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MiR-488 promotes fracture healing by targeting DKK1

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Abstract. – OBJECTIVE: The aim of this study was to evaluate the effect of miR-488 on fracture healing and to investigate the possible underlying mechanism.

PATIENTS AND METHODS: The expression level of miR-488 in clinical cases was detected by quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). After prediction of the potential target of miR-488 in websites, three groups were established to elucidate the effects of miR-488 and Dickkopf1 (DKK1) on osteoblasts function: the miR-NC group (negative control), the miR-488 mimics group (MC3T3-E1 cells transfected with miR-488 mimics) and the mimics + si-DKK1 group (MC3T3-E1 cells transfected with miR-488 mimics and si-DKK1). Subsequently, cell viability, apoptosis rate of osteoblasts and osteogenesis-associated markers were determined respectively.

RESULTS: In the present study, we found that the expression of miR-488 in patients with osteoporosis was significantly lower than that of healthy controls. The potential target of miR-488 was predicted by three public databases. Luciferase reporter gene assay confirmed that DKK1 was a direct target of miR-488. Besides, the overexpression of miR-488 could significantly reduce the transcription and translation levels of DKK1. These results suggested that miR-488 had a negative regulatory effect on DKK1. Subsequent experiments demonstrated that decreased expression of DKK1 induced by miR-488 up-regulation could significantly improve cell viability and suppress cell apoptosis. Meanwhile, the expression levels of osteogenesis-associated markers were remarkably elevated.

CONCLUSIONS: Our research revealed the role of miR-488 in promoting osteoblast function. This indicated that miR-488 could be a potential therapeutic target for fracture healing.

Key Words:
Fragility fractures, Osteoporosis, MiR-488, Osteoblasts, Dickkopf1 (DKK1).

Introduction

Fragility fracture refers to fracture resulted from minor trauma, namely, fractures caused by falls at or below the height of the body's center of gravity. It is also a severe disease commonly found in the elderly. Meanwhile, fragility fractures are known as osteoporotic fractures because they are serious consequences of trauma caused by osteoporosis. Due to a profound impact on social health and economy, the fragility fracture is one of the major public health problems in the world[1,2]. In the United States, about 2 million patients suffer from fragility fractures annually, which will even increase to 3 million by 2025[3]. As for fracture healing, the interaction and synergistic effects of various growth factors as well as other types of cytokines are needed to regulate the activation of cells and the proliferation of osteoblasts[4]. Four conditions for successful fracture healing include appropriate mechanical environment, osteoblasts, bone scaffolds and effective bone formation induced by growth factors[5]. To accelerate the bone regeneration process, many strategies have focused on improving the treatment of fragility fractures. However, multiple defects still exist in the treatment regimens. Therefore, more effective and practical bone regeneration programs need to be investigated.

Micro-ribonucleic acids (miRNAs) are a type of endogenous, highly evolutionarily conserved RNA molecules with 20-24 nucleotides in length. They can regulate the translation of target genes and play various important regulatory roles in cells. Studies have demonstrated that one miRNA may have one or more target genes. Meanwhile, multiple miRNAs can be combined to subtly regulate the expression of one target gene[6,7]. Currently, more than one thousand human miRNAs have been discovered since the first discovery of

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miRNA. A large number of researches have investigated the effects of miRNAs and it is supposed that miRNAs regulate about one-third of human genes. However, the exact biological function of miRNAs remains to be elucidated.

As a member of the miRNA family, miR-488 exhibits unique advantages in the diagnosis and treatment of various diseases, such as panic disorder[8], pigmentation[9], hepatocellular carcinoma[10], and non-small-cell lung cancer (NSCLC)[11]. However, the exact role of miR-488 in regulating osteoblast function during fracture healing as well as the possible underlying mechanism has rarely been reported. Therefore, the aim of this study was to analyze the effect of miR-488 on the biological behaviors in regulating osteoblast function.

Patients and Methods

Clinical Samples

Human blood samples were collected from patients who were diagnosed with osteoporosis according to BMD or BMC. Briefly, osteoporosis was diagnosed if the femoral neck and/or lumbar spine T score was less than –2.5 SD. Meanwhile, other people were enrolled as normal controls. Blood samples were centrifuged at 1000 g for 10 min, and the serum was stored in aliquots at -80°C for subsequent use. Serum level of miR-488 in osteoporosis patients was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). This study was approved by the Ethics Committee of the First People's Hospital of Changzhou. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Mouse osteoblastic cell line MC3T3-E1 was purchased from the Chinese Academy of Sciences (Shanghai, China). All cells were incubated in α-modified minimal essential medium (α-MEM, Gibco-BRL, Grand Island, NY, USA) complemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin and 100 IU/mL penicillin (Invitrogen, Carlsbad, CA, USA) in a 37°C, 5% CO_2 incubator.

Cell Transfection

MC3T3-E1 cells were pre-cultured in 24-well plates for 24 h. MiR-488 mimics and si-Dickkopf1 (DKK1) were synthesized and transfected into osteoblastic cells to analyze the biological function of miR-488. Subsequently, three group were established to study the potential relevance between miR-488 and osteoblasts, including the miR-NC group (negative control), the miR-488 mimics group (MC3T3-E1 cells transfected with miR-488 mimics) and the mimics + si-DKK1 group (MC3T3-E1 cells transfected with miR-488 mimics and si-DKK1). All the stuff was purchased from RiboBio (Guangzhou, China). Cell transfection was performed according to the manufacturer's instructions of Lipofectamine RNAiMAX (Life Technologies, Gaithersburg, MD, USA).

Luciferase Reporter Gene Assay

TargetScan, miRDB and microRNA websites predicted that DKK1 was the target gene of miR-488. MC3T3-E1 cells were co-transfected with pMIR-3'UTR-DKK1/pMIR-3'UTR-Mut DKK1 and miR-488 mimics/negative control (NC). Then the cells were transfected with pMIR-Renilla plasmid (Promega, Madison, WI, USA) and seeded into 12-well plates. The cells were then lysed after transfection. The Luciferase activity was detected by a multi-function microplate reader (Promega Corporation, Madison, WI, USA). The results were normalized to Renilla Luciferase activity.

Cell Proliferation

MC3T3-E1 cells were harvested and inoculated into 96-well plates at a density of 2×10^3 cells for 48 hours. 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (MultiSciences, Hangzhou, China) was added to each well after 4-hour incubation. Subsequently, 150 μL dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added to each well for solubilizing formed formazan. Half an hour later, the absorbance at the wavelength of 490 nm was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

TUNEL Assay

The apoptosis of MC3T3-E1 cells was detected by TUNEL assay according to the manufacturer's instructions (Roche, Basel, Switzerland). Horseradish peroxidase-mediated diaminobenzidine reaction was used to visualize TUNEL-positive cells. After counterstain, randomly selected fields were photographed at a magnification of 200×. Apoptosis index was used to measure the degree of cell apoptosis.

Alkaline Phosphatase (ALP) Activity Analysis

According to the instructions of ALP activity assay kit, the ALP activity was quantified by

enzyme-linked immunosorbent assay (ELISA) (Beyotime, Shanghai, China) using the ALP yellow liquid substrate system. Total protein of MC3T3-E1 cells was extracted, and ALP activity was normalized based on the total amount of protein. Absorbance at the wavelength of 405 nm was measured by an ultraviolet spectrophotometer. Meanwhile, ALP activity was calculated by using the following formula: ALP activity (U/g) = [(absorbance $_{\text{measured tube}}$/absorbance $_{\text{standard tube}}$) × nitrophenol amount$_{\text{standard tube}}$]/total protein (g).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total RNA of cells was extracted according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). SYBR green qPCR assay was used to measure the expression level of DKK1, β-catenin and Bone morphogenetic protein 2 (BMP2). GAPDH was used as an internal reference. Meanwhile, miR-488 expression normalized to U6 was detected by TaqMan miRNA assay (Applied Biosystems, Foster City, CA, USA).

Western Blot

Total protein of MC3T3-E1 cells was extracted by radio-immunoprecipitation assay (RIPA) solution (Beyotime, Shanghai, China). The concentration of each protein sample was determined by the bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). Briefly, an equal amount of total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. After sealing with 5% skim milk, the membranes were incubated with primary antibodies of rabbit anti-mouse β-catenin, BMP2, and β-actin (1:1000) at 4°C overnight. Then the membranes were washed with Tris-Buffered Saline with Tween-20 (TBST) (Beyotime, Shanghai, China), followed by incubation with anti-rabbit secondary antibody (coupled by horseradish peroxidase) at room temperature for 2 h. Immunoreactive bands were exposed by enhanced chemiluminescence method (Thermo Fisher Scientific, Waltham, MA, USA); β-actin was used as an internal reference, and the relative changes in protein expression were detected.

Statistical Analysis

GraphPad Prism 6.02 (GraphPad Software, Inc., La Jolla, CA, USA) and PASW Statistics 18.0 (SPSS Inc., Fayetteville, NC, USA) were used for all statistical analysis. All results were presented as means ± standard deviation (SD). One-way ANOVA was used to compare the differences among groups, followed by Post-Hoc Test (Least Significant Difference). The unpaired *t*-test was used to compare the differences between the two groups. $p<0.05$ was considered statistically significant.

Results

MiR-488 Was Downregulated in Patients with Osteoporosis

To examine the role of miR-488 in osteoporosis, we first detected the expression level of miR-488 expression in clinical patients. Results showed that the expression level of miR-488 in patients with osteoporosis was significantly lower than that of healthy controls (Figure 1).

DKK1 Was a Direct Target of miR-488

To explore the potential target of miR-488, we searched three publicly available algorithms (TargetScan, miRDB and microRNA) and found that DKK1 was a supposed target of miR-488 (Figure 2A). We then established Luciferase reporter vectors containing wild or mutant-type miR-488 seed sequences of the DKK1 3'UTR to confirm whether miR-488 had a regulatory effect on DKK1. The increased expression level of miR-488 by mimics transfection resulted in a significant decrease in the Luciferase activity of wild-type DKK1 3'UTR reporter gene. However, it had no effect on mutant-type (Figure 2B). This

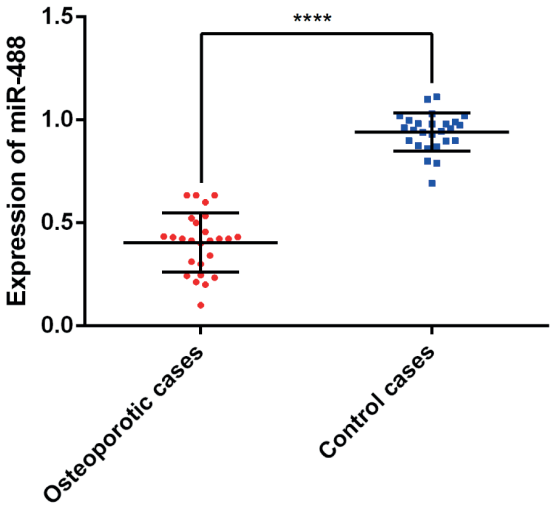

Figure 1. The expression level of miR-488 in clinical cases (****$p< 0.0001$).

suggested that the expression of DKK1 could be regulated by miRNA-488.

MiR-488 Decreased the Expression Level of DKK1

Subsequently, three groups (the miR-NC group, the miR-488 mimics group and the mimics + si-DKK1 group) were established in MC3T3-E1 cells to experiments to further study the correlation between miR-488 and osteoblasts. QRT-PCR results demonstrated that the expression level of DKK1 was remarkably inhibited by the up-regulation of miR-488 in MC3T3-E1 cells (Figure 3B). These data further confirmed that DKK1 could be negatively regulated by miR-488.

MiR-408 Promoted Cell Viability

To examine the function of miR-488 in the proliferation of osteoblasts, MTT assay was performed to detect cell proliferation rate. Results indicated that the proliferation rate of MC3T3-E1 cells was significantly increased after transfection with miR-488 mimics. In contrast, the growth rate was significantly decreased in the mimics+ DKK1 group (Figure 3A).

MiR-488 Inhibited Cell Apoptosis

MiR-488 remarkably decreased the apoptosis level of TUNEL positive cells. However, after increasing the DKK1 siRNA, the apoptosis index of MC3T3-E1 cells was significantly intensified. This suggested that miR-488 could repress the apoptosis rate by targeting DKK1 (Figure 3C).

MiR-488 Increased the Expression of Osteogenic Markers

The expression of ALP begins to increase at the early stage of osteogenic differentiation, which maintains a high concentration throughout the whole process of osteogenesis. Therefore, ALP staining can be used as an early marker of osteogenesis.

Current researches[12,13] have shown that Wnt/β-catenin signaling pathway plays an important role in fracture healing. β-catenin is located at the center of the Wnt/β-catenin signaling pathway. Meanwhile, the number and state of its presence in cells exert a decisive influence on this signal pathway, which is considered as a sign of activation[14,15].

BMP2 also plays a crucial role in bone growth and has a strong bone-inducing activity[16]. Furthermore, BMP2 is a necessary component of the signaling cascade that controls fracture repair[17].

Results indicated that after the transfection of miR-488, the ALP activity was significantly higher than that of the control group. Meanwhile, the expressions of osteogenesis-associated markers, including β-catenin and BMP2, were also remarkably higher. However, the addition of si-

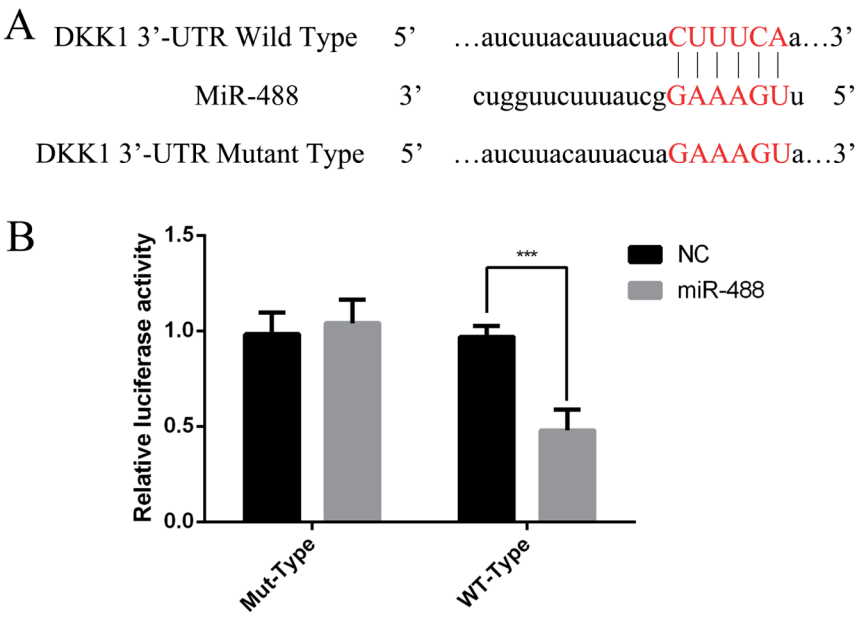

Figure 2. DKK1was a direct and functional target of miR-488. MC3T3-E1 cells were transfected with miR-488 mimics and inhibitor. ***A,*** Diagram of putative miR-488 binding sites of DKK1. ***B,*** Relative activities of Luciferase reporters (***p<0.01).

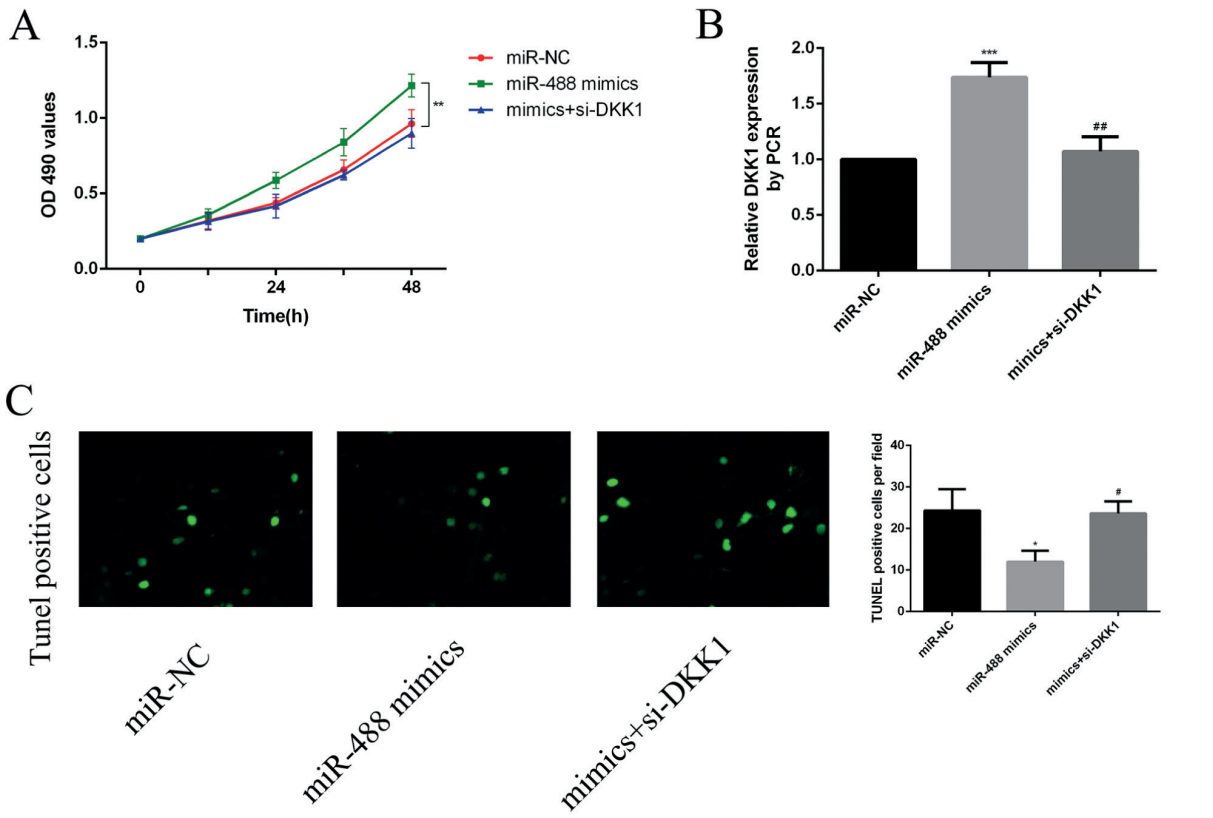

Figure 3. ***A,*** MiR-488 promoted the proliferation of MC3T3-E1 cells. ***B,*** Expression level of DKK1 detected by qRT-PCR. ***C,*** MiR-488 inhibited the apoptosis of MC3T3-E1 cells. Data were presented as means ± standard deviations. (Magnification, x200, **p*<0.05, ***p*<0.01, ****p*<0.001 *vs.* the NC group; #*p*<0.05, ##*p*<0.01 *vs.* the Mimics group).

DKK1 could reverse the effect of miR-488 (Figure 4).

Discussion

Osteoporosis is a metabolic and systemic bone disease characterized by reduced bone mass and deterioration of bone microarchitecture in the body. It can result in increased bone fragility and high possibility of fractures[18,19]. Osteoporotic fracture is known as the most severe complication of osteoporosis. Meanwhile, patients with osteoporosis may fracture in areas with rich cancellous bones due to minimal stress and injuries in the activities of daily living, such as falls. Therefore, osteoporosis is a high-risk factor for fracture in the elderly.

Fracture healing is a complex process, which mainly includes the formation of granulation tissue and bone callus as well as reconstruction of bone. Most fractures heal with completely restored structure and bone function[20,21]. However, about 10% of patients with fractures eventually occur abnormal union. This includes complications like limited and delayed fracture healing and un-united fractures. These complications may eventually lead to a severe public health problem and cause huge economic and social burdens[22].

In recent years, more and more attention has been gradually paid to the regulation of osteoblast differentiation and bone formation, to find new targets or strategies for treating the bone formation. In the medical field, the exploration of new methods for the treatment of osteoporotic fractures remains urgent[23,24].

Studies have shown that miRNA exerts its biological function via regulating target genes. Therefore, identifying target proteins is helpful to understand the possible molecular mechanism of miRNA. MiR-488, a member of the large miRNA family, is involved in the regulation of va-

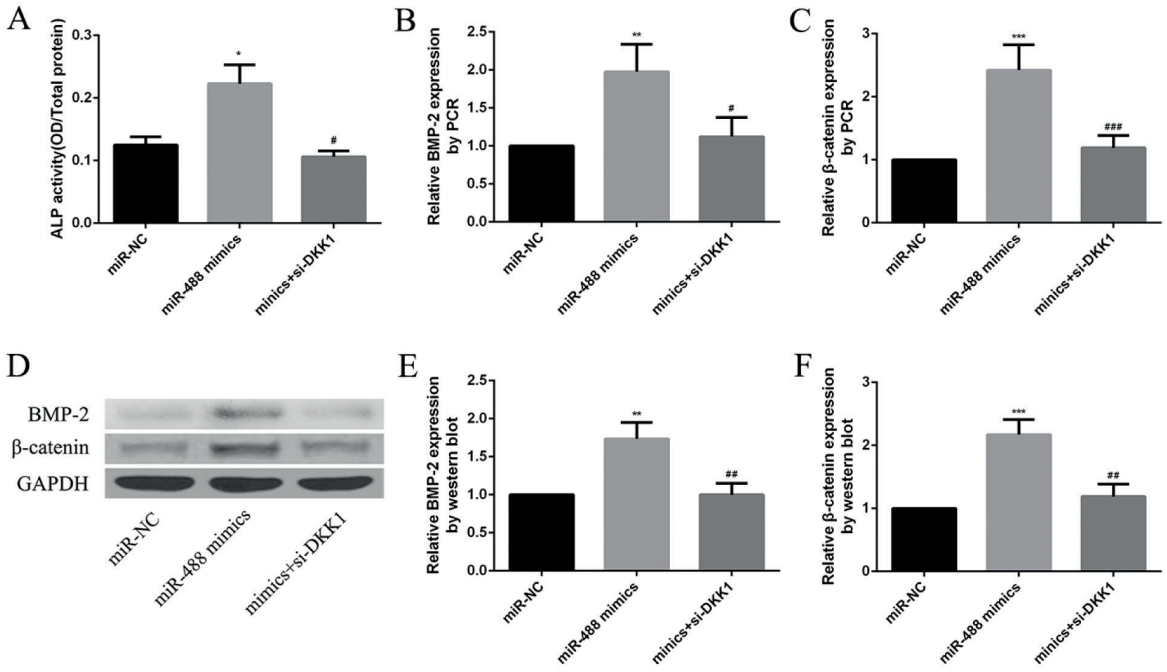

Figure 4. MiR-488 promoted the expressions of osteogenesis-associated markers. ***A,*** Activity of ALP determined by ELISA. ***B,*** and ***C,*** Expression levels of β-catenin and BMP2 detected by qRT-PCR. ***D-F,*** Protein expression levels of β-catenin and BMP2 detected by Western-blot. Data were presented as means ± standard deviations. (Magnification, x200, *p<0.05, **p<0.01, ***p<0.001 *vs.* the NC group; #p<0.05, ##p<0.01, ###p<0.001 *vs.* the Mimics group).

rious genes and signaling pathways in the body[25]. However, the exact mechanism of miR-488 on osteoblast function remains unclear. In this study, we found that the expression of miR-488 in the blood samples of patients with osteoporosis was significantly lower than that of healthy controls. TargetScan and miRDB software predicted that DKK1 was the target gene of miR-488. Subsequent Luciferase reporter gene assay confirmed that miR-488 could bind to DKK1. Besides, overexpression of miR-488 could significantly reduce the transcription and translation levels of DKK1. These results all suggested that DKK1was a target gene of miR-488.

Wnt/p-catenin signaling pathway has been proved to be a key regulatory pathway in the embryonic bone development and formation[26-28]. It is highly conserved in different species and is essential for the development, growth, differentiation and apoptosis of cells. Current studies[29-31] have indicated that the Wnt/β-catenin signaling pathway may play an important role in fracture healing. Under physiological and pathological conditions, Dickkopf-1 (DKK-1) is a regulator in bone remodeling. DKK-1 is also an inhibitor of the Wnt signaling pathway, which is widely used in bone formation research. Kwon et al[32] found that low levels of DKK-1 may weaken the inhibition of the Wnt signaling pathway and enhance osteoblast differentiation as well as new bone formation. Therefore, the classical Wnt signaling pathway and its inhibitor DKK-1 have attracted much attention in osteoblast function.

To further explore the effect of miR-488 on osteoblast function, we investigated the biological function of miR-488 by transfecting miR-488 and/or si-DKK1 in mouse osteoblastic cell line MC3T3-E1. MTT and TUNEL assay were conducted to detect the proliferation ability and apoptosis rate of MC3T3-E1 cells. As expected, the overexpression of DKK1 could effectively reverse the effects of miR-488 on cell proliferation and apoptosis. To comprehensively describe the strength of bone formation ability of cells, classic markers of bone formation including alkaline phosphatase (ALP), β-catenin and bone morphogenetic protein2 (BMP2) were selected and used in this study. Experimental results showed that the secretion of ALP (a marker of bone formation) in osteoblasts transfected with miR-488 was significantly elevated. Meanwhile, increased levels of β-catenin and BMP2 (genes relating to bone

formation) were also found. However, DKK1 could suppress the expressions of these osteogenesis-associated markers. The above results all suggested that miR-488 potentially promoted fracture healing.

Conclusions

We revealed that miR-488 regulated the differentiation of osteoblasts and bone formation by regulating DKK-1. This improved the efficacy of osteoporosis symptoms, thereby providing new targets and strategies for the treatment of osteoporosis-induced fragility fractures.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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