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MicroRNA 98-5p Overexpression Contributes to Delayed Fracture Healing via Targeting BMP-2

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MicroRNAs (miRNAs) are related to the regulation of bone metabolism. Delayed fracture healing (DFH) is a common complication after fracture surgery. The study attempted to examine the role of miR-98-5p and bone morphogenetic protein (BMP)-2 with the onset of DFH. A total of 140 patients with femoral neck fracture were recruited, including 80 cases with normal fracture healing (NFH) and 60 cases with DFH. MC3T3-E1 cells were induced cell differentiation for cell function experiments. Real-time quantitative polymerase chain reaction (RT-qPCR) was carried out to test mRNA levels. Cell proliferation and apoptosis were determined via CCK-8 and flow cytometry assay. Luciferase reporter assay was done to verify the targeted regulatory relationship of miR-98-5p with BMP-2. In comparison with NFH cases, DFH patients owned high levels of serum miR-98-5p and low concentration of BMP-2, and the levels of the two indexes are significantly negatively correlated. Both miR-98-5p and BMP-2 had the ability to predict DFH, while their combined diagnostic value is the highest. BMP-2 was demonstrated to be the target gene of miR-98-5p. Overexpression of BMP-2 reversed the role of miR-98-5p in MC3T3-E1 cell proliferation, apoptosis and differentiation. Increased miR-98-5p and decreased BMP-2 serve as potential biomarkers for the diagnosis of DFH. MiR-98-5p overexpression inhibits osteoblast proliferation and differentiation via targeting BMP-2.

Keywords: biomarker; delayed fracture healing; miR-98-5p/BMP-2; osteoblast differentiation
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Introduction

Delayed fracture healing (DFH) is a common complication after fracture surgery, which is one of the most difficult and complicated diseases in the traumatic orthopedics field (Helbig et al. 2018). DFH occurs in 5%-10% of patients after operation (Pazarci et al. 2019). Age, diabetes, smoking, local infection, nutritional deficiency, soft tissue injury, and compartment syndrome are risk factors for DFH (Schlundt et al. 2015). DFH seriously affects the treatment outcome of patients and can lead to severe disability, decreased quality of life and increased medical burden (Wallimann et al. 2021). In the event of DFH, the only definite and effective treatment is bone graft and internal fixation (Peters et al. 2022). It can lead to severe disability, decreased quality of life and increased medical burden. Studying the mechanism of bone healing at the level of gene and molecular signaling pathways is helpful to find the factors affecting fracture healing. This can help the early diagnosis and intervention of bone healing, and provide a new idea for the clinical diagnosis and therapy of fracture and DFH.

MicroRNAs (miRNAs) are non-coding RNAs with a size of 23 bases. By binding to the 3′ untranslated region

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(UTR) of target genes, miRNAs can inhibit the translation of target genes, and regulate the gene expression at the transcription level (Imakawa et al. 2022). In recent years, miRNAs related to the regulation of osteogenic and osteoclast differentiation have been discovered successively, and have gradually become the focus of current research (Moura et al. 2020; Yang et al. 2020). Studies have shown that miRNAs play an important role in the regulation of cell proliferation, differentiation and apoptosis (Shan et al. 2021), among which the involvement of miRNAs in the mediation of osteoblast differentiation and bone metabolism has been a research hotspot in recent years (Zhang et al. 2017a; Arfat et al. 2018). For example, downregulation of miR-467g can significantly promote the regeneration ability of new bone, and miR-467g can prevent the regeneration of new bone by inhibiting Runx2 signaling pathway (Kureel et al. 2017). MiR-221 can inhibit osteoblast differentiation and bone formation through binding to RUNX2 (Zhang et al. 2017b). Therefore, miRNAs may be a new target for the therapy of bone degenerative diseases and other abnormal bone formation disorders.

MiR-98-5p is one of the most popular members of miRNAs family in the research field of bone remodeling in recent years (Zheng et al. 2019). In osteoporosis, elevated levels of miR-98-5p are detected in the *in vitro* osteoporosis models, and miR-98-5p inhibition can promote the preosteoblast viability and differentiation via activating PI3K/AKT/GSK3β signaling (Zheng et al. 2019). Besides, miR-98-5p also participates in the bone formation and is considered to be a promising target for osteoporosis (Wang et al. 2022). Furthermore, bone morphogenetic protein (BMP)-2 serves as a target gene of miR-98-5p, and can abolish the role of miR-98-5p in preosteoblast proliferation and differentiation (Zheng et al. 2022). BMP-2 is a potent bone-inducing growth factor that can recruit osteoblasts, promote osteoblast differentiation, and promote bone formation (Moon et al. 2014; Ramazzotti et al. 2016).

In view of the previous evidence, this study attempted to clarify the association of miR-98-5p and BMP-2 with the onset of DFH, so as to provide reference for clinical diagnosis and therapy. Furthermore, the underlying mechanism was preliminary verified *in vitro*.

Materials and Methods

Study subjects

A total of 140 patients with femoral neck fracture who received surgical treatment in Zhucheng People's Hospital from May 2019 to October 2021 were selected, including 80 cases with normal fracture healing (NFH) with the mean age of 54.66 ± 8.27 years old (mean ± standard deviation, SD) and 60 cases with delayed fracture healing (DFH) with the mean age of 55.02 ± 9.57 years old. The age showed no significant difference between the two groups ($P > 0.05$). Inclusion criteria: (1) Unilateral femoral neck fracture revealed by X-ray and CT examination; (2) Hospitalized for internal fixation surgery; (3) Give informed consent to this study and sign the consent form. Exclusion criteria: (1) Previous history of fracture or orthopedic surgery; (2) Degenerative, arthritis, bursitis, synovitis, femoral head necrosis, rheumatoid arthritis, bone tumors and other orthopedic diseases; (3) Acute and chronic infections, immune and hematological diseases. The diagnostic criteria (Park et al. 2014) for DFH were: (1) no or minimal callus formation on X-ray examination at 3 months after fracture, and (2) visible sclerosis and gaps in the bone at the broken end. At the reexamination 4 weeks after operation, 3 mL peripheral venous blood was collected, centrifuged at 3,000 r/min at 4°C for 15 min, and the supernatant serum samples were obtained and stored at −80°C.

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Zhucheng People's Hospital. Informed consent was obtained from all individual participants included in the study.

Cell culture and differentiation

BeNa Culture collection (Beijing, China) was the supplier of MC3T3-E1 pre-osteoblasts. The cells were cultured in α-Minimum Essential Medium (MEM), containing 10% fetal bovine serum, 1×10^5 U/L penicillin and streptomycin. The incubator was under aseptic conditions at 37°C with 5% CO_2.

MC3T3-E1 cells in the logarithmic phase were seeded in 6-well plates with the plating density of 6×10^6 per well. When the cells had grown to 80% confluence, α-MEM medium was replaced with the osteogenic induction medium, containing 10% fetal bovine serum, 5 mmol/L L-glycerophosphate, 100 nmol/L dexamethasone and 50 g/L ascorbic acid. The culturation was performed for 14 days to induce osteoblast differentiation.

Cell transfection

For transfection, miR-98-5p mimic and its negative control (miR-NC) were designed and obtained by RiboBio (Guangzhou, China). To overexpress BMP-2, the full length of BMP-2 cDNA was cloned into pcDNA3.1 vector (pcDNA-BMP-2) by Shanghai GenePharm, and the empty vector was identified as the negative control (pcDNA-NC). MC3T3-E1 cells were seeded into 6-well plates with the plating density of 6×10^6 per well, then the cell transfection was done when cells reached the logarithmic growth phase. Lipofectamine 3000 was applied for the cell transfection, mixed with miR-mimic or pcDNA vector, and added into the cell culture medium. After culturation for 6 hours, the medium was replaced with normal medium.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted and reverse transcribed to synthesize cDNA. Subsequently, cDNA was used as template to detect gene mRNA levels by RT-qPCR using Mir-x miRNA qRT-PCR TB Green reagent or TB Green Premix

Ex Taq™ II reagent on an ABI 7500 system (Applied Biosystems, Foster City, CA, USA). 20 μL reaction system was needed, including 1 μL cDNA, 1 μL primers (forward and reverse), 10 μL TB Green and 8 μL deionized water. The primer sequences for miR-98-5p, BMP-2, alkaline phosphatase (ALP), osteocalcin (OCN), and runt-related transcription factor 2 (RUNX2) were recorded in Table 1. U6 or GAPDH were applied for the normalization. $2^{-\Delta\Delta Ct}$ was used for the calculation of mRNA levels under the normalization with U6 or GAPDH. Relative mRNA levels were normalized to respective control groups and expressed as mean and SD.

CCK-8 assay

CCK-8 assay was done for the determination of the cell proliferation. Cells were plated in 96-well plates with the plating density of 4×10^4 per well. After 48 h of transfection, cells were incubated with 100 μL α-MEM medium and 10 μL CCK-8 solution at 37℃ for 30 min, and the absorbance value at 450 nm was detected by fluorescence spectrophotometer.

Flow cytometry assay

The cells were digested by trypsin and harvested by centrifugation. Flow cytometry was performed according to Annexin V/propidium iodide (PI) kit instructions. 5 μL of Annexin V-FITC and PI were mixed and supplemented to the cell medium. The cells were incubated for 15 min avoid light at room temperature, and the apoptosis rate was detected by flow cytometry. Followed by FCM (BD FACS Calibur, BD Biosciences, Franklin Lakes, NJ, USA) using the FL1 channel for Annexin V-FITC and the FL2 channel of PI. Final apoptotic cell number was estimated as a total percentage of early apoptotic cells staining positive for Annexin V and negative for PI and late apoptotic cells positive for both Annexin V and PI. The cell apoptosis rate was calculated as follows: Cell apoptosis rate (%) = (apoptotic cell number / total cell number) × 100.

Luciferase reporter assay

The potential target genes of miR-98-5p were identified by TargetScan database (http://www.targetscan.org/). It was found that the UACCUCA sequence in the 3′-UTR of BMP-2 could bind with the AUGGAGU sequence in miR-98-5p, so BMP-2 was the potential target gene of miR-98-5p. The BMP-2 sequence containing miR-98-5p binding site was synthesized and named as wild-type (BMP-2-wt) sequence. The mutant sequence (BMP-2-mut) was synthesized by the Shanghai Sangon Biotechnology Company (Shanghai, China) using WT sequence as template. The mut or wt sequences were cloned into vector, and co-transfected into MC3T3-E1 cells with miR-98-5p mimic or miR-NC. After 48 h transfection, the luciferase activity of sea kidney was detected in each group.

Statistical analysis

SPSS 21.0 software (IBM SPSS, Armonk, NY, USA) and GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA) was applied for the data analysis and plotting. Differences of categorical variables between groups were evaluated using the $\chi 2$ test, while continuous variables were compared through one-way ANOVA. Outliers were values more than 1.5 times the value of the interquartile range and no outliers were identified. Peason's correlation analysis was performed to evaluable correlation between two variables. Receiver operating characteristic (ROC) curves and area under the ROC curve (AUC) was generated to assess the diagnostic values, and Hosmer-Lemeshow test was used to assess goodness-of-fit. The optimal cut-off point was obtained using the Youden index [maximum (sensitivity + specificity – 1)]. Differences were considered statistically significant when $P < 0.05$.

Table 1. Primer sequences for real-time quantitative polymerase chain reaction (RT-qPCR).

Gene		Sequences (5′-3′)
miR-98-5p	Forward	ATCCAGTGCGTGTCGTG
	Reverse	TGCTTGAGGTAGTAAGTTG
BMP-2	Forward	CGTGAGGATTAGCAGGTCTTTG
	Reverse	TTTCGCTTGACGCTTTTCTC
ALP	Forward	CTCCCAGTCTCATC TCCT
	Reverse	AAGACCTCAACTCCCCTGAA
OCN	Forward	GTG ACGAGTTGGCTGACC
	Reverse	CAAGGGGAAGAGGAAAGAAGG
RUNX2	Forward	CGGAATGCCTCTGCTGTTAT
	Reverse	AGCTTCTGTCTGTGCCTTCT
U6	Forward	CTCGCTTCGGCA GCACA
	Reverse	AACGCTTCAC GAATTTGCGT
GAPDH	Forward	CTCAGACACCATGGGGAAGGTGA
	Reverse	ATGATCTTGAGGCTGTTGTCATA

Results

Basic information comparison of the two study groups

As shown in Table 2, no significant difference was detected in terms of age and sex between the DFH and NFH groups ($P > 0.05$). It can be seen that more smokers were found in DFH group compared with the NFH group ($P < 0.01$), indicating that smoking might be related to the onset of DFH. In addition, in DFH group, more cases were complicated with diabetes, but the difference was not significant ($P > 0.05$). The fracture type of all cases was recorded, and a high proportion of cases had open fracture in the DFH group, while more cases with closed fracture were in the NFH group ($P < 0.01$), illustrating that open fracture might be a risk factor for the occurrence of DFH. In addition, the fracture causes were also summarized, and no significant difference was detected between the DFH and NFH groups ($P > 0.05$).

Comparison of serum miR-98-5p and BMP-2 levels between the NFH and DFH groups

As shown in Fig. 1A, high levels of miR-98-5p were detected in the serum of cases with DFH in comparison with the NFH group ($P < 0.001$). But DFH cases exhibited lower concentration of BMP-2 than the NFH group, and the difference was significant (Fig. 1B, $P < 0.001$). Furthermore, the Pearson's correlation analysis results demonstrated the negative correlation between miR-98-5p levels and BMP-2 concentration (Fig. 1C, $r = -0.778$, $P < 0.001$).

Diagnostic values of miR-98-5p and BMP-2 in DFH

The ROC curve was drawn to evaluate the diagnostic value of miR-98-5p and BMP-2 for DFH. As demonstrated in Fig. 2A, the area under the curve (AUC) of miR-98-5p for predicting the occurrence of DFH was 0.882, with the sensitivity of 81.7% and the specificity of 97.5%. The AUC of BMP-2 was 0.887, and the sensitivity and specificity were 70.0% and 97.5%, respectively (Fig. 2B). Furthermore, their combined diagnostic value was evaluated. It was found that the AUC of miR-98-5p combined with BMP-2 in predicting DFH was 0.944, which was higher than the single indicator (Fig. 2C). The sensitivity (88.3%) was also improved compared with single index.

BMP-2 is a target gene of miR-98-5p in MC3T3-E1 cells

MC3T3-E1 cells were recruited for the cell function experiments. First, the MC3T3-E1 cells were induced differentiation, and levels of *ALP*, *OCN*, and *RUNX2* increased gradually along with the cell differentiation (Fig. 3A). In addition, decreased miR-98-5p and increased BMP-2 were detected in MC3T3-E1 cells as the induction time increases,

Table 2. Clinical data of the two study groups.

Items	NFH group (n = 80)	DFH group (n = 60)	χ^2	*P* value
Sex, n (%)			0.517	0.472
Male	57 (71.25)	46 (76.67)		
Female	23 (28.75)	14 (23.33)		
Age, year (%)			0.513	0.474
≥ 60	26 (32.50)	23 (38.33)		
< 60	54 (67.50)	37 (61.67)		
Smoking, n (%)			7.97	**0.005**
Yes	25 (31.25)	33 (55.00)		
No	55 (68.75)	27 (45.00)		
Complications, n (%)				
Hypertension	16 (20.00)	19 (31.67)	1.065	0.302
Diabetes	19 (23.75)	23 (38.33)	3.472	0.062
Coronary heart disease	11 (13.75)	10 (16.67)	0.229	0.632
Fracture type, n (%)			5.717	**0.017**
Open fracture	33 (41.25)	37 (61.67)		
Closed fracture	47 (58.70)	23 (38.33)		
Fracture causes, n (%)			0.303	0.96
Traffic accident	23 (28.75)	19 (31.67)		
Tumble	33 (41.25)	24 (40.00)		
Falls	17 (21.25)	13 (21.67)		
Exercise	7 (8.75)	4 (6.66)		

NFH, normal fracture healing; DFH, delayed fracture healing.
Data were expressed as number and percentage. Bold fonts represent significant differences.

and the changes of the levels were time-dependent (Fig. 3B, C). Fig. 3D showed the binding sequences of miR-98-5p with BMP-2. Then the luciferase reporter assay demonstrated the restraining role of miR-95-5p in the luciferase activity of cells transfected wide type BMP-2, but had no influence on cells transfected with mutant BMP-2 (Fig. 3E).

Role of miR-98-5p and BMP-2 in osteoblast proliferation, apoptosis and differentiation

To investigate the effect of miR-98-5p and BMP-2 on the function of MC3T3-E1 cells, the level of miR-98-5p was regulated by cell transfection. As shown in Fig. 4A, miR-98-5p mimic transfection led to the increase of miR-98-5p levels, but the influence was abolished by BMP-2 overexpression. CCK-8 assay demonstrated that miR-98-5p overexpression inhibited the cell proliferation of MC3T3-E1, but BMP-2 upregulation reversely promoted the cell proliferation (Fig. 4B). Similarly, enhanced cell apoptosis was detected after miR-98-5p mimic transfection, while BMP-2 overexpression inhibited the cell apoptosis (Fig. 4C). To evaluate the MC3T3-E1 differentiation, the cell differentiation markers' expression was detected. As shown in Fig. 4D, miR-98-5p inhibited the mRNA levels of *ALP*, *OCN* and *RUNX2*, but these trends were reversed by BMP-2 overexpression.

Discussion

DFH is a common complication after fracture surgery, which can lead to severe disability in patients with reduced quality of life and increased medical burden. The evaluation of related indicators of delayed fracture union can help predict the risk of delayed fracture union, and provide a basis for clinical development of treatment strategies. Studies have shown that miRNA is involved in the process of fracture healing, and its abnormal expression is closely associated with DFH (Teng et al. 2018; Guo et al. 2022). The involvement of miRNAs in the regulation of osteoblast differentiation, bone metabolism and bone formation has been a research hotspot in recent years (Takahara et al. 2018, 2020). In the current study, upregulation of miR-

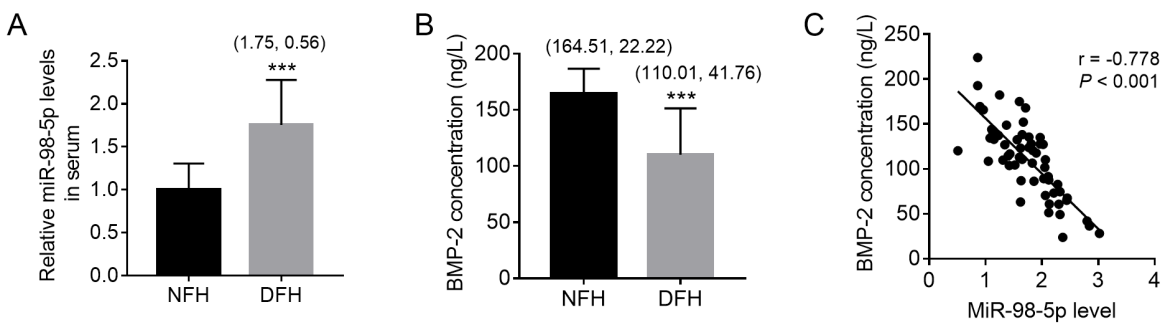

Fig. 1. Serum miR-98-5p and BMP-2 levels between the normal fracture healing (NFH) and the delayed fracture healing (DFH) groups.
A. MiR-98-5p was highly expressed in the serum of DFH patients compared with the NFH. B. Low BMP-2 concentration was detected in DFH cases. C. Negative correlation was identified between miR-98-5p levels and BMP-2 concentration. Data were expressed as mean and standard deviation (SD) for each group (n = 5). ***$P < 0.001$.

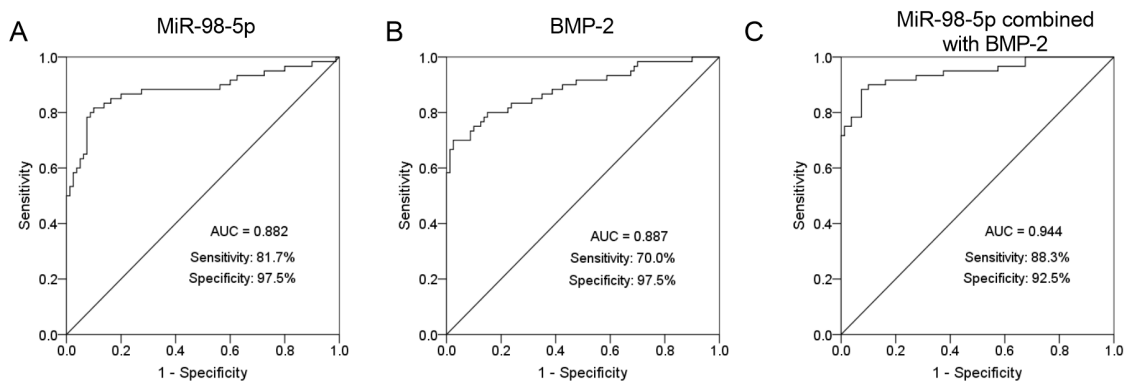

Fig. 2. Diagnostic values of miR-98-5p and BMP-2 in delayed fracture healing (DFH).
A. Receiver operating characteristic (ROC) curve of serum miR-98-5p to distinguish DFH cases from normal fracture healing (NFH) ones. B. ROC curve of BMP-2 to distinguish DFH cases from NFH ones. C. The ROC curve of miR-98-5p combined with BMP-2 in predicting DFH.

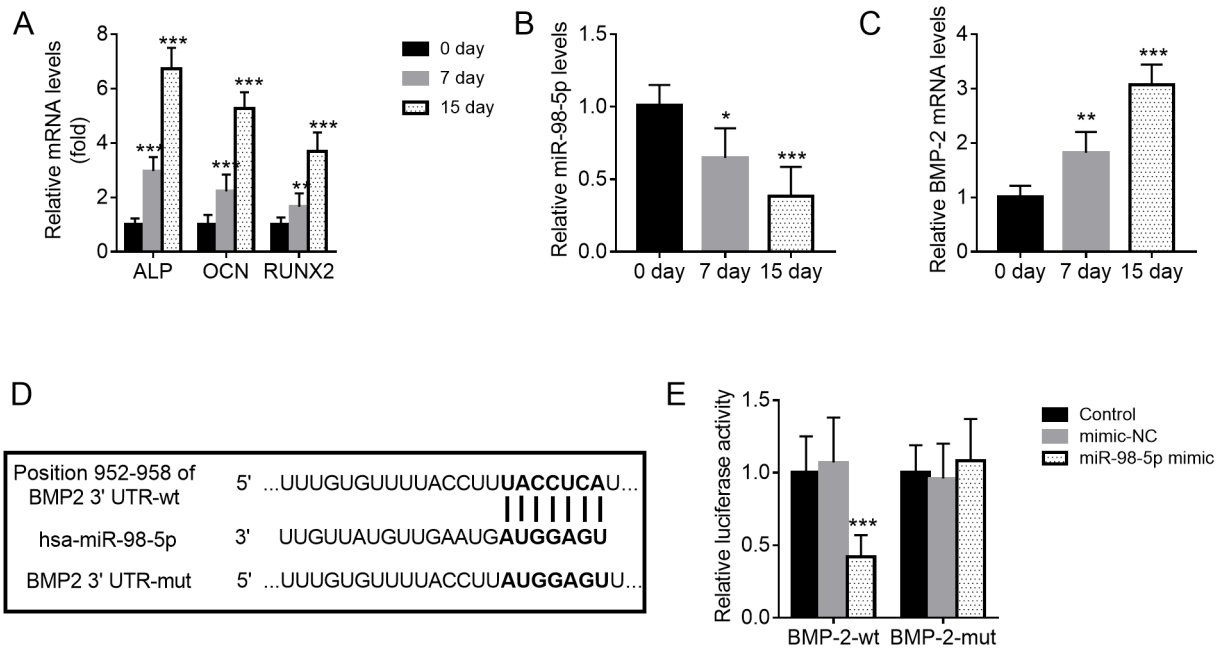

Fig. 3. BMP-2 is a target gene of miR-98-5p in MC3T3-E1 cells.
A. Alkaline phosphatase (*ALP*), osteocalcin *(OCN*), and runt-related transcription factor 2 (*RUNX2*) mRNA levels increased gradually along with cell differentiation. B. miR-98-5p decreased gradually along with the cell differentiation. C. BMP-2 increased gradually along with the cell differentiation. D. TargetScan predicted the binding sequences between miR-98-5p and BMP-2. E. Luciferase activities of cells receiving different treatments. Data were expressed as mean and standard deviation (SD) for each group (n = 5). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

95-5p was detected in the serum of cases suffered from DFH, indicating its potential role in the development of DFH.

Fracture healing is a complex physiological process, and bone regeneration requires the participation of a variety of cytokines. In which, bone morphogenetic protein (BMP-2) plays a significant role (Ouyang et al. 2021). BMP-2 is a member of the transforming factor β superfamily, and is a multifunctional secretory protein (Hara et al. 2017). It can induce the differentiation of bone marrow mesenchymal cells into chondrocytes and osteoblasts, and promote the formation of new bone (Bauge and Boumediene 2015). At the same time, it can also promote the expression of ALP and OCN, promote the expression of bone matrix proteins, and mineralize extracellular matrix, thus promoting the maturation of osteocytes (Krukiewicz et al. 2020). The present results demonstrated the decreasing BMP-2 concentration in DFH cases compared with the NFH ones. BMP-2 plays an important role in fracture healing. The absence of BMP-2 in chondrocytes can lead to the failure of fracture healing initiation or prolong the formation time of cartilage callus, thus affecting the fracture healing process (Gao and Wang 2020). Animal experiments have shown that the administration of recombinant human BMP-2 to mice with ovariectomized fractures can promote the fracture healing (Huang et al. 2018). Gao and Wang (2020) have reported that patients with good recovery after fracture surgery own high levels of BMP-2 than those with poor recovery, and serum BMP-2 level could be used as a marker of recovery after hip replacement for femoral neck fracture. The present results based on ROC curve demonstrated the diagnostic ability of BMP-2 for DFH with the AUC of 0.887. Notably, a significantly negative correlation was detected between serum miR-98-5p levels and BMP-2 concentration. Thus, the diagnostic value of miR-98-5p was also evaluated. It can be seen that the diagnostic ability of miR-98-5p is comparable to that of BMP-2, but its diagnostic sensitivity is slightly higher. However, the sensitivity and specificity of diagnosis were improved after combination of the two factors. The present ROC analysis results showed that miR-98-5p combined with BMP-2 could better predict the risk of DFH after femoral neck fracture surgery, indicating that detection of serum miR-98-5p and BMP-2 could provide effective information for the prevention of DFH and guide clinical treatment.

Fracture healing is a complex and dynamic process, which is regulated by various cell types and cytokines (He et al. 2017). Among them, osteoblasts and osteoclasts play an important role in bone remodeling (He et al. 2021). Osteoblasts are mesenchymal cells that play a major role in bone formation in fracture healing by participating in the formation of new bone (Abdul Rahim et al. 2022). Osteoblasts are important repair cells in the process of fracture healing (Kao et al. 2019). The recovery process of cell

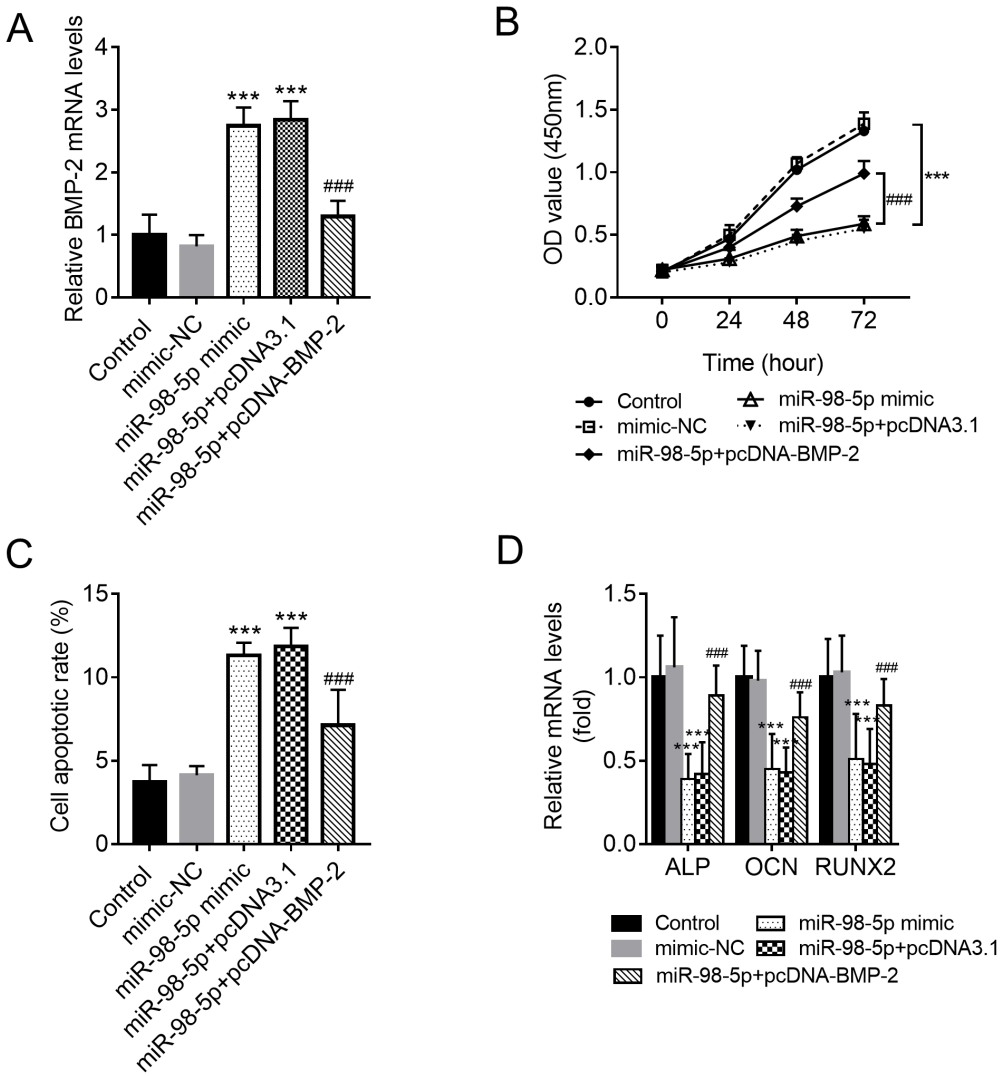

Fig. 4. Role of miR-98-5p and BMP-2 in osteoblast proliferation, apoptosis and differentiation.
A. MiR-98-5p mimic transfection led to the increase of miR-98-5p levels, but the influence was abolished by BMP-2 overexpression. B. MiR-98-5p overexpression inhibited the cell proliferation of MC3T3-E1, but BMP-2 upregulation reversely promoted the cell proliferation. C. Enhanced cell apoptosis was detected after miR-98-5p mimic transfection, while BMP-2 overexpression inhibited the cell apoptosis. D. MiR-98-5p inhibited the mRNA levels of *ALP*, *OCN* and *RUNX2*, but these trends were reversed by BMP-2 overexpression. Data were expressed as mean and standard deviation (SD) for each group (n = 5). $^{***}P < 0.001$ compared with control group; $^{###}P < 0.001$ compared with miR-98-5p mimic group.

migration, differentiation, tissue healing, and the release of cytokines and growth factors after fracture mainly depends on the activity of osteoblasts (Ghiasi et al. 2017). MiRNAs are produced by all cell types, and the same miRNA may be derived from a variety of cell sources, such as endothelial cells, monocytes and macrophages, vascular smooth cells, and platelets and eventually are secreted in blood. In the current study, MC3T3-E1 cells were recruited for cell function experiments. It can be seen that miR-98-5p overexpression inhibited osteoblast proliferation and differentiation, whereas it promoted cell apoptosis. Studies have shown that miRNA can rapidly and effectively degrade the mRNA of target genes or inhibit the translation of target proteins by binding to the target sites, and thus become a new endogenous post-transcriptional regulatory pathway for cell maintenance, proliferation, differentiation, apoptosis and other phenomena (Leng et al. 2020). In the present study, the target relationship was confirmed between miR-98-5p and BMP-2 in MC3T3-E1 cells. Furthermore, the rescue experiments revealed that BMP-2 reversed the role of miR-98-5p in osteoblast proliferation, apoptosis and differentiation.

In conclusion, serum miR-98-5p level is increased and BMP-2 level is decreased in patients with DFH, and the

combined detection of miR-98-5p and BMP-2 can provide reliable information for the evaluation of DFH. MiR-98-5p overexpression inhibits osteoblast proliferation and differentiation via targeting BMP-2. However, the samples size is relatively small, and an external validation of serum miR-98-5p and BMP-2 in a DFH cohort is lacking, which should be performed in future.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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