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Irisin attenuates pyroptosis in high glucose-induced pancreatic β cells via the miR-133a-3p/FOXO1 axis

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Abstract

Introduction: Irisin is closely related to type 2 diabetes mellitus (T2DM) and other metabolic diseases. It can improve the homeostasis of T2DM. MiR-133a-3p is decreased in the peripheral blood of patients with T2DM. Forkhead box protein O1 (FOXO1) is widely expressed in β-cells and affects the occurrence of diabetes through transcriptional regulation and signalling pathway regulation.

Material and methods: The miR-133a-3p inhibitor was constructed to verify the effect of irisin on pyroptosis through miR-133a-3p. Next, we predicted the presence of targeted binding sequences between FOXO1 and miR-133a-3p by bioinformatics software, which was then confirmed with a double fluorescence assay. Finally, the FOXO1 overexpression vector was used to further verify the effect of irisin through the miR-133a-3p/FOXO1 axis.

Results: We first observed that irisin inhibited the protein levels of N-terminal gasdermin D (GSDMD-N) and cleaved caspase-1 and the secretion of interleukins (IL): IL-1β and IL-18 in Min6 cells treated with high glucoes (HG). Irisin inhibited pyroptosis of Min6 cells treated with HG by reinforcing miR-133a-3p. Then, FOXO1 was validated to be the target gene of miR-133a. Both miR-133a-3p inhibitor and overexpression of FOXO1 restrained the force of irisin on pyroptosis in HG-induced Min6 cells.

Conclusion: We explored the protective effect of irisin on HG-induced pyroptosis of islet β-cells *in vitro* and explained its mechanism of inhibiting pyroptosis through the miR-133a-3p/FOXO1 axis, to provide a theoretical basis for finding new molecular targets to delay β-cell failure and the treatment of T2DM.

Key words: *irisin; T2DM; FOXO1; miR-133a-3p; pyroptosis*

Introduction

The incidence of diabetes worldwide is increasing year by year. According to the latest data released by the International Diabetes Federation, there are about 536.6 million adults endured diabetes worldwide in 2021, and this data is increasing year by year [1]. Most patients with diabetes have type 2 diabetes mellitus (T2DM) [2]. Diabetes has become a serious and common chronic disease, which seriously threatens human health and at the same time leads to costly complications, which can be disabling and even life-threatening [1]. Therefore, active prevention and treatment of diabetes has become an important issue for researchers.

T2DM is characterized by insulin resistance and impairment of β cell function. Insulin resistance is a necessary and insufficient condition to induce diabetes, and β-cell dysfunction plays a more crucial role in T2DM [3]. The United Kingdom (UK) Prospective Diabetes Study showed that the number of β cells decreased at a rate of 4% per year [4] in islets of T2DM patients, as disease progressed. β cell pyroptosis is the main cause of β cell failure and functional defects. Cell pyroptosis is a caspase-1-dependent process of programmed cell necrosis, which is mediated by gasdermin D (GSDMD) and associated with the secretion of interleukin 1β (IL-1β) [5]. NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome triggers caspase-1, which cleaves GSDMD protein and causes cell perforation, while pro-IL-1β translates to mature IL-1β secreted from the cell pores, and it eventually leads to the swelling and death of cells [6]. At present, it is believed that there is a chronic systemic low-grade inflammatory response in T2DM, and NLRP3 inflammasome and a variety of inflammatory factors are activated [7]. Human pancreatic islets β cells exposed to high glucose (HG) can induce the production of cytotoxic IL-1β, which then promotes apoptosis of β cells and reduction of insulin secretion [8]. Studies also have found that when β cells have an inflammatory reaction, they can cause β cell pyroptosis and then mediate functional defects of β cells [9].

In recent years, more and more studies have revealed that mirco RNAs (miRNAs) are closely con-

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nected to the pathogenesis of diabetes, including β cell growth and insulin resistance, and miRNAs can become biomarkers of T2DM and its complications [10]. Studies have shown that miR-133a-3p decreased in the peripheral blood of T2DM patients and had a predictive value for the prognosis of patients [11].

Forkhead box protein O1 (FOXO1) is the earliest transcription factor found in the FOXO subfamily. It is located in 13q14.1 and encodes 655 amino acids (AA). It is widely expressed in β cells and affects the occurrence of diabetes through transcriptional regulation and the regulation of signal pathways [12]. FOXO1 regulates β cell proliferation, apoptosis, differentiation, oxidative stress, autophagy, and metabolism, and regulates glucose and lipid metabolism in the liver [13]. In addition, FOXO1 is also closely related to the chronic systemic inflammatory response of diabetes. As a pro-inflammatory factor, it increases the expression of inflammatory factors in diabetes cells and the expansion of inflammatory response [14]. Studies have found that FOXO1 can be associated with cell death through miRNAs. In Parkinson's animal model, miR-135b inhibits cell death by inhibiting the activation of NLRP3 mediated by FOXO1 [15]. Alcohol abnormally regulates miR-148a through FOXO1, so thioredoxin (TXNIP) overexpression promotes liver cell death [16]. Studies on nasopharyngeal carcinoma cells found that the compound cinobufotalin increased the sensitivity of nasopharyngeal carcinoma cells to cisplatin by stimulating PI3K/AKT/c-Myc/p53/miR-133a-3p signalling pathway through FOXO1 [17], However, the power of miR-133a-3p /FOXO1 axis on β-cell failure and dysfunction in islet β cells has not been reported.

Irisin is a muscle factor that can act on white adipose tissue and induce its transformation into brown adipose tissue. It is formed after the decomposition of the extracellular domain of fibronectin type III domain-containing protein (5FNDC5) by proteolytic enzymes, which can improve the homeostasis of obesity and glucose, and is expected to be used as a protein molecule for the treatment of human metabolic diseases [18]. It has been reported that irisin is closely related to metabolic diseases such as obesity and T2DM. The level of serum irisin in T2DM patients is much lower than that in healthy controls [19]. Nevertheless, the pathogenesis of irisin and T2DM, especially the interaction between irisin and islet β cells, needs to be further studied.

Material and methods

T2DM pancreatic β-cell model

The T2DM cell model was constructed by incubating Min6 cells with 25 mmol/L HG for 24 h. The insulin level in the culture medium was detected by enzyme-linked immunosorbent assay (ELISA) to determine the success of the modelling.

ELISA

The supernatants of Min6 cells with different treatments were collected after 48 h of incubation. The insulin levels and secretion of IL-1β and IL-18 in the supernatants were measured using an ELISA kit according to the manufacturer's instructions. The diluted cell supernatant was added to the bottom of the well of the monoclonal antibody-coated plate, without touching the well wall, and mixed gently by shaking. The plates were sealed with the plate sealing membrane and incubated at 37°C for 1 h. Subsequently, the plates were washed 5 times with diluted washing solution. After patting dry, 50 μl of enzyme-labelled solution was added to each well except the blank control well. After incubation at 37°C for 1 h, as above, the plate was fully washed 5 times. After patting dry with absorbent paper, 50 μL of TMB were added to each well. After the reaction was shielded from light for 10 min, 50 μL of stop solution (2 mol/L H_2SO_4) was added. Finally, optical density (OD) values of each well were read at 450 nm.

Western blotting

The Min6 cells were washed twice with 1 mL of pre-cooled phosphate buffered saline (PBS) in each tube at 4°C to wash off the culture medium, and then lysed with radio immune precipitation (RIPA) buffer containing protease inhibitor and placed on ice for 20 min. The protein content was determined by adding bicinchoninic acid (BCA) solution. After denatured by boiling the samples for 5 min, the protein was separated by polyacrylamide gel and then fixed on a polyvinylidene fluoride (PVDF) membrane. After washing with PBS solution, the membranes were blocked with 5% skimmed milk for 1 h at room temperature. Subsequently, the PVDF membranes were incubated overnight at 4°C with suitable dilution of primary antibodies and rinsed with phosphate buffer saline with tween (PBST) buffer 3 times for 10 minutes each time. Next, the membranes were incubated in horseradish peroxidase-labelled secondary antibodies diluted with blocking solution for 1 h at room temperature, shaken slowly, and washed with PBST buffer 3 times. Finally, the pre-mixed enhanced chemiluminescence (ECL) luminescent substrate was added to the membrane for colour reaction.

RT-qPCR

Total RNA was extracted with a Trizol reagent. A NanoDrop 2000 spectrophotometer (Thermo Scientific, United States) was used to determine the concentration and OD260/OD280 of extracted RNA. To determine the integrity of RNA, the 5S rRNA, 18S rRNA, and 28S rRNA bands of total RNA were detected by agarose gel electrophoresis. Immediately thereafter, total RNA was reverse transcribed into complementary DNA (cDNA) using a PrimeScript™ RT Kit (Qiagen, Germany). Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was conducted using an SYBR Green PCR kit (Qiagen, Germany). The PCR procedure was: 95°C for 5 min; 95°C for 10 s, and 60°C for 30 s, for 40 cycles. At the end of the cycle, the specificity of the product was identified by melting curve, the temperature was gradually elevated from 60°C to 97°C, and the fluorescence signal was collected 5 times per degree. The RT-qPCR amplification for each experiment was carried out in triplicate, and the $2^{-\Delta\Delta Ct}$ method [20] was used to calculate the expression of RNA.

Cell transfection

MiR-133a-3p mimics and inhibitors and their negative controls were synthesized by Sangon Biotech (Shanghai, China) according to mouse mature miR sequence: miR-133a (miRBase IDs: MIMAT0000145). The riboFect™ CP Transfection Kit (RiboBio Co., Ltd.) was used to carry out the transfection experiment as per the manufacturer's instructions.

Bioinformatic target prediction

The putative targets of miR-133a-3p were forecasted with the software of TargetScan (http://www.targetscan.org), DIANA

TOOLS (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=site/index), and miRWalk (http://mirwalk.umm.uni-heidelberg.de/) following the manufacturer's instructions.

Dual-luciferase reporter assay

According to the prediction results of bioinformatics analysis, miR-133a-3p may bind to FOXO1 3'UTR. The Dual-Luciferase reporter plasmids of target gene FOXO1 and the mutants mutated with binding site pGL3-FOXO1-WT and pGL3-FOXO1-MUT were constructed, respectively. Subsequently, the 2 correctly sequenced luciferase reporter plasmids WT and MUT were co-transfected into Min6 cells with miR-133a-3p mimics and negative control plasmids, respectively. After 48 h of transfection, the cells were harvested and lysed. After centrifugation, the supernatants were collected, and luciferase activity was measured using a luciferase assay system.

Caspase-1 activity

Caspase-1 activity was detected by the absorbance of peptide nucleic acid (pNA) generated from the degradation of Ac-YVAD-pNA (acetyl-Tyr-Val-Asp p-nitroanilide) catalysed by caspase-1. After different treatments, Min6 cells were collected and lysed with lysis buffer on ice for 15 min. Subsequently, the supernatant was transferred to a prechilled centrifuge tube, and the protein concentration was determined to be 1–3 mg/mL. The cells were incubated with the caspase-3 substrate Ac-YVAD-pNA at 37°C for 1–2 h. When an obvious colour change occurred, the absorbance of pNA at 405 nm was determined using a microplate reader. The absorbance of pNA was calculated as the A405 of the sample minus the A405 of the blank control.

Statistical analysis

The statistical software GraphPad Prism version 8.0 was mainly used for data analysis. The statistics of all experimental results were based on the experimental results of at least 3 repetitions. The results were expressed as means ± standard deviations (SD), and the statistical differences were analysed by Student's t-test or one-way analysis of variance (ANOVA). $p < 0.05$ was considered significant. Image J was used for the semiquantitative analysis.

Results

Irisin inhibited pyroptosis of Min6 cells treated with HG

The T2DM cell model was constructed by incubating Min6 cells with 25 mmol/L HG for 24 h. The insulin level in the culture medium was detected by ELISA to determine the success of modelling (Fig. 1A). Then, the best concentration of irisin used in Min6 cells was determined as 100 ng/mL (Fig. 1B). Both the protein levels of N-terminal gasdermin D (GSDMD-N) and cleaved-caspase-1 were elevated in HG-treated Min6 cells but were significantly reduced in the HG + irisin group (Fig. 1CD). The secretion levels of IL-1β and IL-18 were also increased in HG-treated Min6 cells, but observably reduced in the HG + irisin group (Fig. 1E).

Irisin inhibited pyroptosis of Min6 cells treated with HG through alleviating miR-133a

To evaluate the function of miR-133a-3p in HG-treated Min6 cells, miR-133 expression levels were detected in Min6 cells treated with HG or irisin. The miR-133 expression levels were highly decreased in HG-treated Min6 cells, but they were promoted when irisin was added (Fig. 2A). Then Min6 cells were transfected with miR-133a inhibitor and inhibitor negative control (inhibitor NC) control, which were validated in Figure 2B. The protein expression levels of GSDMD-N and cleaved-caspase-1 were tested under the condition of miR-133a-3p inhibitor. Our results show that in irisin-treated T2DM model cells, the protein levels of GSDMD-N and cleaved-caspase-1 decreased significantly. However, the addition of miR-133a-3p inhibitor significantly restored the protein levels of GSDMD-N and cleaved-caspase-1 (Fig. 2CD). The secretion levels of IL-1 β and IL-18 were also restored under the condition of miR-133a-3p inhibitor in HG-treated Min6 cells (Fig. 2E).

FOXO1 was the target gene of miR-133a

The target gene of miR-133a-3p was predicted using miRNA target prediction analysis tools, and FOXO1 was predicted as the target gene of miR-133a online (Fig. 3A). Importantly, the dual-luciferase reporter experiment was conducted to confirm that FOXO1 is the target gene of miR-133a-3p in HEK293T cells (Fig. 3B). We then transfected Min6 cells with mimics, mimics negative control (NC), inhibitor, or inhibitor NC of miR-133a-3p. Subsequently, the protein expression levels of FOXO1 were detected by Western blotting (WB). After 24 h of transfection, the relative miR-133a-3p protein expression levels of FOXO1 were significantly suppressed in the mimics group and highly promoted in the inhibitor group, compared to their NC groups ($p < 0.01$) (Fig. 3C). Our results suggest that miR-133a-3p adversely regulates the expression of FOXO1.

Irisin inhibited pyroptosis of HG-treated Min6 cells by up-regulating the miR-133a-3p/FOXO1 axis

To further study the effect of miR-133a-3p/FOXO1 in the process of irisin inhibiting pyroptosis of HG-treated Min6 cells, FOXO1 was overexpressed and transfected into irisin-treated HG-Min6 cells. Firstly, the expression levels of FOXO1 were detected in different groups in Figure 4A; the results showed that both miR-133a-3p inhibitor and pcDNA-FOXO1 could counteract the effect of irisin in HG-Min6 cells. The protein levels of FOXO1, GSDMD-N, and cleaved-caspase-1 were also examined, and the results presented a similar trend (Fig. 4B). Moreover, the activity of caspase-1 and the secretion levels of L-1 β and IL-18 also showed bucking effects of miR-133a-3p inhibitor and pcDNA-FOXO1 to irisin (Fig. 4C-D).

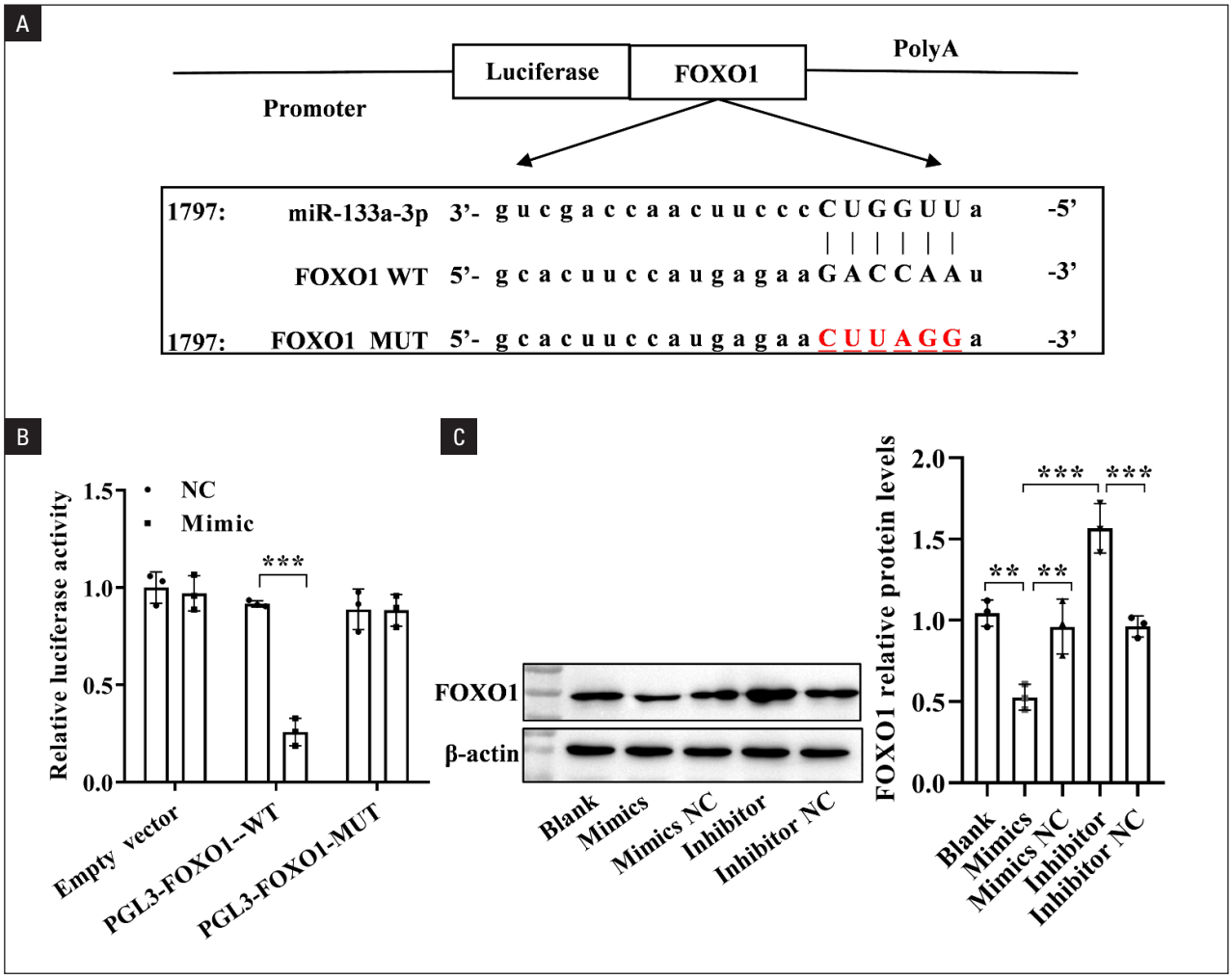

Figure 3. *Forkhead box protein O1 (FOXO1) is the target gene of miR-133a.* **A.** *Prediction of miR-133a binding to FOXO1. FOXO1 was predicted as the target gene of mir-133a-3p online;* **B.** *A dual-luciferase reporter system was used to determine that FOXO1 is the target gene of mir-133a in HEK293T cells;* **C.** *Western blotting was operated to detect protein expression levels of FOXO1 in Min6 cells. **p < 0.01 and ***p < 0.001 show significance.*

Discussion

Impaired insulin secretion has long been considered a key feature in the pathogenesis of T2DM, which is mainly caused by the reduction of the β-cell population and defective β-cell function. Irisin has been shown to have a favourable effect on metabolic diseases, including obesity and T2DM. Although irisin has a promising application prospect, its specific mechanism is still unclear. In this study, we explored the protective effect of irisin on HG-induced islet β-cell pyroptosis in T2DM and explained the mechanism by which irisin inhibited pyroptosis through the miR-133a-3p/FOXO1 molecular axis, so as to provide a theoretical basis for finding new molecular targets to delay β-cell failure and the treatment of T2DM.

Pyroptosis of β cells is an important cause of β-cell failure and functional defects. In the face of insulin resistance, successful β-cell compensation would delay the progression of T2DM. It is currently believed that there is a chronic systemic low-grade inflammatory reaction in T2DM, with NLRP3 inflammasome and multiple inflammatory factors activated [21]. Exposure of mouse or human islet β cells to HG can induce the production of IL-1β, which is toxic to β cells themselves [22]. The secreted IL-1β promotes the apoptosis of β cells and the reduction of insulin secretion through a series of signal transductions [23]. In addition, studies have also found that inflammation of β cells can lead to pyroptosis of β cells and then mediate the occurrence of functional defects of β cells in islets [23]. In our study, IL-1β and IL-18 were secreted with higher levels in HG-induced Min6 cells, and pyroptosis-related GSDMD and caspase-1 were also activated.

Previous studies have revealed that serum irisin levels are decreased in T2DM patients, and irisin has

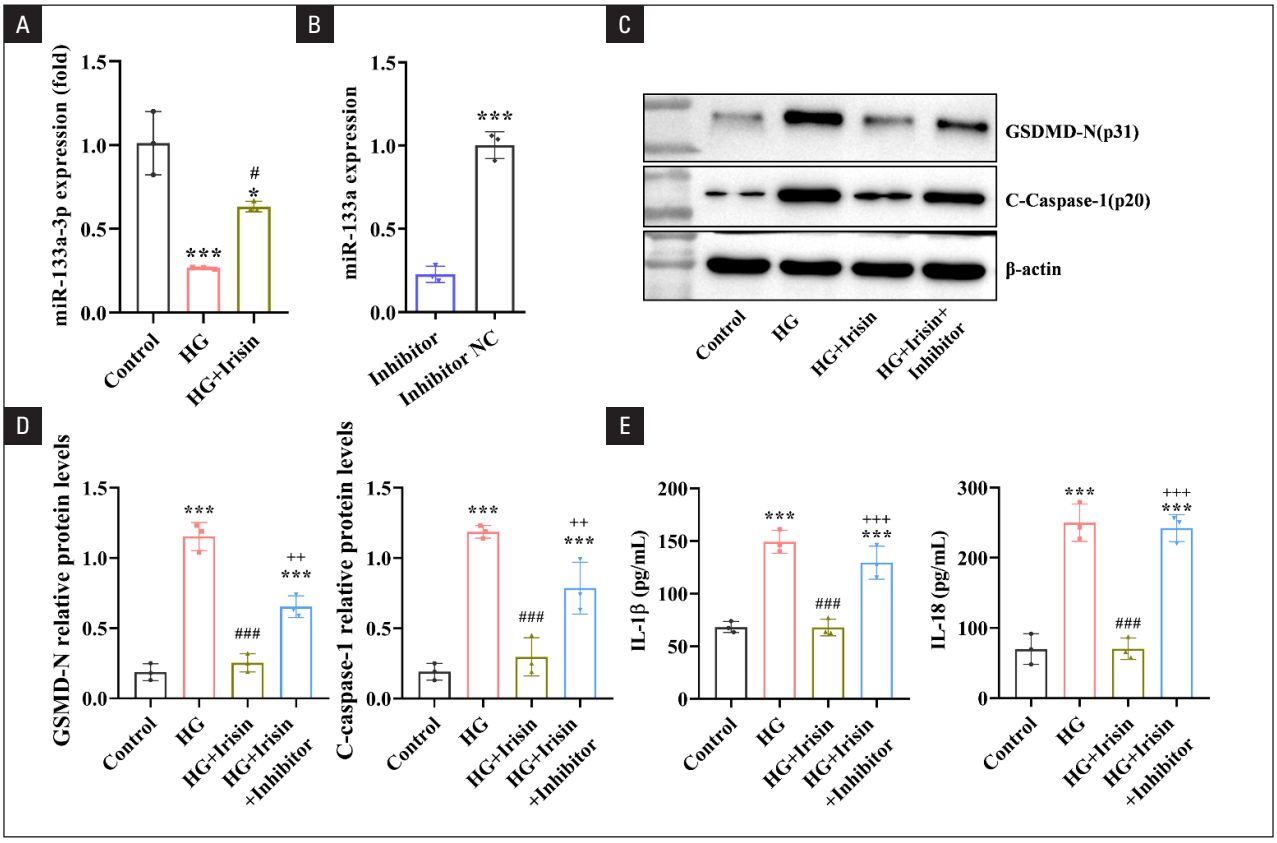

Figure 2. *Irisin inhibits pyroptosis of Min6 cells treated with high glucose (HG) through alleviation of miR-133a.* **A.** *Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed to detect miR-133 expression levels in Min6 cells treated with HG or irisin;* **B.** *RT-qPCR was performed to detect miR-133a expression levels in Min6 cells transfected with miR-133a inhibitor and inhibitor-negative control (Inhibitor NC);* **C.** *The protein expression levels of N-terminal gasdermin D (GSDMD-N) and cleaved-caspase-1 were determined by Western blotting;* **D.** *Semi-quantitative analysis of GSDMD-N and cleaved-caspase-1 protein expression levels;* **E.** *Interleukins (IL): IL-1β and IL-18 protein levels in the cell culture supernatant were determined by enzyme-linked immunosorbent assay (ELISA). *$p < 0.05$ and ***$p < 0.001$ vs. the control group; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs. the HG group; $^{++}p < 0.01$ and $^{+++}p < 0.001$ vs. the HG + irisin group*

the cytoprotective effect, which can promote cell proliferation and inhibit cell apoptosis [24]. The study of Song et al. [25] found that irisin could advance the proliferation of human umbilical vein endothelial cells through extracellular regulated protein kinases (ERK) signalling pathway, partially inhibit the apoptosis induced by HG, and inhibit the apoptosis induced by oxidized low-density lipoprotein in human aortic endothelial cells [26]. Japanese researchers found that irisin could also inhibit cigarette smoke-induced apoptosis of A549 cells [27]. Consistent with the findings of Annalisa [28] and Liu et al. [29], our study also showed that irisin could inhibit the expression levels of GSDMD, caspase-1, IL-1β, and IL-18 related to pyroptosis in mouse islet β cells in vitro.

Current studies have shown that irisin has anti-inflammatory effects which can reduce the inflammation of endothelial cells induced by advanced glycation end products (AGEs) [30] and nerve injury induced by hypoxia and glucose deficiency by inhibiting the reactive oxygen species–NLR family, pyrin domain-containing 3 (ROS-NLRP3) inflammatory pathway [31]. In macrophages stimulated by lipopolysaccharide (LPS), irisin down-regulates the toll-like receptor 4 (TLR4)/myeloid differentiation primary response protein 88 (MyD88) pathway by inhibiting mitogen-activated protein kinases (MAPK) phosphorylation. Thus, the phosphorylation of nuclear facto kappa beta (NF-κB) is inhibited, the transcription of inflammatory factors is reduced, and the secretion is decreased to achieve the purpose of anti-inflammation [32]. To further investigate the anti-inflammatory mechanism of irisin in T2DM and to promote the development of chronic systemic inflammation in diabetes based on FOXO1, we found a targeted binding sequence between FOXO1 and miR-133a-3p by bioinformatics prediction. Double fluorescence experiments confirmed the targeting relationship between FOXO1 and miR-133a-3p, in which miR-133a-3p played a negative regulatory role on FOXO1 expression. Through the recovery test of

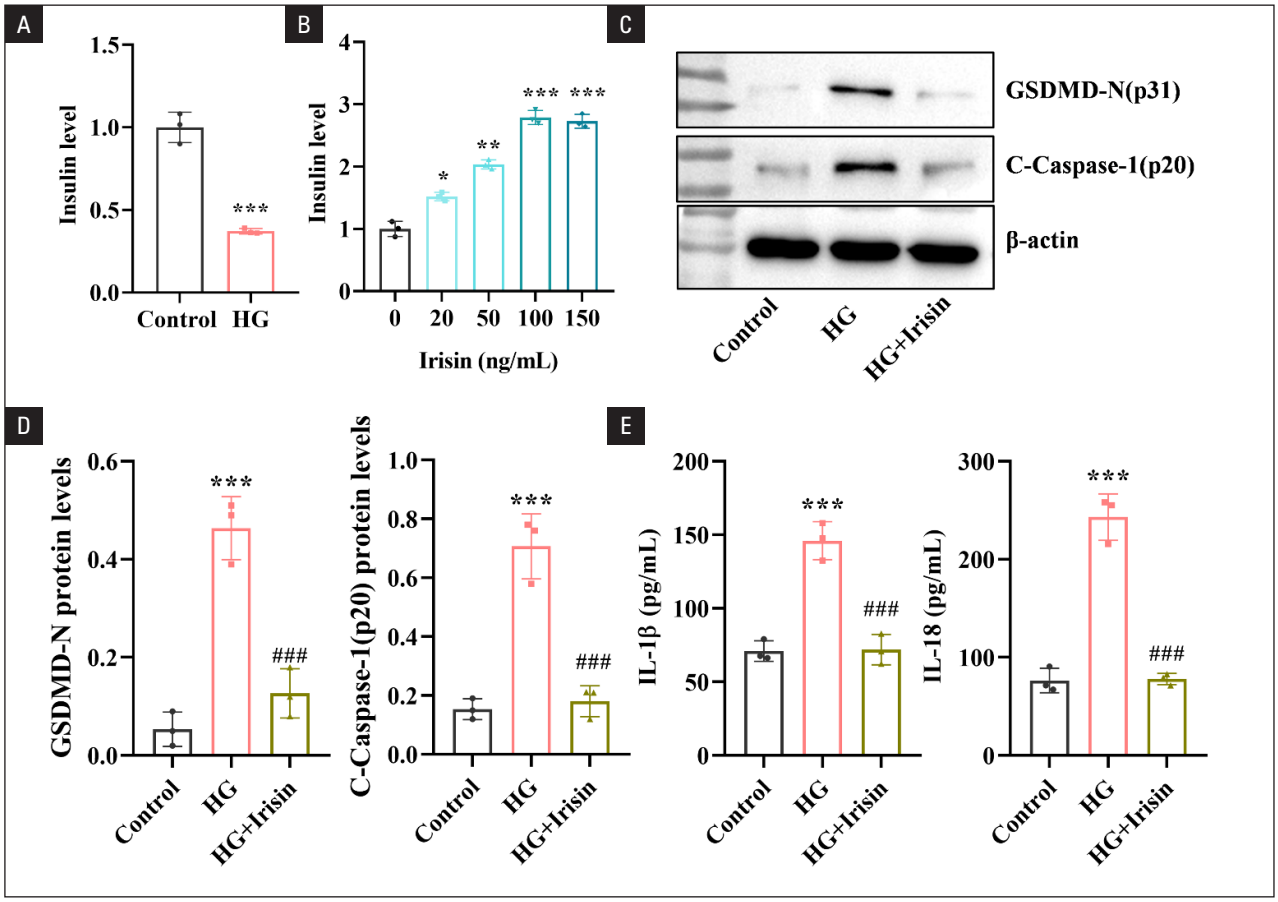

Figure 1. *Irisin inhibits pyroptosis of Min6 cells treated with high glucose (HG).* **A.** *The type 2 diabetes mellitus (T2DM) cell model was constructed by incubating Min6 cells with 25 mmol/L HG for 24 h. The insulin level in the culture medium was detected by enzyme-linked immunosorbent assay (ELISA) to determine the success of modelling;* **B.** *Min6 cells were treated with several concentrations of irisin to determine the best concentration;* **C.** *The levels of N-terminal gasdermin D (GSDMD-N) and cleaved-caspase-1 protein expression levels were detected by western blotting;* **D.** *Semi-quantitative analysis of GSDMD-N and cleaved-caspase-1 protein expression levels;* **E.** *Interleukins (IL): IL-1 β and IL-18 protein levels in the cell culture supernatant were determined by ELISA. *p < 0.05, **p < 0.01 and ***p < 0.001 vs. the control group; ###p < 0.001 vs. the HG group*

FOXO1 overexpression, we found that irisin indeed was essential in inhibiting the development of islet β-cell failure through the miR-133a-3p /FOXO1 molecular axis.

Conclusion

Our study found that the level of irisin in the circulation of T2DM patients was decreased, and its activation effect on the molecular axis of miR-133a-3p/FOXO1 was weakened, leading to the expansion of inflammatory response, and the failure and dysfunction of islet β cells, leading to the progression of the disease. Exogenous upregulation of the level of irisin in the circulation could inhibit inflammation, improve β-cell function, and delay the progression of T2DM. The success of this project will provide a theoretical foundation for finding new molecular targets to delay pancreatic β-cell failure and for the treatment of T2DM.

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None.

Conflicts of interest
The authors declare that they have no conflicts of interest.

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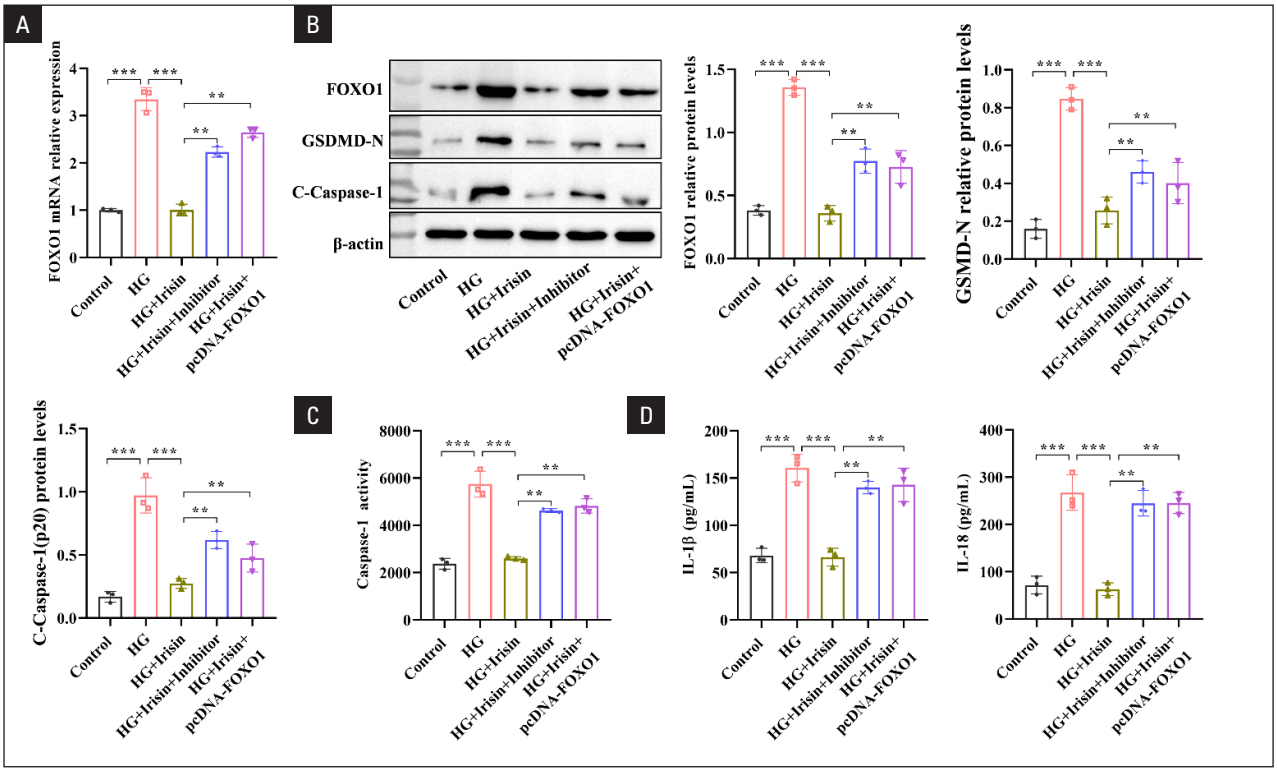

Figure 4. *Irisin inhibits pyroptosis of high glucose (HG)-treated Min6 cells by up-regulating the miR-133a-3p/Forkhead box protein O1 (FOXO1) axis.* **A.** *Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed to detect FOXO1 expression levels in Min6 cells treated with high glucose, irisin, miR-133a-3p inhibitor, or pcDNA-FOXO1;* **B.** *The protein expression levels of FOXO1, N-terminal gasdermin D (GSDMD-N), and cleaved-caspase-1 were determined by Western blotting;* **C.** *The caspase-1 activity was determined;* **D.** *Interleukins (IL): IL-1β and IL-18 protein levels in the cell culture supernatant were determined by enzyme-linked immunosorbent assay (ELISA). **$p < 0.01$ and ***$p < 0.001$ show significance in our study*

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