

Forkhead box O1 promotes INS-1 cell apoptosis by reducing the expression of CD24

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Abstract. Type 2 diabetes seriously affects human health and burdens public health systems. Pancreatic β -cell apoptosis contributes to a reduction in β -cell mass, which is responsible for the occurrence of type 2 diabetes. However, the mechanism that underlies this effect remains unclear. In the present study, the role of forkhead box O1 (Foxo1) was investigated (which is a key regulatory factor in β -cell function) in the apoptotic behavior of β -cells and a potential underlying mechanism was determined. It was demonstrated that Foxo1 overexpression significantly reduced the proliferation of INS-1 cells and increased the apoptosis of INS-1 cells, in contrast to foxm1, foxp, foxa1, foxc and foxb1 overexpression. The present study aimed to investigate potential underlying mechanisms using bioinformatics, including Gene Set Enrichment Analysis, and biological experiments, including flow cytometry, cell counting kit-8, immunofluorescence, western blotting, reverse transcription-quantitative polymerase chain reaction analysis and lentiviral transfection. Further experiments conclusively showed that cluster of differentiation (CD)24 expression was significantly decreased when INS-1 cells were treated with Foxo1. Animal experiments showed high CD24 expression in the pancreatic islets of diabetic Goto-Kakizaki rats. Moreover, Gene Set Enrichment Analysis showed that CD24 expression was associated with the adaptive immune response

of β -cells. Finally, no significant differences in the proliferation and apoptosis of CD24 overexpressing INS-1 cells were observed after Foxo1 treatment. These results suggested that Foxo1 overexpression in β -cells was able to increase apoptosis by inhibiting CD24 expression. This study may provide an approach for the treatment and prevention of type 2 diabetes.

Introduction

Over the past three decades, the number of patients with diabetes mellitus has increased rapidly; 90% of these patients suffer from type 2 diabetes mellitus, rendering type 2 diabetes one of the most serious public health challenges worldwide (1). Type 2 diabetes mellitus is an endocrine system disease that results from β -cell dysfunction. β -cell dysfunction is characterized by the specific absence of the first phase of glucose-induced insulin secretion (2). Pancreatic β -cells are responsible for abnormal glucose metabolism due to defects in insulin secretion or due to a loss of β -cell mass resulting from cell death (3,4). Apoptosis constitutes the primary form of β -cell death (5-7); however, the underlying mechanisms remain unclear.

The transcription factor, forkhead box O1 (Foxo1), is a key regulator of pancreatic β -cell mass; however, the role of Foxo1 in the maintenance of β -cell function remains controversial (8). A previous study provided a mechanism linking glucose- and growth factor receptor-activated pathways to protect β -cells against oxidative damage via Foxo1 (9). Kitamura *et al* (10) reported that Foxo1 could inhibit the expression of the β -cell-specific transcription factor Pdx1 and that this led to the impairment of β -cell neogenesis, which should be responsible for a reduction in β -cell mass. Other studies have also reported that the suppression of Foxo1 expression reduces the expression of apoptotic markers and promotes β -cell survival in type 2 diabetes (9-12). However, further studies are required to determine the role of Foxo1 in β -cells.

Cluster of differentiation (CD)24 is a glycoprotein expressed in a wide variety of human malignancies, such as renal cell carcinoma, β -cell lymphoma, small cell and non-small cell lung carcinoma, epithelial ovarian cancer, and breast cancer (13-16). However, little is known regarding the correlation between CD24 expression and β -cell function.

The aim of the present study was two-fold, to determine whether Foxo1 could promote β -cell apoptosis and to examine

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Abbreviations: Foxo1, forkhead box O1; Foxm1, forkhead box M1; Foxp, forkhead box p2; Foxa1, forkhead box A1; Foxc, forkhead box C; Foxb1, forkhead box b1; CD24, cluster of differentiation 24; ZAP70, ζ -chain-associated protein kinase 70; PTAFR, platelet-activating factor receptor; TMEM14, transmembrane protein 14

Key words: diabetes, Foxo1, CD24, INS-1, apoptosis

the association between Foxo1 and CD24, and the effect of CD24 expression on β -cell function. The results of this study may provide a novel approach for the treatment and prevention of type 2 diabetes.

Materials and methods

Materials. RPMI-1640, HEPES, fetal bovine serum (FBS), L-glutamine, Lipofectamine 2000 transfection reagent, TRIzol reagent, a PureLink RNA Mini kit, and a High Capacity cDNA Reverse Transcription kit were obtained from Invitrogen, Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Sodium pyruvate, β -mercaptoethanol and Cell counting kit-8 were purchased from Sigma-Aldrich (St. Louis, MO, USA). pcDNA3-Foxo1, pcDNA3-Foxm1, pcDNA3-Foxp, pcDNA3-Foxa1, pcDNA3-Foxc and pcDNA3-Foxb1 were purchased from Fujian Funeng Co., Ltd. (Shanghai, China). Rat INS-1 pancreatic β -cells were obtained from the China Center for Type Culture Collection (Shanghai, China). An Apoptosis Detection kit was purchased from KeyGEN Biotech (Shanghai, China). Real-time PCR primers, which included primers against CD24, ZAP70, PTAFR, TMEM14 and SPOCK2, were custom-synthesized by Invitrogen, Thermo Fisher Scientific, Inc. Rabbit polyclonal anti-rat CD24 antibodies were purchased from Santa Cruz Biotechnology, Inc., Dallas, TX, USA (cat. no. sc-11406; dilution, 1:200) and mouse monoclonal anti-rat β -actin primary antibodies were purchased from Abcam, Cambridge, UK (cat. no. ab6276; dilution, 1:10,000). The secondary antibodies were mouse anti-rabbit IgG (dilution, 1:100; cat. no. 211-005-109) and rabbit anti-mouse IgG (dilution, 1:200; cat. no. 315-0005-003) horseradish peroxidase (HRP)-conjugated antibodies, which were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). The present study was performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (17) and the guidelines for animal experiments and associated activities by the ethics committee of Shanghai First Central Hospital, and was approved by the ethics committee of Shanghai First People's Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China).

INS-1 cell culture. Rat INS-1 pancreatic β -cells were cultured in RPMI-1640 medium containing 11 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES, 10% FBS, 2 mM glutamine, and 50 μ M β -mercaptoethanol at 37°C in a 5% CO₂ incubator. The medium was refreshed every 2 days.

INS-1 cell transfections. Prior to transfection, INS-1 cells were seeded in 6-well plates at a density of 2×10^6 cells/well until the cells grew to >80% confluence. Then, the cells were transfected with 2 μ g pcDNA3-Foxo1, pcDNA3-Foxm1, pcDNA3-Foxp, pcDNA3-Foxa1, pcDNA3-Foxc or pcDNA3-Foxb1 using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. Opti-MEM I (50 μ l; Invitrogen; Thermo Fisher Scientific, Inc.) containing 2 μ g plasmid was mixed with Opti-MEM I containing 2 μ l Lipofectamine 2000 and incubated for 20 min at room temperature. The medium was placed in a 6-well plate (100 μ l/well) and cultured at 37°C in a 5% CO₂ incubator for 72 h. pcDNA3 was used as a control. The

transfection medium was replaced with regular growth medium (RPMI-1640 medium with 11 mmol/l D-glucose supplemented with 10% FBS, 100 U/ml penicillin, 10 μ g/ml streptomycin, 10 mmol/l HEPES, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate and 50 μ mol/l β -mercaptoethanol) after 5 h, and the cells were observed at each indicated time point using an inverted fluorescence microscope (DMI 6000B; Leica Microsystems GmbH, Wetzlar, Germany).

Cell counting kit (CCK)-8 tests. INS-1 cells transfected with Foxo1 were seeded in 96-well plates at a density of 1×10^5 cells per well for 24 h. Then, 10, 20 or 40 mM Foxo1 was added, and the cells were continuously cultured for 24 or 72 h. Untreated cells were used as a negative control, and dimethyl sulfoxide-treated cells were used as a positive control. At the indicated times, 10 μ l CCK-8 (Sigma-Aldrich) was added, and the plates were incubated for 3 h. After this period, the absorbance was measured at 450 nm using a microplate reader (Multiskan® Spectrum; Thermo Fisher Scientific, Inc.).

Apoptosis assays. Apoptosis was analyzed using an Annexin V-FITC Apoptosis Detection kit (KeyGEN Biotech) according to the manufacturer's instructions. Foxo1-transfected INS-1 cells were cultured in 6-well plates at a density of 1×10^5 cells per well for 2 days. Adherent INS-1 cells at 80% confluence were passaged with 0.125% trypsin-0.02% EDTA (Invitrogen; Thermo Fisher Scientific, Inc.) and inoculated at a density of 2×10^4 in 24-well culture dishes in growth medium as described above. At the indicated times, the cells were digested with 0.25% trypsin and washed with pre-cooled phosphate-buffered saline (PBS; Gibco; Thermo Fisher Scientific, Inc.). Binding buffer (300 μ l) containing 5 μ l Annexin V-fluorescein isothiocyanate (FITC) was added and incubated at room temperature in the dark for 15 min. Subsequently, 5 μ l propidium iodide (PI) solution was added and incubated at room temperature in the dark for 15 min. The stained cells were measured and analyzed in a BD FACSCalibur™ (BD Biosciences, Franklin Lakes, NJ, USA) by CellQuest software (version 1.1; BD Biosciences).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Rat pancreases were obtained from male Goto-Kakizaki (GK) rats (n=8; age, 7 weeks; weight, 260-300 g) and Sprague Dawley rats (n=8; age, 7 weeks; weight, 260-300 g) serving as a control, which were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). These animals were maintained in a standard animal laboratory with free activity and free access to water and food. They were maintained in a temperature-controlled environment at 22-24°C, relative humidity of 40-60%, with a 12-h light/dark cycle. The rats were fasted for 8 h prior to surgery and were sacrificed by exsanguination under anesthesia with 40 mg/kg sodium pentobarbital (Sigma-Aldrich), and maximal efforts were made to minimize suffering. Rat islet cells were digested using collagenase P (Roche Diagnostics, Basel, Switzerland) and purified by Ficoll density separation with Eurocollins (Mediatech, Inc., Herndon, VA, USA) using a previously described method (18). The cells released were then resuspended in growth medium as described above. The pancreatic islet cells obtained from GK rats (age, 7 weeks)

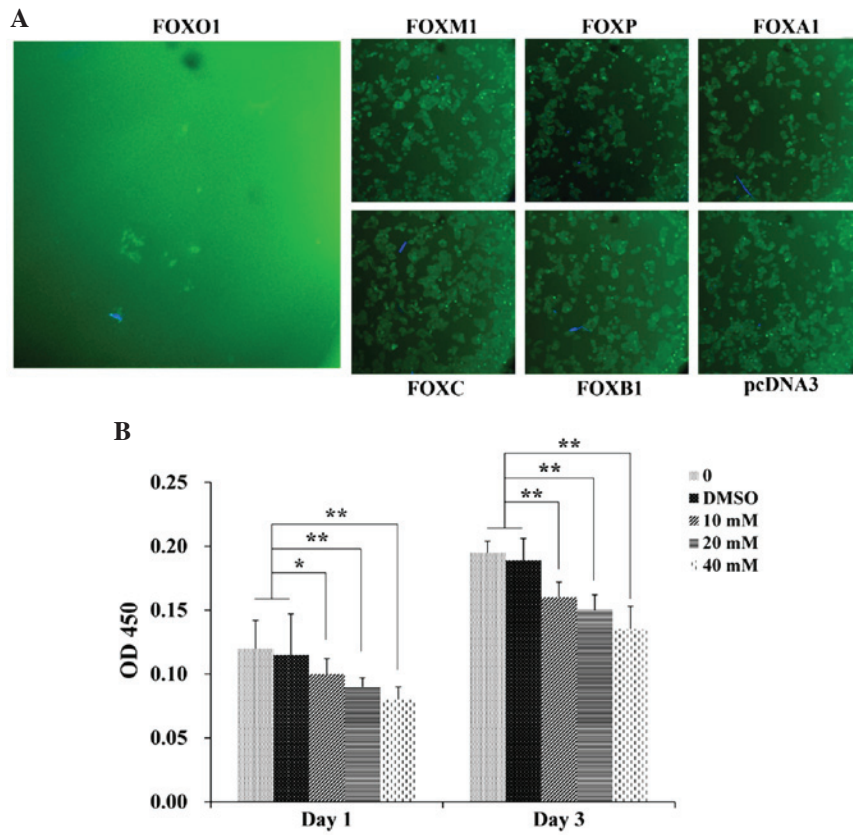


Figure 1. Foxo1 inhibits INS-1 cell proliferation. (A) INS-1 cell proliferation 3 days after treatment with Foxo1, foxm1, foxp, foxa1, foxc, or foxb1 using immunofluorescent assay. Magnification, x100. (B) Proliferation tests of INS-1 cells treated with 10, 20 or 40 mM Foxo1 on days 1 and 3. * $P < 0.05$, ** $P < 0.01$. DMSO, dimethyl sulfoxide; OD, optical density; fox, forkhead box.

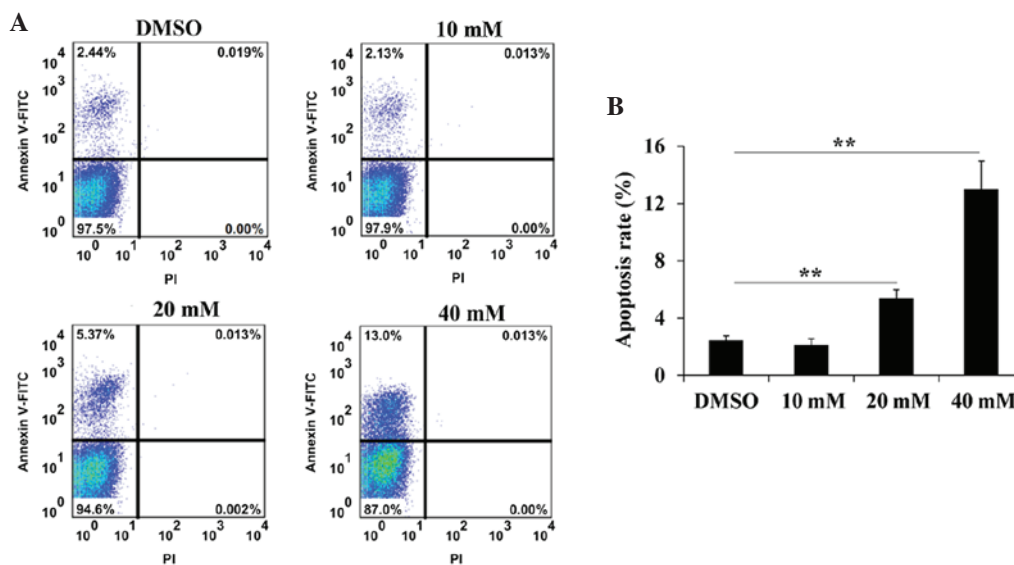


Figure 2. Foxo1 promotes INS-1 cell apoptosis by flow cytometry. (A) The apoptosis rate of INS-1 cells treated with 10, 20 or 40 mM Foxo1; the control group was treated with DMSO. (B) Statistical analysis of INS-1 cell apoptosis after treatment with different concentrations of Foxo1. Fox, forkhead box; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; PI, propidium iodide.

were ground to a powder with liquid nitrogen. Then, the powders of INS-1 cells that received different treatments were lysed using TRIzol reagent, and total RNA was purified using a PureLink RNA Mini kit. The purified RNA (0.5 μ g) was reverse-transcribed into cDNA using a High Capacity cDNA

Reverse Transcription kit at 42°C for 1 h, followed by 85°C for 5 sec. The cDNA produced was diluted 5-fold and used as the PCR template. PCR was performed with SYBR® Premix Ex Taq kit (containing No AmpErase UNG, 0.4 μ l primer mixture (each 10 μ M) and 7.6 μ l double distilled water) to detect CD24,

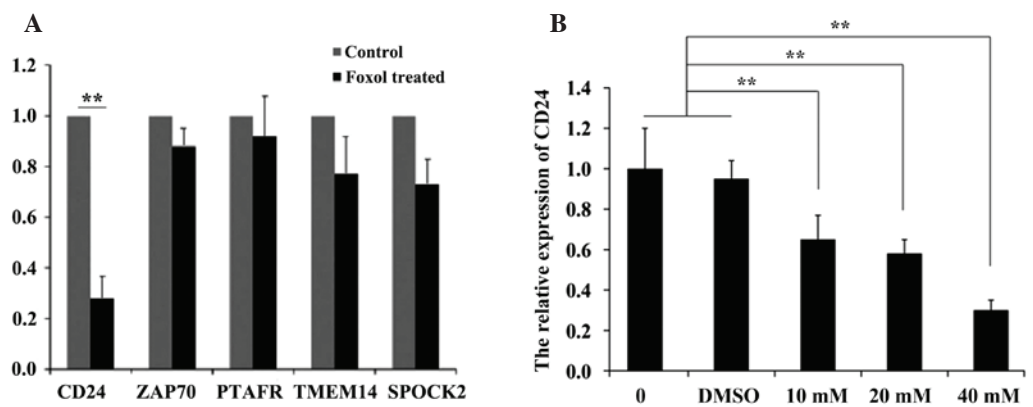


Figure 3. Foxo1 suppresses CD24 expression in INS-1 cells. (A) The expression analysis of five genes in INS-1 cells overexpressing Foxo1 as analyzed by reverse transcription-quantitative polymerase chain reaction. (B) CD24 expression in INS-1 cells following treatment with 10, 20 or 40 mM Foxo1. **P<0.01. FOX, forkhead box; CD, cluster of differentiation; ZAP70, ζ -chain-associated protein kinase 70; PTAFR, platelet-activating factor receptor; TMEM14, transmembrane protein 14; DMSO, dimethyl sulfoxide.

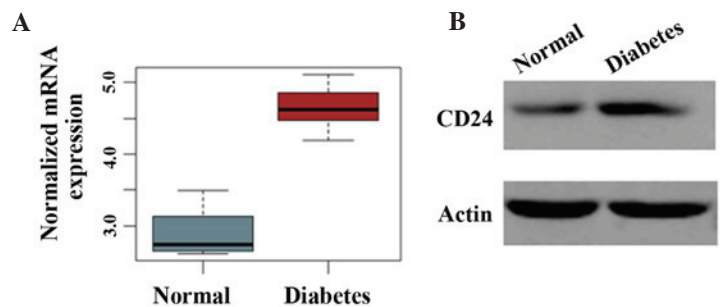


Figure 4. CD24 is highly expressed in the pancreatic islets of diabetic Goto-Kakizaki rats as analyzed by reverse transcription-quantitative polymerase chain reaction and western blotting. CD24 (A) mRNA and (B) protein expression in normal animals and in a diabetes model. CD, cluster of differentiation.

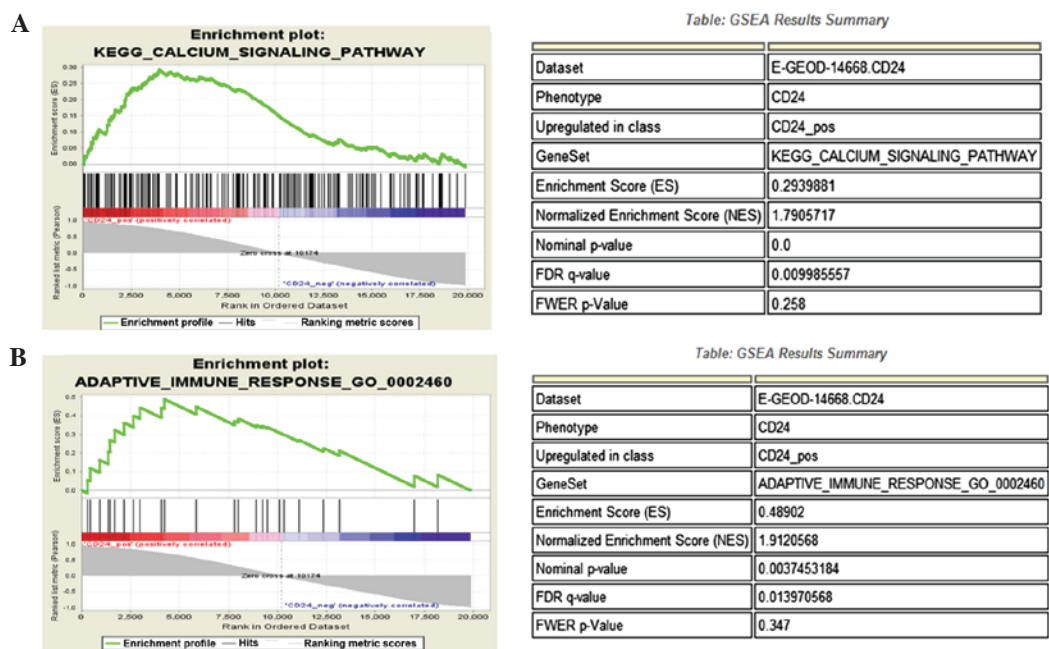


Figure 5. Bioinformatic analysis of the role of CD24 in β -cell function. (A) The role of CD24 in the calcium signaling pathway. (B) The role of CD24 in the adaptive immune response. CD, cluster of differentiation; GSEA, Gene Set Enrichment Analysis.

ζ -chain-associated protein kinase 70 (ZAP70), platelet-activating factor receptor (PTAFR), transmembrane protein 14 (TMEM14) and SPOCK2 mRNA as previous studies have shown these genes are associated with the proliferation and

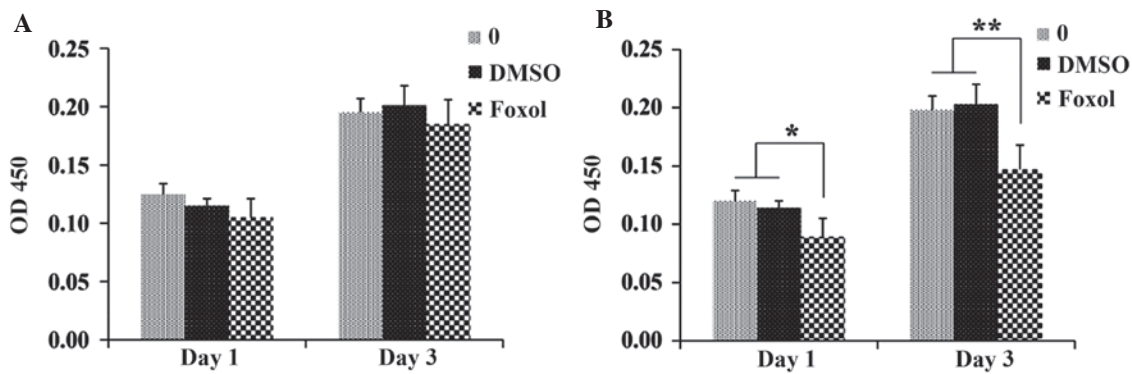


Figure 6. Overexpression of CD24 promotes INS-1 cell proliferation as assayed using a cell counting kit-8. (A) Proliferation tests of INS-1 cells treated with Foxol after the overexpression of CD24 on days 1 and 3. (B) The proliferation test of INS-1 cells treated with Foxol after the reverse transcription of control on days 1 and 3. *P<0.05, **P<0.01. CD, cluster of differentiation; fox, forkhead box; DMSO, dimethyl sulfoxide; OD, optical density.

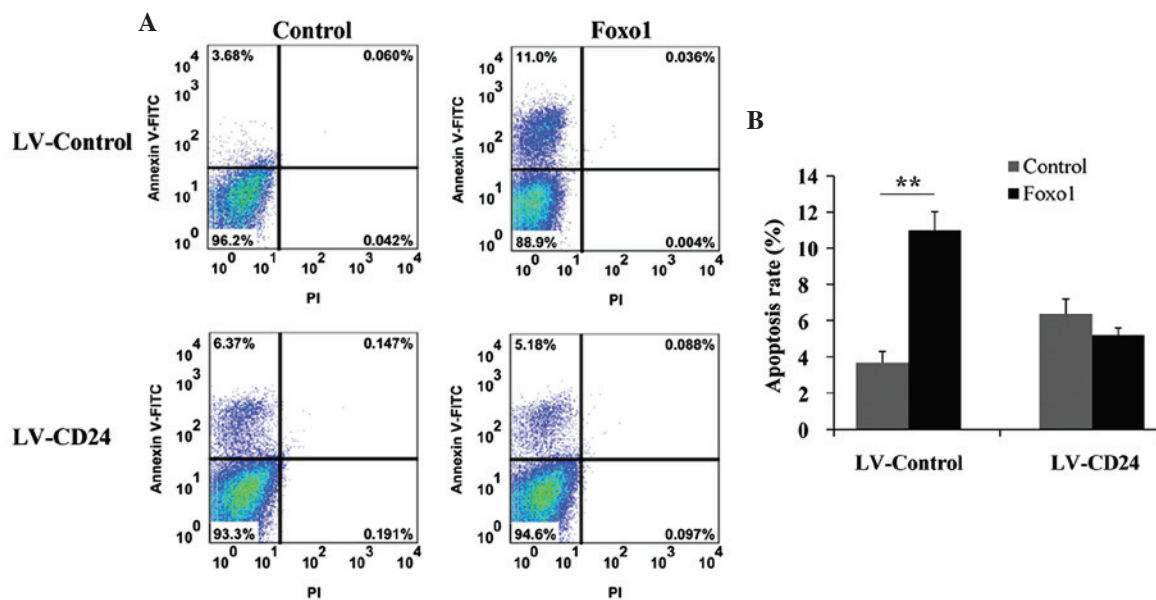


Figure 7. Overexpression of CD24 inhibits INS-1 cell apoptosis. (A) Flow cytometry showed the apoptosis rate of INS-1 cells treated with Foxol after the overexpression of either CD24 or control; DMSO treatment served as a control. (B) Quantitative analysis of apoptosis in INS-1 cells treated with Foxol after overexpression of CD24 or control. **P<0.01. CD, cluster of differentiation; fox, forkhead box; DMSO, dimethyl sulfoxide.

viability of tumor cells (19-23). Reactions were conducted on a DNA Engine Opticon 2 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The thermocycling conditions were as follows: Initiation, 95°C for 2 min; 40 cycles at 95°C for 15 sec and 60°C for 30 sec; and a melting curve stage of 60°C for 20 sec and 95°C for 10 sec. β -actin served as a control to normalize relative expression of the target genes which was calculated using the $2^{-\Delta\Delta C_q}$ method (24).

The primers used were as follows: 5'-TGCTCTACCCA CGCAGATT-3' (sense) and 5'-GGCCAACCCAGAGTT GGAA-3' (antisense) for CD24; 5'-GTTGACTCATCCTCA GAGACGAATC-3' (sense) and 5'-AGGTTATCGCGCTTC AGAA-3' (antisense) for ZAP70; 5'-GCTGCTCATTGG AGGGTGA-3' (sense) and 5'-TGTGTCTCTGTCTGGGTC CT-3' (antisense) for PTAFR; 5'-GATAGTCAGCCCGTACG-3' (sense) and 5'-CGCATCGCCTTATGCGAT-3' (antisense) for TMEM14; 5'-GAGACGAAGTGGAGGATGACTA-3' (sense) and 5'-CTTGCAGATGGAGTCTTTGTTT-3' (antisense) for SPOCK2; 5'-GACTCATCGTCGTA CTCTGCTTGCTG-3'

(sense) and 5'-GGAGATTACTGCCCTGGCTCCTA-3' (antisense) for β -actin.

Western blot analysis. Proteins were extracted from the pancreatic islets as previously described (11), concentration was determined using the Bradford assay and equal quantities of protein (20 μ g) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes and blocked in PBS with Tween 20 (Invitrogen; Thermo Fisher Scientific, Inc.; PBST) containing 5% non-fat milk. The membranes were then incubated with CD24 primary antibodies for 12 h at 4°C. The membranes were washed three times with PBST and incubated with HRP-conjugated secondary antibodies and washed a further three times with PBST. The protein bands were visualized using SuperSignal Pico ECL reagent (Pierce Biotechnology, Inc., Rockford, IL, USA). The membranes were reprobbed with anti- β -actin antibody as loading controls and immunocomplexes were detected with Amersham™ ECL

Prime Western Blotting Detection reagent (GE Healthcare Life Sciences, Chalfont, UK). The immunoblots were scanned and quantified using ImageTool 3.0 software (compdent.uthscsa.edu/dig/itdesc.html), the relative expression level of CD24 was normalized to β -actin expression.

Lentiviral vector carrying CD24 gene and infection. The lentiviral vector carrying the CD24 gene was purchased from Fujian Funeng Co., Ltd. (cat no. 20120810). The lentiviral system was used to produce INS-1 cells that stably overexpressed CD24. INS-1 cells were cultured in 24-well plates. When the cell confluence reached 90%, the lentiviral vector (20 MOI) carrying the CD24 gene was added, and the plates were incubated for the indicated time at 37°C in a 5% CO₂ incubator.

Role of CD24 in β -cells on Gene Set Enrichment Analysis (GSEA). The dataset (E-GEOD-14668.CD24) used in this study was downloaded from the Broad Institute website (<http://www.broadinstitute.org/gsea/index.jsp>), and the entire data set with expression values was uploaded to the GSEA software (25) to explore the role of CD24 in β -cells and interpret the enrichment results.

Statistical analysis. Data processing and statistical analysis were performed using SPSS 19 (IBM SPSS, Armonk, NY, USA). Data are presented as the mean \pm standard error of the mean. Data from two groups were compared using Student's t-test and continuous variables between several groups were compared with one-way analysis of variance. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Foxo1 inhibits INS-1 proliferation. INS-1 cells were transfected with Foxo1, foxm1, foxp, foxa1, foxc or foxb1 to explore which of these factors effectively inhibited proliferation; pcDNA3 treatment served as the control. In all of the six tested FOX family genes, only Foxo1 significantly downregulated the expression of CD24 and inhibited INS-1 cell proliferation ($P < 0.01$). As shown in Fig. 1, it was demonstrated that Foxo1 could effectively suppress the proliferation of INS-1 cells after 3 days of incubation, whereas the foxm1-, foxp-, foxa1-, foxc-, and foxb1-transfected cells did not show similar results. Moreover, the correlation between the concentration of Foxo1 and the proliferation rate of INS-1 cells was investigated as shown in Fig. 1B. The proliferation rate of INS-1 cells decreased with increases in the concentration of Foxo1 on day 1 and day 3, and the proliferation rate of INS-1 cells in the 10 mM Foxo1-treated group was significantly lower than those of the control and DMSO-treated groups on day 1 ($P < 0.05$) and day 3 ($P < 0.01$). A significant difference was also observed between the groups treated with 20 and 40 mM Foxo1 and the control and DMSO groups on day 1 ($P < 0.01$), and similar results were observed on day 3. These results suggested that Foxo1 was able to inhibit INS-1 cell proliferation.

Foxo1 promotes INS-1 cell apoptosis. It was demonstrated that Foxo1 was able to inhibit INS-1 cell proliferation, and whether Foxo1 can promote INS-1 cell apoptosis. As shown

in Fig. 2, the apoptosis rate of INS-1 cells increased with increasing concentrations of Foxo1. The apoptosis rate of INS-1 cells treated with 10 mM Foxo1 was lower than that of the DMSO-treated group; however, this difference was not statistically significant. The apoptosis rate of INS-1 cells treated with 20 and 40 mM Foxo1 was significantly higher than that of the DMSO-treated group ($P < 0.01$). These results revealed that Foxo1 could promote INS-1 cell apoptosis.

Foxo1 suppresses CD24 expression in INS-1 cells. PCR arrays were used to screen the key signaling molecules that participate in this process. Five genes (CD24, ZAP70, PTAFR, TMEM14 and SPOCK2) were selected for analysis, and it was demonstrated that CD24 expression significantly decreased in the Foxo1-treated group compared with that of the DMSO-treated group (Fig. 3A). However, there was no significant decrease in the relative expression of ZAP70, PTAFR, TMEM14 or SPOCK2 ($P = 0.88$, $P = 0.92$, $P = 0.77$ and $P = 0.73$, respectively). CD24 expression was also measured in INS-1 cells after treatment with different concentrations of Foxo1, as shown in Fig. 3B. A negative correlation was identified between the expression of CD24 and the concentration of Foxo1. CD24 expression in the 10, 20 and 40 mM Foxo1-treated groups was significantly lower than that of the control and DMSO-treated groups ($P < 0.01$). These results indicated that Foxo1 could inhibit CD24 expression.

CD24 is highly expressed in a diabetes model. Then, the expression level of CD24 was measured in the pancreatic islets of normal rats and of diabetic GK rats. As shown in Fig. 4, the expression levels of CD24 mRNA and protein were markedly higher in the diabetes model than in normal animals. This finding suggested that CD24 is highly expressed in the diabetic pancreas islet.

Bioinformatic analysis. Bioinformatic analysis indicated that CD24 participates in the calcium signaling pathway (Fig. 5A and B) and is involved in the adaptive immune response of β -cells (Fig. 5C and D).

Overexpression of CD24 promotes INS-1 cell proliferation. Having demonstrated that Foxo1 inhibits INS-1 cell proliferation by suppressing CD24 expression, further studies were performed to evaluate the role of CD24 in INS-1 cell proliferation by reverse-transcribing CD24. Following overexpression of CD24, no significant difference in cell proliferation was observed in the Foxo1-treated group compared with the control and DMSO-treated groups on days 1 and 3. Whereas the proliferation rate was lower in the LV-Control-treated group than in the control and DMSO-treated groups on day 1 ($P < 0.05$), a significant difference in cell proliferation was also observed in the LV-Control-treated group and in the control and DMSO-treated groups on day 3 ($P < 0.01$; Fig. 6). These results revealed that CD24 is important in INS-1 cell proliferation.

Overexpression of CD24 inhibits INS-1 cell apoptosis. The apoptosis rate of INS-1 cells overexpressing CD24 following treatment with Foxo1 was also determined. As shown in Fig. 7, the apoptosis rate in the Foxo1-treated group was significantly

higher than that of the control reverse-transcribed group on day 3 ($P<0.01$). However, no obvious difference in the apoptosis rate was observed between the Foxo1-treated group and the control group over-expressing CD24. These results indicated that CD24 is important in INS-1 cell apoptosis.

Discussion

The prevalence of diabetes mellitus is rapidly increasing worldwide. The global number of patients with diabetes mellitus is projected to rise to 439 million by 2030, and 90% of these patients will have type 2 diabetes mellitus (26). Excessive apoptosis of β -cells is the primary cause of type 2 diabetes; however, the mechanism underlying this apoptosis remains unclear. Foxo1 is a transcription factor that is a member of the FOX family. It is important in multiple biological processes including oxidative stress, apoptosis and cell cycle arrest. Furthermore, Foxo1 is a tumor suppressor, which is down-regulated in multiple types of tumor (27). Recent studies showed that the expression of the other five genes (CD24, ZAP70, PTAFR, TMEM14 and SPOCK2) are correlated with the proliferation and viability of tumor cells (19-23). A widely used cell line for islet β -cell function studies is the INS-1 cell line derived from the original radiation-induced tumor described by Chick *et al* (28). In the present study, a possible apoptotic mechanism in β -cells was identified, where Foxo1 overexpression promotes apoptosis by reducing CD24 expression. Therefore, this study demonstrated the important roles of Foxo1 and CD24 in β -cell apoptosis.

In the adult pancreas, Foxo1 is exclusively expressed in the islet β -cells (29). Foxo1 is a negative regulator of the transcription factor Pdx1, which is crucial in β -cell growth and function (30,31). Foxo1 inactivation leads to increased Pdx1 expression and β -cell proliferation (10). By contrast, Foxo1 activation promotes apoptosis in β -cells. Roy *et al* (32) reported that the suppression of the PI3K/AKT and MEK/ERK pathways activated foxo transcription factors, leading to cell cycle arrest and apoptosis in pancreatic cancer (32). Moreover, McLoughlin *et al* (33) found that Foxo1 enhances skeletal muscle atrophy by promoting skeletal muscle cell apoptosis via DNA binding-dependent and DNA binding-independent mechanisms. These results indicated that Foxo1 is key in the apoptosis of β -cells and of other cells. The results of the present study are consistent with this conclusion; Foxo1 overexpression promoted apoptosis in β -cells, and the inhibitory effects were enhanced with increasing Foxo1 concentrations.

CD24 is a glycoprotein that is expressed at the surface of the majority of β lymphocytes and is essential in the immune system (34). In INS-1 cells treated with Foxo1 following overexpression of the control, proliferation was significantly reduced on day 1 ($P<0.05$) and day 3 ($P<0.01$), and the apoptosis rate was significantly increased ($P<0.01$). However, no significant differences were observed following Foxo1 treatment of cells overexpressing CD24. This suggests overexpression of CD24 may block the effects of Foxo1 to promote INS-1 cell proliferation and inhibit apoptosis. The present results also confirmed this model and it was demonstrated that CD24 was associated with the adaptive immune response of β -cells. Furthermore, a previous study demonstrated that the

expression of genes involved in the final steps of insulin secretion is reduced in patients with type 2 diabetes (35). One of the final steps in insulin secretion is the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels, which triggers the exocytosis of insulin (36), and this calcium-triggered exocytosis results in the release of insulin from the secretory granules, which follows the Ca^{2+} influx through voltage-gated channels (37). Notably, it was demonstrated that CD24 was involved in the calcium signaling pathway, where this protein may regulate β -cell function.

In conclusion, in the present study, it was demonstrated that Foxo1 overexpression was able to promote β -cell apoptosis by decreasing CD24 expression, and the role of CD24 in β -cell function was preliminarily discussed. CD24 is involved in the calcium signaling pathway and in the adaptive immune response of β -cells. However, additional studies are required to clarify the role of CD24 in β -cell function.

Acknowledgements

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