European Review for Medical and Pharmacological Sciences 2019; 23: 8476-8484

The effect of SOX9 on islet β cells in high glucose environment through regulation of ERK/P38 signaling pathway

Y.-N. ZHANG, D.-X. FU, J.-X. XU, G.-Y. WANG

Department of Second Endocrinology, CangZhou Central Hospital, Cangzhou City, Hebei Province, China

Abstract. – OBJECTIVE: Islet beta cells are involved in insulin secretion. SRY-related high mobility group 9 (SX-S9) is involved in the progression of various diseases, but the role of SOX9 in islet β cells remains unclear.

PATIENTS AND METHODS: The islet β cell MIN6 cells were cultured *in vitro* and randomly divided into control group, high glucose group, and SOX9 siRNA group followed by analysis of SOX9 mRNA and protein expression by real-time PCR and Western blot, respectively, cell proliferation by MTT assay, cell apoptosis by flow cytometry, secretion of inflammatory factors TNF-α and IL-2 by ELISA, insulin secretion levels by spectrophotometer, myeloperoxidase (MPO), and superoxide dismutase (SOD) activities, as well as ERK/P38 signaling protein expression by Western blot.

RESULTS: Under high glucose environment, SOX9 mRNA and protein expression were significantly increased, MIN6 cell proliferation was inhibited, apoptosis rate and secretion of TNF-α and IL-2 were increased, along with decreased insulin secretion, increased MPO content, decreased SOD activity and phosphorylation of ERK/P38, compared with control group ($p < 0.05$). However, transfection of SOX9 siRNA reduced SOX9 expression, promoted proliferation of MIN6 cells, decreased apoptotic rate and secretion of TNF-α and IL-2, increased insulin secretion, decreased MPO content, increased SOD and ERK/P38 protein phosphorylation. Compared with high glucose group, the differences were statistically significant ($p < 0.05$).

CONCLUSIONS: The expression of SOX9 is increased under high glucose environment. Down-regulation of SOX9 expression can inhibit islet cell apoptosis, oxidative stress and inflammation, and promote islet cell proliferation and insulin secretion by regulating ERK/P38 signaling pathway.

Key Words:
Diabetes, SOX9, Islet β cells, Apoptosis, Oxidative stress, ERK/P38 signaling pathway.

Introduction

Diabetes mellitus (DM) is an endocrine and metabolic disease. There are nearly 300 million people with diabetes in the world with more than 100 million diabetic patients in China[1,2]. Diabetes is characterized by hyperglycemia that is caused by defects in insulin secretion or/and its biological effects. Hyperglycemia can cause damages to various tissues and multiple organs. Therefore, diabetes is a serious threat to human health[3,4]. Diabetes can lead to a variety of complications, such as diabetic nephropathy, diabetic neuropathy, etc., and has become one of the medical treatment problems[5]. The pathogenesis of diabetes is complicated. Due to diabetes and other factors in the body, the blood vessels are damaged, which leads to the proliferation of extracellular matrix, thickening of the basement membrane, glomerular sclerosis, leading to multiple organ dysfunction and disease progression[6,7]. Glucose and lipid metabolism disorders, inflammation, oxidative stress and apoptosis are all contributing factors to the occurrence and development of diabetes[8]. Abnormal insulin secretion is a key factor in the development of diabetes[9]. With the deepening of research, it is confirmed that islet cell damage plays a key role in the occurrence of diabetes[10]. Islet beta cells are involved in insulin-secreting islet cells, and islet beta cell apoptosis is a key link in the pathogenesis of diabetes[11,12].

SRY-related S-type 9 (SOX9) is a member of the sex-determining region Y family, also known as the sex-determining region Y-boxin 9 gene[13]. SOX9 participates in multiple signaling pathways including ERK/P38 signaling pathway, which is involved in the development and cell differentiation of multiple cell lines and plays a key role in embryogenesis, neurogenesis, neural crest development, and tumorigenesis

Corresponding Author: Yunna Zhang, MD; e-mail: lao612724naez@126.com

and invasion, as well as stem cell self-renewal in mammalian development[14,15]. SOX9 is involved in the occurrence and development of various diseases[16]. However, the role of SOX9 in islet beta cells under high glucose environments has not been elucidated.

Materials and Methods

Main Materials and Instruments

The MIN6 cell line was constructed by our laboratory and stored frozen in liquid nitrogen. Fetal bovine serum (FBS), 1% double antibody Roswell Park Memorial Institute-1640 (RPMI-1640) medium, and trypsin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) membrane was purchased from Pall Life Sciences (Port Washington, NY, USA), Western blot related chemical reagent was purchased from Shanghai Biyuntian Biotechnology Co., Ltd. (Shanghai, China), enhanced chemiluminescence (ECL) reagent was purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK), rabbit anti-mouse SOX9 monoclonal antibody, ERK/P38 monoclonal antibody and phosphorylated monoclonal antibody. Horseradish peroxidase (HRP)-labeled IgG secondary antibody was purchased from Cell Signal Technology (Danvers, MA, USA). TNF-α and IL-2 ELISA kits were purchased from R&D (Minneapolis, MN, USA). SOX9 siRNA was designed and synthesized by Shanghai Jima Gene Co., Ltd (Shanghai, China). The Myeloperoxidase (MPO) activity detection kit and the superoxide dismutase (SOD) activity detection kit were purchased from Wuhan Boster Co., Ltd (Wuhan, China). The Annexin V-PI kit was purchased from BD Corporation (San Jose, CA, USA). The Labsystem Version 1.3.1 microplate reader was purchased from Bio-Rad Corporation (Hercules, CA, USA). The clean workbench was purchased from Suzhou Purification Equipment Factory in Jiangsu Province (Suzhou, China). The RNA extraction kit and the reverse transcription kit were purchased from ABI (Waltham, MA, USA). The Amp PCR System 2400 DNA Amplifier was purchased from PE Applied Biosystems (Foster City, CA, USA). The Melody C6 flow cytometer was purchased from BD Corporation (San Jose, CA, USA). Other commonly used reagents were purchased from Shanghai Shenggong Biological Co., Ltd (Shanghai, China).

MIN6 Islet Cell Grouping and Processing

Liquid nitrogen was used to store human MIN6 cell line, which was thawed in 37°C water bath until the cells were completely thawed, followed by being centrifuged at 1000 rpm for 3 min, resuspended in 1 ml of fresh medium, transferred to a 50 ml cell culture flask containing 4 ml fresh medium and cultured at 37°C with 5% CO_2 for 24-48 h. MIN6 cells were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum, 10 U/mL penicillin and 10 μg/mL streptomycin at 37°C in a 5% CO_2 incubator to maintain a cell number of 1×10^6/ bottle. The subcultured MIN6 cells were diluted to 1×10^6/ml, inoculated in a 35 mm culture dish, and cultured in serum-free RPMI-1640 medium containing 100 ng/ml PMA and 0.3% bovine serum albumin (BSA) for 24 h. In the experiment, 3-8 generation logarithmic growth phase cells MIN6 were randomly divided into 3 groups, control group; high glucose group in which 30 mmol/L glucose was added to the culture medium to prepare high glucose environment to stimulate cultured cells; SOX9 siRNA group in which SOX9 siRNA was transfected into MIN6 cells under a high glucose environment.

SOX9 siRNA Transfection of MIN6 Islet Cells in High Glucose Environment

SOX9 siRNA was transfected into MIN6 cells in a high glucose environment. The SOX9 siRNA sequence was: the upstream sequence 5'-GCTGGGGAGGAATCTTCA-3'; the downstream sequence 5'-GCAGGTGACGGTGGTCA-3. The cell density was fused to 70-80% in a 6-well plate; SOX9 siRNA liposomes were separately added to 200 μl of serum-free medium, mixed well, and incubated at room temperature for 15 min. The mixed lipo2000 was separately mixed with the corresponding dilution and incubated for 30 min at room temperature. The serum of cells was removed, PBS was gently rinsed, 1.6 ml of serum-free medium was added, and each system was added to each system, and cultured in a 5% CO_2 incubator at 37°C for 48 h.

Real Time PCR Detection of SOX9 Expression

RNA of each group of MIN6 islet cells was extracted on ice using TRIzol reagent, and DNA reverse transcription synthesis was performed according to the kit instructions. The primers were designed according to each gene sequence by Primer6.0 and synthesized by Shanghai Yingjun

Biotechnology Co., Ltd. (Table I). Real-time PCR detection of the gene of interest: Reaction conditions: 55°C 1 min, 92°C 30 s, 58-60°C 45 s, 72°C 35 s, a total of 35 cycles. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as a reference. According to the fluorescence quantification, the starting cycle number (CT) of all samples and standards was calculated. Based on the standard CT value, a standard curve was drawn and then the semi-quantitative analysis was carried out by the 2-ΔCt method.

Western Blot Detection of SOX9, ERK/P38 Protein Expression

The MIN6 islet cell protein was extracted: the lysate was added, and the protein was quantified and stored at –20°C for Western blot. The protein was separated on 10% sodium dodecyl sulphate (SDS-PAGE) electrophoresis, transferred to PVDF membrane, blocked with 5% milk, and incubated with the diluted primary antibody of primary anti-SOX9, pERK1/2, ERK1/2, pP38, P38 antibody (1:2000, 1:1000; 1:1000; 1:1500; 1:2000 dilution respectively) at 4°C overnight. After washing with phosphate-buffered saline with Tween® detergent (PBST), the membrane was incubated with 1:2000 diluted goat anti-rabbit secondary antibody for 30 min, followed by being washed with PBST, developed and exposed after addition of enhanced chemiluminescence for 1 min. X-film and strip density measurements were separately scanned using protein image processing system software and Quantity one software. The experiment was repeated four times (n=4).

MTT Assay Analysis of Cell Proliferation

The logarithmic growth phase MIN6 cell line was digested, counted, and transferred to the second-generation inoculation in a 96-well plate, and treated as above. The seeding density was 5×10^3/well, and the culture plate was transferred to a CO_2 incubator for cultivation. Five replicate wells were designed for each group. After adding relevant factors according to each group design, cells were cultured for 24 h followed by addition of 20 µl 5 g/L MTT solution to each group of cells in each well, and culture for 4 h. The supernatant was removed, 150 µl/well of dimethyl sulfoxide (DMSO) was added, and shaken for 10 min. After the purple crystals were fully dissolved, the absorbance (A) value was measured at a wavelength of 492 nm by a microplate reader, and the cell proliferation rate was calculated. The experiment was repeated 3 times (n=3).

Apoptosis Analysis

Each group of cells was treated, and cells were collected by trypsin digestion without ethylenediaminetetraacetic acid (EDTA). The cells were washed twice with PBS (centrifuged at 2000 rpm for 5 min) to collect 5×10^5 cells and 500 µL of Binding Buffer and 5 µL of Annexin V-EGFP was added and mixed followed by addition of 5 µL propidium iodide (PI) for 5 to 15 min at room temperature. After 1 h incubation, cell apoptosis was measured by flow cytometry.

Analysis of TNF-α and IL-2 Level by ELISA

The supernatants of each group were collected to detect changes in the expression of inflammatory factors TNF-α and IL-2 according to the enzyme-linked immunosorbent assay (ELISA) kit instructions. The measurement should be carried out within 15 min after the addition of the stop solution. The linear regression equation of the standard curve is calculated according to the concentration of the standard product and the corresponding OD value, and the corresponding sample concentration is calculated on the regression equation according to the OD value of the sample.

Detection of MPO Activity and SOD Activity

The changes in SOD activity in lung tissues of each group were examined according to the kit instructions. The tissue protein was extracted and washed in a 95°C water bath. After 40 min, it was taken out and rinsed with cold water. After cooling, it was centrifuged at 4000 rpm for 10 min.

Table I. Primer sequences.

Gene	Forward 5′-3′	Reverse 5′-3′
GAPDH	AGTAGTCACCTGTTGCTGG	TAATACGGAGACCTGTCTGGT
SOX9	TCGCTCGATGCCATTCC	ACACGAGTTACTGATT

The ethanol phase in the tissue homogenate was extracted using an ethanol-chloroform mixture (5:3, v/v volume ratio 5:3) for detection of total SOD activity. MPO activity was mainly carried out according to the kit instructions. The tissue was mixed by a vortex mixer and loaded into an Eppendorf (EP) tube. After 95 min of water bathing, after 40 min, it was taken out and rinsed with cold water. After cooling, it was centrifuged at 4000 rpm for 10 min. The sample was mixed in a buffer containing 30 mM H2O2 phosphate, pH 7.0, and the complex was bathed in water for 10 min. The enzyme activity was determined by detecting the reduced absorption optical density of H_2O_2 at a wavelength of 240 nm.

Analysis of Insulin Secretion Levels

The MIN6 cells in each logarithmic growth phase were counted, digested and counted, and the cell density was adjusted. The cells were seeded in a 48-well plate at 500 μL/well for 24 h to remove the cell culture medium. The supernatant was discarded, washed with PBS, and added to 300 μL/well of HBSS buffer for 30 min at 37°C; washed twice with pre-cooled PBS. After incubation overnight at 4°C, the supernatant was centrifuged at 800 r/min at 4°C for 5 min, and the supernatant was stored at –20 °C. The protein content was measured by the bicinchoninic acid (BCA) assay. The unit mass insulin concentration = insulin content / corresponding protein content / well, The unit was 103 IU.L-1.·g-1.Pro.

Statistical Analysis

All data was analyzed using Statistical Package for the Social Sciences (SPSS) 22.0 software (IBM, Armonk, NY, USA). Measurement data were described by mean ± standard deviation (SD). One-way ANOVA was used for comparison of multiple groups of samples with Bonferroni as post-hoc analysis. The *t*-test was used for comparison between the two groups. The test level was α=0.05. $p < 0.05$ was considered statistically significant.

Results

Expression of Islet MIN6 Cells in SOX9 Under High Glucose Environment

Real time PCR and Western blot were used to detect the expression of SOX9 mRNA and protein in islet cells under normal and high glucose environment. The results showed that compared with control group, SOX9 mRNA and protein expression were increased significantly ($p < 0.05$). While transfection of SOX9 siRNA into islet cells MIN6 cells under high glucose environment significantly down-regulate SOX9 mRNA and protein expression ($p < 0.05$) compared with high glucose group (Figure 1).

Effect of SOX9 on Proliferation of Islet MIN6 Cells in High Glucose Environment

In high glucose environment, the proliferation of pancreatic islet MIN6 cells was decreased. Compared with control group, the difference was statistically significant ($p < 0.05$). Transfection of SOX9 siRNA in high glucose environment significantly promoted cell proliferation compared with high glucose group ($p < 0.05$) (Figure 2).

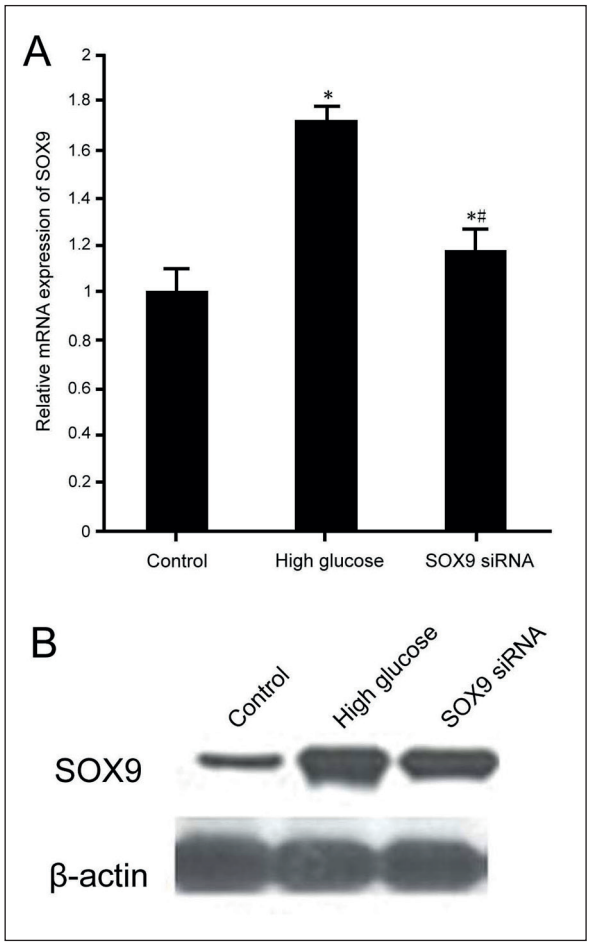

Figure 1. Expression of MIN6 in islet cells of SOX9 in high glucose environment. **A,** Real time PCR detected the expression of SOX9 mRNA, compared with the control group, $^{*}p < 0.05$, compared with the high glucose group, $^{\#}p < 0.05$; **B,** Western blot detection of SOX9 protein expression.

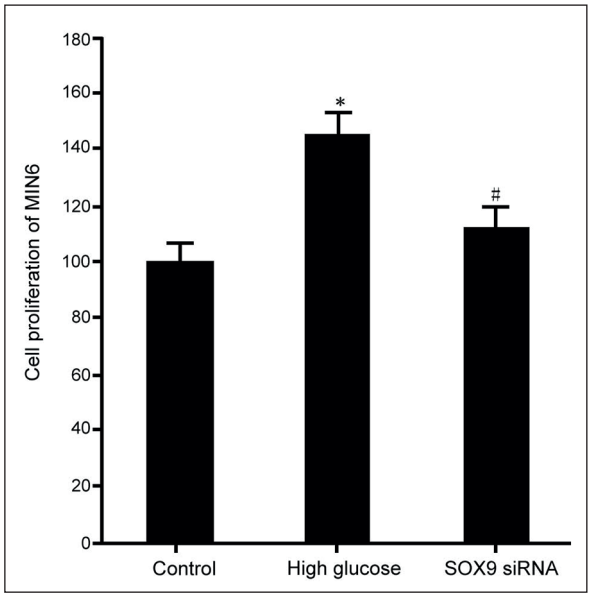

Figure 2. Effect of SOX9 on proliferation of islet MIN6 cells in high glucose environment. Compared with the control group, $*p < 0.05$, compared with the high glucose group, $^{\#}p < 0.05$.

Effect of SOX9 on Apoptosis of MIN6 Cells in High Glucose Environment

Flow cytometry analysis of the effect of SOX9 on apoptosis of islet MIN6 cells in high glucose environment showed that the apoptosis of islet MIN6 cells was significantly increased compared with control group ($p < 0.05$). Transfection of SOX9 siRNA significantly inhibited apoptosis of islet MIN6 cells compared with high glucose group ($p < 0.05$) (Figure 3).

Effect of SOX9 on the Secretion of Inflammatory Factors in MIN6 Cells

The effect of SOX9 on the secretion of inflammatory factors in pancreatic MIN6 cells in high glucose environment was analyzed by ELISA. In high glucose environment, the secretion of inflammatory factors TNF-α and IL-2 was significantly increased in islet MIN6 cells compared with control group ($p < 0.05$). Transfection of SOX9 siRNA significantly inhibited the secretion of inflammatory factors TNF-α and IL-2 ($p < 0.05$) (Figure 4).

Effect of SOX9 on Insulin Secretion of MIN6 Cells

In high glucose environment, the insulin secretion of islet MIN6 cells was decreased. Compared with control group, the difference was statistically significant ($p < 0.05$). However, transfection

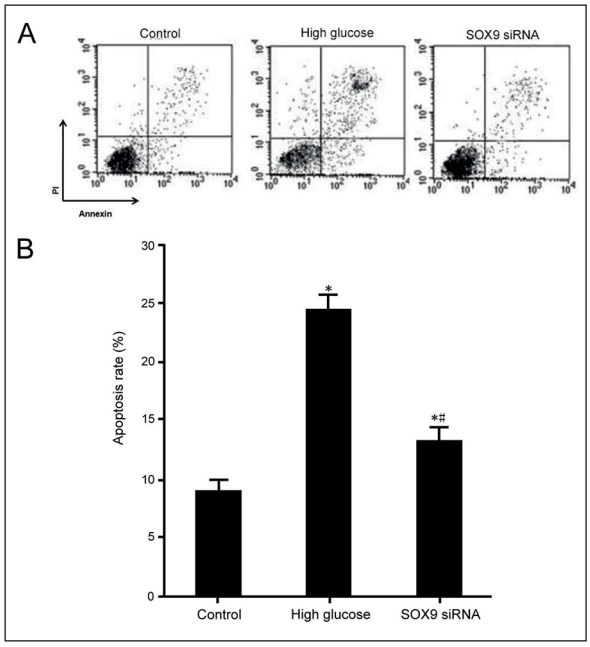

Figure 3. Effect of SOX9 on apoptosis of islet MIN6 cells in high glucose environment. **A,** Flow cytometry analysis of the effect of SOX9 on apoptosis of islet MIN6 cells in high glucose environment; statistical analysis of **B,** cell apoptosis rate compared with control group, $*p < 0.05$, compared with high glucose group, $^{\#}p < 0.05$.

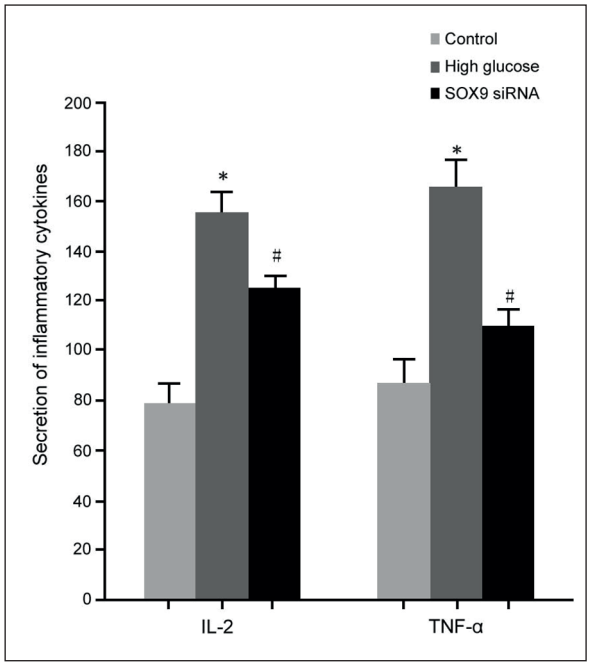

Figure 4. Effect of SOX9 on the secretion of inflammatory factors in islet MIN6 cells in high glucose environment. Compared with control group, $*p < 0.05$, compared with high glucose group, $^{\#}p < 0.05$.

of SOX9 siRNA significantly promoted insulin secretion in MIN6 cells compared with high glucose group ($p < 0.05$) (Figure 5).

Effect of SOX9 on Redox of MIN6 Cells in High Glucose Environment

Under high glucose environment, MPO content in pancreatic islet MIN6 cells was increased and SOD activity was decreased, compared with control group, the difference was statistically significant ($p < 0.05$). After transfection of SOX9 siRNA in high glucose environment, the MPO content was decreased and SOD activity was increased, and the difference was statistically significant compared with high glucose group ($p < 0.05$) (Figure 6).

Effect of SOX9 on ERK/P38 Signaling Pathway in Islet MIN6 Cells

Western blot analysis of the effect of SOX9 on ERK/P38 signaling pathway in pancreatic islet MIN6 cells in high glucose environment showed that in high glucose environment, the phosphorylation of ERK/P38 protein in pancreatic MIN6 cells was decreased. After transfection of SOX9 siRNA in islet MIN6 cells in high glucose environment, ERK/P38 protein phosphorylation level was increased (Figure 7).

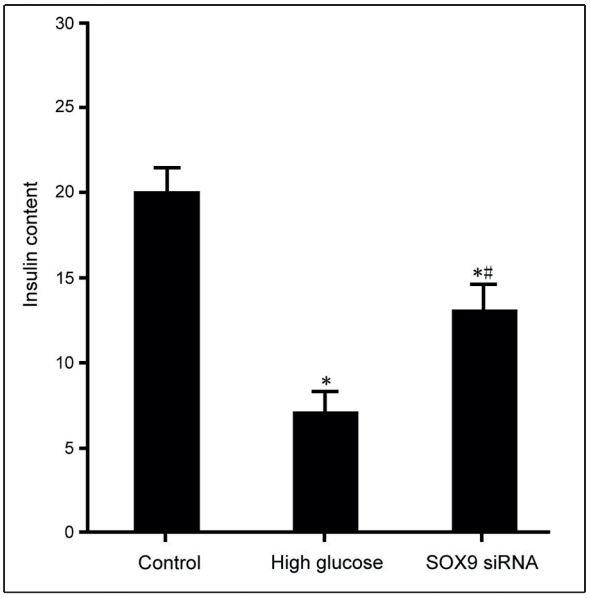

Figure 5. Effect of SOX9 on insulin secretion of islet MIN6 cells in high glucose environment. Compared with control group, $^{*}p < 0.05$, compared with high glucose group, $^{\#}p < 0.05$.

Discussion

The pathogenesis of diabetes has not yet been fully elucidated, and insulin resistance and the number and dysfunction of islet β-cells are the two main links in the development of diabetes[17].

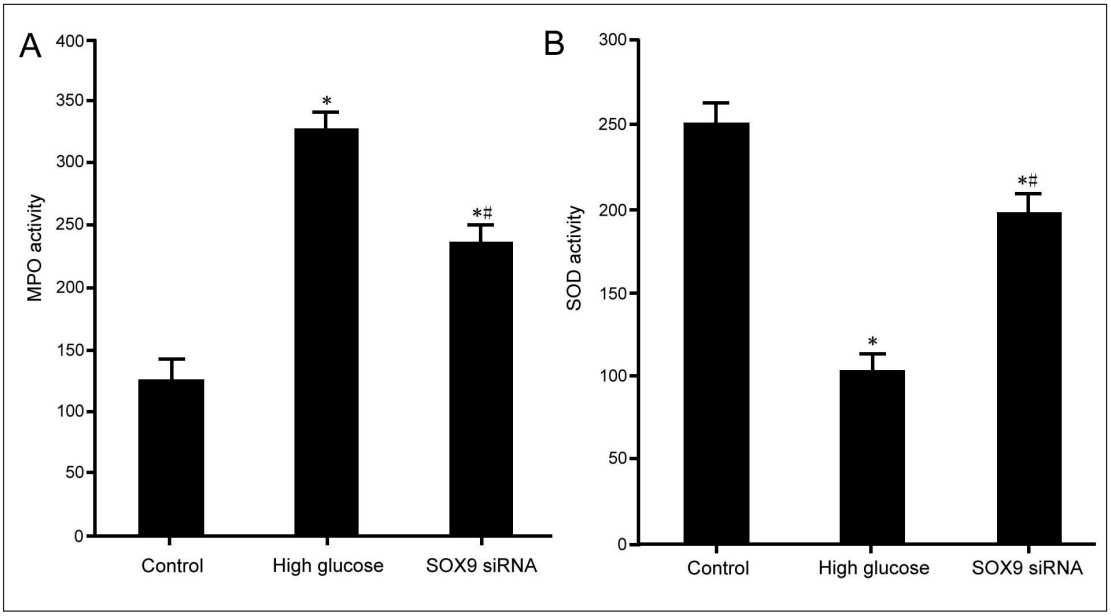

Figure 6. Effect of SOX9 on redox of MIN6 cells in high glucose environment. **A,** MPO content changes; **B,** SOD activity analysis, compared with control group, $^{*}p < 0.05$, compared with high sugar group, $^{\#}p < 0.05$.

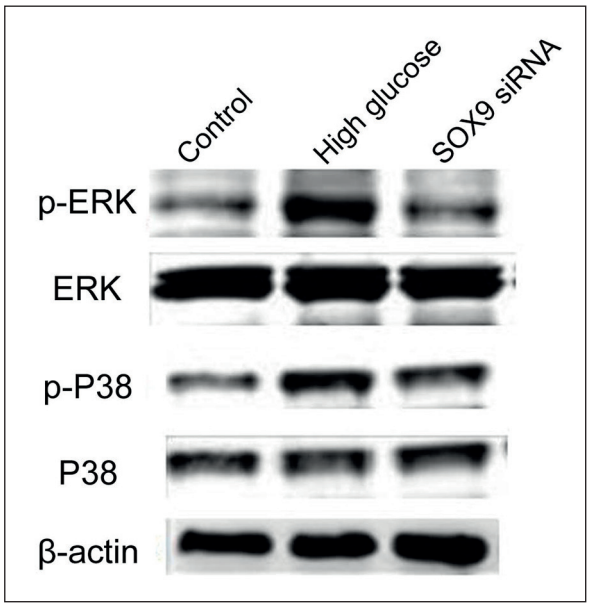

Figure 7. Effect of SOX9 on ERK/P38 signaling pathway in islet MIN6 cells in high glucose environment.

Islet β-cells are a kind of islet cells, which are endocrine cells, accounting for about 70% of the total number of islet cells. They are mainly located in the central part of the islets, and can secrete insulin and regulate blood sugar levels[18]. One of the main functions of islet β cells is to synthesize and secrete insulin, which is a regulator that maintains normal functional metabolism and regulates insulin secretion by blood glucose[19]. High glucose, inflammation, and other pathogenic factors can cause the damage of islet β cell[20]. During fetal development, SOX9 can play a vital role in the development of stem and progenitor cells in the liver, pancreas and hair follicles[21]. Abnormal SOX9 expression can cause skeletal malformations, central nervous system dysfunction and multiple defects in other organs, and even the occurrence of tumors[22]. In this study, it was found that under high glucose environment, the expression of SOX9 in MIN6 cells was increased in pancreatic islet β cells, the proliferation of MIN6 cells was inhibited, and the apoptosis was increased. However, transfection of SOX9 siRNA reversed these changes in high glucose environment, suggesting that increased SOX9 expression in the environment can participate in the regulation of the activity and proliferation of MIN6 cells. In this study, we further analyzed the effects of SOX9 on islet β-cell MIN6 cells and related mechanisms in high glucose environment. The occurrence of diabetes is closely related to oxidative stress, which leads to excessive production of free radicals such as reactive oxygen species, dynamic imbalance of oxidation and antioxidant systems, and thus tissue inflammation and damage. MPO is greatly increased, and SOD activity, which is one of the important antioxidant enzymes for scavenging oxygen free radicals, was decreased. MPO can be released through the cell membrane, thereby aggravating inflammation, leading to apoptosis and damage of MIN6 cells[23,24]. The results showed that SOX9 expression was significantly increased in high glucose environment, SOD activity was decreased, MPO was increased, TNF-α and IL-2 expression was increased, while SOX9 siRNA transfection down-regulated SOX9 expression, promoted SOD activity and decreased MPO and the expression of TNF-α and IL-2, suggesting that SOX9 can participate in the regulation of islet β-cell in high glucose environment by regulating oxidative stress and inflammation. The ERK/P38 signaling pathway can participate in the regulation of redox balance. After activation, it can inhibit external oxidation and chemical substances, thereby inhibiting oxidative stress and exerting a defense effect. It is one of the most important endogenous antioxidant signaling pathways currently considered[25]. This study indicated that ERK/P38 protein phosphorylation was decreased in pancreatic MIN6 cells under high glucose environment; ERK/P38 protein phosphorylation was increased after transfection of SOX9 siRNA in islet MIN6 cells in high glucose environment, suggesting that ERK/P38 signaling pathway plays a key role in islet β-cell MIN6 cells under a high glucose environment. SOX9 can affect the activity of MIN6 by regulating the ERK/P38 signaling pathway.

Conclusions

SOX9 expression is increased under high glucose environment. Down-regulation of SOX9 expression inhibited islet cell apoptosis, oxidative stress and inflammation, and promoted islet cell proliferation and insulin secretion by regulation of ERK/P38 signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Dordevic M, Grdovic N, Mihailovic M, Arambasic Jovanovic J, Uskokovic A, Rajic J, Sinadinovic M, Tolic A, Misic D, Siler B, Poznanovic G, Vidakovic M, Dinic S. Centaurium erythraea extract improves survival and functionality of pancreatic beta-cells in diabetes through multiple routes of action. J Ethnopharmacol 2019; 242: 112043.
2) Cao XY, Liu D, Xia Y, Cai TG, He Y, Liu JL. A novel polysaccharide from lentinus edodes mycelia protects min6 cells against high glucose-induced damage via the mapks and nrf2 pathways. Food Nutr Res 2019; 63.
3) Hu S, de Vos P. Polymeric approaches to reduce tissue responses against devices applied for islet-cell encapsulation. Front Bioeng Biotechnol 2019; 7: 134.
4) Iki M, Fujita Y, Kouda K, Yura A, Tachiki T, Tamaki J, Sato Y, Moon JS, Hamada M, Kajita E, Okamoto N, Kurumatani N. Increased risk of osteoporotic fracture in community-dwelling elderly men 20 or more years after gastrectomy: The fujiwara-kyo osteoporosis risk in men (formen) cohort study. Bone 2019; 127: 250-259.
5) Zhan X, Yan C, Chen Y, Wei X, Xiao J, Deng L, Yang Y, Qiu P, Chen Q. Celastrol antagonizes high glucose-evoked podocyte injury, inflammation and insulin resistance by restoring the ho-1-mediated autophagy pathway. Mol Immunol 2018; 104: 61-68.
6) Kim HL, Kim MA, Park KT, Choi DJ, Han S, Jeon ES, Cho MC, Kim JJ, Yoo BS, Shin MS, Kang SM, Chae SC, Ryu KH. Gender difference in the impact of coexisting diabetes mellitus on long-term clinical outcome in people with heart failure: A report from the korean heart failure registry. Diabet Med 2019.
7) Su ZT, Sauriol L, Bartelt-Hofer J, Brown S, Lew E, Annemans L, Grima DT. The use of computer simulation modelling to estimate complications in patients with type 2 diabetes: Validation of the cornerstone diabetes simulation model. Diabetologia 2018; 61: S138-S138.
8) Du N, Xu Z, Gao M, Liu P, Sun B, Cao X. Combination of ginsenoside rg1 and astragaloside iv reduces oxidative stress and inhibits tgf-beta1/smads signaling cascade on renal fibrosis in rats with diabetic nephropathy. Drug Des Devel Ther 2018; 12: 3517-3524.
9) Qiu DD, Liu J, Shi JS, An Y, Ge YC, Zhou ML, Jiang S. Renoprotection provided by dipeptidyl peptidase-4 inhibitors in combination with angiotensin receptor blockers in patients with type 2 diabetic nephropathy. Chin Med J (Engl) 2018; 131: 2658-2665.
10) Wang D, Jiang L, Feng B, He N, Zhang Y, Ye H. Protective effects of glucagon-like peptide-1 on cardiac remodeling by inhibiting oxidative stress through mammalian target of rapamycin complex 1/p70 ribosomal protein s6 kinase pathway in diabetes mellitus. J Diabetes Investig 2019.
11) O'Brien R, Buckley MM, Kelliher A, O'Malley D. Pi 3-kinase- and erk-mapk-dependent mechanisms underlie glucagon-like peptide-1-mediated activation of sprague dawley colonic myenteric neurons. Neurogastroenterol Motil 2019; 31: 1-11.
12) Rojas J, Bermudez V, Palmar J, Martinez MS, Olivar LC, Nava M, Tomey D, Rojas M, Salazar J, Garicano C, Velasco M. Pancreatic beta cell death: Novel potential mechanisms in diabetes therapy. J Diabetes Res 2018; 2018: 9601801.
13) Wang LH, Zhang ZY, Yu XD, Huang X, Liu Z, Chai YH, Yang L, Wang Q, Li M, Zhao J, Hou J, Li F. Unbalanced yap-sox9 circuit drives stemness and malignant progression in esophageal squamous cell carcinoma. Oncogene 2019; 38: 2042-2055.
14) Ambrozkiewicz MC, Schwark M, Kishimoto-Suga M, Borisova E, Hori K, Salazar-Lazaro A, Rusanova A, Altas B, Piepkorn L, Bessa P, Schaub T, Zhang X, Rabe T, Ripamonti S, Rosario M, Akiyama H, Jahn O, Kobayashi T, Hoshino M, Tarabykin V, Kawabe H. Polarity acquisition in cortical neurons is driven by synergistic action of sox9-regulated wwp1 and wwp2 e3 ubiquitin ligases and intronic mir-140. Neuron 2018; 100: 1097-1115 e1015.
15) Kim AL, Back JH, Chaudhary SC, Zhu Y, Athar M, Bickers DR. Sox9 transcriptionally regulates mtor-induced proliferation of basal cell carcinomas. J Invest Dermatol 2018; 138: 1716-1725.
16) Tan Y, Yao X, Dai Z, Wang Y, Lv G. Bone morphogenetic protein 2 alleviated intervertebral disc degeneration through mediating the degradation of ecm and apoptosis of nucleus pulposus cells via the pi3k/akt pathway. Int J Mol Med 2019; 43: 583-592.
17) Fu JF, Liang L, Gong CX, Xiong F, Luo FH, Liu GL, Li P, Liu L, Xin Y, Yao H, Cui LW, Shi X, Yang Y, Chen LQ, Wei HY. Status and trends of diabetes in chinese children: Analysis of data from 14 medical centers. World J Pediatr 2013; 9: 127-134.
18) He J, Lian C, Fang Y, Wu J, Zhou H, Ye X. The influence of exendin-4 intervention on -obese diabetic mouse blood and the pancreatic tissue immune microenvironment. Exp Ther Med 2016; 12: 2893-2898.
19) Lee E, Lee HS. Peroxidase expression is decreased by palmitate in cultured podocytes but increased in podocytes of advanced diabetic nephropathy. J Cell Physiol 2018; 233: 9060-9069.
20) Cerf ME. Beta cell dysfunction and insulin resistance. Front Endocrinol (Lausanne) 2013; 4: 37.
21) Symon A, Harley V. Sox9: a genomic view of tissue specific expression and action. Int J Biochem Cell Biol 2017; 87: 18-22.
22) Jo A, Denduluri S, Zhang B, Wang Z, Yin L, Yan Z, Kang R, Shi LL, Mok J, Lee MJ, Haydon RC. The versatile functions of sox9 in development, stem cells, and human diseases. Genes Dis 2014; 1: 149-161.

23) Zhou M, Ren H, Han J, Wang W, Zheng Q, Wang D. Protective effects of kaempferol against myocardial ischemia/reperfusion injury in isolated rat heart via antioxidant activity and inhibition of glycogen synthase kinase-3beta. Oxid Med Cell Longev 2015; 2015: 481405.

24) Ertao Z, Jianhui C, Chuangqi C, Changjiang Q, Sile C, Yulong H, Hui W, Shirong C. Autocrine sonic hedgehog signaling promotes gastric cancer proliferation through induction of phospholipase cgamma1 and the erk1/2 pathway. J Exp Clin Cancer Res 2016; 35: 63.

25) Wang BQ, Yang B, Yang HC, Wang JY, Hu S, Gao YS, Bu XY. MicroRNA-499a decelerates glioma cell proliferation while accelerating apoptosis through the suppression of notchl and the mapk-signaling pathway. Brain Res Bull 2018; 142: 96-106.