

Inhibition of miR-98-5p promotes high glucose-induced suppression of preosteoblast proliferation and differentiation via the activation of the PI3K/AKT/GSK3 β signaling pathway by targeting BMP2

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Abstract. Osteoporosis (OP) is a bone metabolic disease, in which low bone mass and the microarchitectural deterioration of bone tissue contribute to the fragility of bones and increase the risk of fracture. The aim of the present study was to determine the role of microRNA (miR)-98-5p in high glucose (HG)-induced preosteoblasts. HG was used to induce preosteoblasts treated in a differentiation medium to establish an *in vitro* OP model. Next, miR-98-5p expression was determined using reverse transcription-quantitative PCR. Following the transfection of an miR-98-5p inhibitor into HG-treated osteoblasts, cell viability was assessed using a Cell Counting Kit-8 assay, while alkaline phosphatase (ALP) activity, differentiation ability and the expression of differentiation-regulated genes osteocalcin and osteopontin were measured using the corresponding ALP, Alizarin red staining, reverse transcription-quantitative PCR and western blotting assays. The association between miR-98-5p and the PI3K/AKT/GSK3 β signaling pathway was determined using western blotting. Next, the binding relationship between miR-98-5p and bone morphogenetic protein 2 (BMP2) was predicted and verified, and the role of BMP2 in the regulation of the PI3K/AKT/GSK3 β signaling pathway was explored using western blotting. The results revealed that miR-98-5p expression was upregulated in HG-induced osteoblasts, and the inhibition of miR-98-5p resulted in enhanced cell viability, alkaline phosphatase activity and differentiation in osteoblasts following HG induction. It was also discovered that miR-98-5p inhibition activated PI3K/AKT/GSK3 β signaling, while knockdown of BMP2, which binds to miR-98-5p, enhanced the activation of this signaling pathway and the differentiation

ability of osteoblasts. In conclusion, the findings of the present study suggested that the inhibition of miR-98-5p expression may activate PI3K/AKT/GSK3 β signaling to promote HG-induced suppression of preosteoblast viability and differentiation by targeting BMP2, which provides a novel insight into future potential molecular markers for OP treatment.

Introduction

Osteoporosis (OP) is a bone metabolic disease, in which low bone mass and microarchitectural deterioration of bone tissue contribute to the fragility of bones and increase the risk of fracture (1,2). Estrogen withdrawal and androgen deficiency are considered to be the two major causes of disease occurrence (3). Aging and changes in lifestyle factors have been discovered to be responsible for the increasingly high prevalence of OP (4). Since it constitutes a major challenge to the health and welfare of elderly individuals, OP has received considerable research attention in developed countries (5). However, little is known regarding the pathogenesis of OP, rendering treatment challenging. As osteoblast differentiation is indispensable for bone regeneration, clarifying the mechanism of osteoblast differentiation is critical to the formulation of therapeutic strategies for OP (6). Increasing evidence has suggested that diabetes may affect bone formation, bone remodeling and wound healing (7,8). High glucose (HG) levels are one of the possible causes for OP and fracture in diabetes mellitus (9). Experiments *in vivo* and *in vitro* have shown that extracellular HG contributed to bone loss and deleterious effects on osteoblast proliferation and function (10,11).

MicroRNAs (miRNAs/miRs) are small endogenous RNA molecules that regulate gene expression at the post-transcriptional level (12,13). They are involved in a large number of biological processes, including cell proliferation, metabolism, stress adaptation, hormone signaling and differentiation (14). An increasing number of reports have indicated the involvement of miRNAs in regulating bone formation to influence the development of OP and bone-related diseases (15,16). For example, miR-218 expression was found to be upregulated during osteoblast differentiation and potentially activated osteoblast differentiation by coordinating with Wnt signaling, indicating its importance in the course of osteogenesis (17,18).

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The inhibitory role of miR-449c-5p in the osteogenic differentiation of human valve interstitial cells was also previously reported (19). In addition, miR-98-5p was demonstrated to regulate osteoblast differentiation in MC3T3-E1 cells by downregulating casein kinase 2-interacting protein-1 (CKIP-1) in bone injury and loss (20); however, its specific role in HG-induced OP has not been reported, to the best of our knowledge.

The PI3K/AKT signaling pathway has been extensively studied, as it is a complex signaling pathway implicated in a large number of biological processes, such as proliferation, angiogenesis, transcription and metabolism (21-23). Not only is it an important intracellular pathway that is often activated to affect the occurrence and progression of cancer, but it was also discovered to be an important metabolic signaling pathway in subchondral bone in osteoarthritis (24). The aim of the present study was to investigate the role of miR-98-5p in HG-induced OP and its possible association with the PI3K/AKT signaling pathway, which may provide novel insights into future targets for OP treatment.

Materials and methods

Bioinformatic analysis. The miR-98-5p and BMP2 target sites were predicted by TargetScan database (http://www.targetscan.org/vert_72/).

MC3T3-E1 cell culture and differentiation. MC3T3-E1 cells, a murine preosteoblast cell line, were obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences and cultured in α -minimum essential medium (α -MEM; Shanghai XP Biomed Ltd.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere at 37°C in 5% CO₂.

The cells were seeded into 6-well plates at a density of 1x10⁵ cells/well and cultured until they reached 80% confluence. For differentiation, the cells were subsequently cultured at 37°C with 5% CO₂ in a differentiation medium [DM; OriCell Basal Medium; Cyagen Biosciences (Guangzhou) Inc.] supplemented with 10% FBS, 50 μ g/ml ascorbic acid and 4 mM β -glycerol phosphate for 7 days (induced 7 day) or 14 days (induced 14 day). The medium was replaced with fresh medium every 2 days. To simulate HG conditions, preosteoblasts were cultured for 14 days at 37°C in 5% CO₂ in DM for differentiation into osteoblasts with 5.5 mM D-glucose or 25 mM D-glucose.

Cell transfection. miR-98-5p mimic, miR-negative control (NC), miR-98-5p inhibitor and inhibitor-NC were purchased from Shanghai Genechem Co., Ltd. Small interfering RNA (siRNA)-bone morphogenetic protein 2 (BMP2) (cat. no. sc-39739) and siRNA-NC were purchased from Santa Cruz Biotechnology Co., Ltd. The primer sequences for the aforementioned oligonucleotides are as follows: miR-98-5p mimic, 5'-UGAGGUAGUAAGUUGUAUUGU U-3'; miR-98-5p inhibitor, 5'-AACAUAACAACUUAUCUACUCA-3'; miR-NC, 5'-UCACAACCUCCUAGAAAGAGUAGA-3'; inhibitor-NC, 5'-CAGUACUUUUGUGUAGUACAA-3'; siRNA-BMP2-1, 5'-CCUAUAUGCUAGAUCUGUACC-3'; siRNA-BMP2-2, 5'-GCACUACUGUUAAGUUCACA-3'; and siRNA-NC, 5'-UUCUCCGAACGUGUC

ACGU-3'. These recombinants (1 μ g) were transfected into MC3T3-E1 cells at 37°C for 48 h using Lipofectamine[®] 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Transfected cells were used for subsequent experiments after 48 h.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. A RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) was used for the reverse transcription of total RNA (0.5-1 μ g) into cDNA according to the manufacturer's instructions. A QuantiMir[™] RT kit (Systems Biosciences) was used for miRNA detection, according to the manufacturer's instructions. qPCR was subsequently performed using a SYBR-Green Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on the ABI 7500 Fast Real-Time PCR Detection system (7500 fast system software; version 2.6.2; Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences used for the qPCR were as follows: miR-98-5p, 5'-TGCTTGAGGTAGTAAGTTG-3' and reverse, 5'-ATCCAGTGCCTGTCTGTG-3'; BMP2 forward, 5'-TGTGGGCCCTCATAAAGAAGCAGA-3' and reverse, 5'-AGCAAGCTGACAGGTCAGAGAACA-3'; runt-related transcription factor 2 (Runx2) forward, 5'-CCCACTTCCTGTGCTCC-3' and reverse, 5'-AGTGAACTCTTGCCCTCGTC-3'; collagen I forward, 5'-TAAGGGTGCAGAGGCGATG-3' and reverse, 5'-GGACCGCTAGGACCAGTTTC-3'; osteopontin (OPN) forward, 5'-TCCAAA GTCAGCCAGGAATCC-3' and reverse, 5'-CGGAGTTGTCTGTGCTCTTCA-3'; osteocalcin (OCN) forward, 5'-CTTGGTGACACCTAGCAGA-3' and reverse, 5'-TTCTGTTTCCTCCCTGCTGT-3'; GAPDH forward, 5'-GGGAACTGTGGCGTGAT-3' and reverse, 5'-GAGTGGGTGTCTGCTGTTGA-3'; and U6 forward, 5'-CTCGCTTCGGCAGCACATATA-3' and reverse, 5'-ACGCTTCACGAATTTGAGTGT C-3'. The following thermocycling conditions were used for the qPCR: Initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 20 sec, annealing at 60°C for 30 sec and extension at 72°C for 60 sec. Relative expression was normalized to the expression levels of GAPDH (mRNA) or U6 (miRNA) using the 2^{- $\Delta\Delta$ C_q} method (25).

Cell Counting Kit-8 (CCK-8) assay. The transfected cells were seeded into a 96-well plate (3x10⁴ cells/well) at 37°C for 14 days, with each well containing 100 μ l culture medium. Next, 10 μ l CCK-8 reagent (Beyotime Institute of Biotechnology) was added into each well and incubated for 4 h at 37°C. Absorbance was measured at a wavelength of 450 nm using a microplate reader (RT-3001; Thermo Fisher Scientific, Inc.).

Detection of alkaline phosphatase (ALP) activity. ALP activity was determined on day 14 after osteogenic induction using an ALP activity kit, according to the manufacturer's instructions (cat. no. P0321S; Beyotime Institute of Biotechnology). Absorbance was measured at a wavelength of 405 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

Alizarin red staining. The MC3T3-E1 cells (2x10⁴ cells/ml) were cultured in 24-well plates supplemented with osteogenic

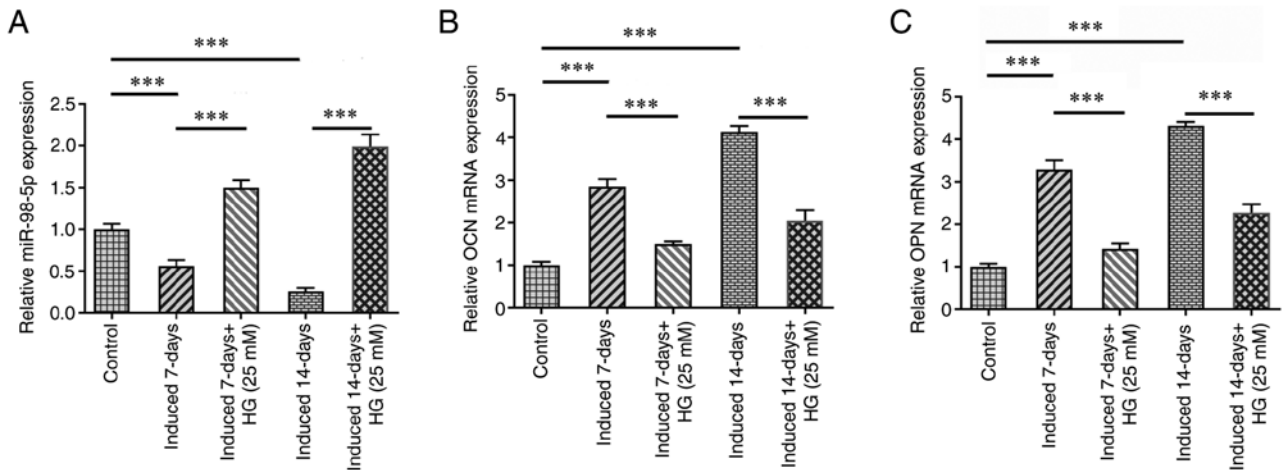


Figure 1. miR-98-5p expression is upregulated during HG-induced preosteoblastic differentiation. The expression levels of (A) miR-98-5p, and differentiation-regulated genes (B) OCN and (C) OPN in the differentiation process of MC3T3-E1 cells after HG induction. Data are presented as the mean \pm SD. *** P <0.001. Induced 7-day, incubation in a DM for 7 days; induced 14-day, incubation in a DM for 14 days; OCN, osteocalcin; OPN, osteopontin; DM, differentiation medium; HG, high glucose.

differentiation induction medium for 2 weeks at 37°C and then mineralized to form opaque calcified nodules. Cells were then fixed with 95% ethanol for 10 min at room temperature, washed with PBS twice and stained with 0.1% alizarin red solution (40 mM) for 15 min at room temperature. Excess stain was washed off with PBS, and cells were observed under an inverted light microscope.

Western blotting. MC3T3-E1 osteoblasts (1×10^5 cells) were seeded into 100-mm dishes and cultured in complete α -MEM until they reached 80% confluence. Cells were lysed by RIPA lysis buffer (Yisheng Biological Technology Co., Ltd.) and the lysates were centrifuged at 20,000 \times g for 30 min at 4°C, and protein concentration was determined using a BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Protein lysates (30 μ g) were separated via 10% SDS-PAGE and transferred onto a PVDF membrane, which were blocked with 5% bovine serum albumin (BSA; Gibco; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. The membranes were subsequently incubated overnight at 4°C with the following primary antibodies: Anti-BMP2 (1:1,000; cat. no. ab214821; Abcam), anti-Runx2 (1:1,000; cat. no. ab236639; Abcam), anti-collagen I (1:1,000; cat. no. ab138492; Abcam), anti-OPN (1:1,000; cat. no. ab63856; Abcam), anti-OCN (1:1,000; cat. no. ab133612; Abcam), anti-phosphorylated (p)-PI3K (1:1,000; cat. no. ab182651; Abcam), anti-PI3K (1:1,000; cat. no. ab86714; Abcam), anti-p-AKT (1:1,000; cat. no. ab38449; Abcam), anti-AKT (1:500; cat. no. ab8805; Abcam), anti-p-GSK3 β (1:1,000; cat. no. ab107166; Abcam), anti-GSK3 β (1:1,000; cat. no. ab280376; Abcam) and anti-GAPDH (1:2,500; cat. no. ab9485; Abcam). Following the primary antibody incubation, the membranes were washed with PBS 3 times for 10 min and incubated with HRP-conjugated goat anti-rabbit or -mouse secondary antibodies (1:1,000; cat. nos. ab205718 and ab6789; Abcam) for 2 h at room temperature. Protein bands were visualized with an ECL kit (Beyotime Institute of Biotechnology) using an enhanced chemiluminescence detection system

(MF-ChemiBIS version 3.2; DNR Bio Imaging Systems). The protein intensity was quantified with ImageJ software v1.8.0 (National Institutes of Health).

Dual luciferase reporter assay. The BMP2 3'-untranslated region, containing wild-type (WT) or mutant (MUT) target sites for miR-98-5p, were amplified via PCR and inserted into a pGL3-control vector (Promega Corporation) to create the wild-type BMP2 (SIRT1-WT) or mutant type SIRT1 (BMP2-MUT) vectors, respectively. MC3T3-E1 cells were seeded into 24-well plates (2×10^5 cells/well) and incubated for 24 h at 37°C in α -MEM supplemented with 10% FBS. Cells were subsequently transiently co-transfected with luciferase reporter vectors, including miR-98-5p mimic and miR-NC, using Lipofectamine 2000. Relative luciferase activity was detected using a Dual Luciferase Reporter assay system (Promega Corporation) according to the manufacturer's instructions and normalized to the *Renilla* luciferase activity.

Statistical analysis. All experiments were repeated at least three times, and data are presented as the mean \pm SD. Statistical analysis was conducted using SPSS 13.0 software (SPSS, Inc.). Statistical differences among different groups were determined using a one-way ANOVA, followed by a Tukey's multiple comparisons test. Unpaired Student's t-test was used to compare the statistical differences between two groups. P <0.05 was considered to indicate a statistically significant difference.

Results

miR-98-5p expression is upregulated during HG-treated preosteoblast differentiation. As shown in Fig. 1A, miR-98-5p expression in differentiated cells for 7 or 14 days was down-regulated compared with that in control cells. In addition, HG-induced osteoblasts exhibited a markedly elevated level of miR-98-5p compared with untreated differentiated osteoblasts. Next, the expression levels of differentiation-regulated genes, OCN and OPN, in HG-induced osteoblasts were

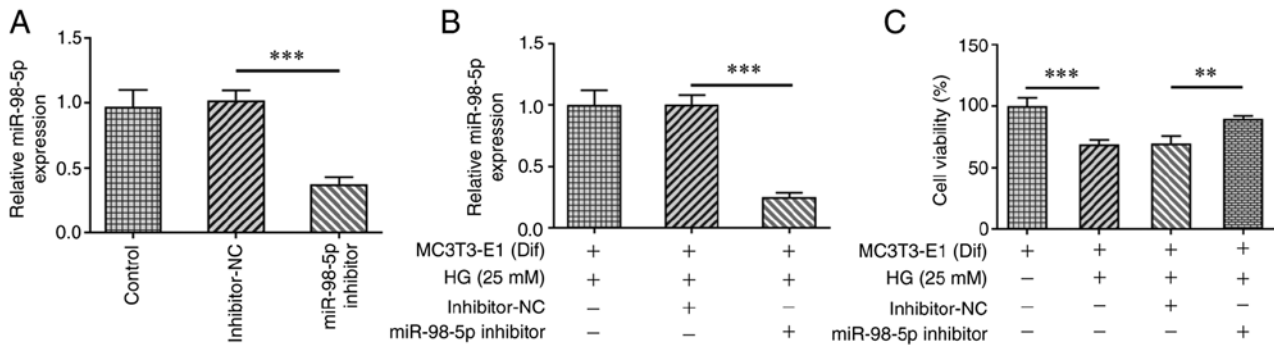


Figure 2. Inhibition of miR-98-5p ameliorates HG-induced osteoblast injury. (A) Expression of miR-98-5p was detected using reverse transcription-quantitative PCR after transfection with a miR-98-5p inhibitor. (B) Expression of miR-98-5p after miR-98-5p inhibitor transfection in HG-induced osteoblasts. (C) Viability of HG-induced osteoblasts after transfection with the miR-98-5p inhibitor. Data are presented as the mean \pm SD. ** P <0.01, *** P <0.001. MC3T3-E1 (Dif), MC3T3-E1 cells that were cultured in a differentiation medium for 14 days. miR, microRNA; HG, high glucose; NC, negative control.

detected. OCN and OPN expression levels were markedly upregulated in differentiated cells compared with the undifferentiated cells (control group), while HG treatment downregulated their expression levels in differentiated cells (Fig. 1B and C). These results suggested the successful establishment of the HG-induced OP model *in vitro*. In addition, the results from the induced 14-day group showed more significant changes in the expression levels of miR-98-5, OCN and OPN; thus, induction for 14 days was selected for subsequent experiments, and this treatment was referred to as MC3T3-E1 (Dif) henceforth.

Inhibition of miR-98-5p expression ameliorates HG-induced osteoblast injury. We hypothesized that high miR-98-5p expression may be associated with HG-induced osteoblast injury; thus, a miR-98-5p inhibitor was transfected into HG-induced MC3T3-E1 cells. The transfection efficiency of the miR-98-5p inhibitor was detected using RT-qPCR (Fig. 2A). Moreover, the mRNA expression levels of miR-98-5p were downregulated by the miR-98-5p inhibitor in HG-treated MC3T3-E1 cells compared with the negative control (Fig. 2B). In addition, the results of the CCK-8 assay demonstrated that the viability of HG-treated MC3T3-E1 cells was suppressed compared with that of the cells without HG induction (Fig. 2C). Notably, the decreased viability of HG-induced MC3T3-E1 cells was partly restored by the miR-98-5p inhibitor. Therefore, miR-98-5p expression inhibition may ameliorate HG-induced osteoblast injury.

miR-98-5p silencing enhances the differentiation of HG-exposed osteoblasts. Subsequently, ALP activity was detected to confirm whether inhibiting miR-98-5p expression could directly influence the differentiation of HG-induced osteoblasts. As shown in Fig. 3A, ALP activity was suppressed following the treatment of MC3T3-E1 cells with HG compared with untreated cells, while this effect was partly abolished by the miR-98-5p inhibitor. Similarly, Alizarin red staining showed that the differentiation of HG-induced MC3T3-E1 cells was restored by the miR-98-5p inhibitor (Fig. 3B). Next, the mRNA and protein expression levels of BMP2, Runx2, OCN, OPN and collagen I were downregulated following HG exposure in differentiated MC3T3-E1 cells, while they were upregulated by the addition of miR-98-5p inhibitor (Fig. 3C and D). These

results indicated that miR-98-5p silencing may enhance the differentiation of HG-induced osteoblasts.

miR-98-5p knockdown activates the PI3K/AKT/GSK3 β signaling pathway. PI3K/AKT has been reported to induce osteoblast proliferation, differentiation and mineralization (26); thus, functional testing was conducted to confirm whether miR-98-5p inhibition could regulate this pathway. HG treatment was found to downregulate the expression ratio of p-PI3K/PI3K, p-AKT/AKT and p-GSK3 β /GSK3 β compared with the MC3T3-E1 (Dif) group, while the miR-98-5p inhibitor subsequently upregulated the expression levels compared with those in the inhibitor-NC group (Fig. 4). These data suggested that the inhibition of miR-98-5p expression may activate the PI3K/AKT/GSK3 β signaling pathway.

miR-98-5p silencing activates the PI3K/AKT/GSK3 β signaling pathway by binding to BMP2. Since previous evidence has suggested that BMP2 participated in the differentiation of osteoblasts by upregulating the PI3K/AKT/GSK3 β signaling pathway (27), it was examined whether miR-98-5p could bind to BMP2. As shown in Fig. 5A, the miR-98-5p mimic was successfully transfected into cells, as demonstrated by the upregulated expression of miR-98-5p compared with the miR-NC group. The binding site of miR-98-5p to BMP2 is shown in Fig. 5B. The results of the dual luciferase reporter assay further verified the binding relationship between miR-98-5p and BMP2, since the relative luciferase activity in osteoblasts co-transfected with the BMP2-WT vector and relative luciferase activity was weaker in the BMP2-WT + miR-98-5p mimic group compared to the BMP2-WT + miR-NC group, while no changes were observed between the BMP2-MUT vectors. (Fig. 5C). siRNA-BMP2 was then successfully transfected into cells and siRNA-BMP2-1 was used in subsequent experiments due to its better knockdown effect (Fig. 5D and E). The results of the CCK-8 assay revealed that BMP2 silencing significantly suppressed the miR-98-5p-mediated increased in the viability of HG-induced osteoblasts (Fig. 5F). Western blotting analysis showed that the PI3K/AKT/GSK3 β signaling pathway activated by the miR-98-5p inhibitor in HG-induced osteoblasts was inactivated by siRNA-BMP2 (Fig. 5G). In addition, siRNA-BMP2 partly abolished the effect of the miR-98-5p inhibitor on enhancing the expression of bone

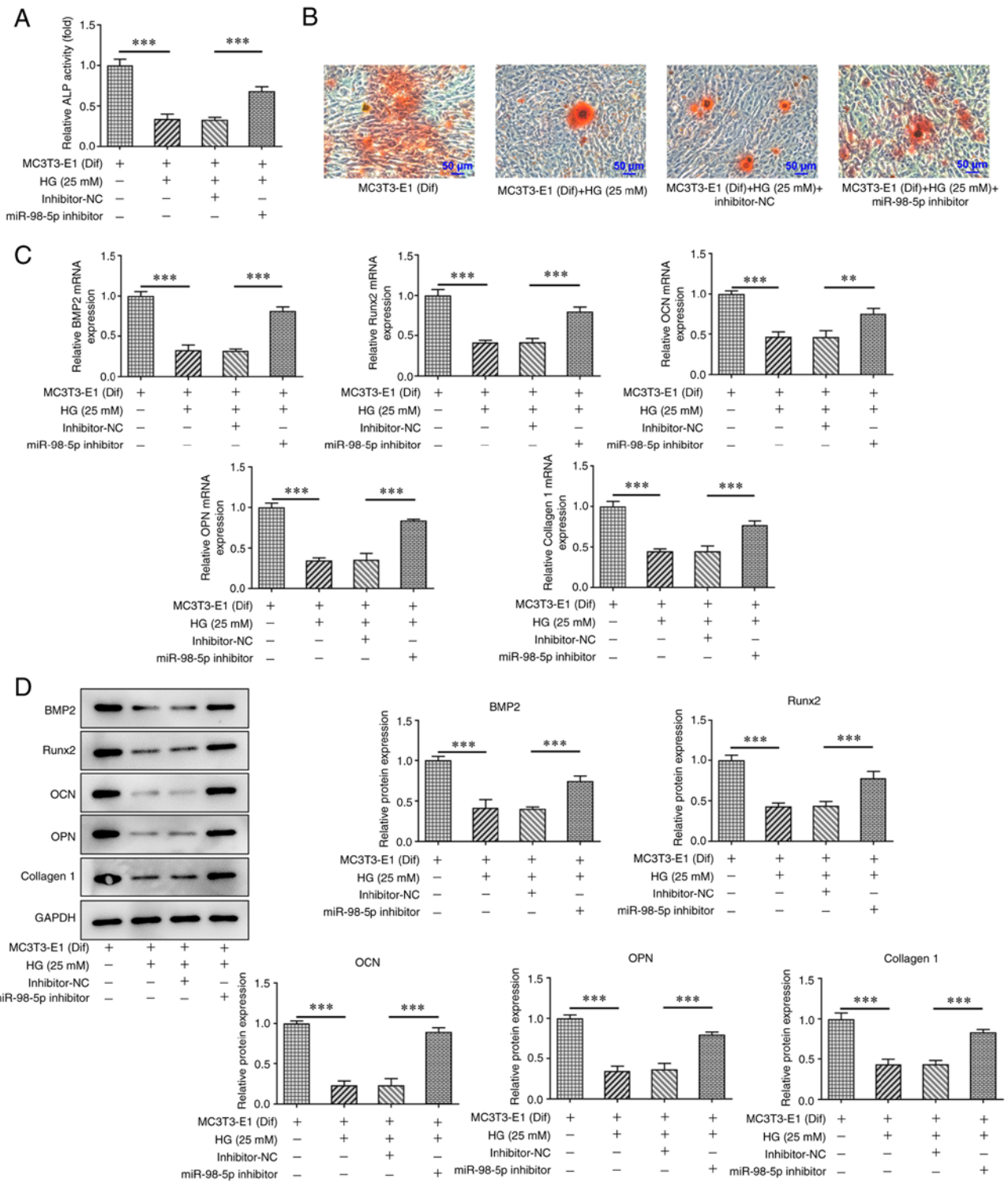


Figure 3. Silencing of miR-98-5p enhances the differentiation of HG-induced osteoblasts. ALP (A) activity and (B) differentiation ability of HG-induced osteoblasts after transfection with the miR-98-5p inhibitor were assessed. Scale bar, 50 μ m. (C) mRNA and (D) protein expression levels of differentiation-regulated factors, BMP2, Runx2, OCN, OPN and collagen I in HG-induced osteoblasts following transfection with a miR-98-5p inhibitor were measured using reverse transcription-quantitative PCR and western blotting, respectively. Data are presented as the mean \pm SD. ***P<0.01; ****P<0.001. MC3T3-E1 (Dif), MC3T3-E1 cells that were cultured in a differentiation medium for 14 days; ALP, alkaline phosphatase; BMP2, bone morphogenetic protein 2; Runx2, Runt-related transcription factor 2; OCN, osteocalcin; OPN, osteopontin; miR, microRNA; HG, high glucose; NC, negative control.

differentiation-regulated genes in HG-induced osteoblasts, suggesting the involvement of BMP2 in activating the PI3K/AKT/GSK3 β signaling pathway (Fig. 5H). These results indicated that miR-98-5p activates the PI3K/AKT/GSK3 β signaling pathway by binding to BMP2.

Discussion

miRNAs are widely known as regulators of numerous pathophysiological processes, such as apoptosis, organogenesis and proliferation (28). In addition, the critical role of miRNAs

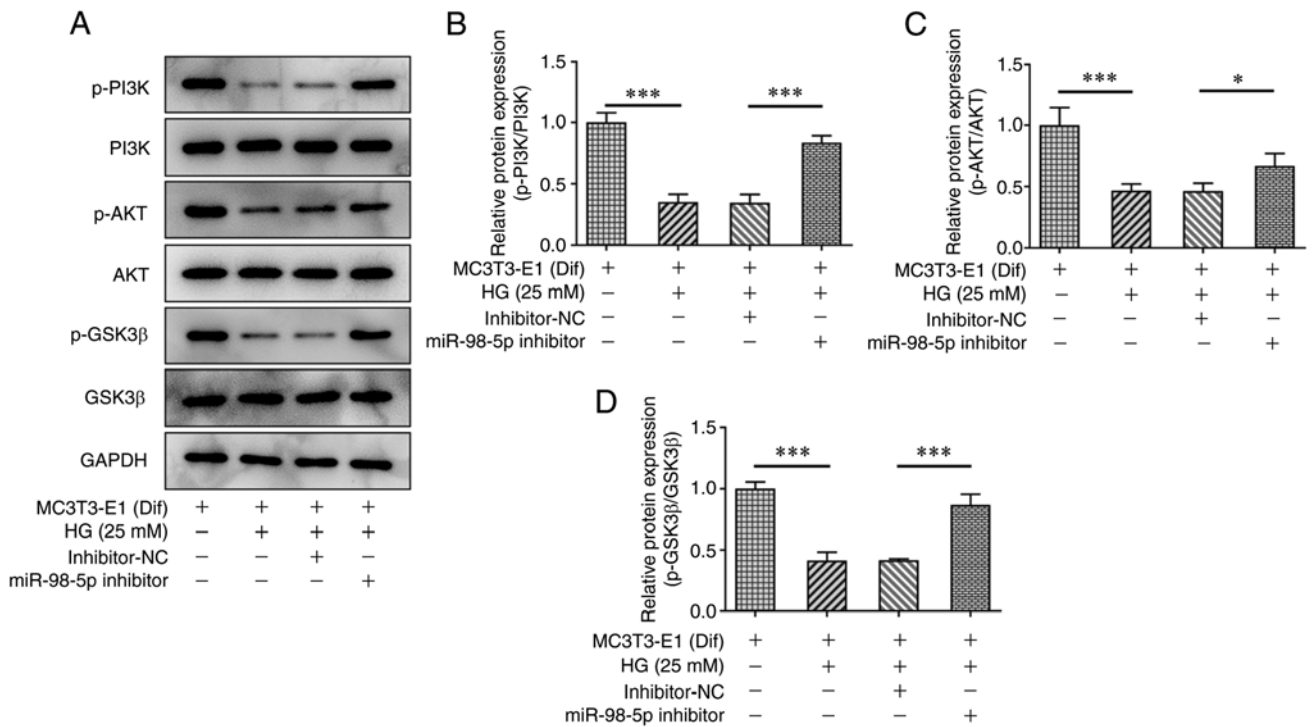


Figure 4. Inhibition of miR-98-5p activates the PI3K/AKT/GSK3 β signaling pathway. (A) Western blotting of proteins. (B) The protein expression levels of p-P13K/P13K after silencing miR-98-5p in HG-induced osteoblasts. (C) The protein expression levels of p-AKT/AKT after silencing miR-98-5p in HG-induced osteoblasts. (D) The protein expression levels of p-GSK3 β /GSK3 β after silencing miR-98-5p in HG-induced osteoblasts. Data are presented as the mean \pm SD. * P <0.05, *** P <0.001. MC3T3-E1 (Dif), MC3T3-E1 cells that were cultured in a differentiation medium for 14 days; miR, microRNA; p-, phosphorylated; NC, negative control; HG, high glucose.

in regulating the development of diseases, including breast cancer, Alzheimer's disease and schizophrenia, cannot be overlooked (29). Previous studies have indicated their role in skeletal development at the post-transcriptional level (30,31). In addition, dysregulated miRNAs were found to be associated with bone degeneration in OP, since the mutations of pre-miR-2861 were found in two adolescents who suffered from juvenile OP (32). The dysregulation of certain mature miRNAs, such as miR-140 and miR-675, in cells that play major roles in bone formation and skeleton remodeling hints at their ability to modulate cell differentiation and bone mass during normal embryonic development (31). miR-2861 accelerated osteoblast differentiation upon BMP2 challenge, and the suppression of miR-2861 expression induced delayed osteoblast differentiation (32). miR-182 also suppressed the expression of forkhead box O1, a transcription factor implicated in oxidation balance in osteoblasts, to inhibit osteoblast proliferation and differentiation (33). In addition, miR-98-5p was demonstrated to promote osteoblast differentiation in MC3T3-E1 cells by regulating casein kinase 2 interacting protein-1 (20). Thus, the present study hypothesized that miR-98-5p may be a potential therapeutic target for OP treatment. MC3T3-E1 cells were used in functional tests of vitamin K2 on osteoporosis in a previous *in vitro* study due to their osteogenic differentiation- and mineralization-related characteristics (34). In the present study, following 7 or 14 days of DM culture, miR-98-5p expression was found to be downregulated in the differentiated MC3T3-E1 cells, which was subsequently abolished following HG induction of the osteoblasts. The HG-induced osteoblast damage was alleviated following the

transfection with the miR-98-5p inhibitor. Furthermore, ALP activity and differentiation-related markers, which were used to detect osteoblast differentiation ability, were inhibited in osteoblasts following HG induction, while transfection with the miR-98-5p inhibitor reversed these effects. Notably, the results of the current are contrary to data from a previous study reporting that miR-98-5p played a promoting role in osteoblast differentiation (20), which may attribute to the multiple biological roles of miR-98-5p under normal and HG conditions. In addition, the present study did not perform miR-98-5p overexpression experiments because the results obtained showed that miR-98-5p silencing significantly promoted cell viability and osteoblast differentiation, which verified our hypothesis. To date, our group have preliminarily studied the role of miR-98-5p in OP, and further miR-98-5p overexpression studies may confirm the findings observed and provide further insight into potential treatment strategies for OP. Thus, miR-98-5p overexpression studies and further exploration of the underlying mechanism in OP will be performed in a future study.

PI3K is a crucial signaling molecule, the activation of which has been shown to regulate numerous cellular processes, including proliferation, migration and protein transport (35). Following the phosphorylation of plasma membrane intrinsic protein (PIP)2 to create PIP3, PIP3 interacts with guanosine triphosphate-binding proteins Rac, protein kinase C or AKT (36). The PI3K/AKT signaling pathway can also control cellular activities, such as cell proliferation and survival (37). The importance of this signaling pathway in osteoblast differentiation has been highlighted in previous studies (38,39). GSK3 β

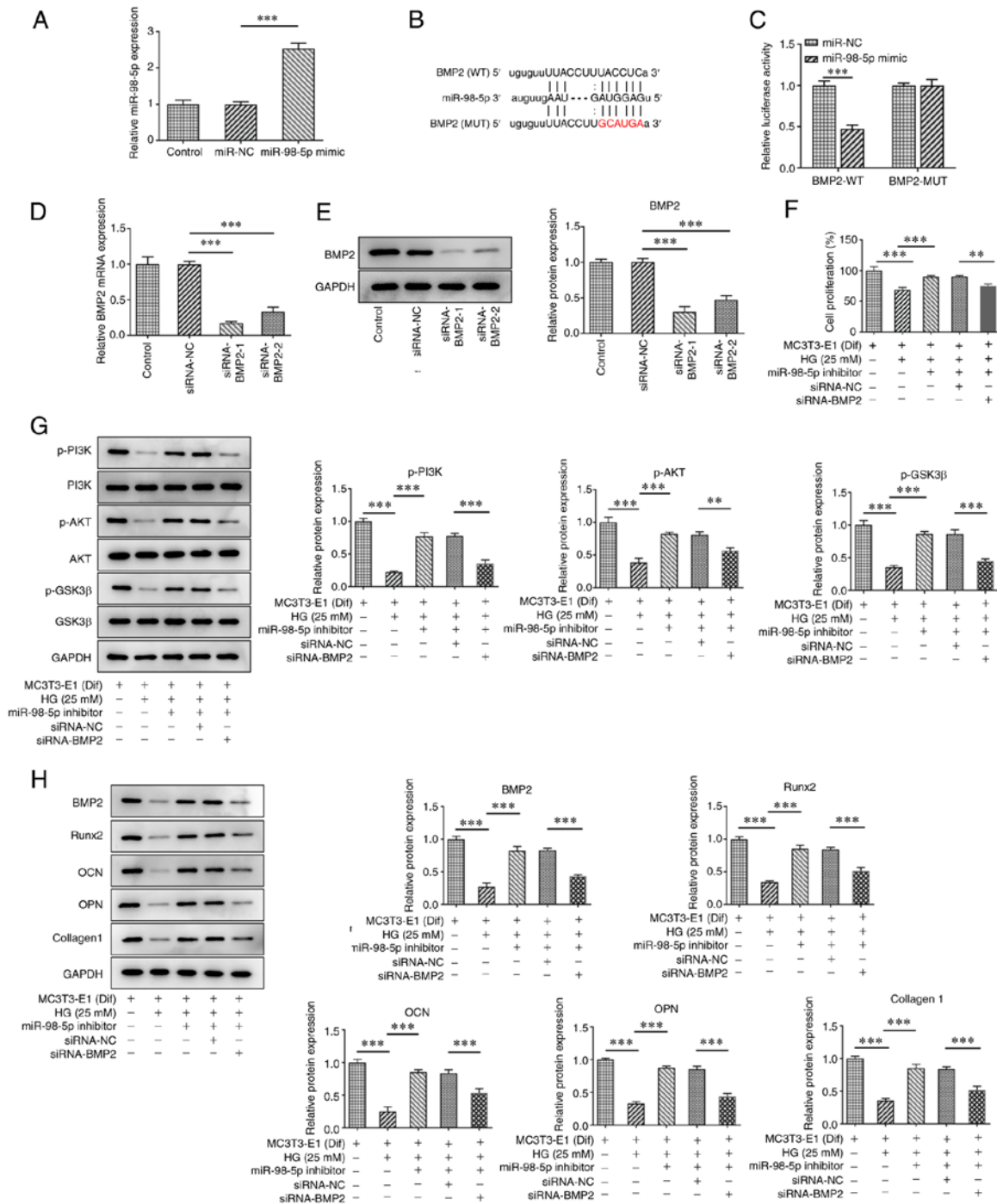


Figure 5. Silencing of miR-98-5p activates the PI3K/AKT/GSK3β signaling pathway by binding to BMP2. (A) Expression of miR-98-5p after transfection with a miR-98-5p mimic. (B) Predicted binding site between miR-98-5p and BMP2. (C) Dual luciferase reporter assay was used for the confirmation of the binding relationship between miR-98-5p and BMP2. (D) mRNA and (E) protein expression of BMP2 after using siRNA to knock down its expression. (F) Cell viability was determined after HG-induced osteoblasts were co-transfected with the miR-98-5p inhibitor and siRNA-BMP2. (G) Protein expression levels of components of the PI3K/AKT/GSK3β signaling pathway after HG-induced osteoblasts were co-transfected with the miR-98-5p inhibitor and siRNA-BMP2. (H) Expression levels of BMP2, Runx2, OCN, OPN and collagen I after HG-induced osteoblasts were co-transfected with the miR-98-5p inhibitor and siRNA-BMP2. Data are presented as the mean ± SD. **P<0.01, ***P<0.001. MC3T3-E1 (Dif), MC3T3-E1 cells that were cultured in a differentiation medium for 14 days; BMP2, bone morphogenetic protein 2; Runx2, Runt-related transcription factor 2; OCN, osteocalcin; OPN, osteopontin; miR, microRNA; NC, negative control; MUT, mutant; WT, wild-type; HG, high glucose; p-, phosphorylated; siRNA, small interfering RNA.

is a multifunctional serine/threonine kinase that can influence energy metabolism, cell proliferation and apoptosis (40). It has also been found to be a critical downstream target of the PI3K/AKT signaling pathway (41). In the present study, the interplay between miR-98-5p and the signaling cascades was further explored. It was found that the p-PI3K/PI3K,

p-AKT/AKT and p-GSK3β/GSK3β ratios were all decreased in HG-induced MC3T3-E1 cells, suggesting the inactivation of the PI3K/AKT/GSK3β pathway. Notably, the activation of this pathway was achieved when the miR-98-5p inhibitor was used to knock down the expression of miR-98-5p in HG-induced MC3T3-E1 cells, which indicated that the activation of the

PI3K/AKT/GSK3 β pathway may be associated with the restoration of HG-induced osteoblast damage. These findings were consistent with those of a previous study, showing that dexamethasone inhibited the cell viability and induced the apoptosis of osteoblasts by suppressing the reactive oxygen species-mediated PI3K/AKT/GSK3 β signaling pathway (40).

BMPs, a group of polypeptides within the TGF superfamily, are critical regulators of multiple biological pathways, including development, cell proliferation and bone formation (42,43). BMP2 has been discovered to promote the differentiation of preosteoblasts into mature osteoblasts by regulating signals that contribute to bone formation (43). BMP2 was also suggested to enhance PI3K/AKT signaling in osteoblast differentiation and combine with miR-98-5p (44). In the present study, RT-qPCR and western blotting analyses showed that BMP2 knockdown suppressed the role of the miR-98-5p inhibitor in activating the PI3K/AKT/GSK3 β signaling pathway. Furthermore, in the current study, the expression levels of osteogenesis- and differentiation-related proteins, including BMP2, Runx2, collagen I, OPN and OCN, were analyzed to reflect the degree of osteoblast differentiation. The Smad pathway, alongside the canonical BMP signaling pathway, has also been shown to be a significant pathway in the process of osteoblast differentiation (45); thus, future studies will also aim to explore the effects of miR-98-5p on the expression levels of components of the Smad pathway. Moreover, the current study determined the role of miR-98-5p and its downstream gene BMP2 and downstream pathway, PI3K/AKT/GSK3 β in OP. However, the study did not reveal how HG regulates the expression of miR-98-5p. The underlying mechanism by which HG regulates the expression of miR-98-5p in diabetes-induced OP will be identified in further studies. Additionally, the effects of various concentrations of glucose on osteoblast viability and differentiation, and the morphological data for these effects, were not analyzed in the present study, and will therefore be a focus of studies in the future.

In conclusion, to the best of our knowledge, the findings of the present study were the first to suggest that the inhibition of miR-98-5p expression may activate the PI3K/AKT/GSK3 β signaling pathway to promote HG-induced preosteoblast viability and differentiation by targeting BMP2. These findings may provide a novel insight into future potential molecular markers for OP treatment.

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Availability of data and materials

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

Authors' contributions

FeZ and FW designed the experiments and made considerable contributions to the writing of the manuscript. FeZ, FuZ and FW performed the experiments and analyzed the data. FW revised the manuscript and supervised the experiments. FuZ and FW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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