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Enhancing Osteoblast Activity and Accelerating Fracture Healing via miR-656-3p Downregulation: A Novel Targeting Strategy Focused on BMP-2 Expression

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Delayed fracture healing (DFH), a common complication of post-fracture surgery, exhibits an incompletely understood pathogenesis. The present study endeavors to investigate the roles and underlying mechanisms of miR-656-3p and Bone Morphogenetic Protein-2 (BMP-2) in DFH. It was recruited 94 patients with normal fracture healing (NFH) and 88 patients with DFH of the femoral neck. Serum miR-656-3p and BMP-2 expressions were quantified using RT-qPCR and the diagnostic potential of them for DFH was evaluated using ROC analysis. Factors influencing fracture healing were identified through logistic regression analysis. Osteogenic differentiation of MC3T3-E1 cells was induced, followed by evaluations of cell proliferation, apoptosis, and differentiation capabilities utilizing CCK-8, flow cytometry, and mRNA expression analysis of osteogenic markers. The targeting relationship between miR-656-3p and BMP-2 was validated through luciferase reporter assays. The levels of miR-656-3p were significantly elevated in DFH patients compared to those with NFH, whereas BMP-2 levels exhibited a decrease, a negative correlation between their expression patterns. Logistic regression analysis revealed that miR-656-3p and BMP-2 serve as influential factors in fracture healing, with their combined assessment exhibiting enhanced predictive value for DFH. Downregulation of miR-656-3p promoted proliferation and differentiation of MC3T3-E1 cells while inhibiting apoptosis. BMP-2, identified as a target of miR-656-3p, negated the effects of miR-656-3p downregulation when BMP-2 expression was inhibited. miR-656-3p modulates osteoblast function by targeting BMP-2, offering novel therapeutic and diagnostic targets for the management of DFH.

Keywords: Delayed fracture healing, MC3T3-E1, osteogenic differentiation, proliferation, apoptosis miR-656-3p, BMP-2.

INTRODUCTION

Fractures, a phenomenon in which the structural integrity of the bone is compromised, triggered by external forces or cumulative strain over time, have a major challenge in their treatment, namely delayed healing (DFH), a complication that accounts for approximately 5 to 10 % of fracture patients[1,2]. DFH not only prolongs the patient's recovery period, accompanied by persistent pain, but it also significantly adds to the financial strain on their families. Therefore, it is important to explore ways to promote early diagnosis, effective prevention and innovative interventional treatments for fractures to improve patient prognosis[3,4]. Fracture healing is a highly complex and finely regulated physiological process involving differentiation of mesenchymal stem cells, maturation of osteoblasts, construction of extracellular matrix and generation of vascular network. Among them, osteoblasts, as a key player in bone formation, play a crucial role in the repair and remodeling of fracture sites[5,6]. In recent years, with the deepening of molecular biology research, scientists have gradually revealed a variety of molecular mechanisms that regulate fracture healing, opening new paths for the innovation of fracture treatment strategies.

MicroRNAs (miRNAs), as an important class of non-coding RNA molecules, play a key role in life activities such as cell proliferation, differentiation, and apoptosis through their unique post-transcriptional regulatory mechanisms. In the field of fracture healing, miRNAs closely related to the regulation of osteogenic differentiation are becoming a hot spot of research[7,8]. For example, miR-214 was found to have a significant

inhibitory effect on bone formation by targeting ATF-4 (activating transcription factor 4), which in turn inhibits the activity of osteoblasts and affects the mineralization process of bone matrix[9,10]. miR-135-5p plays an active role in promoting bone formation by specifically targeting and regulating the expression of HIF1AN, which in turn enhances the differentiation of osteoblasts and contributes to bone growth and repair[11]. miR-656-3p is a recently discovered miRNA that has attracted widespread attention as a novel class of molecules. Some studies have shown that miR-656-3p is able to influence cell cycle progression. For example, overexpression of miR-656-3p was able to inhibit the proliferation and migration ability of placental mesenchymal stem cells in vitro[12]. miR-656-3p is also involved in the regulation of apoptosis. In nasopharyngeal carcinoma cells, miR-656-3p regulates apoptosis by targeting the ELF2 gene[13]. In non-small cell carcinomas, it has likewise been found to regulate apoptosis, invasion, and other malignant behavioral activities of tumor cells[14]. Despite limited direct research on miR-656-3p in delayed fracture healing, its regulatory influence on cell proliferation, differentiation, and apoptosis, coupled with abnormal expression in fracture cases[15], hints at a potential role in the healing process. Specifically, miR-656-3p may regulate proliferation, differentiation and apoptosis at the fracture site to participate in the fracture healing process.

The purpose of this study was to explore the functional role and underlying mechanisms of miR-656-3p in DFH, aiming to establish a theoretical foundation that could guide the development of innovative diagnostic methods and therapeutic interventions for DFH.

MATERIAL AND METHODS

Study subject

There were 88 patients with delayed healing of tibial stem fracture admitted to the Department of Orthopedics of The Second Hospital of Qinhuangdao from 2018 to 2020 were selected as the DFH group, and 94 patients with normal healing of tibial stem fracture during the same period were selected as the NFH group. The diagnostic criteria of the DFH group were that the fresh fracture was still suffering from pain at the fracture site and symptoms of abnormal movement of the end of the bone after the normal healing time of 6 months and the X-rays showed that there was no scab formation at the fracture site, and that there was a persistent fracture line present. In the NFH group, the diagnostic criteria were the formation of a continuous scab at the fracture site on repeat radiographs, the absence of compression, percussion, and conscious pain, normal movement of the fracture site, and the ability to walk independently for at least 3 min(16). Inclusion criteria: 1. patients with tibial stem fracture; 2. patients with first fracture; 3. patients with DFH diagnostic criteria. Exclusion criteria: 1. patients with serious heart, brain, kidney and other organ dysfunction; 2. patients with bone metabolism related diseases; 3. patients with diabetes mellitus; 4. patients with acute and chronic infections; 5. patients who have taken hormones in the past 3 months; 6. patients with tumors and autoimmune diseases; 7. patients with pathological fracture; and 8. patients with serious psycho-neurological pathology who are unable to cooperate with the study.

The Ethics Committee of The Second Hospital of Qinhuangdao reviewed and granted approval for the study, ensuring all patients were notified and provided informed consent.

Cell transfection

During the preparation stage for transfection, MC3T3-E1 cells in logarithmic growth phase were selected, and their culture medium was replaced with serum-free DMEM one hour prior to ensure efficient transfection. Following this, the transfection was conducted in accordance with the guidelines provided by the Lipofectamine 2000 Kit (Invitrogen, USA). Transfection complexes including miR-656-3p mimics (miR-minic), miR-656-3p inhibitors (miR-inhibitor) and their negative controls (miR-NC), as well as miR-656-3p inhibitors combined with BMP-2 knockdown (miR-si-BMP-2) and their respective negative controls (miR+si-NC), were individually introduced into MC3T3-E1 cells. An untreated group of MC3T3-E1 cells served as the control. After 6 hours of incubation in standard conditions, the medium was replaced with standard DMEM containing 10% FBS to support continued cell growth. Following an additional 48 hours of culture, successfully transfected cells were selected for further investigation.

Cell viability

Selected transfected MC3T3-E1 cells from each group were resuspended in DMEM culture medium and evenly seeded into 96-well plates. At 0, 24, 48, and 72 hours, 10 μL of CCK-8 solution (Beyotime, Beijing, China) was added to each well, followed by incubation under standard conditions for 2 hours. Subsequently, 150 μL of dimethyl sulfoxide (DMSO) was added to each well to terminate the reaction.

Finally, the optical density (OD) of each well was measured at 450 nm using a microplate reader, and the relative cell viability was calculated accordingly.

Cell apoptosis

Well-grown MC3T3-E1 cells are selected, washed with PBS, and centrifuged to remove the supernatant. Subsequently, 100μL of binding buffer is added. The cells are then mixed with Annexin V-FITC and PI in the dark at room temperature, as per the instructions of the Apoptosis Detection Kit (BD Pharmingen, USA), and incubated for 15 minutes. Following this, apoptosis is detected using a flow cytometer, and the data is analyzed with FlowJo software.

Osteoblast differentiation

A medium containing L-glycerophosphate (5 mM), dexamethasone (100 nM), and ascorbic acid (50 μg/mL) was prepared for osteogenic differentiation induction (OI). Transfected MC3T3-E1 cells were cultured in this OI medium under standard conditions for two weeks. Samples were collected at days 0, 7, and 14 to assess changes in osteoblastic differentiation markers.

RT-qPCR

On the 4th week post-op morning, 5ml venous blood was drawn from fasting patients and serum was isolated via centrifugation. Total RNA was extracted from the collected patient serum and differently treated MC3T3-E1 cells according to the instruction guide that came with the TRIzol kit (Invitrogen, USA). The extracted total RNA was screened by assay using a UV spectrophotometer to ensure the purity of the total RNA. It was converted to complementary DNA (cDNA) using the instruction manual operation of the reverse transfection kit (Takara, Japan). The cDNA amplification was performed by PrimeScript RT Master Mix (Takara Japan), using a 25 μL reaction system containing 12.5 μL of SYBRPremix ExTaqII, 0.5 μL of upstream primer, 0.5 μL of downstream primer, 1 μL of cDNA, and 0.5 μL of sterilized double-distilled water. The PCR procedure was as follows: 95°C for 10 min, 25 cycles, 95°C for 15 s, 60°C for 60 s. The primer sequences in this experiment were constructed by Shanghai Biotechnology Engineering (Table SI), including miR-656-3p, BMP-2, runt-related transcription factor 2 (RUNX2), osteocalcin (OCN), osteopontin (OPN) and alkaline phosphatase (ALP) mRNA, in which the relative expression of miRNA and mRNA were calculated using the 2-ΔΔCt method with U6 and GAPDH as internal references, respectively.

Dual-luciferase reporter assay

The TargetScan database (http://www.targetscan.org/vert_72/) was used to predict the binding sites of miR-656-3p and BMP-2, and to design the WT-BMP-2 (wild-type) and MUT-BMP-2 (mutant) sequences of the miR-656-3p 3'UTR. These sequences were cloned into pmirGLO vector (Promega, USA) and co-transfected with miR-mimic, miR-inhibitor and miR-NC to MC3T3-E1 cells, respectively. After 48 hours of transfection, the cell fluorescence activity was determined with dual-luciferase reporter system (Promega, USA).

Statistical analysis

The statistical analysis of all data was conducted utilizing Graphpad prism 6. 0 and SPSS 22.0 software. For continuous variables, mean ± standard deviation (x ± sd) was reported, with t-test applied for pairwise comparisons and ANOVA for multiple group comparisons. For categorical data, counts were presented, and $\chi 2$ test was employed for intergroup comparisons. The risk factors of DFH were explored using a multifactorial logistic regression model. The diagnostic efficacy of miR-656-3p and BMP-2 for DFH was evaluated using Receiver Operating Characteristic (ROC) curve analysis. Additionally, the correlations among indices were analyzed through Pearson correlation analysis. Statistical significance was set at $p < 0.05$.

RESULTS

A comprehensive analysis of clinical information from two patient groups, encompassing age, BMI, gender, smoking and alcohol consumption history, fracture location, and fracture type, revealed no statistically significant differences between the two groups (Table SII).

Predictive value of miR-656-3p and BMP-2 to DFH

The study revealed a statistically significant upregulation of miR-656-3p expression in the serum of patients in the DFH group compared to those in the NFD group ($P < 0.001$, Fig. 1a), whereas BMP-2 expression levels demonstrated a pronounced downregulation ($P < 0.001$, Fig. 1b). Further correlation analysis unveiled a negative correlation between miR-656-3p and BMP-2 levels in the serum of DFH patients ($r = -0.562$, $P < 0.001$, Fig. 1c). To ascertain the diagnostic utility of miR-656-3p and BMP-2 as biomarkers for DFH, ROC curve analysis was conducted (Fig. 1d). Although both miR-656-3p

Table SI. — Primer sequences.

Primer name		Primer sequence
BMP-2	forward	5'-CAGAACACAAGTCAGTGGGAGAGC-3'
	reverse	5'-GAGGTGCCACGATCCAGTCATTC-3'
RUNX2	forward	5'-TCTTCACAAATCCTCCCC-3'
	reverse	5'-TGGATTAAAAGGACTTGG-3'
OCN	forward	5'-ACTCGAACGACTCTGATGATGT-3'
	reverse	5'-GTCAGGTCTGCGAAACTTCTTA-3'
ALP	forward	5'-ACGTGGCTAAGAATGTCATC-3'
	reverse	5'-CTGGTAGGCGATGTCCTTA-3'
OPN	forward	5'-ACACTTTCACTCCAATCGTC-3'
	reverse	5'-TGCCCTTTCCGTTGTTGTCC-3'
GAPDH	forward	5'-CTCTGCTCCTCCTGTTCGAC-3'
	reverse	5'-GCGCCCAATACGACCAAATC-3'
miR-656-3p	forward	5'ACACTCCAGCTGGGAATATTATACAGTCA 3'
	reverse	5'CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGAGGUUG 3'
U6	forward	5'-CTCTGCTCCTCCTGTTCGAC-3'
	reverse	5'-GCGCCCAATACGACCAAATC-3'

miR-656-3p: microRNA-656-3p; BMP-2: bone morphogenetic protein 2; RUNX2: runt-related transcription factor 2; OCN: osteocalcin, ALP: alkaline phosphatase; OPN: osteopontin.

Table SII. — Comparison of the baseline data of study objects.

Parameters	NFH	DFH	P value
	(n =94)	(n = 88)	
Age (year)	50.91±12.54	54.01±11.77	0.088
BMI (kg/m^2)	23.70±3.55	23.04±3.02	0.182
Gender			0.478
Female	41	43	
male	53	45	
Smoking			0.796
No	52	47	
Yes	42	41	
Drinking			0.894
No	49	45	
Yes	45	43	
Fracture ide			0.886
Left	46	44	
Right	48	44	
Fracture type			0.518
Open fracture	50	51	
Closed fracture	44	37	

Annotation: BMI, body mass index.

and BMP-2 exhibited diagnostic value when utilized individually, with AUC values of 0.899 and 0.851 respectively, their combined use notably enhanced the diagnostic performance. Specifically, the AUC value increased to 0.938, accompanied by an improvement in diagnostic sensitivity (85.23%) and specificity (92.55%).

Notably, the results of logistic regression analysis identified serum levels of miR-656-3p and BMP-2 as potential risk factors for the development of impaired fracture healing (Table I).

Impact of miR-656-3p Expression on the Behavioral Activities of MC3T3-E1 Cells

An OI model was established using MC3T3-E1 cells, as illustrated in Fig. 2a. With the extension of osteogenic induction duration, a corresponding

Table I. — Logistic regression analysis of risk factors for DFH.

	p-value	OR	95%CI
miR-656-3p	0.030	2.009	1.069-3.775
BMP-2	0.035	0.507	0.269-0.954
Age	0.822	0.931	0.499-1.738
BMI (kg/m^2)	0.863	1.057	0.563-1.985
Gender (male/female)	0.970	0.988	0.531-1.841
Smoking	0.870	1.054	0.564-1.969
Drinking	0.884	1.047	0.567-1.934
Fracture Location,	0.612	0.847	0.446-1.609
Fracture type	0.738	0.897	0.474-1.697

increase in the mRNA expression of osteogenic markers, including RUNX2, OCN, OPN, and ALP, was observed (Fig. 2b). Additionally, a decrease in miR-656-3p expression and a concomitant increase in BMP-2 mRNA expression were noted (Fig. 2c). To investigate the effects of altered miR-656-3p levels on the behavioral activities of MC3T3-E1 cells, successful upregulation and downregulation of miR-656-3p expression were achieved through transfection with miR-656-3p mimics and inhibitors, respectively (Fig. 3a). The findings indicated that upregulation of miR-656-3p significantly inhibited the differentiation potential (Fig. 3b) and proliferative capacity (Fig. 3c) of MC3T3-E1 cells, while promoting their apoptotic abilities (Fig. 3d). Conversely, downregulation of miR-656-3p had opposing effects.

BMP-2 as a Key Target of miR-656-3p in Regulating MC3T3-E1 Cell Functions

Based on the identified binding sites between miR-656-3p and BMP-2, dual-luciferase reporter constructs containing WT-BMP-2 and MUT-BMP-2 sequences were designed and analyzed (Fig. 4a). The results demonstrated that miR-656-3p effectively negatively regulated the luciferase activity of WT-BMP-2 (Fig. 4b), suggesting a direct interaction between miR-656-3p and BMP-2 mRNA. Furthermore, it was found that knockdown of miR-656-3p significantly enhanced BMP-2 mRNA expression (Fig. 4c), further

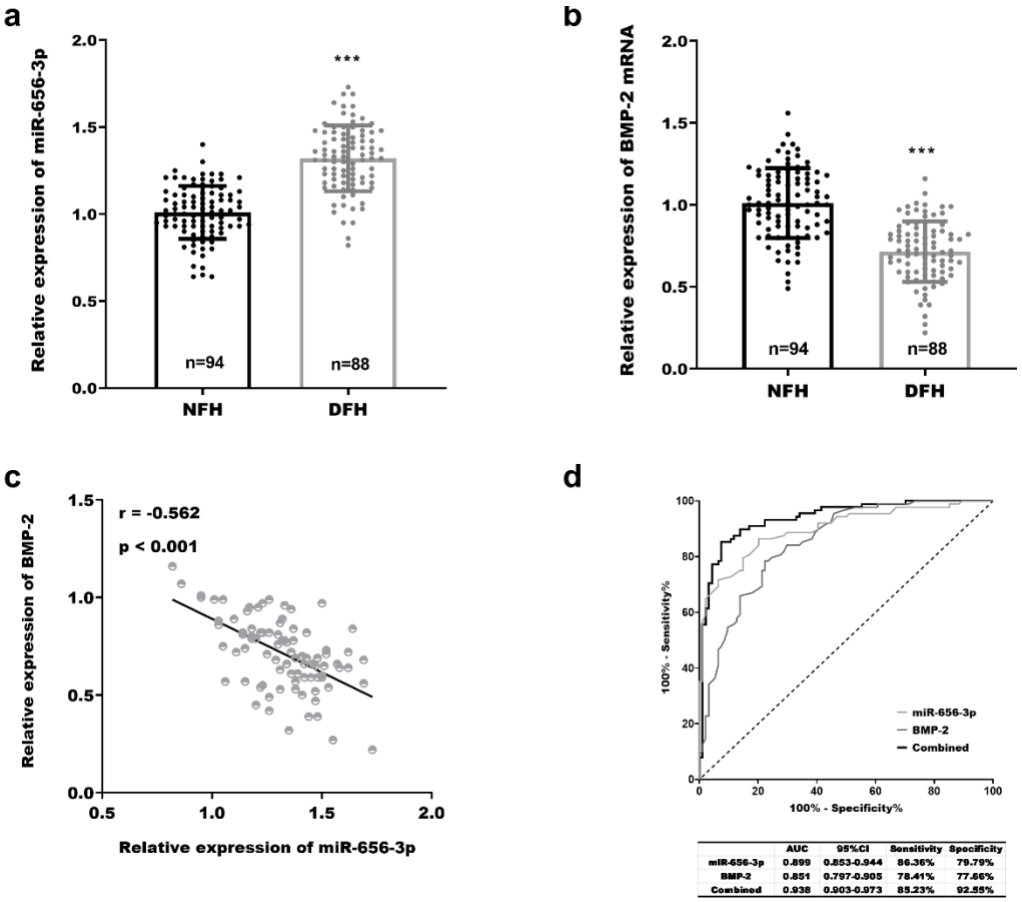

*Fig. 1 — (a) Expression of miR-656-3p in serum samples from DFH and NFH patients; (b) Expression of BMP-2 in serum samples from DFH and NFH patients; (c) Correlation analysis of miR-656-3p and BMP-2 expression in DFH patients; (d) Diagnostic potential of miR-656-3p and BMP-2 for DFH. ***P< 0.001, vs NFH. DFH: delayed fracture union. NFH: normal fracture union.*

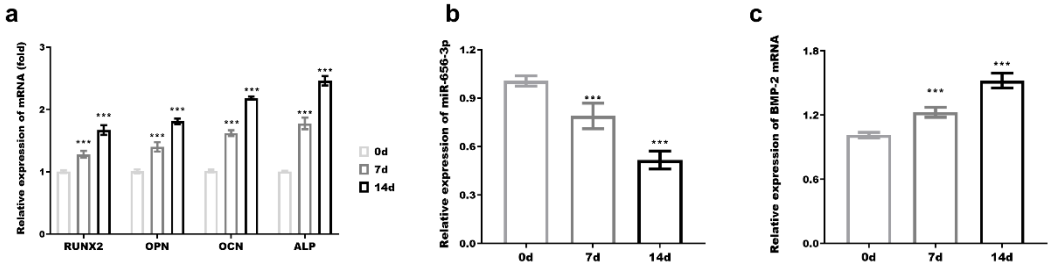

*Fig. 2 — (a) Changes in osteoblast differentiation-related protein markers at different time points; (b) Expression of miR-656-3p during osteoblast differentiation; (c) Expression of BMP-2 during osteoblast differentiation. ***P< 0.001, vs 0d.*

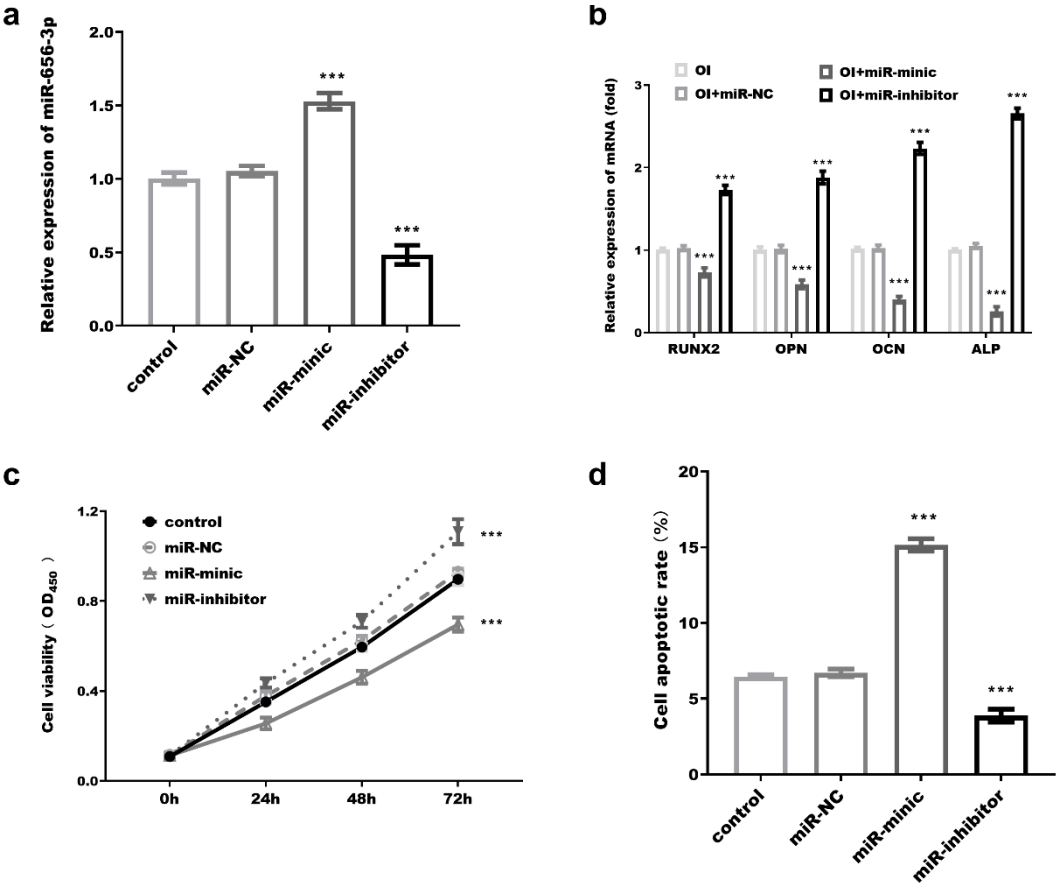

*Fig. 3 — (a) Regulation of miR-656-3p expression; (b) Effect of miR-656-3p expression on osteoblast differentiation; (c) Effect of miR-656-3p expression on osteoblast proliferation; (d) Effect of miR-656-3p expression on osteoblast apoptosis. ***P< 0.001, vs control or OI. OI: osteogenic differentiation induction.*

corroborating the inhibitory effect of miR-656-3p on BMP-2. The positive effects on MC3T3-E1 cell differentiation (Fig. 4d), proliferation (Fig. 4e), and apoptosis (Fig. 4f) of miR-656-3p downregulation were reversed, when BMP-2 was simultaneously silenced.

DISCUSSION

The hallmark of DFH lies in the inability of fractures to heal within the anticipated timeframe, posing significant challenges to orthopedic treatment. Early prediction of DFH facilitates the implementation of targeted interventions, thereby enhancing fracture prognosis and alleviating patient suffering[17]. BMP-2, an efficacious bone inductive cytokine belonging to the TGF-β superfamily, is pivotal for bone formation and fracture healing[18,19]. Consistent with our findings, GAO et al.[20]. reported that serum BMP-2 levels were significantly higher in patients with favorable post-fracture recovery compared to those with poor recovery, suggesting its potential as a biomarker for post-hip replacement recovery in femoral neck fractures.

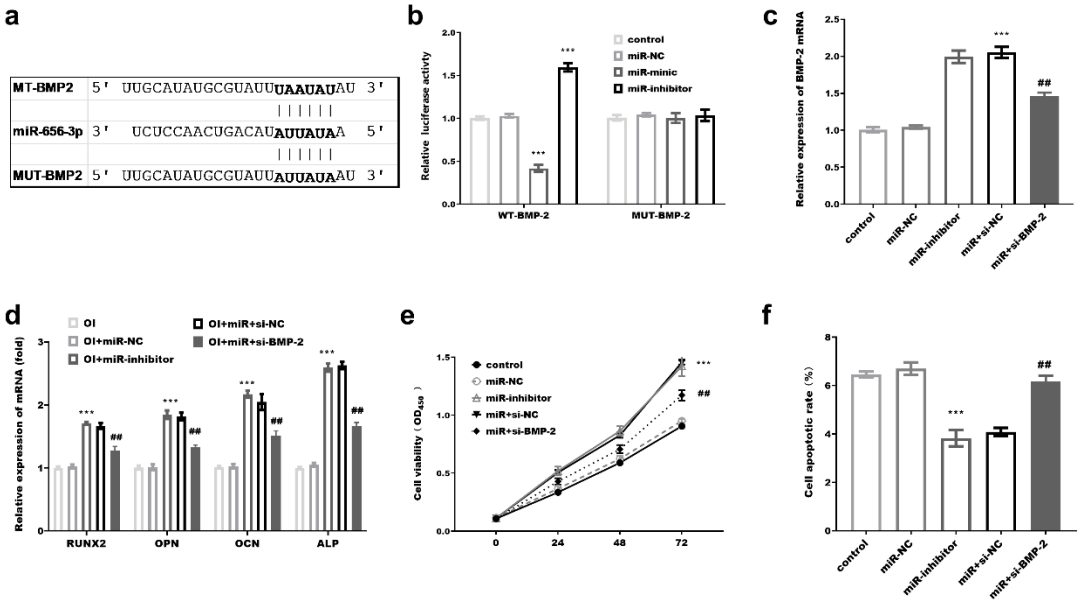

*Fig. 4 — (a) Predicted binding sites between miR-656-3p and BMP-2; (b) Dual-luciferase reporter assay to validate the targeting relationship between miR-656-3p and BMP-2; (c) Effect of inhibiting miR-656-3p expression on BMP-2 levels; (d) Effect of inhibiting BMP-2 expression on osteoblast differentiation (e) Effect of inhibiting BMP-2 expression on osteoblast proliferation; (f) Effect of inhibiting BMP-2 expression on osteoblast apoptosis. ***P< 0.001, vs control or OI; ** P< 0.001, vs miR-inhibitor or OI+ miR-inhibitor. OI: osteogenic differentiation induction.*

Recent advancements in miRNA research have underscored their pivotal role in regulating cellular growth and differentiation, particularly in the context of DFH. Specific miRNAs have been shown to play pivotal roles in fracture healing by finely tuning BMP-2 expression and function[21]. For instance, miR-6979-5p targets BMP-2, positively modulating osteoblast differentiation and influencing fracture healing dynamics[22]. MiR-142-5p, on the other hand, regulates osteogenic differentiation of BMSCs via the BMP-2/SMAD signaling pathway[23]. This study initially uncovers the upregulation of miR-656-3p in DFH and confirms its direct targeting relationship with BMP-2. Prior research has shown that miR-656-3p is upregulated in placental mesenchymal stem cells under hypoxic conditions, enhancing cell survival and migration through targeting HIF-1α[17]. Similarly, in prostate hyperplasia, miR-656-3p exerts a pivotal regulatory role by specifically downregulating CTGF[24]. These discoveries emphasize the crucial function of miR-656-3p in modulating target gene expression. Integrating previous studies on the prognostic value of serum BMP-2[25,26], this study further discovered that a combined assessment of miR-656-3p and BMP-2 serum levels significantly enhances the predictive capability for DFH. This finding not only reveals a novel regulatory axis involving miR-656-3p and BMP-2 but also paves a new avenue for diagnostic and management strategies for DFH.

Osteoblast proliferation is the basis of fracture healing, through which more osteoblasts are formed and participate in the regeneration and repair of bone tissue. Whereas excessive apoptosis of osteoblasts leads to insufficient bone formation and affects the process of fracture healing[27,28]. Wei et al. demonstrated that miR-654-5p overexpression downregulates BMP-2, inhibiting osteoblast proliferation[29]. This study discovered that miR-656-3p downregulation enhances MC3T3-E1 proliferation and anti-apoptosis, an effect reversed by BMP-2 knockdown. Similarly, Luo et al. reported that miR-142 also inhibits osteoblast proliferation and promotes apoptosis via BMP-2[30]. All these demonstrate the regulatory role of miRNAs targeting BMP-2 in osteoblast activity.

Furthermore, osteogenic differentiation is a critical process in the functional maturation of osteoblasts, which can secrete bone matrix and promote bone mineralization, thus accelerating fracture healing[31,32]. ALP, OCN, OPN, and RUNX2 serve as core markers in the osteogenic differentiation process, representing early osteoblast activity, late osteoblast function, bone metabolism regulation, and osteogenic differentiation transcriptional control, respectively. Collectively, they indicate the differentiation progress and functional status of osteoblasts[33]. In this study, OI induction was applied to MC3T3-E1 cells, revealing a gradual increase in mRNA expression of ALP, OCN, OPN, and RUNX2 over time, accompanied by a decrease in miR-

656-3p expression and a concomitant rise in BMP-2. Transfection with a miR-656-3p inhibitor enhanced the differentiation of MC3T3-E1 cells, whereas knockdown of BMP-2 inhibited this enhancement. Similarly, Zhang et al. noted that overexpression of BMP-2 can alleviate the inhibitory effect of upregulated miR-98-5p on osteoblast differentiation[34]. The above evidence revealed that miR-656-3p achieves a dynamic and balanced regulation of osteoblast proliferation, apoptosis, and osteogenic differentiation processes by modulating the expression of BMP-2.

Certainly, this study has limitations, including being based on a specific population and experimental conditions, with a potentially inadequate sample size to comprehensively reflect the universality and variability of miR-656-3p and BMP-2 in DFH. While the combined detection of miR-656-3p and BMP-2 levels enhances DFH prediction accuracy, this finding requires extensive clinical validation to confirm its applicability across different patient groups. Future research should delve deeper into the mechanisms of miR-656-3p and BMP-2, identify other potential regulatory factors and signaling pathways, expand sample validation, explore clinical applications, and develop new diagnostic and treatment strategies to improve DFH prediction and management.

CONCLUSION

In summary, this study uncovers a novel regulatory axis involving miR-656-3p and BMP-2, whose combined assessment significantly enhances the diagnostic value for DFH. By potentially suppressing BMP-2 expression, miR-656-3p modulates osteoblast proliferation, differentiation, and apoptosis, thereby contributing to the pathogenesis of DFH.

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Conflicts of Interest: The authors declare that they have no competing interests.

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