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miR-181a-1-3p affects the healing process of fractures through regulating the expression of DKK2

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Abstract

Introduction and Objective. Delayed fracture healing (DFH) represents a frequently encountered complication in the surgical management of fractures. The aim of the study is to elucidate how miR-181a-1-3p regulates DKK2 expression.

Materials and Method. The study included 95 normal fracture healing (NFH) patients and 70 DFH patients. The expression of miR-181a-1-3p, DKK2, and osteogenic differentiation markers (RUNX2, OCN, OPN) was detected using RT-qPCR. The diagnostic utility of miR-181a-1-3p in DFH was evaluated through ROC curve. Cell proliferation was determined by CCK-8 assay. Detection of ALP activity was performed with an ELISA kit. Dual luciferase reporter assay and RIP assay were employed to validate the targeted binding of miR-181a-1-3p and DKK2.

Results. Serum miR-181a-1-3p expression was up-regulated and DKK2 expression was down-regulated in DFH patients compared to the NFH group. miR-181a-1-3p had high diagnostic value for DFH. Inhibition of miR-181a-1-3p significantly promoted the proliferation of MC3T3-E1 cells, elevated migration, and up-regulated ALP activity and RUNX2, OCN, OPN levels. While over-expression of miR-181a-1-3p produced opposite results. DKK2 was a target gene of miR-181a-1-3p. Knockdown of DKK2 partially reversed the effects of inhibiting miR-181a-1-3p on the proliferation, migration and differentiation of MC3T3-E1 cells.

Conclusions. miR-181a-1-3p regulates osteoblast proliferation, migration and differentiation processes by targeting DKK2, thereby affecting the pathological development of DFH. This research offers a potential theoretical foundation for the early diagnosis and targeted treatment of DFH.

Key words

osteoblast, fracture healing, DKK2, miR-181a-1-3p

INTRODUCTION AND OBJECTIVE

In trauma-related injuries, fractures are the most common type, with their high incidence and complexity having a significantly impact on the physical and mental health of patients [1]. Among them, the femoral neck fracture (FNF) is a common form of proximal femur fracture in clinical practice, accounting for about 53% of fractures in this anatomical region. Alarmingly, the incidence of FNFs has been steadily increasing in recent years [2]. Current clinical treatment for FNF predominantly relies on surgical intervention. However, despite the intrinsic ability of bone to heal, a notable 5%-10% of patients had impaired healing, manifesting as bone non-union or delayed fracture healing (DFH) [3]. Fracture healing is an intricately regulated, dynamic pathological sequence encompassing a myriad of multidimensional biological events. These include the convergent aggregation of mesenchymal stem cells, the differentiation and maturation of osteoblasts, as well as extracellular matrix mineralization and vascularization [4]. Therefore, the search for biomarkers associated with the fracture healing process has profound scientific significance and clinical value for early predictions of healing disorders, and inform the optimization of clinical intervention strategies.

MicroRNAs (miRNAs), as evolutionarily conserved non-coding RNA molecules, play a crucial role as they exert negative regulatory effects by specifically binding to target mRNAs, thereby silencing gene expression [5]. Recent studies have revealed the significant role of miRNAs as important regulators in the cascade of osteoblast differentiation and bone formation [6]. For example, miR-221-3p and miR-222-3p inhibited the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) by regulating IGF-1/ERK signaling pathway [7]. Conversely, miR-140-5p promoted osteogenic differentiation and facilitate post-fracture healing in mouse embryonic mesenchymal stem cells [8].

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Furthermore, the over-expression of miR-210-3p inhibited osteogenic differentiation, migration, and proliferation in MC3T3-E1 cells, marking it as a potential target for regulating osteogenic differentiation [9]. Together, these findings reveal a multidimensional role for ncRNAs in the intricate regulation of bone metabolism. Notably, miR-181a-1-3p showed upregulated expression in miRNA microarray analysis conducted on a rat model of closed transverse femur fracture [10]. This observation may suggest a potential correlation with the regulatory function of this molecule in inflammation. Previous studies showed that miR-181a-1-3p participated in modulating inflammatory responses [11]. In a rat model of spinal cord ischemia-reperfusion injury, sevoflurane pre-treatment was shown to elevate the expression of miR-181a-1-3p [12]. However, the role of miR-181a-1-3p in DFH pathogenesis, its diagnostic significance, and whether it regulates fracture healing by targeting specific mRNAs, still need to be further explored. It is hypothesize that miR-181a-1-3p may impair osteoblast function by inhibiting DKK2, thereby promoting the occurrence of DFH.

In conclusion, this study clarifies the significant potential of miR-181a-1-3p as a diagnostic biomarker for DFH through the assessment of its serum expression in DFH patients, complemented by receiver operating characteristic (ROC) curve analysis. The mechanism by which miR-181a-1-3p regulates osteoblast differentiation was revealed through *in vitro* experiments. These findings not only provide a robust theoretical foundation, but also offer experimental support for the early diagnosis of DFH and the development of targeted intervention strategies for enhancing fracture healing.

MATERIALS AND METHOD

Inclusion of patients. The study received ethical approval from the Ethics Committee of Nanjing Jiangning Hospital. The study included 165 FNF patients who were treated at Nanjing Jiangning Hospital from April 2022 – March 2024. They were divided into two groups based on post-operative healing outcomes: 95 patients were classified in the normal fracture healing (NFH) group and 70 patients in the DFH group. Inclusion criteria: (1) a diagnosis of FNF confirmed by imaging examinations such as X-ray and computed tomography (CT); (2) admission hospitalization for surgical intervention; (3) provision of comprehensive information to patients and their families, who subsequently signed a written informed consent form; (4) availability of complete clinical data. Exclusion criteria: (1) a prior history of fracture or associated surgical interventions; (2) histories of serious life-threatening systemic diseases, such as aematologic diseases, immune system diseases, or malignant tumours; (3) combination of degenerative arthritis, necrosis of the femoral head, synovitis, etc., and other orthopedic underlying diseases. The diagnostic criteria for DFH were established: (1) X-ray assessments conducted 3 months post-fracture revealing little to no bone callus formation; (2) notable osteosclerosis and visible gaps at the fracture sites. Clinical data for all subjects, including age, gender, BMI, smoking, drinking, history of hypertension, history of diabetes, history of heart disease, fracture type and fracture causes, are summarized in Table 1. 5mL of fasting venous blood was collected from all patients. The blood sample was left at room temperature for 30 minutes, then centrifuged at 3,000 rpm for 15 minutes at 4 °C. The resulting supernatant serum was subsequently extracted and stored at -80 °C for further analysis.

Table 1. Comparison of baseline data between the two groups

Items	NFH group (n=95)	DFH group (n=70)	t/χ2	P value
Age, year (%)	59.05±5.54	59.60±5.40	0.012	0.527
Gender, n (%)			0.277	0.599
Male	49 (51.58)	39 (55.71)		
Female	46 (48.42)	31 (44.29)		
BMI (kg/m^2)	23.34±2.67	23.61±2.40	2.727	0.513
Smoking, n (%)			1.761	0.185
Yes	43 (45.26)	39 (55.71)		
No	52 (54.74)	31 (44.29)		
Drinking, n (%)			1.268	0.260
Yes	50 (52.63)	43 (61.43)		
No	45 (47.37)	27 (38.57)		
History of hypertension, n (%)			1.206	0.272
Yes	53 (55.79)	45 (64.29)		
No	42 (44.21)	25 (35.71)		
History of diabetes, n (%)			1.229	0.268
Yes	46 (48.42)	40 (57.14)		
No	49 (51.58)	30 (42.86)		
History of heart disease, n (%)			2.171	0.141
Yes	46 (48.42)	42 (60.00)		
No	49 (51.58)	28 (40.00)		
Fracture type, n (%)			3.153	0.076
Open fracture	37 (38.95)	37 (52.86)		
Closed fracture	58 (61.05)	33 (47.14)		
Fracture causes, n (%)			2.546	0.280
Traffic accident	47 (49.47)	43 (61.43)		
Tumble	33 (34.74)	20 (28.57)		
Others	15 (15.79)	7 (10.00)		

NFH – normal fracture healing; DFH – delayed fracture healing; n – number; BMI – body mass index

Cell culture and osteogenic differentiation induction. The MC3T3-E1 cell line was procured from the BeNa Culture Collection (Beijing, China). Cells were cultivated in α-MEM supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin, all of which were purchased from Gibco, (Carlsbad, CA, USA). Osteogenic differentiation induction experiments were initiated upon achieving 90% confluence of the cells. The osteoblast induction medium comprised 20 mM sodium β-glycerophosphate (Sigma-Aldrich, Burlington, MA, USA), 100 μg/mL ascorbic acid (Sigma-Aldrich) and 10% FBS. In the experimental procedure, the original medium was replaced with osteoblast induction medium, and the cells were subsequently placed in an incubator to continue incubation for 15 days to foster differentiation. Cell samples were collected on day 15 of the induction culture.

Cell transfection. Inhibitor-NC, miR-181a-1-3p inhibitor, mimic-NC, miR-181a-1-3p mimic, si-NC and si-DKK2 were meticulously designed and synthesized by RiboBio (Guangzhou, Guangdong, China). MC3T3-E1 cells in the

logarithmic growth phase were selected for transfection. The aforementioned oligonucleotides were transfected into the cells in accordance with the instructions provided for lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Following the transfection, cells were harvested 24 h later for subsequent experimental analysis.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from serum samples and cells according to the Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) instructions. Extracted RNA was tested for purity using the company's NanoDrop 2000 UV spectrophotometer (Thermo Fisher Scientific), and sample quality was assessed by the A260/A280 ratio. The miRNA in total RNA was reverse transcribed into complementary DNA (cDNA) using the Hairpin-itTM Real-Time PCR miRNAs kit (GenePharma, Shanghai, China). Meanwhile, mRNA was reverse transcribed to obtain the corresponding cDNA template using the HiFiScript cDNA Synthesis Kit (Cwbiotech, Beijing, China). The RT-qPCR amplification of miR-181a-1-3p, DKK2, RUNX2, OCN, and OPN was performed on a LightCycler 96 (Roche, Basel, Switzerland) using TB Green® Fast qPCR Mix (Takara, Tokyo, Japan) as the reaction mix. Expression analysis for miRNA and mRNA was conducted with U6 and GAPDH serving as internal references, respectively. RT-qPCR conditions were 95 °C for 3 min (initial denaturation), followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s. The relative expression of the target genes was calculated using the $2^{-\Delta\Delta Ct}$ method. The primers used in the RT-qPCR are shown in Supplementary Table S1.

Table S1. Primer used for RT-qPCR

Gene Name	PRIMER SEQUENCE	
	Forward (5′-3′)	Reverse (5′-3′)
miR-181a-1-3p	GAACATTCAACGCTGTCGGT	GTTAGCCATAGGGTACAATCAACG
DKK2	GAAGGGTTTTGCTGTGCTCG	ACTTTGCAAGACAGGCCCTT
RUNX2	CCGGAATGCCTCTGCTGTTATGA	ACTGAGGCGGTCAGAGAACAAACT
OCN	TAGCAGACACCATGAGGACCATCT	CCTGCTTGGACATGAAGGCTTTGT
OPN	TGGCTGAATTCTGAGGGACTAAC	TATAGGATCTGGGTGCAGGC
U6	CGCTTCGGCAGCACATATAC	TTCACGAATTTGCGTGTCAT
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

Cell proliferation assay. MC3T3-E1 cells were seeded into 96-well plates (Corning, Corning, NY, USA) at a density of 2×10^3 cells/well. Following incubation periods of 0, 24, 48, and 72 h, 10μL of counting kit-8 (CCK-8) solution (Dojindo Laboratories, Kumamoto, Japan) was added to each well, after which the plates were incubated at 37 °C for 2 h. Subsequently, absorbance values were measured at 450 nm using a microplate reader (Thermo Fisher Scientific) to assess the levels of cell proliferation.

Migration assay. The permeable Transwell inserts (adapted for 24-well plates) from Corning were used for the migration capacity assay. MC3T3-E1 cells were suspended in serum-free medium at a density of 5×10^4 cells per well and seeded into the upper chamber of the Transwell. The lower chamber was supplemented with complete medium containing 10% FBS. Following a 24 h incubation period, the migrated cells were fixed in 4% paraformaldehyde (Solarbio, Beijing, China) for 15 min and then stained with 1% crystal violet (Solarbio). Finally, the stained cells were enumerated using a light microscope (Olympus, Tokyo, Japan).

Alkaline phosphatase activity. The activity of cellular alkaline phosphatase (ALP) was determined using an Alkaline Phosphatase Assay Kit (Beyotime, Shanghai, China). Cells were lysed with cell lysis buffer (Beyotime), and the resulting supernatant was collected. Experiments were performed according to the kit's protocol, with the reaction being halted by adding 0.1 N NaOH, and absorbance was measured at 405 nm.

Dual luciferase reporter assay. Potential target genes of miR-181a-1-3p were identified through the TargetScan online database (https://www.targetscan.org/vert_72/). To verify the target-binding interaction between miR-181a-1-3p and DKK2, luciferase reporter vectors containing wild-type (DKK2-WT) and mutant (DKK2-MUT) DKK2 sequences were constructed. Subsequently, DKK2-WT or DKK2-MUT reporter vectors were co-transfected into MC3T3-E1 cells with miR-181a-1-3p inhibitor or miR-181a-1-3p mimic. After 48 h of transfection, luciferase activity was measured using a reporter gene assay system (Promega, Madison, WI, USA).

RNA immunoprecipitation (RIP) assay. The Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA, USA) was employed to conduct RIP experiments. The procedure commenced with the resuspension of cells in pre-cooled RIP lysate. An anti-argonaute 2 (Ago2) antibody (Abcam, Cambridge, MA, USA) or a negative control immunoglobulin G (IgG) antibody (Abcam) was incubated with magnetic beads. The resulting complexes were combined with cell lysate and incubated at 4 °C for an overnight period. The precipitated complexes were treated with proteinase K. The purified RNA was used for RT-qPCR analysis to detect the enrichment of target RNA.

Statistical analysis. The statistical analysis of the study was conducted using SPSS 27.0 (IBM SPSS, Armonk, NY, USA) and GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). Continuous variables were presented as mean ± standard deviation (SD), and the t-test was applied for comparison between two groups. Categorical variables were analyzed using the χ^2 test. Analysis of variance (ANOVA) was employed to compare the means across multiple groups. The diagnostic efficacy of miR-181a-1-3p for distinguishing DFH was evaluated using receiver operating characteristic (ROC) curve. Pearson analysis was applied to assess the correlation between miR-181a-1-3p and DKK2 expression. All experiments were independently repeated at least 3 times. $P < 0.05$ indicated that the difference was statistically significant.

RESULTS

Clinical information analysis of participants. A comparative analysis of the clinical baseline data between NFH and DFH patients showed that there was no statistical difference between the 2 groups in terms of age, gender, BMI, smoking, drinking, history of hypertension, history of diabetes, history of heart disease, fracture type and fracture

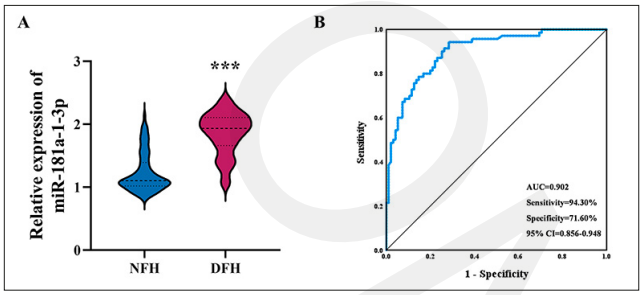

Figure 1. Expression and diagnostic value of serum miR-181a-1-3p. (A) Expression of serum miR-181a-1-3p in 2 patient cohorts (***$P < 0.001$ vs NFH group). (B) ROC curve analysis illustrating the diagnostic effect of miR-181a-1-3p on fracture healing

causes ($P > 0.05$; Tab. 1). These results indicate that the baseline characteristics were well-balanced between the NFH and DFH groups, thereby reinforcing the validity of subsequent comparisons analyses.

Serum miR-181a-1-3p is up-regulated in DFH patients. RT-qPCR assays indicated that serum miR-181a-1-3p expression in DFH patients was significantly higher than that in the NFH group ($P < 0.001$; Fig. 1A). Furthermore, the ROC curve results revealed an area under the curve (AUC) of 0.902 (95% CI: 0.856–0.948) with sensitivity and specificity at 94.30% and 71.60%, respectively (Fig. 1B). This suggested that serum miR-181a-1-3p levels could effectively distinguish DFH patients from NFH controls, indicating that it had a good diagnostic value. These results imply that the abnormal expression of miR-181a-1-3p may be associated with the pathogenesis of DFH.

Regulation of osteoblasts by inhibition or over-expression of miR-181a-1-3p. To investigate the functional mechanism of miR-181a-1-3p in fracture healing, *in vitro* osteogenic differentiation experiments were performed using MC3T3-E1 cells. RT-qPCR results revealed a significant downregulation of miR-181a-1-3p expression following transfection with its inhibition, whereas its expression was markedly up-regulated after over-expression of miR-181a-1-3p ($P < 0.001$; Fig. 2A). CCK-8 assays showed that transfection with miR-181a-1-3p inhibitor significantly promoted MC3T3-E1 cell proliferation ($P < 0.001$), whereas transfection with miR-181a-1-3p mimic inhibited proliferation ($P < 0.001$; Fig. 2B). Transwell assays demonstrated that cell migration was increased or decreased after transfection with miR-181a-1-3p inhibitor or miR-181a-1-3p mimic ($P < 0.001$; Fig. 2C). Additionally, inhibition of miR-181a-1-3p resulted in elevated ALP activity and up-regulated the expression of osteogenic differentiation markers including RUNX2, OCN, and OPN; whereas over-expression of miR-181a-1-3p produced the opposite effects ($P < 0.001$; Fig. 2D-E). These results suggest that miR-181a-1-3p may negatively regulate the fracture healing process by inhibiting the proliferation, migration and differentiation capabilities of osteoblasts.

DKK2 is a target gene of miR-181a-1-3p. To examine the regulatory relationship between miR-181a-1-3p and DKK2 in osteoblasts, the study predicted potential mutual binding sites between these two entities by TargetScan online database (Fig. 3A). To validate the predicted results, the luciferase reporter gene experiments were conducted. The results showed that co-transfection with miR-181a-1-3p inhibitor significantly increased luciferase activity in the DKK2-WT constructs,

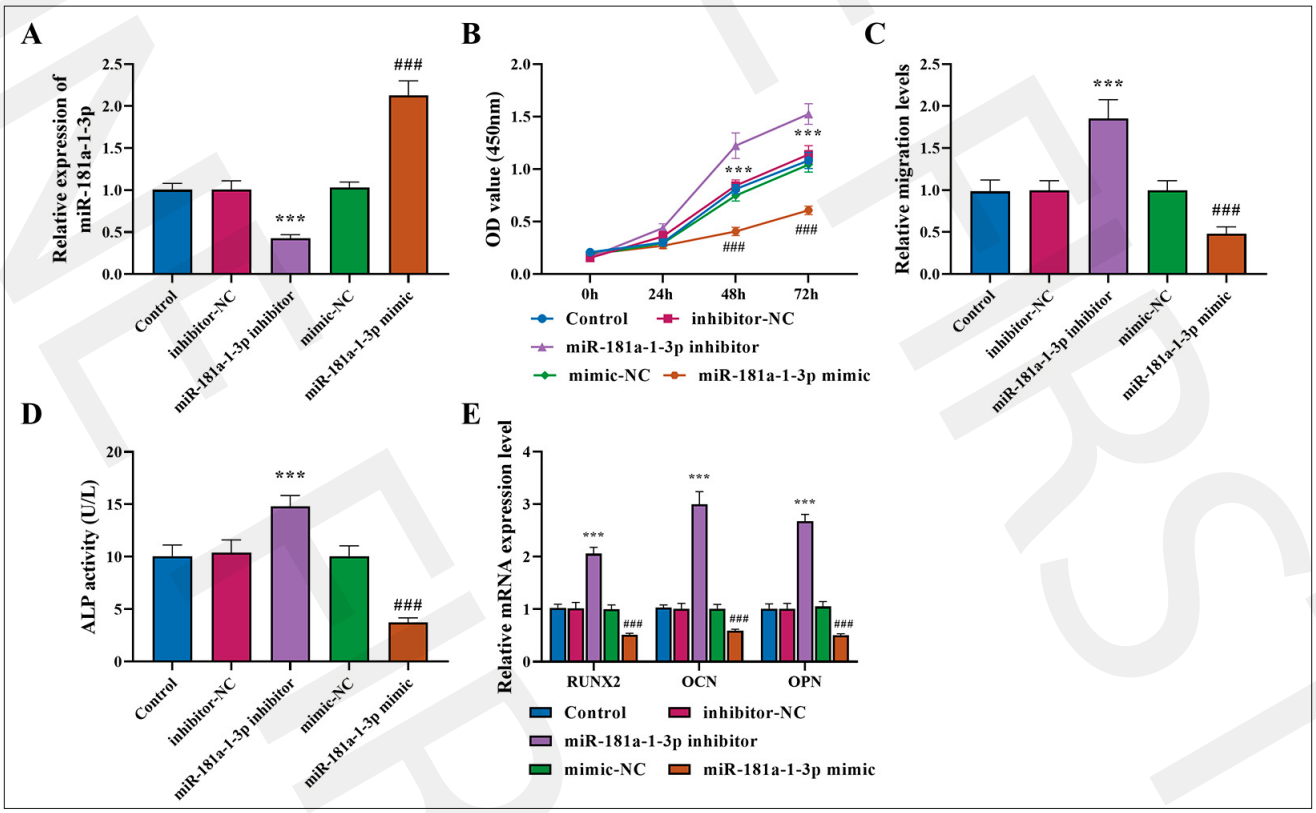

Figure 2. Regulatory effects of inhibition or over-expression of miR-181a-1-3p on MC3T3-E1 cells. Effects of miR-181a-1-3p inhibition or over-expression on the cellular levels of miR-181a-1-3p (A), cell proliferation (B), migration levels (C), ALP activity (D), and the expression of osteogenic differentiation markers (RUNX2, OCN, OPN) (E). ***$P < 0.001$ vs inhibitor-NC group; ###$P < 0.001$ vs mimic-NC group

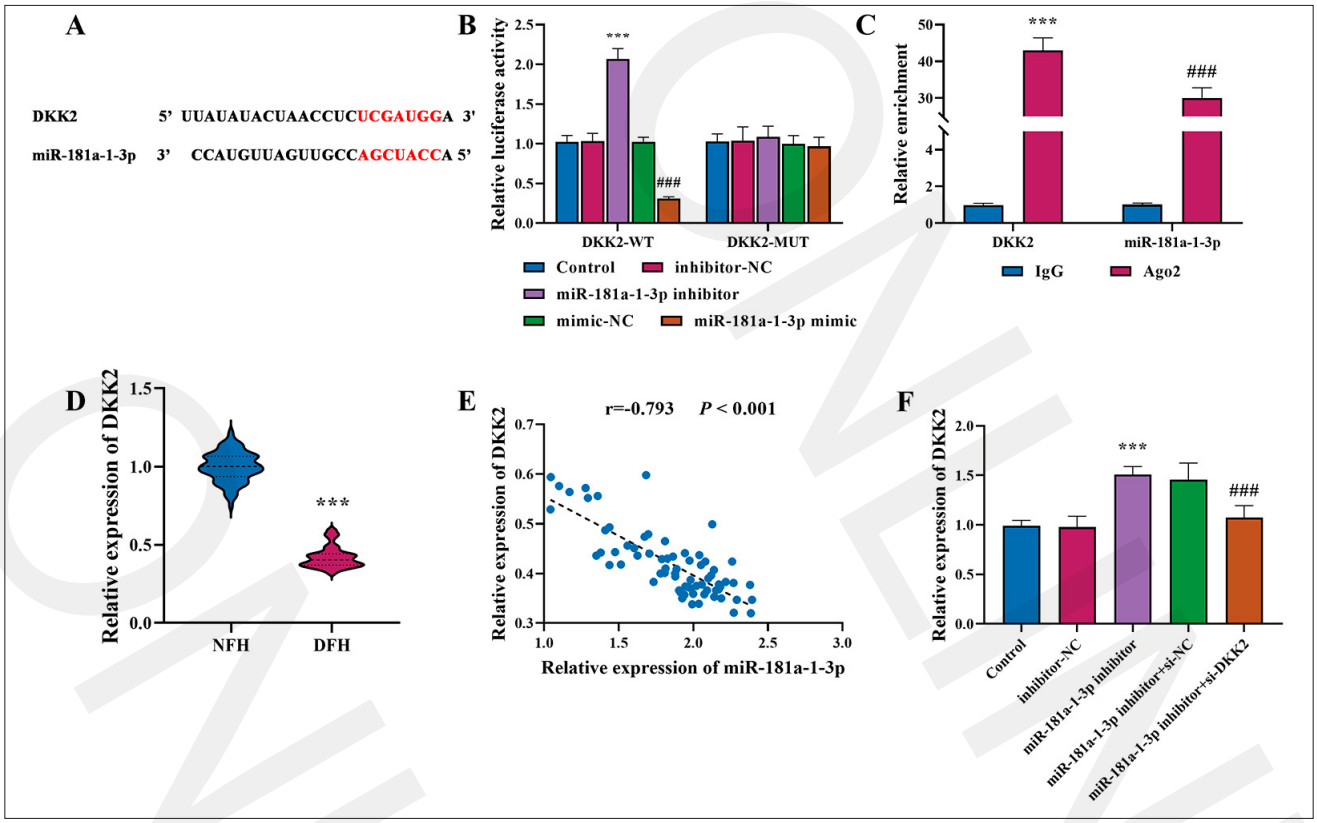

Figure 3. Validation and association analysis of miR-181a-1-3p and its targeting interactions with DKK2. (A) TargetScan has predicted potential binding sites between miR-181a-1-3p and DKK2. The target-binding relationship was analyzed by dual luciferase reporter gene assay (B) and RIP assay (C). (***$P < 0.001$ vs inhibitor-NC group; ***$P < 0.001$ vs IgG group, ###$P < 0.001$ vs mimic-NC group; ###$P < 0.001$ vs IgG group). (D) Expression of serum DKK2 in 2 patient cohorts. (***$P < 0.001$ vs NFH group). (E) Correlation analysis of serum miR-181a-1-3p and DKK2. (F) Effect of co-transfection with miR-181a-1-3p inhibitor and si-DKK2 on cell DKK2 expression. (***$P < 0.001$ vs inhibitor-NC group; ###$P < 0.001$ vs miR-181a-1-3p inhibitor + si-NC group)

while co-transfection with miR-181a-1-3p mimic significantly decreased it ($P < 0.001$; Fig. 3B). There was no significant alteration in luciferase activity following the transfection of the DKK2-MUT construct ($P > 0.05$). RIP assay further confirmed the binding relationship, revealing a significant enrichment of miR-181a-1-3p and DKK2 in the complexes pulled down by the anti-Ago2 antibody ($P < 0.001$; Fig. 3C). In addition, serum DKK2 expression was down-regulated in DFH patients ($P < 0.001$; Fig. 3D). Correlation analysis showed that serum miR-181a-1-3p was negatively correlated with DKK2 levels in DFH patients (r=-0.793; $P < 0.001$; Fig. 3E). Inhibition of miR-181a-1-3p expression resulted in increased intracellular DKK2 levels; further knockdown of DKK2 resulted in decreased cellular DKK2 expression ($P < 0.001$; Fig. 3F).

In summary, these results confirm that DKK2 is a regulatory target of miR-181a-1-3p, and with both factors are involved in DFH disease progression through a negative regulatory mechanism.

Regulation of osteoblasts by miR-181a-1-3p and DKK2. To comprehensively explore the regulatory mechanisms of miR-181a-1-3p and DKK2 in MC3T3-E1 cells, co-transfection of miR-181a-1-3p inhibitor and si-DKK2 was performed. As shown in Fig. 4A, inhibition of miR-181a-1-3p led to a marked enhancement in proliferation, and knockdown of DKK2 resulted in decreased proliferation ($P < 0.001$). The cell migration results showed that transfection with miR-181a-1-3p inhibitor significantly promoted cell migration; however, this pro-migratory effect was partially attenuated by knockdown of DKK2 ($P < 0.001$; Fig. 4B).

In the context of cell differentiation, the differentiation status of MC3T3-E1 cells was assessed by detecting the expression of osteogenic differentiation markers. Post co-transfection with miR-181a-1-3p inhibitor and si-DKK2, the ALP activity of the cells was reduced, and the expression of RUNX2, OCN, and OPN was also suppressed ($P < 0.01$; Fig. 4C-D).

In summary, miR-181a-1-3p and DKK2 play key regulatory roles in the processes of proliferation, migration, and differentiation in MC3T3-E1 cells, collectively contributing to the regulation of fracture healing mechanisms.

DISCUSSION

Osteoblast differentiation and mineralization play a crucial role in the process of bone regeneration, and any disruption in these mechanisms can heighten the risk of fractures [13]. DFH, a common post-operative complication of fractures, not only extends the rehabilitation period but also imposes a significant risk of impaired limb function [3]. The MC3T3-E1 cells, characterized by their stable osteogenic differentiation potential, serve as a reliable *in vitro* model for recapitulating the processes of fracture healing. The discovery of novel biomarkers and the elucidation of their underlying mechanisms are thus pivotal for the early diagnosis and targeted intervention of DFH. This study demonstrated that miR-181a-1-3p regulated osteoblast biological functions through DKK2.

In recent years, the role of miRNAs in the regulation of osteoblast differentiation, bone metabolic homeostasis

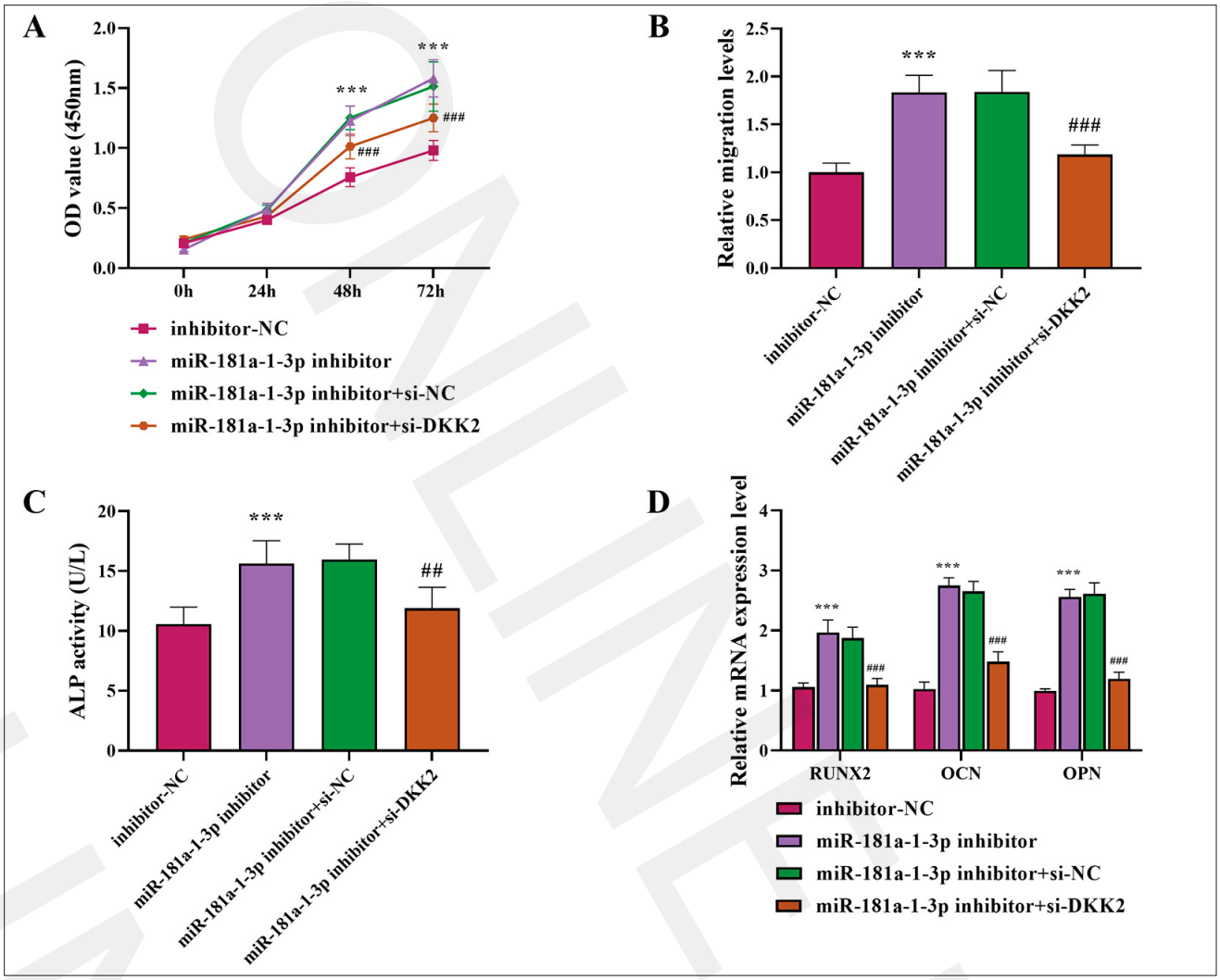

Figure 4. Synergistic regulation of MC3T3-E1 cells by miR-181a-1-3p and DKK2. Effects of co-transfection of the miR-181a-1-3p inhibitor and si-DKK2 on cell proliferation (A), migration levels (B), ALP activity (C), and the expression of osteogenic differentiation markers (RUNX2, OCN, OPN) (D). (***$P < 0.001$ vs inhibitor-NC group, ##$P < 0.01$, ###$P < 0.001$ vs miR-181a-1-3p inhibitor + si-NC group)

and bone tissue formation has become a prominent area of research in the field of bone regeneration [14]. In a previous study, Wang et al. found that miR-654-3p was significantly reduced in DFH patients, and ROC analysis showed that miR-654-3p had a good diagnostic value (AUC=0.858). In contrast, the current study revealed that miR-181a-1-3p was highly expressed in the serum and MC3T3-E1 cells of DFH patients, exhibiting an opposite expression trend to miR-654-3p. ROC analysis further showed that miR-181a-1-3p had a better diagnostic value (AUC=0.902). These findings suggest that miR-181a-1-3p may be a more promising biomarker for DFH.

During the fracture healing process, the number of osteoblasts exhibits a dynamic increase, with their differentiation and activity facilitating the acceleration of new bone formation [15]. Therefore, a systematic assessment of the cellular biological indicators associated with DFH is important for revealing the mechanisms of healing disorders. A fundamental marker of osteogenic differentiation, ALP not only catalyzes the liberation of inorganic phosphate, but also promotes osteoblast proliferation and differentiation through the activation of downstream signalling pathways [16]. The expression of RUNX2, a pivotal regulator of bone formation, is modulated by a variety of miRNAs [17]. OCN is a serum-specific marker reflecting the activity of osteoblasts. OPN plays a dual function in facilitating both of osteoblast differentiation and the mineralization of new bone matrix [18]. A progressive increase in the expression or activity of these markers during osteogenic differentiation is an important indication of successful differentiation. Previous studies confirmed that ALP, OCN and RUNX2 were upregulated during the osteogenic differentiation of MC3T3-E1 cells. Over-expression of miR-98-5p impeded osteoblast proliferation and differentiation while promoting apoptosis [19].

In contrast, inhibition of miR-222-3p enhanced the expression of RUNX2, increased OPN expression and elevated ALP activity, thereby accelerated matrix mineralization [20]. In the current study it is discovered that miR-181a-1-3p is a crucial player in DFH pathogenesis. Inhibition of miR-181a-1-3p promoted proliferation, migration, and differentiation of MC3T3-E1 cell; whereas its over-expression exerted opposite effects. This suggests that miR-181a-1-3p may regulate the biological behaviour of osteoblasts by regulating key osteogenic markers, such as ALP and RUNX2, thereby impacting the fracture healing process.

miRNAs often synergize with mRNAs to orchestrate various biological functions. It was found that DKK2 was a potential target gene for miR-181a-1-3p, utilizing prediction from the TargetScan online database. The Wnt signalling pathway plays a pivotal role in bone formation, with DKK2 is a key regulatory factor in this pathway [21]. Clinical evidence indicates that DKK2 is detectable in serum, and its reduced levels are significantly associated with an increased risk of fractures [22]. Notably, the current study revealed that serum DKK2 expression was significantly down-regulated in DFH patients. When miR-181a-1-3p was inhibited, knockdown of DKK2 inhibited cell proliferation, migration, and osteogenic differentiation capabilities. This phenomenon suggests that normal expression of DKK2 during the NFH phase facilitates accelerated callus formation and mineralization by enhancing osteoblast function, thereby ensuring a seamless process of fracture healing. Furthermore, previous studies demonstrated that miR-27a promoted osteogenic differentiation in MC3T3-E1 cells by targeting DKK2 [23]. Over-expression of DKK2 significantly reversed the pro-osteogenic effects induced by miR-128 [24]. These findings indicate that DKK2 plays a crucial role in the regulation of osteogenesis. DKK2, as a classic antagonist of the Wnt/β-catenin pathway, its down-regulation may relieve the inhibition on the Wnt signal, thereby activating downstream osteogenic transcription programmes such as RUNX2 [21]. This is consistent with the phenomenon of increased RUNX2 expression observed by the authors of the current study. Additionally, the ERK/MAPK pathway is crucial in responding to growth factor stimulation, promoting cell proliferation and differentiation; while the BMP/Smad pathway, especially the Smad5-RUNX2 axis, is the core driving force for mesenchymal stem cells to differentiate into osteoblasts [25]. Whether DKK2 indirectly regulates ERK or Smad signals through interaction with non-classical Wnt pathways or other extracellular ligands warrants further investigation.

Limitations of the study. This study has several limitations. First, this study is a single-centre investigation with a relatively limited sample size, which fails to adequately account for geographic and racial diversity. This limitation may to some extent restrict the generalizability of the findings. Future endeavors will entail conducting a large-scale, multicentre, prospective cohort study encompassing diverse populations to further validate the diagnostic efficacy of miR-181a-1-3p for DFH. Second, the current study primarily focused on cellular-level functional experiments, and therefore lacks validation through *in vivo* models. Subsequent work will involve establishing animal models to provide a more solid foundation for clinical translation.

Finally, although the study preliminarily suggests that miR-181a-1-3p influences the expression of osteogenic differentiation markers by targeting DKK2, the downstream signalling pathways of the miR-181a-1-3p/DKK2 axis remain unclear. Further investigation into whether the miR-181a-1-3p/ DKK2 axis exerts its effects by regulating key signalling pathways will be a crucial direction for elucidating the underlying molecular mechanisms.

CONCLUSIONS

The study confirmes that miR-181a-1-3p is significantly up-regulated in both serum samples and cells from DFH patients. miR-181a-1-3p inhibits the proliferation, differentiation and migration of osteoblasts by targeting DKK2 and regulating its expression. This regulatory mechanism ultimately impacts fracture healing and ultimately leading to the development of DFH. These findings offer a new perspective on the mechanisms of fracture healing and provide a theoretical foundation for clinical diagnosis and treatment of DFH.

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