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The function of miR-4666a-3p in the diagnosis of osteoporotic fractures and the promotion of fracture healing

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Abstract

Introduction and Objective. MicroRNAs (miRNAs) regulate osteogenic differentiation in osteoporotic fractures (OF). The aim of the study is to investigate the diagnostic value and regulatory role of miR-4666a-3p in OF.

Materials and Method. The study is based on clinical samples (89 healthy controls, 79 OP patients, 83 OF patients) and hFOB1.19 cell experiments. RT-qPCR analyzed miR-4666a-3p expression, and logistic regression identified OF risk factors. ELISA measured RANKL and OPG concentrations. *In vitro,* osteoblast differentiation was induced to assess ALP activity, Runx2, Osteocalcin, Col1a1, cell viability (CCK-8), and apoptosis (flow cytometry). Target genes were bioinformatically predicted and confirmed via dual-luciferase assay. Rescue experiments explored the role of GSK3B in miR-4666a-3p-mediated osteogenic regulation. All experiments were repeated at least three times.

Results. Downregulation of miR-4666a-3p could distinguish healthy controls and OP patients, further differentiate between OP and OF patients, and may predict the risk of OF occurrence. During osteoblast differentiation, miR-4666a-3p expression increased, ALP activity elevated, and key marker levels upregulated. Overexpression of miR-4666a-3p promoted osteoblast differentiation and activity, and reduced apoptosis rate, whereas inhibiting miR-4666a-3p had the opposite effect. MiR-4666a-3p negatively regulated the downstream target GSK3B. Overexpression of GSK3B reversed the osteogenic effects of miR-4666a-3p.

Conclusions. Downregulation of miR-4666a-3p may predict OF risk. Its upregulation promoted osteoblast differentiation by targeting GSK3B, potentially facilitating fracture healing.

Key words

osteoporotic fracture, osteoblast differentiation, GSK3B, miR-4666a-3p

INTRODUCTION AND OBJECTIVE

Osteoporotic fractures (OF) refer to a type of fracture that occurs in the human body when it is in a state of osteoporosis (OP) and without external force acting upon it during daily life. These fractures have several characteristics, such as a high risk of re-fracture, a complex process of fracture healing, and extremely poor bone quality [1]. OP is a bone disease characterized by poor bone microstructure and mineralization, reduced bone mineral density, or decreased bone gain. Dual-energy X-ray absorptiometry (DEXA) is the gold standard for measuring bone density. Osteoporosis can be diagnosed when the T value is lower than -2.5. And type I procollagen N-terminal propeptide (P1NP) and type I collagen cross-linked C-terminal peptide (CTX) are reference markers for bone formation and resorption, respectively. Alkaline phosphatase (ALP), osteocalcin, Runt-related transcription factor 2 (Runx2), Collagen Type I Alpha 1 Chain (Col1a1), Receptor Activator of Nuclear Factor κB Ligand (RANKL), and Osteoprotegerin (OPG) also assess bone quality. RANKL promotes bone resorption; OPG inhibits it as a decoy receptor. Their dynamic balance is key to fracture healing [2, 3]. Osteoporosis can lead to an increased risk of fractures, such as fractures in the spine, wrists, pelvis, proximal humerus, or hip, which require hospitalization [4]. OP occurs due to an imbalance between bone resorption and bone formation during remodeling. Postmenopausal women and the elderly are the main groups affected by OP, especially rural elderly people who have been engaged in agricultural labour for a long time. Due to cumulative joint load, pesticide exposure may interfere with bone metabolism, insufficient sunlight leading to vitamin D deficiency, and relatively scarce medical resources in rural areas, the risk of OF is higher, and the consequences are more severe. Mechanisms such as endocrine disorders, inflammatory joint diseases, haematopoietic disorders, and nutritional imbalance are all involved [5]. OF has a high incidence, disability rate, and mortality rate in clinical settings, and the treatment costs are high. Therefore, it is necessary to conduct effective early diagnosis to improve the prognosis of patients.

MicroRNAs (miRNAs) are non-coding small RNAs with a stem-loop structure and a length of approximately 22 nucleotides. They play important regulatory functions in animals and plants, mainly regulating gene transcription by targeting messenger RNA (mRNA) through the 3'

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untranslated region. They play a crucial part in cell viability and apoptosis, as well as in the occurrence of various diseases and physiological processes, and are considered important mediators of epigenetic modifications [6]. With the deepening of research on miRNAs, the functions of miRNAs have been increasingly understood. Some studies have found that specific miRNAs circulating in serum serve as biomarkers for OP, further predicting the occurrence of OF. Evidence indicates that miR-18a-3p accelerates OP development through regulation of glutamate ionotropic receptor AMPA type subunit 1 (GRIA1), suggesting that therapeutic targeting of the miR-18a-3p/GRIA1 interaction could be beneficial for OP treatment [7]. Additionally, Cikedaoerji Na et al. discovered that the upregulation of miR-331-3p can promote OP and also regulate Neuropilin 2, thereby promoting the occurrence of OF [8]. All these studies confirm that many miRNAs play significant roles in osteogenic differentiation or osteoclast activity. Therefore, identifying key miRNA molecules that regulate osteoblast differentiation and exploring their mechanisms is highly important for developing novel therapeutic strategies for OF.

As a member of the microRNA family, miR-4666a-3p has been reported to exhibit differential expression in heart diseases [9, 10]. Analysis and screening of the osteoporosis-related expression profile data in the Gene Expression Omnibus (GEO) database (GSE93883 dataset) revealed a significant downregulation in the expression level of miR-4666a-3p. In recent years, its role in orthopaedic diseases has also gradually gained attention: Studies have shown that miR-4666a-3p has certain diagnostic value for femoral traumatic osteonecrosis [11]. What is more important is that the expression of miR-4666a-3p is significantly downregulated in patients with OP and osteoporotic vertebral fractures [12]. These data suggest that miR-4666a-3p has significant research potential in the field of OP. However, the specific clinical significance and regulatory mechanism of miR-4666a-3p in OP remain unclear. Based on these, this study proposes the hypothesis that miR-4666a-3p is differentially expressed in OF and has certain diagnostic value, and it may be involved in osteoblast differentiation. Therefore, the study detected the expression levels of miR-4666a-3p in the serum of OP patients and patients with OF, and used an osteogenic cell differentiation model to explore its regulatory mechanism in OF systematically.

MATERIALS AND METHOD

Research subjects and sample collection. From June 2023 – December 2024, a total of 162 patients with OP visited Zhucheng People's Hospital, and were included in the study. At the same time, 89 healthy controls who underwent physical examinations during the same period were also included. According to the World Health Organization (WHO) guidelines for the prevention and management of osteoporosis [13], the OP patients were divided into two groups: the fracture group (OF, n = 83) and the non-fracture group (OP, n = 79). The inclusion criteria were patients with primary OP (measured by DEXA with a T value ≤ -2.5 at the hip) who had not received any related drug treatment. Among them, patients in the fracture group needed to have a new, low-energy injury-induced fragility fracture of the hip, vertebrae, distal radius, or proximal humerus through X-ray examinations. The exclusion criteria include fractures caused by high-energy trauma and clearly non-osteoporosis-related factors, as well as patients with a history of anti-osteoporosis treatment, a history of long-term use of drugs that affect bone metabolism, or those with severe endocrine diseases, malignant tumours, and significant organ dysfunction.

Fasting elbow venous blood samples were collected from the patients at the time of admission (within 24–48 hours after the fracture), on the first postoperative day, and on the 7th, 14th, 21st, and 28th postoperative days. The serum was separated and stored at -80 °C for subsequent research, including miR-4666a-3p, Runx2, Osteocalcin, Col1a1, and GSK3B expression levels, as well as RANKL and OPG concentration and ALP activity. The study was conducted with the approval of the Institutional Review Board at Zhucheng People's Hospital, with written informed consent obtained from all participants and their legal guardians.

In vitro, osteoblast differentiation was induced to extract the miR-4666a-3p, Runx2, Osteocalcin, Col1a1 and GSK3B RNA, as well as ALP activity, cell viability (CCK-8), and apoptosis. hFOB1.19 cells were used as a human-derived osteoblast model.

Cell culture and osteoblast differentiation. The cells used in the research were the human osteoblastic cell line (hFOB1.19 cells), provided by the Cell Bank of the Chinese Academy of Sciences. They were cultured in a complete medium (DMEM medium containing 10% FBS and 0.3 mg/mL G418) under a humidified environment of 33.5 °C and 5% CO_2.

After the HFOB1.19 cells reached the appropriate density, they were transferred to a 39 °C culture medium, and the culture medium was substituted with an osteogenic culture medium – DMEM. The DMEM contained 10% FBS, 100 nM dexamethasone, 50 μg/mL ascorbic acid Vc, and 10 mmol/L β-glycerophosphate sodium. Detection was conducted on days 7, and 14 of the differentiation induction.

Cell transfection. For cell transfection, the transfection reagent INTERFERin® (Polyplus-transfection®, France) was used. The miR-4666a-3p mimics, mimics control, inhibitors, and inhibitor control were provided by Qiagen. The overexpression vectors of GSK3B (OE-GSK3B) or OE-NC were provided by Takara Bio.

RNA extraction. The miR-4666a-3p, Runx2, Osteocalcin, Col1a1, and GSK3B RNA in serum and cells were extracted using the TRIzol-based RNA extraction method (BioTeke Corporation, China). The concentration and purity of the extracted RNA were measured using an RNA concentration analyzer (BioSpectrometer® basic, Eppendorf). The purity ratio should be within 1.8–2.0 to be qualified and can be used for subsequent experiments.

Quantitative real-time PCR (RT-qPCR) analysis. Total RNA was reverse-transcribed into cDNA using the miScript II RT Kit (Qiagen, Germany) and HiScript® III RT SuperMix (Vazyme, China). RT-qPCR was performed with the TB Green® Premix Ex Taq™ II (Tli RNaseH Plus, Takara Bio, Japan) on a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, USA). U6 and GAPDH were used as internal reference controls. The primer sequences are as shown in (Suppl. Tab. 1). The reaction conditions were: 95 °C for 10 minutes; 95 °C for 15 seconds, 60 °C for 1 minute, for a total

Supplementary Table 1. Primer sequences used for RT-qPCR

Gene	Sequences (5′-3′)
miR-4666a-3p	F: CATACAATCTGACATGTATTT
	R: AGTGCAGGGTCCGAGGTATT
Runx2	F: GCCGGGAATGATGAGAACTA
	R: GGACCGTCCACTGTCACTTT
Osteocalcin	F: AGGGAGGATCAAGTCCCG
	R: GAACAGACTCCGGCGCTA
Col1a1	F: GCCAAGGCAACAGTCGCT
	R: CTTGGTGGTTTTGTATTCGATGAC
GSK3B	F: GTCCTGGGAACTCCACAAGGG
	R: GTGAAATGTCCTGTTCCTGAC
U6	F: CGCTTCGGCAGCACATATAC
	R: TTCACGAATTTGCGTGTCAT
GAPDH	F: TGCCCAGAACATCATCCCT
	R: TGAAGTCGCAGGAGACAACC

of 40 cycles. The miR-4666a-3p, Runx2, Osteocalcin, Col1a1, and GSK3B expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method.

Alkaline phosphatase (ALP) activity assay. The ALP staining kit (Alkaline Phosphatase Activity Assay Kit, Vazyme) was used to analyze the ALP activity of serum and cells. Serum samples were directly centrifuged to obtain the supernatant for later use; cell samples required thorough lysis with the lysis buffer, followed by centrifugation at 4 °C and 12,000 rpm for 30 minutes to collect the supernatant. Subsequently, the lysate supernatants of serum or cells were subjected to ALP activity assays, according to the instructions of the kit.

Enzyme-linked immunosorbent assay (ELISA) assay. The concentrations of serum RANKL and OPG were detected using an ELISA kit (Abcam, UK), strictly following the instructions: add the serum samples to the pre-coated enzyme-labeled plate, incubate with the specific antibody, and then perform the colour reaction. Measure the absorbance value at 450 nm using an enzyme reader.

Cell viability assay. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay. Transfected cells were plated in 96-well plates, and CCK-8 solution was added at 24, 48, and 72 h post-transfection, followed by a 2-hour incubation. OD450 values were determined using a spectrophotometric plate reader, and the measurement repeated 3 times for each detection well.

Cell apoptosis assay. The apoptosis of cells was detected by using the Annexin V-FITC/PI double staining method combined with flow cytometry. The staining was carried out according to the instructions of the kit, after which the cells were detected and analyzed on the machine to determine the proportion of apoptotic cells.

Dual-luciferase reporter assays. The GSK3B sequence with miR-4666a-3p binding sites (predicted by the miRDB database) was subsequently cloned into a firefly luciferase expression vector (Promega, USA) to generate the recombinant plasmid GSK3B-WT. The binding sites of miR-4666a-3p and GSK3B were mutated to construct the recombinant plasmid GSK3B-MUT. The INTERFERin® transfection reagents were used to co-transfect GSK3B-WT and GSK3B-MUT with the mimics of miR-4666a-3p, the control mimics, the miR-4666a-3p inhibitor, and the inhibitor control with hFOB1.19 cells. The fluorescence intensity was detected using a multi-well plate reader (BioTek, USA).

Statistical analysis. Experiments were repeated 3 times; data are shown as mean ± SD. Intergroup comparisons were analyzed using a two-tailed Student's t-test (for 2 groups) or one-way analysis of variance (ANOVA) (for multiple groups). The data were plotted in chart form using GraphPad. The data in the Tables were analyzed using SPSS. Receiver operating characteristic (ROC) curve analysis was used to evaluate the diagnostic efficacy of miR-4666a-3p for OP and OF. Logistic regression analysis was used to screen out the independent influencing factors for the occurrence of OF; Pearson correlation analysis was used to test the expression correlation between miR-4666a-3p and GSK3B. $P < 0.05$ was considered statistically significant.

RESULTS

Expression and diagnostic role of miR-4666a-3p. Analysis of the clinical data revealed that there were no significant differences in age, body mass index (BMI), or smoking status among healthy controls, OP patients, and OF groups. There were significant differences in T-score, receptor activator of nuclear factor-κB ligand (RANKL), and osteoprotegerin (OPG) levels (Tab. 1). Analysis of the GEO dataset GSE93883 revealed distinct miRNA expression profiles between OF patients and healthy controls (Fig. 1A). The top 20 significantly upregulated and downregulated miRNAs are shown in Fig. 1B. The levels of miR-4666a-3p in the serum of OF patients, OP patients, and healthy controls were determined. It was observed that the abundance of miR-4666a-3p in OP patients was lower than that in healthy controls, while the abundance of miR-4666a-3p in the serum of OF patients was significantly lower than that of OP patients (Fig. 1C). For OF patients, it was found that with the increase of recovery time, the abundance of miR-4666a-3p also gradually increased (Fig. 1D).

Table 1. General information about the study subjects

Features	Healthy Controls (n = 89)	Osteoporosis (n = 79)	Osteoporotic Fractures(n=83)	P values
Age, years	63.72±4.85	64.31 ± 6.15	65.53 ± 6.59	0.221
BMI, kg/m^2	23.68 ± 2.91	23.97 ± 2.78	24.03 ± 2.83	0.126
Drinking (%)	41(46.07)	36(49.37)	39(46.99)	0.528
T-score	-0.02 ± 0.47	-2.98 ± 0.39	-3.13 ± 0.42	0.012
RANKL, pg/mL	247.58±43.26	269.37 ± 46.59	284.87 ± 61.46	0.035
OPG, pg/mL	690.35±170.13	776.28 ± 230.57	785.24 ± 210.78	0.029

BMI – body mass index; RANKL – receptor activator of nuclear factor-κB ligand; OPG – osteoprotegerin

ROC analysis confirmed the diagnostic value of miR-4666a-3p for OP (AUC=0.934, sensitivity=0.838, specificity=0.889) (Fig. 1E) and for OF (AUC=0.909, sensitivity=0.924, specificity=0.838) (Fig. 1F). Logistic regression identified miR-4666a-3p (OR=0.328; p=0.002) as an independent

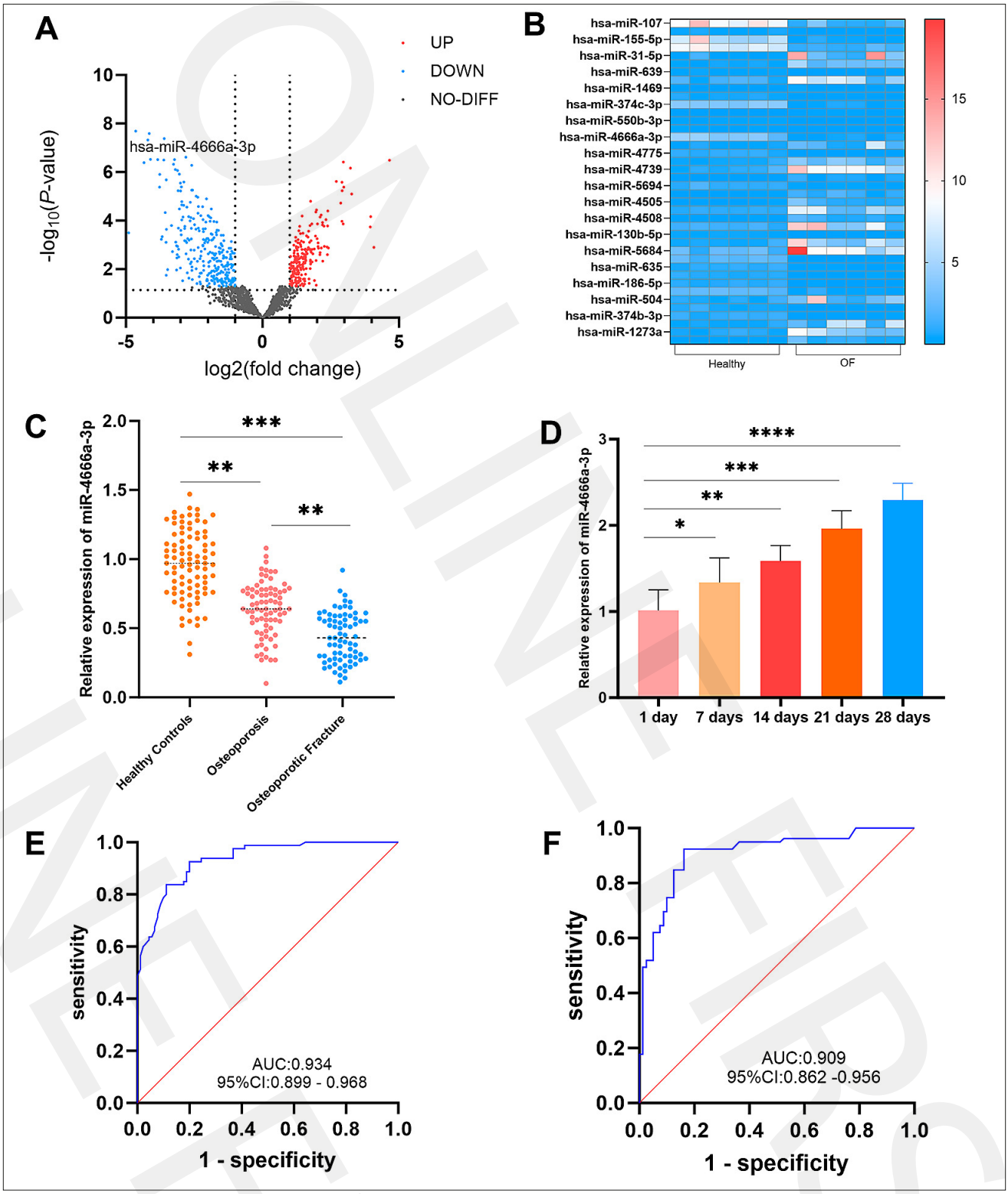

Figure 1. Expression of miR-4666a-3p and its clinical value. (A-B) Volcano plot and heatmap visualization analysis of differentially expressed miR-4666a-3p. (C) The Levels of miR-4666a-3p in healthy controls, patients with osteoporosis, and patients with OF. (D) The levels of miR-4666a-3p change along with the recovery time of OF patients. (E) ROC curve of miR-4666a-3p in diagnosing OP compared with healthy controls. (F) The ROC curve of miR-4666a-3p for diagnosing OP and OF

predictor of OF, indicating that high expression levels confer a 67.2% reduction in fracture risk compared to low expression (Tab. 2).

Expression level of miR-4666a-3p during osteogenic differentiation. The results showed that as the osteogenic induction time increased, the level of miR-4666a-3p also increased (Fig. 2A). The ALP activity (Fig. 2B) and the levels of Runt-related transcription factor 2 (Runx2), osteocalcin, and Collagen type I alpha 1 chain (Col1a1) (Fig. 2C), also increased with the prolongation of differentiation time.

Table 2. Logistic regression analysis of independent factors influencing osteoporotic fractures

Features	OR	95% CI	P values
Age	1.706	0.580-1.995	0.816
BMI	1.122	0.554-1.886	0.944
Drinking	1.125	0.556-1.888	0.938
RANK	1.842	0.770-3.407	0.435
OPG	0.706	0.295-1.691	0.170
miR-4666a-3p	0.328	0.151-1.116	0.002

BMI – body mass index; RANKL – receptor activator of nuclear factor-κB ligand; OPG – osteoprotegerin

Effect of miR-4666a-3p on osteoblast differentiation. To validate the efficiency of miRNA modulation, osteoblasts were transfected with miR-4666a-3p mimic or inhibitor. qRT-PCR results showed that miR-4666a-3p expression was significantly upregulated upon mimic transfection and markedly downregulated upon inhibitor transfection (Fig. 3A), confirming successful transfection. Functional assessment revealed that overexpression of miR-4666a-3p markedly increased ALP activity, whereas knockdown of miR-4666a-3p substantially reduced ALP levels (Fig. 3B). Consistent with this finding, overexpression of miR-4666a-3p upregulated key osteogenic markers (Runx2, Osteocalcin,

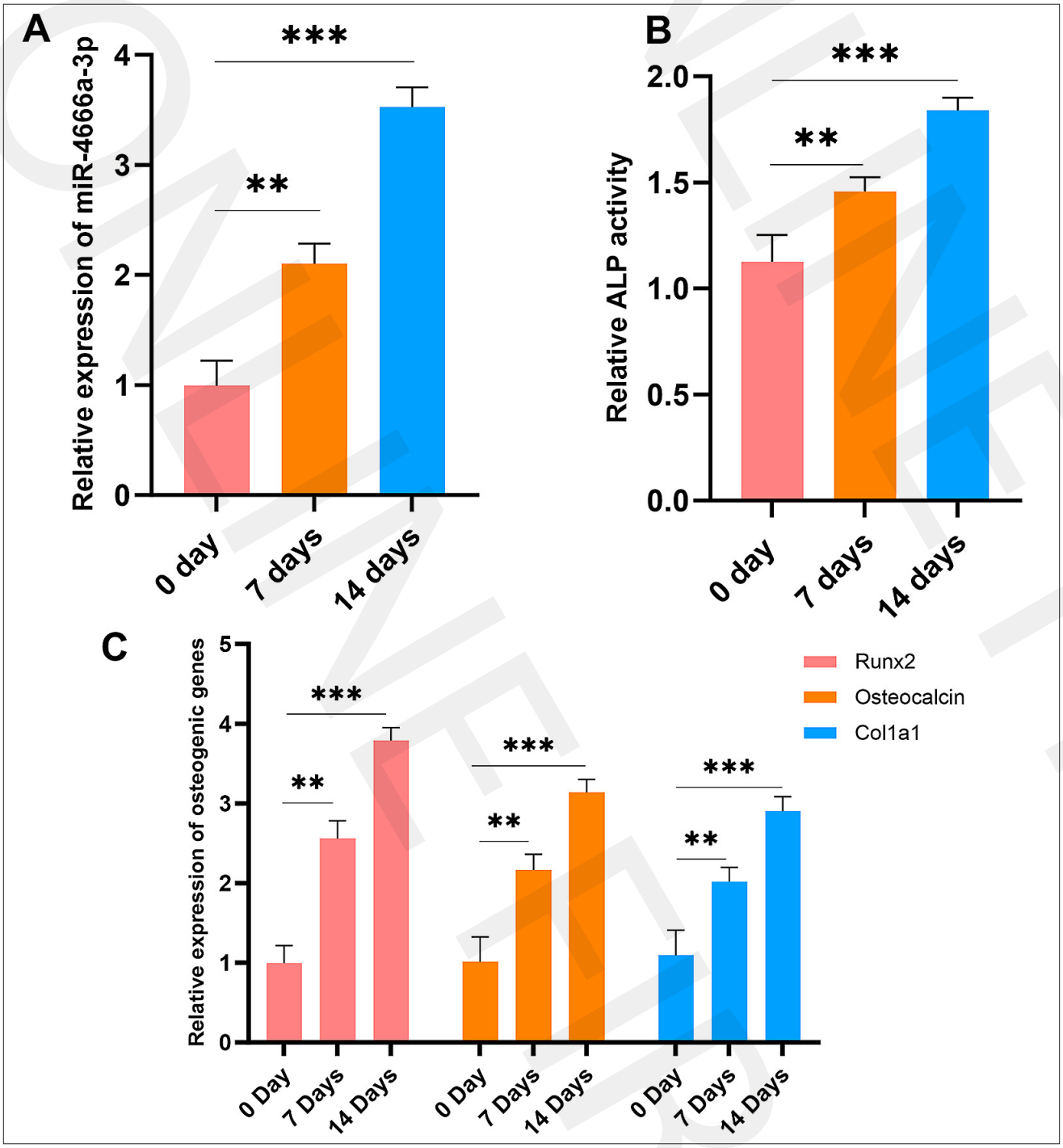

Figure 2. Expression level of miR-4666a-3p during osteogenic differentiation. (A) Level of miR-4666a-3p during the differentiation process of osteoblasts. (B) The ALP activity changes with the differentiation time of osteoblasts. (C) Expression levels of Runx2, Osteocalcin, and Col1a1 during the differentiation process of osteoblasts

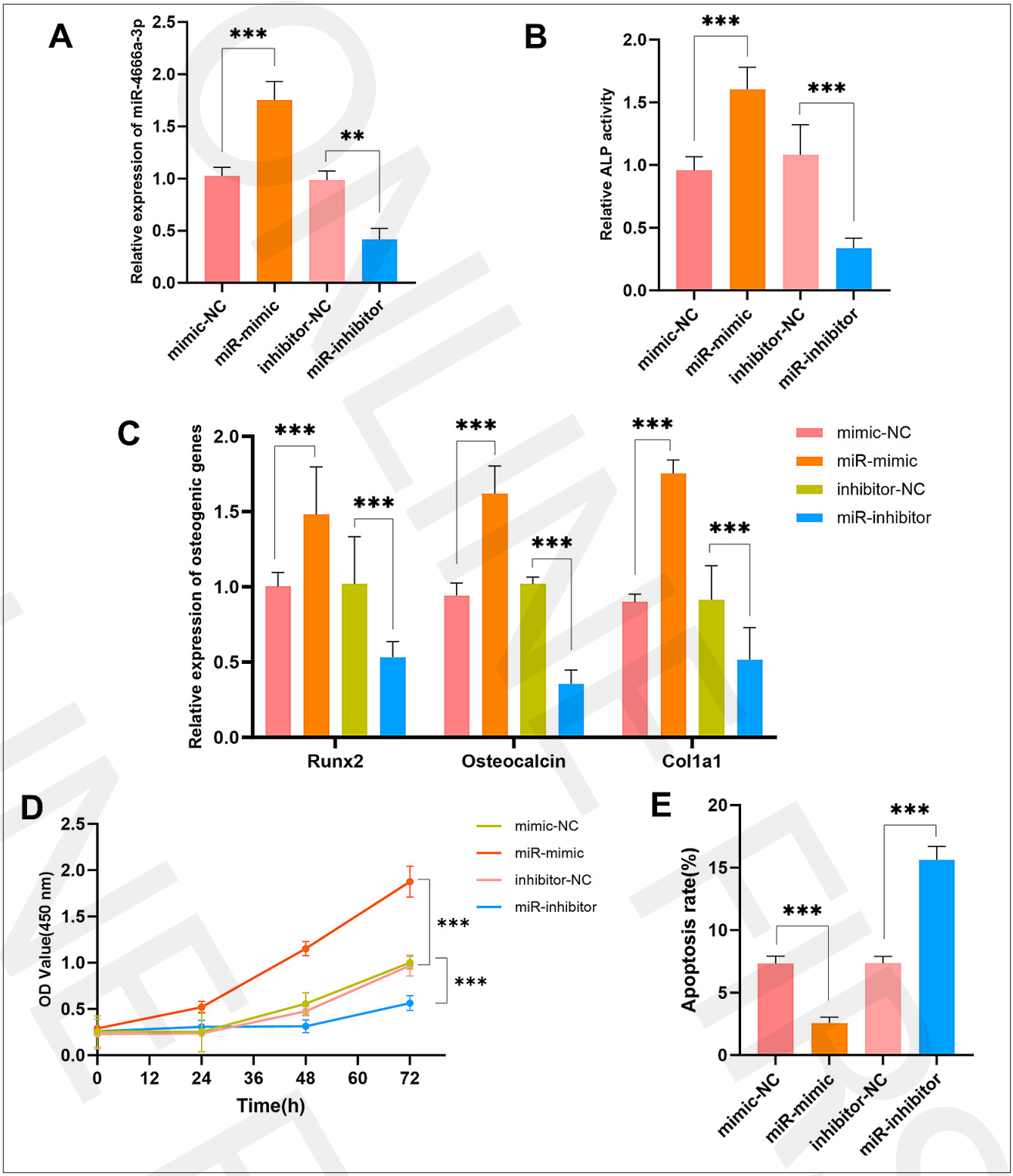

Figure 3. The effect of miR-4666a-3p on osteoblast differentiation. (A) Changes in expression levels after upregulation and downregulation of miR-4666a-3p. (B-E) Overexpression of miR-4666a-3p promotes (B) ALP activity, enhances (C) expression of osteogenic differentiation markers (Runx2, Osteocalcin, and Col1a1), promotes (D) osteoblast proliferation, and inhibits (E) cell apoptosis; inhibition of miR-4666a-3p expression produces the opposite effect

and Col1a1), while its knockdown downregulated these factors (Fig. 3C). Cell viability and apoptosis assays were performed to assess the functional effects of miR-4666a-3p in hFOB1.19 cells. CCK-8 assays showed that miR-4666a-3p overexpression significantly promoted cell viability at 48 and 72 hours compared to mimic-NC controls, while its inhibition markedly reduced viability relative to inhibitor-NC controls (Fig. 3D). Flow cytometry analysis further demonstrated that miR-4666a-3p overexpression decreased apoptosis, whereas its inhibition increased apoptotic rates (Fig. 3E).

MiR-4666a-3p interacts with GSK3B. GSK3B was predicted to be the downstream target of miR-4666a-3p. RT-qPCR showed progressively elevated GSK3B transcript levels in

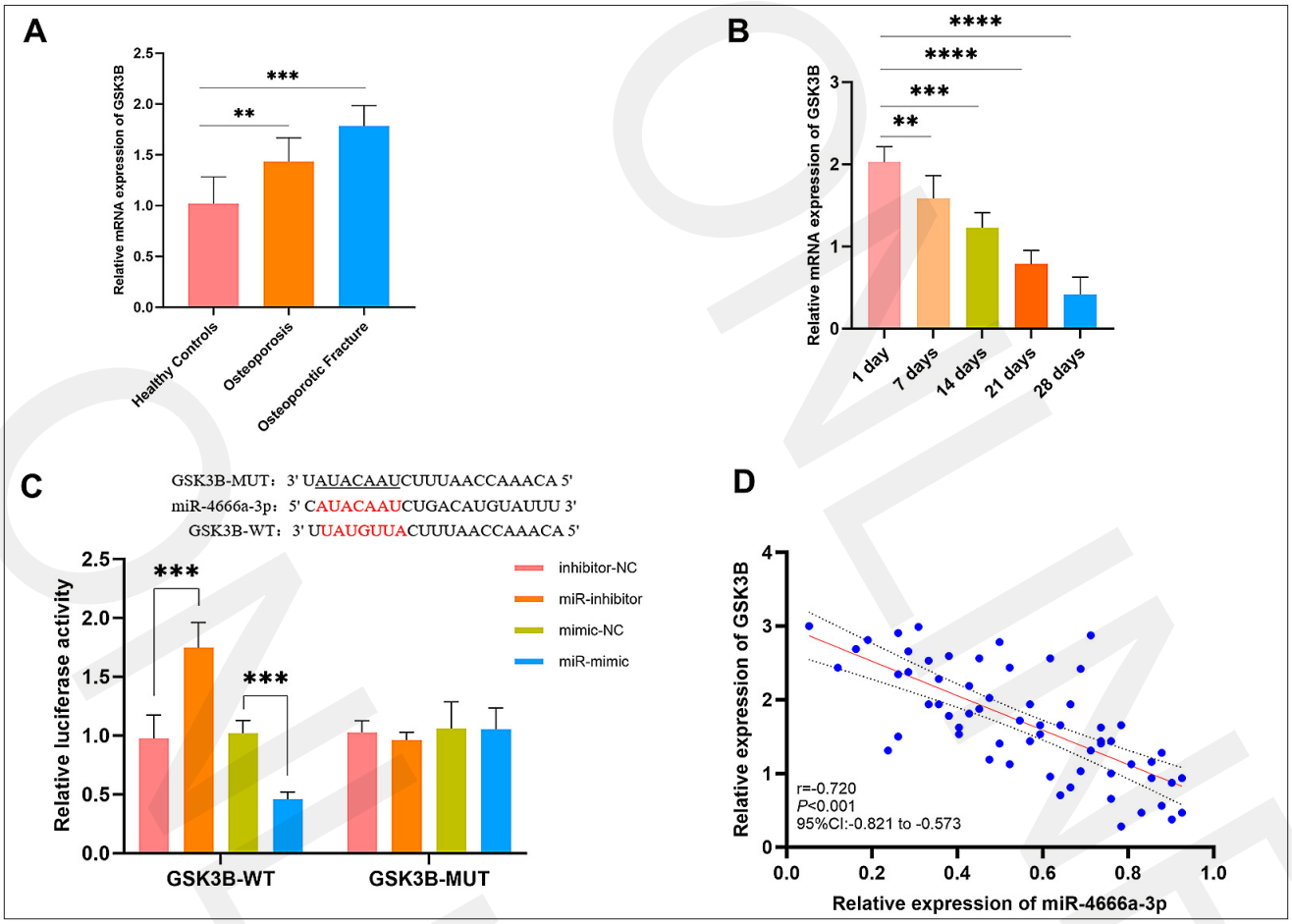

Figure 4. miR-4666a-3p interacts with GSK3B. (A) Expression of GSK3B in healthy controls, OP, and OF. (B) GSK3B expression changes with the recovery time of fracture patients. (C) Dual luciferase assay. (D) Pearson's test for analyzing the correlation between miR-4666a-3p and GSK3B

serum from healthy controls to OP patients, and further increased expression in OF patients (Fig. 4A). Additionally, GSK3B levels gradually decreased during the recovery phase of osteoporotic fractures (Fig. 4B), demonstrating an inverse correlation with miR-4666a-3p expression.

Dual-luciferase reporter assays confirmed direct regulatory interaction. In the GSK3B-WT construct, the miR-4666a-3p inhibitor significantly enhanced the luciferase activity, while the overexpression of miR-4666a-3p inhibited its activity. No effects were observed with the GSK3B-MUT construct (Fig. 4C). Furthermore, Pearson correlation analysis revealed a significant negative correlation between miR-4666a-3p and GSK3B expression (Fig. 4D), collectively establishing GSK3B as a direct target of miR-4666a-3p.

GSK3B reversed the promoting effect of miR-4666a-3p on osteogenic differentiation. To further investigate the regulatory effect of miR-4666a-3p on GSK3B, the interaction between them was examined in hFOB1.19 cells. Overexpression of miR-4666a-3p significantly reduced GSK3B mRNA levels, while simultaneous overexpression of both miR-4666a-3p and GSK3B restored GSK3B expression (Fig. 5A).

Functional rescue experiments demonstrated that GSK3B overexpression reversed miR-4666a-3p's pro-osteogenic effects. ALP activity assays showed that the overexpression of GSK3B significantly weakened the promoting effect of miR-4666a-3p on ALP levels (Fig. 5B). Similarly, qRT-PCR analysis revealed that GSK3B overexpression counteracted miR-4666a-3p-induced upregulation of osteogenic markers (Runx2, Osteocalcin, and Col1a1) (Fig. 5C). The CCK-8 results showed that the overexpression of GSK3B inhibited the phenomenon of enhanced cell viability caused by miR-4666a-3p (Fig. 5D). Flow cytometry analysis indicated that the overexpression of GSK3B restored the decreased cell apoptosis rate caused by miR-4666a-3p (Fig. 5E).

DISCUSSION

OP is the third most common chronic disease worldwide. OF, as its most common complication, often occurs in postmenopausal women and the elderly population, especially among the elderly who have engaged in heavy physical labour for a long time [5, 14]. Numerous investigations have revealed that miRNAs play a crucial regulatory role in the occurrence and development of OF. Previous research has indicated that miR-4666a-3p may play an important role in the pathological process of fracture-related diseases [11]. In the current study, all the enrolled patients were diagnosed through DEXA scans (with a bone hip T value ≤ -2.5), and confounding factors were excluded under the exclusion criteria. On this basis, the expression level of miR-4666a-3p in the serum of OF patients found that its expression was significantly downregulated, and it gradually increases during the recovery process of patients with OF. This result is consistent with the conclusion

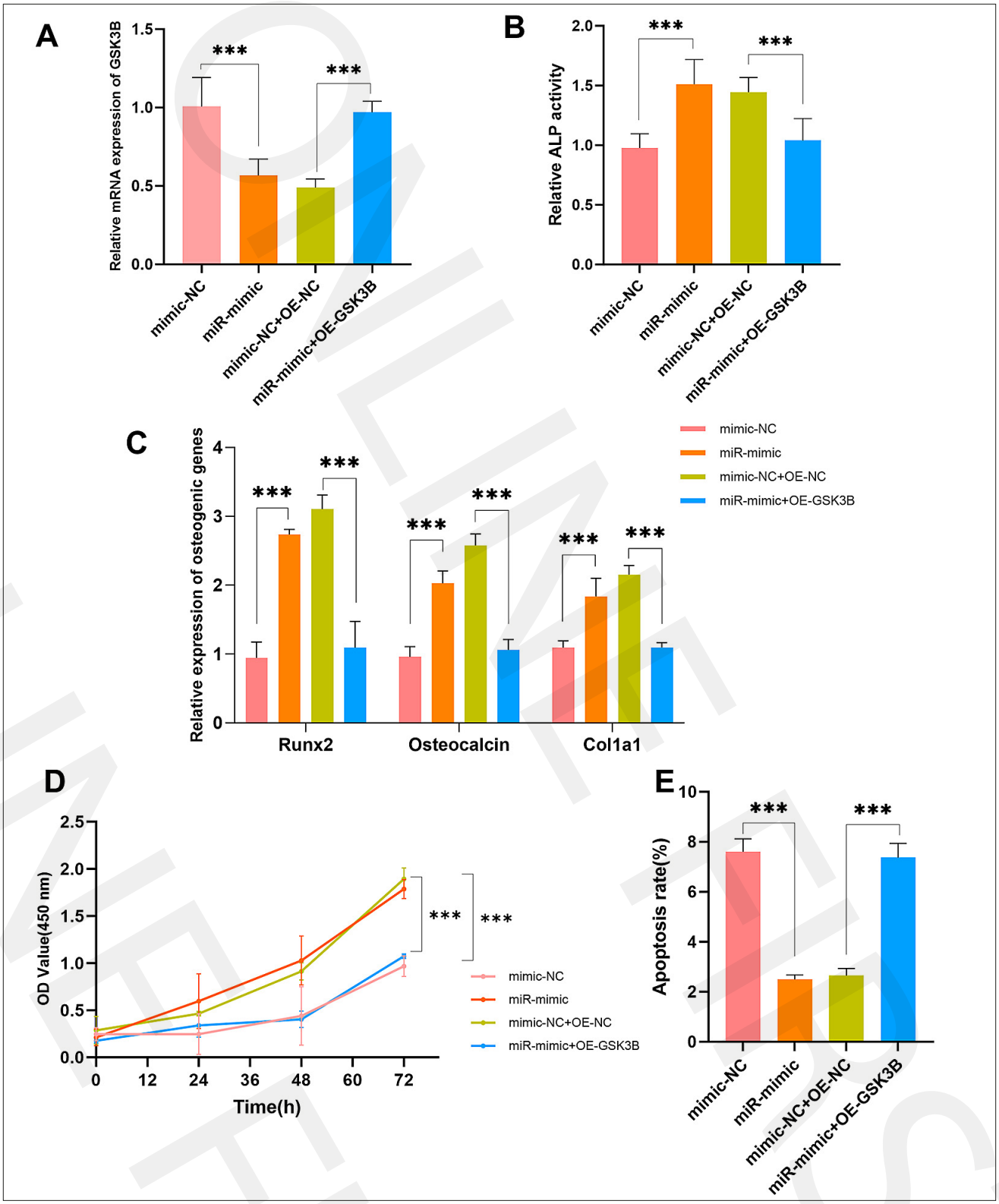

Figure 5. GSK3B reverses the effect of miR-4666a-3p on osteoblasts. (A) GSK3B levels after miR-4666a-3p/GSK3B overexpression. (B-E) Overexpression of GSK3B reduces (B) ALP activity, downregulates (C) the expression of osteogenic differentiation markers (Runx2, Osteocalcin, and Col1a1), inhibits (D) osteoblast activity, and promotes (E) osteoblast apoptosis

of Lei et al. [12] and the expression trend in the GSE93883 database. Meanwhile, the bone density T-score, RANKL, and OPG showed significant differences among the healthy controls, OP, and patients with OF. The T-score of bone density is a core indicator for diagnosing osteoporosis and quantifying the risk of fractures, and is recognized as one of the most critical risk factors for OF [2, 3].

The RANKL/OPG system serves as the core regulatory hub of bone metabolism, directly determining the rate of bone resorption and the balance of bone remodelling [5].

Similar to these key indicators, the differential expression of miR-4666a-3p in OF suggests that it may play an important role in the pathogenesis of OF. Furthermore, miR-4666a-3p can effectively distinguish between patients with OP and OF, and miR-4666a-3p can predict the risk of OF from OP, and is a protective factor for OF. This further supports its possible inhibitory regulatory role in the occurrence and development of OF, and possesses a potential clinical warning and intervention value.

The core pathological mechanism of OF lies in the imbalance of bone metabolism and the destruction of bone microstructure. Among them, the dysfunction of osteoblasts is the key link that drives the insufficient bone formation. hFOB1.19, as a human-derived osteoblast model, provides a crucial research tool for revealing and targeting the intervention of osteoblast function defects in OF [15]. Previous studies have verified gene functions by inducing osteogenic differentiation of hFOB1.19 [16]. The current study conducted osteogenic induction on hFOB1.19 cells. The experiment further revealed that as the induction time increased, the ALP activity and the expression levels of osteogenic-related markers (Runx2, Osteocalcin, Col1a1) all showed a gradient increase over time. An increase in ALP activity indicated active early differentiation of osteoblasts, which can serve as a reference marker for the initiation of early fracture repair in OF [17, 18]. The upregulation of Runx2 and Osteocalcin expression jointly reflects the acceleration of bone formation and mineralization process, which is commonly seen in the reparative response of OF [19]. An increase in Col1a1 expression indicates an increase in type I collagen synthesis, directly supporting the reconstruction of bone matrix and microstructure repair [20]. These indicate that osteoblast differentiation has been successful. Moreover, as the differentiation time is prolonged, the level of miR-4666a-3p significantly increases, which is contrary to the clinical outcome trend, suggesting that miR-4666a-3p may promote osteoblast differentiation. Osteoblast activity is the foundation for maintaining the ability of bone formation and bone strength. Its functional decline directly leads to an increased risk of OF [21]. The Increase in osteoblast apoptosis further weakens the ability of bone formation by reducing the number of functional cells, and is a key pathological mechanism for the progression of OP and the impairment of fracture repair [22]. Functional gain and loss experiments were conducted on miR-4666a-3p. After overexpression of miR-4666a-3p, ALP activity, related marker levels, and cell activity significantly increased, while the apoptosis rate decreased; inhibiting its expression produced the opposite effect. These further confirmed that miR-4666a-3p can stimulate the osteoblast differentiation and activity of HFOB1.19 cells.

MiRNA exerts its regulatory function in diseases by inhibiting the expression of target genes at the post-transcriptional level. This is the core mechanism through which miRNA achieves its regulatory role. The current study predicts that GSK3B – a multifunctional serine/threonine protein kinase – is the downstream target of miR-4666a-3p. GSK3B serves as a key regulator of the Wnt/β-catenin signalling pathway, which plays an essential role in promoting osteogenic differentiation [23]. GSK3B can be targetted by miR-155-5p, which elevates its expression and subsequently suppresses Wnt signalling. This mechanism ultimately attenuates osteogenic differentiation and cell proliferation [24].

The above findings indicate the considerable potential of GSK3B in osteoblasts. The study demonstrates that GSK3B was highly expressed in OP and OF patients, and significantly downregulated during the recovery period of patients with OF, showing a negative correlation with miR-4666a-3p, and that there was a targeted binding relationship between them. Functional restoration experiments further showed that GSK3B could reverse the promoting effect of miR-4666a-3p on osteoblast differentiation and activity. The results suggest that miR-4666a-3p may regulate osteoblasts by inhibiting GSK3B. These preliminarily results reveal the regulatory potential of the miR-4666a-3p/GSK3B axis in osteoblast differentiation. This mechanism may be involved in the pathogenesis of OP, providing a new perspective for the study of its pathological mechanism.

CONCLUSION

In summary, miR-4666a-3p is significantly downregulated in patients with OF and could predict the risk of OF occurrence. During osteogenic differentiation, the expression of miR-4666a-3p is significantly upregulated, which can effectively promote osteoblast differentiation and activity. This effect can be reversed by its target gene GSK3B. These findings suggest that, at both functional and mechanistic levels, miR-4666a-3p may participate in the occurrence and development of OF by targeting GSK3B, providing a new theoretical basis for the clinical management and early intervention research of OF.

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