

miR-205 regulates bone turnover in elderly female patients with type 2 diabetes mellitus through targeted inhibition of Runx2

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Abstract. The present study aimed to explore the expression of microribonucleic acid (microRNA) (miR)-205 in bone tissues and serum of elderly female patients with type 2 diabetes mellitus (T2DM) complicated with osteoporosis (OP), and to investigate the effect of miR-205 on osteogenesis/adipogenesis of bone marrow mesenchymal stem cells (BMSCs) and its mechanism in elderly female mice with T2DM + OP. The bone tissues and serum of 24 female patients with T2DM + OP at the Third Affiliated Hospital of Qiqihar Medical University were collected as the research group, while those of 24 healthy people were collected as the control group. The expression level of miR-205 was detected in both groups via reverse transcription-polymerase chain reaction (RT-PCR). Then the elderly female mouse model of T2DM + OP was established as a model group, while normal mice of the same age were used as the control group. The mice in the model and control groups were transfected with miR-205 mimic, negative control (NC)-mimic, miR-205-inhibitor and NC-inhibitor. Alizarin red S (ARS) staining and RT-PCR were conducted after osteogenic induction for 21 days, and oil red O (ORO) staining and RT-PCR were performed after adipogenic induction for 24 days. The overexpression of miR-205 inhibited osteogenic differentiation and promoted adipogenic differentiation of BMSCs in elderly female mice with T2DM + OP, while knockdown of miR-205 promoted osteogenic differentiation and inhibited adipogenic differentiation of BMSCs in elderly female mice with T2DM + OP. In addition, miR-205 was able to directly suppress the expression of its target gene RUNX family transcription factor 2 (*Runx2*). The expression level of miR-205 was obviously increased in female patients with T2DM + OP and the elderly female mouse model of

T2DM + OP. In addition, miR-205 was able to regulate the osteogenic/adipogenic differentiation of BMSCs, and miR-205/Runx2 may be a new method and target for the treatment of female patients with T2DM + OP.

Introduction

The ovarian function and estrogen level continuously decline in women relative to age, ultimately leading to osteoporosis (OP) (1). OP is one of the common complications of patients with type 2 diabetes mellitus (T2DM), causing great morbidity and seriously affecting the quality of life of patients (2-4). Currently, the main therapeutic methods for elderly patients with T2DM and OP include calcium and vitamin D supplements, physical exercise, specified diet and high quality nursing, yet the occurrence and progression of OP in elderly patients with T2DM cannot be effectively controlled (5,6). Therefore, it is of great clinical significance to explore new therapeutic methods and drugs for patients with T2DM and OP.

Microribonucleic acids (microRNAs or miRs) are a type of single-stranded, non-coding and small-molecule RNAs approximately 18-25 nucleotides in length, which are involved in the occurrence and progression of various diseases (7,8). It has been reported that some miRs can participate in bone turnover, bone metabolism and bone development, thereby regulating the occurrence and progression of OP (9-11). However, whether there is a certain association between miRs and T2DM + OP has not yet been reported.

In previous experiments, several miRNAs related to OP have been screened, and it was found that miR-205 had a certain association with T2DM + OP in elderly patients (9-11). In the present study, therefore, the expression of miR-205 in bone tissues and serum of elderly female patients with T2DM + OP was explored, and the effect of miR-205 on osteogenesis/adipogenesis of bone marrow mesenchymal stem cells (BMSCs) and its specific mechanism in elderly female mice with T2DM + OP were investigated, so as to provide new insight for the clinical treatment of elderly female patients with T2DM + OP.

Materials and methods

Patients and data. A total of 24 female patients with T2DM + OP treated at The Third Affiliated Hospital of Qiqihar Medical

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University (Qiqihar, Heilongjiang, China) from October 2016 to June 2017 were selected. The patients were aged from 60 to 80 years with an average age of 72.42 ± 13.41 years. All patients met the clinical diagnostic criteria for T2DM and the WHO diagnostic criteria for OP. WHO diagnostic criteria for T2DM included: Plasma glucose ≥ 11.1 mmol/l, fasting plasma glucose (FPG) ≥ 7.0 mmol/l, or oral glucose tolerance test 2-h postprandial blood glucose (OGTT2hPG) ≥ 11.1 mmol/l at any time. WHO diagnostic criteria for OP included: A bone mineral density (BMD) measured by dual-energy X-ray absorptiometry to be 2.5 standard deviations below that in the local region and of the same gender and ethnic group (T value ≤ -2.5). According to studies (12-17), when studying orthopedic-related diseases, the selected controls are usually patients admitted to the hospital for trauma. In this study, trauma that required emergency surgery due to multiple trauma to the bone was defined as severe trauma. At the same time, 24 patients with normal blood glucose and BMD who were treated at this hospital due to severe trauma were selected as the control group. During surgery of the elderly female patients with T2DM + OP and patients in the control group, the bone tissues were collected from the femur or tibia and immediately stored in liquid nitrogen. In addition, the fasting venous blood was collected in both groups in the morning, and the serum was separated and stored in a refrigerator at -80°C .

Exclusion criteria included patients with T1DM, special type of diabetes or autoimmune diseases, patients who presented with severe hematological diseases, those who received estrogen, calcitonin, vitamin K and other drugs that may affect bone metabolism for a long period prior to the study, those complicated with severe kidney disease, tumors or gout, patients who presented with other congenital OP, those who had poor mental or consciousness state or failed to cooperate in the study.

Establishment of an elderly female mouse model of T2DM + OP. All mice were maintained at a temperature between 20 and 26°C , with a humidity of 40 to 60%, and a 12-h light/12-h dark cycle, and free access to food and water. Animal health and behavior were monitored each morning and evening.

A total of 54 C57BL/6J female mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. for this study, and 24 C57BL/6J female mice, 8 weeks of age, were used as the controls. Bone tissue and serum samples were collected from the mice. Thirty C57BL/6J female mice, age 16 months were used to construct the models. One week after acclimatization, a mouse model of osteoporosis was constructed. All mice were anesthetized with 50 mg/kg of 1% sodium pentobarbital. After anesthesia, under sterile conditions, ophthalmic scissors, ophthalmic tweezers, hemostatic forceps and other instruments were used to remove the bilateral ovaries of the mice. After suture, the mice were rested on a clean table, until they could move freely. Bilateral ovaries of the mice were removed. After 4 weeks of breeding, if the mice exhibited phenotypes such as reduced bone density, increased bone separation, and decreased number of trabeculae, the osteoporosis mouse model was deemed as successfully constructed. At the same time, the mice were fed with high-fat and high-glucose diets for 6 weeks, and

2% streptozotocin (STZ) solution (pH 4.4) was intraperitoneally (i.p.) injected (35 mg/kg/time) after fasting for 12 h, so as to establish the T2DM model. A FPG level of >11.1 mmol/l within 2 weeks after STZ injection indicated T2DM. Finally, 24 mice met the criteria, and the elderly female mouse model of T2DM + OP was achieved after 8 weeks. The bone tissues and serum samples were collected in both groups for subsequent experiment. Once it was clear that the 6 mice did not meet the requirement of subsequent experiment, the experiments on the 6 mice were stopped immediately, and euthanasia was performed. In 'Guide for the Care and Use of Laboratory Animals' of the National Institutes of Health, endpoints for experiments are defined. It is highlighted that euthanasia is performed to reduce animal pain and suffering before death. Dying is the state before the death of the animal, which can replace death as the experimental endpoint. The criteria for the dying end point include the following: i) weight loss; ii) body temperature reduction; iii) obvious inactivity or remain motionless; iv) arched back posture; v) hair stains; vi) tumor ulceration and bleeding; vii) dyspnea. In this experiment, we found that 6 mice did not meet the requirement of subsequent experiment, and because of turbid hair and arched back, they were euthanized. The other 6 mice were sacrificed by injection of 200 mg/kg sodium pentobarbital.

To summarize, in the present study, 54 female C57BL/6J mice were utilized, and 24 8-week-old control mice were also sacrificed by cervical dislocation after anesthesia. Thirty 16-month-old mice were used to construct the model of type 2 diabetes with osteoporosis; among them, 6 mice did not meet the standard and were euthanized, and 24 mice were sacrificed by cervical dislocation after anesthesia.

Total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from bone tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and the miRNA was extracted from serum using a serum miRNA extraction kit (Foregene). The RNA purity and concentration were detected using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.). Then RNA was synthesized into cDNA using the RevertAid™ First Strand cDNA Synthesis kit (Fermentas) and amplified using the SYBR Green Master Mix (Roche) on the ABI7500 PCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.) to detect the expression of the target gene. All primer sequences used in this experiment are shown in Table I.

Separation and culture of mouse BMSCs. After the successful establishment of the elderly female mouse model of T2DM + OP, mouse bone marrow mesenchymal stem cells (BMSCs) were extracted according to a previous study (18). First, the mice were anesthetized by an i.p. injection of sodium barbiturate (Sigma-Aldrich; Merck KGaA), and then the mice were sacrificed by cervical dislocation. The bilateral femur and tibia were isolated under sterile conditions; muscle tissue was removed, and the femur and tibia were exposed. Then BMSCs were cultured in high-glucose DMEM (Corning) containing 10% fetal bovine serum (FBS) (Cyagen), 100 U/ml penicillin (Beyotime) and 100 $\mu\text{g/ml}$ streptomycin (Beyotime). The medullary cavity was rinsed repeatedly, the cells were extracted out of the marrow cavity, centrifuged with

Table I. Primer sequences.

Gene	Forward (5'-3')	Reverse (5'-3')
Murine miR-205	CACGTCCCAGGCTCCA	CGGGCATCGAAACTGCCAATT
Human miR-205	CTTGTCCTTCATTCCACCGGA	TGCCGCCTGAACTTCACTCC
Murine <i>U6</i>	GCGCGTCGTGAAGCGTTC	GTGCAGGGTCCGAGGT
Human <i>U6</i>	CGCTTCGGCAGCACATATA	TTCACGAATTTGCGTGTTCAT
Murine <i>ALP</i>	AGAACCCCAAAGGCTTCTTC	CTTGGCTTTTCCCTTCATGGT
Murine <i>OCN</i>	AGGGCAGCGAGGTAGTGAA	TCCTGAAAGCCGATGTGGT
Murine <i>PPARγ</i>	TCTGAGTCTGTATGGAGTGACAT	CCAAGTCGTTACATCTAGTTCA
Murine <i>FABP4</i>	TGCCTTTGTGGGAACCTG	CCTGTCTGTCTGCGGTGAT
Murine <i>Runx2</i>	TCTACTATGGCACTTCGTCAGG	GCTTCCATCAGCGTCAACAC
Murine <i>β-actin</i>	TAAAGACCTCTATGCCAACACAGT	CACGATGGAGGGGCCGACTCATC

U6, RNA, U6 small nuclear 1; *ALP*, alkaline phosphatase, biomineralization associated; *OCN*, osteocalcin [also known as bone γ -carboxyglutamate protein (*BGLAP*)]; *PPAR γ* , peroxisome proliferator-activated receptor- γ ; *FABP4*, fatty acid binding protein 4; *Runx2*, runt-related transcription factor 2.

a high-speed centrifuge (Thermo Fisher Scientific, Inc.) for 5 min at 200 x g, and the cell pellet was placed in cell bottles. Then the cell pellet was placed in an incubator (Thermo Fisher Scientific, Inc.) with 5% CO₂ at 37°C. BMSCs were purified by differential adherence method; culture medium was replaced every three days, and non-adherent cells were removed; the splitting ratio was 1:3. The above steps were repeated and passed to the third generation for subsequent experiments. The study was approved by the Animal Ethics Care Committee of Qiqihar Medical University (QMU-AECC-2019-51).

Cell transfection. The third-generation BMSCs in the logarithmic growth phase were inoculated into a 24-well plate (2x10⁴/cm²), and transiently transfected using the transfection reagent X-treme (Vazyme). After transfection with the miR-205 mimic (final concentration: 50 nM) and miR-205 inhibitor (final concentration: 100 nM) for 24 h, subsequent experiments were performed. The sequences were: miR-205 mimic, 5'-UCCUUC AUUCCACCGGAGUCUG-3' and NC-mimic, 5'-UUCUCC GAACGUGUCACGUTT-3'; miR-205 inhibitor, 5'-CAGACU CCGGUGGAAUGAAGGA-3' and NC-inhibitor, 5'-CAGUAC UUUUGUGUAGUACAA-3'.

CCK-8 assay. BMSCs in the third-generation logarithmic growth phase were selected and seeded in 96-well plates at a density of 1x10⁴ cells/cm². The cells were transfected with miR-205. After 24, 48 and 72 h, 10 μ l of CCK-8 solution and 100 μ l of serum-free medium were added to each well. The cells were cultured for 2 h in an incubator, and the absorbance value (OD value) of each well was measured at 450 nm using a microplate reader (Tecan).

Osteogenic and adipogenic induction of BMSCs. The third-generation BMSCs in the logarithmic growth phase were inoculated into a 24-well plate (2x10⁴/cm²) and transfected with miR-205. Then osteogenic induction medium containing 10% FBS, 10 mmol/l sodium 3-phosphoglycerate, 10 mol/l dexamethasone and 50 μ g/ml ascorbic acid was added for osteogenic induction for 21 days, and the original medium was replaced with fresh medium once every 3 days. After that,

alizarin red S (ARS) staining was performed. First, the induction medium was discarded, and the cells were washed with PBS repeatedly, fixed with 4% paraformaldehyde for 30 min, stained with ARS dye (Cyagen) for 30 min and washed again with PBS, followed by observation under an inverted microscope (magnification, x10).

In addition, the third-generation BMSCs in the logarithmic growth phase were inoculated into a 24-well plate and transfected with miR-205. Then adipogenic induction medium containing 1 μ M of dexamethasone, 200 μ M of indometacin, 0.5 mM of IBMX and 10 μ M of insulin for adipogenic induction for 24 days, and the original medium was replaced with the fresh medium once every 3 days. After that, oil red O (ORO) staining was performed. First, the induction medium was discarded, and the cells were washed with PBS 3 times, fixed with 4% paraformaldehyde at room temperature for 30 min, stained with ORO dye (Cyagen) for 30 min and washed again with PBS, followed by observation under the inverted microscope (magnification, x10).

Target prediction. The target genes of miR-205 were predicted and analyzed using the online websites TargetScan (http://www.targetscan.org/vert_72/) and miRanda (<http://www.microna.org/src/jmiranda/>).

Western blotting. The total protein was lysed using protein lysis buffer (Beyotime) and centrifuged at 2,500 x g and 4°C for 5 min. The supernatant was transferred into another EP tube and cryopreserved in a refrigerator at -80°C. The protein concentration was measured using the Bradford protein quantification kit, and loading buffer was added to the samples and boiled. After 12.5% SDS-PAGE, the protein samples were transferred onto a PVDF membrane, sealed with skim milk for 2 h, and incubated with the primary antibodies, Runx2 (dilution 1:1,000, cat. no. AF2593; Beyotime) or GAPDH (dilution 1:1,000, cat. no. AF1186; Beyotime) on a shaker at 4°C overnight. On the next day, the protein samples were incubated with the anti-rabbit secondary antibody (dilution, 1:500; cat. no. A0279; Beyotime) at room temperature. After 1 h, the fluorescence signals were collected using the luminescence

detector and analyzed by Image-Pro Plus 6 software (Media Cybernetics).

Luciferase reporter assay. The miR-205 precursor sequences and the 3'-untranslated region (3'UTR) of the mouse *Runx2* gene were cloned into the psi-CHECK2 vector through PCR, enzyme digestion, ligation and transformation, and 0.5 μg of 3'UTR and 1 μg of miR were co-transfected into BMSCs. After 48 h, the cells were collected and lysed. According to the instructions of the dual-luciferase reporter gene kit (Promega), the firefly luciferase activity and *Renilla* luciferase activity were detected for luciferase reporter assay.

Statistical methods. All data are expressed as mean \pm standard error of measurement, and a t-test was performed for the comparison of sample means. GraphPad 7.0 software (GraphPad Software, Inc.) was used for the statistical processing of all data, and the t-test for statistical analysis. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ were indicative of statistically significant differences as shown in the figures and defined in the figure legends.

Results

Expression of miR-205 is increased in elderly female patients with T2DM + OP. Compared with the control group, the expression level of miR-205 was significantly increased in the bone tissues and serum of elderly female patients with T2DM + OP ($P = 0.0098$ and $P = 0.001$) (Fig. 1A and B).

Expression of miR-205 is increased in the elderly female mouse model of T2DM + OP. The expression level of miR-205 was higher in the bone tissues, serum and BMSCs of the elderly female mice with T2DM + OP than this level in the control group (Fig. 2A-C).

Effect of miR-205 on BMSC viability in the elderly female mice with T2DM + OP. To investigate the effect of miR-205 overexpression on the biological function of BMSCs in elderly female type 2 diabetic mice with OP, the cells were transfected with the miR-205 mimic and the transfection was deemed successful (Fig. 3A). CCK-8 assay was performed to explore the effect of miR-205 on the cell viability of BMSCs extracted from the elderly female mice with T2DM + OP. The results showed that overexpression of miR-205 significantly inhibited the viability of BMSCs in the elderly female mice with T2DM + OP when compared to the negative control (NC) group (Fig. 3B). To investigate the effect of miR-205 knockdown on the biological function of BMSCs in elderly female type 2 diabetic mice with OP, the cells were transfected with the miR-205 inhibitor and the transfection was deemed successful (Fig. 3A). Knockdown of miR-205 significantly increased the viability of BMSCs in elderly female mice with T2DM + OP when compared to the NC group (Fig. 3C).

Effects of overexpression of miR-205 on osteogenic/adipogenic differentiation of BMSCs in elderly female mice with T2DM + OP. The results of ARS staining showed that overexpression of miR-205 obviously inhibited osteogenic differentiation of BMSCs in the elderly female mice with

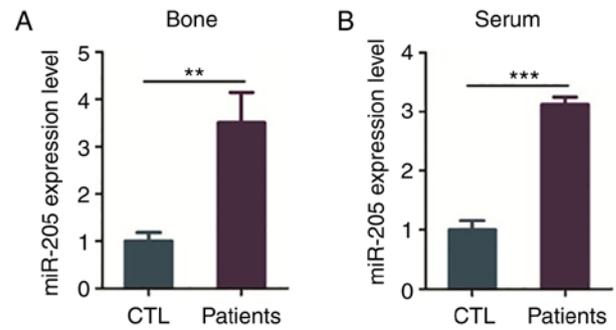


Figure 1. Expression level of miR-205 in the elderly female patients with T2DM + OP. Expression level of miR-205 in (A) bone tissues and (B) serum. ** $P < 0.01$ and *** $P < 0.001$, compared to the control (CTL). T2DM, type 2 diabetes mellitus; OP, osteoporosis.

T2DM + OP (Fig. 4A). In addition, the results of RT-PCR revealed that overexpression of miR-205 obviously reduced the expression of the osteogenesis-related genes, alkaline phosphatase, biomineralization associated (*ALP*) and osteocalcin (*OCN*) in BMSCs of elderly female mice with T2DM + OP (Fig. 4B).

Moreover, BMSCs of elderly female mice with T2DM + OP were transfected with miR-205 mimic and negative control (NC)-mimic, and ORO staining and RT-PCR were carried out after adipogenic induction for 24 days. The results of ORO staining showed that overexpression of miR-205 obviously promoted adipogenic induction of BMSCs in elderly female mice with T2DM + OP (Fig. 4C). In addition, the results of RT-PCR revealed that overexpression of miR-205 obviously increased the expression of adipogenesis-related genes *PPAR γ* and *FABP4* in BMSCs of elderly female mice with T2DM + OP (Fig. 4D).

Effects of knockdown of miR-205 on osteogenic/adipogenic differentiation of BMSCs in elderly female mice with T2DM + OP. BMSCs of elderly female mice with T2DM + OP were transfected with miR-205 inhibitor and NC-inhibitor, and ARS staining and RT-PCR were performed after osteogenic induction for 21 days. The results of ARS staining showed that knockdown of miR-205 markedly promoted osteogenic differentiation of BMSCs in elderly female mice with T2DM + OP (Fig. 5A). In addition, the results of RT-PCR revealed that knockdown of miR-205 significantly upregulated the expression of osteogenesis-related genes *ALP* and *OCN* (Fig. 5B).

Moreover, BMSCs of elderly female mice with T2DM + OP were transfected with miR-205 inhibitor and NC-inhibitor, and ORO staining and RT-PCR were conducted after adipogenic induction for 24 days. The results of ORO staining showed that the knockdown of miR-205 markedly suppressed adipogenic induction of BMSCs in elderly female mice with T2DM + OP (Fig. 5C). In addition, the results of RT-PCR revealed that the knockdown of miR-205 significantly downregulated the expressions of adipogenesis-related genes *PPAR γ* and *FABP4* (Fig. 5D).

miR-205 inhibits *Runx2* expression in a targeted manner. The target genes of miR-205 were predicted using online websites

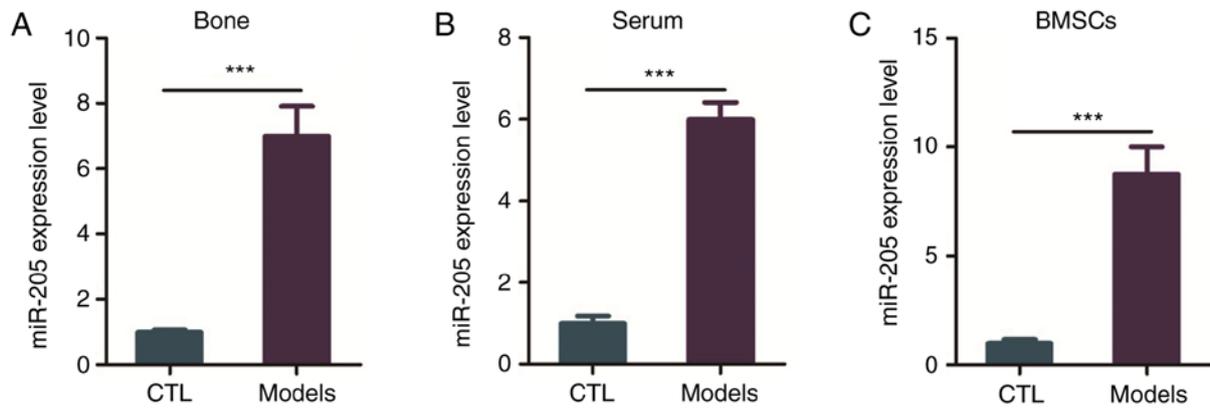


Figure 2. Expression level of miR-205 in the elderly female mouse model of T2DM + OP. Expression level of miR-205 in (A) bone tissues, (B) serum and (C) BMSCs. ***P<0.001, compared to the control (CTL). T2DM, type 2 diabetes mellitus; OP, osteoporosis; BMSCs, bone marrow mesenchymal stem cells.

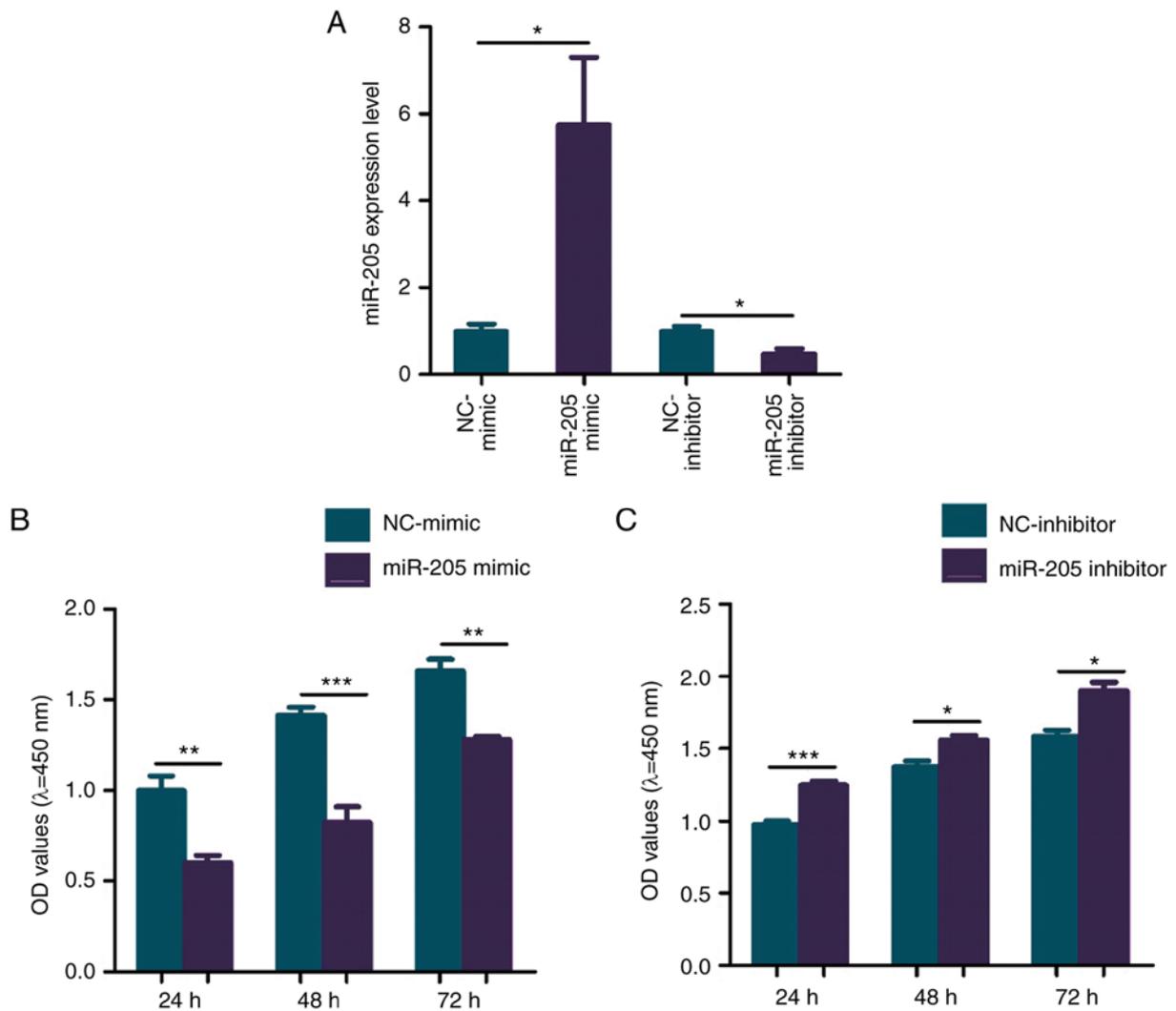


Figure 3. Effect of overexpression and knockdown of miR-205 on the viability of BMSCs in female mice with T2DM + OP. (A) Transfection efficiency of miR-205 mimic and inhibitor compared with the NC groups. (B and C) CCK-8 assay was used to detect the cell viability (OD value) of each group of BMSCs. *P<0.05, **P<0.01 and ***P<0.001 compared to the relative NC group. T2DM, type 2 diabetes mellitus; OP, osteoporosis; BMSCs, bone marrow mesenchymal stem cells; OD, optical density; NC, negative control.

TargetScan and miRanda, and the binding sites for miR-205 and Runx2 are shown in Fig. 6A. Luciferase reporter assay confirmed that there was a regulatory relationship between

miR-205 and Runx2 (Fig. 6B). BMSCs of elderly female mice with T2DM + OP were transfected with miR-205 mimic, NC-mimic, miR-205 inhibitor and NC-inhibitor, after which

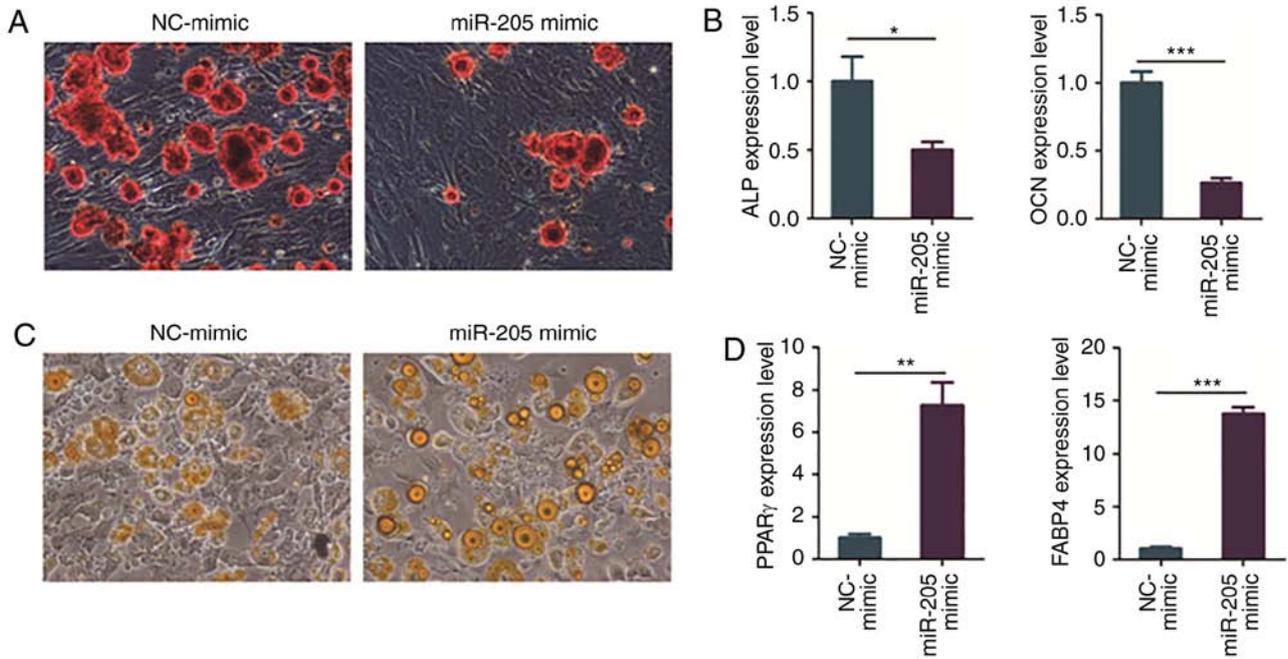


Figure 4. Overexpression of miR-205 inhibits osteogenic differentiation and promotes adipogenic differentiation of BMSCs in elderly female mice with T2DM + OP. (A) Osteogenic differentiation of BMSCs as detected via ARS staining. (B) Expression levels of osteogenesis genes, *ALP* and *OCN*, as detected via RT-PCR. (C) Adipogenic differentiation as detected via ORO staining. (D) Expression levels of adipogenesis genes, *PPAR γ* and *FABP4*, as detected via RT-PCR. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the NC-mimic group. BMSCs, bone marrow mesenchymal stem cells; T2DM, type 2 diabetes mellitus; OP, osteoporosis; ARS, alizarin red S; ORO, oil red O; *ALP*, alkaline phosphatase, biomineralization associated; *OCN*, osteocalcin; *PPAR γ* , peroxisome proliferator-activated receptor- γ ; *FABP4*, fatty acid binding protein 4.

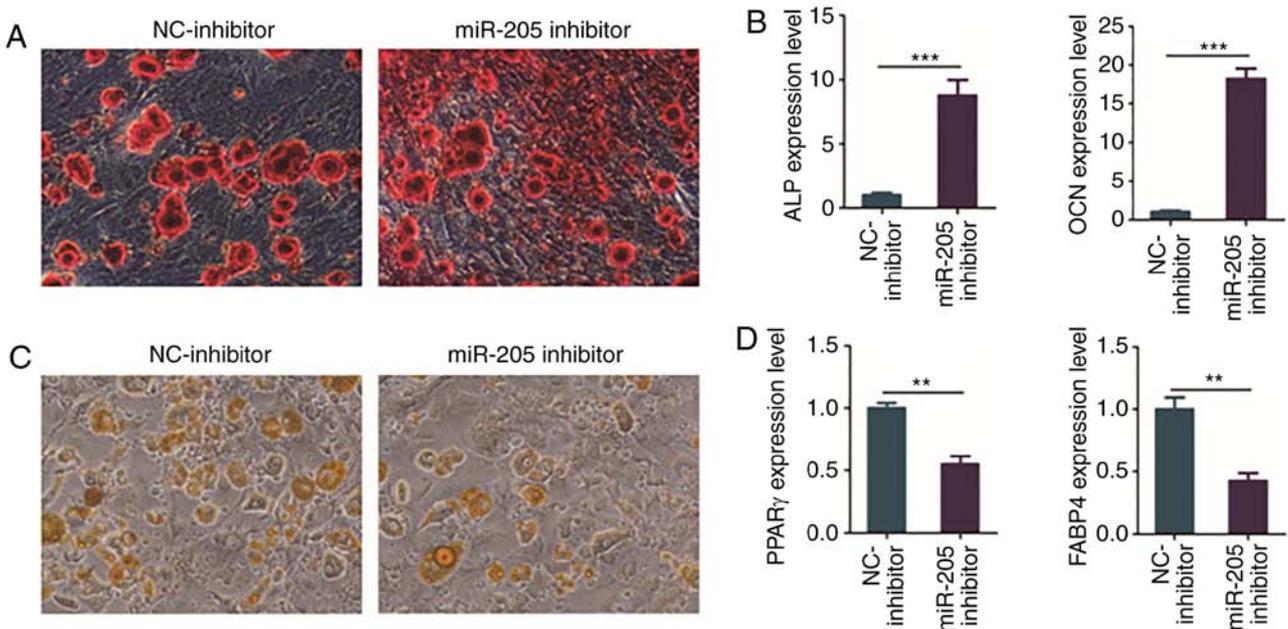


Figure 5. Knockdown of miR-205 promotes osteogenic differentiation and inhibits adipogenic differentiation of BMSCs in elderly female mice with T2DM + OP. (A) Osteogenic differentiation of BMSCs detected via ARS staining. (B) Expression levels of osteogenesis genes, *ALP* and *OCN*, as detected via RT-PCR. (C) Adipogenic differentiation as detected via ORO staining. (D) Expression levels of adipogenesis genes, *PPAR γ* and *FABP4*, as detected via RT-PCR. ** $P < 0.01$ and *** $P < 0.001$ compared to the NC-inhibitor group. BMSCs, bone marrow mesenchymal stem cells; T2DM, type 2 diabetes mellitus; OP, osteoporosis; ARS, alizarin red S; ORO, oil red O; *ALP*, alkaline phosphatase, biomineralization associated; *OCN*, osteocalcin; *PPAR γ* , peroxisome proliferator-activated receptor- γ ; *FABP4*, fatty acid binding protein 4.

the mRNA and protein levels of *Runx2* were determined. It was found that overexpression of miR-205 significantly suppressed the mRNA expression of *Runx2*, while knockdown

of miR-205 significantly increased the mRNA expression of *Runx2* (Fig. 6C). As shown in Fig. 6D, the results of western blotting were consistent with those of RT-PCR. Overexpression

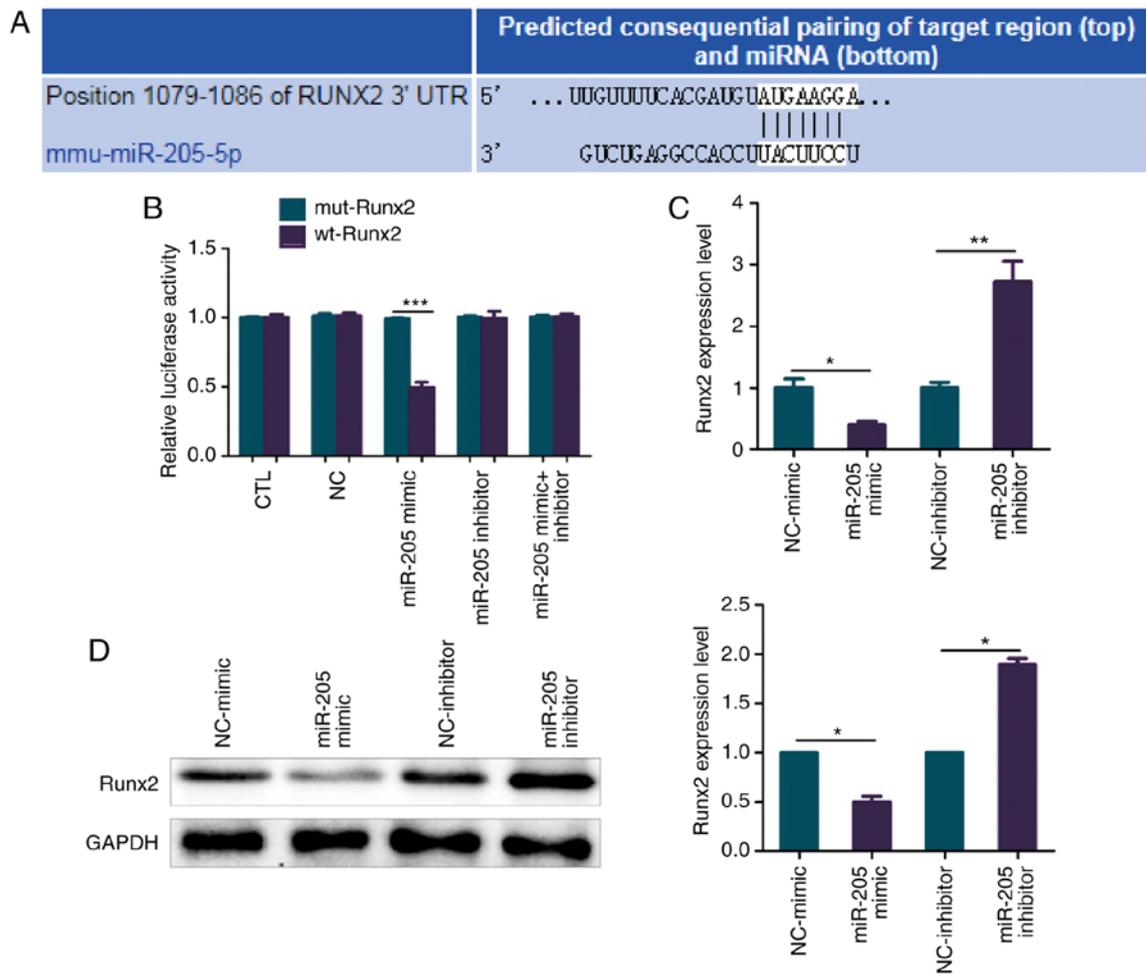


Figure 6. *Runx2* is a direct target gene of miR-205. (A) Binding sites for Runx2 and miR-205. (B) Correlation between miR-205 and Runx2 as detected via luciferase reporter assay. (C) *Runx2* mRNA level as detected via RT-PCR. (D) Runx2 protein level as detected via western blotting. *P<0.05, **P<0.01 and ***P<0.001. Runx2, RUNX family transcription factor 2; CTL, control; NC, negative control.

of miR-205 significantly suppressed the protein expression of Runx2, while knockdown of miR-205 significantly increased the protein expression of Runx2 (Fig. 6D).

Discussion

Osteoblasts are the most important cells in the bone microenvironment, accounting for approximately 95% of the total cell population in bone tissues, and they can determine the proportion of components in bone tissues, whose main source is bone marrow mesenchymal stem cells (BMSCs) (19,20). Under certain conditions, BMSCs can differentiate into osteoblasts, adipocytes and chondrocytes, and there is a certain dynamic balance between osteogenic and adipogenic differentiation of BMSCs under normal conditions, which plays an important role during bone development (21-23).

Studies have found that miRNAs, as ubiquitous molecules that regulate gene expression, play important regulatory roles in bone development and bone formation (24). For example, Yang *et al* found that miR-21 was able to facilitate bone formation and bone regeneration through PTEN/PI3K/Akt/HIF-1 α (25). Therefore, whether there is an miRNA able to regulate osteogenic/adipogenic differentiation of BMSCs in elderly female patients with T2DM + OP was explored in the present study.

In previous studies, the expression levels of miRNAs (miR-205, miR-188, miR-23a, miR-26a and miR-214) related to bone development and bone formation were detected in elderly female patients with T2DM + OP (9-11), and the results showed that the expression of miR-205 was significantly upregulated in bone tissues and serum of elderly female patients with T2DM + OP, thus miR-205 was selected as the object of further study. It is reported that miR-205 can regulate the biological functions of a variety of cancer cells and participate in bone formation. However, the correlation between miR-205 and osteogenic/adipogenic differentiation of BMSCs in elderly female patients with T2DM + OP has been sparsely studied. Therefore, the expression of miR-205 in bone tissues, serum and BMSCs of elderly female mice with T2DM + OP and its effect on osteogenic/adipogenic differentiation of BMSCs was further investigated. It was found that the expression of miR-205 was obviously increased in elderly female patients with T2DM + OP and elderly female mice with T2DM + OP, which provides an important target and marker for the early prediction and diagnosis of T2DM + OP in the elderly. Hu *et al* (26) found that the miR-205 expression obviously declined during osteogenic differentiation of normal BMSCs, consistent with our conjecture. In the present study, overexpression of miR-205 inhibited osteogenic differentiation and promoted adipogenic differentiation

of BMSCs in elderly female mice with T2DM + OP, while knockdown of miR-205 promoted osteogenic differentiation and inhibited adipogenic differentiation of BMSCs in elderly female mice with T2DM + OP. Hu *et al* (26) found that miR-205 can weaken osteogenic differentiation and enhance adipogenic differentiation of normal BMSCs. Therefore, we conclude that miR-205 inhibits osteogenic differentiation and promotes adipogenic differentiation of BMSCs in normal mice and elderly female mice with T2DM + OP. In addition, the results of luciferase reporter assay, RT-PCR and western blotting confirmed that miR-205 could directly inhibit the expression of its target gene *Runx2*. *Runx2* is an important regulator of osteogenic differentiation and is able to regulate the transcription of various osteogenesis-related genes (27,28). Inhibiting *Runx2* expression may affect osteogenic differentiation of BMSCs, thereby impacting the formation of osteoblasts and ultimately affecting bone development and bone formation (29). We will reorganize the phenotype of BMSCs *in vitro* in future research, and perform subcutaneous implantation experiments of BMSCs from nude mice with different expression levels of miR-205. We suspect that the subcutaneous implantation of BMSCs with overexpressing miR-205 can inhibit the formation of subcutaneous bone in nude mice, and the subcutaneous implantation of BMSCs with silenced miR-205 can promote the formation of subcutaneous bone in nude mice. Therefore, it was found in the present study that miR-205 is involved in the osteogenic/adipogenic differentiation of BMSCs in elderly female mice with T2DM + OP by targeted inhibition of the expression of its target gene *Runx2*.

In conclusion, the expression level of miR-205 is obviously increased in female patients with T2DM + OP and elderly female mouse model of T2DM + OP. In addition, miR-205 can regulate the osteogenic differentiation of BMSCs, and miR-205/*Runx2* may serve as a new method and target for the treatment of female patients with T2DM + OP, which provides theoretical support and experimental basis for the research on bone loss in female patients with T2DM + OP.

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Availability of data and materials

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

Authors' contributions

GZ, HL, WZ, ML, LT and WJ conceived and designed this study. GZ, HL, WZ, ML, WJ and XL conducted the data collection, analysis and summary. GZ, HL, WZ, LT, WJ and XL were responsible for data analysis and interpretation. GZ wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the

research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Animal Ethics Care Committee of Qiqihar Medical University (QMU-AECC-2019-51).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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