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Original Article

miR-153-3p inhibits osteogenic differentiation of BMSCs by down-regulating the expression of RUNX2 in a high glucose environment

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Abstract: To study the effect of miR-153-3p on the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) in a high glucose environment and its potential mechanism. The results showed that high glucose inhibited the osteogenic differentiation of BMSCs, and the expression of miR-153-3p increased during osteogenic differentiation. Further experiments found that in BMSCs induced by high glucose, overexpression of miR-153-3p inhibited the osteogenic differentiation of BMSCs, and the expressions of osteogenesis-related genes bone sialoprotein, Collagen I and alkaline phosphatase were down-regulated, while silencing of miR-153-3p alleviated the inhibition effect. The dual-luciferase reporter gene assay confirmed that the 3'-untranslated region (3'-UTR) of runt related transcription factor 2 (RUNX2) had a targeted binding site with miR-153-3p and a negative regulatory effect. Molecular studies further confirmed that miR-153-3p inhibited the osteogenic differentiation of BMSCs by targeting the 3'-UTR of RUNX2. In conclusion, our study found that as one key regulator of high glucose affecting the osteogenic differentiation of BMSCs, miR-153-3p may play a negative regulatory role by inhibiting the expression of RUNX2.

Keywords: miR-153-3p, osteogenic differentiation, BMSCs, high glucose, RUNX2

Introduction

Diabetes as chronic systemic metabolic disease includes type1 diabetes mellitus (T1DM) and type2 diabetes mellitus (T2DM), and is characterized by abnormal glucose metabolism. It is often accompanied by various complications such as periodontal disease, neuropathy, retinopathy, kidney disease, cardiovascular disease and skeletal abnormalities, which seriously affect the quality of life of patients. Among them, osteoporosis is one of the more common complications of skeletal system abnormalities in diabetic patients, which is caused by abnormal glucose metabolism and is often accompanied by a higher risk of fracture [1]. Although T1DM and T2DM may have different effects on bone by different mechanisms, patients with both show greater bone fragility [2]. For example, compared with normal people, T1DM patients have decreased bone mineral density due to insufficient insulin synthesis in the body. Intensive insulin therapy can restore bone anabolism in T1DM patients and stabilize bone mineral density [3]. The bone mineral density of T2DM patients is comparable to that of normal people or even slightly higher, but their bones still show many structural features that are prone to fracture, such as increased cortical pores, reduced cortical area and reduced bone strength [4].

In recent years, with the development of oral implant dentures, the clinical demand for implant treatment is also increasing. However, for dental implant patients with T2DM and osteoporosis, continuous hyperglycemia or blood glucose fluctuations can significantly

increase the level of oxidative stress in the body, aggravate the inflammatory response, impair bone regeneration and delay bone healing, which may affect osseointegration [5]. Previous study has confirmed that diabetic patients have a higher risk of implant failure than non-diabetic patients, and are more likely to have implant loosening or loss [6]. Therefore, studying the pathogenesis, possible prevention and treatment methods for diabetic osteoporosis will help reduce postoperative complications of oral implant repair, and maintain or improve the long-term clinical efficacy of implant dentures in patients with diabetes, especially those with osteoporosis, and effectively reduce the healthcare cost and trauma of patients.

As the main source of osteoblasts, bone marrow mesenchymal stem cells (BMSCs) are important for bone remodeling and regeneration [7]. Under the guidance of specific transcription factors, they can be induced to differentiate into osteoblasts, chondroblasts, fibroblasts and some mesodermal cells [8]. Study had shown that high glucose can inhibit the osteogenic differentiation of BMSCs by various pathways. For example, nucleic acids, phospholipids and non-enzymatic glycosylation of proteins result in the formation of advanced glycation end-products (AGEs) in hyperglycemic states [9], and the accumulation of AGEs promotes chronic inflammation in diabetic patients, inhibiting osteoblast mineralization and negatively regulating bone formation [10]. However, the exact mechanism by which high glucose affects the osteogenic differentiation of BMSCs has not been fully studied.

MicroRNAs exert negative regulatory effects by binding to the 3'-untranslated region (3'-UTR) of target mRNA, which results in degradation or translational repression of mRNA [11, 12]. Study has suggested that miRNAs are important in bone formation and osteogenic differentiation processes, and dysregulation of miRNAs is also related to bone disease [13]. The miR-137-3p could directly inhibit runt related transcription factor 2 (RUNX2) and CXCL12, and silencing its expression could promote osteogenesis and angiogenesis in vivo and in vitro, thereby preventing hormone-induced femoral head necrosis [14]. MiR-376c-3p inhibited the osteogenic differentiation of BMSCs by targeting the osteogenesis-related gene IGF1R [15]. In addition, microRNAs are involved in high glucose-mediated bone formation. High glucose and high fat environment can induce the increased expression of miR-155, and silencing this gene could promote the osteogenic differentiation and upregulate the expression of SIRT1 [16]. Wang et al. confirmed that high glucose might promote miR-214-3p expression to inhibit the osteogenic differentiation of BMSCs in TIDM patients [17]. Gene miR-153-3p was the main target of our study. It plays a role in SULF2-induced differentiation of hepatic stellate cells into cancer-associated fibroblasts by TGFβ1/SMAD3 signaling pathway, thereby promoting the development of liver cancer [18]. Besides, lncRNA KCNQ1OT1 upregulated HIF-1α by targeting miR-153-3p to promote proliferation, migration and invasion of retinoblastoma cells [19]. However, few reports have been done on bone metabolism. Previous study showed that by kdm6a-induced H3K27me3 demethylation, the osteogenic differentiation of periodontal ligament stem cells could be inhibited by miR-153-3p [20]. However, whether high glucose could induce miR-153-3p to affect BMSCs osteogenesis has not been reported yet.

Therefore, this study mainly explored the effect of miR-153-3p on the osteogenesis of BMSCs in a high glucose environment. Meanwhile, the miR-153-3p expressions in the process of osteogenic differentiation in the medium with different glucose concentrations were measured, and the potential mechanism of its effect on the osteogenic differentiation of BMSCs was further explored, so as to find a new treatment method for improving the osseointegration of diabetic patients.

Materials and methods

Cell separation and culture

This study has been approved by the Animal Care Welfare Committee of Guizhou Medical University (Approval No. 2201420). BMSCs were obtained from the femur and tibia of 1-2-week-old SD rats (Experimental Animal Center of Guizhou Medical University) [21]. The BMSCs were resuspended in complete medium, containing 89% DMEM basal medium (Gibco, Carlsbad, CA, USA), 1% penicillin-streptomycin (Sigma-Aldrich, USA), and 10% fetal bovine serum (Biological Industries, Israel), and then incubated in a humidified incubator con-

Table 1. The transfection gene sequences

Description of gene	Sequence of gene
Rno-miR-153-3p mimics	(sense): 5'-UUGCAUAGUCACAAAAGUGAUC-3'
	(antisense): 5'-UCACUUUUGUGACUAUGCAAUU-3'
MicroRNA mimics N.C.	(sense): 5'-UUCUCCGAACGUGUCACGUTT-3'
	(antisense): 5'-ACGUGACACGUUCGGAGAATT-3'
Rno-miR-153-3p inhibitor	5'-GAUCACUUUUGUGACUAUGCAA-3'
MicroRNA inhibitor N.C.	5'-CAGUACUUUUGUGUAGUACAA-3'

taining 5% CO_2 at 37°C. Half of the medium was changed after 24 h, the whole medium was changed after 72 hours, and then changed every 2-3 days. After the cells were grown and fused to 80-90%, subculture was carried out, and the cells were subcultured at 1:2 or 1:3 ratio.

Flow cytometry analysis of stem cell surface markers

BMSCs were trypsinized and washed twice with pre-cooled PBS, then 1.0×10^6 cells were put into the flow sample tube and incubated with CD29, CD90, CD45, CD11b primary antibodies (BioLegend, San Diego, CA, USA) at room temperature for 15-25 min in dark. After incubation, unbound antibody was washed away with PBS. The fluorescently labeled samples were tested by Flow cytometry.

Osteogenic and adipogenic differentiation of BMSCs

To simulate the high glucose environment in vivo, the third-generation BMSCs were treated with high glucose (25 mM) as the cell growth environment, and a normal glucose (5.5 mM) group was used as the control. To induce osteogenic differentiation of cells, the osteogenic induction solution (0.01 M β-sodium glycerophosphate, 50 μg/mL vitamin C, 10 mM dexamethasone) was added to the culture medium with different glucose concentrations. After 21 days, 4% paraformaldehyde was applied to fix cells for 30 min, and 0.2% alizarin red was used to stain for 30 min. The cells were observed under a microscope and photographed. Cetylpyridinium chloride solution was used for semi-quantitative calcium nodules, and OD values were measured at 570 nm.

Referring to the kit instructions (Cyagen, China, RASMX-90031), BMSCs were placed in a special adipogenic induction medium for 21 days. Then, 4% paraformaldehyde was applied to fix cells for 30 min, and Oil Red O was used to stain for 30 min. The cells were observed under a micrroscope and photographed.

CCK-8 assay

The BMSCs (5,000/well) were incubated for 24, 48, 72 and 96 h. Subsequently, 100 μL of premix solution (90 μL of basal medium and 10 μL of cck-8 solution) was added and incubated for 1 h. A microplate reader (SYNERGYH4) was used for measuring the absorbance value (OD) at 450 nm.

Cell transfection

After the third-generation BMSCs (with density of 1×10^5 cells/mL) were grown and fused to 80-90%, the cells were starved overnight. Subsequently, mimic and inhibitor of miR-153-3p, and their respective negative controls were transfected with Lipofectamine 2,000 (Invitrogen, Carlsbad, CA, USA), respectively. The transfection efficiency of cells was tested by qRT-PCR (See **Table 1**). To silence the expression of RUNX2, siRUNX2 and its negative control (HANBIO, Shanghai, China) were transfected into BMSCs, respectively.

Real-time quantitative reverse transcription PCR (qRT-PCR)

According to the kit instructions, Trizol reagent (Invitrogen) was used to extract total cellular RNA from BMSCs. Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.) was applied for testing the concentration and purity of RNA. The first-strand cDNAs was synthesized by using PrimeScriptTM RT reagent kit (Takara, Dalian, China), and the TB Green Premix Ex TaqTM II kit (Takara) was applied for fluorescence quantitative PCR detection by ABI 7500 System instrument (Applied Biosystems, Carlsbad, CA, USA). cDNA synthesis and real-time PCR detection of miRNA were carried out by the miDETECTA TrackTM miRNA qRT-PCR Starter Kit (RIBOBIO, Guangzhou, China). The internal reference genes for mRNA and miR-153-3p were β-actin and U6, respectively (See **Table 2**). The fold change in expressions was calculated by $2^{-\Delta\Delta Ct}$ method.

Table 2. The primer sequences

Description of primer	Sequence of primer
RUNX2 primer (Forward)	5'-CTTCGTCAGCGTCCTATCAGTTCC-3'
RUNX2 primer (Reverse)	5'-TCCATCAGCGTCAACACCATCATTC-3'
ALP primer (Forward)	5'-GGCGTCCATGAGCAGAACTACATC-3'
ALP primer (Reverse)	5'-CAGGCACAGTGGTCAAGGTTGG-3'
BSP primer (Forward)	5'-AGCGGAGGAGACAACGGAGAAG-3'
BSP primer (Reverse)	5'-GTCGTGGTGCCATAACTGGTCAG-3'
CollagenI primer (Forward)	5'-TGTTGGTCCTGCTGGCAAGAATG-3'
CollagenI primer (Reverse)	5'-GTCACCTTGTTCGCCTGTCTCAC-3'
β-actin primer (Forward)	5'-CTGTGTGGATTGGTGGCTCT-3'
β-actin primer (Reverse)	5'-CAGCTCAGTAACAGTCCGCC-3'
U6 primer (Forward)	5'-CTCG CTTCGGCAGCACA-3'
U6 primer (Reverse)	5'-AACGCTTCACGAAT TTGCGT-3'

Alkaline phosphatase (ALP) activity

BMSCs in osteogenic induction medium with different glucose concentrations were taken, and total cell protein was extracted. Alkaline phosphatase (ALP/AKP) activity detection kit (Nanjing Jiancheng, Nanjing, China) was applied to measure the ALP activity in cells according to the instructions. Three sub-wells were set for each group, and the OD value at 510 nm was detected by a microplate reader.

ALP staining

After 7 days of osteogenic induction, the BMSCs were fixed with 4% paraformaldehyde for 30 min, and BCIP/NBT alkaline phosphatase ester color development kit (Beyotime, Shanghai, China) was used for ALP staining according to the instructions.

Western blot assay

The cells were lysed by RIPA buffer whole cell lysate (Solarbio, Beijing, China), and centrifuged at 12,000 g/min at 4°C for 10 min. The total protein was collected, 5x loading buffer was added, and the protein was denatured by boiling in a metal bath at 100°C for 10 min. The protein concentration was tested by the BCA Protein Assay Kit (Solarbio). Thereafter, 30 μg sample was separated in SDS-PAGE gel (10% separating gel, 5% stacking gel), transferred onto PVDF membrane, and incubated with primary antibodies (anti-ALP (1:1000, ab65-834, Abcam, UK), anti-Collagen I (1:1,000, ab260043, Abcam, UK), anti-RUNX2 (1:1500, mAb#12,556, Cell Signaling Technology, MA, USA) and anti-bone sialoprotein (BSP; 1:1000, DF7738, Affinity, Jiangshu, China)) overnight at 4°C. The anti-β-actin (1:2000, 20536-1AP, Proteintech, Wuhan, China) was used as internal reference group. Subsequently, protein samples were incubated with goat anti-rabbit IgG secondary antibody (1:5000, SA00001-2, Proteintech) for 1 h at room temperature. Finally, protein bands were exposed by a luminescence detector with ECL enhanced chemiluminescence kit (Millipore, Billerica, MA, USA) according to the instructions, and the gray value of each band was obtained by ImageJ software.

Dual-luciferase reporter gene assay

The RUNX2 target fragment with a binding site to miR-153-3p was cloned into the 3' non-coding region of the luciferase gene in the pGL3 luciferase reporter plasmid vector. At the same time, the target fragment was mutated and cloned into another plasmid vector to construct wild-type and mutant plasmids (constructed by Shanghai Genechem Co., Ltd). The Lipofectamine 2,000 (Invitrogen) was used for transfection, and miR-153-3p mimic or its negative control and pGL3-wt RUNX2 or pGL3-mut RUNX2 were co-transfected into HEK293T cells. After 48 h, the dual luciferase reporter gene assay kit (Yeasen Biotechnology, 11402-ES60, Shanghai, China) was used to test firefly and Renilla luciferase activities based on protocol, and the ratio was calculated.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism 9.0 (GraphPad Software). The measurement data were expressed as mean ± SD and analyzed by Student's t-test or one-way analysis of variance (ANOVA) with Tukey post hoc test. $P<0.05$ was considered as statistically significant.

Results

Culture and identification of BMSCs

In this study, BMSCs were successfully isolated from SD rats. Cell adherence was spindle-shaped and grew in a swirling pattern (**Figure 1A**). By alizarin red staining, the formation of

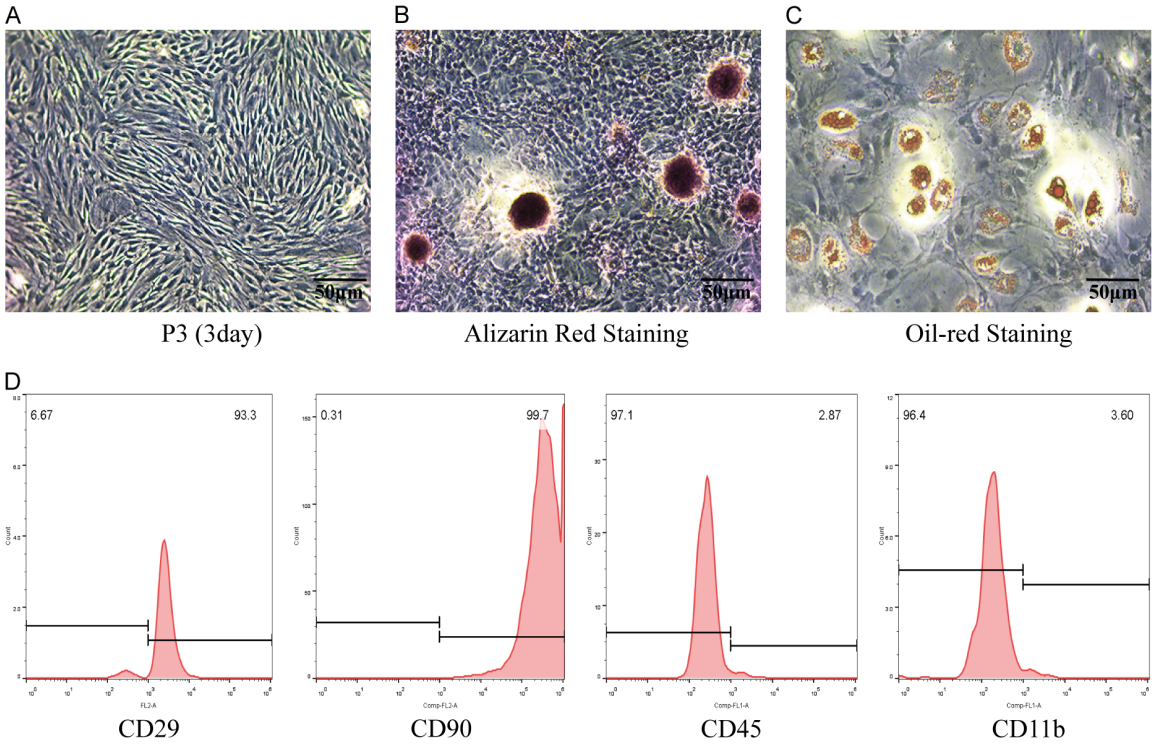

Figure 1. Culture and identification of BMSCs. A: BMSCs cultured to the third day of P3 generation. B: Calcium nodules detected by alizarin red staining. C: Lipid droplets detected by oil red staining. D: The stem cell surface markers CD29 and CD90 were positive, and CD45 and CD11b were negative. BMSCs: bone marrow mesenchymal stem cells.

red mineralized nodules was found at 21 days (**Figure 1B**). By oil red O staining, lipid droplet formation was found at 21 days of adipogenic induction (**Figure 1C**). Results of Flow cytometry showed that the stem cell surface markers including CD29 and CD90 were positive, and CD45 and CD11b were negative (**Figure 1D**).

High glucose induces up-regulation of miR-153-3p expression in BMSCs

The results of qRT-PCR showed that the expressions of ALP, RUNX2, BSP and Collagen I decreased in a high glucose environment (**Figure 2A**), and the protein expressions also showed the same trend (**Figure 2B**, **2C**), indicating that high glucose could inhibit the formation of osteogenesis, which was similar to the results reported in the previous study [22]. Under the simulated high glucose environment in vitro, the expression profile of miR-153-3p was measured on 0, 3, 7 and 14 days after the osteogenic induction. Compared with the high glucose group, the miR-153-3p in the normal glucose group gradually decreased in a time-dependent manner, especially on the 14th day (**Figure 2D**), which showed the same trend as the osteogenic differentiation of BMSCs, indicating that miR-153-3p may contribute to the process of osteogenic differentiation under high glucose environment and has a positive correlation with osteogenesis.

Osteogenic differentiation inhibited by high glucose-induced miR-153-3p

The miR-153-3p expression was promoted by high glucose, which was positively related to the osteogenic differentiation process, suggesting that it may participate in and affect the osteogenic differentiation process.

The intracellular expression of miR-153-3p was greatly increased after transfection of miR-153-3p mimic, and was significantly reduced after transfection with miR-153-3p inhibitor (**Figure 3A**). The results of cell proliferation experiments showed that the cell proliferation ability was attenuated in the miR-153-3p mimic group compared with that of the high glucose group, while slightly increased after transfection with miR-153-3p inhibitor, suggesting that high glucose could improve the proliferation ability (**Figure 3B**). It is speculated that glucose

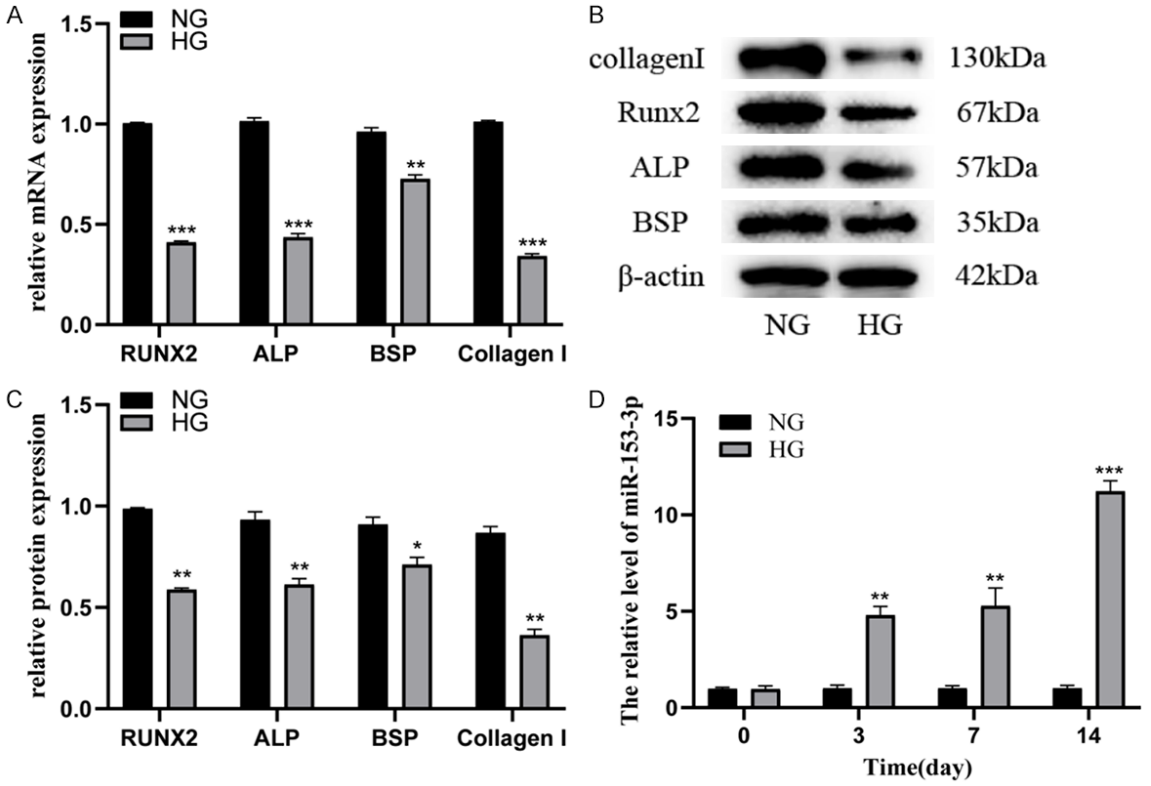

Figure 2. High glucose induced up-regulation of miR-153-3p expression in BMSCs. A-C: Effects of high glucose (25 mM) on the mRNA and protein expressions of osteogenesis-related genes in BMSCs compared with those of the normal control group. mRNA expression was measured by qRT-PCR, and protein expression was measured by Western blot. D: The expression of miR-153-3p in high glucose-induced BMSCs on 0, 3, 7, and 14 days, respectively (measured by qRT-PCR). All data are expressed as mean ± SEM, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. NG. BMSCs: bone marrow mesenchymal stem cells; NG: normal glucose; HG: high glucose; RUNX2: runt related transcription factor 2; ALP: alkaline phosphatase; BSP: bone sialoprotein; qRT-PCR: quantitative real-time PCR.

is the central energy source of all cells. High concentrations of glucose can provide more energy for cell growth within a certain range. The results of the ALP activity experiment showed that compared with the high glucose group, the ALP activity of BMSCs in the normal glucose group was higher, miR-153-3p mimic could attenuate the ALP activity of the cells, and inhibitor increased ALP activity in cells (**Figure 3C**). In addition, alizarin red staining results found that miR-153-3p mimic group showed reduced calcium deposition and mineralized matrix area compared with the high glucose group and the negative control group, and the ALP staining results showed the same decreasing trend (**Figure 3D**, **3E**), indicating that the ability of in vitro osteogenic differentiation was weakened. The mRNA and protein levels of osteogenesis-related genes ALP, BSP and Collagen I were further analyzed. qRT-PCR analysis indicated that miR-153-3p mimic significantly attenuated the expressions of these three osteogenic genes (**Figure 3F-H**), and results of Western blot showed the same trend (**Figure 3I**, **3J**), suggesting that miR-153-3p overexpression can inhibit the osteogenic differentiation.

Inhibitory effect of high glucose on osteogenic differentiation of BMSCs attenuated by silencing miR-153-3p

Alizarin red staining found that compared with the normal glucose group, the number of mineralized nodules and the area of mineralized matrix were reduced in BMSCs, while the addition of miR-153-3p inhibitor significantly weakened the inhibitory effect of high glucose treatment on the osteogenic differentiation, and ALP staining showed the same trend. (**Figure 4A**, **4B**). Results of qRT-PCR showed that the inhibitor could restore the decreased expression of ALP, BSP and Collagen I induced by high glucose stimulation (**Figure 4C-E**), and the protein results from Western blot showed the same trend (**Figure 4F**, **4G**). Our results sug-

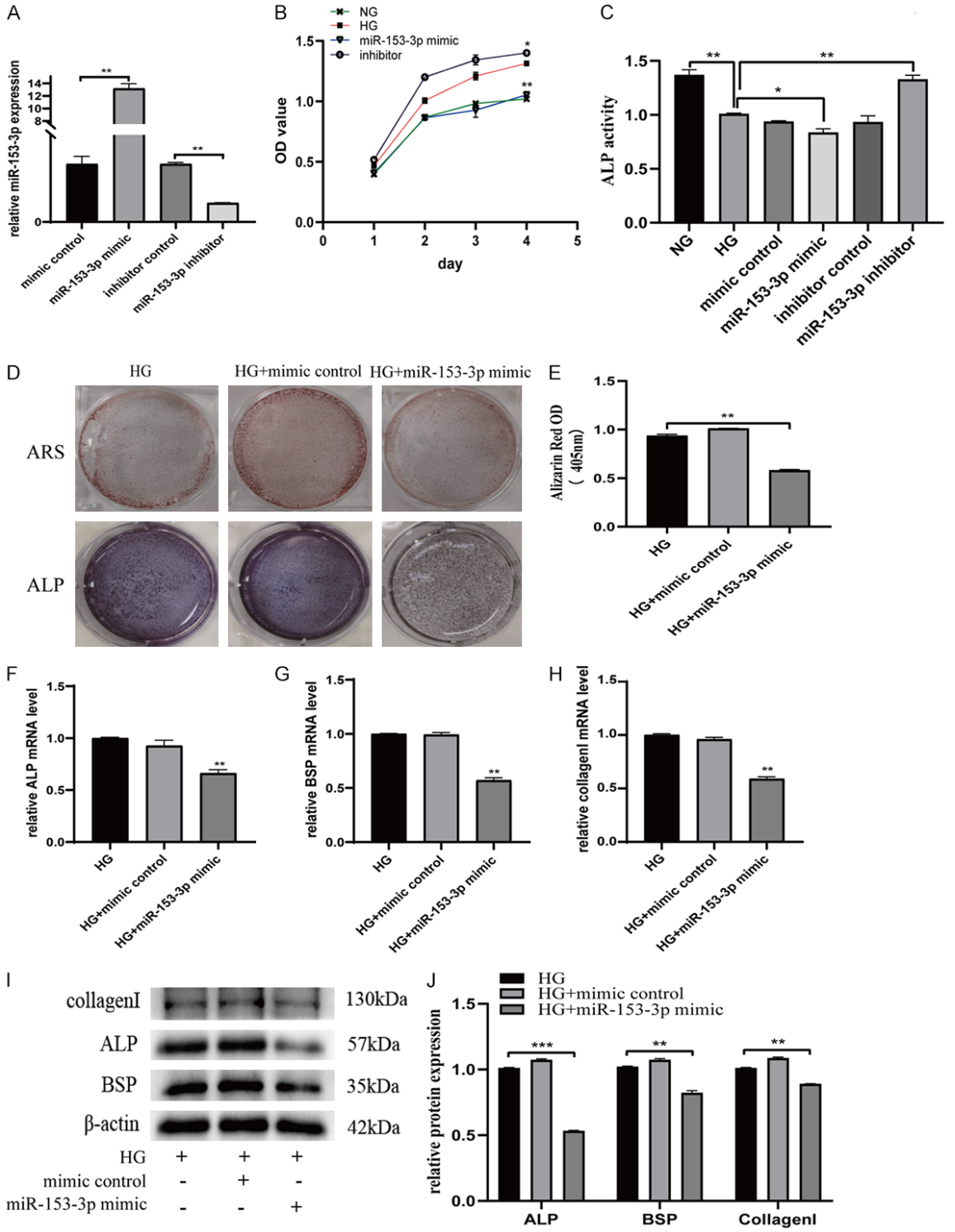

Figure 3. High glucose-induced miR-153-3p inhibited the osteogenic differentiation of BMSCs. A: qRT-PCR detection of cell transfection efficiency. B: The effect of CCK-8 on cell proliferation after treatment of BMSCs with miR-153-3p mimic or inhibitor. C: ALP activity assay to detect changes in ALP activity after 7 days of osteogenic induction. D, E: Alizarin red staining and ALP staining of calcium deposits in BMSCs after treatment with miR-153-3p mimic or mimic negative control in osteogenic medium. F-J: The mRNA and protein expression levels of osteogenesis-related genes BSP, Collagen I and ALP were detected by qRT-PCR and Western blot, respectively. All data are presented as mean ± SEM, $^{*}P<0.05$, $^{**}P<0.01$ vs. HG. RUNX2: runt related transcription factor 2; ALP: alkaline phosphatase; ARS: alizarin red staining; BSP: bone sialoprotein; BMSCs: bone marrow mesenchymal stem cells.

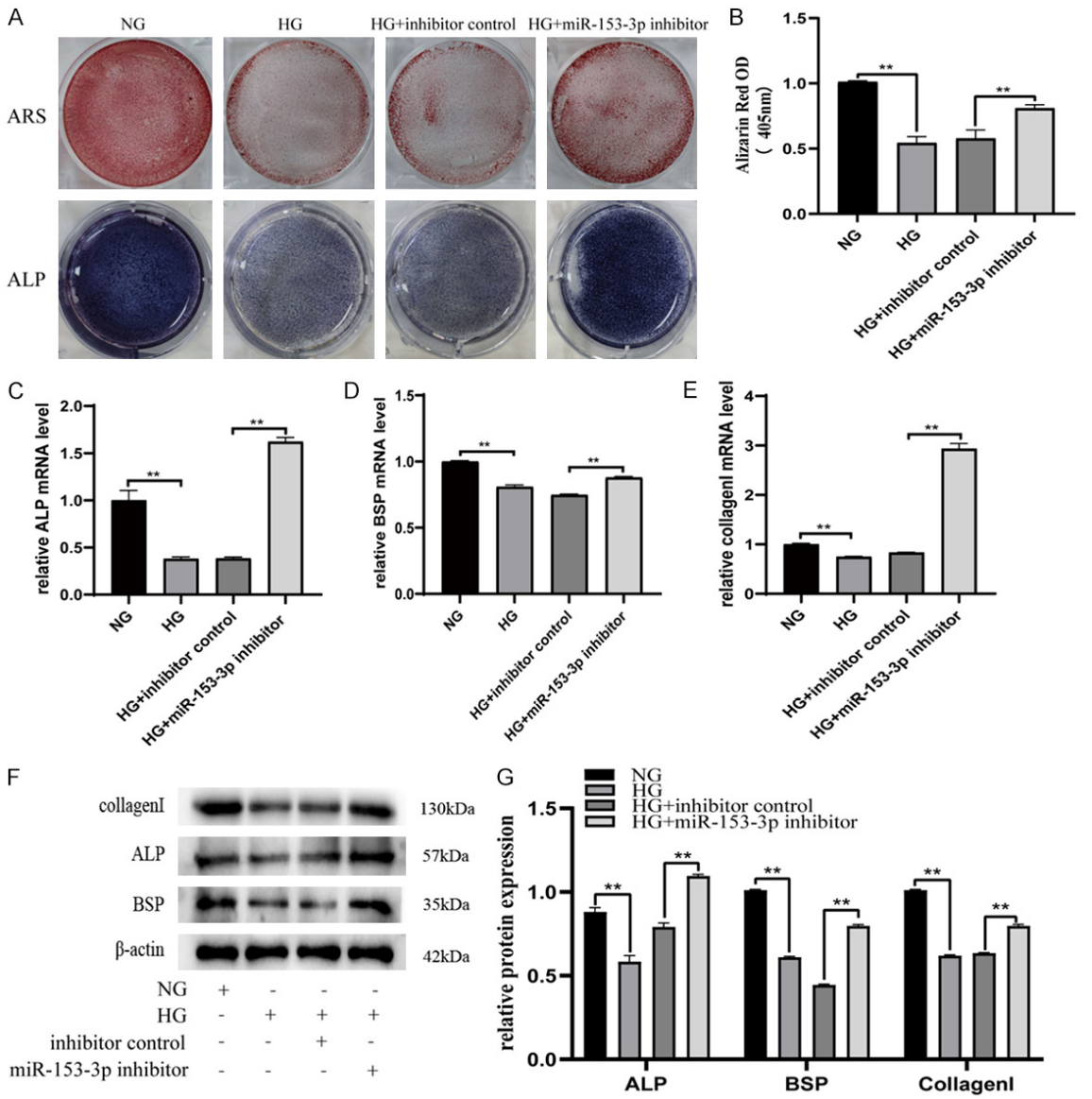

Figure 4. Silencing of miR-153-3p could attenuate the inhibitory effect of high glucose on the osteogenic differentiation of BMSCs. A, B: Changes in Alizarin red staining and ALP staining of calcium deposits in BMSCs after high glucose treatment with miR-153-3p inhibitor or inhibitor negative control. C-G: The mRNA and protein expression levels of osteogenesis-related genes BSP, collagen I and ALP were detected by qRT-PCR and Western blot, respectively. All data are presented as mean ± SEM, $^{**}P<0.01$. ALP: alkaline phosphatase; BSP: bone sialoprotein; BMSCs: bone marrow mesenchymal stem cells.

gested that miR-153-3p contributed to the osteogenic differentiation disorder, and silencing this gene could attenuate the inhibitory effect of high glucose on osteogenic differentiation.

High glucose-induced miR-153-3p targets RUNX2 specifically

The potential targets of miR-153-3p were predicted by software (miRBase, TargetScan). As shown in **Figure 5A**, a potential binding site was found for the miR-153-3p sequence in the 3'-UTR region of the transcription factor RUNX2. The mimic could significantly reduce the luciferase activity of pGL3-Runx2-3'-UTR-wt, while the luciferase activity of pGL3-Runx2-3'-UTR-mut mutant showed no significant inhibition (**Figure 5B**).

The mimic could significantly inhibit the mRNA and protein levels of endogenous RUNX2 mea-

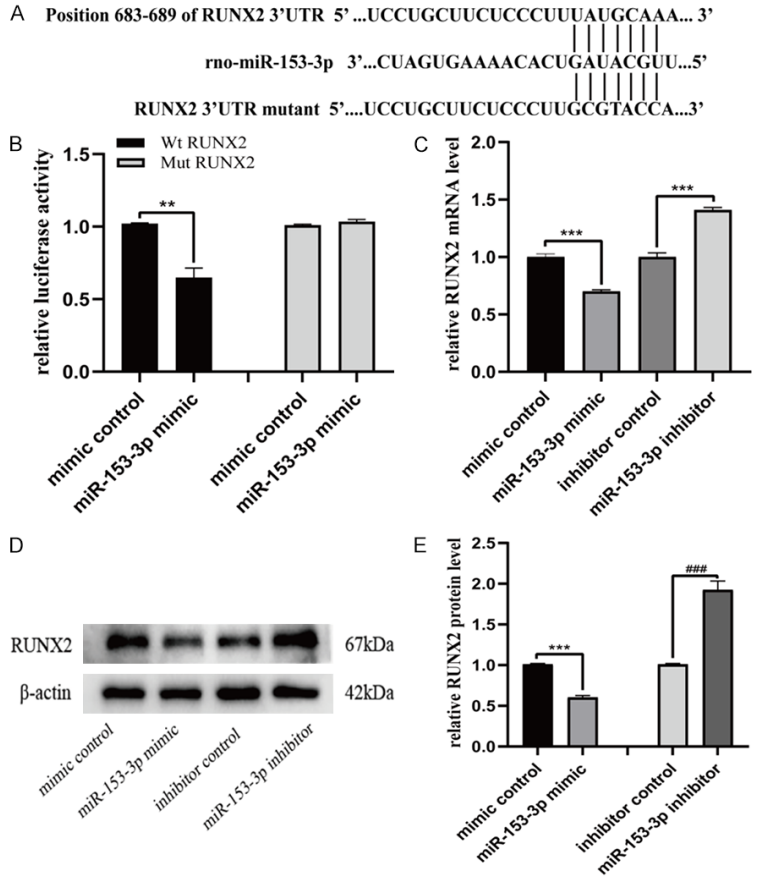

Figure 5. High glucose-induced miR-153-3p specifically targets RUNX2. A: Schematic illustration of the luciferase reporter gene design of the 3'-UTR region of RUNX2 with a binding site to miR-153-3p. B: The effect of miR-153-3p mimic and luciferase reporter plasmid on luciferase activity after co-transfection into HEK293T cells, respectively. C-E: Changes in mRNA and protein expression of RUNX2 after miR-153-3p mimic, miR-153-3p inhibitor and their corresponding negative controls treated BMSCs. **P<0.01, $^{###}$P<0.001, ***P<0.001. RUNX2: runt related transcription factor 2; 3'-UTR: the 3'-untranslated region; BMSCs: bone marrow mesenchymal stem cells.

sured by qRT-PCR and Western blot, while miR-153-3p inhibitor could up-regulate the levels (**Figure 5C-E**), which finding is consistent with the hypothesis. It showed that the inhibition of RUNX2 by miR-153-3p occurred at the transcriptional level and the translational level.

miR-153-3p inhibits osteogenic differentiation of BMSCs by inhibiting the expression of endogenous RUNX2

Under a high glucose condition, alizarin red staining indicated that miR-153-3p inhibitor could enhance the osteogenic differentiation, and silencing of RUNX2 could attenuate this effect. ALP staining showed the same trend (**Figure 6A**, **6B**), indicating that the osteogenic capacity was inhibited. Similarly, the miR-153-3p inhibitor could enhance the mRNA expression of osteogenic genes ALP, BSP and Collagen I in BMSCs, while silencing RUNX2 decreased the mRNA levels of these genes (**Figure 6C-E**). The same trend of protein levels was found in Western blot assay (**Figure 6F**, **6G**). All results showed that in a high glucose environment, osteogenic differentiation is attenuated by miR-153-3p through inhibiting the activity of transcription factor RUNX2, which may be a direct target gene for miR-153-3p.

Discussion

Osteoporosis is one of the common metabolic complications in patients with diabetes, mainly due to the imbalance of bone metabolism. Osteoporosis occurs when the bone formation in the body is slower than bone resorption. BMSCs have multi-directional differentiation potential, self-renewal ability, can differentiate into osteoblasts under the induction of specific conditions, and are involved in bone remodeling [23]. However, in a rat model of T2DM induced by high-fat and low-dose STZ, BMSCs were found to have impaired osteogenic potential, with reduced dellular metabolic activity, mineralization, ALP activity and osteogenic gene expression [24], which may be due to persistent hyperglycemia in vivo affecting the osteogenic differentiation ability, and ultimately causing the occurrence of T2DM-related bone complications. The high glucose can inhibit the osteogenic differentiation of BMSCs through multiple pathways, associating with a series of skeletal complications [25]. For example, through the lncRNA AK028326/CXCL13 pathway, high glucose can prevent the osteogenic differentiation of mesenchymal stem cells [26]. Therefore, studying the molecular mechanism of inhibiting

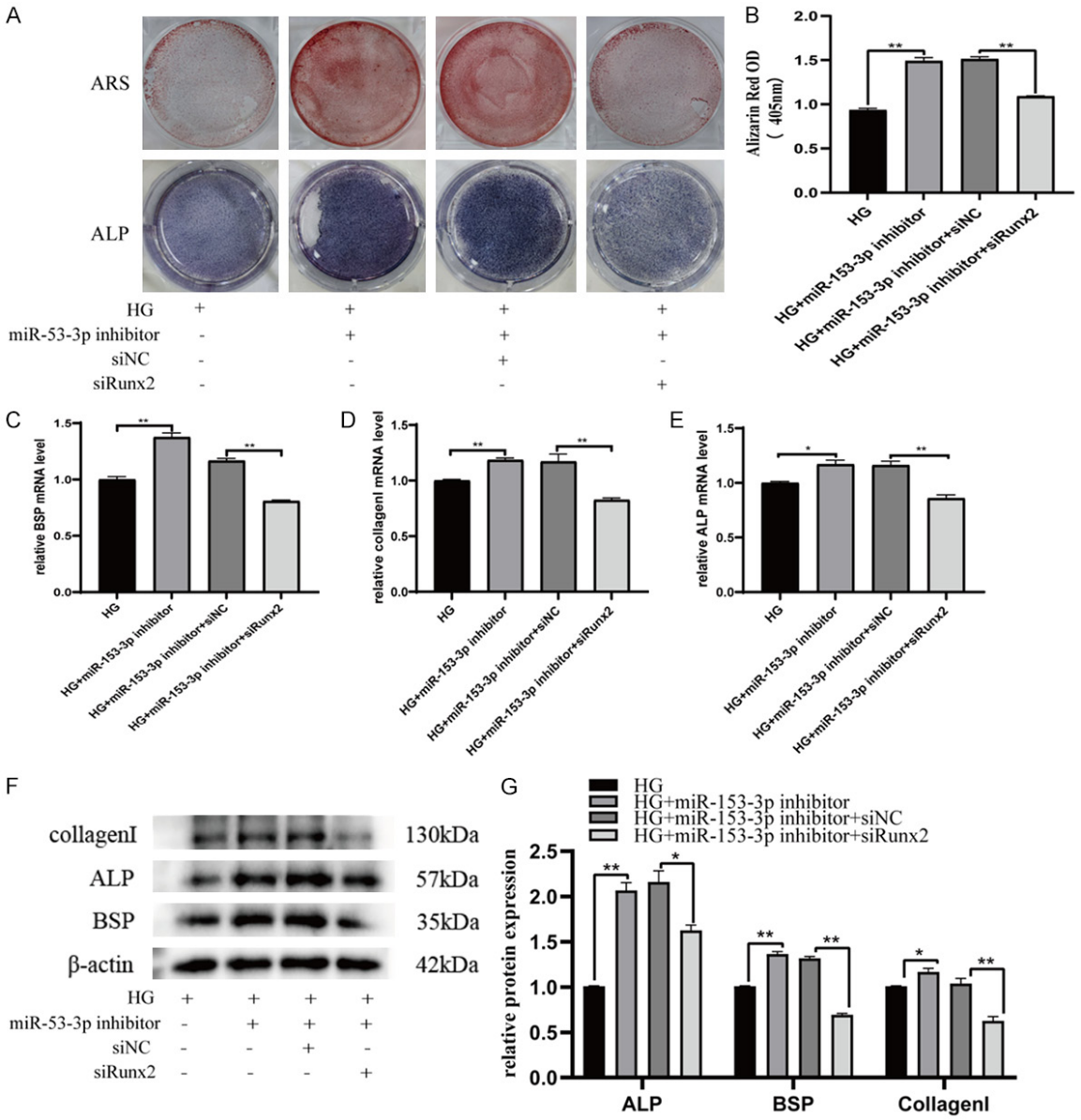

Figure 6. MiR-153-3p inhibited the osteogenic differentiation of BMSCs by inhibiting the expression of endogenous RUNX2. A, B: Alizarin red staining and ALP staining showing changes of calcium deposits in BMSCs after treatment with miR-153-3p inhibitor and RUNX2 siRNA in high-glucose osteogenic medium. C-G: The mRNA and protein expression levels of osteogenesis-related genes BSP, Collagen I, and ALP were measured by qRT-PCR and Western blot, respectively. All data were expressed as mean ± SEM, $^{*}P<0.05$, $^{**}P<0.01$. ALP: alkaline phosphatase; BSP: bone sialoprotein; RUNX2: runt related transcription factor 2; BMSCs: bone marrow mesenchymal stem cells.

the osteogenic differentiation will contribute to developing new therapeutic methods and reducing osteoporosis complications.

Study has indicated that miRNAs are needed in osteogenic differentiation [13]. Furthermore, multiple findings support that high glucose is a key regulator of miRNA expression in diabetes [27]. The miRNAs are related to regulating the occurrence and development of osteogenic differentiation in a high glucose environment [28]. Therefore, our study focused on whether the miR-153-3p is involved in the interaction between high glucose and osteogenic differentiation of BMSCs. According to reports, this gene is currently mainly studied in tumors, but less in the direction of stem cell osteogenesis. In this study, the effect of high glucose on

BMSCs and the expression changes of miR-153-3p during osteogenesis were tested by simulating a high glucose environment in vivo. Our results showed that high glucose inhibited the osteogenic differentiation, which is consistent with previous findings [29]. Besides, the miR-153-3p expression gradually increased during osteogenic differentiation in a time-dependent manner. In addition, overexpression of miR-153-3p could reduce the mineralization ability of BMSCs in vitro, and decrease the activity of ALP, the accumulation of mineralized matrix, and the expressions of osteogenic genes BSP, Collagen I and ALP. Inhibition of miR-153-3p reduced the inhibitory effect of high glucose-induced osteogenic differentiation. This trend is consistent with Jiang's findings [18]. Therefore, it was speculated that high glucose-induced miR-153-3p might have a negative regulatory effect on the osteogenic differentiation. Since miRNA mainly binds to the 3'-UTR region of its target gene mRNA and regulates gene expression [29], the software miRBase and TargetScan were used to predict the target gene of miR-153-3p, and found a potential binding site of transcription factor RUNX2. RUNX2 is an important nuclear transcription factor in the early stages of osteogenic differentiation, which upregulates the expression of bone matrix protein genes such as CollagenI, OPN, BSP and OCN [30]. Study has shown that RUNX2 is down-regulated in osteoporosis as a typical marker of osteoporosis [31]. In addition, ALP is also one of the early markers of osteogenic differentiation, as the mineralization of the extracellular matrix occurs, the enzyme can provide inorganic phosphate by hydrolyzing pyrophosphate and promote physical mineralization [32]. The results confirmed that miR-153-3p binds to RUNX2, which suggests that RUNX2 may be one of the target genes of miR-153-3p. Molecular studies further confirmed that high glucose-induced miR-153-3p reduces RUNX2, thereby inhibiting the osteogenic differentiation.

This study provided experimental support for miR-153-3p as a new therapeutic target for diabetic osteoporosis. However, the effect of miR-153-3p on osteogenesis was studied only from the cellular level, and animal-level evidence is still lacking. From the perspective of miRNA, this study revealed the possible mechanism of high glucose inhibiting the osteogenic differentiation, which was a complex process, involving the strict regulation of numerous transcription factors, signaling molecules and various signaling pathways. Further research is needed on other signaling pathways and detailed molecular regulatory mechanisms of miR-153-3p in regulating the osteogenic differentiation of BMSCs in a high glucose environment.

Conclusion

In conclusion, our study showed that high glucose-induced miR-153-3p could inhibit the osteogenic differentiation of BMSCs, and silencing miR-153-3p could alleviate the high glucose-induced osteogenesis disorder, which may be associated with the down-regulation of the expression of transcription factor RUNX2.

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Disclosure of conflict of interest

None.

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