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MiR-203 is involved in osteoporosis by regulating DKK1 and inhibiting osteogenic differentiation of MSCs

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Abstract. – OBJECTIVE: To investigate whether miR-203 is involved in the osteogenic differentiation of rat mesenchymal stem cells (MSCs) by regulating DDK1, thus participating in the pathogenesis of osteoporosis.

PATIENTS AND METHODS: miR-203 expression in serum samples of 60 osteoporosis patients and 60 normal subjects was detected using Real-time fluorescence quantitative polymerase chain reaction (qRT-PCR) assay. MSCs were isolated from bone marrow of rats and then identified. Subsequently, the effects of miR-203 and DKK1 on osteogenic differentiation were estimated by alkaline phosphatase (ALP) activity, alizarin red staining, ALP staining, respectively. Expression levels of osteogenic-specific genes were detected by Western blot. Rescue experiments were conducted to confirm whether miR-203 could promote osteogenic differentiation of MSCs by inhibiting DKK1.

RESULTS: Serum level of miR-203 in osteoporosis patients was significantly lower than that of the normal subjects. Overexpressed miR-203 in MSCs enhanced ALP activity, expression of osteogenic marker genes and the number of calcified cells. Additionally, miR-203 could bind to DKK1. The regulatory effect of miR-203 on osteogenic differentiation in MSCs was reversed by DKK1.

CONCLUSIONS: MiR-203 promotes the differentiation of rat MSCs into osteoblast-like cells, which may be associated with the regulation of DKK1 expression.

Key Words:
MiR-203, MSCs, Osteogenic differentiation, DKK1, Osteoporosis

Introduction

Osteoporosis is a systemic metabolic bone disease characterized by reduced bone mass and microstructure abnormalities of bone tissue[1]. Evidence has shown that osteoporosis can inhibit the differentiation of bone marrow stem cells into osteoblasts and promote osteoclast differentiation[2]. Reduced bone mass and bone strength further lead to osteoporosis development[3]. MicroRNAs (miRNA) are small non-coding RNA containing about 22 nucleotides[4,5] that play a vital regulatory role in the differentiation and development of cells and tissues[6]. MiRNAs are found not only in tissues and cells, but also in the circulatory system. Researches have shown that, in addition to tumors, circulating miRNAs are abnormally expressed in many diseases. It was found that miRNAs can affect the development and progression of liver cancer through various pathways, such as regulation of cell proliferation or apoptosis[7]. Specifically, miR-203 exerts its anti-cancer and pro-apoptotic potentials in epithelial tumors[8]. Bone marrow derived-mesenchymal stem cells (BMSCs) have the potential of multi-directional differentiation. Advantages of simple culture, rapid *in vitro* proliferation and cryopreservation of BMSCs endow them with potential clinical application value[9]. In this study, based on the detection of miR-203 in osteoporosis patients, we used the mesenchymal stem cells (MSCs) cultured *in vitro* as research object. We aim to explore the impact of miR-203 on osteogenic differentiation of MSCs, providing a new direction for elucidating the pathogenesis of osteoporosis.

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Patients and Methods

Patients

60 osteoporosis patients and 60 normal volunteers were selected from our hospital. All the subjects were female and no significant difference in age was found between the two groups. In the fasting state and quiet environment, venous blood of the subjects in osteoporosis group and control group was collected in the early morning. This study was approved by the Ethics Committee of People's Hospital of Rizhao. Signed written informed consents were obtained from all participants before the study.

Bone Mineral Density Measurement

Spiral scanning was completed by Toshiba Aquilion16 row CT scanner and CT volume data were sent to QCT dedicated workstation. The bone mineral density (BMD) was calculated using Mindways QCT analysis software through selecting ROI (region of interest) in the coronal and sagittal plan by multi-plane reorganization.

MSCs Isolation and Culture

Femur and tibia bone marrow of anesthetic Sprague Dawley (SD) rats were obtained and prepared into single cell suspension. After centrifugation, the supernatant was discarded and the cells were seeded in culture flasks at 37°C, 5% CO_2 saturated humidity incubator. Osteogenic induction was performed with culture medium containing 1 mmol/L dexamethasone, 1 mmol/L sodium β-glycerophosphate and 50 mmol/L ascorbic acid.

MSCs Identification

Third-passage MSCs isolated from rats were collected for identification. The cells were digested, centrifuged at 4°C, washed with phosphate-buffered saline (PBS), and sequentially added with monoclonal antibodies CD29 and CD45. Cells were then resuspended in 500 μL of PBS and assayed by flow cytometry.

MSCs Mineralization Ability Detection

Osteoblasts were cultured, digested and seeded in 6-well plates. After the cells were cultured for 14 days, they were fixed with Ob fixer. Subsequently, cells were washed three times with PBS and alizarin red S stain was added for 30 min incubation. The excessive stain was washed away with distilled water and the cells were examined under optical microscope.

ALP Vitality Testing

After cell culture for 7 days, culture medium of the MSCs was discarded. The cells were then fixed with 4% paraformaldehyde and 5% citric acid for 30 s and incubated with mixture of 0.2% naphthol and 0.2% Nitrogen salt for 15 min. After the working solution was discarded, the cells were observed under microscope.

MSCs Osteogenic Ability Analysis

MSCs were treated in different ways for 7 days and their osteogenic ability was analyzed using ALP staining assay. All the staining steps were performed according to the instructions. Incubation solution was added to the coverslips within the 6-well plate at 37°C, and then the coverslips were rinsed. Hematoxylin counterstain dye was used to re-dye the cells. After rinse and air dry, the MSCs were observed under optical microscope.

RNA Extraction and Real-Time Fluorescence Quantitative Polymerase Chain Reaction (qRT-PCR)

The RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA, USA), chloroform and isopropanol, and then stored at -80°C after the RNA concentration of each sample was determined. Reverse transcription was performed to collect complementary Deoxyribose Nucleic Acid (cDNA). 1 μL cDNA sample of each treatment was taken for qRT-PCR according to SYBR Green method instruction (MedChem Express, Monmouth Junction, NJ, USA).

Primer sequences were as follows: DKK1 (F: 5'-CAGTGCCACCTTGAACTCAGT-3', R: 5'-CCGCCCTCATAGAGAACTCC-3'), miR-203 (F:5'-GTCGTACCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTAGT-3', R: 5'-GCCCGTGAAATGTTTAGGACCAC-3').

Cell Transfection

MiR-203 mimics or miR-203 inhibitor was transfected into the third-passage MSCs with good growth. The sequences of miR-203 mimics and inhibitor were as follows: miR-203 mimics (5'-GUGAAAUGUUUAGGACCACUAG-3', 5'-AGUGGUCCUAAACAUUUCACUU-3'); miR-203 inhibitor (5'-CUAGUGGUCCUAAACAUUUCAC-3').

Luciferase Reporting Assay

The wild-type sequence DKK1 (WT 3'UTR) and the mutant-type sequence DKK1 (MUT

3'UTR) were constructed. The MSCs were seeded in 96-well plates when their confluency reached 80%. The miR-203 mimics or negative controls were co-transfected with the constructed wild-type DKK1 or mutant-type DKK1, respectively. 48 hours after transfection, the dual luciferase-reporting assay was performed to detect the fluorescence intensity.

Western Blotting

After protein sample of cultured MSCs was extracted, it was boiled and stored at -20°C. The protein sample was taken out and dissolved when used. Each protein sample was added in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel in electrophoresis system. After the gel was transferred into polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland), the membrane was blocked. Membranes were then incubated with primary antibody overnight, and incubated with secondary antibodies. Finally, image exposure was performed to observe the protein expression.

Statistical Analysis

All experiments were repeated three times. Results were analyzed by statistical product and service solutions (SPSS 19.0, IBM, Armonk, NY, USA) statistical software. The measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Comparison between groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p < 0.05$ indicated the difference was statistically significant.

Results

The Expression of miR-203 in Peripheral Blood of Osteoporosis Patients was Significantly Lower Than That of Normal Group

We detected miR-203 expression in 60 osteoporosis patients and 60 normal subjects by qRT-PCR assay. The indexes of age, height and weight of the subjects had no significant differences between the two groups. The results indicated that serum level of miR-203 in osteoporosis patients was significantly decreased compared with that of the normal subjects ($p < 0.001$) (Figure 1A). Further analysis demonstrated that BMD of the subjects in the control group was higher than that in osteoporosis group ($p < 0.01$) (Figure 1B), indicating that decreased expression of miR-203 may be involved in the development of osteoporosis.

Phenotype Identification of Bone Marrow Mesenchymal Stem Cells

We selected bone marrow-derived MSC as a cell model to study osteoporosis *in vitro*. MSCs grew into a long fusiform shape with strong refraction on the fourth day (Figure 2A). After cell passage, a large number of calcified nodules of MSCs in the osteogenic induction medium were revealed by alizarin red staining, whereas similar phenotype was not observed in control group (Figure 2B). Immunophenotypic characteristics of the third-passage MSCs were detected by flow cytometry. The results showed that CD29-positive rate was 99.21% and CD45-neg-

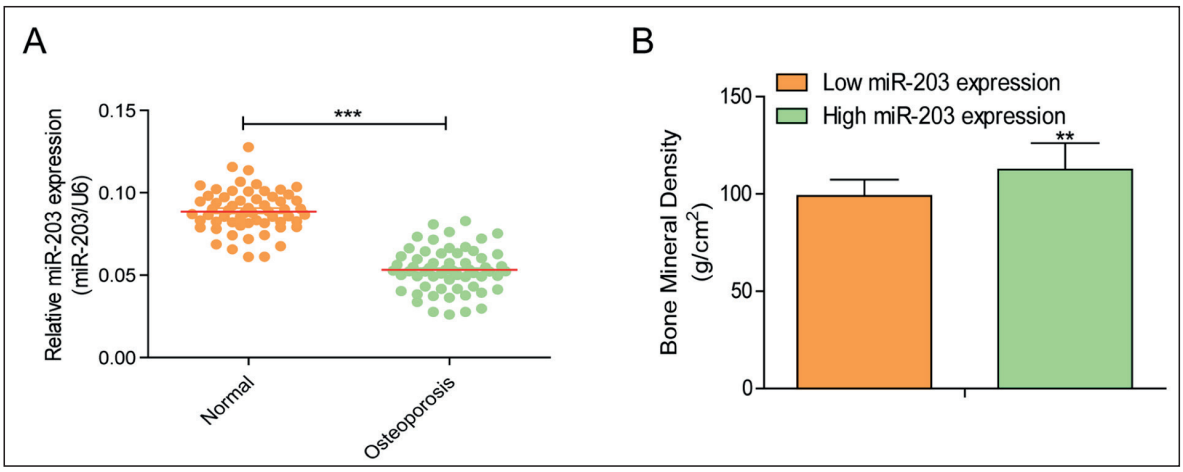

Figure 1. The expression of miR-203 in peripheral blood of osteoporosis patients is significantly lower than that of normal group. ***A,*** The expression of miR-203 in the peripheral blood of 60 osteoporotic patients is significantly lower than that of the 60 normal individuals ($p < 0.001$). ***B,*** BMD of osteoporosis patients with high expression of miR-203 is higher than those with low expression of miR-203 ($p < 0.01$).

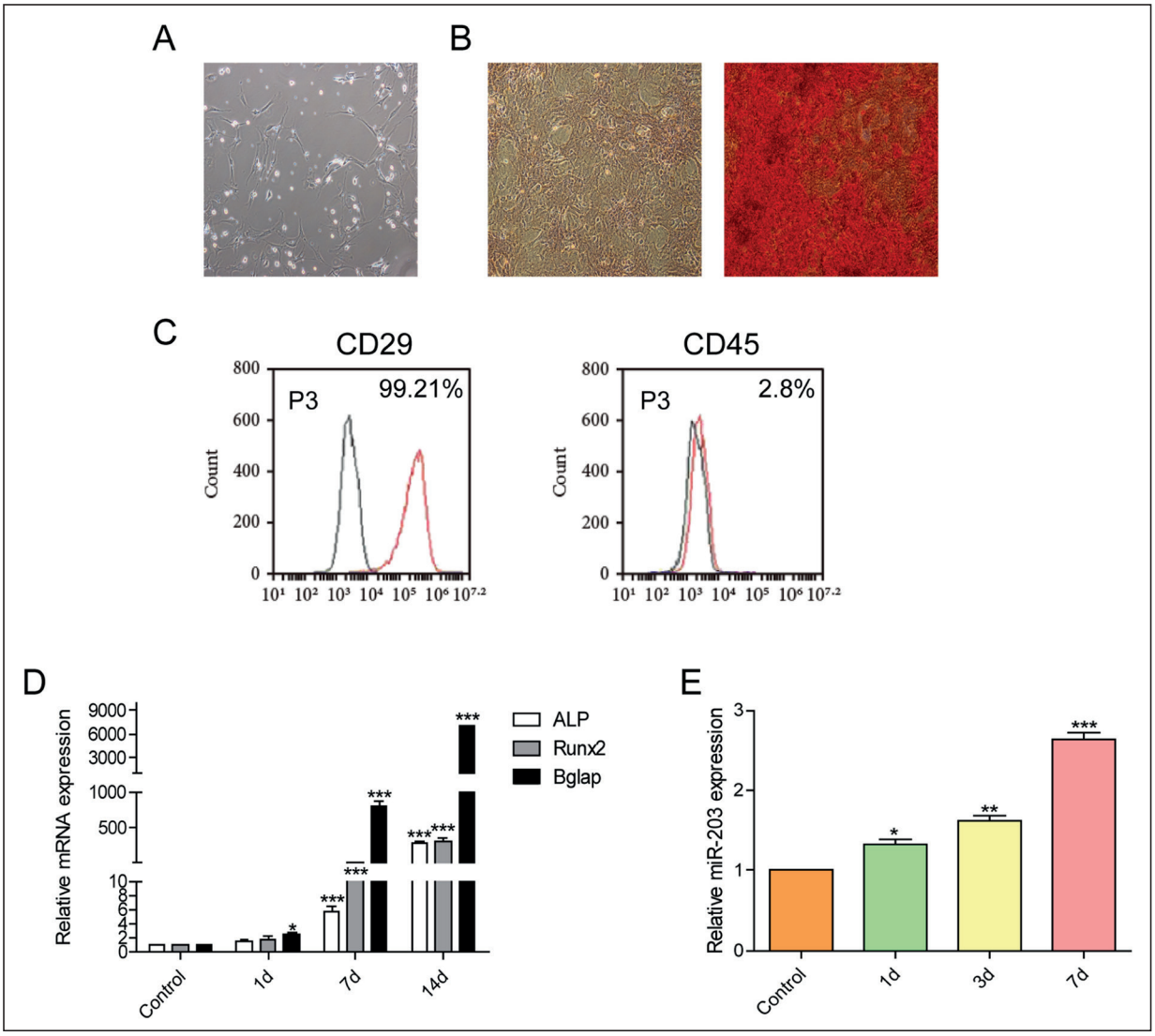

Figure 2. Phenotypic characterization of bone marrow MSCs is shown. ***A,*** MSCs grew into a long fusiform shape with strong refraction on the fourth day. ***B,*** After cell passage, significant calcified nodules of MSCs in the osteogenic induction medium were revealed by alizarin red staining, whereas the control group did not show such change. ***C,*** Immunophenotypic characteristics of the third-passage MSCs were detected by flow cytometry. The CD29-positive rate was 99.21% and CD45-negative rate was 2.8%, which were consistent with the immunophenotypic characteristics of bone marrow MSCs. ***D,*** On the first day, only expression of BGLAP was found increased. On the 7th day, the expression of all the marker genes including ALP, RUNX2 and BGLAP were significantly higher than that before induction of differentiation. ***E,*** The expression of miR-203 was slightly elevated on the first day of osteogenic differentiation ($p < 0.05$) and gradually increased over time.

ative rate was 2.8%, which were consistent with the immunophenotypic characteristics of bone marrow MSCs (Figure 2C). The expression levels of osteoblast marker genes ALP, RUNX2 and BGLAP were detected at 1 day, 7 days and 14 days after induction of differentiation. On the first day, only BGLAP expression was found to be increased. On the 7th day, the expressions of all the marker genes including ALP, RUNX2, and BGLAP, were significantly higher than that before induction of differentiation, which were gradually increased in a time-dependent manner (Figure 2D). The expression level of miR-203 was detected at the same time points. It was found that the expression of miR-203 was slightly elevated on the first day of osteogenic differentiation ($p < 0.05$) and gradually increased over time (Figure 2E). The above results showed that osteogenic differentiation was successfully induced in our model. Meanwhile, with the time of osteogenic

differentiation extended, the expression of miR-203 also gradually increased.

MiR-203 Promoted Osteogenic Differentiation of MSCs

MiR-203 expression was significantly enhanced after miR-203 mimics transfected into MSCs, while opposite result was observed after miR-203 inhibitor was transfected (Figure 3A). Subsequently, the effect of miR-203 on ALP activity in MSCs was examined. It was found that decreased expression of miR-203 dramatically weakened the ALP activity, while increased expression of miR-203 enhanced ALP activity conversely (Figure 3B). After overexpression of miR-203, the expressions of osteogenesis-related genes, including ALP, BGLAP and RUNX2, were significantly enhanced. On the contrary, reverse results were observed after knockdown of miR-203 (Figure 3C). In addition, miR-203 overexpression in MSCs up-regulated the expressions of osteoblast-associated proteins RUNX2,

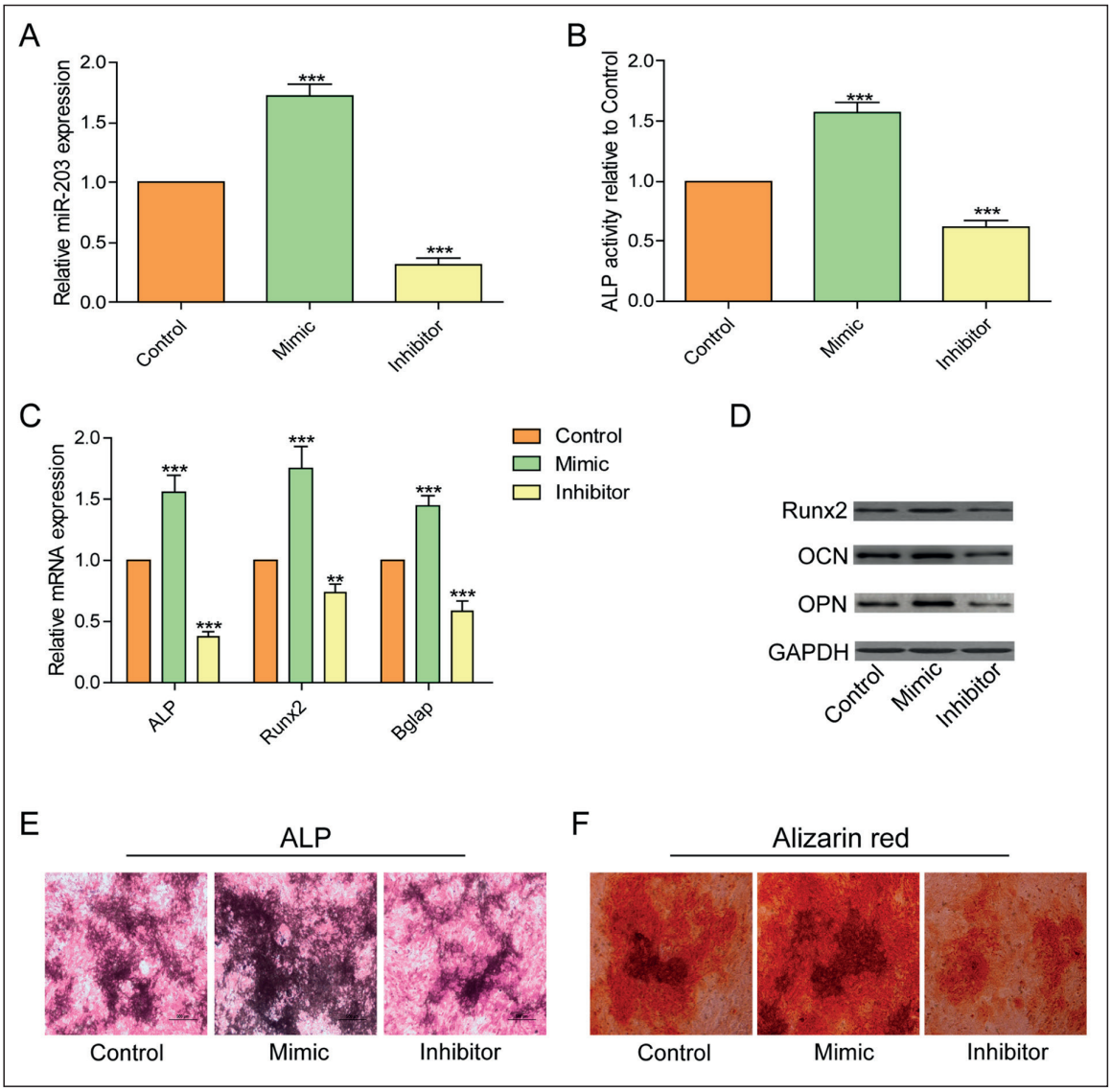

Figure 3. MiR-203 promotes osteogenic differentiation of MSCs. ***A,*** The expression of miR-203 was significantly up-regulated after miR-203 mimics transfected in MSCs, while opposite result was observed after miR-203 inhibitor transfection. ***B,*** Down-regulated expression of miR-203 significantly decreased ALP activity, while up-regulated expression of miR-203 enhanced ALP activity conversely. ***C,*** After overexpression of miR-203, the expressions of osteogenesis-related genes, including ALP, BGLAP and RUNX2, were significantly enhanced. On the contrary, reverse results were observed after knockdown of miR-203. ***D,*** Overexpression of miR-203 enhanced the expressions of osteoblast-associated proteins RUNX2, OCN and OPN, while knockdown of miR-203 reduced the expressions of above proteins.

OCN and OPN, while miR-203 knockdown reduced the above proteins (Figure 3 D). Furthermore, the MSCs treated with different ways for 7 days were used for ALP staining. The results showed that the higher expression level of miR-203 enhanced the degree of bone differentiation, whereas knockdown of miR-203 decreased the degree (Figure 3E). The results demonstrated that miR-203 level might be correlated with bone differentiation. After osteogenic induction for 14 days, the mineralized nodules were observed by alizarin red staining under inverted microscope (Figure 3F). The result was consistent with that on the 7th day, indicating that miR-203 inhibits osteogenic calcified nodules. All the above data suggested that miR-203 promotes the expressions of osteogenic genes and may promote osteogenic differentiation of MSCs.

MiR-203 Promoted Osteogenic Differentiation of MSCs by Degrading DKK1

MiRNAs can bind to the mRNA of target gene, thereby affecting disease development. Therefore, we predicted the target gene of miR-203 by bioinformatics and verified by functional analysis. Lastly, DKK1 was selected as the target gene of miR-203 for following experiments. To further verify whether miR-203 can bind to DKK1, DKK1-WT 3'UTR and DKK1-MUT 3'UTR were constructed (Figure 4A left). The activity of luciferase in the DKK1-WT 3'UTR group was decreased after miR-203 mimic was transfected. However, no significant change was observed in the DKK1-MUT 3'UTR group (Figure 4A right). Moreover, the overexpression of miR-203 significantly down-regulated gene expression of DKK1, whereas inhibition of miR-203 significantly up-regulated DKK1 gene expression (Figure 4B). Subsequently, protein level of DKK1 was examined, in accordance with that of mRNA level (Figure 4C). We next examined the effect of miR-203-DKK1 axis on osteogenic differentiation of MSCs. After overexpression of miR-203, the expressions of osteoblast-associated genes ALP, BGLAP and RUNX2 were significantly enhanced, which was reversed by DKK1 overexpression (Figure 4D). Meanwhile, overexpressed miR-203 improved the expressions of osteoblast-associated proteins RUNX2, OCN and OPN, which was also reversed by DKK1 overexpression (Figure 4E). These results indicated that miR-203 promotes osteogenic differentiation of MSCs by inhibiting DKK1.

Discussion

Osteoporosis is a systemic disease characterized by decreased bone formation, increased bone resorption and increased bone fragility, leading to an increased risk of fracture. Decreased BMD is one of the criteria for the diagnosis of osteoporosis currently[10]. The occurrence of osteoporosis is considered as the result of both genetic and environmental factors[11,12]. Bae et al[13] have demonstrated that differentially expressed miRNAs and their decreased affinity to target mRNAs would affect its downstream pathway, thus increasing the susceptibility to osteoporosis.

MiRNA is a class of non-coding RNA with 18-25 bases in length[14]. MiR-203, located at 14q32.33, was found to be down-regulated in esophageal adenocarcinoma[15], malignant pleural mesothelioma[16], liver cancer[17] and bladder cancer[18] in recent studies. In our work miR-203 expression in enrolled osteoporosis patients was also significantly down-regulated.

Bone marrow-derived mesenchymal stem cells (BMSCs) are widely observed in connective tissues, such as fat, muscle and blood[19]. BMSCs play a critical role in the repair and reconstruction of bone in osteoporosis patients[21]. BMSCs are easy to culture *in vitro* and can differentiate into osteoblasts under osteogenic induction[21]. Therefore, in this report, we used BMSCs as cell model to study the relationship between miRNA and osteoporosis. MiR-203 mimics or inhibitor was transfected into BMSCs, respectively to detect the osteogenic differentiation ability and expressions of osteogenic-related genes. The findings showed that miRNA-203 was highly correlated to osteogenic differentiation ability of BMSCs.

DKK1 is one of the Wnt signaling inhibitors[22], which inhibits the canonical Wnt signaling pathway by binding to the Wnt complex receptor LRP5/6[23]. In this study, miR-203 promoted osteogenic differentiation and inhibited DDK1 expression.

In summary, miR-203 expression was down-regulated in osteoporosis patients compared with that in normal controls. MiR-203 could promote the differentiation of MSCs into osteoblasts by inhibiting DDK1, which may provide theoretical basis and research direction for future exploring the mechanism of osteoporosis.

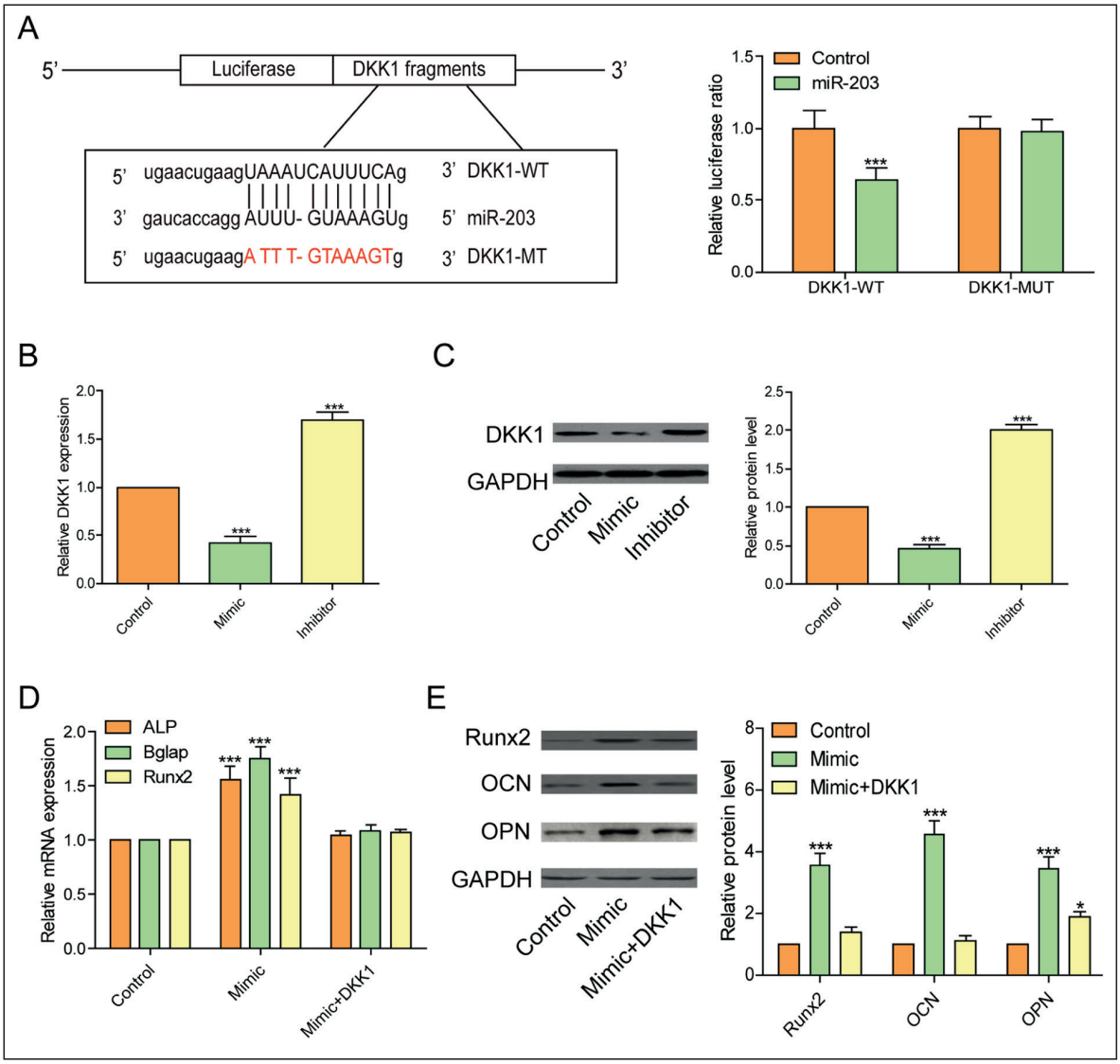

Figure 4. MiR-203 promotes osteogenic differentiation of MSCs by degrading DKK1. ***A,*** Binding site of miR-203 to DKK1 and luciferase reporter assay were shown. ***B,*** Overexpression of miR-203 significantly down-regulated the gene expression of DKK1. On the contrary, inhibition of miR-203 significantly up-regulated DKK1 expression. ***C,*** Overexpression of miR-203 significantly down-regulated the protein expression of DKK1. On the contrary, inhibition of miR-203 significantly up-regulated DKK1 expression. ***D,*** After overexpression of miR-203, the expressions of osteoblast-associated genes ALP, BGLAP and RUNX2 were enhanced significantly, which were reversed by DKK1 overexpression. ***E,*** Overexpression of miR-203 improved the expressions of osteoblast-associated protein RUNX2, OCN and OPN, which were reversed by DKK1 overexpression.

Conclusions

We have demonstrated that miR-203 can promote the differentiation of rat MSCs into osteoblast-like cells, which may be related to the regulation of DKK1 expression.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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