Role of nucleolar protein NOM1 in pancreatic islet β cell apoptosis in diabetes

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Abstract. Diabetes is a metabolic disease that results from impairment in insulin secretion. The present study aimed to investigate the potential role of NOM1 in the function of pancreatic islet β cells and insulin secretion. MIN6 cells isolated from mice were transfected with siRNA-NOM1 to assess the influence of NOM1 on the expression of the cell apoptosis-associated proteins, such as caspase-3. In addition, MIN6 cells were cultured in medium containing different glucose concentrations in order to assess the sensitivity of MIN6 cells to glucose. The effect of NOM1 expression and glucose on MIN6 cell proliferation was also analyzed using an MTT assay. Furthermore, the mRNA expression levels of insulin 1 and 2 in MIN6 cells were detected using reverse transcription-quantitative polymerase chain reaction, while the expression levels of various cell apoptosis-associated proteins, Bcl-2 and Bax, were analyzed using western blot analysis. Compared with the control group, downregulation NOM1 and high glucose concentration of 25 mM significantly increased the cleaved caspase-3 level in MIN6 cells (P<0.05). In addition, downregulation of NOM1 significantly inhibited the MIN6 cell proliferation ability and reduced the insulin 2 mRNA expression (P<0.05). NOM1 knockdown also resulted in significantly increased Bax2 level and decreased Bcl-2 level in MIN6 cells (P<0.05). However no significant difference in insulin mRNA expression was observed between the control and siRNA-NOM1-transfected group (P>0.05). In conclusion, the present study suggested that NOM1 expression may be affected by blood glucose, and that NOM1 may be associated with pancreatic islet β cell apoptosis. In addition, NOM1 may serve a pivotal role in diabetes by affecting insulin synthesis and secretion in pancreatic islet β cells.

Introduction

Diabetes is a group of metabolic diseases, which is characterized by high levels of blood glucose and blood fat over a long period of time (1). Previous studies have indicated that the glycolipids joint toxicity results in increased oxidative stress and endoplasmic reticulum stress, as well as in protein misfolding, thus leading to the activation of pancreatic islet β cell apoptosis (2,3). Insulin, which is only secreted by pancreatic islet β cells, is an essential hormone for metabolic processes, including the metabolism of glucose, amino acids and fatty acids in the liver, muscles, adipose tissues and the brain (4,5). Increased blood sugar levels induce the transcription, synthesis and secretion of insulin, while genetic and environmental factors result in impaired insulin function and secretion (6,7). Therefore, a defects in insulin secretion is the major pathophysiological mechanism of diabetes. However, the mechanisms through which high blood sugar leads to pancreatic islet β cell damage and further promotes the progression of diabetes remain unclear.

Nucleolar protein NOM1, which contains an MIF4G domain and an MA3 domain, was first isolated from the bone marrow of children with acute myeloid leukemia (8,9). It has been previously demonstrated that proteins containing the MIF4G or MA3 domains are associated with the cell proliferation and growth, and serve pivotal roles in cell apoptosis and protein translation (10). NOM1 is highly conserved in a variety of species, including in yeast and in humans (11). Recently, the role of NOM1 has been reported to be associated with pancreatic development by regulating ribosome biogenesis in zebrafish (12), indicating that NOM1 may be involved in diabetes progression. However, the correlation between the NOM1 gene and diabetes has yet to be fully described.

In the present study, we analyzed the effect of NOM1 expression on pancreatic islet β cell apoptosis using a gene knockdown method under the condition of high glucose concentration. This study aimed to investigate the effect of NOM1 on pancreatic islet β cell proliferation and insulin expression during diabetes progression. Thus, the current study may provide a basis for understanding the pathogenesis of diabetes and developing therapeutic targets for diabetes in clinical practice.

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Table I. Primers used for targets quantitative polymerase chair reaction amplification in this study.

Gene	Primer	Sequence (5'-3')
Insulin 1	Sense	GAAGTGGAGGACCCACAAGTG
	Antisense	CTGAAGGTCCCCGGGGCT
Insulin 2	Sense	AGCCCTAAGTGATCCGCTACAA
	Antisense	AGTTGCAGTAGTTCTCCAGCTG
β-actin	Sense	GACGTTGACATCCGTAAAGA
	Antisense	GCCAGAGCAGTAATCTCCTT

Materials and methods

Cell culture. The mouse derived pancreatic β cells, MIN6, were purchased from the American Type Culture Collection (Masassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 6 mg/ml penicillin G, 5 mg/ml streptomycin sulfate, 2 mM L-glutamine and 50 μM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) at 37˚C and 5% CO_2.

MIN6 cell response to high glucose concentration. MIN6 cells were cultured in DMEM supplemented with 5 mM glucose (Sigma-Aldrich) and seeded in 6-well plates at a density of $1x10^6$ cells/well. Subsequent to culturing for 3 days, the cells were washed with serum-free and glucose-free medium, and then were incubated in glucose-free RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with glucose concentrations between 5 and 25 mM glucose. The medium was also supplemented with 0.2% bovine serum albumin, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Sigma-Aldrich). Subsequently, the expression levels of NOM1 and a cell apoptosis-associated protein caspase-3 in MIN6 cells was measured by reverse transcription-quantitative polymerase chair reaction (RT-qPCR) and western blot analysis after 72 h.

Cell proliferation assay. The effects of NOM1 and glucose on MIN6 cell proliferation ability were assessed using MTT assay, according to a previously described method (13). Briefly, MIN6 cells transfected with small interfering RNA (siRNA)-NOM1 or its negative control (GenePharma, Shanghai, China) at the logarithmic stage were cultured in DMEM mixed with 10% FBS. Transfection was performed using Lipofectamine 2000 regent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Cells were planted into 96-well plates at a density of $5x10^3$ cells per well. After culturing for 24 h, the cells were centrifuged at 225 x g for 10 min at 4˚C, and then the supernatant was removed. Next, 20 μl MTT was added to each well and then cultured for 4 h, followed by addition of 150 μl dimethyl sulfoxide to the cells for 10 min. The absorbance of cells in each well was observed at 570 nm under an absorption spectrophotometer (Hitachi U-3110; Hitachi, Tokyo, Japan). All experiments were conducted in triplicate.

Glucose-stimulated insulin secretion. MIN6 cells were transfected with siRNA-NOM1, and then incubated for 72 h at 37˚C. The MIN6 cells were then washed three times with phosphate-buffered saline (PBS) buffer (pH 7.4) and incubated for 2 h in Krebs-Ringer bicarbonate HEPES buffer (KRBH-BSA; including 120 mM NaCl, 4 mM KH_2PO_4, 20 mM HEPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM $NaHCO_3$ and 0.5% BSA, pH 7.4) containing 5 mM glucose. Subsequently, the medium was replaced with fresh KRBH-BSA supplemented with 5 and 25 mM glucose, followed by incubation for 1 h. Finally, the secreted insulin was detected using a radioimmunoassay kit (EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions (14).

RT-qPCR. The mRNA expression levels of insulin in MIN6 cells treated with different concentration of glucose and siRNA-NOM1 were detected as previously described (15). Briefly, MIN6 cells from different groups were collected at 48 h, ground in liquid nitrogen and then washed three times with PBS buffer (pH 7.4). Total RNA from MIN6 cells was extracted using TRIzol extraction reagent (Invitrogen; Thermo Fisher Scientific, Inc.) as previously described (16), and then RNase-free DNase I (Promega Corp., Madison, WI, USA) was added to remove DNA from the sample. The concentration and purity of extracted RNA were detected using a SMA4000 UV-Vis spectrophotometer (Merinton, Shanghai, China) at 260 nm. The purified RNA of 0.5 μg/μl was reverse transcribed into cDNA synthesis with the PrimeScript 1st Strand cDNA Synthesis kit (Takara, Dalian, China). Primers used for target amplification are shown in Table I, and all primers were synthesized by GenePharma. The mRNA expression levels were then detected using SYBR Green Real-Time PCR Master Mix (Invitrogen; Thermo Fisher Scientific, Inc.) in a total volume of 20 μl containing 1 μl cDNA from the PCR product, 10 μl SYBR Premix EX Taq, 1 μl of each primer (10 μM) and 7 μl double-distilled H_2O. The PCR reaction was performed at 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Melting curve analysis of the amplification products was subsequently performed at the end of each PCR analysis in order to confirm that only one product was amplified and detected. Data were analyzed according to the $2^{-\Delta\Delta Cq}$ method (17). β-actin was selected as the internal control.

Western blot analysis. Cell apoptotic proteins from MIN6 cells were investigated with western blotting analysis as
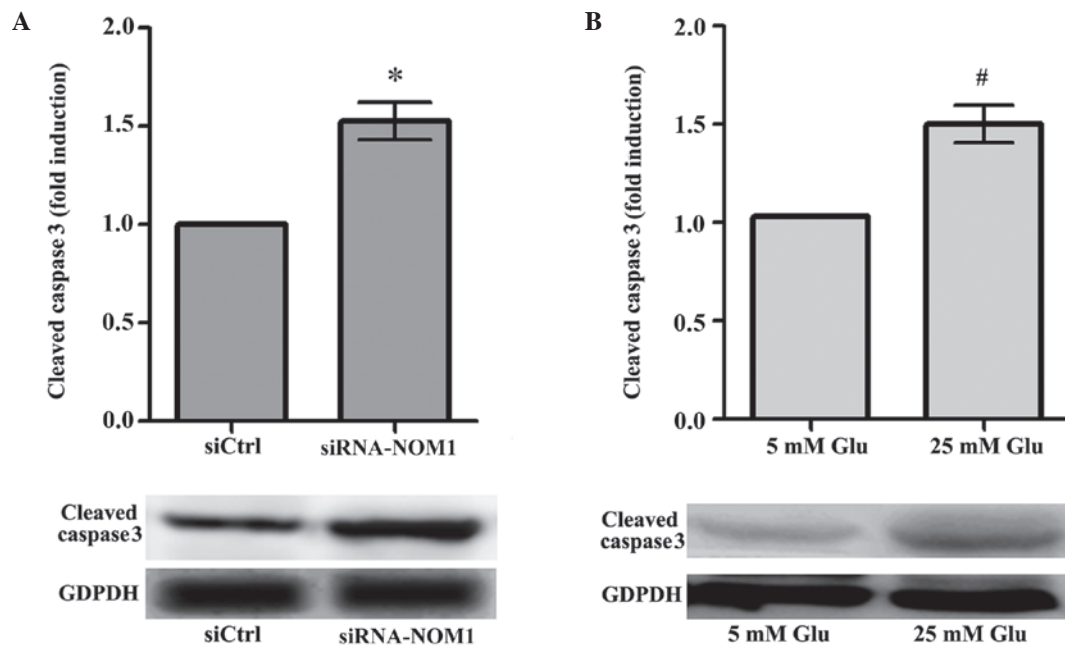
Figure 1. Effects of NOM1 expression and glucose concentration on MIN6 cells. (A) Downregulation of NOM1 increases the expression of the apoptotic factor, cleaved caspase-3, in MIN6 cells. (B) Effect of different glucose concentrations on the expression of cleaved caspase-3 (apoptotic factor) in MIN6 cells transfected with siRNA-NOM1. *P<0.05 vs. siCtrl; #P<0.05 vs. 5mM Glu. siRNA, small interfering RNA.

previously described (18). Briefly, MIN6 cells transfected with siRNA-NOM1 and treated with glucose (5 or 25 mM) were cultured for 48 h and then lysed with radioimmunoprecipitation assay buffer (Sangon Biotech Co. Ltd., Shanghai, China) containing phenylmethylsulfonyl fluoride. Next, the samples were centrifuged at 225 x g for 5 min at 4˚C. The supernatant was collected to measure the concentration of proteins using a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc., Rockford, IL, USA). Subsequently, a total of 20 µg protein per cell lysate was subjected to 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (EMD Millipore). The membrane was then blocked in Tris-buffered saline-Tween 20 (TBST) mixed with 5% non-fat milk for 1 h. Subsequently, the sample was incubated overnight at 4˚C with rabbit anti-human antibodies against NOM1 (cat. no. HPA019866; dilution 1:100; Sigma-Aldrich), B-cell lymphoma 2 (Bcl-2; cat. no. PRS3335; dilution 1:100; Sigma-Aldrich), Bcl-2-associated X protein (Bax2; cat. no. B3428; dilution 1:100; Sigma-Aldrich) and cleaved caspase-3 (cat. no. ab2302; dilution 1:1,000; Abcam, Cambridge, UK), followed by incubation with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (cat. no. ab6721; dilution, 1:1,000; Abcam) at room temperature for 1 h. The membrane was then washed three times with 1X TBST buffer for 10 min. Finally, detection was performed on X-ray films following addition of a chromogenic substrate BeyoECL Plus (Beyotime Institute of Biotechnology, Haimen, China) using an enhanced chemiluminescence method. GAPDH (cat. no. ab181602; 1:1,000 dilution; Abcam) was selected as the internal control.

Statistical analysis. All data in the present study are presented as the mean ± standard error of the mean. Statistically significant differences between two groups were analyzed using Student's t-test. P<0.05 was considered to demonstrate a statistically significant difference.

Results

Effects of NOM1 and glucose on MIN6 cells. The effect of NOM1 in MIN6 cells was analyzed using transfection of cells with a siRNA-NOM1 vector, and measured by RT-qPCR and western blot. Compared with the control group (siCtrl), downregulation of NOM1 expression resulted in a significantly increase in the expression of cleaved caspase-3 in MIN6 cells (P<0.05; Fig. 1A), suggesting that NOM1 may inhibit the expression of cell apoptosis-associated factors in MIN6 cells. In addition, the effect of different concentrations of glucose on MIN6 cells was analyzed (Fig. 1B). The results showed that, following treatment with 25 mM glucose, the cleaved caspase-3 expression in MIN6 cells significantly increased compared with that in the 5 mM glucose treatment group (P<0.05). Thus, a high glucose concentration may lead to increased expression of MIN6 cell apoptosis-associated factors.

Cell proliferation assay. siRNA-NOM1 was transfected into MIN6 cells treated with different concentrations of glucose (5 and 25 mM), and the cell proliferation ability of MIN6 cells in each group was assessed using an MTT assay (Fig. 2). When treated with 5 mM glucose, downregulation of NOM1 expression significantly decreased the cell proliferation ability of MIN6 cells compared with the negative control (P<0.05). A similar tendency was observed when MIN6 cells were treated with 25 mM glucose. These results indicated that NOM1 may serve a crucial role in the regulation of MIN6 cell proliferation ability.
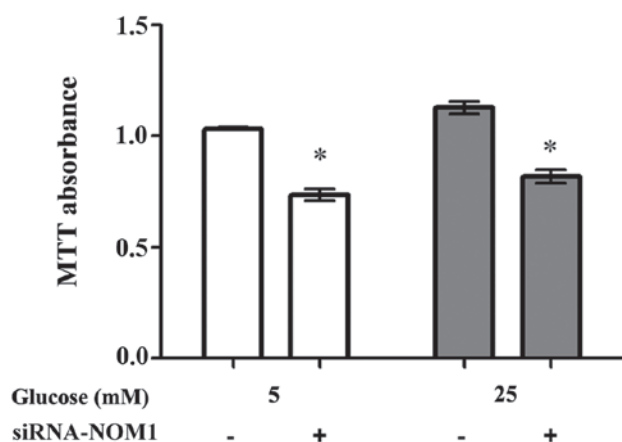
Figure 2. Effect of NOM1 on MIN6 cell proliferation ability. *P<0.05 vs. 5 or 25 mM glucose group without siRNA-NOM1. siRNA, small interfering RNA.
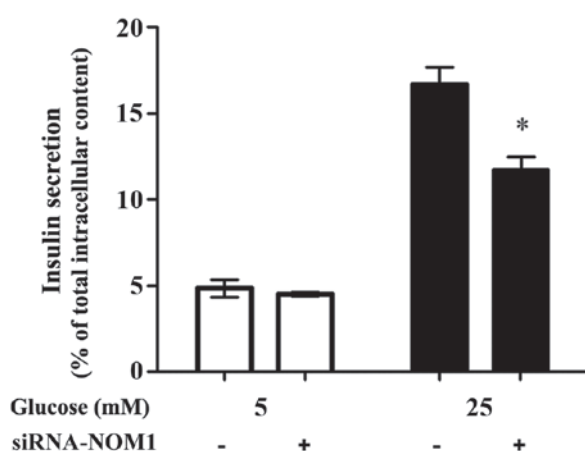

Figure 3. Insulin secretions in MIN6 cells from different group. MIN6 cells were transfected with siRNA-NOM1 and treated with different glucose concentration. *P<0.05 vs. 25 mM glucose group without siRNA-NOM1. #P<0.05 vs. 5 mM Glu. siRNA, small interfering RNA.
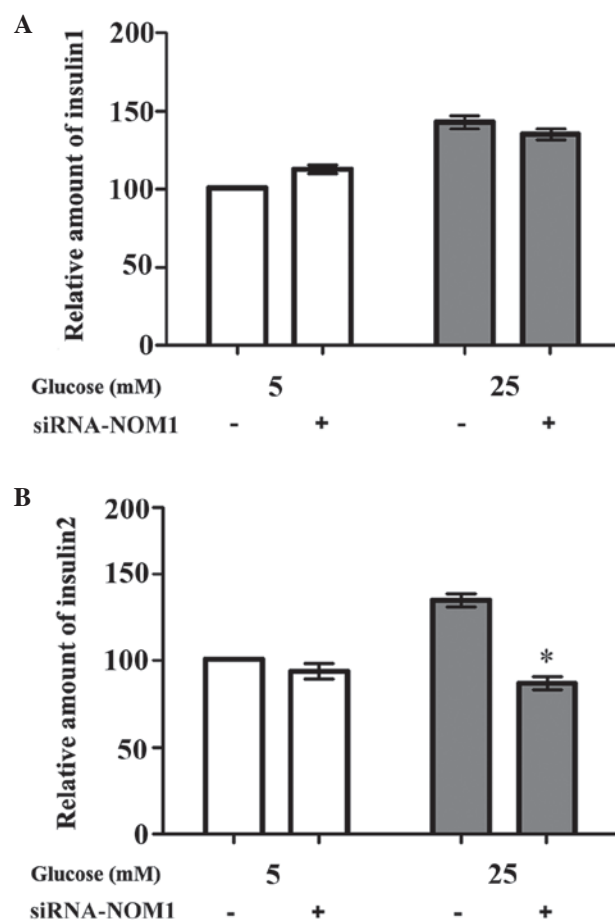

Figure 4. Reverse transcription-quantitative polymerase chain reaction analysis demonstrating the relative mRNA expression levels of (A) insulin 1 and (B) insulin 2 in MIN6 cells. *P<0.05 vs. 25 mM glucose group without siRNA-NOM1. siRNA, small interfering RNA.

Glucose-stimulated insulin secretion. As shown in Fig. 3, when MIN6 cells were treated with 5 mM glucose, no significant difference was observed in insulin secretion between the siCtrl and siRNA-NOM1 groups. When MIN6 cells were treated with 25 mM glucose, insulin secretion in MIN6 cells transfected with siRNA-NOM1 was significantly decreased when compared with that in the cells transfected with siCtrl (P<0.05; Fig. 3). However, insulin secretion in MIN6 cells transfected with siRNA-NOM1 was significantly higher in cells treated with 25 Mm glucose compared with those treated with 5 mM glucose.

Insulin 1 and insulin 2 mRNA expression levels in MIN6 cells. RT-qPCR analysis was used to detect the mRNA expression levels of insulin 1 and insulin 2 in the different transfection and glucose treatment groups (Fig. 4). The results showed that there was no significant difference in insulin 1 mRNA expression among the control and siRNA-NOM1-transfected groups, upon treatment of MIN6 cells with 5 or 25 mM glucose (P>0.05; Fig. 4A). However, when MIN6 cells were treated with 25 mM glucose after transfection for 48 h, the insulin 2 mRNA expression in the siRNA-NOM1-transfected group

was significantly reduced compared with the control group (P<0.05; Fig. 4B). However, there was no significant difference in insulin 2 mRNA expression between the two groups when MIN6 cells were treated with 5 mM glucose.

Western blot analysis. The expression levels of NOM1 and the cell apoptosis-associated proteins caspase-3, Bcl-2 and Bax in MIN6 cells from each experimental group were detected using western blotting analysis (Fig. 5). The results demonstrated that, when MIN6 cells were treated with 5 mM glucose, there were no significant differences in the levels of cell apoptotic proteins, including Bcl-2, Bax2, and NOM1, between the control and transfection groups (P>0.05). By contrast, when cells were treated with 25 mM glucose, NOM1 expression in the siRNA-NOM1 group was significantly reduced compared with the control group (P<0.05; Fig. 5A), whilst there was no significant difference in Bcl-2 expression among the different transfection groups (P>0.05; Fig. 5B). In addition, Bax2 expression in the siRNA-NOM1 group was significantly increased compared with the control group (P<0.05; Fig. 5C).

Discussion

Diabetes, characterized by increased levels of glucose in the blood and urine, is a metabolic disorder that involves
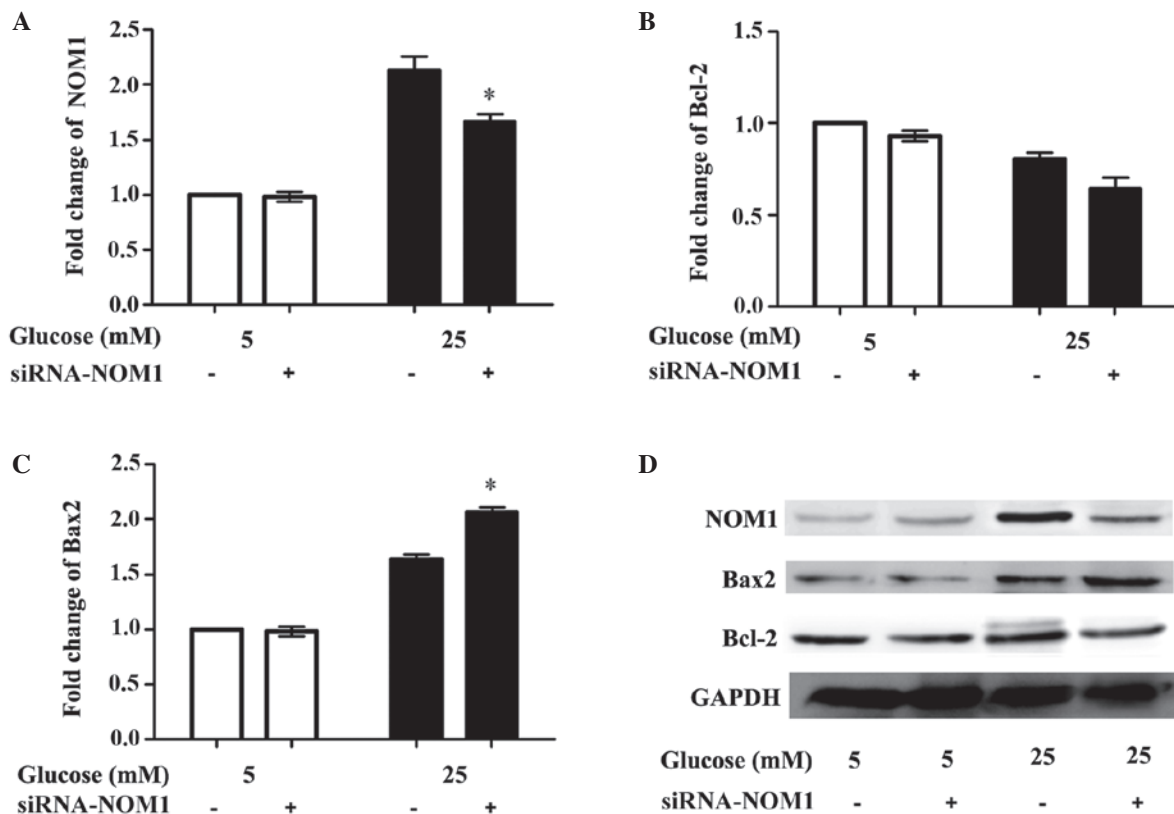
Figure 5. Western blot analysis was used to investigate the expression levels of cell apoptotic proteins in MIN6 cells. The expression levels of (A) NOM1, (B) Bcl-2, and (C) Bax2 in MIN6 cells with different glucose concentrations (5 or 25 mM) and transfected with or without siRNA-NOM1. (D) Western blots of the various proteins are shown. *P<0.05 vs. 25 mM glucose group without siRNA-NOM1. #P<0.05 vs. 5 mM Glu. siRNA, small interfering RNA.

the disturbance of sugar, fat and protein metabolisms resulting from impairment in the secretion and function of insulin (19,20). The present study analyzed the role of NOM1 expression in the apoptosis and insulin secretion of pancreatic islet MIN6 β cells. The results showed that knockdown of NOM1 was able to significantly inhibit MIN6 cell proliferation and significantly contribute to MIN6 cell apoptosis through increasing cleaved caspase-3 and Bax2 expression levels, but decreasing Bcl-2 expression (P<0.05). Furthermore, NOM1 knockdown was found to significantly decrease insulin secretion and the expression of insulin 2 mRNA in MIN6 cells cultured with a high glucose concentration (25 mM; P<0.05).

Since diabetes indicates a sensitivity to high sugar levels, the current study investigated the effect of glucose in NOM1-expressing cells by indirectly assessing the cleaved caspase-3 level in MIN6 cells (21). The results demonstrated that a high glucose concentration of 25 mM led to increased caspase-3 expression in MIN6 cells; since NOM1 may be negatively correlated to the cleaved caspase-3 level, the NOM1 expression was affected by 25 mM glucose in MIN6 cells. Insulin is a secreted peptide that regulates homeostasis in mammals, while insulin biosynthesis is controlled by glucose at many levels (22). Panda et al observed that the mRNA level of mouse insulin 2 was correlated with insulin expression (23). In the present study, NOM1 knockdown significantly decreased the insulin 2 mRNA expression in MIN6 cells, implying that downregulation of NOM1 may suppress insulin expression in MIN6 cells.

Pancreatic islet β cell proliferation is positively correlated to insulin secretion during diabetes progression (24). In the present study, knockdown of NOM1 significantly inhibited MIN6 cell proliferation, indicating that upregulation of NOM1 may promote MIN6 β cell proliferation. In addition, it has been previously demonstrated that caspase family proteins are cysteine proteases and they are widely involved in cell apoptosis (25). Caspase-3 is the apoptotic factor that can be activated by upstream key cellular proteins, such as cytochrome c and other signal proteins (26). Furthermore, Bcl-2 and Bax2 are two cell apoptosis-associated proteins that belong to the Bcl-2 family protein (27). Overexpression of anti-apoptotic Bcl-2 or downregulation of pro-apoptotic Bax2 has been shown to inhibit apoptosis in various cells (28). Therefore, the Bcl-2/Bax ratio has become an index for cell apoptosis in a number of diseases. For instance, Federici et al have proved that caspase-3 expression and Bcl-2/Bax ratio were altered during human pancreatic islets cell apoptosis induced by Arg[972] polymorphism (29). The present study revealed that NOM1 knockdown was able to significantly increase cleaved caspase-3 expression and decrease Bcl-2/Bax2 ratio in MIN6 cells, suggesting that upregulation of NOM1 may inhibit MIN6 β cell apoptosis.

In conclusion, the results presented in the current study suggested that NOM1 may serve pivotal roles in pancreatic islet β cell proliferation and insulin secretion by affecting cell apoptosis. The present study may provide a basis for the understanding of pathogenesis and development of diabetes therapies in clinical practice. However, further experimental studies are required for in-depth investigation of the underlying mechanism.

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