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Effect of miR-654-3p targeting EMP1 on osteoblast activity and differentiation in delayed fracture healing

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Abstract

Background Delayed fracture healing (DFH) is a common postoperative complication in fracture patients, and a validated serum marker may aid in the clinical management and improve the prognosis of fracture patients. In this study, we investigated the diagnostic role and potential regulatory mechanisms of miR-654-3p in DFH.

Methods 73 patients with DFH and 75 patients with normal fracture healing (NFH) were included. Expression of miR-654-3p and EMP1 and several mRNA markers of osteogenic differentiation were evaluated by RT-qPCR. The diagnostic value of miR-654-3p and EMP1 alone and in combination was assessed using ROC curves. Cell proliferation capacity was assessed by CCK-8 and apoptosis rate by flow cytometry. DLR experiments demonstrated the targeting relationship between miR-654-3p and EMP1.

Results Levels of miR-654-3p were found to be significantly lower in DFH compared to NFH. Following cell differentiation treatment, miR-654-3p levels increased and EMP1 levels decreased. Furthermore, a negative correlation was identified between miR-654-3p and EMP1 target binding and expression levels. The combination of miR-654-3p and EMP1 holds significant diagnostic value for DFH. miR-654-3p high expression can inhibit EMP1 levels, which promotes cell proliferation, increases osteoblast activity and levels of differentiation markers, and decreases the rate of apoptosis.

Conclusion miR-654-3p and EMP1 are aberrantly expressed in DFH, and both have high diagnostic value for DFH. miR-654-3p is involved in the proliferation, differentiation, and apoptotic activities of osteoblasts by regulating the level of EMP1, thus affecting the progression of DFH.

Keywords miR-654-3p, Delayed fracture healing, EMP1

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Background

Fracture healing is a dynamic and complex disease state and the normal healing time is usually less than 4 months [1, 2]. Delayed fracture healing (DFH) is defined as a fracture that has not reached the mark of complete fracture healing within the time required for normal healing [3]. DFH is a common complication in fracture patients after surgery. It seriously affects the patient's quality of life and can even lead to disability [4]. Survey data show that delayed fracture healing occurs in approximately 5–10% of fracture patients [5]. Therefore, the search for an effective diagnostic marker that can differentiate between DFH and NFH can help in the early detection and diagnosis of DFH and the timely adjustment of clinical treatment protocols.

MicroRNA (miRNA) is a class of non-coding, single-stranded RNA molecules of approximately 22 nucleotides in length [6–9]. It has been shown to be closely associated with bone development and formation [10–13]. Furthermore, it has been demonstrated that miRNAs are capable of regulating the fracture healing process by affecting the physiological activities of osteoblasts and osteoclasts (e.g. differentiation, proliferation, etc.) through various mechanisms [14]. Xin et al. found that miR-214 can regulate fracture healing by inhibiting the SOX4 signaling pathway [15]. Mizuno et al. also identified the ability of miR-125b to regulate the differentiation functions of osteoblasts through downstream target genes. Therefore, it can be concluded that miRNAs have the potential to serve as serum markers for the prediction of DFH.

Table 1 Clinical data of the study subjects

Indicators	NFH	DFH	*P* value
	(*n* = 75)	(*n* = 73)	
Age (year)	41.64 ± 12.22	43.90 ± 10.74	0.234
BMI (kg/m^2)	23.99 ± 1.88	24.37 ± 2.10	0.244
Gender			0.615
Female	38	40	
Male	37	33	
Smoking			0.63
No	33	35	
Yes	42	38	
Alcohol intake			0.636
No	30	32	
Yes	45	41	
Fracture Side			0.622
Left	39	35	
Right	36	38	
Osteosynthesis method			0.622
Open reduction	36	38	
Losed reduction	39	35	
Severity of fracture			0.706
Complete fracture	30	27	
Incomplete fracture	45	46	

miR-654-3p plays a regulatory role in a variety of diseases [16]. Existing studies have demonstrated that miR-654-3p is aberrantly expressed in bone nonunion and osteogenesis imperfecta and plays an important role in the regulation of these two diseases [17]. However, the expression and regulatory mechanisms of miR-654-3p in delayed fracture healing are currently unclear. In this study, We examined miR-654-3p levels in patients with DFH and used bioinformatics to search for downstream target genes of miR-654-3p, in order to explore the potential regulatory mechanism of the miR-654-3p target gene signaling pathway on delayed fracture healing.

Materials and methods

Participants

The present study encompasses a cohort of 73 patients diagnosed with DFH and 75 patients with normal fracture healing (NFH) from January 2022 to November 2023 in Yidu Central Hospital of Weifang were included. The inclusion criteria were as follows: (a) fulfilment of the diagnostic criteria for fracture; (b) fulfilment of the criteria for delayed fracture healing: no obvious signs of healing for 4 consecutive months; (c) aged ≥ 18 years; (d) treated with internal fixation. Exclusion criteria: (a) accompanied by other parts of the trauma; (b) accompanied by metabolic bone disease, osteoarthritis and other bone disease patients. The clinical data of the two groups of patients were collected and the specific information is shown in Table 1.

The study was approved by the Ethics Committee of Yidu Central Hospital of Weifang and adhered to the Declaration of Helsinki. Furthermore, all patients and their families signed an informed consent form.

RT-qPCR

At the 4-week postoperative follow-up, 5 mL of fasting peripheral venous blood was collected from the patients. The Trizol reagent (Thermo Fisher, USA) was then utilised for the extraction of total RNA from serum. Subsequently, cDNA was extracted using a reverse transcription kit (Takara, Dalian, China) was utilised to extract cDNA from the serum. The cDNA was then subjected to PCR, with U6 serving as an endogenous reference for miR-654-3p, and ALP, OCN, Runx2 mRNA according to the instructions of the KAPA SYBR Rapid One-Step Kit (Roche, Switzerland). Results were normalized using the $2^{-\Delta\Delta CT}$ method.

Cell culture and transfected

The selection of MC3T3-E1 cells were selected for culture and osteogenic differentiation induction (OI) is a pivotal step in this study. The cells were cultivated in MEM-α medium containing nucleosides and GlutaMAX additive. The medium contained 10% FBS (Gibco, USA)

and was incubated at 37 ℃ with a CO_2 concentration of 5%.

The miR-654-3p inhibitor and empty vector oligonucleotides (miR-NC), as well as blank small interfering RNA (EMP1-NC) and si-EMPI were purchased from RiboBio (Guangzhou, China). The transfection of cells into differentiated cells was conducted in strict accordance with the stipulated protocol of the Lipofectamine 3000 kit (Invitrogen, USA).

Cell viability assay

A volume of 200 μL of cell suspension was inoculated into 96-well plates with three composite wells per group. The cells were then subjected to a culture period of 24 h, 48 h, and 72 h, respectively. Then, cells were incubated for 1 h with 10 μL of CCK-8 solution. The cells were removed and shaken for 10 s on an enzyme marker in order to measure OD 450 nm.

Cell apoptosis assay

Subsequent to the differentiation and transfection of MC3T3-E1 cells, the cells were harvested and washed thrice with Phosphate Buffered Saline (PBS). Subsequently, 100 μL of binding buffer was then added. Annexin V-fuorescein isothiocyanate and 5 μL PI were added to the cells and incubated for 15 min in the dark. The suspension was transferred to a flow-through tube under conditions that avoided light, and the apoptosis rate was measured by flow cytometry. The mRNA expression of Bax, Bcl-2, and Caspase-3 was detected by RT-qPCR.

Western blotting assay

The treated cells were incubated in lysis buffer and centrifuged (12,000 rpm) after 5 min to collect the lysis buffer. Protein content was assessed using a bicinchoninic acid (BCA) kit (Beyotime, P0010). Thirty μg of protein was up-sampled per lane and the protein was subsequently separated by 10% SDS-PAGE. It was then electrotransferred onto a PVDF membrane (Millipore). After blocking with 5% skimmed milk for 2 h, the membrane was incubated overnight. The EMP1 antibody was diluted with the blocking solution. The PVDF membrane was immersed in the anti-incubation solution and incubated at 4 ℃ overnight. Treat PVDF membrane with HRP-conjugated secondary antibody for 2 h at room temperature. Membranes were detected using the BeyoECL Plus Kit (Beyotime, P0018).

Bioinformatics analysis

miRDB, Targetscan and miRWalk databases were used to predict miR-654-3p downstream target genes (screening condition: absolute value of Total context + + score ≥ 0.4).

Dual-luciferase reporter assay (DLR) assay

Gene plasmids were designed on the basis of bioinformatic analysis of the two binding sites. Wild-type recombinant EMP1 (WT-EMP1) and mutant recombinant EMP1 (MT-EMP1) plasmids were constructed. Subsequently, miR-NC, miR-mimic and miR-inhibitor were transfected using Lipofectamine 3000 kit (Invitrogen, USA) and detected using DLR kit (Beyotime, Shanghai).

RIP assay

Cells were resuspended with RIP lysate equal to the volume of cells and incubated with antibody-coupled magnetic beads at 4 °C overnight. After washing, RNA was purified and reverse-transcribed into cDNA following the RT-qPCR assay procedure to detect target levels.

Statistical analysis

The results obtained are expressed as the mean ± standard deviation. All experiments were repeated at least three times with parallel testing. The patients' clinical physiological data were analyzed and tabulated using SPSS, and the rest of the data were analyzed and plotted using GraphPad. ROC survival assessed was used to determine the diagnostic role of miRNAs and mRNAs. A p-value of below 0.05 indicates that the difference between the two groups is statistically significant.

Results

Comparison of patients' clinical data

The study found no statistically significant differences between the two groups in terms of age, BMI, sex, smoking status, alcohol consumption, side of fracture, osteosynthesis method, and fracture severity ($P > 0.05$, Table 1).

Expression and diagnostic role of miR-654-3p

Levels of miR-654-3p were found to be markedly lower in the DFH group than in the NFH group ($P < 0.001$, Fig. 1A). The diagnostic significance of miR-654-3p for DFH was predicted by ROC curve. The AUC of the ROC curve was 0.858 (95% CI 0.797–0.918), which was found to be capable of distinguish the DFH patients from the NFH group (Fig. 1B).

Effects of osteogenic differentiation induction on miR-654-3p levels and cell function

Levels of MiR-654-3p in differentiated cells were significantly increased ($P < 0.001$, Fig. 2A). Furthermore, levels of osteoblast activity and differentiation markers were increased ($P < 0.001$, Fig. 2B), the proliferative capacity of cells was decreased, the rate of apoptosis was increased, the levels of pro-apoptotic factors were increased, and inhibitory apoptotic factors levels were decreased ($P < 0.001$, Fig. 2C and E).

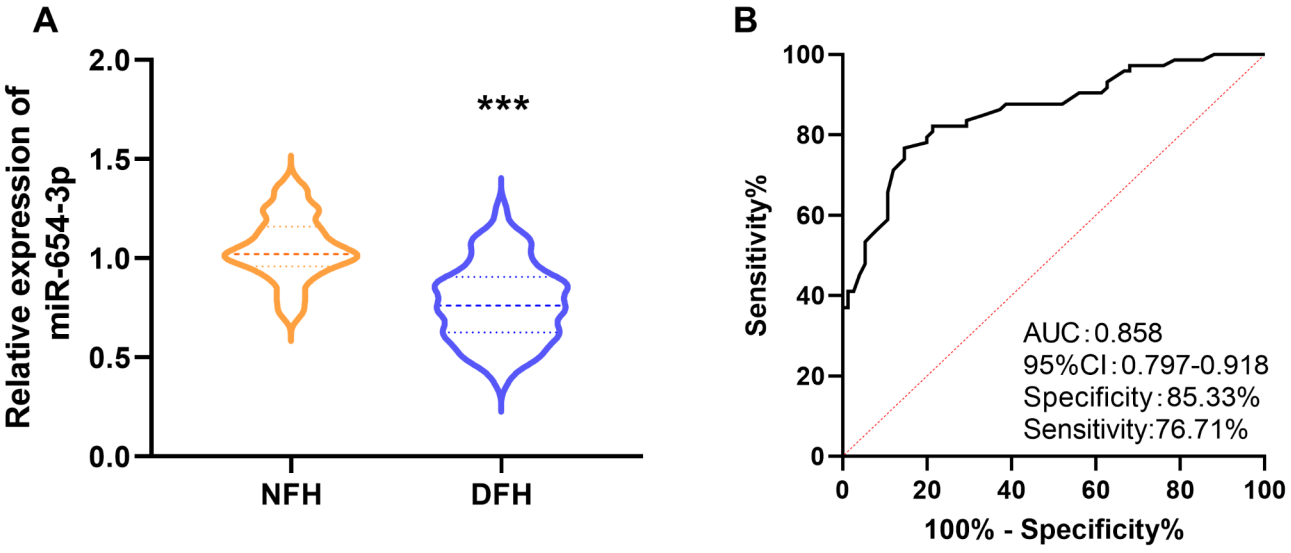


Fig. 1 Expression and diagnostic value of miR-654-3p. (**A**) Comparison of miR-654-3p in DFH and NFH patients (*** $P < 0.001$ vs. NFH); (**B**) Diagnostic value of miR-654-3p in patients with DFH (AUC = 0.858,95% CI: 0.797–0.918)

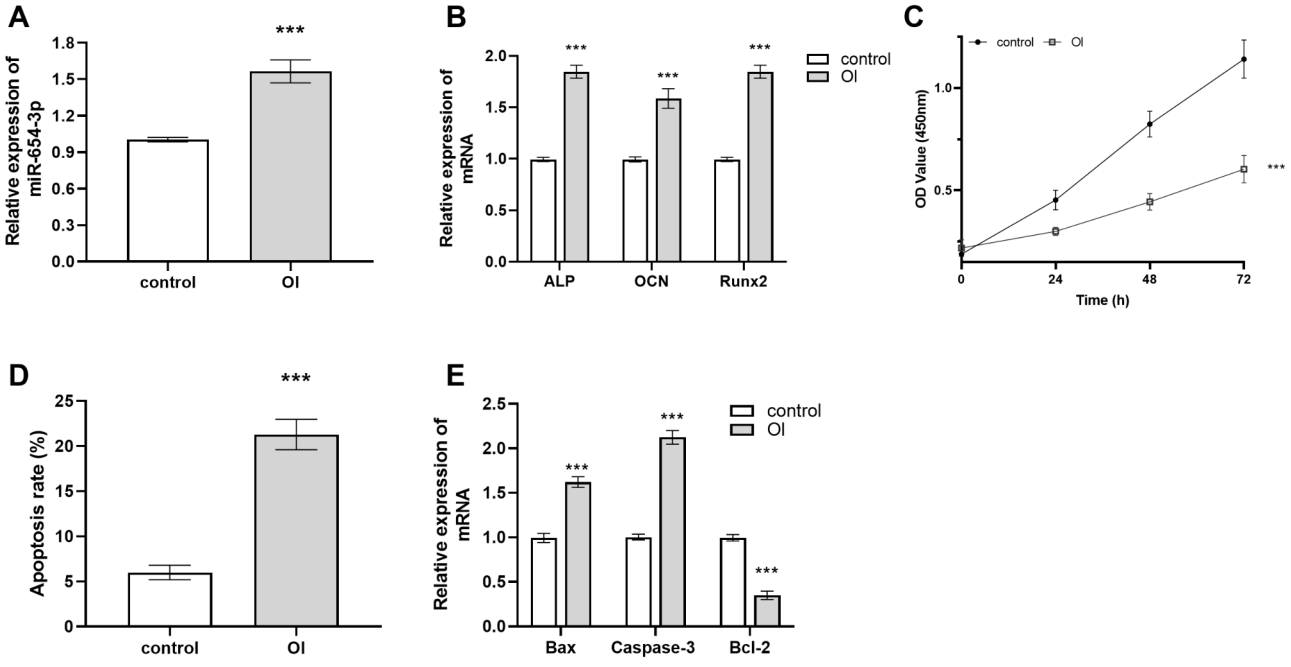


Fig. 2 Effects of differentiation on gene expression and cells. (**A**) Differentiation treatment increased miR-654-3p levels in cells; (**B**) Differentiation treatment increased ALP, OCN, and Runx2 levels in cells; (**C**) Decreased cell proliferation capacity due to differentiation treatment; (**D**) Elevated apoptosis due to differentiation treatment; (**E**) Differentiation treatments resulted in elevated pro-apoptotic factors and decreased inhibitory apoptotic factors (*** $P < 0.001$ vs. control)

Effect of miR-654-3p levels on osteoblast differentiation and apoptosis

The transfection of inhibitors into differentiated cells resulted in a decrease in miR-654-3p levels, and mimics increased miR-654-3p levels ($P < 0.001$, Fig. 3A). miR-654-3p downregulation decreased the levels of markers such as ALP, decreased cell proliferation, and increased apoptosis, with increased pro-apoptotic factors and decreased apoptosis suppressor factors. Conversely, an increase in miR-654-3p levels led to a substantial enhancement in osteoblast proliferation and activity and decreased apoptosis ($P < 0.001$, Fig. 3B-E).

MiR-654-3p target genes and its diagnostic value

As illustrated in Fig. 4A, the seven target genes of miR-654-3p overlapping in the three databases. Of these,

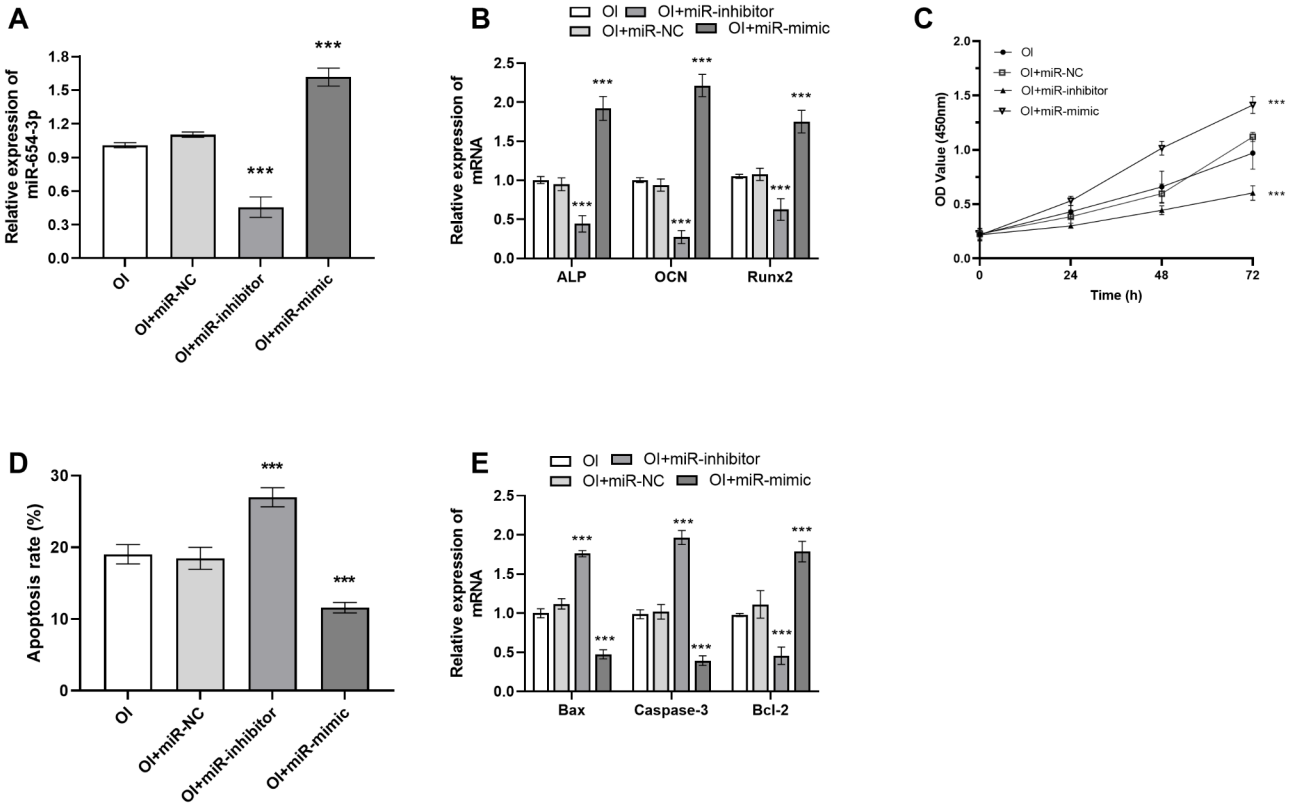


Fig. 3 Effect of miR-654-3p levels on fracture healing. (**A**) Effect of different transfection on miR-654-3p levels; (**B**) Inhibition of miR-654-3p levels decreases the levels of markers of osteoblast activity and differentiation, and increasing miR-654-3p expression increases these markers levels; (**C**) High expression of miR-654-3p enhances cell proliferation; **D and E.** Low expression of miR-654-3p increases apoptosis, promotes levels of pro-apoptotic factors and decreases expression of anti-apoptotic factors (*** $P < 0.001$ vs. OI)

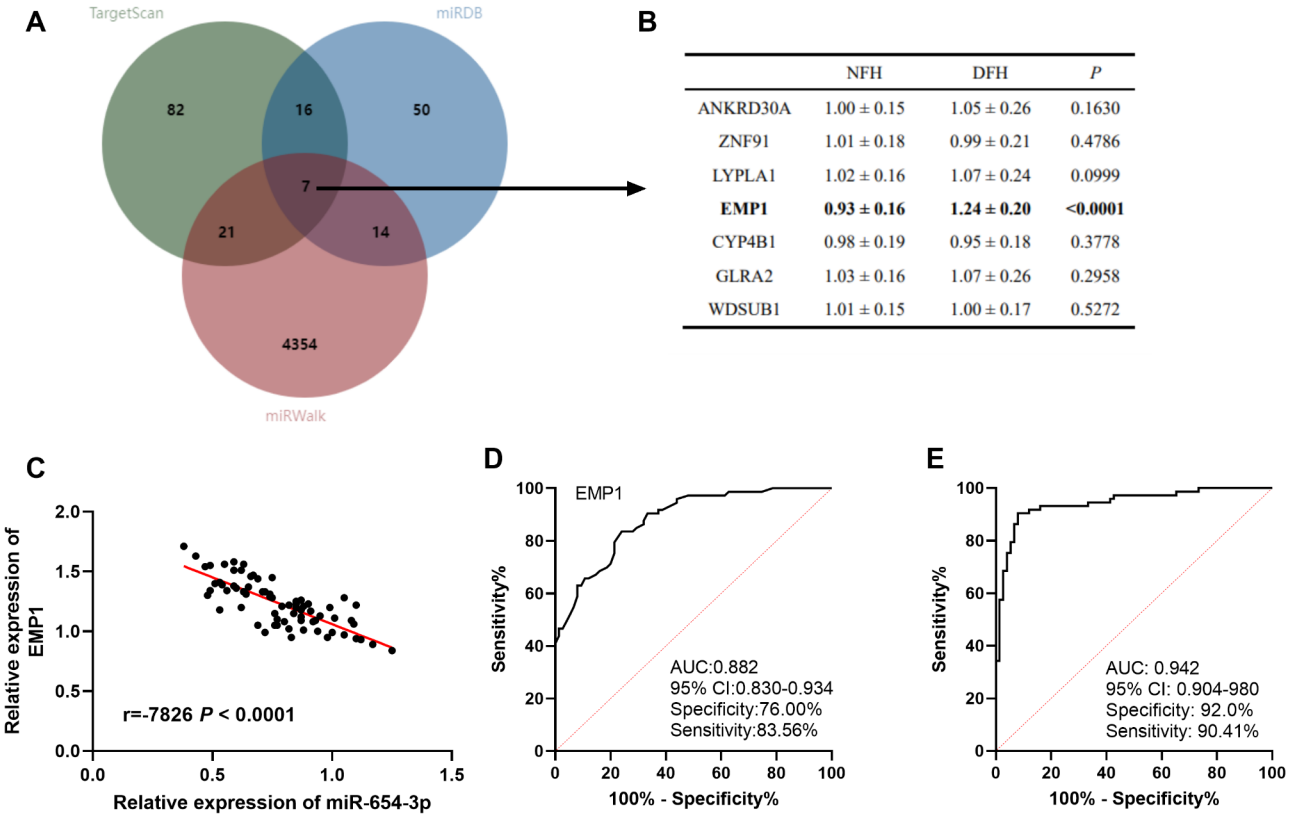
only EMP1 had significantly different expression levels in DFH and NFH ($P < 0.001$, Fig. 4B). Furthermore, a negative correlation was observed between the level of miR-654-3p and EMP1 expression (Fig. 4C). the AUC of the ROC curve was 0.882 (95% CI 0.830–0.934), which could differentiate the DFH patients from the NFH group (Fig. 4D). Figure 4E shows that the AUC of miR-654-3p combined with EMP1 for the diagnosis of DFH was 0.942, with a specificity of 92.0% and a sensitivity of 90.41%, which were higher than those of miR-654-3p and EMP1 alone. Therefore, the combined diagnosis of the two is the most effective.

Regulatory role of miR-654-3p and EMP1

Figure 5A depicts the putative target base between EMP1 and miR-654-3p. The expression of EMP1 in cells decreased after differentiation treatment (Fig. 5B). The data in Fig. 5C confirm the specific binding of miR-654-3p to EMP1. In WT-EMP1, increased levels of miR-654-3p were observed to decrease luciferase activity, whereas inhibition of miR-654-3p expression significantly increased activity ($P < 0.001$). Adjusting EMP1 level did not affect miR-654-3p expression, whereas inhibiting the level of miR-654-3p resulted in an increase in the level of EMP1, suggesting that miR-654-3p can regulate EMP1 expression, but EMP1 cannot regulate the expression of miR-654-3p (Fig. 5D and E). The protein expression of EMP1 showed the same trend; inhibiting the level of miR-654-3p increased the content of EMP1 protein, while knocking down EMP1 decreased its protein expression content (Fig. 5F). In addition, the results of RIP experiments also confirmed the binding relationship between miR-654-3p and EMP1 (Fig. 5G).

Effects of miR-654-3p and EMP1 on cellular

As illustrated in Fig. 6A, the inhibition of miR-654-3p levels resulted in a decrease in ALP, OCN, and Runx2 mRNA levels, and knockdown of EMP1 reversed this effect ($P < 0.001$). The reduced expression of miR-654-3p was found to result in reduced cell proliferation. In contrast, silencing of EMP1 increased cell proliferation ($P < 0.001$, Fig. 6B). Inhibition the expression of miR-654-3p has been demonstrated to increase apoptosis, while knockdown of EMP1 reversed this effect ($P < 0.001$, Fig. 6C). Inhibition of miR-654-3p expression led to an increase in pro-apoptotic factors levels (Bax and Caspase-3 mRNA) but decreased Bcl-2 expression, while silencing of EMP1 reversed this effect ($P < 0.001$, Fig. 6D).



	NFH	DFH	*P*
ANKRD30A	1.00 ± 0.15	1.05 ± 0.26	0.1630
ZNF91	1.01 ± 0.18	0.99 ± 0.21	0.4786
LYPLA1	1.02 ± 0.16	1.07 ± 0.24	0.0999
EMP1	**0.93 ± 0.16**	**1.24 ± 0.20**	**<0.0001**
CYP4B1	0.98 ± 0.19	0.95 ± 0.18	0.3778
GLRA2	1.03 ± 0.16	1.07 ± 0.26	0.2958
WDSUB1	1.01 ± 0.15	1.00 ± 0.17	0.5272

Fig. 4 Binding and regulation of miR-654-3p with EMP1. (**A**) Seven target genes of miR-654-3p overlapping in three databases; (**B**) Expression of seven downstream target genes in DFH and NFH; (**C**) miR-654-3p showed negative correlation with EMP1 expression level; (**D**) Diagnostic value of EMP1 in patients with DFH; (**E**) Combined diagnostic of EMP1 and miR-654-3p in patients with DFH

Discussion

DFH is a complication that can arise following fracture surgery that reduces the quality of life of patients and, in severe cases, can lead to disability [18]. Therefore, the prediction of delayed healing in fracture treatment can guide the clinic in implementing effective interventions to prevent the occurrence of DFH [19]. MiRNAs are a class of endogenous non-coding RNAs that have the capacity to regulate gene expression in a post-transcriptional manner. They play a crucial role in a wide range of physiological processes, and are potential predictive or diagnostic biomarkers for many diseases [20]. In recent years, miRNAs have been found to be key regulators in the complex process of fracture healing [21], and have been reported as miR-214 [22], miR-7212-5p [23], and so on.

In our study, miR-654-3p expression was reduced in DFH patients. This showed an opposite trend to other miRNAs associated with DFH, such as elevated expression of miR-545-3p in DFH as reported by Kang et al. [24]. And The results of our ROC suggest that miR-654-3p has a better diagnostic role. miRNA usually act to fulfill their potential roles with mRNAs. We predicted the potential target genes of miR-654-3p through several databases and identified seven potential target genes. Further analysis revealed a significant difference in the expression of EMP1 in the two groups of patients, so we focused on EMP1.

The downstream target gene of miR-654-3p, EMP1, is a member of the EMP family, which is a hotspot of researchers' attention because it is mainly involved in physiological activities. For example, EMP1 has been demonstrated to promote the proliferation and invasion of ovarian cancer cells as reported by Liu et al. [25]. Our results showed that EMP1 was highly expressed in the DFH group and was of good value for the diagnosis of DFH. The combination of miR-654-3p and EMP1 led to the diagnosis of DFH with an AUC of 0.942, which was of high diagnostic and predictive significance, suggesting that it may be involved in the pathological process of FH.

The FH process comprises three main events: inflammatory, repair and remodeling [26]. The differentiation and activity of osteoblasts play an important role in FH [27]. miRNAs are able to regulate many physiological activities of osteoblasts, including cell migration, differentiation, proliferation and apoptosis [28]. microRNAs have been demonstrated to regulate gene expression at the post-transcriptional level and regulate

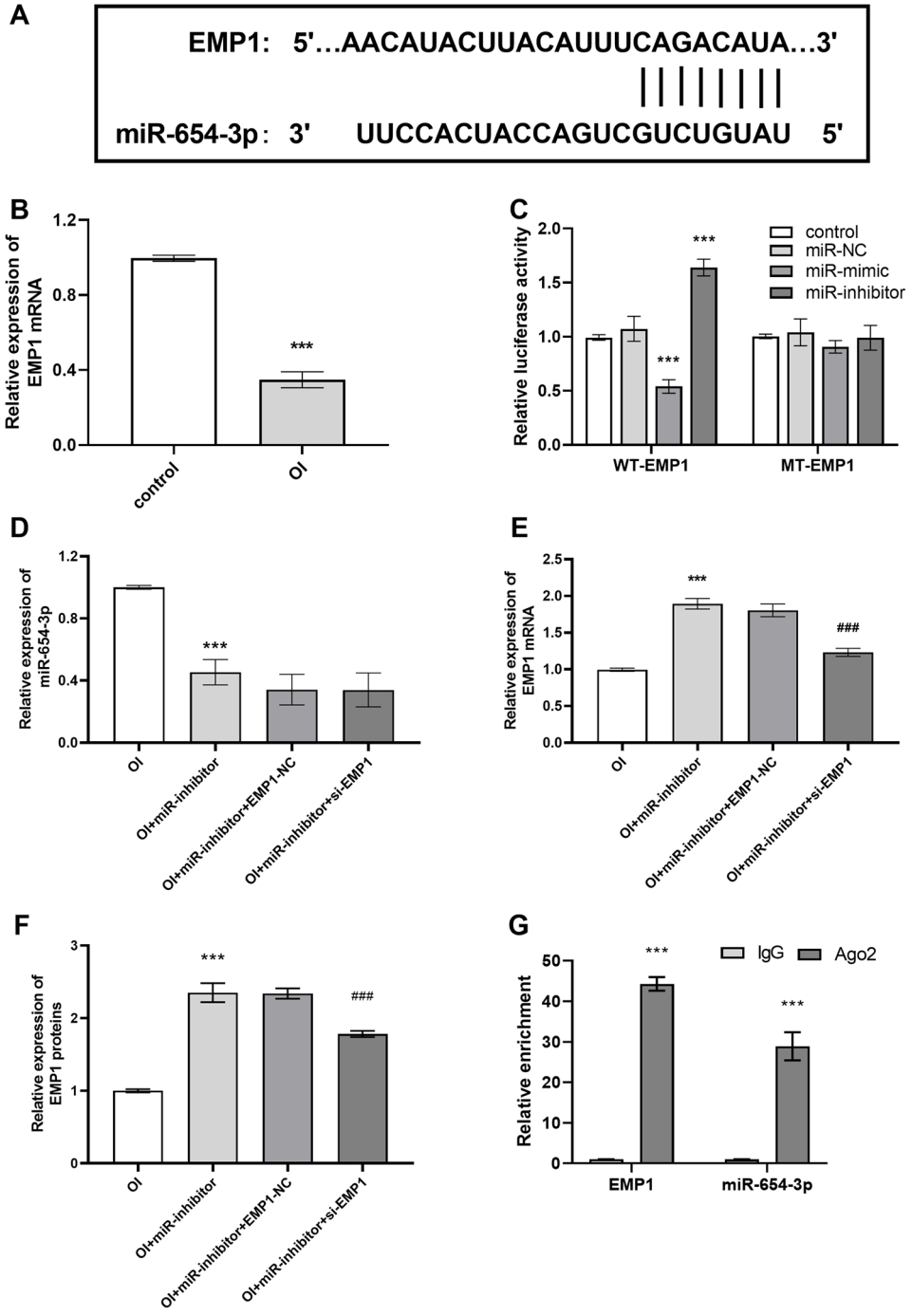


Fig. 5 Regulatory role of miR-654-3p and EMP1. (**A**) Predicted binding sites of miR-654-3p and EMP1; (**B**) Differentiation treatment reduced EMP1 levels in cells; (**C**) DLR tests confirm target recognition relationship of miR-654-3p with EMP1; (**D**) Effect of transfection on miR-654-3p levels; (**E**) Effect of transfection on EMP1 levels; (**F**) Effect of transfection on EMP1 protein content; (**G**) Both miR-654-3p and EMP1 were predominantly enriched in anti-Ago-2 (*** $P < 0.001$ vs. control or OI, ### $P < 0.001$ vs. OI + miR-inhibitor + EMP1-NC)

bone formation and remodeling [29]. Cell differentiation and activity help to accelerate new bone formation. Biomarkers of osteoblast activity and function include alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx2) and osteocalcin (OCN) are biomarkers of osteoblast activity and function [30]. The primary function of ALP is to catalyse the hydrolysis of phosphate esters during the process of osteogenesis, thereby releasing the inhibition of bone salt formation by phosphate and to promote osteoblast proliferation and differentiation, and it is an indicator of the response to osteogenic activation [31, 32]. Runx2 is implicated in the process of

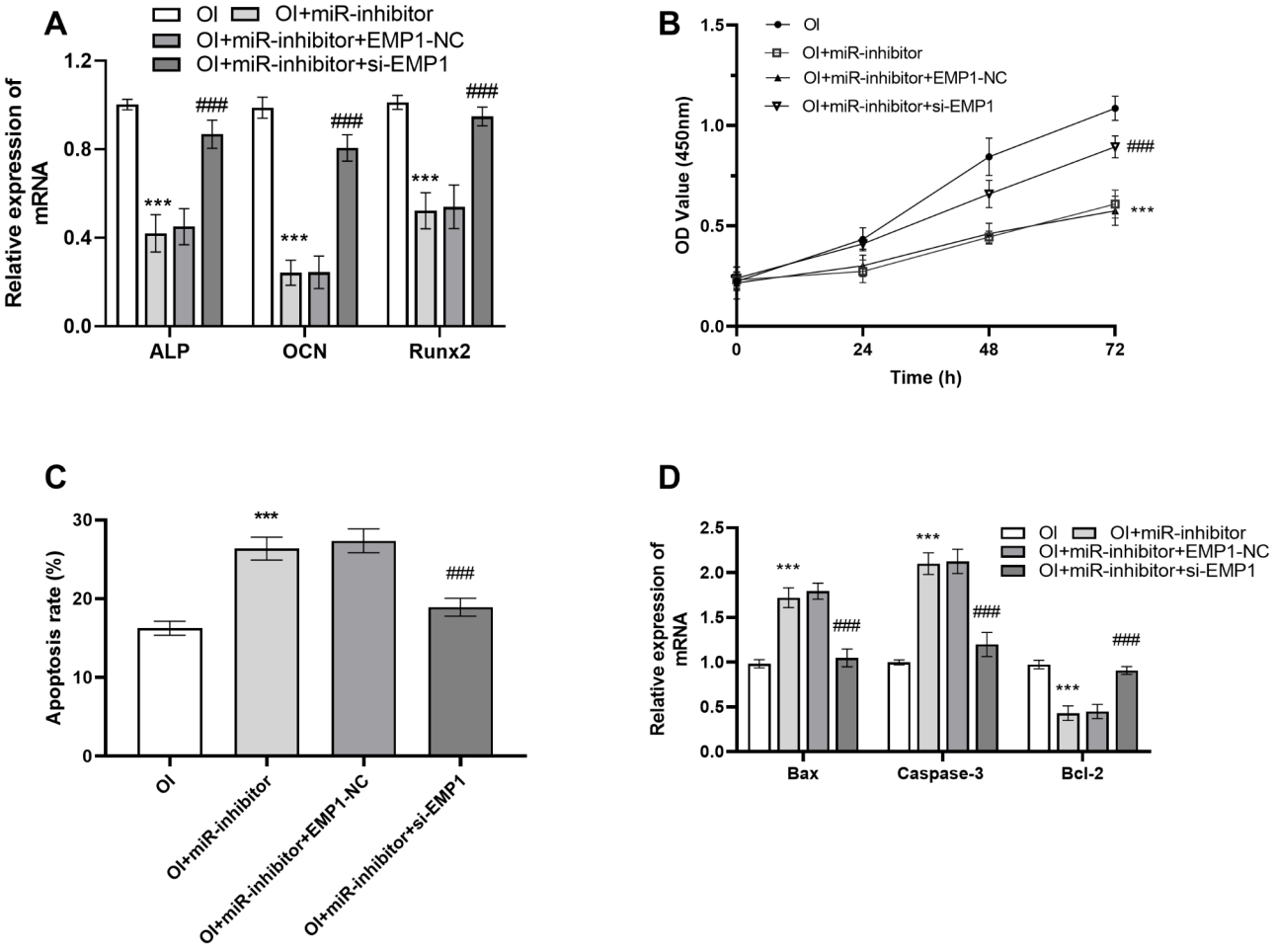


Fig. 6 Effects of miR-654-3p and EMP1 on cellular. (**A**) Effect of aberrant expression of EMP1 and miR-654-3p on ALP, OCN, and Runx2 levels; (**B**) Effect of aberrant expression of EMP1 and miR-654-3p on cell proliferation; (**C**) Regulation of apoptosis rate by miR-654-3p and EMP1; (**D**) Regulation of Bax, Caspase-3, and Bcl-2 mRNA levels by miR-654-3p and EMP1; (*** $P < 0.001$ vs. OI, ### $P < 0.001$ vs. OI + miR-inhibitor + EMP1-NC)

osteoblast differentiation and is a key regulator of osteoblast differentiation in MSCs [33]. OCN is frequently utilised as a serum marker of bone formation in osteoblasts [34]. Like miR-190a-5p reported by Lee et al. [35], our study has the same finding: aberrant levels of miRNAs and mRNAs modulates the levels of these markers and the physiological activity of osteoblasts.

In the present study, it was observed that inhibition of miR-654-3p levels resulted in elevated EMP1 expression levels. This, in turn, led to a decline in osteoblast proliferation capacity and a subsequent decrease in ALP, OCN and Runx2 levels. Elevated levels of EMP1 have been demonstrated to impede osteoblast proliferation and decrease osteoblast activity and differentiation capacity. This suggests that EMP1 is a factor that inhibits fracture healing. In addition, elevated levels of EMP1 lead to an increase in the rate of apoptosis. Studies of its apoptosis-promoting mechanism have shown that EMP1 promotes apoptosis by increasing the expression of pro-apoptotic factors (including Bax and caspase-3) and decreasing the levels of anti-apoptotic factor (Bcl-2). The knockdown of EMP1 has been demonstrated to reverse these deleterious effects. In contrast, miR-654-3p could promote fracture healing by decreasing the expression level of EMP1, promoting osteoblast proliferation and differentiation, and decreasing the rate of osteoblast apoptosis.

However, there are some potential limitations of this study: firstly, existing studies suggest that BMP2 or FGFR2 are signalling pathways involved in DFH. miRNAs are often involved in regulating the process of DFH through these signalling pathways. For example, MiR-223-3p directly targets FGFR2 in FH [36], and MicroRNA 98-5p overexpression promotes delayed FH by targeting BMP-2 [37]. Therefore, we speculate that miR-654-3p and EMP1 may also regulate DFH by participating in DFH-related signalling pathways; however, the specific signalling pathways involved in DFH by miR-654-3p and EMP1 need to be thoroughly investigated; In addition, the isoforms of EMP1 are also important for its involvement in DFH and need to be explored in subsequent

experiments. Secondly, more abundant experimental models (e.g., in vivo models) are needed to further confirm our findings from various aspects; and lastly, the current study available results and data need to be validated in a larger cohort.

Conclusion

In conclusion, miR-654-3p and EMP1 have high diagnostic value for DFH. Silent EMP1 expression may contribute to fracture healing. Increasing miR-654-3p levels resulted in a decrease in EMP1 levels, which improved cell proliferation and promoted osteogenic differentiation, decreased apoptosis and facilitated fracture healing.

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Author contributions
S.T. W and M.W. W designed the research study. S.L. S, X.S L and D.Z. L performed the research., S.T. W, M.W. W, S.L. S, X.S L and D.Z. L analyzed the data. S.T. W and M.W. W wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

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

Declarations

Ethics approval and consent to participate
The experimental protocol was reviewed and approved by the Ethics Committee of Yidu Central Hospital of Weifang, and all the participating patients signed informed consent.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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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. 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.
3. Benshabat D, Factor S, Maman E, Khoury A, Krespi R, Ashkenazi I et al. Addition of bone marrow aspirate concentrate resulted in high rate of healing and good functional outcomes in the treatment of clavicle fracture nonunion: a retrospective case series. J Clin Med. 2021;10(20).
4. Qin Q, Gomez-Salazar M, Cherief M, Pagani CA, Lee S, Hwang C, et al. Neuron-to-vessel signaling is a required feature of aberrant stem cell commitment after soft tissue trauma. Bone Res. 2022;10(1):43.
5. Ye Z, Liu Y, Song J, Gao Y, Fang H, Hu Z, et al. Expanding the therapeutic potential of salvia miltiorrhiza: a review of its Pharmacological applications in musculoskeletal diseases. Front Pharmacol. 2023;14:1276038.
6. Yu DL, Yu ZG, Han GS, Li J, Anh V. Heterogeneous types of miRNA-disease associations stratified by Multi-Layer network embedding and prediction. Biomedicines. 2021;9(9).
7. 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.
8. 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.
9. Selvakumar SC, Preethi KA, Sekar D. MicroRNA-510-3p regulated vascular dysfunction in preeclampsia by targeting vascular endothelial growth factor A (VEGFA) and its signaling axis. Placenta. 2024;153:31–52.
10. Mohanakrishnan V, Balasubramanian A, Mahalingam G, Partridge NC, Ramachandran I, Selvamurugan N. Parathyroid hormone-induced down-regulation of miR-532-5p for matrix metalloproteinase-13 expression in rat osteoblasts. J Cell Biochem. 2018;119(7):6181–93.
11. 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.
12. Selvakumar SC, Preethi KA, Sekar D. MicroRNAs as important players in regulating cancer through PTEN/PI3K/AKT signalling pathways. Biochim Et Biophys Acta Reviews cancer. 2023;1878(3):188904.
13. Levine B, Kroemer G. Biological functions of autophagy genes: A disease perspective. Cell. 2019;176(1–2):11–42.
14. Komatsu DE, Duque E, Hadjiargyrou M. MicroRNAs and fracture healing: Preclinical studies. Bone. 2021;143:115758.
15. Xin Z, Cai D, Wang J, Ma L, Shen F, Tang C, et al. MiR-214 regulates fracture healing through inhibiting Sox4 and its mechanism. J Musculoskel Neuronal Interact. 2020;20(3):429–36.
16. Wu C, Zhang XC, Chen LR, Huang HZ, Wu WY, Wang Y, et al. Pyroptosis and mitochondrial function participated in miR-654-3p-protected against myocardial infarction. Cell Death Dis. 2024;15(6):393.
17. Wei J, Chen H, Fu Y, Zhang B, Zhang L, Tao S, et al. Experimental study of expression profile and specific role of human MicroRNAs in regulating atrophic bone nonunion. Medicine. 2020;99(36):e21653.
18. Wallimann A, Magrath W, Thompson K, Moriarty T, Richards RG, Akdis CA, et al. Gut microbial-derived short-chain fatty acids and bone: a potential role in fracture healing. Eur Cells Mater. 2021;41:454–70.
19. Wu Y, Chao J, Bao M, Zhang N. Predictive value of machine learning on fracture risk in osteoporosis: a systematic review and meta-analysis. BMJ Open. 2023;13(12):e071430.
20. Han H, Wang X, Li W, Liu J, Fan Y, Zhang H, et al. Identification and characterization of LncRNAs expression profile related to goat skeletal muscle at different development stages. Animals: Open Access J MDPI. 2022;12:19.
21. Bourgery M, Ekholm E, Fagerlund K, Hiltunen A, Puolakkainen T, Pursiheimo JP, et al. Multiple targets identified with genome wide profiling of small RNA and mRNA expression are linked to fracture healing in mice. Bone Rep. 2021;15:101115.
22. Breulmann FL, Hatt LP, Schmitz B, Wehrle E, Richards RG, Della Bella E, et al. Prognostic and therapeutic potential of MicroRNAs for fracture healing processes and non-union fractures: A systematic review. Clin Translational Med. 2023;13(1):e1161.
23. Mi B, Xiong Y, Yan C, Chen L, Xue H, Panayi AC, et al. Methyltransferase-like 3-mediated N6-methyladenosine modification of miR-7212-5p drives osteoblast differentiation and fracture healing. J Cell Mol Med. 2020;24(11):6385–96.
24. Kang R, Huang L, Zeng T, Ma J, Jin D. Long non-coding TRPM2-AS regulates fracture healing by targeting miR-545-3p/Bmp2. J Orthop Surg Res. 2024;19(1):466.
25. Liu Y, Ding Y, Nie Y, Yang M. EMP1 promotes the proliferation and invasion of ovarian cancer cells through activating the MAPK pathway. OncoTargets Therapy. 2020;13:2047–55.
26. Manso G, Elias-Oliveira J, Guimarães JB, Pereira ÍS, Rodrigues VF, Burger B, et al. Xenogeneic mesenchymal stem cell biocurative improves skin wounds healing in diabetic mice by increasing mast cells and the regenerative profile. Regenerative Therapy. 2023;22:79–89.
27. Yao Z, An W, Moming A, Tuerdi M. Long non-coding RNA TUG1 knockdown repressed the viability, migration and differentiation of osteoblasts by sponging miR-214. Experimental Therapeutic Med. 2022;23(3):203.

28. Wu L, Fu W, Cao Y, Zhao S, Zhang Y, Li X et al. Inhibiting miR-618 promotes keratinocytes proliferation and migration to enhance wound healing in mice. Int J Mol Sci. 2024;25(14).
29. Xie H, Liu M, Jin Y, Lin H, Zhang Y, Zheng S. miR-1323 suppresses bone mesenchymal stromal cell osteogenesis and fracture healing via inhibiting BMP4/SMAD4 signaling. J Orthop Surg Res. 2020;15(1):237.
30. Ma ZP, Zhang ZF, Yang YF, Yang Y. Sesamin promotes osteoblastic differentiation and protects rats from osteoporosis. Med Sci Monitor: Int Med J Experimental Clin Res. 2019;25:5312–20.
31. Cheng X, Zhao C. The correlation between serum levels of alkaline phosphatase and bone mineral density in adults aged 20 to 59 years. Medicine. 2023;102(32):e34755.
32. Bin-Bin Z, Da-Wa ZX, Chao L, Lan-Tao Z, Tao W, Chuan L, et al. M2 macrophagy-derived Exosomal miRNA-26a-5p induces osteogenic differentiation of bone mesenchymal stem cells. J Orthop Surg Res. 2022;17(1):137.
33. Zhang Q, Wen H, Liao G, Cai X. Tendon stem cells seeded on dynamic chondroitin sulfate and Chitosan hydrogel scaffold with BMP2 enhance tendon-to-bone healing. Heliyon. 2024;10(4):e25206.
34. Teng C, Tong Z, He Q, Zhu H, Wang L, Zhang X et al. Mesenchymal stem Cells-Hydrogel microspheres system for bone regeneration in calvarial defects. Gels (Basel Switzerland). 2022;8(5).
35. Lee SK, Jung SH, Song SJ, Lee IG, Choi JY, Zadeh H, et al. miRNA-Based early healing mechanism of extraction sockets: miR-190a-5p, a potential enhancer of bone healing. Biomed Res Int. 2022;2022:7194640.
36. Wang B, Wu W, Xu K, Wu H. MicroRNA-223-3p is involved in fracture healing by regulating fibroblast growth factor receptor 2. Bioengineered. 2021;12(2):12040–8.
37. 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.

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