MicroRNA-18 promotes apoptosis of islet β-cells via targeting NAV1

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Abstract. The detailed pathogenesis of diabetes mellitus (DM) remains to be fully elucidated. The purpose of the present study was to explore the role of microRNA (miR)-18 in DM and its underlying mechanisms, providing novel ideas for the treatment of the disease. After inflammatory factor-mediated induction, miR-18 expression in the islet β-cell line MIN6 was detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). miR-18 mimics and miR-18 inhibitor were then constructed and transfected into MIN6 cells. The mRNA levels of pro-insulin in MIN6 cells were also detected by RT-qPCR. Released insulin levels and insulin secretion function of MIN6 cells were accessed by ELISA and glucose-stimulated insulin secretion assay, respectively. Apoptosis of MIN6 cells was detected by a terminal deoxy-nucleotidyl transferase-mediated deoxyuridinetriphosphate nick end labeling assay and western blot analysis of apoptotic proteins. The binding interaction of miR-18 and neuron navigator 1(NAV1), a constituent of the phosphoinositide 3-kinase (PI3K)/AKT pathway, was assessed using a dual-luciferase reporter gene assay. Finally, the regulatory effect of miR-18 on the PI3K/AKT pathway was determined by western blot analysis. After induction of inflammatory factors in MIN6 cells, miR-18 expression was markedly upregulated. Transfection with miR-18 mimics inhibited pro-insulin levels, as well as insulin production and secretion capacity. miR-18 knockdown partially abrogated the inhibited insulin secretion capacity induced by interleukin-1β (IL-1β) treatment. In addition, apoptosis of MIN6 cells was increased by miR-18 mimics. The dual-luciferase reporter gene assay confirmed the direct binding of miR-18 to NAV1. Western blot analysis suggested that miR-18 markedly inhibited the PI3K/AKT pathway in MIN6 cells. In conclusion, miR-18 expression is upregulated by IL-1β induction in islet β-cells. It was demonstrated that miR-18 promotes apoptosis of islet β-cells at least partially by inhibiting NAV1 expression and insulin production via suppression of the PI3K/AKT pathway.

Introduction

Diabetes mellitus (DM) is a metabolic disease characterized by hyperglycemia due to impaired insulin secretion and/or insulin dysfunction (1). In recent years, the incidence of DM has markedly increased and endangers human health. DM is affected by multiple factors, including genetic factors, autoimmune system defects and viral infections. Pancreatic islet β-cell dysfunction decreases insulin secretion and elevates blood glucose levels, eventually resulting in the occurrence of DM (2,3). DM is thought to be closely associated with islet inflammation caused by immune dysregulation. It has been suggested that inflammatory factors involved in the inflammatory response exert a crucial role in the occurrence and development of DM by inducing islet β-cell apoptosis and insulin secretion defects (4). The islet cells do not release inflammatory factors; however, macrophages that infiltrate islet cells release inflammatory factors, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), which may damage islet β cells. Activated T cells also produce inflammatory factors and induce apoptosis, leading to the death of islet β-cells (5). Studies have indicated that insulin induces AKT phosphorylation through the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway, which promotes glucose uptake and synthesis of glycogen (6,7). The synthesis of glycogen further affects the absorption and metabolism of blood glucose and increases insulin sensitivity.

MicroRNAs (miRNAs/miRs) are a class of highly evolutionarily conserved, single-stranded, non-coding RNAs. miRNAs degrade mRNA or inhibit translation of target genes via binding to target mRNAs (8). A complex regulatory network is formed by an individual miRNA with multiple target genes, to participate in cell differentiation, proliferation, apoptosis and metabolism (9,10). Hence, miRNAs are important in the differentiation, development, regulation of the quantity and maintenance of the function of islets (11,12). A previous study demonstrated that neuron navigator 1 (NAV1) was elevated under high-glucose conditions in isolated human

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pancreatic islets, indicating that NAV1 was involved in the pathogenesis of diabetes mellitus (13). It was also reported that miR-18 is involved in the regulation of the occurrence, invasion and metastasis of multiple tumor types (14). However, few studies have explored the function of miR-18 in islet β-cells. The present study aimed to explore the role of miR-18 in diabetes mellitus and the underlying mechanisms, providing novel ideas for the treatment of diabetes.

Materials and methods

Cell culture and transfection. The mouse islet β-cell line MIN6 was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare, Little Chalfont, UK) containing 15% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 50 μM β-mercaptoethanol, 100 U/ml penicillin, 10 μg/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate and 25 mM glucose. Cell passage was performed every 4 days. MIN6 cells were induced with a mixture of cytokines for 24 h at 37°C, including IL-1β (10 ng/ml; cat. no. GMP-101-1B; PrimeGene, Shanghai, China), TNF-α (10 ng/ml; cat. no. GMP-103-01; PrimeGene) and IFN-γ (10 ng/ml; cat. no. 224-09; PrimeGene). Prior to transfection, cells were seeded in 24-well plates for 24 h until the cell confluency reached 80-90%. miRNA negative control or miR-18 mimics and inhibitor (GenePharma, Shanghai, China) were diluted with serum-free and antibiotic-free medium and then mixed with Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The following sequences were used: miRNA negative control forward, 5'-UUCUCCGAACGUGUCCGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3'; miR-18 mimics forward, 5'-UAAGGUGCAUCUAGUGCAGAUAG-3' and reverse, 5'-AUCUGCACUAGAUGCACCUUAUU-3'; miR-18 inhibitor forward, 5'-CUAUCUGCACUAGAUGCACCUUA-3', and reverse, 5'-AUGUACGGUAUAUAGACCUGCGA-3'. After the mixture was maintained at room temperature for 20 min, it was added to each well, and the cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C for 4-6 h, then the medium was replaced. The subsequent experiments were performed after 24 h of transfection.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted with 400 μl TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and 80 μl chloroform was added, followed by centrifugation at 4°C and 4,500 x g/min for 15 min. The supernatant was collected and incubated with 200 μl isopropanol, followed by another round of centrifugation at 4°C and 4,500 x g/min for 15 min. The RNA extract was further purified using 75% ethanol. The RT procedure was performed using the Takara PrimeScript RT Master Mix kit (Takara Bio Inc., Tokyo, Japan) according to the manufacturer's protocol. RT-qPCR was performed using SYBR® Green Master Mix (Takara Bio Inc.) according to the manufacturer's protocol. The primer sequences used in this experiment were as follows: Proinsulin forward, 5'-GCAGCCTTTGTGAACCAACAC-3' and reverse, 5'-CCCCGCACACTAGGTAGAGA-3'; GAPDH forward, 5'-ACCCACTCCTCCACCTTTGA-3' and reverse, 5'-CTGTTGCTGTAGCCAAATTCG

T-3'; NAV1 forward, 5'-GCCTCAGACAATCTCAGTTCAG-3' and reverse, 5'-ACCACTGTCGTACTCCAGTTTT-3'; B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax) forward, 5'-CATATAACCCCGTCAACGCAG-3' and reverse, 5'-GCAGCCGCCACAAACATAC-3'; Bcl-2 forward, 5'-GTCTTCGCTGCGGAGATCAT-3' and reverse, 5'-CATTCCGATATACGCTGGGAC-3'; miR-18 forward, 5'-CAGTAAAGGTAAGGAGAGCTCAATCTG-3' and reverse, 5'-CATACAACCACTAAGCTAAAGAATAATCTGA-3'. The qPCR was performed according to the miScript SYBR Green PCR kit instructions (Qiagen GmbH, Hilden, Germany). The relative RNA expression levels were analyzed using the $2^{-\Delta\Delta Cq}$ method (15).

Glucose-stimulated insulin secretion (GSIS) assay. MIN6 cells were washed with Krebs-Ringer bicarbonate HEPES (KRBH) buffer once and incubated with 0.2 ml glucose-free KRBH buffer for 1 h. After discarding the supernatant, MIN6 cells were maintained in low-glucose KRBH buffer for 1 h, followed by incubation with high-glucose KRBH buffer for another hour. Prior to the GSIS assay, 200 μl 10% ethanol hydrochloride solution was added in each well and released insulin levels were detected by ELISA.

ELISA. Corresponding reagents were prepared and placed at room temperature. Sample or standard solution (100 μl) was added in the sample wells or standard wells, respectively. Corresponding antibodies, including proinsulin antibody (1:500; cat. no. ab8304; Abcam, Cambridge, MA, USA) and insulin antibody (1:500; cat. no. ab100578; Abcam) were then added for incubation at room temperature for a total of 2 h. Subsequently, horseradish peroxidase-labeled antibodies were added (1:1,000; cat. no. ab150074; Abcam). After incubation for 1 h at room temperature, 100 μl substrate was added, followed by colour reaction in the dark for 10 min. The absorbance at the wavelength of 450 nm was detected by a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay. MIN6 cells were fixed in 4% formaldehyde and then washed with PBS containing 2% hydrogen peroxide at room temperature. Two drops of TdT enzyme buffer (Beyotime Institute of Biotechnology, Haimen, China) were then added to the cells and allowed to react at room temperature for 1 h prior to termination. The cells were incubated in TdT buffer for 1 h at 37°C. After washing with PBS for 3 times, cells were incubated with the peroxidase-labeled anti-digoxigenin antibody (1:200; cat. no. ab150155; Abcam) in a wet box at room temperature for 30 min. TUNEL staining was observed and recorded under an optical microscope (IX70; Olympus, Tokyo, Japan).

Dual-luciferase reporter gene assay. Through a bioinformatics prediction (genemania.org), NAV1 was screened out as a target gene of miR-18. MIN6 cells were inoculated into a 48-well plate and cell density was allowed to reach 40-60% on the next day. The cells were transfected using Lipofectamine 2000. Transfection experiments were performed using NC mimics or microRNA-18 mimics, NC inhibitor or microRNA-18 inhibitor, NAV1-WT or NAV1-MUT plasmid (0.2 μg; cat. no. k801-200; AmyJet Scientific, Inc., Wuhan,
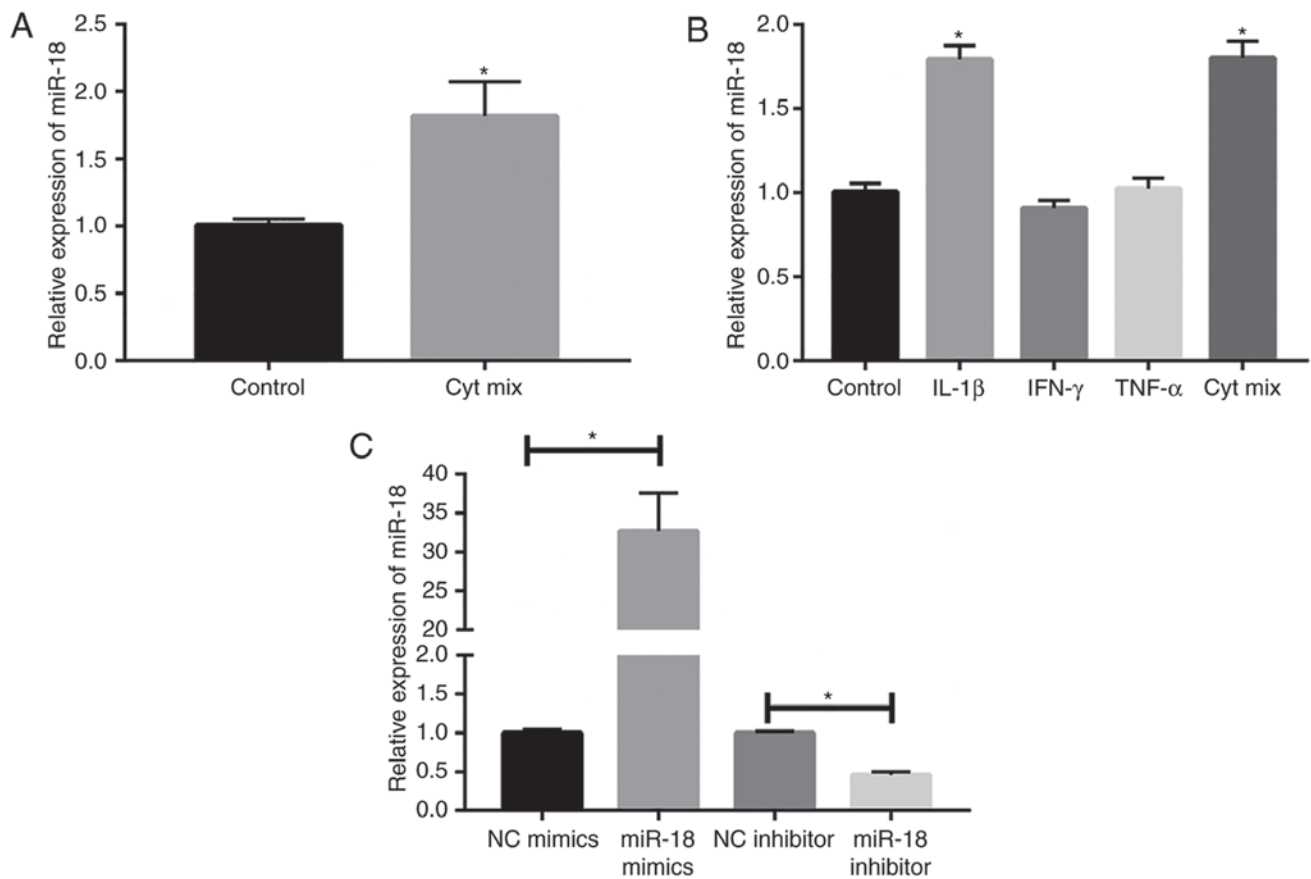
Figure 1. Inflammatory factors promote miR-18 expression in islet β-cells. (A) miR-18 expression was markedly elevated in cytokine-induced MIN6 cells. (B) miR-18 levels were upregulated by induction with IL-1β, TNF-α and IFN-γ or their combination. (C) The upregulation or knockdown efficacies of miR-18 mimics and inhibitor in MIN6 cells. *P<0.05 vs. Control. miR, microRNA; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; NC, negative control; Cyt mix, mixture of cytokines IL-1β, TNF-α and IFN-γ.

China), and an internal reference plasmid PRL-SV40 (0.004 μg; cat. no. k803-500; AmyJet Scientific, Inc.). At 24 h after cell transfection, cells were lysed using a dual luciferase reporter gene assay system solution (Promega Corporation, Madison, WI, USA) to detect fluorescence intensity. Comparison with *Renilla* luciferase activity was used for normalization.

Western blot analysis. Total protein was extracted from treated cells with radioimmunoprecipitation assay buffer, BCA method (Beyotime Institute of Biotechnology) was used for quantification of total protein. A total of 10 μl protein was loaded and separated by SDS-PAGE (12% gel) electrophoresis and transferred to a polyvinylidene difluoride membrane (Roche, Basel, Switzerland). Membranes were washed with Tris-buffered saline containing Tween-20 (TBST), and blocked with 5% skimmed milk with TBST at 25°C for 1 h. After incubation with primary antibodies, including NAV1 (cat. no. ab65166), PI3K (cat. no. ab151549), phosphorylated (p)-PI3K (cat. no. ab182651), AKT (cat. no. ab8805), p-AKT (cat. no. ab38449), Bcl-2 (cat. no. ab59348), BAX (cat. no. ab32503) and GAPDH (cat. no. ab8245; all from Abcam) at 4°C overnight and secondary antibodies (1:1,000; cat. no. ab6940; Abcam). Enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.) was used to detect the signal on the membrane. Quantity One (version 4.0; Bio-Rad

Laboratories) was used for quantification of western blotting signals.

Statistical analysis. The SPSS 21.0 statistical software package (IBM Corp., Armonk, NY, USA) was used for data analysis. Values are expressed as the mean ± standard deviation. Comparison between multiple groups was performed using one-way analysis of variance followed by a least significant difference post-hoc test. P<0.05 was considered to indicate statistical significance.

Results

Inflammatory factors promote miR-18 expression in islet β-cells. Multiple inflammatory factors are involved in the occurrence and progression of DM through a complex regulatory network. In the present study, MIN6 cells were induced with a mixture of cytokines (IL-1β, TNF-α and IFN-γ). After 24 h of incubation, miR-18 expression was markedly elevated in MIN6 cells (Fig. 1A). To further examine the effect of inflammatory factors on miR-18 levels, MIN6 cells were induced with 10 ng/ml IL-1β, TNF-α, IFN-γ or a combination of these cytokines. The results indicated that the levels of miR-18 were upregulated by induction with IL-1β or a combination of these cytokines, suggesting that IL-1β markedly induced miR-18 expression, while TNF-α and IFN-γ did not (Fig. 1B).
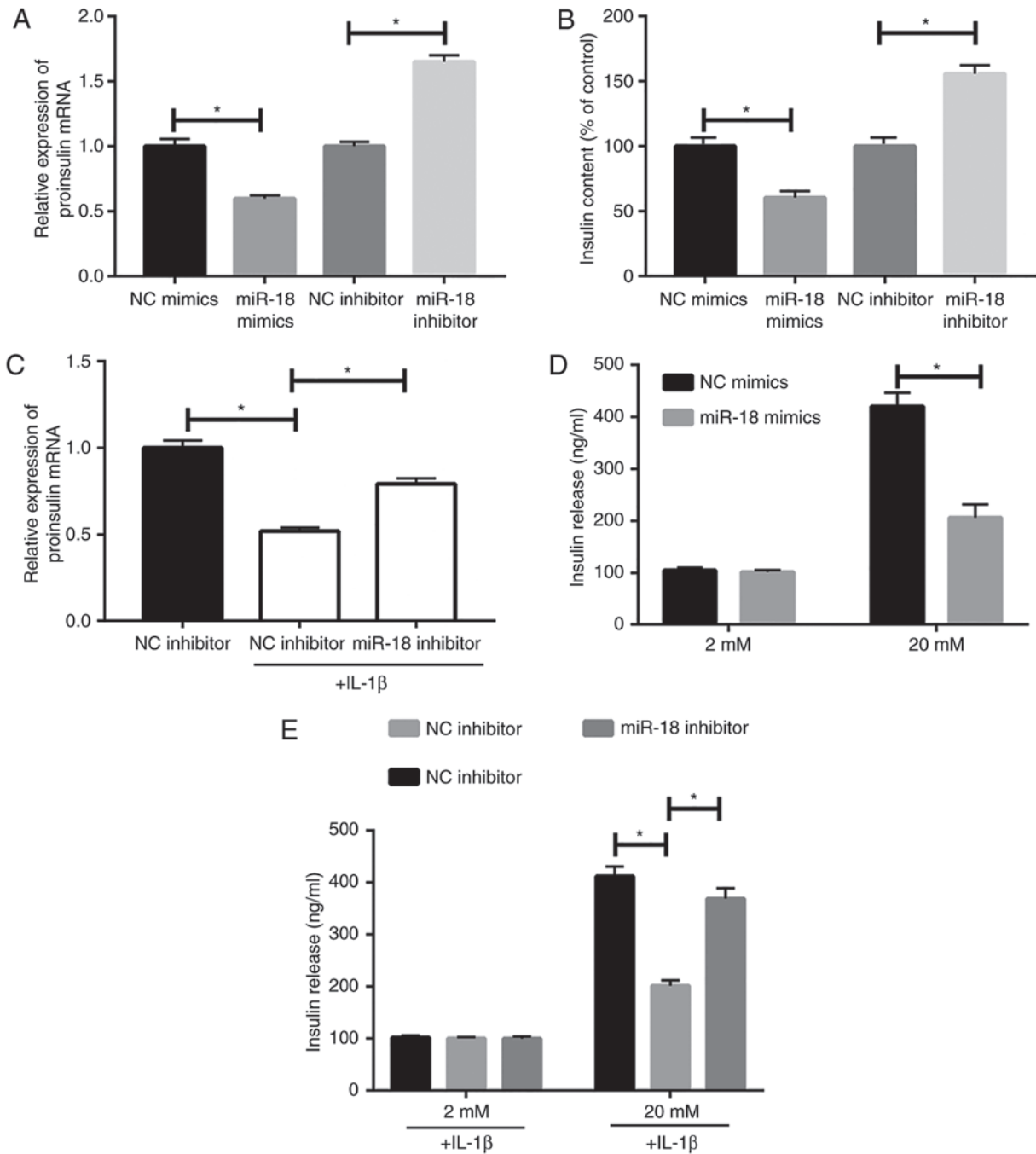
Figure 2. miR-18 inhibits insulin production by islet β-cells. (A) Reverse transcription-quantitative polymerase chain reaction analysis indicated that miR-18 mimics caused a downregulation of pro-insulin levels in MIN6 cells. (B) miR-18 mimics caused a reduction of released insulin levels as detected by ELISA. (C) miR-18 knockdown partially abrogated the IL-1β-induced decrease in the levels of pro-insulin. (D) A glucose-stimulated insulin secretion assay indicated the inhibitory effect of miR-18 on insulin secretion. (E) The suppressed insulin secretion capacity in IL-1β-induced MIN6 cells was partially abrogated by miR-18 knockdown. *P<0.05. miR, microRNA; IL, interleukin; NC, negative control.

Subsequently, miR-18 mimics and inhibitor were constructed and their transfection efficacies in MIN6 cells were verified by RT-qPCR (Fig. 1C).

miR-18 inhibits insulin production. The results of the RT-qPCR analysis indicated that transfection of miR-18 mimics caused a downregulation of proinsulin levels in MIN6 cells (Fig. 2A). Furthermore, miR-18 mimics reduced released insulin levels, as detected by ELISA (Fig. 2B). To confirm the regulatory effect of miR-18 on islet β-cells, MIN6 cells were induced

with 10 ng/ml IL-1β after transfection with miR-18 inhibitor. The results demonstrated that miR-18 knockdown partially abrogated the IL-1β-induced reduction of proinsulin (Fig. 2C). The GSIS assay indicated an inhibitory effect of miR-18 on insulin secretion (Fig. 2D). However, the suppressed insulin secretion capacity in IL-1β-induced MIN6 cells was partially abrogated by miR-18 knockdown (Fig. 2E).

miR-18 promotes apoptosis of islet β-cells. The TUNEL assay indicated that miR-18 overexpression promotes apoptosis of
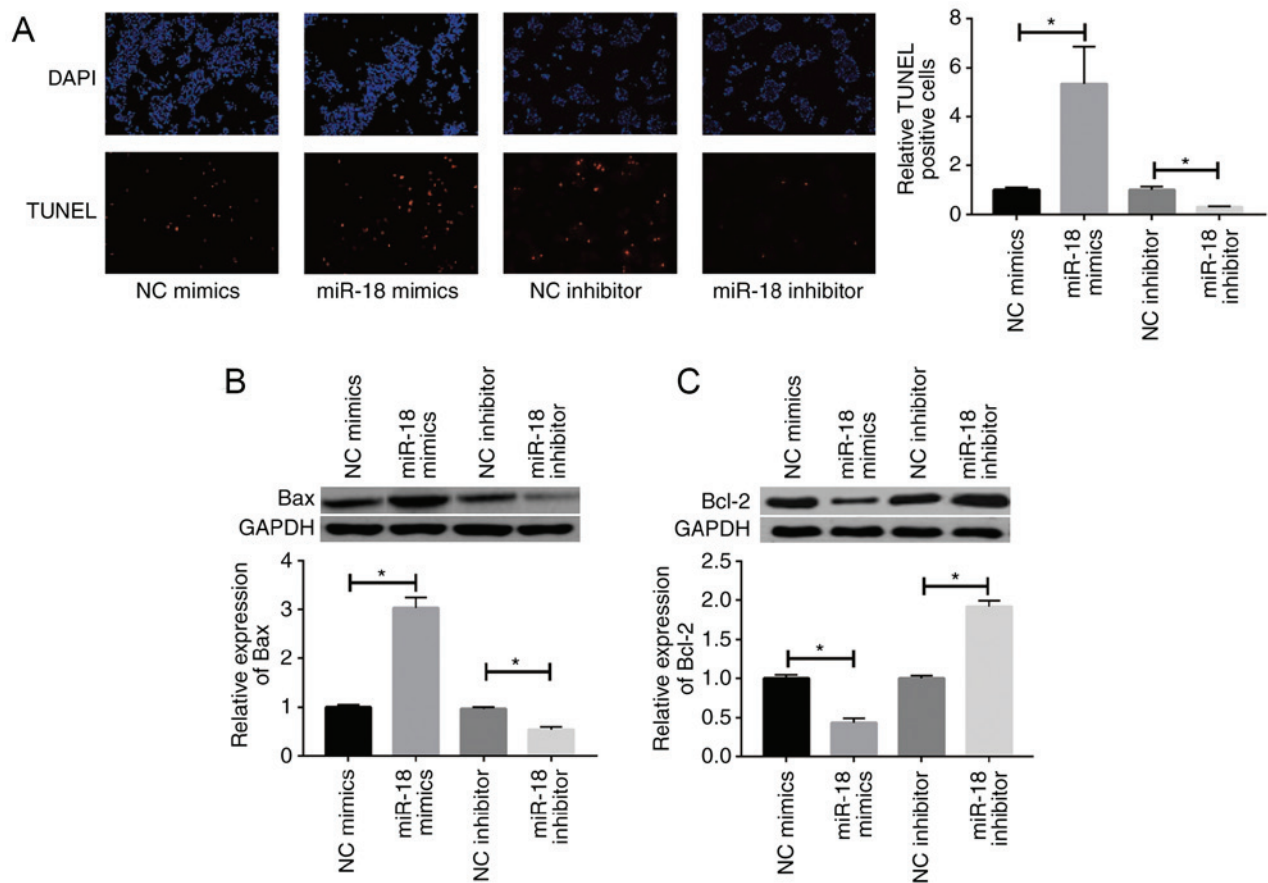
Figure 3. miR-18 promotes apoptosis of islet β-cells. (A) A TUNEL assay indicated that miR-18 mimics promoted apoptosis of islet β-cells, whereas miR-18 knockdown achieved the opposite result (magnification, x100). Western blot analysis indicated that transfection of miR-18 mimics into MIN6 cells (B) markedly upregulated the pro-apoptotic gene Bax and (C) downregulated the anti-apoptotic gene Bcl-2. *P<0.05. miR, microRNA; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick end labeling; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; NC, negative control.

islet β-cells, whereas miR-18 knockdown produced the opposite results (Fig. 3A). miR-18 mimics caused a marked upregulation of the pro-apoptotic gene Bax, while downregulating the anti-apoptotic gene Bcl-2 in MIN6 cells (Fig. 3B and C).

miR-18 inhibits NAV1 expression in islet β-cells. Through a bioinformatics prediction, NAV1 was screened out as a target gene of miR-18. Wild-type NAV1 and mutant-type NAV1 sequences were then constructed to verify the binding interaction of miR-18 and NAV1 (Fig. 4A). Decreased luciferase activity was identified in MIN6 cells co-transfected with miR-18 mimics and wild-type NAV1 reporter plasmid. However, no significant change in luciferase activity was identified after co-transfection of miR-18 mimic and mutant-type NAV1 reporter plasmid (Fig. 4B). Furthermore, cells co-transfected with miR-18 inhibitor and wild-type NAV1 reporter plasmid exhibited a higher luciferase activity compared with those co-transfected with miR-18 inhibitor and mutant-type NAV1 reporter plasmid (Fig. 4C). To further verify the interaction between miR-18 and NAV1, the expression levels of NAV1 were detected after transfection of miR-18 mimics or inhibitor. The results indicated that mRNA and protein levels of NAV1 were negatively regulated by miR-18 (Fig. 4D and E).

miR-18 inhibits the PI3K/AKT pathway in islet β-cells. It was speculated that the PI3K/AKT pathway is involved in the

regulation of islet β cells. The results indicated that miR-18 mimics caused a marked downregulation of the levels of p-AKT and p-PI3K, as well as the p-AKT/AKT and the p-PI3K/PI3K ratio, in MIN6 cells (Fig. 5A). miR-18 knockdown produced the opposite results (Fig. 5B).

Discussion

Epidemiological studies have indicated that the increased incidence of DM worldwide is closely associated with the increased number of obese individuals. Obesity is involved in the pathogenesis of DM through inflammation (16-18). It is currently thought that islet-β cell dysfunction has a key role in the occurrence and development of DM. Dysfunctional β-cells and apoptosis are the leading causes of DM. Relative studies have proved the significant role of cytokines in regulating islet cell function, of which TNF-α and IL-1β are crucial in the pathogenesis of DM. TNF-α is produced by macrophages and adipose cells. It promotes lipid decomposition and release of free fatty acids, thus leading to insulin resistance. TNF-α is also an important cytokine involved in cell apoptosis. It is the initiator of the classic death receptor and caspase-8 apoptotic pathway. Abundant peroxynitrite and free radicals are further released to promote cell apoptosis (19). IL-1β inhibits insulin secretion and damage islet cell function through nuclear factor-κB, c-Jun N-terminal kinase and suppressor of cytokine
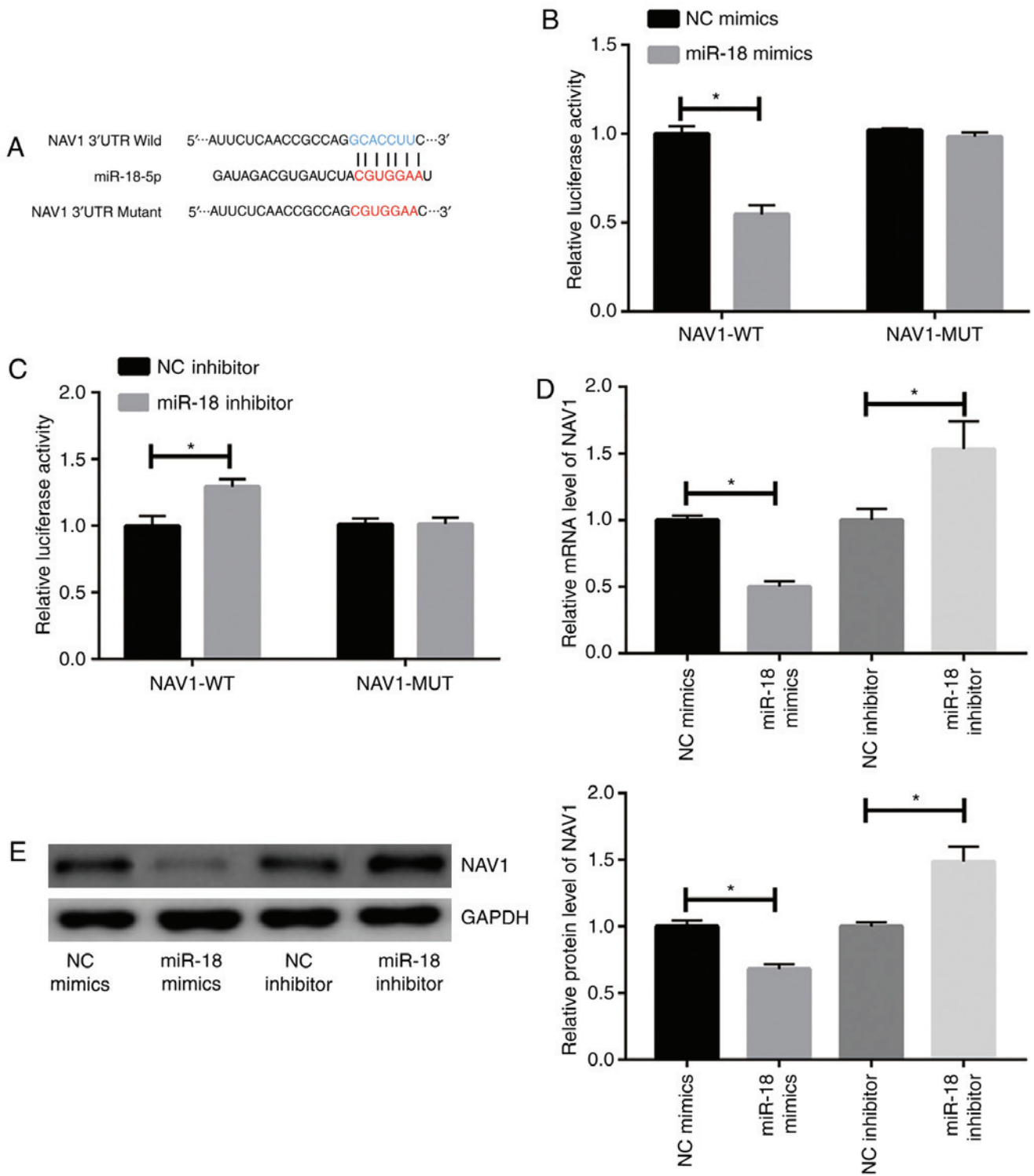
Figure 4. miR-18 inhibits NAV1 expression in islet β-cells. (A) Complementary binding sequence for miR-18-5p in the 3'-UTR of NAV1 and its mutant-type NAV1 sequence. (B) Luciferase reporter plasmids driven by the abovementioned WT and MUT fragments of NAV1 were co-transfected with miR-18 mimics or NC mimics into MIN6 cells. Decreased luciferase activity was identified in the WT group but not in the MUT group in the presence of miR-18 mimics. (C) Cells co-transfected with miR-18 inhibitor and WT NAV1 exhibited lower luciferase activity compared with those co-transfected with miR-18 inhibitor and MUT-type NAV1. (D) The mRNA and (E) protein levels of NAV1 were negatively regulated by miR-18. *$P<0.05$. miR, microRNA; NAV1, neuron navigator 1; UTR, untranslated region; WT, wild-type; MUT, mutant; NC, negative control.

signaling-3 pathways, eventually leading to the occurrence of DM (20). A variety of inflammatory factors, including TNF-α, IL-1β and INF-γ, are involved in the development of DM (21). In the present study, IL-1β treatment caused a marked upregulation of miR-18 expression in MIN6 cells.

Various studies have focused on the effects of a high-fat diet in gene transcription and protein translation. Although post-transcriptional and translational regulations are also crucial, their regulatory effects on β-cell damage induced by a high-fat diet have been rarely reported. miRNAs exert
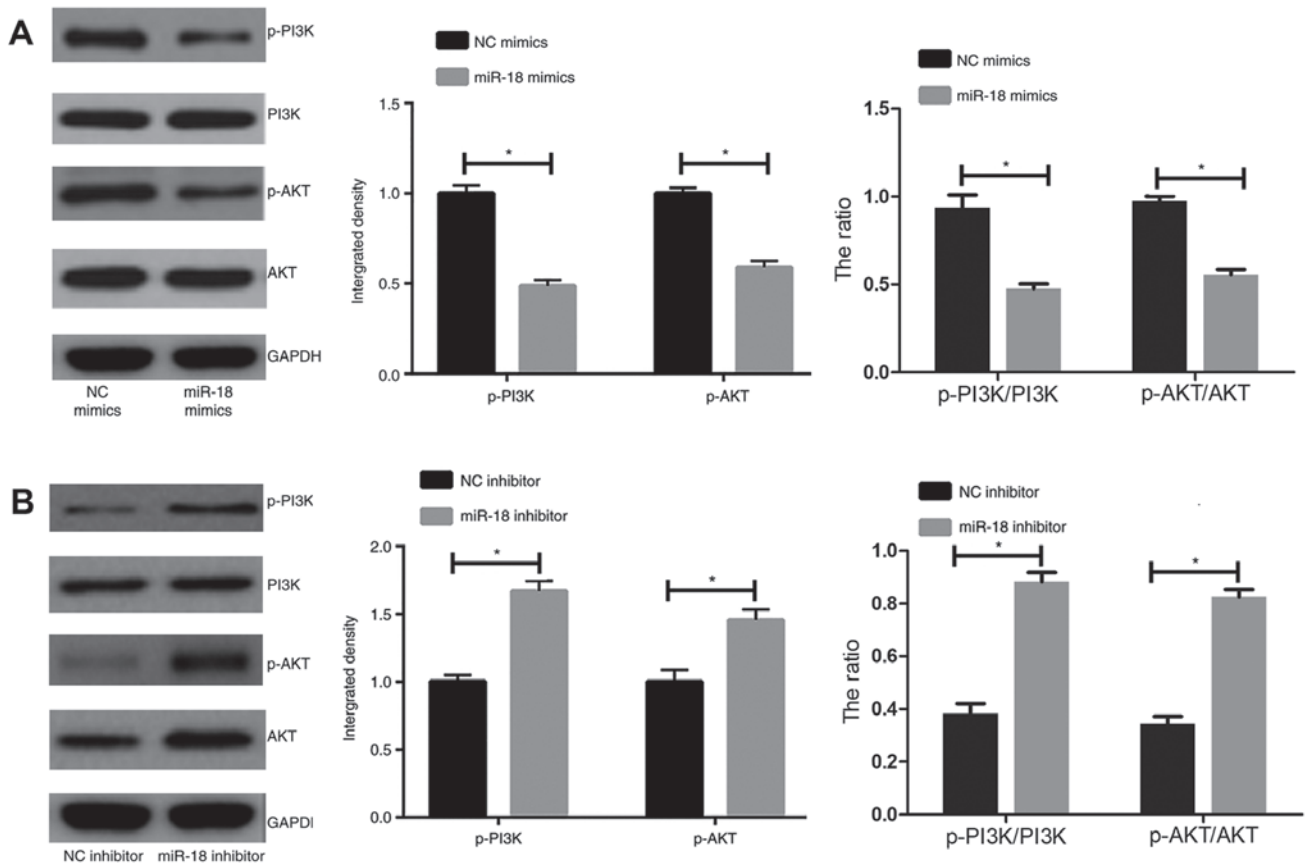
Figure 5. miR-18 inhibits the PI3K/AKT pathway in islet β-cells. (A) miR-18 mimics cause a marked decrease of p-AKT/AKT and p-PI3K/PI3K in MIN6 cells. (B) miR-18 knockdown caused a significant upregulation of levels of-AKT/AKT and p-PI3K/PI3K in MIN6 cells. *P<0.05. miR, microRNA; p-PI3K, phosphorylated phosphoinositide 3-kinase; NC, negative control.

their crucial roles by regulating their target genes at the post-transcriptional and translational levels. It has been predicted that >60% of the human genome is regulated by miRNAs (22). Previous studies have indicated that certain miRNAs regulate the differentiation and development of mouse islet β-cells (23). Knockdown of miR-146a or miR-34a in mouse islet β-cells was demonstrated to remarkedly decrease palmitate-induced apoptosis, while it did not affect the insulin release function (24). The present study indicated that inflammatory factors cause an upregulation of miR-18 expression in islet β-cells and that miR-18 markedly inhibits insulin production.

Studies have suggested that the number of islet β-cells is progressively reduced during the disease course of DM, which may be explained by the excessive apoptosis occurring (25). The amount of islet β-cells is markedly decreased in patients with type 2 DM. Abundant apoptosis is observed in DM patients, whereas proliferation of islet β-cells is under normal control, indicating the significant role of apoptosis in the occurrence and progression of DM. Studies on human islet amyloid polypeptide transgenic mice have indicated that the increase in β-cell apoptosis exceeds the increase in cell replication, leading to β-cell loss (26). Furthermore, high levels of cell apoptosis are encountered at the early stage of DM. Other studies indicated that the apoptotic rate of β-cells in patients with type 1 or type 2 DM is 3-10 times higher than that of healthy controls (4,12), while the proliferation

rate of β-cells is maintained at a normal level. In the present study, miR-18 markedly promoted MIN6 cell apoptosis.

miR-18 is widely expressed in human and mouse tissues, which serves as a tumor-suppressor gene via targeting K-Ras (27). The present study demonstrated that NAV1 is a target gene of miR-18. The PI3K/AKT pathway is one of the classical signaling pathways involved in suppression of apoptosis and promotion of proliferation (28). In the present study, miR-18 was indicated to regulate DM development via inhibiting the PI3K/AKT pathway.

In conclusion, miR-18 expression is upregulated by IL-1β induction in islet β-cells. miR-18 promotes apoptosis of islet β-cells, at least in part, by inhibiting NAV1 expression and insulin production via suppression of the PI3K/AKT pathway. However, miR-18 has multiple target genes and furthermore, the association between NAV-1 and the apoptosis of islet β-cell requires further investigation. In addition, the present study only provided evidence from *in vitro* experiments, and a further *in vivo* study may be required.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HF and MS designed the study; LC, ZW and LS performed the experiments; ZW and LS collected the data; HF and MS analyzed the data; and HF and LC prepared the manuscript. 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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