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Oleic acid increases the transcriptional activity of FoxO1 by promoting its nuclear translocation and β-catenin binding in pancreatic β-cells

Magdalena Jazurek-Ciesiolka[a,1,2], Justyna Janikiewicz[a,2], Pawel Dobrzyn[b], Anna Dziewulska[a], Kamil Kozinski[a], Agnieszka Dobrzyn[a,*]

[a] Laboratory of Cell Signaling and Metabolic Disorders, Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland
[b] Laboratory of Medical Molecular Biochemistry, Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland

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ABSTRACT

In the setting of metabolic overload, chronic elevations of free fatty acids in blood and tissues are associated with pancreatic β-cell lipotoxicity and failure. Ultimately, obesity combined with insulin resistance increases the dysfunctional demand of β-cells and contributes to the development of type 2 diabetes. Forkhead box O1 (FoxO1) is a potent transcriptional regulator of pancreatic β-cell function and tolerance to lipid stress. The present study examined the effects of stearoyl-CoA desaturase 1 (SCD1)-metabolized precursors and products, notably oleic acid, on the compensatory capacity of β-cells and their relationship with regulation of the FoxO1 and Wnt pathways. The trioleate-induced compromise of insulin sensitivity blunted the compensatory response of pancreatic β-cells in primary rat islets. These events were associated with increases in the nuclear accumulation and transcriptional activity of FoxO1. Such effects were also observed in INS-1E cells that were subjected to oleate treatment. The overexpression of human SCD1 that was accompanied by endogenously generated oleic acid also led to an increase in the nuclear abundance of FoxO1. The mechanism of the oleate-mediated subcellular localization of FoxO1 was independent of the fatty acid receptor GPR40. Instead, the mechanism involved diversion of the active β-catenin pool from an interaction with transcription factor 7-like 2 toward FoxO1-mediated transcription in β-cells. Our findings identify a unique role for oleic acid in the compensatory response of pancreatic β-cells and emphasize the importance of FoxO1 in β-cell failure in obesity-induced insulin resistance.

1. Introduction

Obesity is a major risk factor for the development of peripheral insulin resistance and impairments in pancreatic β-cell function [1]. Pancreatic β-cells develop an adaptive mechanism to compensate for high metabolic challenge through the expansion of β-cell mass and increase in insulin secretion. When periods of accelerated fat deposition in islets are sustained, the insulin demand of peripheral tissues overwhelms the secretory capacity of the pancreatic β-cell population, and insufficient compensation can lead to type 2 diabetes (T2D) [2,3]. The molecular regulators of β-cell adaptation remain incompletely defined. Increases in circulating levels of plasma fatty acids (FAs) and disturbances in lipid metabolism suggest that free FAs are plausible regulators [3,4]. The effects of lipids on pancreatic β-cell function depend on both the time of deposition and level of FA desaturation [5]. Chronic exposure to FAs results in a reduction of β-cell mass, blunts insulin biosynthesis and secretion, and leads to β-cell loss [4]. Saturated FAs (SFAs) were found to have more severe effects on the insulin secretory capacity and survival of pancreatic β-cells than monounsaturated fatty acids (MUFAs) [6]. Stearoyl-CoA desaturase 1 (SCD1) is the rate-limiting enzyme that catalyzes the biosynthesis of MUFAs by introducing a *cis*-double bond to a fatty-acyl CoA. The preferred desaturation

Abbreviations: BrdU, 5-bromo-2-deoxyuridine; FAs, fatty acids; FoxO1, Forkhead box O1; GPR40, G-protein-coupled receptor 40; GSIS, glucose-stimulated insulin secretion; GSK3β, glycogen synthase kinase-3β; HF, high-fat; JNK, c-Jun N-terminal kinase; MUFAs, monounsaturated fatty acids; Pdx1, pancreatic and duodenal homeobox 1; SCD1, stearoyl-CoA desaturase 1; SFAs, saturated fatty acids; T2D, type 2 diabetes; TCF7L2, transcription factor 7-like 2; TO, trioleate; TS, tristearate; TUG761, G-protein-coupled receptor 40 antagonist

* Corresponding author at: Laboratory of Cell Signaling and Metabolic Disorders, Nencki Institute of Experimental Biology, 3 Pasteur St., 02-093 Warsaw, Poland.
E-mail address: a.dobrzyn@nencki.gov.pl (A. Dobrzyn).
[1] Present address. Department of Molecular Biomedicine, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland.
[2] These authors contributed equally to this work.

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substrates are palmitic acid (16:0) and stearic acid (18:0), which are converted to palmitoleate (16:1n-7) and oleate (18:1n-9), respectively [7]. The resulting MUFAs constitute major structural components of cellular membranes and lipid signaling molecules [8]. Increases in SCD1 activity were reported to protect common experimental β-cell models from lipotoxicity and improve their secretory function [9–11]. Defects in glucose-stimulated insulin secretion (GSIS) and decline in cell survival were reported in numerous pancreatic β-cell lines as well as rodent and human islets under oleate exposure [12–14]. Conversely the protective effect of oleate against palmitate-induced toxicity on pancreatic β-cells was linked to the activation of the pro-survival ER stress response [15]. In spite of that, little is known about the exclusive role of oleic acid in the management of the pancreatic β-cells adaptation.

FoxO1 is a member of the forkhead box O family and potent repressor and activator of the transcription of target genes through its forkhead DNA-binding domain. Multiple biological processes in pancreatic β-cells, including proliferation, the stress response, differentiation, and apoptosis, are under the control of FoxO1 [16]. The expression profile of FoxO1 in islets closely parallels the expression profile of pancreatic and duodenal homeobox 1 (Pdx1) during pancreas development [17]. In the mature pancreas, FoxO1 protein is localized predominantly in the cytoplasm of β-cells because of continuous insulin production that activates Akt signaling [18]. Under conditions of normoglycemia, the subcellular localization and transcriptional activity of FoxO1 are regulated primarily by Akt phosphorylation at three conserved serine/threonine residues, leading to the nuclear exclusion and subsequent degradation of FoxO1 [19,20]. The exposure of pancreatic β-cells to oxidative stress or elevations of glucose concentrations led to the nuclear redistribution of FoxO1 in βTC-3 cells and pancreatic islets [21,22]. Conversely, the transcriptional activity of FoxO1 was positively regulated by stress-activated c-Jun N-terminal kinase (JNK) via the promotion of its import into the nucleus [14,23].

Pancreas-specific transgenic mice that expressed constitutively active FoxO1 exhibited impairments in glucose tolerance and insulin secretion, a marked decrease in β-cells, abnormal islet architecture, and β-cell compensation [21,24,25]. Intriguingly, the overexpression of kinase-negative protein kinase Cδ in β-cells protected mice from high-fat (HF) diet-induced β-cell failure through a mechanism that involved the inhibition of FoxO1 protein [26]. Pancreas-specific *FoxO1* knockout mice were characterized by an increase in β-cell mass, mild improvements in glucose tolerance, and a high abundance of pancreatic duct cells, indicating an acceleration of β-cell neogenesis [27].

Abnormalities in Wnt signaling were also attributed to pancreatic β-cell dysfunction and the development of T2D [28]. We recently showed that the activation of Wnt signaling in a pre-diabetic state was correlated with the adaptation of β-cells to systemic insulin resistance, whereas a lack of Wnt activators in systemic circulation during the progression of T2D was observed in parallel with dysfunctional pancreatic islets [29]. The acylation of Wnt proteins with SFAs and MUFAs is required for both Wnt secretion and signaling [30,31]. Interestingly, SCD1 is necessary to produce active, lipid-modified Wnt proteins [32]. Although the critical role of FoxO1 in pancreatic β-cell function has been established, its crosstalk with Wnt signaling has not yet been clarified.

FoxO1 serves as an important regulator of the compensatory β-cell response to insulin resistance [12], and FoxO1 mediates the FA-induced dysfunction of pancreatic β-cells [14,33,34]. In the present study, we investigated whether metabolic precursors and products of SCD1 activity affect β-cell adaptation and FoxO1-dependent transcription status. We found that trioleate-derived insulin resistance was not followed by a compensatory response of pancreatic β-cells. This phenomenon was associated with an increase in the nuclear accumulation of FoxO1 and sequestration of the active β-catenin pool from an interaction with transcription factor 7-like 2 (TCF7L2) toward FoxO1-mediated transcription. Altogether, our data underscore the importance of oleate in the shift of the transcriptional balance between FoxO1 and Wnt/β-catenin signaling to orchestrate the compensatory response of pancreatic β-cells, with the aim of preserving their function under conditions of obesity-related insulin resistance.

2. Materials and methods

2.1. Animals

Male Wistar rats, 6 weeks of age ($n = 8$), were fed ad libitum standard laboratory chow, a HF diet (Ssnif diet D12492, E15742-34, fat content: 60 Kcal%), a tristearate (TS) diet (tristearin content: 20 Kcal %), or a trioleate (TO) diet (triolein content: 20 Kcal%) for 12 weeks (Ssniff EF, Soest, Germany). Food consumption was monitored daily, and body weight was recorded once per week. Intraperitoneal glucose tolerance tests (2 g/kg body weight) were performed after overnight fasting. Glucose levels were measured at the indicated time points using glucose strips with an Optium Xido glucose meter. At the end of each diet, the rats were anesthetized with pentobarbital and sacrificed by cervical dislocation. Blood was collected aseptically by direct cardiac puncture and centrifuged at 13,000 $\times g$ for 5 min at 4 °C to collect plasma. Fasting plasma insulin levels were measured using a rat/mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Millipore, Billerica, MA, USA). All of the animal procedures were approved by the First Local Ethical Committee for Animal Experiments in Warsaw.

2.2. Materials

Primary antibodies against AKT, phosphorylated AKT (pAKT; Ser473), pFoxO1 (Ser256), and insulin were obtained from Cell Signaling Technology (Hertfordshire, UK). Peroxidase-conjugated β-actin and β-catenin antibodies were purchased from Sigma (St. Louis, MO, USA). Antibodies against the active form of β-catenin and against FoxO1 were obtained from Millipore (Billerica, MA, USA). TCF7L2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). FITC-conjugated anti-5-bromo-2-deoxyuridine (BrdU) antibody was obtained from BD Biosciences (San Jose, CA, USA). Fetal bovine serum (FBS), tetramethylrhodamine-labeled anti-rabbit secondary antibody, DAPI reagent, and pcDNA3 empty vector were obtained from Life Technologies (Carlsbad, CA, USA). The pTOP-flash-luciferase reporter plasmid that contained multiple copies of an optimal TCF-binding site and mutated pFOP-flash plasmid were a gift from Randall Moon (Addgene, Cambridge, MA, USA). The pRL-CMV-luciferase plasmid was obtained from Promega (Madison, WI, USA). The other chemicals were purchased from Sigma unless otherwise specified. The pMafA construct that contained the mouse MafA promoter was kindly provided by Prof. T. Kitamura (Gunma University, Japan), The pcDNA3 vector that carried human SCD1 was a generous gift from Prof. J.M. Ntambi (University of Wisconsin, Madison, WI, USA). The GPR40 receptor antagonist TUG761 was provided by Prof. Evi Kostenis (University of Bonn, Germany).

2.3. 2-Deoxyglucose uptake in rats

After overnight fasting, the rats were injected with 3 μCi 2-deoxy-D-[^{3}H]glucose and 0.5 μCi [^{14}C]mannitol (Perkin Elmer, Waltham, MA, USA) per 100 g body weight through the femoral vein with or without 0.075 U/g body weight of insulin. The visceral adipose tissue was collected 25 min later. The tissue samples were digested with 1 M NaOH, followed by neutralization with 1 M HCl and the addition of scintillation cocktail. 2-Deoxyglucose uptake was calculated as the difference between the radioactivity of total adipose tissue and radioactivity of the adipose tissue extracellular space. The glucose uptake values were normalized per gram of the protein.

2.4. Islet isolation and insulin secretion assay

The pancreas was collected for islet isolation as described previously [29]. Briefly, 7 ml of ice-cold 1.4 mg/ml collagenase solution was introduced into the pancreas via intraductal infusion. The perfused pancreas was removed, transferred to a 50-ml conical tube that was prefilled with 8 ml of cold collagenase enzyme solution, and incubated for 20–30 min at 37 °C. The islets were then purified by Histopaque-1077 density centrifugation. Prior to the experiments, the islets rested overnight in RPMI-1640 medium that was supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C under a 95% air and 5% CO_2 atmosphere. After resting, the islets were hand-picked and incubated in Krebs Ringer bicarbonate secretion buffer (25 mM HEPES, 114 mM NaCl, 23 mM $NaHCO_3$, 5 mM KCl, 2 mM $CaCl_2$•$2H_2O$, 1.2 mM $MgSO_4$, and 0.1% bovine serum albumin [BSA]) that contained 2.75 or 16.5 mM glucose at 37 °C for 45 min. Aliquots of the incubation media were collected to determine insulin concentrations using a rat/mouse insulin ELISA kit (Millipore) according to the manufacturer's instructions.

2.5. Pancreatic islet immunohistochemistry

Isolated pancreatic islets were fixed in Bouin's solution for 2 h and maintained in 4% paraformaldehyde. The tissue was then embedded in paraffin and cut into 5 μm sections. For histological analysis, the sections were subjected to hematoxylin and eosin staining. Indirect immunofluorescence was detected using rabbit anti-insulin and rabbit anti-FoxO1 antibodies. To assess pancreatic β-cell proliferation, the measurement of BrdU incorporation into cellular DNA was evaluated by incubating the islets with 10 μM BrdU for 18 h. Direct immunofluorescence was detected using mouse FITC-conjugated anti-BrdU antibody. The nuclei were stained with TO-PRO-3 reagent (Invitrogen, Carlsbad, CA, USA) prior to visualization under a Leica TCS SP5 spectral confocal microscope.

2.6. INS-1E cell culture and chemical treatment

Rat insulin-producing INS-1E cells (donated by Prof. Pierre Maechler, University of Geneva, Switzerland) were cultured in complete RPMI-1640 that was supplemented with heat-inactivated 5% fetal bovine serum, 10 mM HEPES, 50 μM 2-mercaptoethanol, 2 mM glutamine, 1 mM sodium pyruvate, 100 IU penicillin/ml, and 100 μg streptomycin/ml until 80–90% confluence at 37 °C with 5% CO_2. All of the experiments were performed between passages 72 and 110. To analyze the effects of FAs on FoxO1 regulation, INS-1E cells were exposed to 0.4 mM albumin-bound sodium salts of palmitate, stearate, or oleate for 6 h. For control samples, corresponding concentrations of albumin were used. Alternatively, to investigate the role of GPR40 receptors in the oleate-induced activation of FoxO1, INS-1E cells were pre-incubated for 1 h with the specific GPR40 receptor antagonist TUG761 [35] and then co-supplemented with 0.4 mM oleic acid. To determine the role of β-catenin in the oleate-induced activation of FoxO1, INS-1E cells were incubated with the glycogen synthase kinase-3β (GSK3β) inhibitor lithium chloride (LiCl) [36] for 8 h. Oleic acid was added for the last 6 h prior to sample collection. Additional experiments were performed by transfecting 4 μg of empty pcDNA3 vector or pcDNA3 that encoded human SCD1 with Lipofectamine 2000 (Invitrogen) for 6 h according to the manufacturer's instructions.

2.7. Luciferase reporter assays

INS1-E cells were transiently transfected with 1 μg pTOP-flash (TCF reporter gene plasmid) or 1 μg pFOP-flash (mutated plasmid). Alternatively, for MafA transcriptional activity, a firefly luciferase reporter construct that contained the mouse MafA promoter was introduced to the cells. All of the reporter plasmids were transfected in combination with 25 ng pRL-CMV using Lipofectamine 2000 as described above. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity is expressed as relative light units, measured as pMafA or the quotient of pTOP-flash and pFOP-flash values, normalized to renilla luciferase luminescence.

2.8. INS-1E immunostaining

INS-1E cells were grown on 0.001% poly-L-ornithine-precoated coverslips and fixed in 4% paraformaldehyde for 15 min. After fixation, the cells were permeabilized with 0.1% Triton-X-100 and then blocked with 1% BSA before labeling with anti-FoxO1 antibody. Nuclei were stained with DAPI (Invitrogen, Carlsbad, CA, USA), and images were acquired using a Leica TCS SP5 confocal microscope.

2.9. Western blot analysis

INS-1E cells and pancreatic islets were solubilized in ice-cold lysis buffer that contained 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 1% Triton-X-100, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM $NaWO_4$, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, and 1.4 μg/ml aprotinine. Cytoplasmic and nuclear fractions of INS-1E cells were prepared with NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The purities of cytoplasmic and nuclear fractions were confirmed by immunostaining with anti-GAPDH and anti-PARP antibodies, respectively. Protein concentrations were determined using the Bio-Rad protein assay (Hercules, CA, USA) with BSA as the standard. Clarified protein extracts (20 μg) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore), which were blocked with 5% skim milk and probed with appropriate antibodies. Protein levels of Pdx1, β-catenin, TCF4, FoxO1, and Akt (Santa Cruz Biotechnology) and the extent of phosphorylation of Akt (Ser473) and FoxO1 (Ser256) were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore) as recommended by the manufacturer.

2.10. Co-immunoprecipitation

INS-1E cells were lysed in precipitation lysis buffer (20 mM Tris [pH 8.0], 75 mM NaCl, 15 mM $MgCl_2$, 1 mM EDTA, 0.5% Nonidet P-40, 10% glycerol, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, and 1.4 μg/ml aprotinine). Precleared lysates that contained 500 μg protein were agitated overnight at 4 °C with 1 μl of anti-active-β-catenin and 20 μl of Protein A/G PLUS-agarose beads (Santa Cruz Biotechnology). The immunoprecipitates were washed with lysis buffer, resuspended in 1× sample buffer, and boiled for 5 min at 95 °C. The samples then underwent SDS-PAGE for Western blotting with anti-FoxO1 and anti-TCF7L2 antibodies.

2.11. Gene expression analysis

Total cellular RNA was prepared from INS-1E cells and pancreatic islets using TRI reagent (Sigma). DNase-treated RNA was reverse transcribed with the RevertAid H Minus First Stand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using an Applied Biosystems 7500 Fast Instrument with SYBR green (Thermo Scientific). The quantification of gene expression was normalized to β-actin or GAPDH using the $\Delta\Delta C_t$ method. Specific primer sequences are presented in Supplementary Table 1.

2.12. Statistical analysis

The data are expressed as mean ± SD. Statistical comparisons were

performed using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls post hoc test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Insulin resistance that was induced by the TO diet was not followed by pancreatic islet adaptation

Lipotoxicity is a well-known contributor to systemic insulin resistance, dysfunctional pancreatic islet adaptation, and subsequent β-cell failure [3]. To induce insulin resistance, the rats were fed a HF diet, high-TO diet, or high-TS diet for 12 weeks. In response to glucose challenge, HF- and TO-fed rats exhibited a significantly impaired glycemic profile compared with their normal chow-fed littermates (Fig. 1A). Interestingly, TS-fed rats maintained normal glucose tolerance (Fig. 1A). Moreover, HF- and TO-fed animals developed weight gain and an increase in adiposity (Supplementary Fig. 1A and B). This effect was followed by alterations of insulin-stimulated 2-deoxyglucose uptake in adipose tissue (Fig. 1B). Control littermates and TS-fed rats exhibited a > 5-fold higher increase in glucose uptake, whereas HF-fed animals exhibited a > 4-fold decrease in glucose uptake. No insulin-

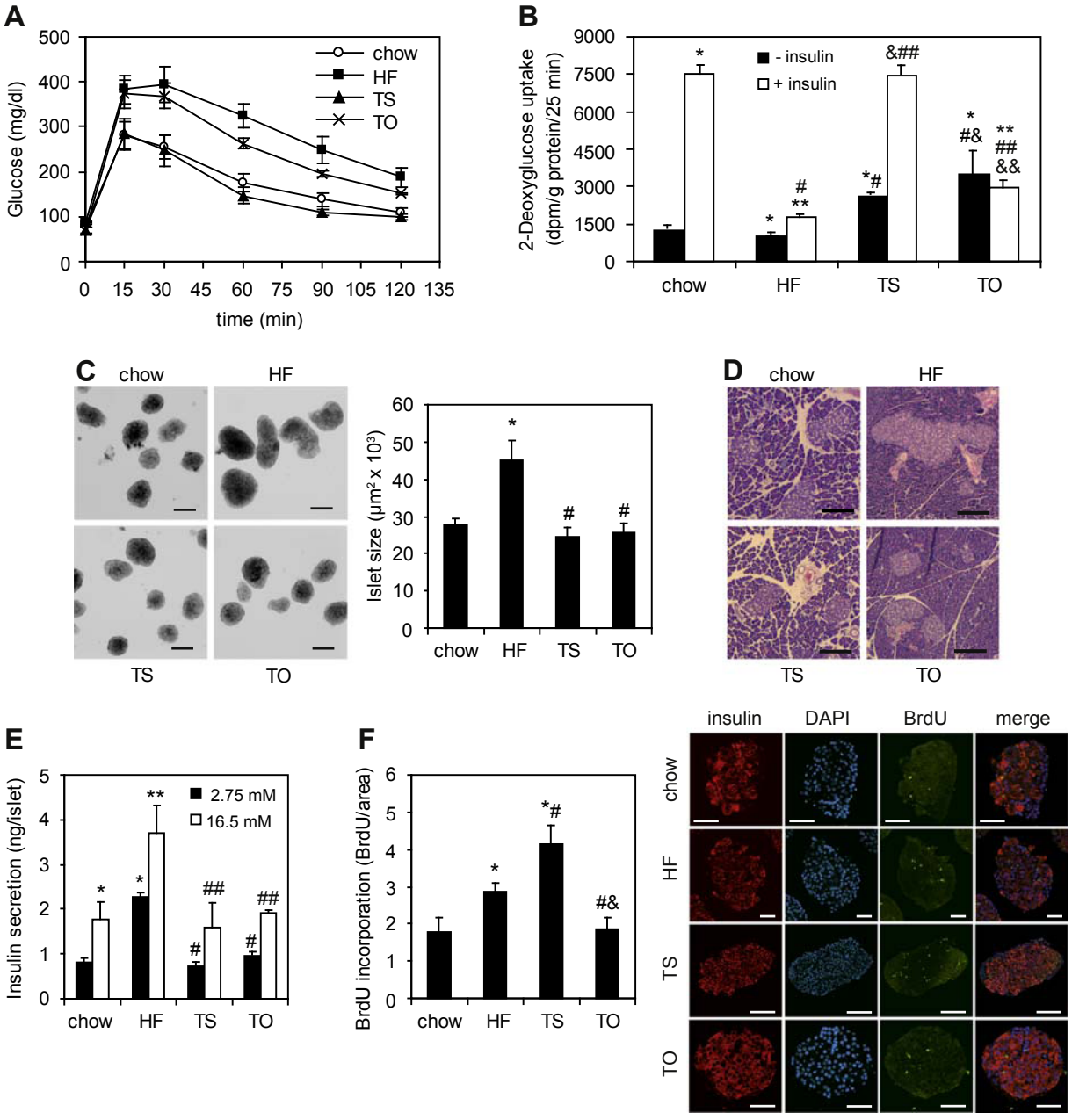

Fig. 1. Effect of dietary-delivered fatty acids on glucose metabolism and β-cell adaptation. The tests were performed in male rats that were fed a normal chow diet, high-fat (HF) diet, tristearate (TS) diet, and trioleate (TO) diet and islets that were isolated from these animals ($n = 8$/group). (A) Glucose tolerance test. The rats received a glucose injection after overnight fasting. (B) Basal and insulin-stimulated 2-deoxy-D-glucose uptake in adipose tissue. The data are expressed as mean ± SD. $^{*}p < 0.05$, vs. chow -insulin; $^{\#}p < 0.05$, vs. HF–insulin, $^{\&}p < 0.05$, vs. TS –insulin, $^{**}p < 0.05$, vs. chow + insulin; $^{\#\#}p < 0.05$, vs. HF + insulin, $^{\&\&}p < 0.05$, vs. TS + insulin. (C) Islet morphology and size. Scale bars = 20 μM. The data are expressed as mean ± SD. $^{*}p < 0.05$, vs. chow; $^{\#}p < 0.05$, vs. HF. (D) Hematoxylin and eosin staining. Scale bars = 200 μM. (E) Glucose-stimulated insulin secretion. The data are expressed as mean ± SD. $^{*}p < 0.05$, vs. 2.75 mM chow; $^{\#}p < 0.05$, vs. 2.75 mM HF, $^{**}p < 0.05$, vs. 16.5 mM chow; $^{\#\#}p < 0.05$, vs. 16.5 mM HF. (F) Incorporation of 5-bromo-2-deoxyuridine (BrdU) in islets. The data are expressed as mean ± SD. $^{*}p < 0.05$, vs. chow; $^{\#}p < 0.05$, vs. HF; $^{\&}p < 0.05$, vs. TS. Representative images are presented (scale bar = 10 μm).

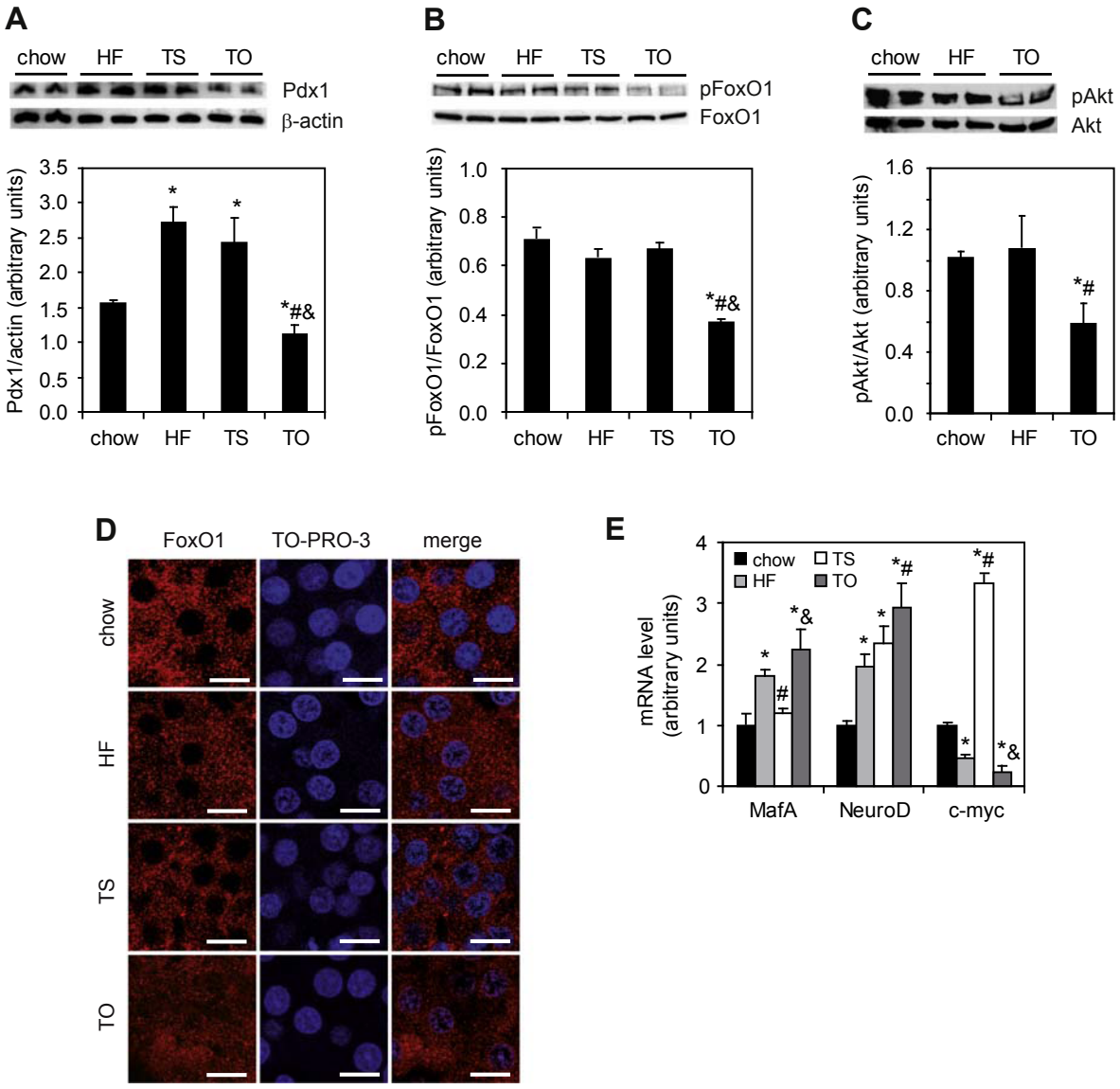

Fig. 2. Trioleate diet alters effectors of β-cell identity and increases the nuclear accumulation and activity of FoxO1. (A) Pdx1 protein, (B) FoxO1 protein and phosphorylation (Ser256), and (C) Akt protein and phosphorylation (Ser473) levels were assessed by Western blot. The data are expressed as mean ± SD (male, $n = 8$/group). $^{*}p < 0.05$, vs. chow; $^{\#}p < 0.05$, vs. HF; $^{\&}p < 0.05$, vs. TS. (D) Immunofluorescence staining of FoxO1 in pancreatic islet sections from rats that were fed a chow diet, high-fat (HF) diet, tristearate (TS) diet, and trioleate (TO) diet. Scale bars = 20 μM. (E) *MafA*, *NeuroD*, and *c-myc* mRNA levels in islets were determined by real-time RT-PCR. The data are expressed as mean ± SD (male, $n = 8$/group); $^{*}p < 0.05$, vs. chow; $^{\#}p < 0.05$, vs. HF; $^{\&}p < 0.05$, vs. TS.

stimulated increase in 2-deoxyglucose uptake was observed in the TO-fed group (Fig. 1B). These results indicate that the HF and TO diets led to a decrease in insulin sensitivity.

To maintain normoglycemia during metabolic challenge, pancreatic β-cells undergo an adaptation process that involves an increase in islet size, the expansion of β-cell mass, and the hypersecretion of insulin [1]. To examine whether changes in insulin sensitivity in HF- and TO-fed animals accounted for alterations of β-cell function and architecture, we morphometrically analyzed isolated islets. The size and mass of pancreatic islets significantly increased in HF-fed rats, with no changes in TO-fed rats (Fig. 1C, D). Only islets from HF-fed animals exhibited significantly higher GSIS and alterations of basal insulin secretion (Fig. 1E), which were consistent with their higher plasma insulin levels (Supplementary Fig. 1C) and elevation of total pancreatic insulin content (Supplementary Fig. 1D). We then performed an in vitro BrdU-labeling assay to determine the rate of β-cell proliferation (Fig. 1F). Unsurprisingly, islets that were derived from HF-fed rats had a significant 2-fold higher occurrence of BrdU and insulin double-positive cells compared with islets from normal chow-fed littermates. The proliferation rate was unaffected by the TO diet. Although significant insulin resistance was observed in the TO-fed group, the size and mass of islets, insulin secretion (Fig. 1C, D, E), plasma insulin levels, and the rate of β-cell proliferation (Supplementary Fig. 1C and Fig. 1F) were significantly lower than in HF-fed animals, but no differences compared with normal chow-fed littermates were observed. Moreover, dysfunction of β-cells after TO feeding was not related to the process of apoptosis (data not shown). Altogether, these data suggest that TO diet-induced insulin resistance was not followed by the compensatory adaptation of pancreatic β-cells.

3.2. Trioleate diet increases FoxO1 activity and its nuclear accumulation in pancreatic islets

Pdx1 is a crucial determinant of β-cell identity, function, and survival, thus playing an important role in islet adaptation to systemic insulin resistance [37,38]. We found that the TO diet resulted in the collapse of β-cell adaptation. We thus further investigated the level of

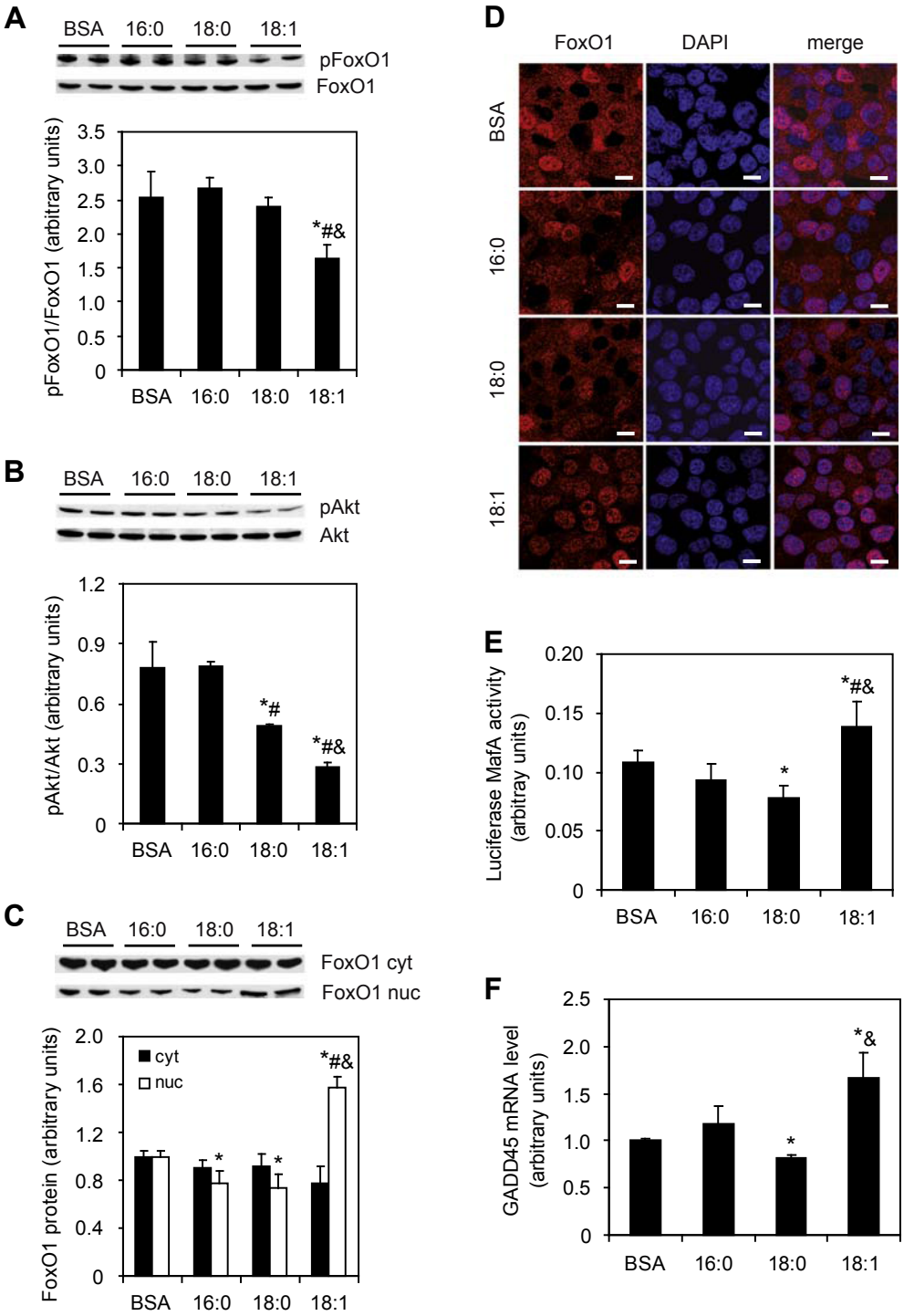

Fig. 3. Nuclear translocation of FoxO1 and promoter activity of *MafA* are elevated in INS-1E cells treated with oleate. INS-1E cells were incubated with BSA (control), 0.4 mM palmitate (16:0), 0.4 mM stearate (18:0), or 0.4 mM oleate (18:1) for 6 h. (A, B) Western blot analyses of (A) FoxO1 protein and phosphorylation (Ser256) and (B) Akt protein and phosphorylation (Ser473). The data are expressed as the mean ± SD from three independent experiments. $^{*}p < 0.05$, vs. BSA; $^{\#}p < 0.05$, vs. palmitate; $^{\&}p < 0.05$, vs. stearate. (C) Nuclear and cytoplasmic levels of FoxO1 protein. The data are expressed as the mean ± SD from three independent experiments. $^{*}p < 0.05$, vs. BSA nucleus; $^{\#}p < 0.05$, vs. palmitate nucleus; $^{\&}p < 0.05$, vs. stearate nucleus. (D) Immunofluorescence staining of FoxO1. (E) *MafA* promoter activity. INS-1E cells were transiently co-transfected with a plasmid that carried the *MafA* promoter and pRL-CMV as an internal control. Relative luciferase activity was derived from firefly/renilla ratios. (F) *GADD45* mRNA levels. The data are expressed as the mean ± SD from three independent experiments. $^{*}p < 0.05$, vs. BSA; $^{\#}p < 0.05$, vs. palmitate; $^{\&}p < 0.05$, vs. stearate.

Pdx1 protein in islets that were isolated from the experimental animal groups. Pdx1 levels significantly increased in islets from HF- and TS-fed rats, whereas Pdx1 levels decreased by 30% in islets from TO-fed animals compared with their normal chow-fed littermates (Fig. 2A).

Another factor that regulates β-cell adaptation and identity is FoxO1 [16], the activity of which is downregulated by phosphorylation-dependent nuclear exclusion through the Akt pathway [39]. We found markedly lower levels of pFoxO1 (Fig. 2B) and pAkt (Fig. 2C) in the TO-fed group. The decrease in FoxO1 phosphorylation suggests its nuclear translocation and increase in transcriptional activity. Therefore, we performed anti-FoxO1

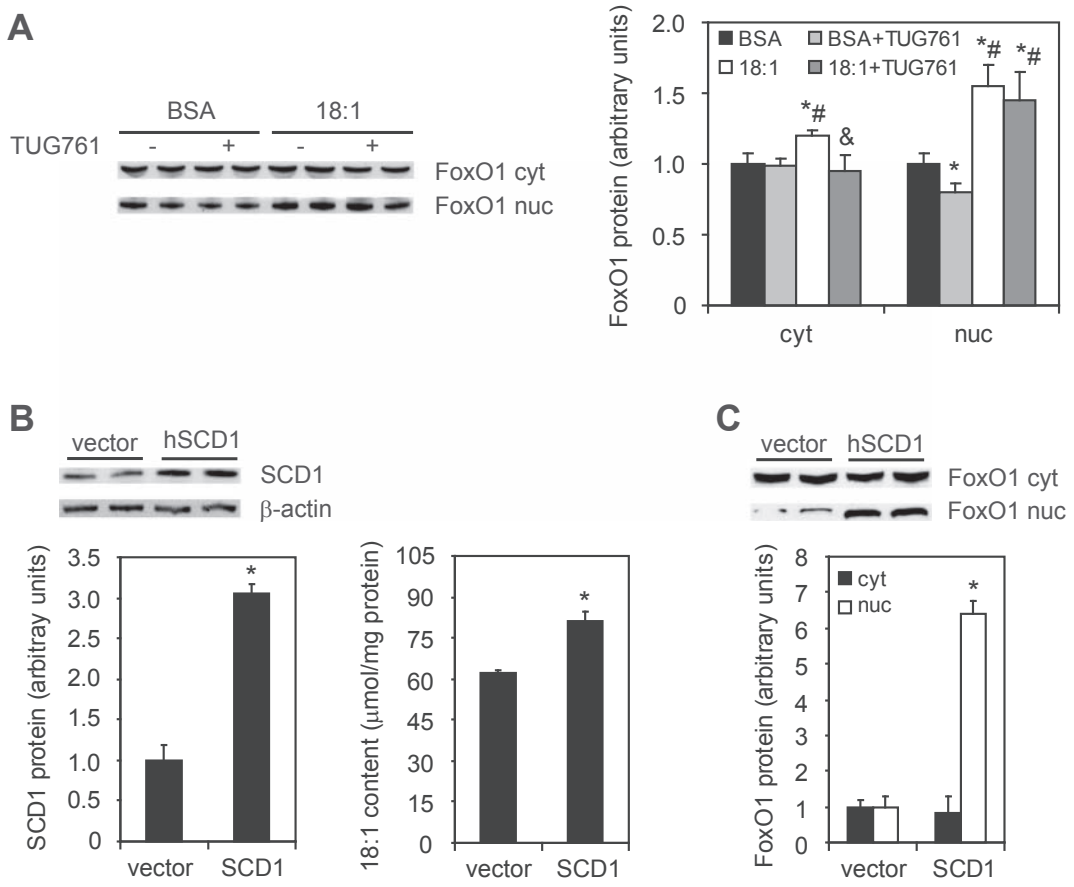

Fig. 4. Effect of GPR40 receptor inhibition and SCD1 overexpression on the intracellular distribution of FoxO1 in oleate-treated INS-1E cells. (A) Cells were pretreated for 1 h with 100 μM TUG761 (a specific inhibitor of GPR40 receptors) and then co-incubated with BSA (control) or oleate for an additional 6 h. The distribution of FoxO1 in cytoplasmic and nuclear fractions was assessed by immunoblotting. The data are expressed as the mean ± SD from three independent experiments. $^{*}p < 0.05$, vs. BSA; $^{\#}p < 0.05$, vs. BSA + TUG761; $^{\&}p < 0.05$, vs. oleate. INS-1E cells were transfected with an empty vector or a plasmid that carried hSCD1. (B, C) Western blot analyses of SCD1 levels and content of endogenously synthesized oleic acid (B) and cellular distribution of FoxO1 (C). The data are expressed as the mean ± SD from three independent experiments. $^{*}p < 0.05$, vs. empty vector.

immunohistochemistry in isolated islets. Indeed, in TO-fed animals, we observed a significant increase in the presence of FoxO1 in the nucleus (Fig. 2D). To corroborate these findings, we profiled expression of the FoxO1-targeted genes *MafA* and *NeuroD*. As shown in Fig. 2E, *MafA* and *NeuroD* gene expression increased in HF- and TS-fed rats, but the highest increase was observed in TO-fed animals compared with the normal chow-fed group. These findings indicated that the TO diet increased the nuclear localization and activity of FoxO1.

3.3. Oleate increases transcriptional activity of FoxO1 in INS-1E cells

To elucidate the molecular mechanism that is involved in TO diet-induced FoxO1 regulation, we treated INS-1 pancreatic β-cells with palmitic acid (16:0), stearic acid (18:0), and oleic acid (18:1). As shown in Supplementary Fig. 2, INS-1E cells that were incubated with stearic acid exhibited a significant increase in glucose-stimulated insulin secretion, whereas incubation with palmitic acid and oleic acid led to a decline in high glucose-induced insulin release compared with untreated controls. The effect of oleic acid treatment on the secretory capacity of β-cells in vitro was more severe than in islets from TO-fed animals.

The intracellular distribution of FoxO1 is determined by its Akt-dependent phosphorylation. Therefore, we also analyzed the phosphorylation status of FoxO1 and Akt in INS-1E cells. Significant decreases in pFoxO1 (Fig. 3A) and pAkt (Fig. 3B) were observed in oleate-treated cells compared with BSA controls. Oleic acid also increased FoxO1 accumulation in the nuclei of INS-1E cells by 60%, detected by subcellular fractioning and immunostaining, respectively (Fig. 3C, D). Interestingly, stearic acid treatment reduced FoxO1 levels in nuclear fractions of INS-1E cells by 40% (Fig. 3C, D). To investigate whether oleate-induced FoxO1 nuclear accumulation is followed by an increase in the transcriptional activity of FoxO1, we analyzed the promoter activity of *MafA*. The luciferase reporter assay revealed a significant increase in the promoter activity of *MafA* with oleate treatment (Fig. 3E). The mRNA level of the FoxO1 target gene *GADD45* also significantly increased in INS-1E cells that were treated with oleic acid (Fig. 3F).

3.4. Exogenously and endogenously delivered oleate regulates the subcellular localization of FoxO1 independently of the GPR40 receptor

Accumulating evidence indicates that many molecular actions of FAs are mediated by their interactions with GPR40 G-protein-coupled receptors [40]. To test the hypothesis that the oleate-induced increase in FoxO1 activity is modulated by the GPR40 signaling pathway, we used the specific GPR40 receptor antagonist TUG761 [35]. TUG761 decreased the amount of nuclear FoxO1, whereas GPR40 receptor inhibition did not abolish the effect of oleic acid on the nuclear accumulation of FoxO1 in INS-1E cells (Fig. 4A). These data clearly indicate that the oleate-mediated induction of FoxO1 occurs independently of GPR40 receptor signaling.

To investigate whether endogenously synthesized oleic acid affects the intracellular distribution and activity of FoxO1, INS-1E cells were transiently transfected with a pCMV6 plasmid that carried human SCD1

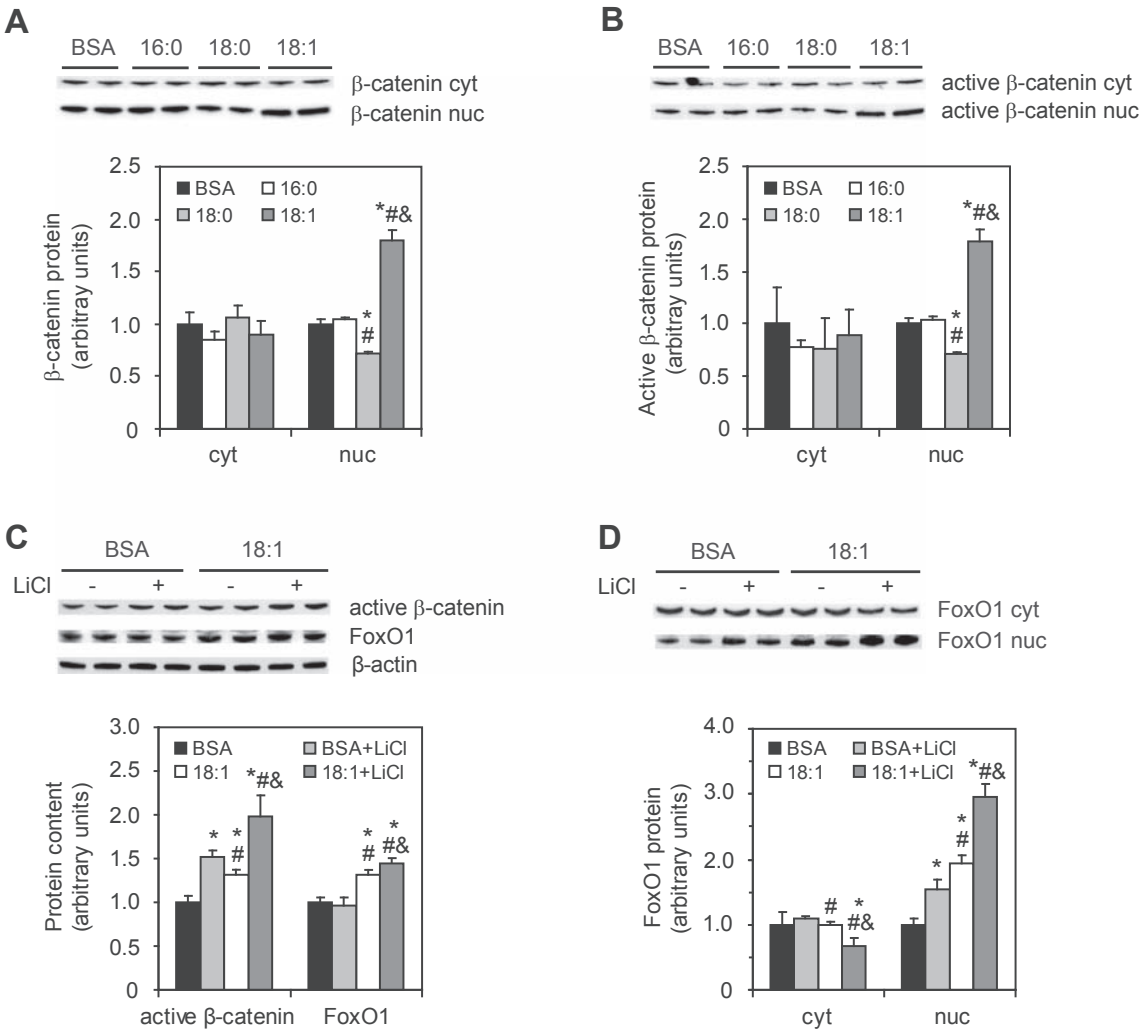

Fig. 5. Oleic acid treatment increases the nuclear translocation of β-catenin in INS-1E cells. INS-1E cells were incubated with BSA (control), 0.4 mM palmitate (16:0), 0.4 mM stearate (18:0), or 0.4 mM oleate (18:1) for 6 h. Nuclear and cytoplasmic levels of total β-catenin (A) and the active form of β-catenin (B) were evaluated by immunoblotting. The data are expressed as the mean ± SD from three independent experiments. $^{*}p < 0.05$, vs. BSA; $^{\#}p < 0.05$, vs. palmitate; $^{\&}p < 0.05$, vs. stearate. INS-1E cells were then pretreated for 2 h with LiCl (an inhibitor of GSK3β) and then co-incubated with BSA (control) or oleic acid for an additional 6 h. (C, D) Protein levels of active β-catenin and FoxO1 (C) and the nuclear and cytoplasmic levels of FoxO1 (D) were assessed by Western blot. The data are expressed as the mean ± SD from three independent experiments; $^{*}p < 0.05$, vs. BSA; $^{\#}p < 0.05$, vs. BSA + LiCl; $^{\&}p < 0.05$, vs. oleate.

(hSCD1), a key enzyme that is responsible for the synthesis of oleic acid (Fig. 4B). The overexpression of hSCD1 increased FoxO1 accumulation > 6-fold in nuclear fractions of INS-1E cells (Fig. 4C). These results suggest that endogenously synthesized oleic acid, similar to exogenously delivered oleate, is a potent regulator of FoxO1 activity.

3.5. Nuclear accumulation of β-catenin is increased by oleate treatment in INS-1E cells

The transcriptional activity of FoxO1 also depends on its binding to β-catenin, the major effector of the canonical Wnt signaling pathway that is responsible for regulating pancreatic β-cell insulin secretion and proliferation [36,41,42]. To examine whether FAs affect the subcellular localization of β-catenin, INS-1E cells were treated with palmitate, stearate, and oleate. As shown in Fig. 5A and B, palmitate treatment did not alter the cellular accumulation of β-catenin, whereas stearate treatment significantly decreased the cellular accumulation of β-catenin in the nuclear fraction. Oleate treatment induced the accumulation of total and active forms of β-catenin in the nucleus of INS-1E cells.

To investigate whether the effect of oleate on β-catenin accumulation influences the cellular distribution of FoxO1, we used LiCl, an inhibitor of GSK3β, a key regulator of intracellular β-catenin stabilization [43]. As expected, LiCl treatment increased active β-catenin levels, and this effect was further augmented by the addition of oleic acid (Fig. 5C). Moreover, simultaneous treatment with LiCl and oleic acid elevated FoxO1 levels in nuclear fractions of INS-1E cells (Fig. 5D). Altogether, these data suggest that the oleate-derived nuclear accumulation of FoxO1 is accompanied by an increase in the nuclear translocation of β-catenin.

3.6. Oleate favors β-catenin-FoxO1 binding over β-catenin-TCF interaction

β-catenin is a potent regulator of transcriptional activity through direct interactions with either TCF (its main partner in Wnt signaling) or FoxO1 [28,44]. To elucidate whether the molecular interplay between β-catenin and FoxO1 affects TCF7L2 binding, we performed an immunoprecipitation assay in INS-1E cells. Interestingly, oleate treatment increased the interaction between β-catenin and FoxO1 and simultaneously decreased the binding of β-catenin with TCF7L2 (Fig. 6A). This effect was further translated into lower TCF7L2 transcriptional activity (Fig. 6B) and lower mRNA expression of *cyclin D1* and *c-myc*, target genes of TCF7L2, in oleate-treated INS-1E cells and pancreatic islets from TO-fed rats, respectively (Fig. 2F, 6C). Palmitate and stearate did not affect the interaction of β-catenin with TCF7L2 or

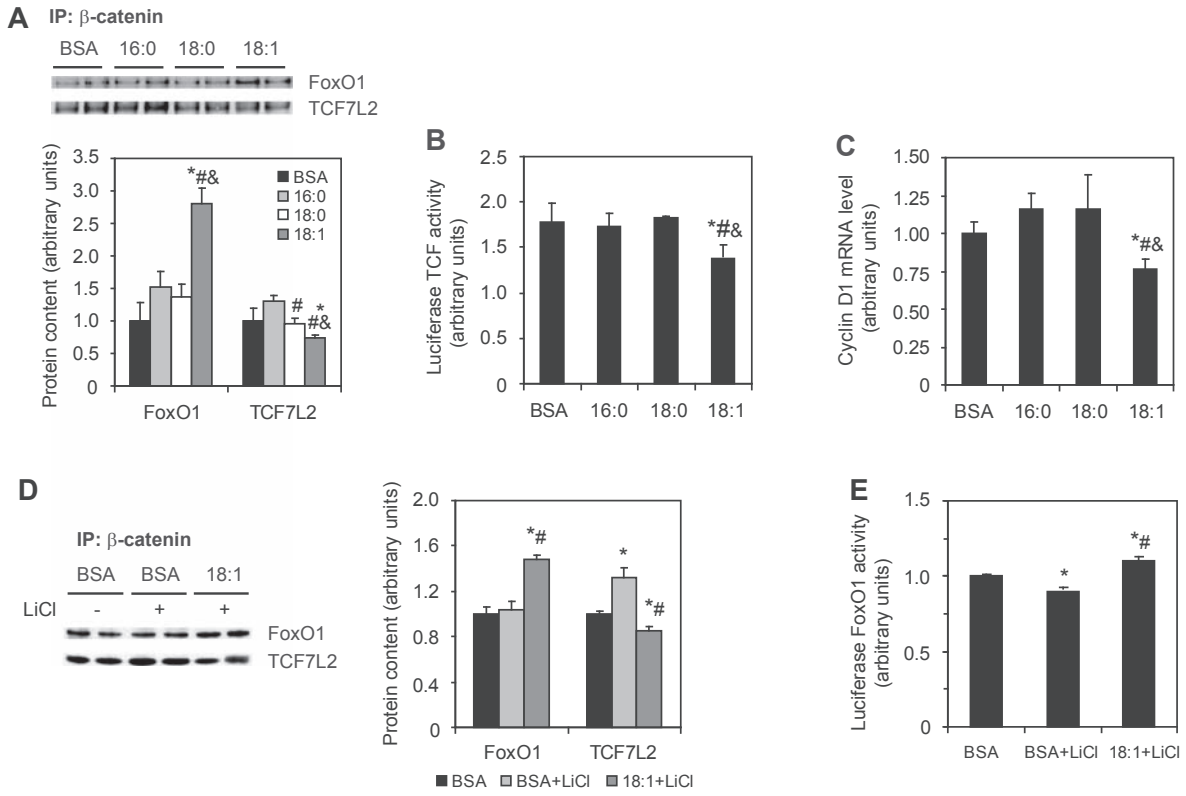

Fig. 6. Interaction between FoxO1 and β-catenin is increased by oleate treatment in INS-1E cells. Experiments were performed in INS-1E cells that were incubated with BSA (control), 0.4 mM palmitate (16:0), 0.4 mM stearate (18:0), or 0.4 mM oleate (18:1) for 6 h. (A) The interaction between the active form of β-catenin and either FoxO1 or TCF7L2 was analyzed. (B) Transcriptional activity of TCF7L2. (C) *CyclinD1* mRNA level. The data are expressed as the mean ± SD from three independent experiments. $^{*}p < 0.05$, vs. BSA; $^{\#}p < 0.05$, vs. palmitate; $^{\&}p < 0.05$, vs. stearate. INS-1E cells were also pretreated for 2 h with LiCl (an inhibitor of GSK3β) and then co-incubated with BSA (control) or oleic acid for an additional 6 h. (D) Binding of the active form of β-catenin to FoxO1 or TCF7L2 under conditions of LiCl and oleate treatment was compared by coimmunoprecipitation. (E) Transcriptional activity of FoxO1. The data are expressed as the mean ± SD from three independent experiments. $^{*}p < 0.05$, vs. BSA; $^{\#}p < 0.05$, vs. oleate + LiCl.

FoxO1 in INS-1E cells (Fig. 6A-C).

To test the hypothesis that oleic acid shifts the active β-catenin pool interaction with TCF7L2 toward an interaction with FoxO1, we treated INS-1E cells with LiCl to activate Wnt signaling. LiCl increased the β-catenin-TCF7L2 interaction, whereas the binding of β-catenin with FoxO1 remained unaffected (Fig. 6D). Interestingly, the addition of oleate shifted the β-catenin interaction with TCF7L2 toward an interaction with FoxO1 (Fig. 6D). This effect was followed by an increase in the transcriptional activity of FoxO1 in INS-1E cells that were simultaneously treated with oleate and LiCl (Fig. 6E). Collectively, these results indicate that oleic acid shifts active β-catenin from an interaction with TCF7L2 toward an interaction with FoxO1, thus affecting its transcriptional activity.

4. Discussion

The molecular mechanisms and signaling networks by which pancreatic β-cells compensate for obesity and lipid-derived insulin resistance remain poorly understood. Long-chain FAs were proposed to be novel regulators of FoxO1-mediated transcription in β-cells [14], but no direct mechanism has yet been identified. Our previous study showed that a decrease in SCD1 activity was associated with the dysfunction of INS-1E β-cells [45] and a significant decrease in Wnt-regulated gene expression [29]. Therefore, the present study explored the causal link between the compensatory capacity of β-cells in response to SCD1-metabolized FAs and the FoxO1 and Wnt pathways. We found that both the HF and TO diets induced insulin resistance, but only the HF-fed group maintained an increase in islet size and ability to compensate for impairments in insulin action. The effect of impairments in β-cell adaptation was associated with increases in the nuclear accumulation and transcriptional activity of FoxO1, and these effects were also observed in INS-1E cells that were treated with oleate. We also found that oleate mediated the subcellular localization of FoxO1 independently of the main FA interaction receptor GPR40 and shifted the β-catenin interaction with TCF toward FoxO1-mediated transcription in β-cells. Thus, the present data indicate that FoxO1 is a link between the oleic acid-mediated downregulation of transcription in β-cells and abnormalities in pancreatic islet adaptation to systemic insulin resistance.

Recent data indicate that FoxO1 signaling is an integral part of the β-cell response to diet-induced insulin resistance [12,13]. The nuclear redistribution of FoxO1 after oleate treatment has been previously observed in various β-cell lines [14,33,34]. However, the precise mechanism of such an interrelation has not been previously explored. In the present study, we found that oleic acid treatment resulted in the nuclear translocation of FoxO1 in pancreatic islets. Our observations are consistent with the findings of Okamoto et al., in which the constitutive nuclear expression of FoxO1 prevented β-cell hyperplasia and the expansion of β-cell mass in response to insulin resistance [12]. Both the TO diet and oleic acid treatment of INS-1E β-cells decreased the phosphorylation of Akt and FoxO1, indicating alterations of the intracellular localization of FoxO1. These findings were corroborated by the significant increase in the nuclear accumulation and transcriptional activity of FoxO1. We initially speculated that the oleate-mediated induction of FoxO1 might occur via the binding of medium- and long-chain FAs to GPR40. GPR40 receptors are highly expressed in rodent primary β-cells, human islets, and β-cell lines [46] and also necessary for the FA-mediated potentiation of GSIS [40,47,48]. In the present study, oleate activated FoxO1 target genes through a mechanism that is

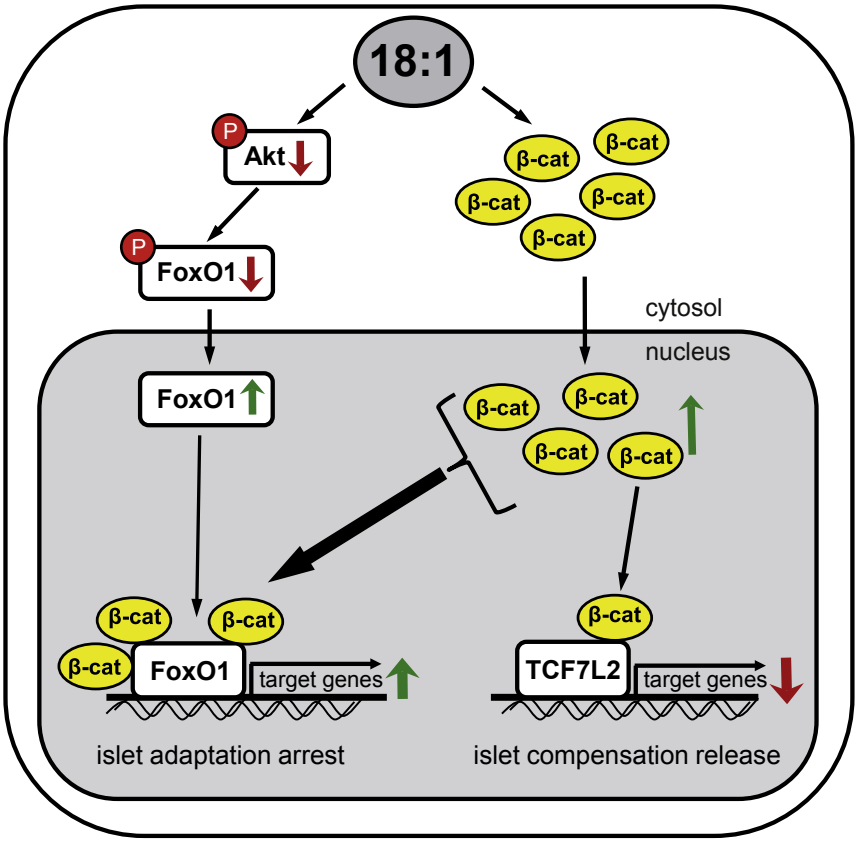

Fig. 7. Effect of oleic acid on the intracellular localization and transcriptional activity of FoxO1 during pancreatic β-cell adaptation to insulin resistance. Oleate supplementation drives insulin resistance and compromises the compensatory capacity of β-cells. This effect coincides with elevations of the nuclear translocation and transcriptional activity of FoxO1. Oleate diverts the restricted pool of β-catenin from TCF7L2- to FoxO1-mediated transcription. This shift of the transcriptional balance may be responsible for suppressing beneficial Wnt signaling in the compensatory response of pancreatic β-cells toward FoxO1 activation. The unsuccessful attempt of islets to recover may lead to β-cell failure and the onset of diabetes.

distinct from GPR40 signaling, in which GPR40 antagonism with TUG761 had no effect, suggesting the existence of an alternative regulatory pathway that is independent of membrane receptors.

The activity of FoxO1 is dynamically regulated at multiple levels, primarily by protein-protein interactions [49]. The pathophysiological contribution of the interaction between FoxO1 and β-catenin was convincingly demonstrated in various mammalian cells that were subjected to oxidative stress [12]. The stability and intracellular localization of β-catenin are determined by its degradation rate, which is regulated by canonical Wnt signaling [44]. The Wnt/β-catenin pathway detrimentally influences development of the pancreas and pancreatic β-cell function [50]. Within the nucleus, β-catenin becomes a component of the bipartite transcription factor with TCF to induce the transcription of Wnt target genes [51]. In our previous study, we demonstrated that the accumulation of active β-catenin was correlated with higher insulin secretion and an increase in β-cell proliferation in pre-diabetic animals. In fully developed T2D, in contrast, the downregulation of Wnt signaling coincided with dysfunctional pancreatic islets and a lack of their adaptation capacity [29]. The present study extended these findings, in which the nuclear localization of FoxO1 and β-catenin and their mutual binding were elevated by oleate exposure, and such an association consequently restrained TCF-dependent transcription in INS-1E cells. The reservoir of β-catenin is limited, and its association with FoxO1 functionally competes with TCF to activate transcription, which has been hypothesized to be a cellular stress defense mechanism [52]. The process of β-catenin diversion from TCF to FoxO1 has been previously reported [41,52–55], but such a shift was not reported in islets. Oxidative stress promoted the association between β-catenin and FoxO1, which was inversely related to TCF-mediated transcription and osteoblastogenesis [53]. A similar shift that affected hepatic gluconeogenesis was promoted by starvation in the liver [54] and observed in liver-specific dominant-negative TCF7L2 mouse hepatocytes and Hepa1-6 cells [55]. The present study provides evidence that activation of the Wnt pathway by LiCl is not sufficient to reverse the oleate-mediated effect on β-catenin unleash from TCF7L2, suggesting that the mechanism is independent of GSK3 regulation.

FoxO transcription factors favor cell cycle arrest, and this function is enhanced by β-catenin [36]. Our study suggests that oleate antagonizes Wnt signaling by diverting β-catenin from TCF- to FoxO1-mediated transcription in INS-1E cells, what may represent an initial cellular defense mechanism against metabolic stress. Our data support the hypothesis that intact Wnt signaling is critical for a proper pancreatic β-cell compensatory response to lipid-derived insulin resistance [50]. The pace of cellular proliferation is crucial for sustaining an appropriate mass of fully functional β-cells [39]. The overexpression of FoxO1 accelerates the proliferation of β-cells through a cyclin D1-dependent mechanism when they are exposed to low nutrient conditions [56]. However, under conditions of metabolic stress, such as oleate exposure, the activation of FoxO1 leads to the suppression of β-cell proliferation. Instead, signaling through the insulin/PI3K/Akt cascade restrains FoxO1 activation and promotes protection against FA-induced apoptosis in pancreatic β-cells [33]. The FoxO1-induced halt of β-cell replication may allow time for the repair of damaged DNA or cellular lipid detoxification. Interestingly, the nuclear localization of FoxO1 was followed by increases in the expression of *MafA*, *NeuroD*, and *GADD45* target genes. Thus, βTC-3 and INS-1E cells remained protected from oxidative stress and nitric oxide-induced DNA damage, respectively [22,57]. All aforementioned genes and the promoter activity of *MafA*

were highly elevated in islets and INS-1E cells that were challenged with oleate. However, an increased *MafA* gene expression alone is insufficient to sustain islet adaptation in the conditions of oleate overload and simultaneous downregulation of Wnt signaling. Thus, FoxO1 appears to possess a dual mode of action between proliferation and stress resistance to preserve β-cell adaptation.

In conclusion, the present study provides experimental support for the essential role of oleate in the management of physiological β-cell adaptation to insulin resistance through control of the subcellular localization of FoxO1. Our studies establish a novel mechanism by which oleic acid orchestrates crosstalk in gene-expression regulation between FoxO1 and Wnt signaling in pancreatic β-cells. While the role of oleate in the regulation of pancreatic β-cell survival remains under stormful discussions [58–61], our results bring new insights to the relevance of aforementioned fatty acid for islets functioning in the context of obesity-related T2D development. The present data suggest that oleate halts the compensatory response of β-cells by sequestering the limited pool of β-catenin from the interaction with TCF7L2 toward FoxO1-mediated transcription (Fig. 7). Our data provide a basis for evaluating new therapeutic interventions that target FoxO1 to protect against the failure of β-cell adaptation in at-risk obese individuals.

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Author contributions

A.Do., M.J., P.D. contributed to the conception and design of the study. M.J., J.J., P.D., A.Dz., K.K., A.Do. were involved with data acquisition, analysis and interpretation. A.Do, M.J., J.J., P.D. wrote and proofread the manuscript. All authors contributed critically to revision and approval of the manuscript.

Disclosure statement

None.

Transparency document

The Transparency document associated with this article can be found, in online version.

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