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miR-20b-3p regulates bone healing via GFI1

FANGYUAN ZHANG[1], NA ZHAO[2], XI WU[3], RUI CHEN[3], SHAOCHUN JING[4]

[1]Department of Trauma Surgery, First Hospital of Qinhuangdao, Qinhuangdao 066000, China; [2]Operating Room Nursing, The Second Hospital of Qinhuangdao, Qinhuangdao 066600, China; [3]Department of Surgery, First Hospital of Qinhuangdao, Qinhuangdao 066000, China; [4]Department of Traumatic Arthropathy, The Second Hospital of Qinhuangdao, Qinhuangdao 066600, China.

Correspondence at: Shaochun Jing, Department of Traumatic Arthropathy, The Second Hospital of Qinhuangdao, No. 133, Chaoyang South Street, Changli County, Qinhuangdao City, 066600, Hebei Province, China. Tel: +86-0335-2989337. Email: shaochunjing96@163.com.

ABSTRACT

Background and study aims: As severe intra-articular injuries of the distal tibia, Pilon fractures are frequently complicated by delayed bone consolidation and nonunion. Early prediction of healing outcomes remains challenging due to the lack of reliable molecular markers. This study aimed to determine the diagnostic utility of serum miR-20b-3p for Pilon fracture consolidation and to elucidate its mechanistic role in modulating osteoblast activity via GFI1.

Materials and methods: The clinical cohort comprised 183 individuals treated for closed, unilateral Pilon fractures. Circulating miR-20b-3p was quantified using qRT-PCR, with its diagnostic capacity evaluated using receiver operating characteristic (ROC) curves. Mechanistically, a dual-luciferase reporter system confirmed the direct targeting of GFI1 by miR-20b-3p. For in vitro analyses, MC3T3-E1 pre-osteoblasts were transfected with specific miR-20b-3p mimics or inhibitors. Subsequently, cellular proliferation and osteogenic marker transcripts were evaluated utilizing CCK-8 assays and qRT-PCR, respectively.

Results: Circulating miR-20b-3p was significantly reduced among patients who failed to heal normally ($P < 0.001$). ROC analysis revealed that miR-20b-3p had good diagnostic performance for predicting delayed union, with an AUC of 0.890. GFI1 was identified as a direct target of miR-20b-3p. In vitro, overexpression of miR-20b-3p significantly promoted MC3T3-E1 cell proliferation and upregulated osteogenic markers, whereas inhibition of miR-20b-3p exerted the opposite effect. The pro-osteogenic effects of miR-20b-3p were reversed by GFI1 overexpression.

Conclusions: miR-20b-3p may serve as a diagnostic biomarker for Pilon fracture healing and facilitate osteoblast proliferation and differentiation by targeting GFI1.

Keywords: miR-20b-3p, Pilon fracture, fracture healing, diagnostic biomarker, osteoblast.

INTRODUCTION

Pilon fractures are severe intra-articular fractures of the distal tibia that directly compromise the articular surface of the ankle joint[1,2]. Pilon fractures are associated with a high rate of complications, including delayed union and nonunion, particularly in complex or infected cases[3]. Such fractures are particularly challenging to repair, with significantly higher rates of nonunion and delayed union compared to other fracture types[4]. Healing outcomes after pilon fractures remain highly variable, with a significant risk of complications and nonunion depending on both fracture characteristics and treatment strategy[5]. Early and accurate prediction of healing outcomes would greatly enhance clinical decision-making, helping to reduce patient burden and optimize medical resource allocation[6].

Although existing evaluation tools such as X-ray/CT and bone metabolism markers are widely used, they cannot capture early molecular-level healing signals, leading to diagnoses that often lag behind pathological progression[7]. Therefore, there is an urgent need to identify molecular markers that reflect early healing processes. Fracture healing is a complex biological process that depends on preservation of the local vascular and cellular environment[8]. Exploring the mechanisms of fracture healing from the perspective of post-transcriptional regulation has become an important direction in orthopedic translational medicine research.

The regulation of the balance between osteogenesis and bone resorption during fracture healing fundamentally depends on post-transcriptional gene modulation[9]. MicroRNAs (miRNAs) are key endogenous regulators that modulate target mRNA stability or

inhibit their translation, thereby directing bone repair at multiple stages[10-12]. Several microRNAs have been shown to regulate fracture healing and osteogenic differentiation, including miR-656-3p, which has been reported to modulate osteoblast activity through BMP-2 signaling[13].Their regulatory effects span the entire healing process, from early cell proliferation[14] to osteogenic differentiation and matrix mineralization[9]. This multidimensional role highlights their potential to accelerate callus formation and improve repair quality. Accordingly, identifying miRNAs with multiple regulatory functions in Pilon fracture healing is a critical strategy to address healing challenges.

Located on human chromosome Xq26.2[15], miR-20b-3p is a multifaceted regulator involved in both tumor suppression and tissue regeneration. It inhibits osteosarcoma proliferation by targeting BAMBI or KIF23[16,17] and promotes angiogenesis in diabetic foot and retinopathy[18].Additionally, its enrichment in healthy plasma exosomes supports myelin regeneration and distal blood perfusion[19]. A multi-omics study revealed that miR-20b-3p is characteristically downregulated during abnormal fracture repair compared to normal bone and callus tissue[20]. However, its diagnostic value and specific mechanism of action in Pilon fracture healing remain unexplored.

While previous studies have extensively explored the role of various microRNAs in fracture healing, such as miR-122-5p[21] and miR-376a-3p[22], the specific added value of our study lies in the dual evaluation of miR-20b-3p's clinical diagnostic potential and its mechanistic role. We specifically selected miR-20b-3p because bioinformatics analysis suggested its strong interaction with GFI1, a known inhibitor of osteogenesis. By integrating a relatively large clinical cohort of Pilon fractures with in vitro functional assays, this study aims to address the current gap in the literature by providing both predictive diagnostic value and incremental mechanistic insight into the miR-20b-3p/GFI1 regulatory axis during delayed healing.

In silico screening highlighted GFI1 as a crucial target of miR-20b-3p. Notably, GFI1 functions as a recognized antagonist of osteogenesis under various pathological skeletal conditions[23]. Mechanistically, GFI1, with its highly conserved SNAG domain, can recruit histone deacetylases (such as HDAC1) to the promoter region of the osteogenic core transcription factor RUNX2. This spatial recruitment leads to epigenetic silencing of local chromatin, thus blocking RUNX2-mediated downstream osteogenic gene cascades and consequently impeding the process of fracture repair[24].

Based on this, the present study aimed to explore the regulatory role of the miR-20b-3p/GFI1 axis in bone repair using clinical samples and in vitro cell models of Pilon fractures. Specifically, we evaluated its effect on cell proliferation and osteogenic differentiation. Ultimately, this study provides experimental evidence to understand the molecular mechanisms of abnormal healing. Furthermore, it offers preliminary insights for future clinical interventions and prognostic evaluation.

MATERIALS AND METHODS

Study subjects

A retrospective analysis was conducted on 183 individuals who underwent open reduction and internal fixation (ORIF) for unilateral closed Pilon fractures at our center. At the final clinical evaluation (conducted at a minimum of 6 months postoperatively), the study population was stratified into a successful consolidation arm (n = 106) and a delayed/non-healing arm (n = 77), dictated by clinical assessments and Radiographic Union Scale for Tibial Fractures (RUST) criteria. The study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of The Second Hospital of Qinhuangdao before the study began. Written informed consent was collected from all participants.

Successful healing was defined as definitive bone union within six months postoperatively. This was evidenced by blurred or absent fracture lines and continuous bridging callus approximating normal bone density on radiographs (RUST score $\geq$ 10), alongside an absence of pain upon weight-bearing, local tenderness, or abnormal movement. Conversely, delayed union or nonunion was diagnosed if healing signs were absent beyond six months. This included distinct fracture gaps, a lack of callus, or medullary sclerosis, paired with persistent, activity-related local pain.

Exclusion criteria comprised: (1) systemic metabolic bone disorders (e.g., severe diabetes, rheumatoid arthritis, hyperthyroidism); (2) previous or pathological fractures at the affected site; (3) recent (within six months) prolonged administration of drugs altering bone remodeling, such as bisphosphonates or corticosteroids; and (4) pre-existing limb dysfunctions, malignancies, or severe organ failure.

Clinical data collection and serum sample processing

Patient characteristics, encompassing age, gender, BMI, and substance use history (smoking and

alcohol), were retrieved from institutional medical records. Fasting blood samples (5 mL) were obtained on the morning of postoperative day 7. Samples were allowed to clot for 30 minutes in sterile tubes, and spun at 3,000 rpm (4°C, 10 min). The resulting serum supernatant was then partitioned into sterile Axygen microcentrifuge tubes and cryopreserved at –80°C for downstream molecular assays.

Cell line selection and routine culture

For in vitro mechanistic assays, the murine pre-osteoblastic MC3T3-E1 cell line (ATCC, USA) was maintained in α-MEM (Gibco, USA) containing 10% FBS and 1% penicillin/streptomycin (both Gibco, USA). The medium pH was strictly regulated at 7.3 ± 0.2. Cultures were incubated in a humidified atmosphere at 37°C with 5% CO_2. Fresh medium was supplied every alternate day. When cells reached approximately 80% confluence, they were detached using 0.25% trypsin (Sigma-Aldrich, USA) and subcultured. Only exponentially growing cells from passages 3 through 6 were utilized for downstream experiments.

Cell transfection and osteogenic induction

MC3T3-E1 cells were transfected using Lipofectamine 3000 (Invitrogen, USA) with miR-20b-3p mimics, inhibitors, or corresponding negative controls (NC) synthesized by Sangon Biotech (Shanghai, China).
Briefly, cells were plated in 24-well plates with MEMα containing 10% FBS. Once cells reached the logarithmic growth phase, target oligonucleotides were complexed with the transfection reagent and applied to the cultures. Six hours post-transfection, the medium was refreshed. Following a 48-hour recovery period, the cultures were transitioned to an osteogenic differentiation medium comprised of α-MEM, 10% FBS, 50 μg/mL ascorbic acid, and 10 mM β-glycerophosphate (Sigma-Aldrich). Cells were maintained in this induction environment for 7 days before harvest.

For co-transfection assays to assess GFI1 function, cells received the miR-20b-3p mimic alongside either a GFI1 overexpression plasmid (OE GFI1; GenePharma, China) or an empty vector control (OE NC). Similar to the previous protocol, osteogenic induction was initiated 48 hours post-transfection and continued for 7 days.

qRT-PCR

Cellular RNA was isolated utilizing TRIzol reagent (Thermo Fisher Scientific, USA). Following quantification and purity verification (A260/A280 ratios between 1.8 and 2.0) via a NanoDrop 2000 spectrophotometer, samples underwent reverse transcription. For miR-20b-3p profiling, a dedicated miRNA assay kit (Takara Bio, Japan) was employed, with expression normalized against U6. For mRNA quantification, transcripts of GFI1 and osteoblastic indicators (RUNX2, COL-1, OCN) were reverse-transcribed and amplified utilizing the PrimeScript RT reagent kit alongside SYBR Premix Ex Taq (Takara Bio), setting GAPDH as the endogenous control. Final relative expression folds were computed applying the $2^{-\Delta\Delta Ct}$ algorithm.

CCK-8 assay

Cellular proliferation kinetics following miR-20b-3p modulation were assessed via the Cell Counting Kit-8 (CCK-8; Dojindo, Japan). Post-transfection with the respective mimics, inhibitors, or controls, MC3T3-E1 cells were plated in 96-well formats at a density of 3×10^3 cells/well and cultured under standard conditions (37°C, 5% CO_2). To track growth trajectories, 10 μL aliquots of the CCK-8 solution were introduced into the wells at 0, 24, 48, and 72 hours. After a 2-hour dark incubation at 37°C, the optical density (OD) at 450 nm was recorded employing a Bio-Rad microplate reader (USA).

Dual-luciferase reporter assay

Computational forecasting utilizing TargetScan highlighted the 3′-UTR of GFI1 as a highly probable binding target for miR-20b-3p. DNA sequences containing either the wild-type (WT-GFI1) or mutated (MUT-GFI1) target sites were synthesized and inserted into pGL3 luciferase vectors (Promega, USA).

Reporter assays were executed in 24-well plates utilizing actively dividing MC3T3-E1 cells. The experimental setup involved the liposome-mediated delivery of the synthesized reporter constructs, paired with either the specific mimic or miR-NC. Transfection efficiency was standardized across wells by incorporating a Renilla luciferase vector into the delivery complexes. Following a two-day incubation period, cellular extracts were subjected to luminescence profiling with a dual-luciferase kit (Promega). Results were derived by dividing the firefly readout by the corresponding Renilla activity.

Statistical analysis

Clinical datasets and in vitro outputs were statistically processed utilizing IBM SPSS (version 26.0) and GraphPad Prism (version 8.0), respectively, with

the threshold for statistical significance predefined at a two-tailed $P < 0.05$. Continuous data passing the Shapiro-Wilk normality assumption were summarized as means ± standard deviations. Group comparisons for these parametric datasets were conducted via unpaired Student's t-tests, or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons for scenarios involving three or more groups. Categorical metrics were presented as constituent ratios and compared applying either Pearson's chi-square or Fisher's exact tests. Independent predictors of bone consolidation were isolated through multivariate logistic regression models (where the relative expression of miR-20b-3p, calculated via the $2^{-\Delta\Delta Ct}$ method, was entered as a continuous un-transformed variable), reporting adjusted odds ratios (aOR) alongside 95% confidence intervals (CI). Furthermore, linear associations were evaluated using Pearson's approach, while the diagnostic efficacy of the target biomarker was quantified by deriving the area under the receiver operating characteristic (ROC) curve.

RESULTS

Analysis of clinical data and baseline characteristics of patients with Pilon fracture

The study cohort comprised 183 surgically managed cases of Pilon fractures. Based on radiographic evidence of consolidation, the cohort was stratified into a successful union arm (n = 106) and a delayed healing arm (n = 77). Baseline profiling (Table I) revealed no statistically significant discrepancies between the cohorts regarding mean age (48.91 ± 4.09 vs. 49.21 ± 3.65 years, P = 0.606) or BMI (23.11 ± 2.23 vs. 23.28 ± 2.53 kg/m², P = 0.638). Furthermore, variables including substance use (smoking, P = 0.234; alcohol consumption, P = 0.176) and injury laterality (P = 0.595) were evenly distributed. Consequently, the baseline homogeneity between these groups effectively minimizes potential confounding biases in our downstream analyses.

Relationship between miR-20b-3p expression and delayed union of Pilon fracture and its diagnostic potential

Subsequent qRT-PCR profiling of patient sera revealed a significant reduction in circulating miR-20b-3p among the delayed union cohort relative to normally healed individuals (Fig. 1A, P < 0.001). To appraise the diagnostic utility of this differential expression, we subsequently mapped ROC curves (Fig. 1B). The results showed that the area under the curve (AUC) of miR-20b-3p for predicting delayed union of Pilon fracture was 0.890. Based on the maximum Youden index, the optimal cutoff value was determined, yielding a diagnostic specificity of 87.0% and sensitivity of 80.2% (P < 0.001). These results suggest that miR-20b-3p has potential clinical value as a molecular marker for early identification of poor fracture healing.

We performed multivariate logistic regression to pinpoint independent determinants of Pilon fracture consolidation (Table II). Strikingly, among the comprehensive clinical variables assessed, circulating miR-20b-3p emerged as the sole significant factor inversely associated with delayed union risk when evaluated as an un-transformed continuous variable (OR = 0.034, 95% CI: 0.014–0.082, P < 0.001). Given that the data represent raw relative expression, a 1-unit increase constitutes a substantial biological shift, which mathematically corresponds to this strong calculated protective effect against delayed healing.

Regulatory effect of miR-20b-3p on osteoblast proliferation and osteogenic differentiation markers

To investigate the functional implications of miR-20b-3p in vitro, we established robust gain- and loss-of-function models in MC3T3-E1 cells utilizing

Table I. — General information of the study subjects.

	Normal healing (n=106)	Delayed healing (n=77)	P value
Age (years)	48.91 ± 4.09	49.21 ± 3.65	0.606
BMI (kg/m²)	23.11 ± 2.23	23.28 ± 2.53	0.638
Gender (male / female)	51 / 55	40 / 37	0.608
Smoke (no / yes)	59 / 47	36 / 41	0.234
Alcohol (no / yes)	63 / 43	38 / 39	0.176
Fracture side (left / right)	62 / 44	42 / 35	0.595

BMI: body mass index.

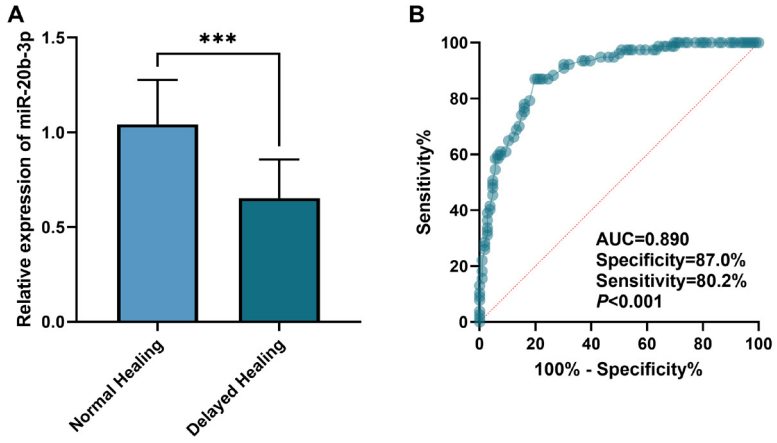


*Fig. 1 — Assessment of miR-20b-3p as a potential biomarker for impaired Pilon fracture healing. Differential expression levels of miR-20b-3p in the systemic circulation of the normal union cohort versus the delayed healing cohort (A). ROC curve analysis of miR-20b-3p for distinguishing delayed healing patients from normal healing controls (B). ***P < 0.001.*

Table II. — The associations between subject characteristics and delayed healing of Pilon fracture.

	OR	95% CI	P value
miR-20b-3p	0.034	0.014-0.082	<0.001
Age (years)	1.650	0.738-3.690	0.223
BMI (kg/m^2)	1.279	0.576-2.840	0.546
Gender (male / female)	0.601	0.264-1.370	0.226
Smoke (no / yes)	1.544	0.691-3.449	0.290
Alcohol (no / yes)	1.789	0.801-3.996	0.156
Fracture side (left / right)	1.659	0.726-3.790	0.230
BMI, body mass index.			

specific mimics and inhibitors, respectively (Fig. 2A). Analysis of proliferation kinetics via CCK-8 assay (Fig. 2B) revealed that miR-20b-3p overexpression markedly accelerated the cell growth trajectory. Specifically, by the 72-hour mark, the OD450 values in the mimic-transfected cohort were significantly elevated relative to the negative control (NC) group ($P < 0.001$). Conversely, the suppression of miR-20b-3p led to a pronounced attenuation of cellular proliferative capacity.

Following a 7-day osteogenic induction period, we evaluated the transcriptional profiles of key bone-forming markers (Fig. 2C–E). miR-20b-3p overexpression was found to robustly potentiate the expression of the master osteogenic transcription factor RUNX2, the structural matrix protein COL-1, and the late-stage mineralization indicator OCN ($P < 0.05$ vs. NC group). Conversely, silencing of miR-20b-3p significantly compromised the mRNA abundance of these markers ($P < 0.05$). Collectively, these findings underscore that miR-20b-3p exerts a dual stimulatory effect, driving both proliferative expansion and osteogenic maturation in pre-osteoblasts.

miR-20b-3p mediates negative regulation by directly targeting GFI1

To decode the molecular circuitry through which miR-20b-3p accelerates recovery, we utilized computational forecasting to identify GFI1 as a high-probability candidate target (Fig. 3A). Validation via dual-luciferase reporter assays (Fig. 3B) demonstrated that miR-20b-3p mimicry specifically repressed the bioluminescence of the GFI1-WT construct ($P < 0.01$ vs. miR-NC), whereas the inhibitory effect was completely abolished in the GFI1-MUT group. This targeted interaction was further substantiated by clinical evidence: GFI1 transcripts were markedly enriched in the serum of the delayed healing cohort (Fig. 3C, $P < 0.001$). Moreover, Pearson correlation analysis (Fig. 3D) revealed a robust inverse relationship between circulating miR-20b-3p and GFI1 levels ($r = –0.825$, $P < 0.001$), supporting their antagonistic roles in fracture resolution.

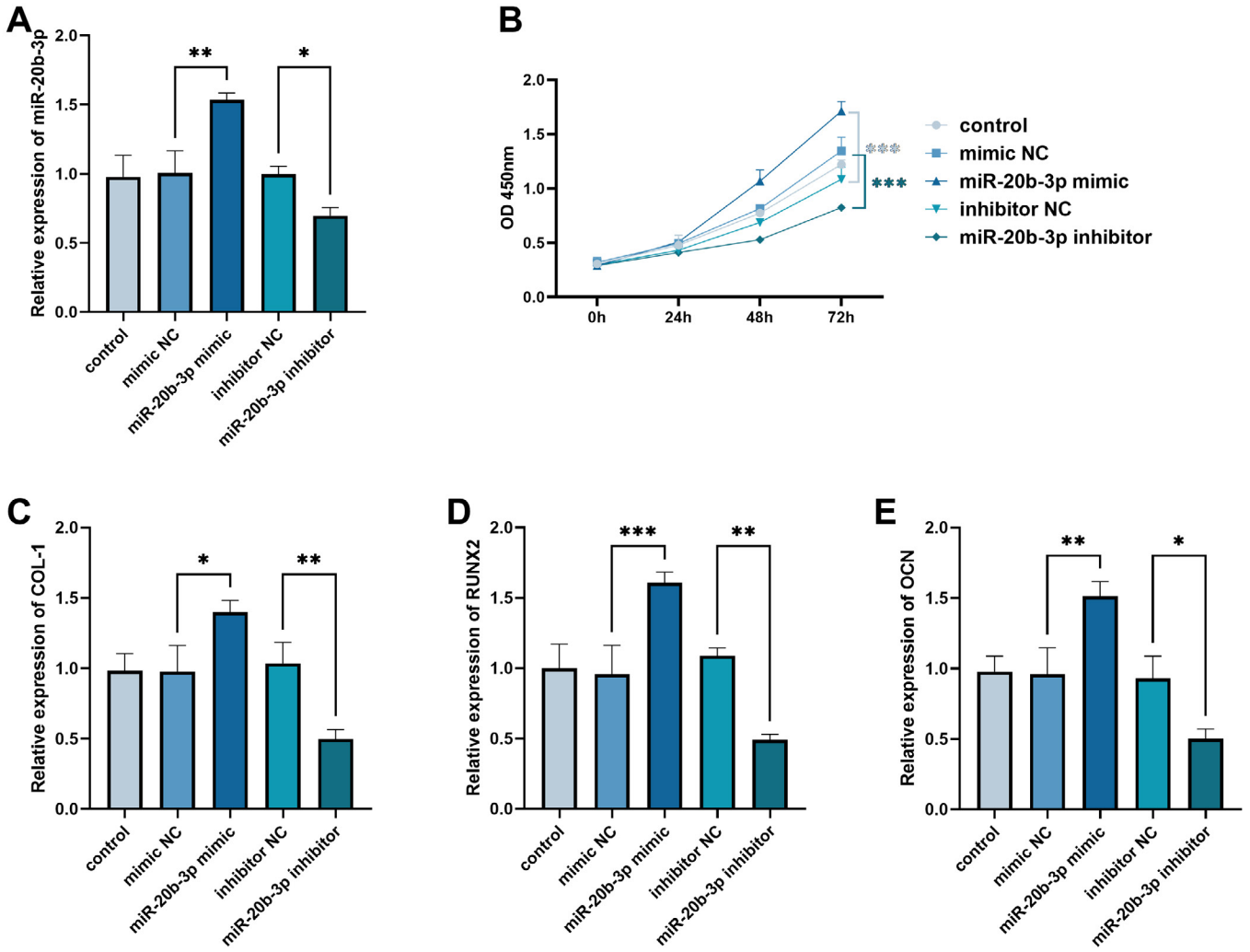


*Fig. 2 — miR-20b-3p promotes proliferation and osteogenic differentiation of MC3T3-E1 cells. qRT-PCR verification of miR-20b-3p transcript levels across the specified experimental cohorts: Control, mimic/inhibitor negative controls (NC), and miR-20b-3p gain/loss-of-function groups (A). Longitudinal assessment of MC3T3-E1 viability at designated intervals (0–72 h) following targeted transfection (B). Relative expression of COL-1 (C), RUNX2 (D), and OCN (E) in the indicated groups. *P < 0.05, **P < 0.01, ***P < 0.001.*

Experimental validation of the miR-20b-3p/GFI1 axis in regulating osteogenic function

To ascertain if GFI1 acts as a functional mediator of miR-20b-3p-driven osteogenesis, we conducted a co-transfection rescue assay. qRT-PCR profiling (Fig. 4A) demonstrated that the exogenous introduction of OE GFI1 significantly restored GFI1 mRNA abundance in miR-20b-3p-mimic-transfected cells ($P < 0.01$ vs. the mimic + OE NC group). This targeted elevation confirmed the successful implementation of our rescue model, enabling subsequent functional assessments. At the functional level (Fig. 4B), OE GFI1 significantly attenuated the promoting effect of miR-20b-3p mimic on cell proliferation ($P < 0.001$). At the gene expression level, the rescue experiment results showed (Fig. 4C–E) that overexpression of GFI1 effectively reversed the upregulation of RUNX2, COL-1, and OCN induced by miR-20b-3p mimic ($P < 0.01$), with the expression of these osteogenic markers returning to levels close to baseline.

DISCUSSION

Pilon fractures are mostly caused by high-energy injuries, and their prognosis is highly complex. Severe axial impact not only causes joint surface crushing, but also leads to serious microenvironmental instability of local soft tissue[25-27]. Fracture healing is influenced by multiple patient- and injury-related factors, which complicates early prediction of outcomes[28]. In this context, relying solely on imaging follow-up often provides delayed information[29]. This study showed that miR-20b-3p was downregulated in delayed healing. Our findings are consistent with previous

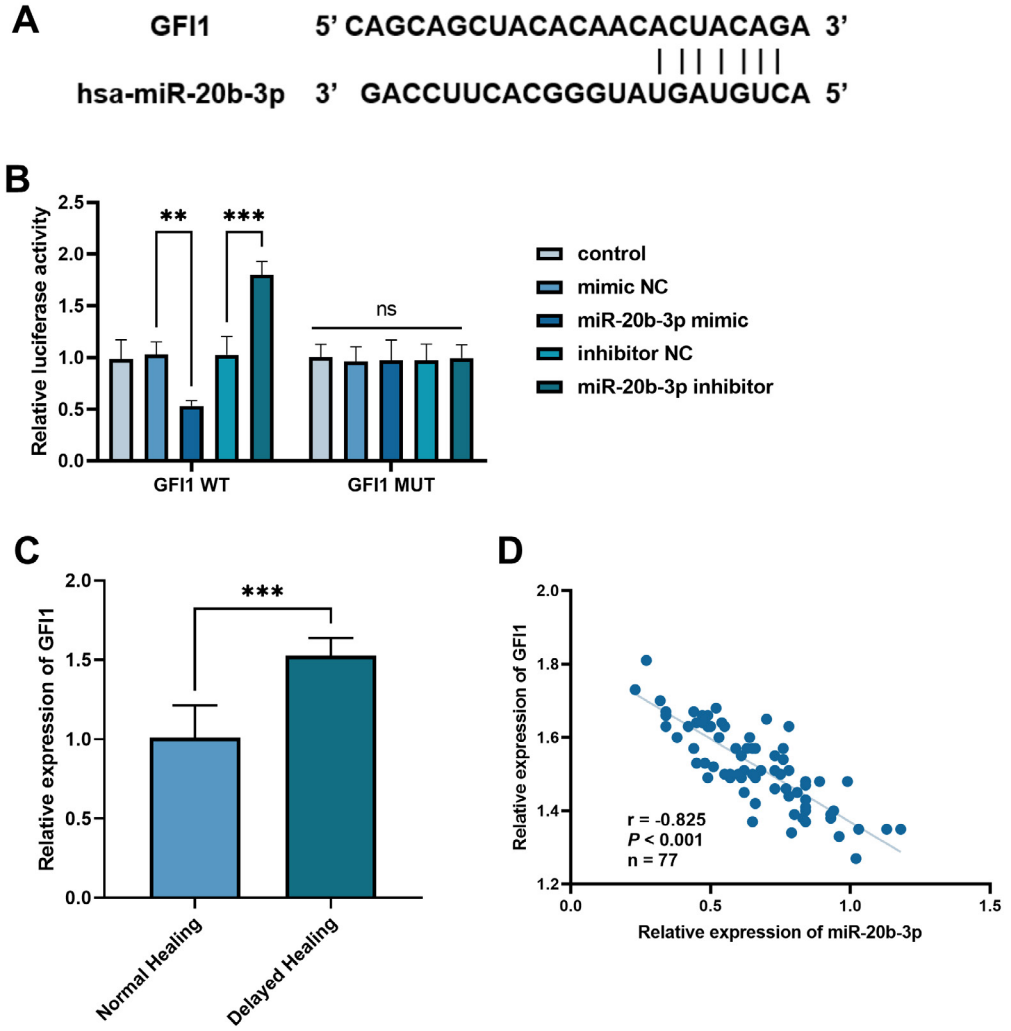


*Fig. 3 — miR-20b-3p directly targets GFI1 in Pilon fracture healing. Predicted binding site of miR-20b-3p on the GFI1 3'-UTR (A). Dual-luciferase reporter assay showing the interaction between miR-20b-3p and GFI1 (B). Relative expression of GFI1 in serum from normal healing and delayed healing groups (C). Correlation between miR-20b-3p and GFI1 expression in Pilon fracture patients (D). ns: not significant, **P < 0.01, ***P < 0.001.*

studies demonstrating that microRNAs regulate osteoblast function and fracture healing through specific target genes, highlighting the relevance of miRNA–gene regulatory axes in bone repair[13].

Unlike imaging examinations, which depend on callus morphology, circulating miRNAs reflect molecular changes in the bone healing microenvironment and offer early warning potential. Previous studies have reported that the loss of key miRNAs induces bone repair stagnation[30]. For example, miR-122-5p[21] and miR-376a-3p[22] have been linked to fracture outcomes. Consistent with these reports, this study showed that miR-20b-3p was significantly downregulated in the delayed healing group, and its diagnostic performance (AUC = 0.890) suggested a comparable clinical value. Early identification of sustained low miR-20b-3p expression after surgery might provide a reference for timely adjustment of treatment strategies to reduce the risk of nonunion.

Fracture repair is a highly collaborative process involving cell proliferation and differentiation. First, the recruitment of the initial callus is highly dependent on the robust proliferation of endogenous osteoprogenitor cells[31,32]. In the CCK-8 assay, we observed a significant enhancing effect of miR-20b-3p on cell growth, suggesting that miR-20b-3p may provide an adequate cell source for bone repair by promoting the proliferation of osteoprogenitors. Secondly, high-quality bone healing requires a complete sequence from early lineage orientation to late mineralization[33,34]. Under osteogenic induction conditions, miR-20b-3p upregulated the expression of the osteogenic marker genes RUNX2, COL-1, and OCN. This multi-stage regulatory feature

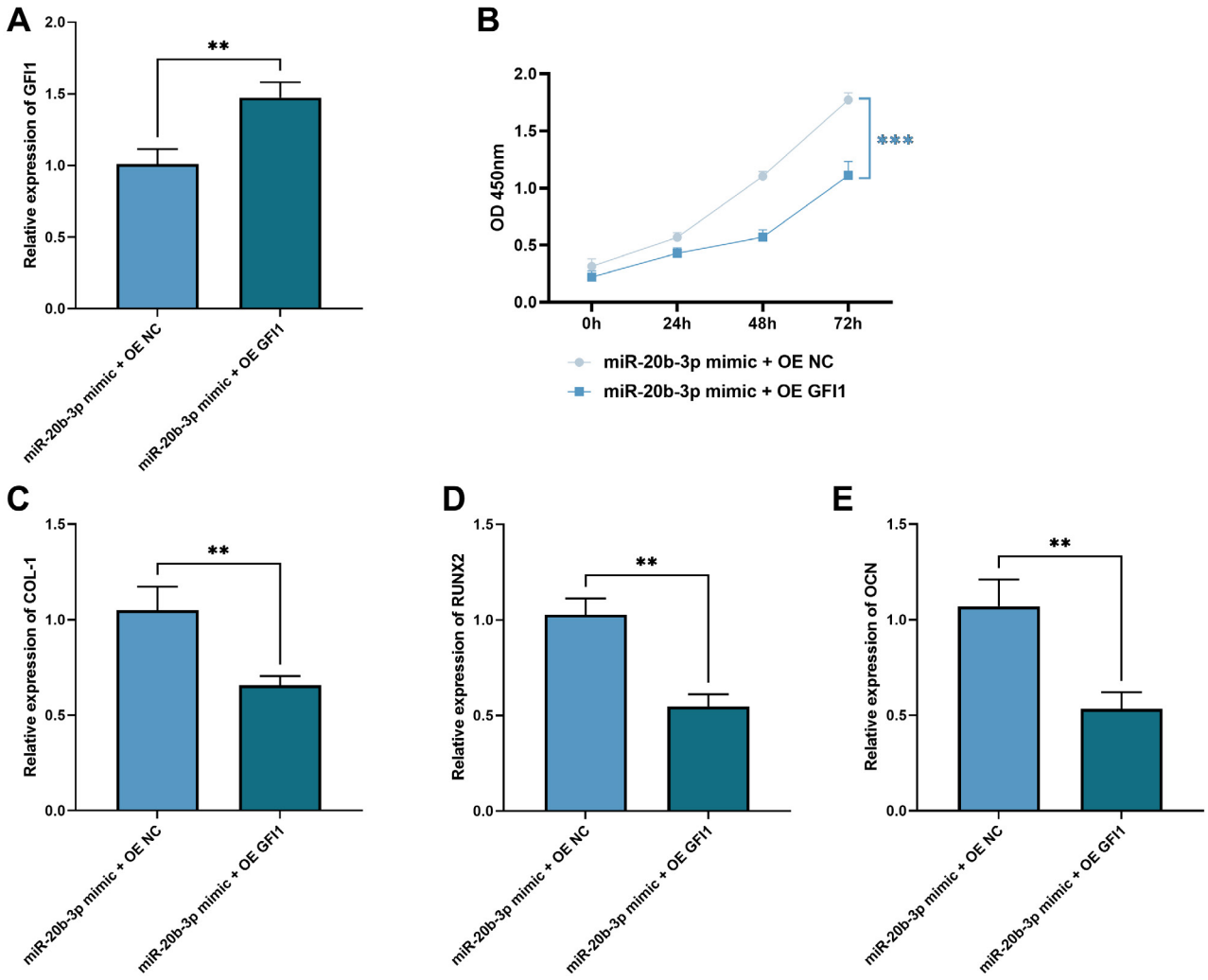


*Fig. 4 — GFI1 overexpression reverses miR-20b-3p-mediated promotion of osteogenic function in MC3T3-E1 cells. Relative expression of GFI1 in miR-20b-3p mimic + OE NC and miR-20b-3p mimic + OE GFI1 groups (A). Cell viability in rescue groups at 0, 24, 48, and 72 h (B). Relative expression of COL-1 (C), RUNX2 (D), and OCN (E) in the rescue groups. **P < 0.01, ***P < 0.001.*

suggests that miR-20b-3p is involved not only in the initiation of osteogenesis but also in the maturation and mineralization of the bone matrix, making it a potential biological target to accelerate the healing of high-energy Pilon fractures.

Mechanistically, our findings further highlight the miR-20b-3p/GFI1 regulatory axis. GFI1 functions as a recognized antagonist of osteogenesis by antagonizing the BMP pathway or directly inhibiting RUNX2 transcriptional activity[23,35]. This study confirmed that the pro-osteogenic effect of miR-20b-3p was essentially mediated by suppressing GFI1 expression and thereby relieving its negative regulation on osteogenesis. The high expression of GFI1 in patients with healing disorders is highly consistent with its role as a key negative regulatory node in pathological tissues, which provides a molecular explanation for the repair failure caused by miR-20b-3p deficiency. However, downstream signaling pathways were not investigated in the present study, and these mechanistic conclusions remain preliminary.

Despite the insights provided by this study, several limitations must be acknowledged. First, the clinical design was retrospective and lacked a validation cohort; therefore, it is premature to present miR-20b-3p as a clinically ready biomarker. Second, all functional data were derived from in vitro MC3T3-E1 cells, and the absence of in vivo validation is a major limitation. The current in vitro model cannot fully recapitulate the complex multicellular interactions within the Pilon fracture microenvironment, such as the intricate crosstalk between vascular endothelial cells and osteoblasts. Future studies using in vivo animal models and prospective cohorts are warranted to delineate the broader functional role of the miR-20b-3p/GFI1 axis in bone repair.

At the clinical level, while a detection strategy based on miR-20b-3p could potentially be incorporated into early evaluation systems for fracture healing, its dynamic changes in relation to prognosis require further verification. Furthermore, combining single-cell sequencing or multi-omics approaches to analyze the target networks of this axis at different stages of fracture repair may provide a more comprehensive view of its functional role. These investigations could ultimately provide a foundation for optimizing individualized treatment strategies for Pilon fractures.

CONCLUSIONS

In summary, this study demonstrates that miR-20b-3p is significantly downregulated in patients with delayed healing of Pilon fractures and exhibits good diagnostic performance for predicting healing outcomes. Mechanistically, our findings suggest that miR-20b-3p may directly target GFI1 to facilitate osteoblast proliferation and differentiation. Collectively, these results highlight the potential of miR-20b-3p as a potential prognostic marker for Pilon fracture consolidation and a candidate molecular target for future therapeutic investigations. Given the study's limitations, including its retrospective nature and the use of an in vitro model, further prospective cohort studies and in vivo validations are essential to confirm these clinical and biological implications.

Ethics approval: The study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of The Second Hospital of Qinhuangdao before the study began.

Consent to participate: Written informed consent was collected from all participants.

Consent for publication: Not applicable.

Availability of data and materials: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' Contribution: Conceptualization, F.Z. and S.J.; Data curation, N.Z., R.C. and X.W.; Formal analysis, R.C. and X.W.; Funding acquisition, S.J.; Investigation, R.C. and X.W.; Methodology, F.Z., N.Z. and X.W.; Project administration, S.J.; Resources, N.Z.; Software, F.Z. and N.Z.; Supervision, S.J.; Validation, F.Z. and N.Z.; Visualization, F.Z.; Writing - original draft, F.Z.; Writing - review & editing, S.J.

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