Effect of Bone Marrow Stromal Cells Derived miR-26b on Chondrocytes of Osteoporosis Rats

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Abstract. ***Objective***. Osteoporosis is a common bone disease. miR-26b regulates OA-induced osteogenesis and induces osteoporosis. miR-26b is elevated in bone marrow stromal cells (BMSCs) during bone formation; however, we haven't fully revealed whether it is directly involved in this process, which was the aim of this study. ***Methods***. An oophorectomized rat model of osteoporosis was used. BMSCs were detected by electron microscopy of exosomes, and mir-26b levels were detected by RT-PCR. The correlation between mir-26b and sirt2 was detected by bioinformatics and luciferase activity analysis. Bone microstructure and cartilage moisture content were also measured. The proliferation ability of mir-26b and sirt2 on chondrocytes was detected by cell viability test and flow cytometry. ***Results***. Western blotting further proved that the surface markers of isolated granular exosomes were positive for CD63 and CD81. Further analysis showed that exosomes' diameters ranged from 50 to 150 nm. Mir-26b is elevated in BMSC, and its mimics can promote proliferation. Luciferase showed that mir-26b targets sirt2 and the effect of elevated mir-26b on chondrocytes was completely reversed by silencing sirt2. The proliferation ability of C28/I2 chondrocytes in Mir MICs group was lower than other two groups, while that in Mir inhibition group had stronger proliferation ability than in the Mir NC group. mir-26b was highly expressed in BMSC, indicating that mir-26b comes from secretion of BMSC. ***Conclusion***. Mir-26 is highly expressed in OP. mir-26b can therefore target sirt2 to promote proliferation and inhibit apoptosis of OP chondrocytes. It may be a new scheme for treatment of OP in the future.

Key words: mir-26b, sirt2, osteoporosis.

Introduction

Osteoporosis (OP) is one of the high-incidence chronic diseases, which severely affects middle-aged and elderly people [1,2]. The number of OP patients worldwide has exceeded 200 million statistically, and it is still on an increasing trend. OP could be possible in all age groups, and its etiology hasn't been well researched [3-5]. Clinically, it is believed that the occurrence of OP is potentially associated with endocrine hormones, organ dysfunction, blood system disease, drug use, etc., and isusually accompanied by varying levels of pain and spinal degeneration [6-8]. OP patients are prone to fractures and bone injuries, which cause serious damage to patient's normal life [9]. The recovery from OP is an extremely lengthy process, with an extremely low cure rate [10]. For more severe OP patients, surgical treatment is required but still has challenges such as high infection rate and high recurrence rate [11]. Fully exploring the OP pathogenesis is key to its treatment. The unbalanced differentiation of bone marrow stromal cells (BMSCs) may lead to pathological diseases, including OP. The source of BMSCs is extensive and has a great differentiation potential [12]. Therefore, BMSCs are the listed stem cells for treatment of many adult and childhood diseases, such as sickle cell disease, rheumatic disease, lymphoma, and heart failure. The osteogenic differentiation has the ability to affect the intensity of heart failure.

MicroRNA (miRNA) is a macro auto coding RNA [13]. miRNA is involved in bone formation by BMSCs. miR-21 can reverse OP by regulating

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RECK, while miR-34 can target Tgif2 and inhibit OP by antagonizing osteoclast formation [14]. It is worth noting that miR-26b is expressed abnormally in osteosarcoma [15], while its effect in OP is still essentially unknown. Therefore, we in this study hypothesized that miR-26b has the same important function. Besides, we also found that the deacetylase SIRT2 is a key gene in OP that is found on human chromosome 10q21.3 [16] and considered in previous studies to be a potential target for treatment of diabetes complicated with OP [17]. Therefore, we speculated that miR-26b might be involved in bone differentiation and growth, thereby reducing the inhibitory effect of BMSCs in osteogenesis.

Materials and Methods

Animal information. 40 SPF Wistar rats were involved in this study, weighing 200±20g, acquired from Experimental Animal Center of our hospital, under license SYXK (Shanghai) 2020-0003. The mice were grouped as model group and control group.

This study was approved by the Ethics Committee of Wenzhou Traditional Chinese Medicine Hospital Affiliated to Zhejiang University of Traditional Chinese Medicine.

Cell data. Human normal chondrocytes C28/I2 were from ATCC and cultured in high-sugar DMEM, 95% air, 37°C.

Model construction. Group A mice were put in the control group, and group B was experimental mice which had ovariectomy, 10 mice/group. The mice in B group were anesthetized with 3% sodium pentobarbital (30 mg/kg, Sigma Aldrich, USA), and their ovaries were removed through a retroperitoneal approach.

BMSCs isolation and identification. BMSCs were cultured in exosomal-deficient FBS to shield off the interference from bovine exosomes. The FBS exosomes were removed by 1×105g ultracentrifugation at 4°C for 16 hours, and then we collected the FBS supernatant and filtered with a 0.22μm filter. mBMSCs and hBMSCs were cultured, then collected, centrifuged at 300g for 10min, 2000g for 15min, 12,000g for 30min, and then centrifuged at 4°C at 4×103g for 1h with ultrafilter to make the supernatant. Finally, we extracted the EXO Quick-TC™ Exosome. According to the instructions, collected blood in EDTA-K2 anticoagulant was centrifuged at 3000g for 15 min at 4°C to collect supernatant which was mixed with ExoQuick exosome precipitation solution, and GAPDH was a positive control and an internal control of exosomes. Exosomes were stored at -80°C for subsequent experiments. Western blotting analysis further proved the positive surface markers of isolated granular exosomes, including CD63 and CD81.

TEM assessed exosomes. After washing, 10 μL of exosome suspension was placed on a 300×copper electron microscope grid. The samples were then incubated for five minutes, and exosomes were counter-stained with 2% uranium oxalate followed by washing and TEM (JEM-2100, Jeol, Japan) analysis.

Sample collection. After three weeks, all rats were anaesthetized and decapitated to obtain 5mL of rat abdominal artery blood, which was placed in a coagulation tube. After 30-minute standing, it was centrifuged (2000×g, 4°C), and their serum was collected and stored at -80°C, followed by removal of the surrounding muscle tissue in the right posterior femur and soaking in the fixative. The cartilage tissue was obtained from knee joint, rinsed with PBS (Gibco, USA), and cut into 1mm3 pieces, followed by Trypsin (0.25%) digestion and centrifugation (200×g) for 10 min to obtain chondrocytes. The samples were passaged with 10% fetal bovine serum culture medium.

Bone microstructure detection. Micro-CT scanner analyzed the microstructure of rat femur. The scanning system was calibrated with 70kV, 85mA and 1000/180° projections within 350 ms. The assessed indicators were bone mineral density (BMD), bone trabecular thickness (Tb.Th), and bone trabecular number (Tb.N).

Cartilage water content detection. The knee joint cartilage scale was taken and weighed, followed by drying it with blast heating machine (60°C) for 12 hours and then weighing it again. Cartilage water content was then calculated as follows; Cartilage water content=(the first weight-the second weight)/first weight×100%.

Real-time PCR detection. RNA was extracted for qRT-PCR analysis with conditions: 10 min 95°C, then 40 cycles of 95°C 15 seconds and 60°C 1 min. The ΔΔCt method was used to calculate the difference between groups.

Liposome transfection of siRNA. We adopted one-step cloning, by adding the same overlapping sequence to the 5'end of the miR-26b gene primer. According to the requirements by the lentivirus packaging, the miR-26b gene needed to be inserted in front of the cPPT/CTS site. The linearization of the vector was achieved by single enzyme digestion with SwaI or PacI. Primer design: 1. The size of the overlapping sequence was 15-20bp,

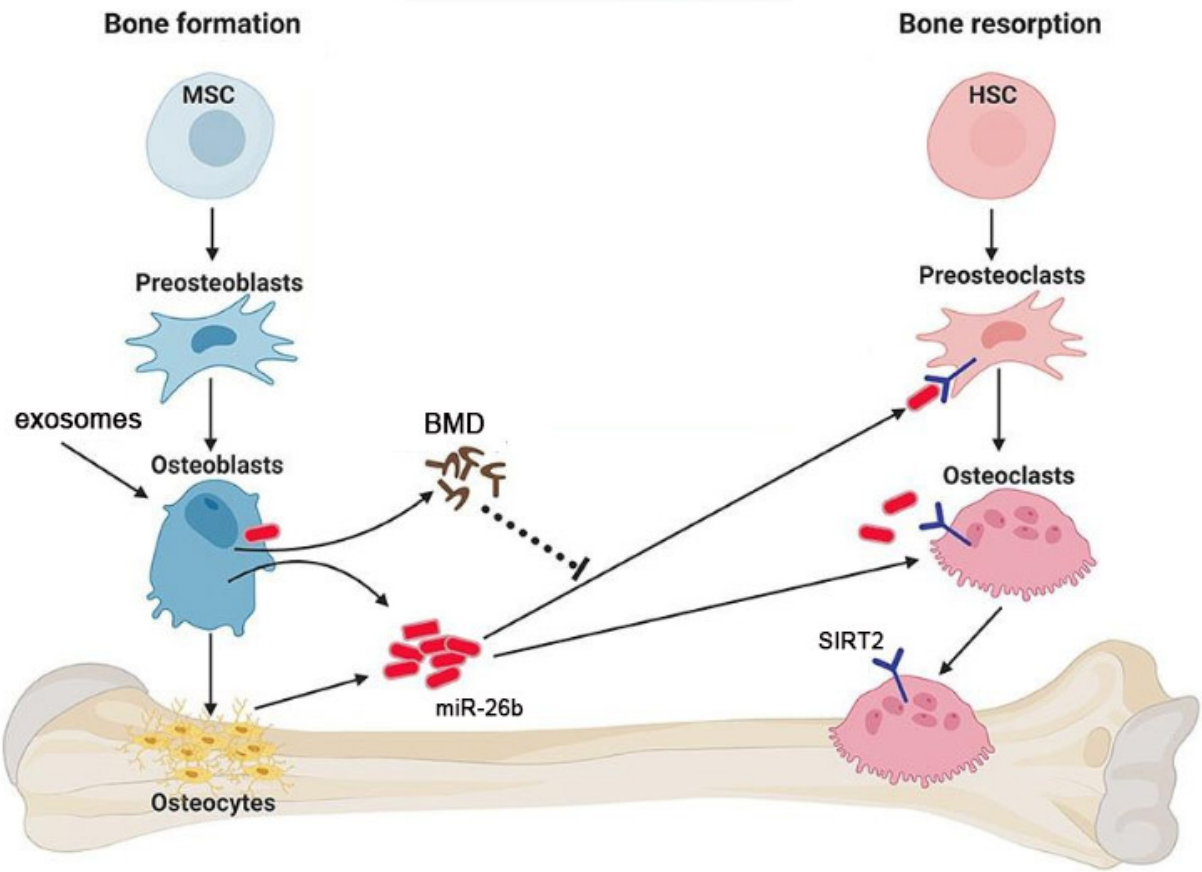

Figure 1. *Schematic diagram of research route ideas.*

and the restriction site was excluded; 2. PacI single restriction enzyme digestion was taken as an example. The upstream and downstream primers for miR-26b gene were: pcDNA3.1-miR-26b WT, Upstream: TCTGCCATAGCAAAACAAG, Downstream: CTGGTCGAGCTGGACGGCGACG, pcDNA3.1-miR-26b Upstream: TCTGCCATAGCAAAACAAGC Downstream: CTGGTCGAGCTGGACGGCGACG. After designing the primers, the miR-26b gene was amplified by PCR, and the gel recovered product, and the vector digested product was recombined. When stable transformation was needed, the recombinant plasmid was used to package the lentivirus in the follow-up experiment.

Cell viability test. The cells were adjusted to 5×104 cells/mL and inoculated in a 96-well plate, followed by the addition of 20μL of MTT solution (5 mg/mL) (Gibco, USA) after culture and the addition of 200μL of DMSO after four hours. Microplate reader (570 nm) detected absorbance value.

Flow Cytometry Detection of Apoptosis. Different concentrations of therapy group were used to treat the cells for a specified time. Cell PI dye was added after cells were attached to the wall. Apoptosis ratio was detected by flow cytometry on the machine and expressed as average ± standard deviation. Flow cytometry reagents were purchased from Miltenyi Biotech, Germany.

Luciferase reporter gene detection. The SIRT2 fragment and predicted miR-26b binding site or mutated miR-26b binding site were transfected into HEK293T cells and then detected by enzyme activity analysis.

Statistical methods. The experimental results were recorded as (mean±standard deviation) and analyzed by SPSS21.0 software. $P<0.05$ was considered significant. Independent t test; single-factor variance and LSD test were used among multiple groups, including repeated variance and Bonferroni test.

Results

Up-regulation of miR-26b during osteogenesis from BMSC. A schematic diagram of the research approach is shown in **Figure 1**. BMSCs were first obtained from chondrocyte C28/I2 cell supernatant by using exosome isolation reagent. The collected BMSCs were identified by TEM, NTA and Western blotting analysis. TEM showed round or

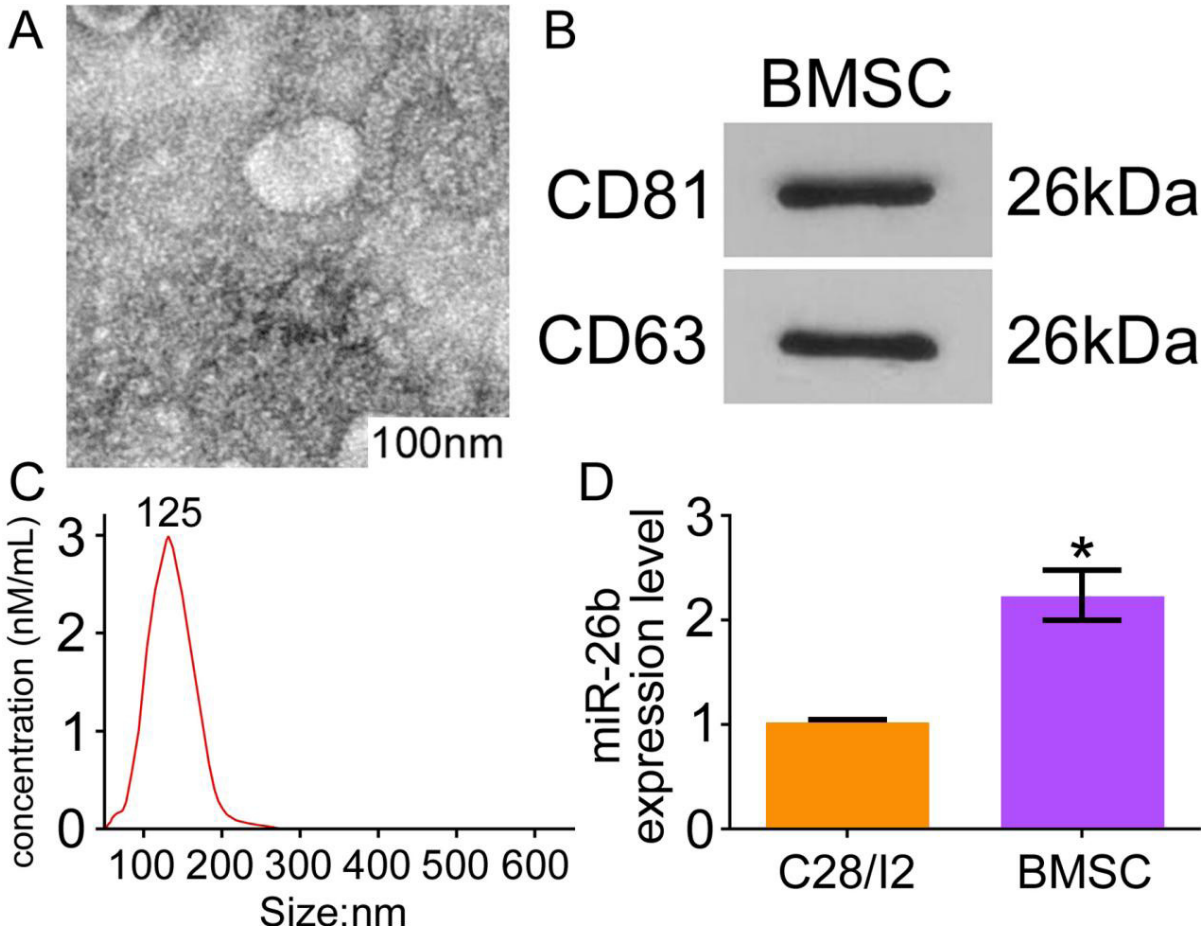

Figure 2. *Up-regulation of miR-26b during bone formation from BMSCs.* ***(A)*** *shows the results of electron microscopy,* ***(B)*** *shows the Western-blot identification of BMSC expression,* ***(C)*** *shows the particle size in BMSC, and* ***(D)*** *shows the expression of miR-26b in BSMC. (Scale 1: 100nm).*

cup-shaped exosomal structure **(Figure 2A)**. Western blotting analysis further detected the positive surface markers of isolated granule exosomes, including CD63 and CD81 **(Figure 2B)**. The diameter of exosomes ranged from 50-150 nm **(Figure 2C)**. RT-PCR analysis further showed that miR-26b was highly expressed in chondrocytes **(Figure 2D)**.

miR-26b regulates the expression of SIRT2 by targeting. Through the TargetScan online website, we first discovered the complementary sites where miR-26b binds to SIRT2 **(Figure 3A)**. SIRT2-WT activity of miR-mimics was lower than miR-NC group (P<0.001, **Figure 3B**). We observed that SIRT2 protein expression in C28/I2 and chondrocytes transfected with miR-26b mimic and SIRT2 in miR-mimics group were highest (P<0.05, **Figure 3C&D**).

Effect of miR-26b on bone structure. The BMD, Tb.Th and Tb.N of the model group were (0.44±0.13) g/cm3, (0.47±0.11) mm, (0.23±0.14)/mm, respectively, which were lower than control group (P<0.001). Cartilage water content in the model group was (3.64±2.17) % **(Figure 4A-C)**, which was higher than the cartilage water content of control group, P<0.001 **(Figure 4D)**.

Effect of miR-26b on chondrocytes by regulating SIRT2 expression. The proliferation ability of C28/I2 and chondrocytes in the miR-mimics group was lower than other two groups, while proliferation ability of miR-inhibition group was better than miR-NC group (P<0.05, **Figure 5A&B**). miR-mimics group had the highest apoptotic rate, and miR-inhibition group's apoptotic rate was lower than miR-NC group's (P<0.05, **Figure 5C**). The proliferation ability of chondrocytes in the RNA-sh

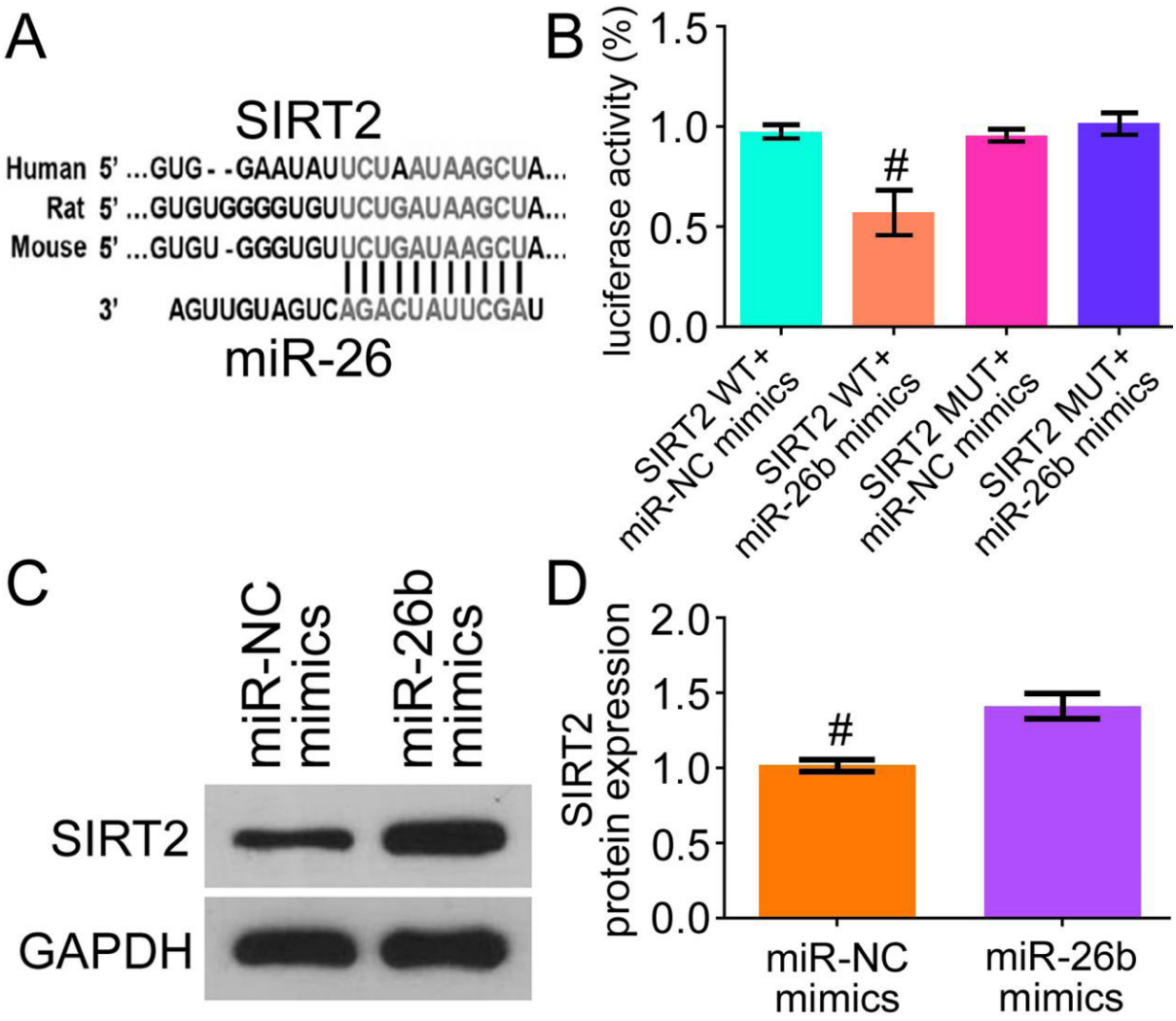

Figure 3. *miR-26b regulates the expression of SIRT2 by targeting.* ***(A)*** *shows the Target Scan detection of the expression of each SIRT2 of miR-26b,* ***(B)*** *shows the luciferase activity analysis,* ***(C)*** *shows the Western-blot detection of protein expression, and* ***(D)*** *shows statistical results. ($^{\#}p<0.05$).*

group C28/I2 was higher than that of the other two groups, while proliferation ability of RNA-si group was weaker than in the RNA-NC ($P<0.05$, **Figure 5D&E**). The RNA-sh group had the lowest apoptotic rate, while the RNA-si group had higher apoptosis than RNA-NC group ($P<0.05$, **Figure 5F**).

Discussion

OP is one of the high-incidence diseases that inflicts the normal life of middle-aged and elderly people. In previous studies, miR-26b's effect on osteoblasts has been persistently confirmed [18], but its mechanism and pathway remain unclear.

BMSCs can promote cancer progression and proliferation in general. Exosomes are released by many cells and exert varying biologic functions. For the purpose of identifying hypoxic BMSC-derived exosomes, we selected a common exosomal miRNA overlap. The composition of exosomal microRNAs evolve with the outside environments, suggesting that their selectiveness of loading into exosomes. miRNA proportion in exosomes is higher than that in donor cells, and RNA-binding protein is a key factor in exosomal biosynthesis. It forms a complex with RNA then becomes exosomes. We identified the source of miR-26b and secretion by BMSC through electron microscopy and protein expression, and we also found a complementary site where

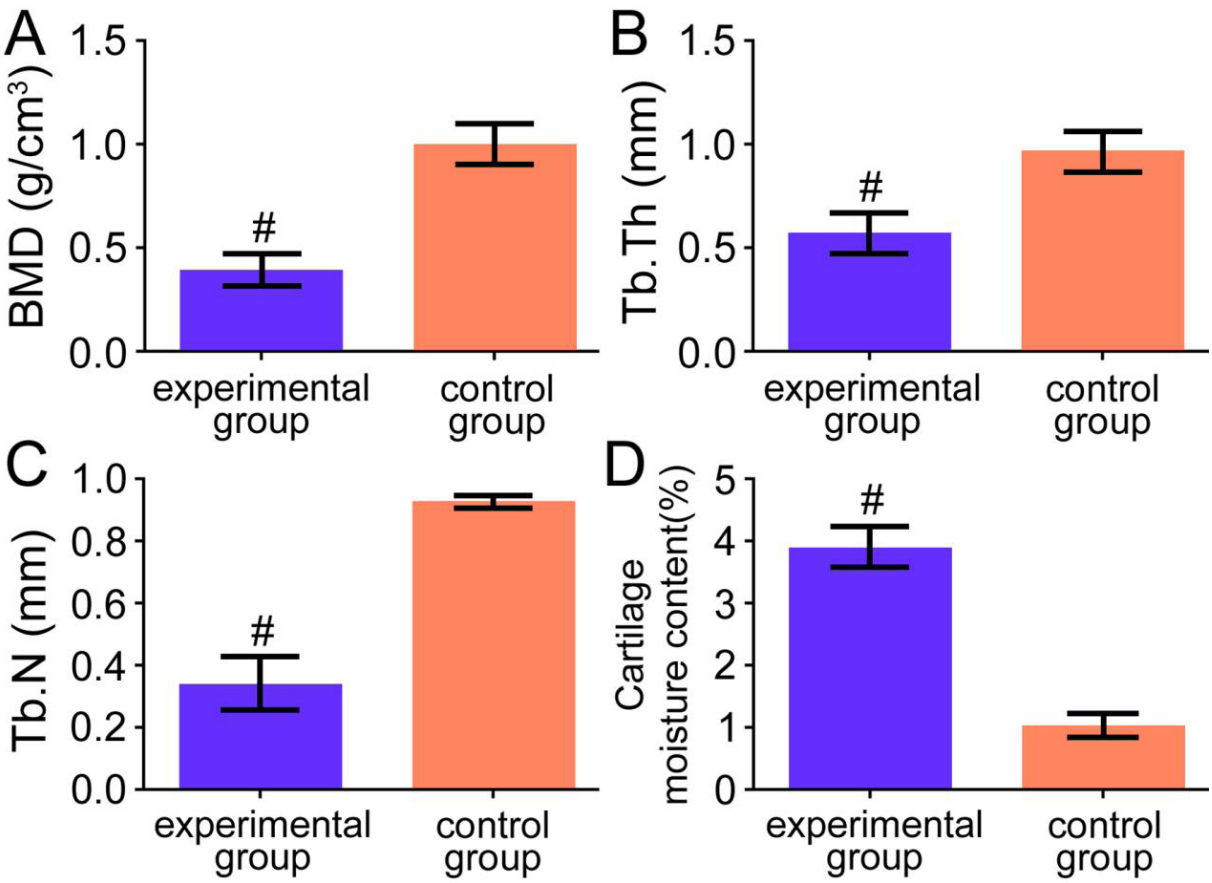

Figure 4. *The effect of miR-26b on bone structure.* ***(A-C)*** *show the expression of BMD, Tb.Th, and Tb.N, respectively, and* ***(D)*** *shows comparison of cartilage water content. (#p<0.05).*

they bind through the online target gene prediction website. Therefore, we have detected whether a relationship exists between miR-26b and SIRT2 through a dual-luciferin reporter enzyme experiment. The fluorescent activity of SIRT2-WT was inhibited after miR-mimics were transfected, suggesting their targeting relationship. By detecting the protein expression of SIRT2 in chondrocytes transfected with miR-mimics, miR-inhibition, and miR-NC, it was found that miR-26b increased and SIRT2 decreased. Thus, we now know that miR-26b can target SIRT2 expression, which is consistent with expression of the two in the above-detected OP [19]. We compared the bone microstructure and cartilage water content of normal rats, BMD, and Tb.Th, and Tb.N of model group were obviously lower than control group, while cartilage water content was higher [20,21]. The cartilage water content should be low in normal circumstances [22]. However, in this study, the water content of articular cartilage in model group was significantly increased, and OP may be the reason. The original normal structure of the bone was destroyed, and the synovium secreted a large amount of synovial fluid, which lowers osmotic pressure in joint cavity. Thus, synovial fluid that cannot be absorbed or exchanged is accumulated in the cartilage tissue, oppressing the cartilage nerve, causing damage and necrosis. Moreover, the proteoglycan in cartilage matrix is negatively charged and has strong water absorption, which results in increased water content of the cartilage.

In previous studies, we found that miR-26b also increased in prostate cancer, pancreatic cancer and other diseases, while SIRT2 decreased in non-alcoholic fatty liver, renal tubulointerstitial fibrosis, etc., which can also support our test result. In the

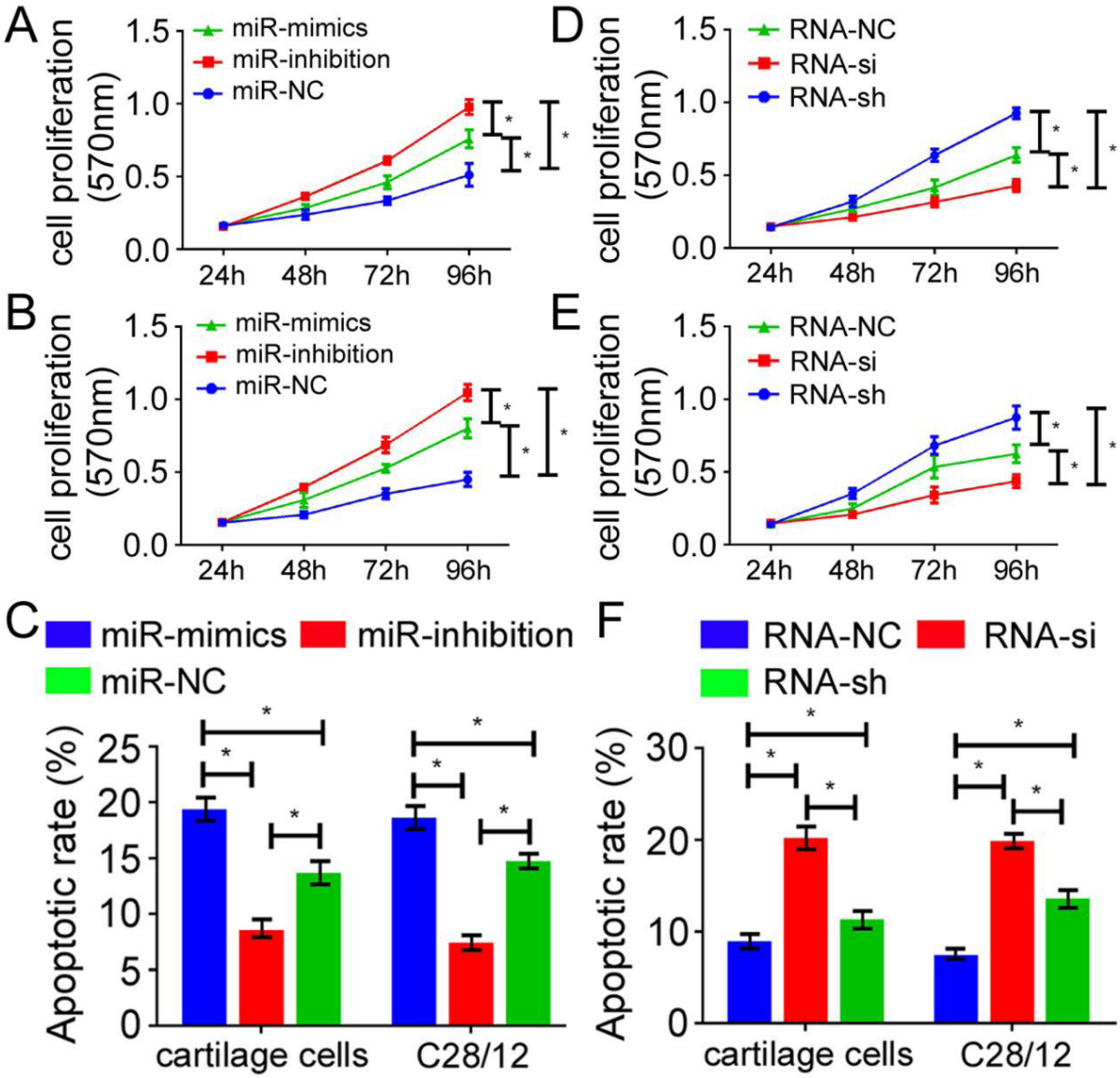

Figure 5. *Effect of miR-26b on chondrocytes by regulating the expression of SIRT2.* ***(A)*** *shows the growth curve for miR-26b expression in C28/I2;* ***(B)*** *shows the growth curve for miR-26b expression in chondrocytes;* ***(C)*** *shows the apoptosis rate of miR-26b expression in cells;* ***(D)*** *shows It is the growth curve for SIRT2 expression in C28/I2;* ***(E)****shows the growth curve for SIRT2 expression in chondrocytes; Figure F shows the apoptosis rate of SIRT2 expression in cells. (*p<0.05).*

miR-26b-intervened cells, we found that miR-26b was inhibited, while SIRT2 was increased, which confirms and indicates that OP has an impact on miR-26b and SIRT2. This result is also in line with analysis of biological effects of miR-26b and SIRT2 in previous studies [23-25]. However, since human samples were not included in this experiment, we need to further explore the specific mechanism in human tissues. Additionally, the OP model rats were established by intragastric administration of retinoic acid. There were also various modeling methods such as ovariectomized OP rats and high glucose-induced OP rats in previous studies. Thus, we can't exclude the errors in miR-26b or SIRT2 in different modular schemes. For these limitations, we will carry out more complete experimental analysis to obtain more effective experimental results for clinical reference.

Conclusion. miR-26b was highly expressed in BMSCs, indicating, that miR-26b is derived from secretion of BMSCs; miR-26b was also highly

expressed in OP; and miR-26b can target SIRT2 to promote proliferation of OP chondrocytes and inhibit apoptosis. It may be an ideal plan for OP treatment.

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