Abnormal expression of TRIAP1 and its role in gestational diabetes mellitus-related pancreatic β cells

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Abstract. Gestational diabetes mellitus (GDM) is a disease that is typically characterized by insulin resistance and pancreatic β cell dysfunction. Currently, the role of TP53-regulated inhibitor of apoptosis 1 (TRIAP1) in the process of GDM remains to be elucidated. Therefore, the present study investigated the effects of TRIAP1 on GDM-related pancreatic β cells. Reverse transcription-quantitative PCR and western blot assays were conducted to analyze the expression levels of TRIAP1 in the peripheral blood of patients with GDM and subjects with healthy pregnancies. Subsequently, TRIAP1 small interfering RNA (siRNA), control siRNA, TRIAP1 plasmid and control plasmid were transfected into INS-1 cells to assess the effects of TRIAP1 on pancreatic β cells. ELISA was used to assess the total insulin content and insulin secretion of pancreatic β cells. MTT and flow cytometry assays were performed to determine the viability and apoptosis of pancreatic β cells. The results demonstrated that TRIAP1 expression was downregulated in peripheral blood samples from patients with GDM. Transfection with TRIAP1 siRNA significantly decreased the levels of total insulin content and reduced insulin secretion in pancreatic β cells. In addition, downregulation of TRIAP1 in pancreatic β cells significantly induced cell apoptosis and reduced cell viability. Accordingly, transfection of INS1 cells with TRIAP1 siRNA increased the levels of the apoptosis-associated genes apoptotic protease-activating factor 1, caspase-3, caspase-7 and caspase-9. However, transfection of the cells with TRIAP1 plasmid resulted in the opposite effects. TRIAP1 increased the growth of pancreatic β cells and their ability to secrete insulin, thus playing a protective role in GDM. The findings verified the effects and the underlying mechanism of TRIAP1 in pancreatic β cells and may provide additional clinical applications for the therapy of GDM.

Introduction

Gestational diabetes mellitus (GDM) is a type of diabetes characterized by abnormal insulin deficiency and pancreatic β cell dysfunction that occurs in pregnant females with a prevalence of 6-20% worldwide (1,2). It is one of the most common diseases that occurs during pregnancy, normally leading to serious complications, such as cardiovascular illness, type 2 diabetes and obesity, as well as an increase in fetal morbidity and mortality (3,4). During the process of GDM, insulin secretion is defective and pancreatic β cells are unable to compensate for insulin resistance, inevitably leading to hyperglycemia (5). Recently, lifestyle habits, including diet and physical activity, have been suggested as key factors that can prevent the symptoms and development of GDM in females (6). However, in patients with GDM where lifestyle management cannot result in the recommended glycaemia, additional therapeutic treatments, including exercise and insulin therapy, are required to control GDM (7,8). Therefore, it is crucial to identify additional therapeutic strategies for GDM.

Dysfunction of pancreatic β-cells have been reported to be a central component in the pathogenesis and development of GDM (9,10). Reduction of pancreatic β cell function is characterized by increased rates of cell apoptosis (11) and defects in insulin generation and secretion (12). During pregnancy, insulin resistance results in increments of the insulin requirement (13), while insulin secretion by β cells is reduced under the stimulation of prolactin and placental lactogens, and fetuin generation and secretion (12). During pregnancy, insulin resistance results in increments of the insulin requirement (13), while insulin secretion by β cells is reduced under the stimulation of prolactin, placental lactogens and fetal growth (14-16). It was shown that hyperglycemia participated in the induction of pancreatic β cell apoptosis (17) and insulin secretion (18). Therefore, investigating preventive mechanisms that can maintain glucose homeostasis by protecting and improving the functions of pancreatic β cells is a significant research topic in the field of maternal medicine. However, to the best of our knowledge, the cellular and molecular mechanisms of pancreatic β cell dysfunction remain to be elucidated.

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TP53-regulated inhibitor of apoptosis 1 (TRIAP1) is a small conserved protein containing 76 amino acids that was initially identified as a p53-induced cell survival factor (19,20). It was previously reported that TRIAP1 results in cell death resistance via a mitochondrial-dependent pathway (21,22). It was also shown that TRIAP1 can modulate apoptotic pathways by interacting with Hsp70-binding protein 1, prevent the formation of apoptotic protease-activating factor 1 (APAF1), release of cytochrome c and induction of caspase-9, which reduced the formation of the apoptosome complex (23). These molecular events lead to insulin resistance by suppressing apoptosis and allowing DNA damage repair (23). Due to the significant function of TRIAP1 in apoptosis and the interaction between pancreatic β cell apoptosis and GDM, the current study explored the involvement of TRIAP1 in the regulation of GDM.

Materials and methods

Clinical sample collection. Peripheral blood samples were collected from 30 female patients with GDM (age range, 24-37 years) and 30 female subjects that were undergoing healthy pregnancies (age range, 23-36 years) at the Obstetrics and Gynecology Hospital of Fudan University Hospital (Shanghai, China) between December 2017 and December 2018. The blood samples were separated by centrifugation (1,000 x g for 10 min at 4˚C) and stored in liquid nitrogen instantly for subsequent experiments. There were no statistically significant differences in age, gestational week and weight between patients with GDM and subjects with healthy pregnancies. The present study was approved by the Ethics Committee of the Obstetrics and Gynecology Hospital of Fudan University, and written informed consent was acquired from all participants prior to their enrollment.

Females with a fasting plasma glucose (FPG) level of ≥4.4 mmol/l but ≤5.1 mmol/l underwent a 75 g oral glucose tolerance test (OGTT). In such cases, a diagnosis of GDM was made when at least one glucose value was elevated (FPG ≥5.1 mmol/l, 1-h OGTT ≥10.0 mmol/l or 2-h OGTT ≥8.5 mmol/l). Pregnant females with the following conditions were excluded from the present study: i) abnormal blood lipid (low-density lipoprotein cholesterol, triglyceride and high-density lipoprotein cholesterol) levels, hypertension and chronic kidney and liver diseases; ii) endocrine diseases, including obesity, diabetes, thyroid disease, osteoporosis, adrenal cortical disease and hyperthyroidism prior to pregnancy; iii) pregnant females currently undergoing long-term drug treatments (such as sodiumvolethohyrine) and iv) other pregnancy complications (pre-eclampsia, pregnancy-induced hypertension or pregnancy with chronic nephritis).

Cell culture and transfection. INS-1 cells were obtained from American Type Culture Collection. The cells were cultured and maintained in RPMI-1640 medium (HyClone; Cytiva) supplemented with 10% fetal bovine serum (HyClone; Cytiva), 11.1 mmol/l glucose, 50 mM b-mercaptoethanol and 1% penicillin/streptomycin. The cells were maintained in 5% CO₂ at 37°C.

TRIAP1-small interfering RNA (siRNA; 0.2 µM; 5'-AGG CAUGCACGGACAUGAATT-3') or control-siRNA (0.2 µM; 5'-GCACCACGTGACGGAGCGT-3'), 1 µg TRIAP1 CRISPR activation plasmid (cat. no. sc-427287-ACT) or control-plasmid (cat no. sc-437275) were purchased from Santa Cruz Biotechnology, Inc. Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for transfection according to the manufacturer's instructions. Following transfection, the cells were cultured for 48 h and collected for further experiments. Cells without any treatment were used as the control.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from peripheral blood and INS-1 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA concentration was detected using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.) at wavelengths of 260 and 180 nm. Subsequently, 800 ng RNA was reversed transcribed into cDNA using HiScript™ Q RT SuperMix (Vazyme Biotech Co., Ltd.). The following temperature conditions for reverse transcription were as follows: 70°C for 5 min, 37°C for 5 min and 42°C for 60 min. qPCR was performed using an ABI Prism 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the SYBR-Green Real-Time PCR kit (Toyobo Life Science) for the detection of RNA expression levels. The following thermocycling conditions were used for the qPCR: Initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. GAPDH was used as the internal control and the relative expression levels of the transcripts were calculated using the 2−ΔΔCq method (24). Primer sequences were listed as following: TRIAP1 forward, 5'-GCACCGACCTCTTCA AGC-3' and reverse, 5'-CCATGAACTCCAGTCTTCA3'; GAPDH forward, 5'-CTTTTGATACGTGGAAGGACTC-3' and reverse, 5'-GTAGGCGCCGGATGATTTCT-3'; APAFI forward, 5'-AACCAGGATGGTGCACTCCA-3' and reverse, 5'-ACTGAAACCAATGCCACTCC-3'; caspase-3 forward, 5'-GAAGTGGAGCTGAGTCTGTCT-3'; reverse, 5'-GCACAGCCTCCTGGAAGGAGCA-3' and reverse, 5'-GCAGGGCCCTACCTCTGACTTATC-3' and caspase-9 forward, 5'-TTCCCAGGTCTTTTCCTCTG-3' and reverse, 5'-CTTTCACCGAAGACGATT-3';

Western blot analysis. To obtain total protein, INS-1 cells and peripheral blood samples were lysed with RIPA buffer containing protease inhibitors (Beyotime Institute of Biotechnology) for 30 min and subsequently centrifuged at 12,000 x g for 3 min at 4°C. Protein concentration was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Subsequently, the proteins were mixed with 4X loading buffer, boiled at 95°C for 10 min and centrifuged at 10,000 x g at 4°C for 1 min. Equal amounts of protein (20 µg) were loaded on 10% SDS gels and analyzed by PAGE. The gels were transferred to PVDF membranes, and the membranes were blocked with 5% non-fat milk for 1 h at room temperature. The membranes were washed with PBS-0.1% Tween-20 (PBST) three times and subsequently incubated overnight at 4°C with the following primary antibodies: TRIAP1 (cat. no. 515801; 1:1,000; Santa Cruz Biotechnology, Inc.).
Biotechnology, Inc.), APAF1 (cat. no. 8723; 1:1,000; Cell Signaling Technology, Inc.), caspase-3 (cat. no. 14220; 1:1,000; Cell Signaling Technology, Inc.), caspase-7 (cat. no. 12827; 1:1,000; Cell Signaling Technology, Inc.), caspase-9 (cat. no. 9508; 1:1,000; Cell Signaling Technology, Inc.) and GAPDH (cat. no. 5174; 1:1,000; Cell Signaling Technology, Inc.). The following day, the membranes were washed with PBST four times and subsequently incubated with horse-radish peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibody (cat. no. 7074; 1:2,000; Cell Signaling Technology, Inc.) and the relative band intensities were analyzed using ImageJ software version 1.48u (National Institutes of Health).

Cell viability assay. INS-1 cells were transfected with TRIAP1-siRNA, control-siRNA, TRIAP1-plasmid and control-plasmid, and then seeded into 96-well plates at a density of 1x10^4 cells/well. Following incubation for 48 h at 37°C, 10 µl MTT solution (5 mg/ml; Sigma-Aldrich; Merck KGaA) was added to each well and the cells were cultured for 4 h at 37°C. Following incubation, DMSO (200 µl; Sigma-Aldrich; Merck KGaA) was added to each well and the plate was shaken for 10 min. Finally, the absorbance was measured using a Neo Multi-Mode Reader (BioTek Instruments, Inc.) at a wavelength of 570 nm.

Flow cytometry analysis. To detect cell apoptosis, INS-1 cells were transfected with TRIAP1-siRNA, control-siRNA, TRIAP1-plasmid and control-plasmid, and then cultured for 48 h. Following transfection, the cells were cultured with normal glucose (3.3 mM) or high glucose (16.7 mM) for 1 h. Subsequently, the total insulin content was extracted following sonication of the cells in acid ethanol (2% H_2SO_4 in 75% ethanol), accompanied with three cycles of freezing and thawing. The cells were centrifuged at 500 x g at 4°C for 5 min and the supernatant was used to determine insulin release levels using ELISA kit (cat. no. PI606; Beyotime Institute of Biotechnology) according to the manufacturer's instructions.

Statistical analysis. Statistical analyses were performed with SPSS 21.0 software (IBM Corp.). The derived values were expressed as the mean ± SD. One-way ANOVA with Tukey's post hoc test was used for comparisons between multiple groups. Unpaired Student's t-test was used to analyze the difference between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

TRIAP1 expression is downregulated in peripheral blood samples from patients with GDM. To examine the pathological relevance of TRIAP1 in GDM, the expression levels of TRIAP1 were detected in peripheral blood samples obtained from 30 patients with GDM and subjects with healthy pregnancies by RT-qPCR and western blot assays. As shown in Fig. 1A-C, the mRNA and protein expression levels of TRIAP1 were significantly lower compared with samples from healthy pregnancies.

Downregulation of TRIAP1 suppresses insulin secretion and total insulin content. To determine whether TRIAP1 is involved in regulating pancreatic β cell function, control-siRNA and TRIAP1-siRNA were transfected into INS-1 cells and the transfection efficiency was confirmed by RT-qPCR and western blot assays. As shown in Fig. 2A and B, transfection of cells with TRIAP1-siRNA significantly downregulated the mRNA and protein expression levels of TRIAP1 compared with the control-siRNA group. In addition, the results presented in Fig. 2C demonstrated that downregulation of TRIAP1 could inhibit the total insulin content in INS-1 cells compared with control-siRNA group. It has been frequently reported that high glucose can promote insulin secretion (25).
In the present study, the data showed that downregulation of TRIAP1 significantly inhibited insulin release under high and low glucose conditions in INS-1 cells compared with control-siRNA groups (Fig. 2D). The aforementioned results indicated that downregulation of TRIAP1 expression suppressed total insulin content and reduced insulin release in INS-1 cells under glucose stimulation.

**Downregulation of TRIAP1 effects INS-1 cell viability and apoptosis.** Subsequently, the effects of TRIAP1 on the viability and apoptosis of INS-1 cells were investigated. Control-siRNA and TRIAP1-siRNA were transfected into INS-1 cells for 48 h, and MTT and flow cytometry analyses were performed to detect the viability and apoptosis of INS-1 cells, respectively. As shown in Fig. 3A-C, downregulation of TRIAP1 significantly decreased cell viability and significantly increased the cell apoptotic population compared with their respective control-siRNA groups. Furthermore, the expression levels of cell apoptosis-associated genes, including APAF1, caspase-3, caspase-7 and caspase-9, were determined by RT-qPCR and western blotting. The findings demonstrated that the protein and mRNA expression levels of APAF1, caspase-3, caspase-7 and caspase-9 were significantly increased in the TRIAP1-siRNA-transfected group compared with the control-siRNA group (Fig. 3D-H). The aforementioned results indicated that TRIAP1-siRNA suppressed insulin secretion by inhibiting cell viability, promoting cell apoptosis and regulating the expression of apoptosis-associated genes in INS-1 cells.

**Upregulation of TRIAP1 promotes insulin secretion and total insulin content.** Subsequently, the regulation of TRIAP1 expression was investigated with regard to the metabolic function of pancreatic β cells. INS-1 cells were transfected with TRIAP1-plasmid and control-plasmid and the transfection efficiency was evaluated by RT-qPCR and western blot assays. As shown in Fig. 4A and B, the mRNA and protein expression levels of TRIAP1 were significantly increased in the TRIAP1-plasmid group compared with the control-plasmid group. The results also demonstrated that upregulation of TRIAP1 expression could significantly increase the total insulin content in INS-1 cells compared with control-plasmid group (Fig. 4C). Upregulation of TRIAP1 expression further significantly promoted insulin secretion under high and low glucose conditions in INS-1 cells compared with the control-plasmid group (Fig. 4D). These data suggest that TRIAP1 serves an essential role in regulating insulin secretion of pancreatic β cells.

**Upregulation of TRIAP1 influences of INS-1 cell viability and apoptosis.** To further investigate the role of TRIAP1 in the regulation of proliferation and apoptosis of INS-1 cells, control-plasmid and TRIAP1-plasmids were transfected into the cells for 48 h. MTT assay and flow cytometry analysis
indicated that upregulation of TRIAP1 significantly increased cell viability and significantly decreased apoptosis of INS-1 cells (Fig. 5A-C) compared with the control-plasmid groups. Western blotting and RT-qPCR results demonstrated that the protein and mRNA expression levels of APAF1, caspase-3, caspase-7 and caspase-9 were significantly decreased in the TRIAP1-plasmid-transfected group compared with the control-plasmid group (Fig. 5D-H).

Taken together, these findings demonstrated that TRIAP1 could influence insulin secretion by regulating cell viability, cell apoptosis and the expression levels of apoptosis-associated genes in INS-1 cells.

Discussion

GDM is a common condition in which hyperglycemia occurs in females during pregnancy (26), which usually leads to an enhanced risk of adverse outcomes for mothers and newborns (27). It was reported that during pregnancy, strict control of blood glucose can ameliorate potential poor health outcomes of the mothers and newborns during pregnancy (28). Individualized management, including exercise, dietary guidance and encouragement for physical activity, was suggested to improve glycemic control in patients with GDM (29). When lifestyle management fails to retain adequate glycemic levels, alternative therapeutic strategies, such as exercise and insulin therapy, may be used for GDM (7,8). Recently, a study demonstrated that pancreatic β cell dysfunction plays an important role in the pathogenesis of GDM (30). TRIAP1 is a small conserved protein containing 76 amino acids that is considered a regulator of cell death (19,20). However, the role of TRIAP1 in pancreatic β cells remains to be investigated. Therefore, the aim of the present study was to assess the regulatory effects of
TRIAP1 in GDM and its functions in pancreatic β cells. It was hypothesized that TRIAP1 played critical roles in regulating pancreatic β cell function.

In the present study, RT-qPCR and western blot assays were used to determine the levels of TRIAP1 in GDM, and the data revealed that TRIAP1 expression was downregulated in peripheral blood samples from patients with GDM compared with those obtained from control subjects. Since the present study focused on the investigation of TRIAP1 in GDM, a control group for patients with diabetes was not included in the current study (31). However, the number of samples of the present study is too small, and large sample needs to be included. Additionally, the association between clinicopathological characteristics of patients with GDM and TRIAP1 expression was not analyzed in the present study. These were the limitations of the present study and will be further studied in the future.

To further investigate the effects of TRIAP1 in GDM, TRIAP1-siRNA and control-siRNA were transfected into INS-1 cells. The transfection efficiency was confirmed by RT-qPCR and western blot assays, and the results indicated that TRIAP1-siRNA significantly downregulated the mRNA and protein expression levels of TRIAP1. Pancreatic β cells are a distinct population of cells that produce insulin in order to fulfill metabolic demands (32). The lack of adaptation of pancreatic β cells to peripheral insulin resistance may be the main cause of GDM (33,34). Therefore, ELISA was performed to evaluate insulin secretion and total insulin content in INS-1 cells under high and low glucose conditions. ELISA results revealed that TRIAP1-siRNA significantly reduced the total insulin content and insulin release in INS-1 cells. However, further confirm the results, observation of changes in cell and insulin secretion by setting different time gradients and concentration gradients in cell experiments is required, which will be investigated this in the future.

A previous study demonstrated that inhibition of TRIAP1 in RPMI8226 cells increased the proportion of apoptotic cells, increased the expression levels of APAF1 and caspase-9, and induced caspase-9 and caspase-3/7 activities (35). Therefore, the effects of TRIAP1 knockdown on the proliferation and apoptosis of INS-1 cells was investigated in the present study. In line with previous studies (19,35,36), the results indicated that TRIAP1-siRNA may lead to the induction of apoptosis and to the inhibition of INS-1 cell proliferation. The data revealed that TRIAP1-siRNA promoted apoptosis of pancreatic β cells. Furthermore, the results from the RT-qPCR and western blot assays demonstrated that TRIAP1-siRNA significantly increased the expression levels of the apoptosis-associated genes APAF1, caspase-3, caspase-7 and caspase9 in INS-1 cells.

Accordingly, TRIAP1 overexpression enhanced insulin secretion and total insulin content in INS-1 cells, as demonstrated by transfection of the cells with TRIAP1-plasmid. In
addition, TRIAP1 overexpression significantly inhibited the induction of INS-1 cell apoptosis and promoted the viability of INS-1 cells. Moreover, the results demonstrated the repressive effects of TRIAP1 overexpression on the expression levels of APAF1, caspase-3, caspase-7 and caspase-9 in INS-1 cells. The aforementioned findings demonstrated that TRIAP1 expression was downregulated in patients with GDM and that it may play an important role in pancreatic β cell function by regulating insulin secretion, as well as proliferation and apoptosis of pancreatic β cells.

Taken together, the present study revealed a novel role of TRIAP1 in GDM. This protein may serve as a potential target for the therapy of GDM. The present study revealed that TRIAP1 could regulate insulin secretion and the proliferation and apoptosis of pancreatic β cells. This indicated a protective role of TRIAP1 in GDM and suggested novel possibilities for the use of targeted therapy against this disease. Although previous studies have analyzed the predictive and diagnostic biomarkers for GDM (37,38), however, whether TRIAP1 plays a prognostic or diagnostic role in GDM needs further research, which will be performed in the future.

In conclusion, TRIAP1 increased the growth of pancreatic β cells and their ability to secrete insulin, which in turn resulted in a protective effect in GDM. Therefore, TRIAP1 may be considered a promising therapeutic target for GDM treatment.
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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

LXL contributed to the conception and design of the study, data acquisition, analysis and interpretation. KHY, FY, YX and LLC contributed to data acquisition and performed statistical analysis. JS contributed to data acquisition, data analysis and prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Obstetrics and Gynecology Hospital of Fudan University (Shanghai, China) and written informed consent was acquired from all participants prior to their enrollment.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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