152 can inhibit osteoblast proliferation, differentiation and induce apoptosis by negatively regulating RUNX2.

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