Original Article

miR-296-3p Controls Osteogenic Proliferation and Differentiation by Targeting ICAT and Is Involved in Fracture Healing

(fragility fractures / miR-296-3p / ICAT / BMSCs)

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Abstract. Fragility fractures have been a cause for concern because of their high incidence. For the prevention and treatment of osteoporotic fractures, it is important to understand how to promote bone formation and increase bone mass. This study investigated miR-296-3p expression and function in fragility fracture. The study enrolled 98 patients with hip fractures, 90 patients with wrist fractures and 35 healthy controls. RT-qPCR was used to detect the miR-296-3p level changes before and after surgery in fracture patients and during the differentiation of human bone mesenchymal stem cells (BMSCs). The starBase bioinformatics database was used for prediction of the miR-296-3p target gene, and dual luciferase report was used for verification of the target relationship. Our results demonstrated that miR-296-3p levels are up-regulated in fracture patients, while they gradually decrease during human BMSC differentiation. The up-regulation of miR-296-3p inhibited the proliferation and differentiation ability of human BMSCs, while inhibition of its expression had the opposite effects. miR-296-3p negatively regulates osteogenic differentiation, and over-expression of inhibitor of β-catenin and TCF (ICAT) could counteract the negative regulatory effect. miR-296-3p targets ICAT and affects the expression of key proteins in the Wnt/β-catenin signalling pathway. In conclusion, miR-296-3p can regulate the division and differentiation of osteoblasts by affecting the expression of ICAT and participate in fracture healing.

Introduction

Fragility fracture is the breaking of a bone due to its own fragility under normal or light external force. The main cause of fragility fractures is osteoporosis (OP) (Dimai and Fahrleitner-Pammer, 2022). OP is common in middle-aged and elderly people, especially menopausal women. In 2016, the prevalence of OP in people over 60 years old in China was 36 %, with 23 % in men and 19 % in women (Tian et al., 2017). OP is initially asymptomatic until fragility fractures occur. Additionally, once a fragility fracture happens, the risk of refracture markedly increases (Rinonapoli et al., 2021). OP and fractures seriously impact the lives of elderly people. Osteoblasts are key cells in bone formation, development and growth. They synthesize, secrete and mineralize the bone matrix for bone reconstruction. The activity, proliferation and differentiation of these cells are crucial factors affecting bone formation (Abdelmagid et al., 2014; Blair et al., 2017). Therefore, promoting bone formation to increase bone mass is significant for the prevention and treatment of osteoporotic fractures.

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Abbreviations: ALP – alkaline phosphatase, BMD – bone mineral density, BMSCs – bone mesenchymal stem cells, ICAT – inhibitor of β-catenin and TCF, MSCs – mesenchymal stem cells, OA – osteoarthritis, OP – osteoporosis, TGF-β – transforming growth factor β.

MicroRNAs (miRNAs) have been shown to be essential for pre-osteoblast proliferation and differentiation (Vimalraj and Selvamurugan, 2013; Papaioannou et al., 2014; Narayanan et al., 2019). Pre-osteoblasts are produced from multipotent mesenchymal stem cells (MSCs) and can differentiate into osteoblasts upon receiving osteogenic signals (Foroutan, 2016). Therefore, the bone-forming differentiation process involves two stages of directional differentiation of MSCs into pre-osteoblasts and further differentiation into mature osteoblasts. Several studies have found that miRNAs can influence BMSCs by various pathways to differentiate into osteoblasts, promote bone formation, improve bone quality and reduce symptoms of OP (Iaquinta et al., 2021).

Studies have shown that miR-296 is abnormally expressed during the process of osteoclast formation (Ma et al., 2016). Research has shown that miR-296-3p is related to chondrocyte apoptosis and participates in the pathogenesis of osteoarthritis (OA) (Zhou et al., 2021). Research also indicates that miR-296-3p/FOSL1 is linked to the differentiation of dental pulp stem cells into bone cells (Liu et al., 2023). Previous studies have shown that miR-296-3p is associated with osteogenic differentiation. However, the specific expression of miR-296-3p in osteoporotic fractures and its influence on osteogenic differentiation during fracture healing still need to be studied.

Therefore, the expression and role of miR-296-3p in fragility fractures will be assessed in this study.

Material and Methods

Clinical specimens

This study selected 98 hip fracture patients and 90 wrist fracture patients at Zunyi Medical University Affiliated Hospital as the experimental group. Inclusion criteria: patients with clinical manifestations of fragility fracture and a clear imaging diagnosis. Patients with severe hepatic and renal dysfunction, coagulopathy, arthritis and systemic immune disorders were excluded. Additionally, 35 healthy persons of similar age who underwent a physical examination at the same time were selected as controls, excluding those with osteoporosis, malignant tumours and neurological disorders. All patients with fragility fractures underwent a bone mineral density evaluation via CT examination to determine the severity and position of the fractures. The experimental group received open reduction surgery and was then treated with plaster or stent fixation.

All subjects had morning fasting blood samples, and serum was separated for later use. All subjects consented to this study and signed an informed consent form. This study received approval from the Affiliated Hospital of Zunyi Medical University Medical Ethics Committee.

Cell culture and transfection

Human BMSCs were purchased from Saiye Biotechnology (Guangzhou, China). BMSCs were seeded in α-MEM medium containing 10 % foetal bovine serum (FBS) (SenBeiJia Biological Technology Co., Ltd., Nanjing, China), at 37 °C and 5 % CO_2 in a 100 % humidity incubator for subculture. The cells were cultured to the 3rd generation before being seeded in 96-well plates. According to the treatment, they were divided into untreated group, mimic negative control (NC group), inhibitor NC group, miR-296-3p mimic group and miR-296-3p inhibitor group. The untreated group was not transfected. The NC groups were transfected with miRNA-NC. The miR-296-3p mimic group was transfected with miR-296-3p mimics to mimic high expression of miR-296-3p, and the miR-296-3p inhibitor group was transfected with miR-296-3p inhibitor to mimic low expression of miR-296-3p. The transfection plasmids were constructed by Nanjing Yaoshunyu Biotechnology Co., Ltd. The cell recovery experiment was divided into untreated group, miR-296-3p mimic transfected group and mimic NC group. The recombinant vector pcDNA3.1-ICAT (OE-ICAT) group and pcDNA3.1 vector (OE-NC) group were also transfected.

Osteogenic induction

Osteogenic induction was performed in cells in the osteogenic induction groups and each transfection group. The culture medium of cells was replaced with osteogenic differentiation medium (α-MEM medium containing 0.2 % ascorbic acid, 0.01 % dexamethasone, 1 % β-glycerophosphate sodium, 1 % penicillin-streptomycin and 10 % FBS) when the cell confluence reached 80 %. The cells were then cultured at 37 °C in a 5 % CO_2 incubator.

Real-time quantitative PCR

Total RNA was extracted from serum and BMSCs according to the instructions of the TRIzol kit (Fanshi Biotechnology Co., Ltd., Shanghai, China). The cDNA was synthesized using a reverse transcription kit (Ita Biotechnology Co., Ltd., Beijing, China). Amplification was performed according to the real-time PCR kit instructions (Fusheng Industrial Co., Ltd., Shanghai, China). The gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method with β-actin as the internal reference.

Cell proliferation assay

The cell counting kit-8 (CCK-8) (Huzhen INDUSTRIAL Co., Ltd., Shanghai, China) was used to estimate cell viability. Transfected cells were transferred to 96-well plates and incubated at 37 °C for 24, 48 and 72 hours. Then, 10 µl of CCK-8 solution was added. The mixture was incubated at 37 °C for 1 hour, and the absorbance at 450 nm was measured.

Detection of alkaline phosphatase (ALP) activity

Two hundred µl of lysis buffer was added to the transfected cells in 96-well plates. The cells were processed according to the instructions of the ALP detection kit. The ALP activity of BMSCs in each group was calculated by measuring the absorbance of BMSCs at a wavelength of 520 nm using a microplate reader.

Luciferase activity assay

The bioinformatics database starBase (https://rnasysu.com/encori/ago) predicted the target gene of miR-296-3p. Using psiCHECK2, both psiCHECK2-ICAT-WT and psiCHECK2-ICAT-mut reporter vectors were constructed. The cells were seeded in 96-well plates and transfected with either wild-type or mutant reporter vectors. After 24 hours, following the dual luciferase reporter gene reagent instructions, the Renilla and firefly luciferase intensity was detected in each well.

Western blot

Proteins were extracted from the transfected cells and their concentrations were determined by the BCA method. Ten µl of protein samples were subjected to 10 % SDS-PAGE electrophoresis and then transferred to PVDF membranes by the semi-dry method. The membranes were blocked with 5 % skim milk at room temperature for 2 hours. Rabbit antibodies against β-catenin (1 : 1000), cyclin D1 (1 : 1000), c-Myc (1 : 1000) and β-actin (1 : 1000) (Cell Signaling Technology, Danvers, MA) were added and incubated at 4 °C for 12–14 hours. The membranes were washed with TBST for five times. Goat anti-rabbit IgG secondary antibody (1 : 2000) (Cell Signaling Technology) was added to the blocking solution and incubated at room temperature for 2 hours, followed by five washes with TBST. The ECL exposure solution was dropped onto the membrane and exposed in the gel imaging system. The grey values of each antibody band were analysed using Image J 1.8.0 software and the grey value of the target protein was normalized with β-actin.

Statistical analysis

The SPSS 26.0 software was used for data analysis. The GraphPad Prism 9.0 software was employed for analysis and charting. The data were expressed as mean ± SD after testing for normality and homogeneity of variance. The Student's *t*-test was used for comparison between two groups. For normally distributed data, one-way analysis of variance (ANOVA) was employed to compare differences between the groups. Subsequently, Dunnet's test was used to further compare whether there were differences between each pair of groups. The non-parametric Kruskal-Wallis test was used to compare the differences between groups when the data were not normally distributed. $P < 0.05$ was used to determine statistical significance.

Results

Clinical data of subjects

Age, sex and BMI did not differ significantly between healthy persons and hip and wrist fracture patients ($P > 0.05$). Nevertheless, bone mineral density (BMD) T-scores of patients with hip and wrist fractures were markedly different from those of the healthy group ($P < 0.001$). The T-scores of the healthy controls were all greater than -1, indicating normal bone mineral density, while the T-scores of the patients with hip and wrist fractures were less than –2.5, indicating OP (Table 1).

Changes in miR-296-3p during BMSC differentiation

To explore the role of miR-296-3p in fracture, we first examined the levels of miR-296-3p in patients with fractures and in healthy individuals. We found that miR-296-3p levels were markedly increased in the hip and wrist fracture patients (Fig. 1A**)**. To further clarify the relationship between miR-296-3p and fracture healing, we measured the miR-296-3p levels in fracture patients after surgical intervention. We found that the miR-296-3p levels in hip and wrist fracture patients gradually decreased over time postoperatively, reaching their lowest level at four weeks postoperatively (Fig. 1B–C). We further detected changes in the miR-296-3p levels during BMSC differentiation. The findings showed that miR-296-3p expression gradually declined during the osteogenic differentiation of BMSCs (Fig. 1D). Furthermore, the osteogenesis-related genes increased over time of induction as the BMSCs were induced to differentiation

Table 1. General information on the subjects

	Control (N = 35)	Hip fracture (N = 98)	Wrist fracture (N = 90)	P value
Age (years)	64.82 ± 9.15	65.68 ± 9.30	66.48 ± 9.93	0.681
Gender (M/F)	14 / 21	45 / 53	40 / 50	0.211
BMI (kg/m^2)	23.54 ± 1.61	24.15 ± 1.98	23.92 ± 2.06	0.089
T-score	–0.23 ± 0.81	–3.20 ± 1.23	–3.21 ± 1.27	P < 0.001***

BMI, body mass index. *** $P < 0.001$

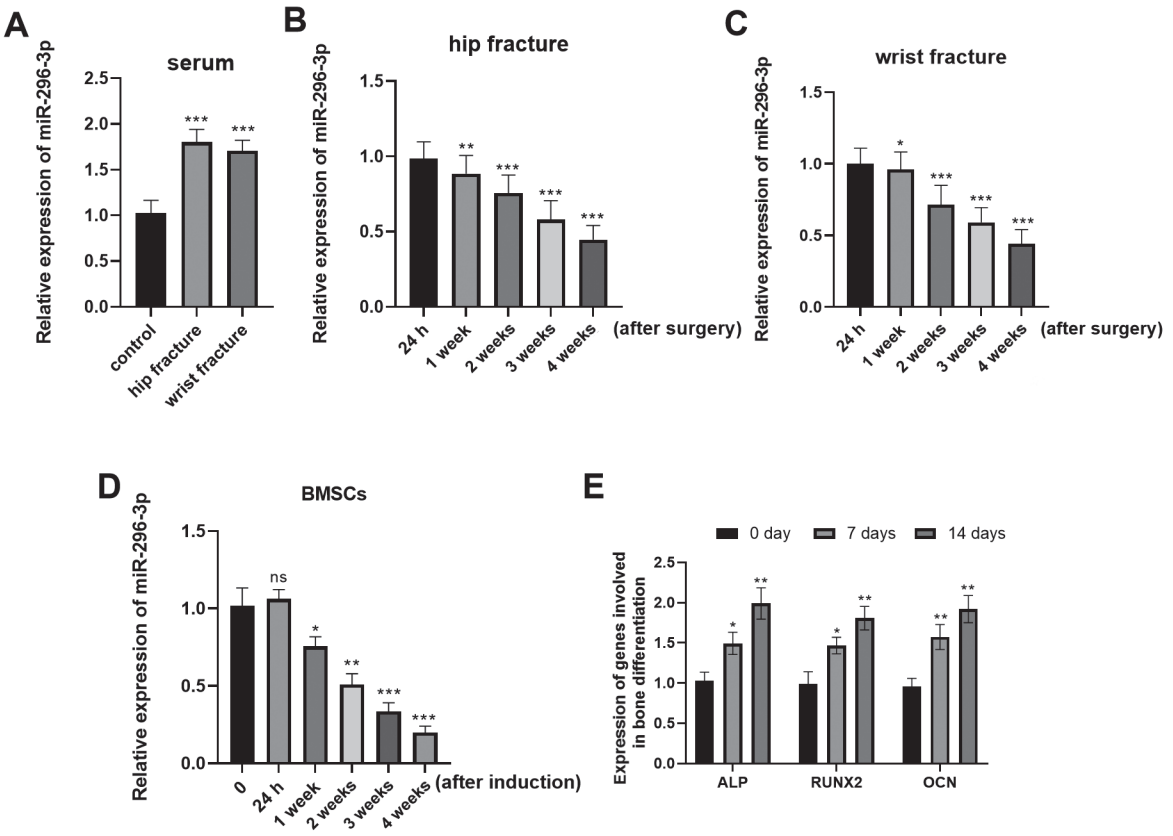

Fig. 1. The levels of miR-296-3p change during the differentiation of BMSCs. Compared with healthy controls, the miR-296-3p level was markedly elevated in the hip and wrist fracture patients (**A**). The miR-296-3p level in hip and wrist fracture patients gradually decreased over time postoperatively, reaching its lowest level at four weeks postoperatively (**B–C**). miR-296-3p expression gradually decreased during the osteogenic differentiation of BMSCs (**D**). The expression of osteogenesis-related genes gradually increased during the osteogenic differentiation of BMSCs. (**E**). ns $P < 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

(Fig. 1E). This is an indication that miR-296-3p may be part of the fracture healing process.

Role of miR-296-3p in the proliferation and osteogenic differentiation of BMSCs

To elucidate the function of miR-296-3p in bone fracture, BMSCs expressing aberrant miR-296-3p were obtained by transfecting cells. According to Figure 2A, compared with the control group, the expression level of miR-296-3p in the cells transfected with miR-296-3p mimics was significantly increased. In contrast, transfection of miR-296-3p inhibitor significantly reduced the expression level of miR-296-3p. This indicated successful transfection. As shown in Figure 2B, the cell proliferation assay indicated that the growth of BMSCs transfected with the miR-296-3p mimic was inhibited compared to the NC control, and the proliferation of BMSCs transfected with the miR-296-3p inhibitor was markedly enhanced compared to the NC control. As shown in Figure 2C, compared to the control group, the ALP activity of BMSCs transfected with miR-296-3p mimic was inhibited, while the ALP activity of BMSCs transfected with miR-296-5p inhibitor was enhanced. Figure 2D shows detection of the levels of genes related to osteogenic differentiation. We found that up-regulation of miR-296-3p markedly decreased mRNA expression of RUNX2 and OCN, while knockdown of miR-296-3p markedly increased mRNA expression of RUNX2 and OCN. These results suggested that overexpression of miR-296-3p inhibited the proliferation and osteogenic differentiation of BMSCs.

Targeting the relationship between miR-296-3p and ICAT

The bioinformatic analysis showed that there are complementary binding sites between the 3' UTR of the ICAT mRNA and miR-296-3p (Fig. 3A). The dual luciferase reporter assay showed that the miR-296-3p mimic inhibited wild-type reporter vector luciferase activity, whereas the miR-296-3p inhibitor promoted the wild-type reporter vector luciferase activity (Fig. 3B**)**. The RT-qPCR and Western blot assays demonstrated that the

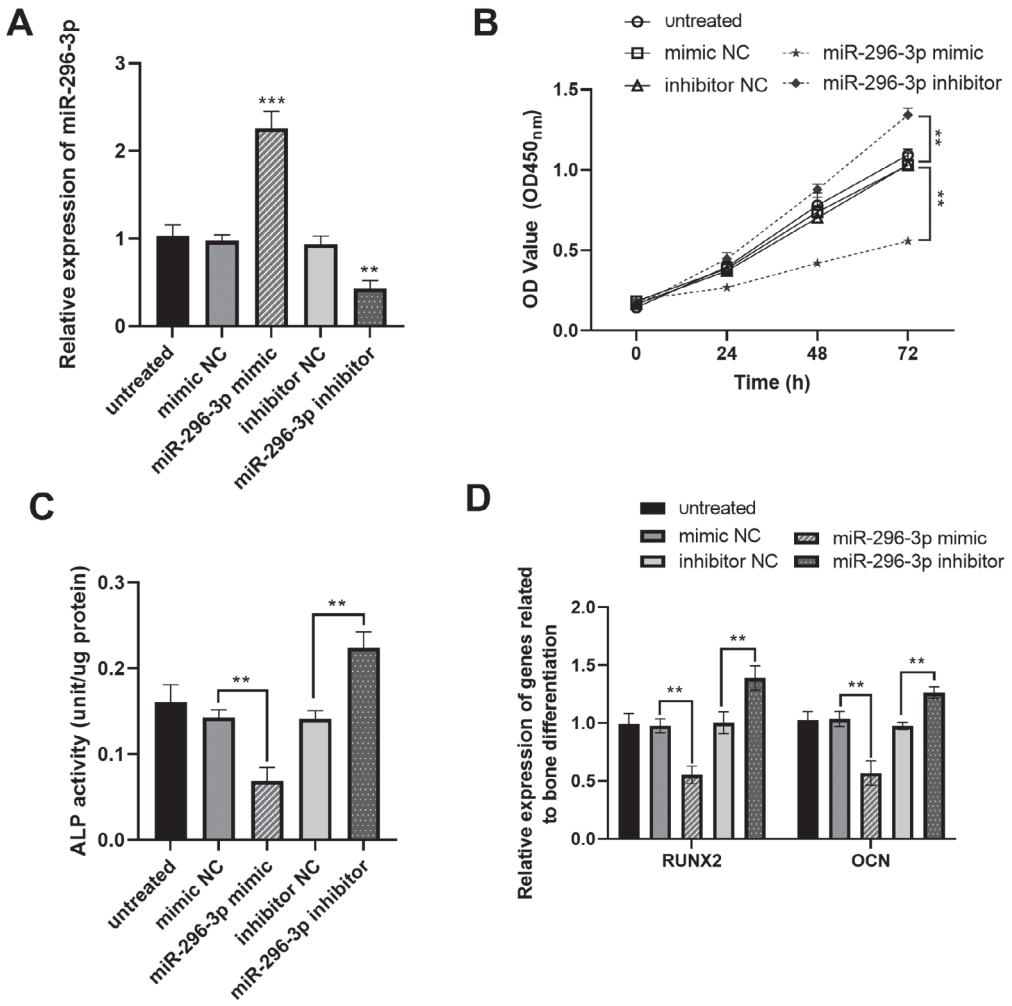

Fig. 2. Role of miR-296-3p in the proliferation and osteogenic differentiation of BMSCs. The miR-296-3p expression level in cells transfected with miR-296-3p mimic was markedly higher than that in the control group, whereas the miR-296-3p expression level in cells transfected with the miR-296-3p inhibitor was markedly lower than that in the control group (**A**). The proliferation and ALP activity of BMSCs were inhibited by up-regulation of miR-296-3p, while inhibition of its expression had the opposite effects (**B–C**). Up-regulation of miR-296-3p suppressed the mRNA levels of RUNX2 and OCN, whereas down-regulation of miR-296-3p had the opposite effect (**D**). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

up-regulation of miR-296-3p suppressed the ICAT level, while the knockdown of miR-296-3p had the opposite effect (Fig. 3C–E). Furthermore, the expression of ICAT in patients with hip fracture and wrist fracture was markedly higher than in healthy controls ($P < 0.001$) (Fig. 3F). There was a marked negative correlation between miR-296-3p and ICAT expression using Pearson correlation analysis (Fig. 3G–H**)**. The level of ICAT expression gradually increased during the differentiation of the BMSCs (Fig. 3I).

miR-296-3p participates in BMSC differentiation by targeting ICAT

The cell recovery assay further verified the role of miR-296-3p targeting ICAT in fractures. RT-qPCR and Western blot assays revealed that the level of miR-296-3p was significantly elevated in BMSCs transfected with miR-296-3p mimic, while the expression of ICAT was significantly decreased. However, after co-transfection with miR-296-3p mimic and e-ICAT, there was no difference in the level of miR-296-3p, but the expression of ICAT significantly increased (Fig. 4A–D). Meanwhile, miR-296-3p mimic + OE-NC transfection inhibited cell viability, whereas miR-296-3p mimic + OE-ICAT transfection alleviated this inhibitory effect (Fig. 4E**)**. Additionally, transfection of BMSCs with miR-296-3p mimic reduced the levels of osteogenic differentiation-related gene markers, while transfection of miR-296-3p mimic + OE-ICAT alleviated the inhibitory effect of miR-296-3p on the osteogenic differentiation of BMSCs (Fig. 4F).

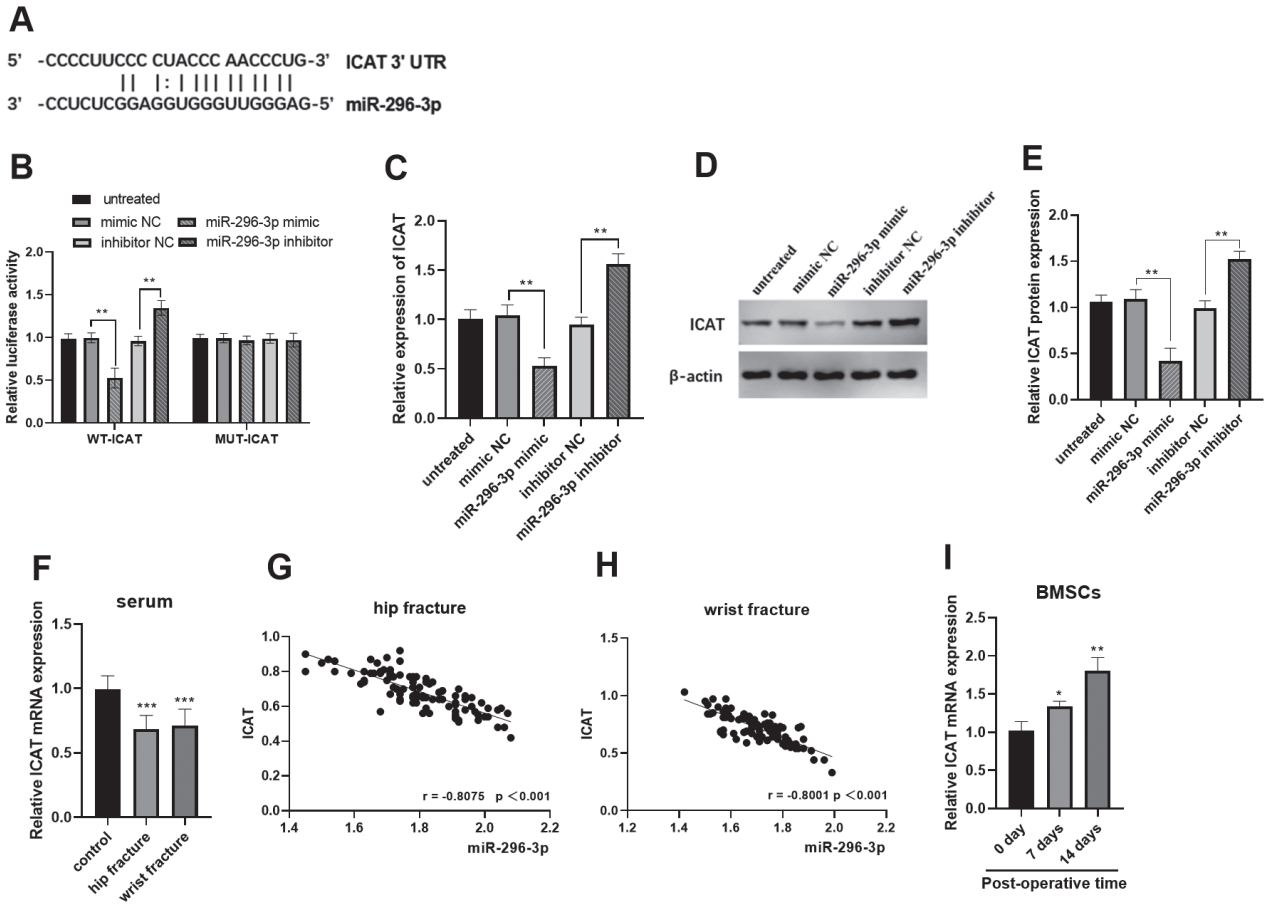

Fig. 3. Targeted association of miR-296-3p with ICAT. There are complementary binding sites between the 3' UTR of the ICAT mRNA and miR-296-3p (**A**). The miR-296-3p mimic inhibited wild-type reporter vector luciferase activity, whereas the miR-296-3p inhibitor promoted wild-type reporter vector luciferase activity. However, there was no effect on the mutated reporter vector (**B**). The RT-qPCR and Western blot assays demonstrated that the up-regulation of miR-296-3p suppressed the ICAT level, while the knockdown of miR-296-3p had the opposite effect (**C–E**). The expression of ICAT in patients with hip fracture and wrist fracture was markedly higher than in healthy controls ($P < 0.001$) (**F**). There was a marked negative correlation between miR-296-3p and ICAT expression using Pearson correlation analysis (**G–H**). The level of ICAT expression gradually increased during the differentiation of the BMSCs (**I**). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Effect of miR-296-3p targeting ICAT on the expression of key proteins in the Wnt/β-catenin pathway

The results of Western blot assay on the effect of miR-296-3p targeting ICAT on the expression of key proteins in the Wnt/β-catenin signalling pathway are shown in Figures 4H and 4G. miR-296-3p significantly inhibited the expression of key proteins in the Wnt/β-catenin signalling pathway (β-catenin, cyclin D1, c-Myc), while ICAT could partially reverse this inhibitory effect.

Discussion

Differentiation of osteoblasts is key to bone healing. Osteoblasts, derived from undifferentiated multipotent MSCs, are crucial in the bone formation process (Donsante et al., 2021). BMSCs can differentiate into various cell types under the influence of numerous factors (Bäckesjö et al., 2009). The irreplaceable value of BMSCs in bone remodelling has been demonstrated in previous studies (Wei et al., 2024). The induction of osteogenic differentiation of BMSCs can be effective in reducing the risk of osteoporotic bone fractures. Recent studies on osteoblast differentiation have shown that miRNAs, a class of post-transcriptional regulatory RNAs, are critical in controlling this process (Jiménez-Ortega et al., 2024). miRNAs are biological regulators that control various processes by silencing specific genes (Sereno et al., 2020). Research suggests that miRNAs are involved in regulating the osteogenic differentiation of BMSCs, but the specific mechanism is not yet fully understood (Zhao et al., 2021; Qiu et al., 2022).

Previous studies demonstrated the up-regulation of miR-485-5p in OP and its inhibition during osteogenic differentiation (Zhang et al., 2018). Studies have shown

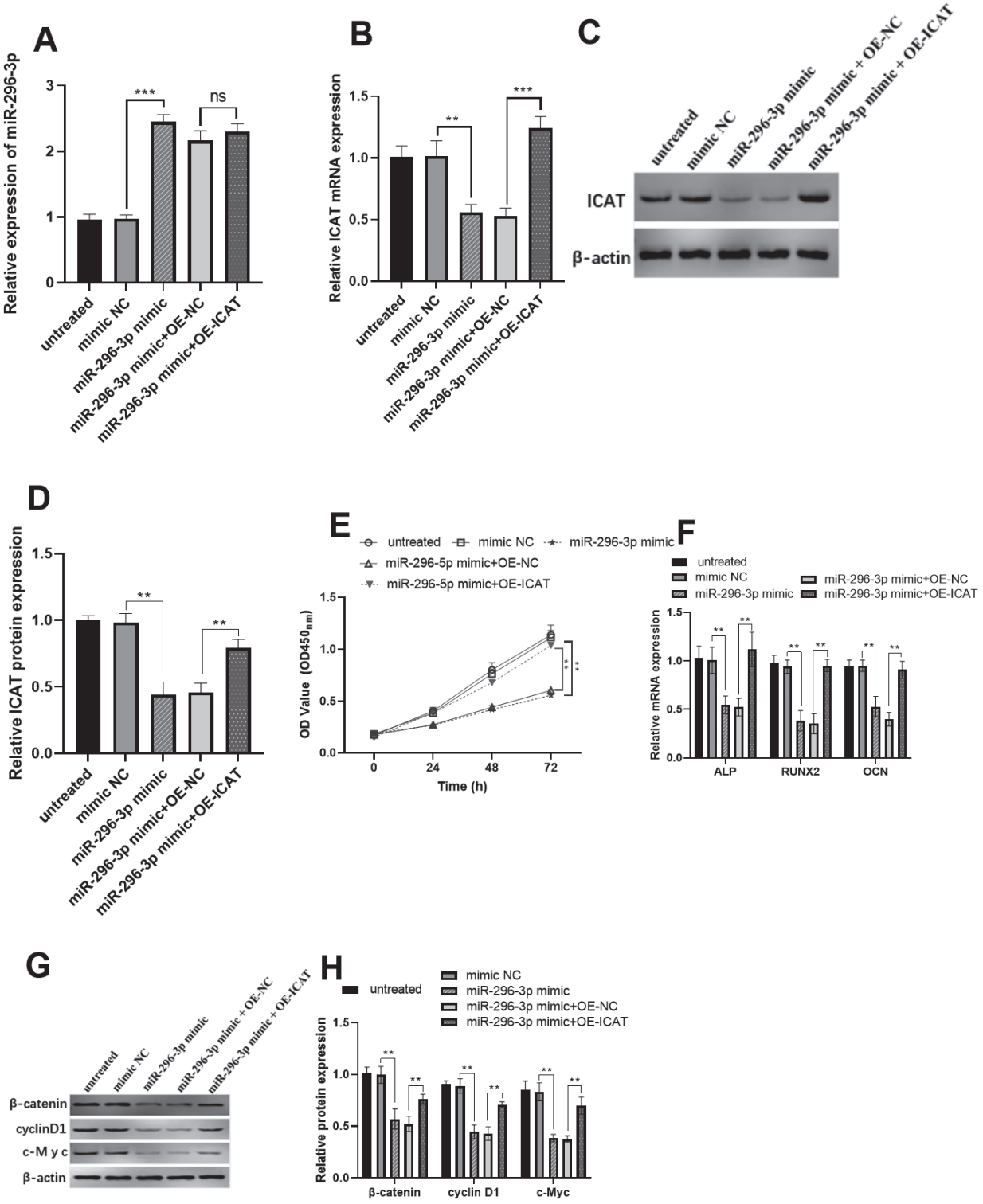

Fig. 4. miR-296-3p is involved in BMSC proliferation and differentiation by targeting ICAT. RT-qPCR and Western blot assays revealed that the level of miR-296-3p was significantly elevated in BMSCs transfected with miR-296-3p mimic, while the expression of ICAT was significantly decreased. However, after co-transfection with miR-296-3p mimic and e-ICAT, there was no difference in the level of miR-296-3p, but the expression of ICAT significantly increased (**A–D**). miR-296-3p inhibited cell viability, whereas ICAT alleviated this inhibitory effect (**E**). miR-296-3p reduced the levels of osteogenic differentiation-related gene markers, while ICAT alleviated the inhibitory effect of miR-296-3p on the osteogenic differentiation of BMSCs (**F**). miR-296-3p significantly inhibited the expression of key proteins in the Wnt/β-catenin signalling pathway (β-catenin, cyclin D1, c-Myc), while ICAT could partially reverse this inhibitory effect (**G–H**). ns $P > 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

that miR-183 is elevated in OP and promotes its development (Qin et al., 2021). Elevated miR-214 levels have been linked to reduced bone formation in bone samples of older people with fractures (Wang et al., 2013). Our study is the first to show that serum miR-296-3p levels are markedly elevated after hip and wrist fractures and that the miR-296-3p levels decrease progressively over time after the treatment procedure. This suggests that miR-296-3p may be a part of the process of healing bone fractures. Studies have shown that up-regulation of miR-139-5p can inhibit the osteogenic differentiation of human BMSCs (Long et al., 2017). In osteoporotic BMSCs, over-expression of miR-4739 markedly inhibited osteogenic differentiation (Li et al., 2021). In BMSCs isolated from OP patients, the expression of miR-100-5p was up-regulated. Over-expression of miR-100-5p inhibited the proliferation and osteogenic differentiation of human BMSCs (Wang et al., 2022). Likewise, our results showed that miR-296-3p levels gradually decreased during human BMSC differentiation. Over-expression of miR-296-3p inhibited the proliferation, ALP activity and differentiation ability of human BMSCs, while inhibition of its expression had the opposite effect. Taken together, our findings indicate a potential function for miR-296-3p during fracture healing.

Studies have shown that miR-296-3p targets ICAT to enhance the proliferation of glioblastoma cells (Zhou et al., 2020). Over-expression of ICAT has been implicated in promoting osteogenic differentiation in previous studies (Kim et al., 2008). The E2 promoter binding factor 1 (E2F1) can promote the differentiation and adipogenesis of pre-adipocytes through the activation of ICAT (Chen et al., 2020). Our study demonstrated that miR-296-3p negatively regulated osteogenic differentiation, and over-expression of ICAT could counteract the negative regulatory effect. This suggests that miR-296-3p may regulate osteoblast differentiation by acting on ICAT. However, the specific mechanism and pathway are still unclear. Related studies have reported that during the differentiation process of chondrocytes, transforming growth factor β (TGF-β) can promote chondrocyte proliferation, suppress cell differentiation and rapidly induce the expression of self-renewal-related protein β-catenin (Lin et al., 2022). ICAT can down-regulate the promoter activity of cell cycle regulatory protein D1 (cyclin D1) as a β-catenin inhibitor (Jiang et al., 2017). It has been concluded that ICAT may play a role in promoting chondrocyte differentiation through a specific linkage. Studies have shown that miR-216a-3p inhibits osteogenic differentiation through Wnt3a in the Wnt/β-catenin signalling pathway (Liang et al., 2022). Our results indicate that miR-296-3p targets ICAT and affects the expression of key proteins in the Wnt/β-catenin signalling pathway. Therefore, we hypothesized that miR-296-3p regulates the expression of ICAT to affect the osteogenic differentiation of human BMSCs, which also involves the Wnt/β-catenin signalling pathway. However, the specific regulatory mechanism of this phenomenon still needs to be further explored.

However, there are still some shortcomings in this experiment. These include selection bias in the selection of subjects, lack of long-term follow-up of subjects and lack of protein or animal experiments. In the next stage, we will improve the experimental design, increase the sample size and further investigate the specific mechanism of miR-296-3p in the regulation of fracture healing to provide more experimental data to guide future fracture treatment.

Taken together, our study suggests that miR-296-3p may be involved in fracture healing. Additionally, miR-296-3p can target ICAT to regulate osteoblast differentiation. The feasibility of translating miR-296-3p as a promising potential therapeutic target into clinical practice requires further investigation and validation.

Conflict of interests

The authors declare that they have no competing interests.

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