Journal of Orthopaedic Surgery and Research

RESEARCH

Open Access

Serum miR-519d-3p and BMP2: potential early diagnostic markers and their mechanism in delayed fracture healing

Jing Xiang[1†], Lina Huang[2†], Chuangye Qu[3], Weibing Bao[4], Wenqi Wang[4], Xiaozhong Zhu[4*] and Yong Deng[5*]

Abstract

Background Delayed fracture healing (DFH) affects patients' quality of life, and there are limitations in diagnosis by CT scan. The purpose of the study is to evaluate the potential and mechanism of clinical application of miRNAs in DFH for early diagnosis and intervention.

Methods Serum samples were obtained from delayed and normal fracture healing patients and the levels of miR-519d-3p and BMP2 were measured by RT-qPCR, and the value of both in the diagnosis of DFH was assessed by ROC curve. Cell viability and apoptosis were monitored using CCK8 kit and flow cytometry, respectively, and mRNA expression of osteogenesis and apoptosis-related genes were detected by RT-qPCR. The molecular interactions were verified using luciferase reporter gene system and RIP technique.

Results Up-regulation of miR-519d-3p expression and down-regulation of BMP2 in the serum of fracture patients four weeks after surgery can be used as an early warning marker of DFH and a risk factor for poor fracture healing. Further studies showed that overexpression of miR-519d-3p markedly inhibited the expression of RUNX2, OCN and ALP and prevented osteoblast differentiation. Meanwhile, it inhibited cell viability, promoted apoptosis, upregulated Bax and Cleaved-caspase-3 mRNA expression, and downregulated Bcl-2 expression. BMP2, targeted by miR-519d-3p, enhanced osteogenesis and reversed the inhibitory of action miR-519d-3p.

Conclusions Serum miR-519d-3p and BMP2 can be used as early diagnostic markers for DFH. miR-519d-3p inhibited osteogenesis by targeting BMP2, which may slow down fracture healing.

Keywords Delayed fracture healing, Osteogenic differentiation, Growth, Apoptosis, miR-519d-3p, BMP2

[†]Jing Xiang and Lina Huang should be considered joint first author

*Correspondence:
Xiaozhong Zhu
Zhuxiaozhong08@163.com
Yong Deng
18208305371@163.com
[1]Department of Rehabilitation, The Affiliated Hospital of Southwest Medical University, Luzhou 646000, Sichuan, China
[2]Department of Rehabilitation Medicine, The Affiliated Hospital of Youjiang Medical University for Nationalities; Guangxi Key Laboratory for Preclinical and Translational Research on Bone and Joint Degenerative Diseases, Baise 533000, China
[3]Department of Orthopedics, Lanzhou Petrochemical General Hospital (The Fourth Affiliated Hospital of Gansu University of Traditional Chinese Medicine), Lanzhou 730060, Gansu Province, China
[4]Department of Radiology, Gansu Provincial Hospital of Traditional Chinese Medicine (The First Affiliated Hospital of Gansu University of Traditional Chinese Medicine), No. 418 Guazhou Road, Qilihe District, Lanzhou 730050, Gansu Province, China
[5]Department of Bone and Joint Surgery, The Affiliated Hospital of Southwest Medical University, No. 25, Taiping Street, Jiangyang District, Luzhou City 646000, Sichuan Province, China

Background

Fractures, a highly prevalent traumatic condition, are a major concern in medical practice. Fracture healing, a complex biological process, has long been a focal point of clinical research and treatment efforts. Extensive research has been carried out globally to develop effective therapeutic strategies that can accelerate the recovery process from this type of trauma [1–3]. Delayed fracture healing (DFH) is a prevalent complication in fracture management, with its diagnosis and treatment posing numerous challenges [4, 5]. Traditional computed tomography (CT) diagnostic techniques, while invaluable for the anatomical assessment of fractures, exhibit a notable delay in monitoring the fracture healing process [6]. This delay stems from the reliance on late-stage imaging findings, such as the disappearance of the fracture line and the formation of callus, which limits the accuracy of early diagnostic information [7, 8]. Consequently, DFH is often detected belatedly, precluding timely and effective interventional therapies. This lag not only impairs the progression of fracture healing but may also augment the patient's susceptibility to suffering and complications. Hence, the identification of an early and precise diagnostic method for DFH is of paramount importance.

Recent advances in molecular biology have highlighted the potential of serum microRNAs (miRNAs) as non-invasive biomarkers for various conditions, including fracture healing. Serum miRNA offers several diagnostic advantages, including relative stability in blood, ease and speed of detection, and non-invasive acquisition [9]. It is widely used in bone related diseases, such as tendon injury [10, 11], osteoarthritis [12, 13], osteoporosis [14] and so on Significantly, changes in serum miRNA expression may be closely associated with the fracture healing process, potentially serving as an effective indicator for early diagnosis of delayed fracture healing (DFH) [15]. Several scientific studies have indicated that specific miRNAs like miR-98-5p [16], miR-181a-5p [17], and miR-214-3p [18] have great potential in predicting the risk of DFH and exploring therapeutic strategies. miRNAs, although being endogenous non-coding small molecule RNAs, can precisely regulate gene expression at the post-transcriptional level by interacting with specific sequences in target mRNAs, thereby affecting cellular physiological functions and complex pathological processes [19, 20]. Bone morphogenetic protein-2 (BMP2), an efficacious osteoinductive cytokine, occupies a pivotal regulatory position in the processes of osteoblast differentiation, proliferation, and bone matrix synthesis [21, 22]. Evidence indicates that specific miRNAs play pivotal roles in fracture healing by regulating BMP2 expression. For example, the downregulation of miR-214-3p has been demonstrated to augment BMP2 expression, thereby enhancing fracture healing outcomes [23]. miR-6979-5p exhibits an inhibitory effect on osteogenic differentiation and bone formation by suppressing BMP2 expression [24]. Therefore, in-depth exploration and analysis of the miRNA regulatory network that modulates BMP2 expression is crucial for comprehensively understanding the underlying molecular mechanisms of bone formation and exploring related diagnostic and therapeutic strategies.

Investigations revealed a study suggesting that miR-519d-3p promotes transforming growth factor (TGF)-small molecule-mediated postoperative epidural scarring through inhibition of bone morphogenetic proteins and activin membrane-bound inhibitor [25]. Given that BMP2 is one of the major members of the TGF - β superfamily, this hints at a possible connection between BMP2 and miR-519d-3p. On the other hand, in studies of orthopaedic-related diseases, there is evidence that miR-519d-3p is strongly associated with the development of post-traumatic osteoarthritis [26] and bone cancer [27]. These investigations hint at a potential connection between miR-519d-3p and the onset of bone-related disorders. Despite the established roles of other miRNAs in fracture healing, the specific mechanisms by which miR-519d-3p influences DFH and its relationship with BMP2 remain poorly understood. Addressing this gap is crucial for developing targeted diagnostic and therapeutic strategies.

Therefore, the present study aims to evaluate the accuracy and reliability of miR-519d-3p in predicting fracture healing by analyzing its expression levels in patients with DFH compared to those with normal healing (NFH). This research could provide valuable insights into early diagnosis and intervention strategies for DFH.

We hypothesize that elevated levels of miR-519d-3p in the serum of DFH patients are inversely correlated with BMP2 expression and that this relationship may contribute to impaired fracture healing. To test this hypothesis, we employed a cohort study design to compare serum levels of miR-519d-3p and BMP2 between DFH and NFH patients, utilizing RT-qPCR for quantification due to its sensitivity and specificity. The findings of this study are expected to elucidate the role of miR-519d-3p in fracture healing and its potential as a biomarker for DFH, thus paving the way for future research and clinical applications.

Methods

Study design and purpose

This study aimed to investigate the diagnostic potential and functional roles of miR-519d-3p and BMP2 in DFH. Serum samples from DFH and normal healing patients were analyzed to assess the diagnostic value of miR-519d-3p and BMP2 using receiver operating characteristic (ROC) curve analysis. To elucidate the underlying

mechanisms, in vitro experiments were conducted to evaluate the effects of miR-519d-3p and BMP2 on cell viability, apoptosis, and osteogenic differentiation, complemented by molecular interaction validation through luciferase reporter assays and RNA immunoprecipitation (RIP). The study design integrated clinical sample analysis with functional experiments to provide a comprehensive understanding of miR-519d-3p and BMP2 in DFH pathogenesis and their potential as therapeutic targets.

Ethics approval

This study adheres to the principles of the Declaration of Helsinki, has been approved by the Ethics Committee of Gansu Provincial Hospital of Traditional Chinese Medicine, and informed consent has been obtained from patients and their families.

Study subject

A cohort of 152 patients with traumatic tibial fractures admitted to the Gansu Provincial Hospital of Traditional Chinese Medicine between 2020 and 2022 was selected for this study. Inclusion criteria encompassed: (1) X-ray-confirmed traumatic tibial fractures; (2) incident fractures; and (3) patients with normal communication abilities and autonomous consciousness. Exclusion criteria included: (1) coexisting bone diseases or pathological fractures; and (2) other conditions such as hypertension, hyperlipidemia, hyperglycemia, immune disorders, cardiovascular, cerebrovascular, renal, or other organ dysfunction, and malignancy. Based on healing status, patients were categorized into delayed healing (DFH, $n=77$) and normal healing (NFH, $n=75$) groups. DFH was diagnosed by the absence of callus formation and persistence of a fracture line on X-ray six months post-normal healing time, accompanied by pain and abnormal bone end movement at the fracture site. NFH was diagnosed by continuous callus formation on X-ray six months post-normal healing time, absence of tenderness, percussion pain, or conscious pain, normal fracture site appearance, and the ability to walk independently for at least three minutes [28].

Sample size

Utilize G*Power (v3.1) to ascertain the initial sample size for the study, considering an effect size of 0.5, statistical power of 0.80, and α level of 0.05. Drawing from Lu et al. [29], with an equal allocation ratio (N2/N1:1) and an anticipated attrition rate of 10% plus margin for error, each group necessitates at least 74 participants. Both study groups fulfilled or surpassed this threshold, thereby guaranteeing the study's validity and reliability.

Clinical data and serum sample collection

The researchers gathered clinical data pertaining to gender, age, BMI, smoking history, alcohol consumption history, fracture side, osteosynthesis method, and severity of fracture. Postoperatively, a 10 mL sample of fasting peripheral venous blood was collected from all patients at week 4. The blood was centrifuged at 3000 rpm for 10 min, and the supernatant was obtained as serum, which was then stored at -80 ℃.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was employed to assess molecular expression in cellular groups and patient sera. Initially, collected samples underwent sequential addition of Trizol and chloroform (Thermo Fisher Scientific, USA), followed by the collection of supernatant after lysis and separation. The precipitated fraction, obtained by mixing with isopropanol, was subsequently washed and dried. RNA was solubilized in RNase-Free water and its quality evaluated using a spectrophotometer. For mRNA analysis, cDNA synthesis was performed using the SuperScript III First-Strand Synthesis System Kit (Invitrogen, USA) with Oligo(dT) primers to selectively reverse transcribe polyadenylated mRNA. For microRNA (miR-519d-3p) analysis, cDNA was synthesized using the miRcute miRNA First-Strand cDNA Synthesis Kit (Tiangen, China) with specific stem-loop primers designed for miRNA reverse transcription. This cDNA served as a template for PCR amplification, conducted under the specified conditions. Relative mRNA expression levels of BMP2, runt-related transcription factor 2 (RUNX2), osteocalcin (OCN), alkaline phosphatase (ALP), B-cell lymphoma 2 (Bcl-2), cleaved cysteinyl aspartate specific proteinase 3 (Cleaved-caspase-3), and Bcl-2-associated X protein (Bax) were determined using the $2^{-\Delta\Delta Ct}$ method, with GAPDH as the internal control. For miR-519d-3p expression, U6 served as the internal reference. Primer sequences were shown in Table S1. Gene accession number and length of the amplification product was shown in Table S2.

Cell culture and transfection

For the experiments, Mouse Embryo Osteoblast Precursor Cells (MC3T3-E1, Meilian Biotechnology Co., Ltd, Shanghai, China) were utilized. Frozen cells were resuscitated and placed in DMEM medium. The DMEM culture medium used includes 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, with all reagents purchased from Thermo Fisher Scientific Company (USA). Then, they were transferred to an incubator (set at 37 °C, 5% CO_2) for incubation. Subsequent passages were conducted to expand the cell population for further experimental requirements.

To regulate molecular expression, well-grown MC3T3-E1 cells were selected for transfection experiments. Based on the experimental grouping, the cells were transfected with purchased miR-519d-3p mimic, miR-519d-3p inhibitor, miR-519d-3p negative control plasmid, pcDNA3.1 empty vector, and pcDNA3.1 + BMP2 vector using the Lipofectamine 2000 Transfection Kit (Invitrogen, USA). Following a 48-hour incubation period, molecular expression was assessed to screen the transfected cells.

Osteoblast differentiation

MC3T3-E1 cells, with their molecular weights successfully modulated, were seeded into six-well plates at a density of 1×10^4 cells per milliliter. Once the cells adhered to the plates, the culture medium was replaced with osteogenic differentiation-inducing medium (Gibco, USA), which comprised L-glycerophosphate, dexamethasone, and ascorbic acid. They were then maintained in an incubator for 15 days. Cells from three distinct time points—without induction, at the midpoint of induction, and at the end of induction—were harvested separately. The expression levels of differentiation marker molecules were assessed in these cells to identify those that had successfully undergone osteogenic differentiation.

Dual-luciferase reporter assay

A luciferase reporter gene assay was conducted to confirm the binding interaction predicted by TargetScan 7.0 (http://www.targetscan.org/vert_72/), which identified the binding sites between miR-519d-3p and BMP2. Specifically, two vectors, wild-type (BMP2-WT) and mutant (BMP2-MT), were constructed based on the presence or absence of the 3′ UTR fragment encompassing the binding site. These vectors were then co-transfected with miR-mimic, miR-inhibitor, and miR-NC into MC3T3-E1 cells using a standard transfection protocol. Following a 48-hour incubation period, luciferase activity was measured in each cell group using the Dual Luciferase Reporter System (Promega, Madison, WI, USA).

RIP analysis

To identify the incorporation of miR-519d-3p and BMP2 within immunoprecipitated complexes, the EZ-Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) was employed. MC3T3-E1 cells underwent lysis through the use of RIP lysis buffer, followed by incubation with magnetic beads that had been coated with either Ago2 or IgG antibodies, maintained at a temperature of 4 °C. After this step, the quantitative expression levels of miR-519d-3p and BMP2 were evaluated using RT-qPCR techniques.

Cell viability

Differentiation-successfully-induced MC3T3-E1 cells were resuspended in DMEM culture medium and seeded into 96-well plates (density: 1×10^4 cells /well). At the specific time point of 72 h, 10 μL of CCK-8 solution (Beyotime, Beijing, China) was added. After an additional 2-hour incubation, the reaction was terminated by the addition of 150 μL of dimethyl sulfoxide. The absorbance at 450 nm was then measured using a microplate reader, and the percentage of cell viability was calculated.

Cell apoptosis

Flow cytometry was employed to evaluate the apoptosis of MC3T3-E1 cells in each group, as detailed by Zhang et al. [30] The experiment utilized an apoptosis detection kit supplied by Soleibao (Beijing, China). Initially, the cells were subjected to staining. To 100 μL of cell suspension, 5 μL of Annexin V-FITC (with a concentration of 1 μg/mL) and 5 μL of propyl iodide (PI) staining solution (with a concentration of 50 μg/mL) were added, followed by incubation in the dark for 15 min. After cell staining, analysis was performed using a flow cytometer (BD FACSVerse), with laser wavelengths set at 488 nm and 561 nm. Representative flow cytometry images, along with a detailed gating strategy, were included to enhance the clarity of the findings. Specifically, cells were first gated based on forward scatter (FSC) and side scatter (SSC) to exclude debris and aggregates. Subsequently, a gate was set on the Annexin V-FITC versus PI plot to differentiate viable cells (Annexin V-/PI-), early apoptotic cells (Annexin V+/PI-), late apoptotic cells (Annexin V+/PI+), and necrotic cells (Annexin V-/PI+). Eventually, the proportion of apoptotic cells was calculated.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism 6.0 and SPSS 22.0 software, chosen for their widespread use in biomedical research and their user-friendly interface and functionality. Categorical variables are expressed as number and chi-square test is used. Continuous variables that conformed to normal distribution were expressed as mean ± standard deviation. The choice of t-test and ANOVA is due to their suitability for comparing mean differences between two or more groups, and their applicability to data that meet the characteristics of normal distribution. Post-hoc analyses (Tukey's tests) were performed following ANOVA to determine specific differences between groups. A multifactorial logistic regression analysis was employed to identify factors that impact fracture healing. The diagnostic accuracy of miR-519d-3p and BMP2 in DFH was assessed through ROC curve analysis. Furthermore, Pearson correlation analysis was performed to explore the correlation between miR-519d-3p and BMP2 expression levels.

Table 1 Comparison of the baseline data of study objects

Parameters	NFH	DFH	*P* value
	(*n* = 75)	(*n* = 77)	
Age (year)	39.08 ± 11.95	41.19 ± 11.42	0.266
Gender (Female/male)	32/43	40/37	0.252
BMI (kg/m^2)	24.29 ± 1.79	23.79 ± 1.90	0.097
Smoking			0.420
No	39	35	
Yes	36	42	
Drinking			0.608
No	31	35	
Yes	44	42	
Fracture Side			0.417
right	40	36	
left	35	41	
Osteosynthesis method			0.509
Open reduction	33	38	
Losed reduction	42	39	
Severity of fracture			0.633
Complete fracture	35	46	
Incomplete fracture	40	31	

Annotation: BMI, body mass index

All statistical tests used are clearly stated above, with statistical significance determined at a p-value threshold of < 0.05.

Results

Predictive value of DFH

No statistically significant differences were observed between the DFH and NFH cohorts in aspects such as gender, age, BMI, past smoking and alcohol use, consumption history, fracture side, osteosynthesis method, and severity of fracture. ($P > 0.05$; see Table 1). Serum miR-519d-3p expression was elevated in the DFH group compared to the NFD group ($P < 0.001$, as shown in Fig. 1A). The ROC curve AUC for miR-519d-3p is 0.865, with a sensitivity of 74.03% and specificity of 84% (as shown in Fig. 1B). In contrast, BMP2 expression was reduced in the DFH group ($P < 0.001$, as shown in Fig. 1C), yielding an AUC of 0.854 with a diagnostic sensitivity of 74.03% and specificity of 81.33% (as shown in Fig. 1D). An inverse relationship was observed between miR-519d-3p and BMP2 levels in DFH patients (r = -0.741, $P < 0.001$; as shown in Fig. 1E). The combination of miR-519d-3p and BMP2 enhanced diagnostic efficacy in DFH patients, increasing sensitivity to 88.31% (as shown in Fig. 1F). Logistic regression analysis further indicated

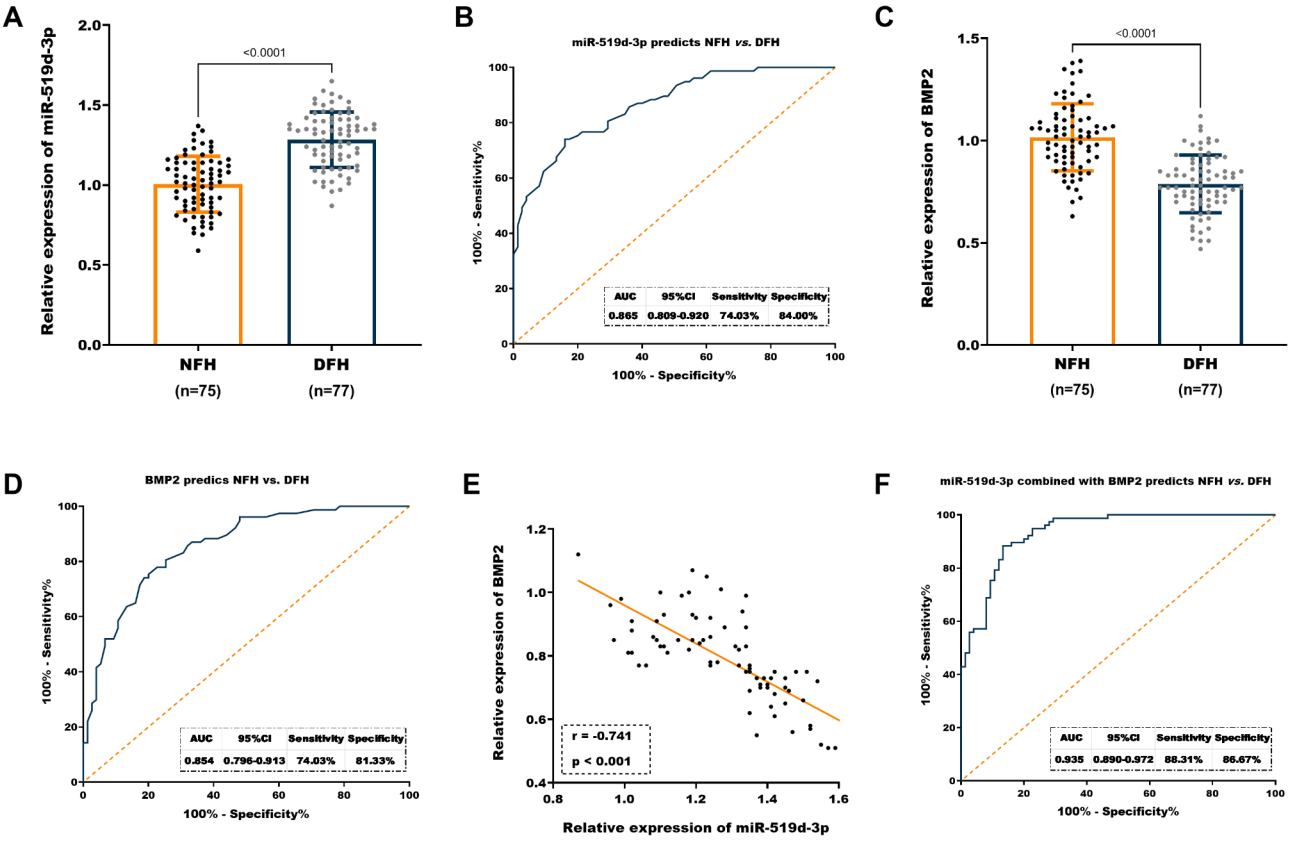

Fig. 1 Expression and Diagnostic Utility of miR-519d-3p and BMP2 in DFH. miR-519d-3p was overexpressed in DFH (**A**) and it can distinguish DFH from BFH patients (**B**). BMP2 was reduced in DFH (**C**), which can differentiate DFH from BFH patients (**D**). miR-519d-3p and BMP2 was negatively correlated in DFH patients (**E**). Combined use of miR-519d-3p and BMP2 enhanced the monitoring of DFH occurrence (**F**)

Table 2 Logistic regression analysis of risk factors for DFH

	p-value	OR	95%CI
miR-519d-3p	0.007	2.717	1.309–5.64
BMP-2	0.012	0.399	0.195–0.82
Age	0.941	1.027	0.505–2.089
Gender (male/female)	0.399	0.739	0.366–1.493
BMI (kg/m2)	0.134	0.581	0.286–1.182
Smoking	0.811	1.092	0.529–2.257
Dringking	0.286	1.484	0.718–3.064
Fracture Side	0.375	1.386	0.674–2.852
Osteosynthesis method	0.618	1.195	0.593–2.407
Severity of fracture	0.617	1.196	0.594–2.407

that miR-519d-3p upregulation ($P=0.007$, OR = 2.717) and BMP2 downregulation ($P=0.012$, OR = 0.399) were associated with adverse fracture healing outcomes (as shown in Table 2).

Expression and interaction of miR-519d-3p and BMP2

During osteogenic differentiation induction in MC3T3-E1 cells, the mRNA expression levels of osteogenic markers RUNX2, OCN, and ALP increased progressively with induction time as shown in Fig. 2A. Concurrently, miR-519d-3p expression decreased as shown in Fig. 2B, whereas BMP2 mRNA expression exhibited an opposite trend as shown in Fig. 2C). To confirm the binding interaction between miR-519d-3p and BMP2, two experiments were performed, yielding results that demonstrated capacity of miR-519d-3p to regulate the luciferase activity of BMP2-WT as depicted in Fig. 3A ($P<0.01$). Furthermore, both miR-519d-3p and BMP2 were enriched in Ago2 as shown in Fig. 3B ($P<0.001$). Additionally, transfection of miR-519d-3p mimics into osteogenically induced MC3T3-E1 cells resulted in miR-519d-3p overexpression ($P<0.001$), while overexpression of BMP2 did not affect the expression of miR-519d-3p ($P>0.05$) as shown in Fig. 3C. miR-519d-3p upregulation suppressed BMP2 expression as shown in Fig. 3D ($P<0.001$).

Regulation of bone induction by miR-519d-3p and BMP2

Further investigation into the impact of miR-519d-3p and BMP2 expression on osteogenic induction in MC3T3-E1 cells revealed that miR-519d-3p overexpression repressed the expression of osteogenic differentiation markers as shown in Fig. 4A ($P<0.001$), hindered the cells viability as shown in Fig. 4B ($P<0.001$). The effect of miR-519d-3p up-regulation on apoptosis-related proteins, as shown in Fig. 4C, was up-regulated by the apoptotic factors Bax and Cleaved-caspase-3 mRNA and down-regulated by the anti-apoptotic factor Bcl-2 ($P<0.001$). Figure 4D more visually showed that apoptosis rate was upregulated after miR-519d-3p upregulation ($P<0.001$). Conversely, overexpression of BMP2 counteracted the upregulation of miR-519d-3p to some extent and showed a positive promotion of osteogenesis in MC3T3-E1 cells.

Discussion

DFH critically impairs patients' functional recovery, yet current clinical assessment heavily relies on CT scans that detect morphological changes only after substantial bone remodeling occurs. This limitation underscores the urgent need for biomarkers capable of dynamically reflecting biological alterations during early fracture healing. Recently, miRNAs have emerged as novel biomarkers demonstrating a close correlation with fracture healing status and their ability to dynamically mirror the biological fluctuations during the healing process [31, 32]. Given this backdrop, the present study is committed to exploring the potential and mechanism of action of miRNAs in the clinical application of DFH, with the intention of precisely reflecting the biological alterations within the body during the early phase of DFH and providing a scientific underpinning for early prediction and intervention.

miR-519d-3p, as a novel molecular entity, has been found to have significant implications in various diseases. In the context of fracture healing, compared with previous studies that mainly focused on traditional biomarkers, our discovery of miR-519d-3p's role in early DFH

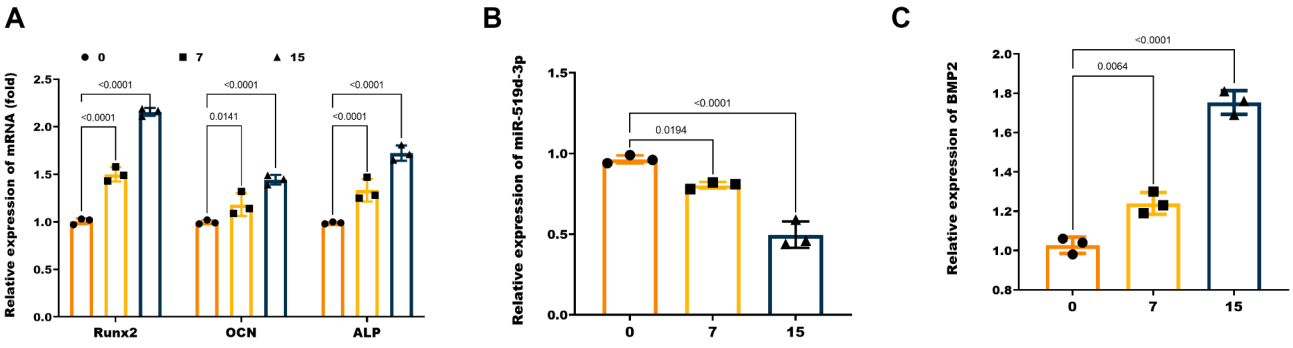

Fig. 2 Induction of Osteoblast Differentiation. Osteogenic differentiation-related mRNA expression was successfully upregulated by the induction medium (**A**). During osteoblast differentiation, the expression of miR-519d-3p was upregulated (**B**) while the expression of BMP2was downregulated (**C**)

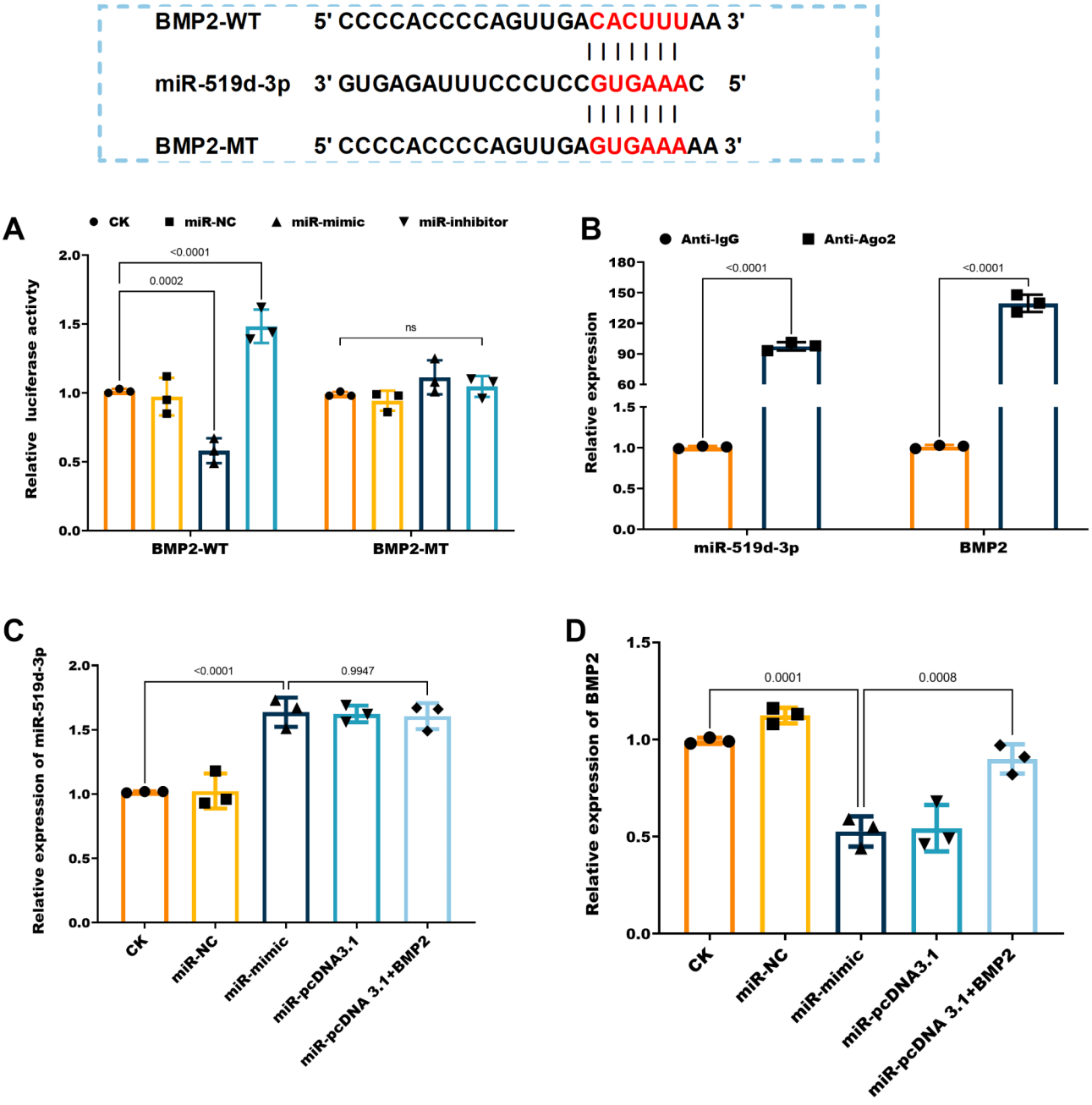

Fig. 3 Interaction between miR-519d-3p and BMP2.Dual luciferase reporter assay (**A**) and RIP experiment (**B**) indicated a binding relationship between miR-519d-3p and BMP2. Transfection with miR-mimic effectively overexpressed miR-519d-3p (**C**) and inhibited BMP2 expression (**D**)

prediction is innovative. For example,, in cervical cancer [33] and colorectal cancer [34], the expression of miR-519d-3p shows trends related to disease progression and patient prognosis. In the current research on fracture healing, we observed that the upregulated expression of miR-519d-3p four weeks after surgery in fracture patients could serve as an early-warning sign for the prediction of DFH and represents a risk factor for poor fracture healing. However, it should be noted that the specificity value of miR-519d-3p in diagnosing DFH is relatively low. This finding contrasts with its high specificity as a biomarker in bone synovitis [26], demonstrating the complexity of using miR-519d-3p alone for DFH diagnosis. To address this, we integrated BMP2. As a well-established osteogenic factor, many studies have suggested that BMP2 may play a crucial role in fracture healing [35, 36]. Our data confirm that combining miR-519d-3p and BMP2 significantly enhances prediction accuracy, offering a novel strategy for early DFH detection.

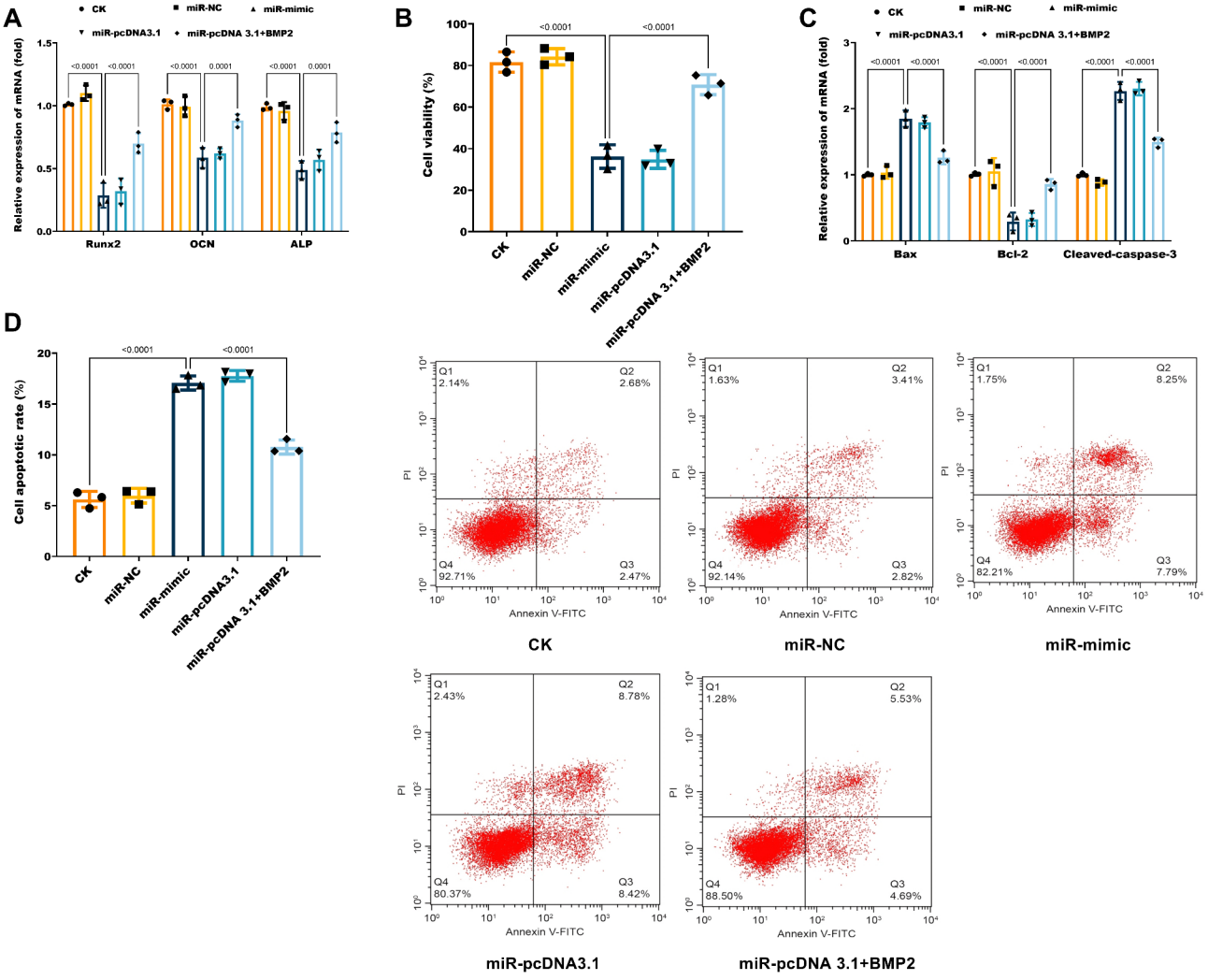

Fig. 4 Regulation of Osteoblasts by miR-519d-3p and BMP2. miR-519d-3p overexpression inhibited osteogenic differentiation markers (**A**), osteoblast viability (**B**), and upregulated apoptotic proteins and downregulated anti-apoptotic proteins (**C**), promoting apoptosis (**D**). BMP2 upregulation counteracts these effects of miR-519d-3p

Osteoblast differentiation constitutes a crucial aspect of the fracture healing process and entails a complex sequence of molecular events. Markers like RUNX2, OCN, and ALP are significant in osteoblast differentiation [37, 38]. In this research, it was observed that overexpression of miR-519d-3p notably suppressed the expression of RUNX2, OCN, and ALP, thereby impeding the osteoblast differentiation process. This finding aligns with the general understanding of factors affecting osteoblast differentiation in previous studies. However, the specific role of miR-519d-3p in this process provides new insights into the molecular regulation mechanism of fracture healing. Additionally, the growth and apoptotic conditions of cells following osteogenic differentiation are vital for fracture healing. Cell growth facilitates the synthesis and accumulation of the bone matrix, while apoptosis might disrupt the bone repair process [39]. The current study's outcomes discovered that miR-519d-3p overexpression curbed cell viability and enhanced apoptosis. This finding aligns with its reported inhibitory effects in cardiomyocytes [40]and lung cancer cells [41], but diverges from its promotional role in osteoarthritis [26], suggesting context-dependent functionality. These results not only validate its role in apoptosis regulation but also emphasize its potential to disrupt bone matrix synthesis—a critical step in fracture repair.

Numerous studies have verified that BMP2 can promote osteoblast differentiation and osteogenesis in vitro. It initiates the osteogenic response by activating downstream signaling pathways [42, 43]. Our study adds to this paradigm by revealing that miR-519d-3p directly targets BMP2, and BMP2 can reverse the inhibitory effects of miR-519d-3p. This interaction suggests a regulatory loop wherein miR-519d-3p overexpression suppresses

BMP2, leading to impaired Smad phosphorylation and osteogenesis-related gene transcription [44, 45]. Such a mechanism may explain why miR-519d-3p upregulation correlates with delayed healing, positioning it as a potential therapeutic target.

Although this study provides novel insights into miR-519d-3p and BMP2 in fracture healing, several limitations warrant attention. First, while we identified targeting relationship between miR-519d-3p and BMP2, the downstream molecular interactions—such as Smad proteins dynamics, transcription factors recruitment, and crosstalk with pathways like Wnt or Notch—remain unexplored. Future studies should employ techniques like chromatin immunoprecipitation (ChIP) or RNA sequencing to map these networks. Second, our cohort lacked stratification by fracture type (e.g., stress vs. pathological fractures) or patient demographics (e.g., age, gender), which may influence miR-519d-3p/BMP2 dynamics. For instance, aging is known to alter miRNA expression and BMP2 responsiveness [46, 47], et its impact here is unclear. Third, while we validated our findings in vitro, in vivo models incorporating longitudinal CT and the molecular analyses are needed to assess clinical translatability.

To address these gaps, we propose the following research directions: (1) Investigate miR-519d-3p/BMP2 crosstalk with Wnt and Notch pathways using co-culture systems or genetic knockout models. (2) Evaluate the long-term effects of miR-519d-3p inhibition on fracture healing in aged or comorbid animal models. (3) Develop a multi-omics approach (e.g., miRNA-mRNA-protein networks) to identify ancillary biomarkers and therapeutic targets. (4) Conduct clinical trials stratifying patients by fracture type and comorbidities to refine diagnostic thresholds for miR-519d-3p/BMP2.

Conclusions

In conclusion, this study initially discovers that the expression levels of miR-519d-3p and BMP2 in serum can serve as early prediction markers for predicting if fracture patients might have a risk of delayed healing. Precisely, miR-519d-3p suppresses the osteoblast differentiation and osteogenesis process by targeting BMP2, which could potentially slow down the fracture healing. This finding provides novel biomarker candidates for the early diagnosis of DFH and also creates new research directions for devising intervention strategies to enhance fracture healing.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13018-025-05695-2.

Supplementary Material 1

Supplementary Material 2

Acknowledgements
Not applicable

Author contributions
Jing Xiang: Formal analysis; Methodology; Software; Validation; Writing– draft & editing.Lina Huang: Methodology; Software; Supervision; Visualization; Writing– draft & editing.Chuangye Qu: Conceptualization; Data curation; Software; Writing– draft.Weibing Bao: Investigation; Resources; Validation. Wenqi Wang: Resources; Software; Supervision; Visualization.Xiaozhong Zhu: Conceptualization; Formal analysis; Methodology; Project administration; Writing– review & editing.Yong Deng: Formal analysis; Methodology; Project administration; Visualization; Writing– review & editing.

Funding
The authors report no funding.

Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
This study adheres to the principles of the Declaration of Helsinki, has been approved by the Ethics Committee of Gansu Provincial Hospital of Traditional Chinese Medicine, and informed consent has been obtained from patients and their families.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 24 December 2024 / Accepted: 7 March 2025
Published online: 21 March 2025

References

1. Migliorini F, Cocconi F, Vecchio G, Schäefer L, Koettnitz J, Maffulli N. Pharmacological agents for bone fracture healing: talking points from recent clinical trials. Expert Opin Investig Drugs. 2023;32(9):855–65.
2. Khanna A, Gougoulias N, Maffulli N. Intermittent pneumatic compression in fracture and soft-tissue injuries healing. Br Med Bull. 2008;88(1):147–56.
3. Martinez de Albornoz P, Khanna A, Longo UG, Forriol F, Maffulli N. The evidence of low-intensity pulsed ultrasound for in vitro, animal and human fracture healing. Br Med Bull. 2011;100:39–57.
4. Saul D, Khosla S. Fracture healing in the setting of endocrine diseases, aging, and cellular senescence. Endocr Rev. 2022;43(6):984–1002.
5. Cheng C, Shoback D. Mechanisms underlying normal fracture healing and risk factors for delayed healing. Curr Osteoporos Rep. 2019;17(1):36–47.
6. Fisher JS, Kazam JJ, Fufa D, Bartolotta RJ. Radiologic evaluation of fracture healing. Skeletal Radiol. 2019;48(3):349–61.
7. Schwarzenberg P, Maher MM, Harty JA, Dailey HL. Virtual structural analysis of tibial fracture healing from low-dose clinical CT scans. J Biomech. 2019;83:49–56.
8. Perlepe V, Michoux N, Heynen G, Vande Berg B. Semi-quantitative CT assessment of fracture healing: how many and which CT reformats should be analyzed? Eur J Radiol. 2019;118:181–6.
9. Yao J, Xin R, Zhao C, Yu C. MicroRNAs in osteoblast differentiation and fracture healing: from pathogenesis to therapeutic implication. Injury. 2024;55(4):111410.
10. Giordano L, Porta GD, Peretti GM, Maffulli N. Therapeutic potential of MicroRNA in tendon injuries. Br Med Bull. 2020;133(1):79–94.
11. Gargano G, Oliviero A, Oliva F, Maffulli N. Small interfering RNAs in tendon homeostasis. Br Med Bull. 2021;138(1):58–67.

12. Oliviero A, Della Porta G, Peretti GM, Maffulli N. MicroRNA in osteoarthritis: physiopathology, diagnosis and therapeutic challenge. Br Med Bull. 2019;130(1):137–47.
13. Gargano G, Oliva F, Oliviero A, Maffulli N. Small interfering RNAs in the management of human rheumatoid arthritis. Br Med Bull. 2022;142(1):34–43.
14. Gargano G, Asparago G, Spiezia F, Oliva F, Maffulli N. Small interfering RNAs in the management of human osteoporosis. Br Med Bull. 2023;148(1):58–69.
15. Groven RVM, van Koll J, Poeze M, Blokhuis TJ, van Griensven M. MiRNAs related to different processes of fracture healing: an integrative overview. Front Surg. 2021;8:786564.
16. Zhang YB, Guo XQ, Wang GG, Pu HB. MicroRNA 98-5p overexpression contributes to delayed fracture healing via targeting BMP-2. Tohoku J Exp Med. 2024;263(1):17–25.
17. Guo X, Zhang J, Han X, Wang G. LncRNA SNHG1 delayed fracture healing via modulating miR-181a-5p/PTEN Axis. J Invest Surgery: Official J Acad Surg Res. 2022;35(6):1304–12.
18. Teng JW, Ji PF, Zhao ZG. MiR-214-3p inhibits β-catenin signaling pathway leading to delayed fracture healing. Eur Rev Med Pharmacol Sci. 2018;22(1):17–24.
19. Gao M, Zhang Z, Sun J, Li B, Li Y. The roles of circRNA-miRNA-mRNA networks in the development and treatment of osteoporosis. Front Endocrinol. 2022;13:945310.
20. Doghish AS, Elballal MS, Elazazy O, Elesawy AE, Shahin RK, Midan HM, et al. MiRNAs as potential game-changers in bone diseases: future medicinal and clinical uses. Pathol Res Pract. 2023;245:154440.
21. Yun HM, Kim B, Jeong YH, Hong JT, Park KR. Suffruticosol A elevates osteoblast differentiation targeting BMP2-Smad/1/5/8-RUNX2 in pre-osteoblasts. Biofactors. 2023;49(1):127–39.
22. Yue B, Zhang W, Li M, Xu L. WTAP increases BMP2 expression to promote osteoblast differentiation and inhibit osteoblast senescence via m(6)A methylation of Sp1. Molecular genetics and genomics. MGG. 2024;299(1):109.
23. Chen J, Yang Y. LncRNA HAGLR absorbing miR-214-3p promotes BMP2 expression and improves tibial fractures. Am J Translational Res. 2021;13(10):11065–80.
24. Xiong Y, Chen L, Yan C, Endo Y, Mi B, Liu G. The LncRNA Rhno1/miR-6979-5p/BMP2 Axis modulates osteoblast differentiation. Int J Biol Sci. 2020;16(9):1604–15.
25. Yang L, Gao Q, Lv F, Guo M, Zhao D. miR–519d–3p promotes TGFβ/Smad mediated postoperative epidural Scar formation via suppression of BAMBI. Mol Med Rep. 2019;20(4):3901–9.
26. Gao J, Xia S. Reduced miR-519d-3p levels in the synovium and synovial fluid facilitate the progression of post-traumatic osteoarthritis by targeting VEGF. Experimental Therapeutic Med. 2021;22(6):1478.
27. Wang J, Zhang Z, Qiu C, Wang J. MicroRNA-519d-3p antagonizes osteosarcoma resistance against cisplatin by targeting PD-L1. Mol Carcinog. 2022;61(3):322–33.
28. Hellwinkel JE, Working ZM, Certain L, García AJ, Wenke JC, Bahney CS. The intersection of fracture healing and infection: orthopaedics research society workshop 2021. J Orthop Research: Official Publication Orthop Res Soc. 2022;40(3):541–52.
29. Lu Z, Li X, Xuan R, Song Y, Bíró I, Liang M et al. Effect of heel lift insoles on lower extremity muscle activation and joint work during barbell squats. Bioeng (Basel Switzerland). 2022;9(7).
30. Zhang W, Cui SY, Yi H, Zhu XH, Liu W, Xu YJ. MiR-708 inhibits MC3T3-E1 cells against H(2)O(2)-induced apoptosis through targeting PTEN. J Orthop Surg Res. 2020;15(1):255.
31. Liu W, Li L, Rong Y, Qian D, Chen J, Zhou Z, et al. Hypoxic mesenchymal stem cell-derived exosomes promote bone fracture healing by the transfer of miR-126. Acta Biomater. 2020;103:196–212.
32. Zhang Z, Wang L, Zhang F, Jing S, Cen M. Functional mechanism and clinical implications of mir-1271-5p in Pilon fracture healing processes. J Orthop Surg Res. 2024;19(1):782.
33. Jiang L, Shi S, Li F, Shi Q, Zhong T, Zhang H et al. miR–519d–3p/HIF–2α axis increases the chemosensitivity of human cervical cancer cells to cisplatin via inactivation of PI3K/AKT signaling. Mol Med Rep. 2021;23(5).
34. Chen R, Zhou S, Chen J, Lin S, Ye F, Jiang P. LncRNA BLACAT1/miR-519d-3p/CREB1 Axis mediates proliferation, apoptosis, migration, invasion, and Drug-Resistance in colorectal Cancer progression. Cancer Manage Res. 2020;12:13137–48.
35. Yu YH, Wilk K, Waldon PL, Intini G. In vivo identification of Bmp2-correlation networks during fracture healing by means of a limb-specific conditional inactivation of Bmp2. Bone. 2018;116:103–10.
36. Tsuji K, Bandyopadhyay A, Harfe BD, Cox K, Kakar S, Gerstenfeld L, et al. BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. Nat Genet. 2006;38(12):1424–9.
37. Wang T, Zhang X, Bikle DD. Osteogenic differentiation of periosteal cells during fracture healing. J Cell Physiol. 2017;232(5):913–21.
38. Chen S, Ma H, Li M, Jia Z, Chen X, Bu N. Long noncoding RNA NORAD promotes fracture healing through interacting with osteoblast differentiation via targeting miR-26a. Biomed Res Int. 2023;2023:9950037.
39. Zhang Y, Yuan Q, Wei Q, Li P, Zhuang Z, Li J, et al. Long noncoding RNA XIST modulates microRNA-135/CREB1 axis to influence osteogenic differentiation of osteoblast-like cells in mice with tibial fracture healing. Hum Cell. 2022;35(1):133–49.
40. Zhang D, Wang B, Ma M, Yu K, Zhang Q, Zhang X. LncRNA HOTAIR protects myocardial infarction rat by sponging miR-519d-3p. J Cardiovasc Transl Res. 2019;12(3):171–83.
41. Yu X, Jiang Y, Hu X, Ge X. LINC00839/miR-519d-3p/JMJD6 axis modulated cell viability, apoptosis, migration and invasiveness of lung cancer cells. Folia Histochem Cytobiol. 2021;59(4):271–81.
42. Zhou L, Wang J, Mu W. BMP-2 promotes fracture healing by facilitating osteoblast differentiation and bone defect osteogenesis. Am J Translational Res. 2023;15(12):6751–9.
43. Khanka S, Somani C, Sharma K, Sharma S, Kumar A, Chattopadhyay N et al. Litsea glutinosa extract promotes fracture healing and prevents bone loss via BMP2/SMAD1 signaling. J Endocrinol. 2024;261(2).
44. Yang X, Mou D, Yu Q, Zhang J, Xiong Y, Zhang Z, et al. Nerve growth factor promotes osteogenic differentiation of MC3T3-E1 cells via BMP-2/Smads pathway. Annals Anat = Anatomischer Anzeiger: Official Organ Anatomische Gesellschaft. 2022;239:151819.
45. Moon SH, Kim I, Kim SH. Mollugin enhances the osteogenic action of BMP-2 via the p38-Smad signaling pathway. Arch Pharm Res. 2017;40(11):1328–35.
46. Wang Y, Sun L, Kan T, Xue W, Wang H, Xu P et al. Hypermethylation of Bmp2 and Fgfr2 Promoter Regions in Bone Marrow Mesenchymal Stem Cells Leads to Bone Loss in Prematurely Aged Mice. Aging and disease. 2024.
47. Halloran D, Pandit V, MacMurray C, Stone V, DeGeorge K, Eskander M et al. Age-Related Low Bone Mineral Density in C57BL/6 Mice Is Reflective of Aberrant Bone Morphogenetic Protein-2 Signaling Observed in Human Patients Diagnosed with Osteoporosis. International journal of molecular sciences. 2022;23(19).

Publisher's note