

MicroRNA-335-5p inhibits osteoblast apoptosis induced by high glucose

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Abstract. Diabetic osteoporosis represents a serious health condition with increasing incidence. Previous studies have shown that microRNA (miR)-335-5p is highly expressed in MC3T3-E1 osteoblasts and promotes their differentiation via downregulating the expression of dickkopf-1 (DKK1). The present study investigated the effects of miR-335-5p on apoptosis of osteoblasts induced by high glucose (HG), as well as the underlying molecular mechanisms. MC3T3-E1 osteoblasts were transfected with miR-335-5p mimics or control miR and cultured under HG conditions for seven days. Reverse-transcription PCR and showed that, compared with the control group, the expression levels of miR-335-5p were significantly downregulated in the HG group. However, no significant differences were observed in the mRNA expression levels of DKK1 between these groups. Furthermore, flow cytometric analysis showed that the apoptotic rate was increased by >2-fold in the HG group compared with that in the control group, while miR-335-5p overexpression significantly decreased the apoptotic rate in these model cells by ~40%. In addition, western blot analysis revealed that the protein expression levels of DKK1 and caspase-3 were significantly elevated in the HG group, which was significantly inhibited by overexpression of miR-335-5p. These results may indicate that miR-335-5p overexpression inhibited HG-induced apoptosis of MC3T3-E1 osteoblasts through decreasing the protein expression levels of DKK1. The results of the present study suggested that miR-335-5p may represent a potential target for the treatment of diabetic osteoporosis.

Introduction

Diabetes and osteoporosis are common metabolic diseases, and are closely related. The effect of diabetes on bone metabolism is predominantly characterized by increased bone resorption, reduced bone formation and bone mineral content, and is prone to fracture, leading to osteoporosis (1). MicroRNAs (miRs) are a class of small, non-coding RNAs, 22-24 nucleotides in length, which post-transcriptionally regulate gene expression through specifically binding to the 3'-untranslated region (UTR) of target mRNAs and inducing either translational repression or mRNA cleavage (2-4). miRs are present in almost all eukaryotes, regulating approximately one-third of protein-coding genes and participating in various pathophysiological processes, including proliferation, differentiation, apoptosis, immunology, metabolism, growth and development (5-8).

miR-335-5p is encoded by the genomic region of chromosome 7q32.2 in humans (9). It has been shown that miR-335-5p suppresses the invasion and metastasis of tumor cells and regulate cytoskeleton dynamics in mouse oocytes (10,11). miR-335-5p is highly expressed in MC3T3-E1 osteoblasts and promotes their differentiation via downregulating the expression of dickkopf-1 (DKK1), which was demonstrated by Zhang *et al* (10) using a luciferase reporter assay as well as loss- and gain-of-function studies. As an inhibitor of the Wnt signaling pathway, DKK1 has key roles in several pathogenic processes, for example, cancer of the pancreas, stomach, liver, breast and cervix (12). miR-335-5p has been shown to influence osteoblast functions through the MAPK, FAK and ErbB signaling pathways (11). However, the effects of miR-335-5p and DKK1 on osteoblast apoptosis induced by high glucose (HG) and the underlying molecular mechanisms have remained elusive.

In the present study, the expression levels of miR-335-5p in MC3T3-E1 osteoblasts cultured under HG conditions were detected, and the effects of miR-335-5p overexpression on HG-induced apoptosis of osteoblasts as well as the expression levels of DKK1 in these cells were assessed.

Materials and methods

Cell line and culture. The MC3T3-E1 osteoblast cell line was purchased from Shanghai Fuxiang Biotechnology Co., Ltd.

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(Shanghai, China). Cells were cultured in α -modified essential medium (α -MEM; Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin/streptomycin antibiotics (Gibco). These cells were divided into the following groups: i) The control group, in which cells were cultured with 5.5 mmol/l glucose (Sigma-Aldrich, St. Louis, MO, USA); ii) the HG group, in which cells were cultured with 22.0 mmol/l glucose; iii) the miR-335-5p overexpression group, in which cells were transfected with agomir-335-5p (5'-UCAAGAGCAAUAACGAAAAAUGU-3'; synthesized by Shanghai GenePharma Co., Ltd., Shanghai, China) to overexpress miR-335-5p and then cultured with 22.0 mmol/l glucose; and iv) the transfection control group, in which cells were transfected with agomir negative control (NC; 5'-UUCUCCGAACGUGUCACGUTTACGUGACACGUCGGAATT-3'; Shanghai GenePharma Co., Ltd.) and cultured with 22.0 mmol/l glucose. Cells in the transfection groups were first transfected with the optimal doses of agomir-335-5p (100 pmol and 5 pmol for six-well and 96-well plates, respectively) for 48 h using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, and then cultured in α -MEM containing glucose for seven days.

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol reagent (Invitrogen). Reverse transcription was performed with the Superscript First-Strand Synthesis System (Invitrogen). qPCR was performed using the Supermix PreMix (SYBR Green; Invitrogen). The PCR amplification mixture contained 5 μ l SYBR Green, 0.4 μ l forward primer, 0.4 μ l reverse primer, 2 μ l cDNA and 2.2 μ l DEPC water. Sequences of the primers used are listed in Table I. PCR thermal cycling was performed on a Bio-Rad T100 thermo cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) as follows: Denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec, 58°C for 20 sec and 72°C for 30 sec, and a final extension at 72°C for 10 min. U6 and GAPDH were used as the controls for miR and mRNA detection, respectively. The relative expression levels of target RNAs were calculated using the $2^{-\Delta\Delta C_q}$ method (13).

Flow cytometry. Cell apoptosis was detected using flow cytometry. In brief, $\sim 1 \times 10^6$ cells were harvested and re-suspended in 1 ml binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl_2 ; Cube Biotech, Monheim, Germany]. An Annexin V-FITC/PI Apoptosis Detection kit (Invitrogen) was used. These cells were stained with 20 μ l Annexin V-fluorescein isothiocyanate and 25 μ l propidium iodide for 15 min at room temperature for 15 min. The fluorescence signals were detected using a flow cytometer (EPICS Altra; Beckman Coulter, Miami, FL, USA).

Prediction of miR-335-5p binding site in DKK1. Prediction of the binding site for miR-335-5p in DKK1 was performed using a combination of the following computational algorithms: Targetscan (<http://www.targetscan.org/>), microRNA (<http://www.microrna.org>) and miRBase Targets

(<http://www.mirbase.org/>). As shown in Fig. 1., bioinformatics analysis revealed that the 3'UTR of DKK1 mRNA contains a sequence which may be targeted by miR-335-5p. miR-335-5p has been confirmed to directly target DKK1 in MC3T3-E1 osteoblasts by a previous study (10).

Western blot analysis. Cells were lysed with radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). After centrifugation at $4,795 \times g$ for 15 min, the protein concentration in the supernatant was determined. A total of 1 μ l supernatant was added to 96-well plates and then 19 μ l PBS and 200 μ l BCA working fluid (A:B=50:1, BCA Protein Assay kit; Beyotime Institute of Biotechnology) was added to each well and then incubated at 37°C for 30 min. The OD value (A562 value) of each well was detected using a Bio-Rad 680 enzyme standard instrument (Bio-Rad Laboratories, Inc.), and then drawing a standard curve to calculate the protein concentration. Subsequently, 20 μ g protein was separated by 12% sodium dodecyl sulfate polyacrylamide gel (Beyotime Institute of Biotechnology) electrophoresis and then electronically transferred onto a polyvinylidene difluoride membrane (Beyotime Institute of Biotechnology). After blocking with 5% bovine serum albumin at 37°C for 2 h, the membrane was incubated with mouse monoclonal anti-DKK1 antibody (cat. no. ab61275; Abcam, Cambridge, UK; 1:200 dilution), rabbit polyclonal anti-caspase-3 antibody (cat. no. 9662; Cell Signaling Technology, Beverly, MA, USA; 1:500 dilution) or rabbit polyclonal GAPDH (cat. no. 10494-1-AP; Proteintech, Wuhan, China; 1:500 dilution) at 4°C overnight. Subsequently, the membrane was incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (Beyotime Institute of Biotechnology; cat. no. A0208; 1:2,000 dilution) and HRP-labeled goat anti-mouse IgG (Beyotime Institute of Biotechnology; cat. no. A0216; 1:2,000 dilution) secondary antibodies at 4°C for 2 h. The blots were visualized using enhanced chemiluminescence reagent (ECL Western Blotting Substrate kit; Pierce Biotechnology, Inc., Rockford, IL, USA). The Gel Doc XR imaging system (Bio-Rad Laboratories, Inc.) was used to capture images of the blots and Fusion FX5 software (Vilber, Lourmat, Marne-la-Vallée, France) was used for densitometric analysis.

Statistical analysis. Values are expressed as the mean \pm standard deviation. Statistical analysis was performed using the SPSS 19.0 software (International Business Machines, Armonk, NY, USA). Student's t-test was used for pair-wise comparison, and one-way analysis of variance was used for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference between values.

Results

HG decreases miR-335-5p in MC3T3-E1 osteoblasts, while not affecting DKK1 mRNA. To investigate the expression levels of miR-335-5p and DKK1 mRNA in MC3T3-E1 osteoblasts, RT-qPCR analysis was performed. Compared with the control group, the expression of miR-335-5p was significantly downregulated in the HG group ($P < 0.05$) (Fig. 2A). Transfection with miR-335-5p led to a significant

Table I. Primer sequences for reverse-transcription quantitative polymerase chain reaction.

Gene	Sequences
miR-335-5p	Forward: 5'-GCGTCAAGAGCAATAACG-3' Reverse: 5'-GTGCAGGGTCCGAGGT-3'
DKK1	Forward: 5'-CTCATCAATTCCAACGCGATCA-3' Reverse: 5'-GCCCTCATAGAGAACTCCCG-3'
U6	Forward: 5'-TGCAGAGGATCTAATT-3' Reverse: 5'-GAAAGACCAGTCCAAGTCC-3'
GAPDH	Forward: 5'-AGGTCGGTGTGAACGGATTG-3' Reverse: 5'-TGTAGACCATGTAGTTGAGGTCA-3'

DKK1, Dickkopf-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; miR, microRNA.

elevation of the expression levels of miR-335-5p in these cells cultured under HG conditions ($P<0.05$) (Fig. 2A). However no significant differences were observed in the mRNA expression levels of DKK1 between these groups ($P>0.05$) (Fig. 2B). These results suggested that miR-335-5p is significantly decreased in MC3T3-E1 osteoblasts cultured under HG conditions, and that transfection with miR-335-5p mimics efficiently increased miR-335-5p levels. As expected, the mRNA levels of DKK1 were not affected by miR-335-5p, as the latter interferes with the translation of DKK1 mRNA but not with DKK1 gene transcription.

miR-335-5p mimics inhibit HG-induced apoptosis of MC3T3-E1 osteoblasts. To investigate the effects of miR-335-5p on MC3T3-E1 osteoblast apoptosis, Annexin V/PI double staining and flow cytometric analysis were utilized. The results showed that, compared with the control group, the apoptotic rate in the HG group was increased by >2-fold ($P<0.05$) (Fig. 3). However, overexpression of miR-335-5p significantly decreased the apoptotic rate in these model cells by ~40% ($P<0.05$) (Fig. 3). These results indicated that miR-335-5p overexpression inhibited HG-induced apoptosis in MC3T3-E1 osteoblasts.

miR-335-5p mimics reduce HG-induced increases in the protein expression of DKK1 and caspase-3 in MC3T3-E1 osteoblasts. To investigate the protein expression levels of DKK1 and the apoptotic protein caspase-3 in MC3T3-E1 osteoblasts, western blot analysis was performed. Compared with the control group, the protein expression levels of DKK1 and caspase-3 were significantly elevated in the HG group ($P<0.05$), and were be significantly downregulated following overexpression of miR-335-5p in these model cells ($P<0.05$) (Fig. 4). These results suggested that miR-335-5p reduced the HG-induced protein expression of DKK1 and caspase-3 in MC3T3-E1 osteoblasts, which may have contributed to the inhibition of HG-induced apoptosis in these cells.

Discussion

Diabetes mellitus and osteoporosis are common metabolic diseases, and the incidence of diabetic osteoporosis has been increasing over the past few years (14). Osteoblasts are the



Figure 1. Predicted binding sites of miR-335-5p in the 3'-untranslated region of DKK1. DKK1, dickkopf-1; miR, microRNA; mmu, musculus.

primary functional cells in bone formation, which synthesize and secrete bone matrix. Osteoblasts have key roles in the mineralization and remodeling processes of bone. The dysfunction and/or loss of osteoblasts leads to increases in bone resorption and relative decreases in bone formation, resulting in a decrease of bone mass and an increase of the risk of bone fracture as well as osteoporosis (15).

miR-335-5p, a sub-type of miR-335, is encoded by chromosome 7q32.2 in humans. It has been shown that miR-335-5p is involved in the regulation of various pathophysiological processes (16-20). Li *et al* (16) have reported that miR-335-5p regulates the invasion and metastasis of gastric cancer cells. Furthermore, Cui *et al* (17) indicated that miR-335-5p regulates cytoskeletal dynamics in oocytes. In addition, miR-335-5p has been found to influence the function of white adipose tissues in patients with diabetes (18) and during the aging process (19), and to be a promising therapeutic target for wet age-associated macular degeneration (20). Besides, miR-335-5p is highly expressed in MC3T3-E1 osteoblasts, which promotes cell differentiation by downregulating DKK1 expression (10). The present study revealed that, compared with the control group, the apoptotic rate and the expression levels of caspase-3 were significantly increased in the HG group, which was consistent with the findings of a previous study (21). Furthermore, the expression of miR-335-5p in the HG group was significantly lower than that in the control group, indicating that miR-335-5p may regulate osteoblast function. DKK1 is an inhibitor of the Wnt signaling pathway. While Wnt/ β -catenin signaling promotes the differentiation of mesenchymal stem cells into osteoblasts and stimulates the proliferation and maturation of osteoblasts (22-24), DKK1 overexpression decreases the levels of β -catenin and subsequently inhibits osteoblast differentiation and induces apoptosis (24,25). Target searches using computational algorithms have predicted that DKK1 mRNA is a target of miR-335-5p with specific binding sites

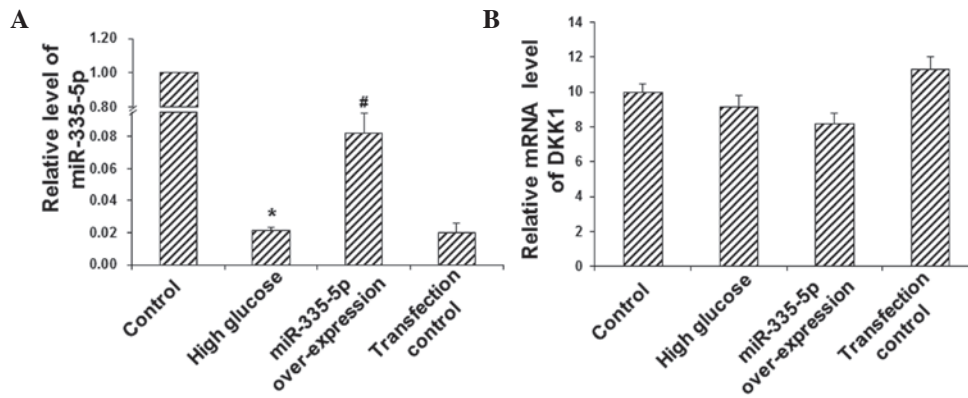


Figure 2. Expression levels of (A) miR-335-5p and (B) DKK1 mRNA in MC3T3-E1 osteoblasts cultured under high-glucose conditions were detected using reverse-transcription polymerase chain reaction analysis. Values are expressed as the mean \pm standard deviation of three independent experiments. * P <0.05 vs. control group; # P <0.05 vs. high glucose group. miR, microRNA.

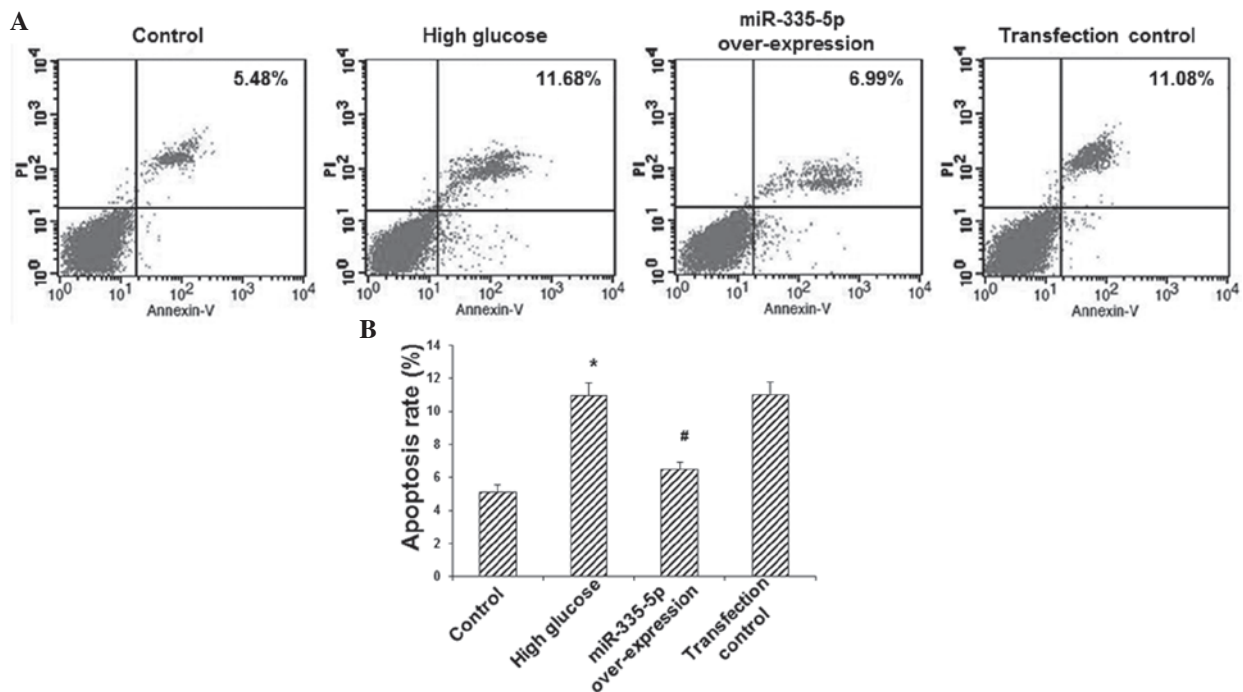


Figure 3. Effects of miR-335-5p on apoptosis of MC3T3-E1 osteoblasts. (A) The cellular apoptosis of MC3T3-E1 osteoblasts was detected using flow cytometry. (B) Apoptotic rates were determined by quantification of PI and Annexin-V-positive cells. Values are expressed as the mean \pm standard deviation of three independent experiments. * P <0.05 vs. control group; # P <0.05 vs. high glucose group. miR, microRNA; PI, propidium iodide.

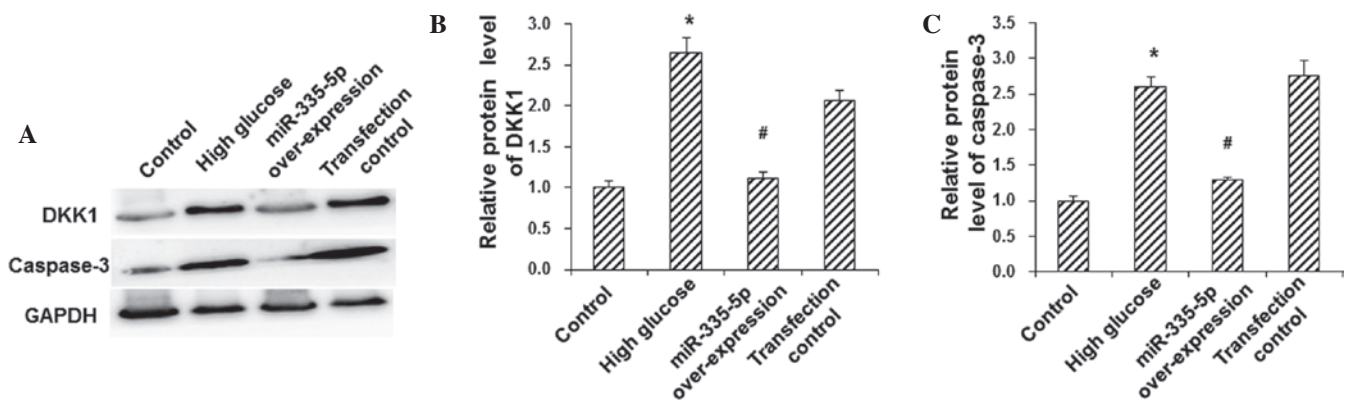


Figure 4. Protein expression levels of DKK1 and caspase-3 in MC3T3-E1 osteoblasts. (A) The protein expression levels of DKK1 and caspase-3 in MC3T3-E1 osteoblasts were detected with western blot analysis. (B and C) Protein expression levels of (B) DKK1 and (C) caspase-3 were quantified by densitometric analysis. Values are expressed as the mean \pm standard deviation of three independent experiments. * P <0.05 vs. control group; # P <0.05 vs. high glucose group. DKK1, dickkopf-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

in its 3'-UTR. The results of the present study indicated that the protein but not the mRNA expression levels of DKK1 were significantly elevated in the HG group compared with those in the control group, which may be attributed to the post-transcriptional regulation of miRNAs.

To verify whether miR-335-5p affects the function of MC3T3-E1 osteoblasts by regulating the expression levels of DKK1, these cells were subjected to vector-mediated overexpression of miR-335-5p. Of note, transfection with miR-335-5p significantly reduced the HG-induced increases of the apoptotic rate as well as the protein expression of DKK1 and caspase-3 in MC3T3-E1 cells. These results indicated that miR-335-5p may inhibit the apoptosis of osteoblasts through downregulating the protein expression levels of DKK1.

In conclusion, the present study demonstrated that HG conditions reduced the expression of miR-335-5p in MC3T3-E1 osteoblasts and thereby upregulated the protein expression levels of DKK1, ultimately inducing the cellular apoptosis. However, overexpression of miR-335-5p partly reversed HG-induced DKK1 overexpression and osteoblast apoptosis. These findings provided a basis for the upregulation of miR-335-5p as a means of prevention and treatment of diabetic osteoporosis.

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