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ORIGINAL ARTICLE

PRMT1 promotes glucose toxicity-induced β cell dysfunction by regulating the nucleo-cytoplasmic trafficking of PDX-1 in a FOXO1-dependent manner in INS-1 cells

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Abstract Protein *N*-arginine methyltransferase-1 (PRMT1), the major asymmetric arginine methyltransferase, plays important roles in various cellular processes. Previous reports have demonstrated that levels and activities of PRMT1 can vary in animals with type 2 diabetes mellitus. The aim of this study was to assess the expression and mechanism of action of PRMT1 during glucose toxicity-induced β cell dysfunction. Liposome-mediated gene transfection was used to transfect INS-1 cells with siPRMT1, which inhibits PRMT1 expression, and pALTER–FOXO1, which overexpresses forkhead box protein O1 (FOXO1). The cells were then cultured in media containing 5.6 or 25 mmol/L glucose with or without the small molecule PRMT1 inhibitor AMI-1 for 48 h. The protein levels of PRMT1, the arginine methylated protein α-metR, FOXO1, Phospho-FOXO1, pancreas duodenum homeobox-1 (PDX-1), and the intracellular localization of PDX-1 and FOXO1 were then measured by western blotting. FOXO1 methylation was detected by immunoprecipitated with anti-PRMT1 antibody and were immunoblotted with α-metR. The levels of insulin mRNA were measured by real-time fluorescence quantitative PCR. Glucose-stimulated insulin secretion (GSIS) and intracellular insulin content were measured using radioimmunoassays. Intracellular Ca^{2+} ($[Ca^{2+}]i$) was detected using Fura-2 AM. Intracellular cAMP levels were measured using ELISA. Chronic exposure to high glucose impaired insulin secretion, decreased insulin mRNA levels and insulin content, increased intracellular $[Ca^{2+}]i$ and cAMP levels, and abolishes their responses to glucose. Inhibiting PRMT1 expression improved insulin secretion, increased mRNA levels and insulin content by regulating the intracellular translocation of PDX-1 and FOXO1, decreasing the methylation of FOXO1, and reducing intracellular $[Ca^{2+}]i$ and cAMP concentrations. Transient overexpression of constitutively active FOXO1 in nuclear reversed the AMI-1-induced improvement of β cell function without changing arginine methylation. It is concluded therefore that PRMT1 regulates GSIS in INS-1 cells, through enhanced methylation-induced nuclear localization of FOXO1, which subsequently suppresses the nuclear localization of PDX-1. Our results suggest a novel mechanism that might contribute to the deficient insulin secretion observed under conditions of chronically hyperglycemia.

Keywords Protein *N*-arginine methyltransferase-1 · Forkhead box protein O1 · Glucose-stimulated insulin secretion · β cell dysfunction · Ca^{2+}

Abbreviations

PRMT1	Protein *N*-arginine methyltransferase-1
FOXO1	Forkhead box protein O1
PDX-1	Pancreas duodenum homeobox-1
GSIS	Glucose-stimulated insulin secretion

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AMI-1 7,7′-Carbonylbis[azanediyl]bis[4-hydroxynaphthalene-2-sulfonic acid]
α-metR Anti-mono and dimethyl arginine protein

Introduction

Type 2 diabetes mellitus (T2DM) results from the failure of pancreatic β cells to secrete sufficient amounts of insulin to compensate peripheral insulin resistance and to maintain normal glucose homeostasis [1]. The loss of β cell function in type 2 diabetes occurs when β cells are chronically exposed to elevated levels of circulating glucose, a state defined as glucotoxicity [2]. The loss of glucose-stimulated insulin secretion (GSIS) and increased basal secretion are major characteristics of type 2 diabetes-associated β cell dysfunction [3]. Glucose is the main physiological stimulus of insulin secretion from pancreatic β cells. The exocytosis of insulin granules is triggered by an elevation of the cytoplasmic-free Ca^{2+} concentration ($[Ca^{2+}]i$) and is further amplified by intracelluar level of cyclic AMP (cAMP). The stimulus-secretion coupling involves metabolism of the glucose, which leads to an increase in cytoplasmic ATP/ADP ratio and closure of ATP-sensitive K^+ (K_{ATP}) channels on the plasma membrane. The resulting depolarization triggers Ca^{2+} influx through voltage-dependent Ca^{2+} channels and the exocytosis of insulin secretory granules [4]. In addition to Ca^{2+}, which is the primary triggering signal, cAMP is the most important regulator of exocytosis in β cells. cAMP is a ubiquitous intracellular messenger that is involved in the regulation of a wide variety of processes in many cell types [5]. cAMP potently enhances Ca^{2+} signals and exocytosis both via protein kinase A (PKA)-dependent and PKA-independent mechanisms in β cells [6].

T2DM is a disease of multifactorial inheritance in patients, caused by both expression of susceptibility genes and exposure to environmental factors. Recent studies have revealed that epigenetic mechanisms might provide the link between genetic pre-disposition and environmental factors via DNA methylation and histone modifications [7, 8]. Volkmar et al. [9] recently identified 254 gene promoters that were differentially methylated in the islets of diabetic patients, and identified a T2DM-related differential methylation pattern in diabetic islets. Protein *N*-arginine methyltransferases (PRMTs) are enzymes that are responsible for the transfer of a methyl group from *S*-adenosylmethionine (SAM) to arginine residues [10, 11]. Protein *N*-arginine methyltransferase-1 (PRMT1) is a dominant type I enzyme that performs as much as 85 % of all cellular PRMT activity in mammalian cells [12]. It has a wide substrate spectrum, including both histone and non-histone substrates [13, 14]. Recent accumulated evidence has revealed an important role of PRMT1-mediated methylation in various cellular processes, including transcriptional regulation, histone function, DNA repairing, and intracellular protein–protein interactions [10, 11]. PRMT1 has also been linked to oncogenesis [15, 16] and cardiovascular diseases [11]. Recent studies have revealed that the function and expression of PRMT1 are dysregulated in the livers of type 2 diabetic Goto–Kakizaki rats and during diabetic retinopathy [17, 18]. We previously demonstrated that PRMT1 expression was increased in pancreatic islet of streptozotocin-induced diabetic rats; such increase was partially reversed by treatment with the anti-hyperlipidemic drug probucol. The direct effect of PRMT1 on β cell function is however yet to be elucidated. Therefore, the aim of the current study was to explore the effect of PRMT1 on INS-1 cells after prolonged glucotoxicity, as well as the cellular mechanism of PRMT1 action.

Pancreas duodenum homeobox-1 (PDX-1) is a transcription factor that plays a pivotal role in pancreatic development and β cell function [19, 20] by regulating the expression of *insulin* and islet-specific genes via interactions with their promoter regions. β Cells chronically exposed to high glucose conditions either in vivo or in vitro exhibit decreased nuclear PDX-1 expression [21–24]. In addition, PDX-1 KO heterozygous mice expressed reduced levels of PDX-1, resulting in impaired glucose tolerance [25, 26]. These findings suggest that PDX-1 is an important regulator of β cell function. Mechanically, PDX-1 has a functional nuclear localization signal [27, 28]. The activity of transcription factors is regulated by their intracellular localization; therefore, factors that stimulate nuclear import can regulate gene expression [25, 26]. Consistent with this, a previous study reported that PDX-1 was imported to the nucleus from the cytoplasm after acute stimulation of cells by high level of glucose [29].

The forkhead transcription factor FOXO1 plays a key role in a variety of physiological functions including apoptosis, cellular proliferation, and glucose metabolism by regulating the transcription of various target genes [30, 31]. Gain-of-function FOXO1 mutations in pancreatic β cells and the liver increase hepatic glucose production via glycogenesis and abrogate β cell ability in compensation, resulting in diabetes [32]. In β cells, the main target of FOXO1 is PDX-1 [32–35]. FOXO1 inhibits *PDX-1* gene transcription by binding to its promoter in pancreatic β cells [32, 33]. In addition, the nuclear localization of PDX-1 and FOXO1 is mutually exclusive, suggesting that FOXO1 might play a role in the deterioration of β cell function by competing with PDX-1 in nuclear. A recent study has also demonstrated that PRMT1-dependent arginine modification of FOXO1 modulates the subcellular

location of FOXO1 in the liver, which regulates transcriptional activity of FOXO1 [36]. Nevertheless, the relevance of PRMT1, FOXO1, and PDX-1 in β cell function has not yet been clarified.

The present study was designed to further explore the effects of PRMT1 on the regulation of pancreatic β cell function, and assess the underlying mechanisms involving expression of FOXO1 and PDX-1 during hyperglycemia-induced pancreatic β cell dysfunction in INS-1 cells.

Materials and methods

Materials

RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA), and glucose-free RPMI-1640 was obtained from Gibco (Carlsbad, CA, USA). AMI-1 (7,7′-carbonylbis[azanediyl]bis[4-hydroxynaphthalene-2-sulfonic acid]) was obtained from Sigma-Aldrich (St. Louis, MO, USA). PRMT1-siRNA was purchased from GeneChem (Shanghai, China), and pALTER-FOXO1 was a kind gift from Dr. Unterman (University of Illinois, Chicago, IL, USA). It was a mammalian cell expression vector by subcloning the constitutively active FOXO1 cDNA in pFB-12A2 into the *Xba*I–*Acc*I site in pAlter-MAX (Promega) downstream from the CMV promoter [37]. Fura-2 acetoxymethyl ester (AM) was purchased from Molecular Probes Inc. (Eugene, OR, USA). Anti-actin HRP-conjugated IgG secondary antibodies were purchased from Beijing 4A Biotech Co. Ltd (Beijing, China). Enhanced chemiluminescence (ECL) reagents were obtained from Beyotime (Shanghai, China). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). The radioimmunoassay (RIA) kit for rat insulin was purchased from Millipore (Boston, MA, USA). INS-1 cells generated and first described by Asfari et al. [38] were a generous gift from Dr. Sun (Dalian Medical University, Dalian, China).

INS-1 cell culture

INS-1 cells were grown in a humidified atmosphere containing 95 % air and 5 % CO_2 in RPMI-1640 medium containing 11.1 mM glucose, 10 % FBS, 1 mM pyruvate, 10 mM HEPES, 50 μM 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were then transfected with siPRMT1 or the indicated plasmid, and subsequently cultured in RPMI-1640 medium containing 5.6 or 25 mM glucose (5.6 G and 25 G, respectively) and/or 100 μM AMI-1. For transfection with siPRMT1 (target sequence 5′-CCAACGCCTGCCTCATAAA-3′) or pALTER-FOXO1, INS-1 cells grown in 6-well plates were transfected using Lipofectamine 2000 following the manufacturer's instructions, and the media were replaced 6 h after transfection. Seventy-two hours after transfection, the cells were treated with 25 mM glucose and/or AMI-1 (100 μM) for an additional 48 h, and then harvested for the assays described below.

Real-time fluorescence quantitative PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol (Invitrogen), and 1 mg of RNA was used for the synthesis of cDNA following the instructions provided with the kit. qRT-PCR was performed by monitoring the increase in fluorescence of SYBR Green dye using a real-time detection system. After a 1-min denaturation at 95 °C, 39 cycles of denaturation, annealing, and elongation were performed (95 °C for 10 s, 60 °C for 30 s, and 65 °C for 5 s, respectively). The relative quantification was performed by calculating the ratio of the PCR products, which was derived from the level of the gene of interest divided by the level of β-actin. These experiments were repeated three times. The sequences of the primers (Sangon, Shanghai, China) used in this study were as follows: PRMT1 (forward: 5′-GAGTTCACCCGATGCCACAAG-3′, reverse: 5′-TCCGGTAGTCGGTGGAACAAG-3′); insulin (forward: 5′-CACCCAAGTCCCGTCGTGAAGT-3′, reverse: 5′-GATCCACAATGCCACGCTTCTG-3′), and β-actin (forward: 5′-GAGAGGGAAATCGTGCGTGAC-3′, reverse: 5′-CATCTGCTGGAAGGTGGACA-3′).

GSIS assay

INS-1 cells seeded in 6-well plates were cultured, transfected, and treated as described above. After pre-incubation for 1 h in sugar-free Krebs–Ringer bicarbonate buffer (KRBB), cells were then treated in KRBB with low (4 mM) or high (16.7 mM) concentrations of glucose for 1 h. Supernatants were then obtained and frozen at −70 °C for the subsequent determination of insulin concentrations. Insulin levels were measured using RIA (Millipore) and were normalized to the protein concentration in each well, which was determined using a bicinchoninic acid assay. The RIA data shown are representative of three independent experiments, each performed in quadruplicate.

Intracellular insulin content measurements

INS-1 cells seeded in 6-well plates were cultured, transfected, and treated as described above. Cells were then treated with hydrochloric acid ethanol solution (75 % anhydrous ethanol, 1.5 % hydrochloric acid, and 23.5 % ultrapure water) at 4 °C overnight. Insulin levels were measured as described in "GSIS assay" section. The RIA

data shown are representative of three independent experiments, each performed in quadruplicate.

Measurement of intracellular-free Ca^{2+}

Standard extracellular saline (SES) was prepared as follows: 138 mM NaCl, 5.6 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES, and 5.6 mM D-glucose were dissolved in double-distilled water, and adjusted to pH 7.35 using NaOH; the osmolarity was then adjusted to 295 mosmol l^{-1} using double-distilled water. INS-1 cell were incubated in SES supplemented with 1 μM Fura-2 AM, 2 % FBS, and 0.02 % Pluronic F-127 for 30 min at 22 °C. Imaging was performed using a microscope equipped with an epifluorescence illuminator (Nikon) and a CCD camera (HQ2, Photometrics Inc.). The relative Ca^{2+} concentrations were quantified by determining the ratio of the emitted fluorescence intensities at excitation wavelengths of 340 and 380 nm (F340/F380), by the equation described by Grynkiewicz et al. [39] with dissociation constant (K_d) of 224 nmol/L.

Measurement of intracellular cAMP content

INS-1 cells were washed three times with PBS, incubated with HCl (100 μL, 1 mol/L) for 10 min at room temperature. They were then centrifuged 600×*g*, 10 min and the same amount of NaOH as the HCl was added to the supernatants. The concentration of cAMP in all samples was quantified using an ELISA (R&D Systems). The experiment was repeated four times.

Western blotting

Cells in each group were harvested and divided into two equal amounts: one half was used to extract total protein, and the other was used to extract nuclear and cytoplasmic proteins. Fifty micrograms each whole cell extracts, 30 μg of cytoplasmic extracts, or 20 μg of nuclear extracts were resolved by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis and transferred to polyvinylidene fluoride membranes (Amersham Pharmacia). Membranes were then immunoblotted with specific antibodies as follows: rabbit polyclonal anti-PRMT1 (1:200; Santa Cruz), rabbit polyclonal anti-PDX-1 (1:200; Santa Cruz), rabbit polyclonal anti-FOXO1 (1:200; Santa Cruz), rabbit polyclonal anti-Phospho-FOXO1 (Ser256) (1:1,000; cell signaling), mouse monoclonal anti-lamin B (1:200; Santa Cruz), and mouse anti-mono and dimethyl arginine (α-metR; 1:1,000; Abcam). Signals were visualized using an ECL detection system after incubation with HRP-conjugated secondary antibodies. All immunoblots shown are representative of three independent experiments.

Co-immunoprecipitation

INS-1 cell protein extracts were prepared as described previously. Proteins were incubated with 50 μL lysis buffer (250 mm NaCl, 5 mm EDTA, 50 mm HEPES (pH 7.5), 0.1 % Nonidet P-40, 0.5 mm DTT, 0.1 % protease inhibitor mixture (Sigma)) was added to the lysates for immunoprecipitation. With protein A- or protein G-Sepharose for 1 h at 4 °C than centrifuged 3,000×*g*, 1 min the supernate incubated 5 μg of rabbit polyclonal anti-PRMT1 (Santa Cruz) or rabbit polyclonal anti-FOXO1 (Santa Cruz) for overnight at 4 °C as input. Pellets were resuspended with wash buffer (20 mm Tris (pH 7.4), 300 mm NaCl, 0.1 % Nonidet P-40, 1 mm DTT, 5 mm EDTA, 25 % glycerol) for 2 h at 4 °C. After washing three times take in loading buffer (Invitrogen) and boiled for 5 min at 95 °C. Samples were analyzed by SDS-PAGE and Bands were visualized by ECL Plus detection reagent as described 2.7

Statistical analysis

Results are expressed as mean ± standard error of the mean ($\bar{x} \pm s$). Differences between means were evaluated using Student's *t* tests or one-way analysis of variance (ANOVA). A *P* value of <0.05 was considered to be statistically significant.

Results

siPRMT1 restored glucotoxicity-induced stimulation of $[Ca^{2+}]i$ and cAMP and INS-1 cells function

RNA interference was used to investigate the role of PRMT1 in high glucose-induced INS-1 cell function. INS-1 cells were transfected with PRMT1-siRNA to silence PRMT1 expression. Seventy-two hours after transfection, total RNA was harvested and analyzed using qRT-PCR. As shown in Fig. 1, PRMT1-siRNA effectively inhibited the expression of *PRMT1* mRNA by ~70 % (Fig. 1a). The transfected cells were then incubated with 25 mM glucose for 48 h. $[Ca^{2+}]i$ were detected using Fura-2 AM, intracellular cAMP levels were measured using ELISA, and insulin secretion and intracellular insulin content were quantified using RIA. As shown in Fig. 1, both basal $[Ca^{2+}]i$ and cAMP content were increased in response to 25 mM glucose treatment. However, high glucose culture abolishes both $[Ca^{2+}]i$ and cAMP responses to glucose, whereas silencing PRMT1 expression

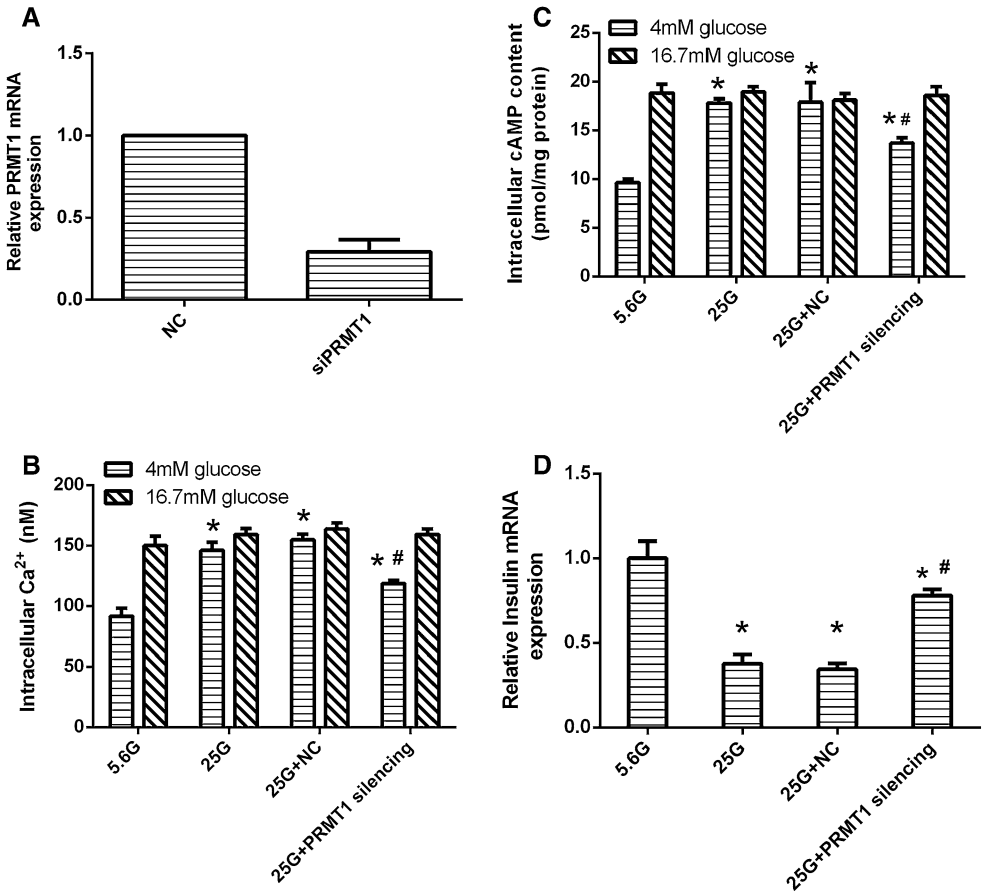


Fig. 1 The effects of high glucose and siPRMT1 on $[Ca^{2+}]i$ and cAMP content. INS-1 cells were transfected with PRMT1-siRNA to silence PRMT1 expression and compared with cells transfected by NC-siRNA (scrambled negative control siRNA). After 72 h transfection, cell total RNA was then harvested and analyzed by RQ-PCR (**a**), or further cultured in medium containing 25 mM glucose for 48 h. Then cell total RNA was then harvested and analyzed by RQ-PCR (**d**) or cultured in media containing 4 mM glucose or 16.7 mM glucose for 1 h. Levels of $[Ca^{2+}]i$ (**b**) were detected by Fura-2 AM; intracellular cAMP (**c**) were measured by ELISA as described in "Measurement of intracellular-free Ca^{2+}" and "Measurement of intracellular cAMP content" sections. Data was expressed as mean ± standard error of the mean (SEM) of three independent experiments. $^{*}P < 0.05$ versus 5.6 G; $^{\#}P < 0.05$ versus 25 G

Table 1 Effects of high glucose, siPRMT1 on the GSIS and insulin content of INS-1 cells (±*s*)

Groups	BIS (4 mM glucose) (ng/mg protein/h)	GSIS (16.7 mM glucose) (ng/mg protein/h)	Insulin content (ng/mg protein)
5.6 G	40.0 ± 7.5	209.0 ± 18.8	1,391.5 ± 160.9
25 G	100.5 ± 8.1*	107.5 ± 15.2*	605.5 ± 96.5*
25 G + NC	100.0 ± 11.5*	107.5 ± 10.5*	577.8 ± 74.6*
25 G + PRMT1 silence	59.8 ± 8.9*,#	143.3 ± 12.5*,#	1,054.0 ± 132.4*,#

Experiments were performed four times. 5.6 G, cells cultured in media containing 5.6 mM glucose; 25 G, cells cultured in media containing 25 mM glucose. 25 G + NC, cells transfected with scrambled negative control siRNA, then cultured in media containing 25 mM glucose; 25 G + PRMT1 silence, cells transfected with PRMT1-siRNA, then cultured in media containing 25 mM glucose

BIS basal insulin secretion, *GSIS* glucose-stimulated insulin secretion

* $P < 0.05$ versus the 5.6 G group; # $P < 0.05$ versus the 25 G group

partially reduced the high glucose-mediated increase in basal $[Ca^{2+}]i$ and cAMP content and *restored* both $[Ca^{2+}]i$ and cAMP responses to glucose (Fig. 1b, c). In addition, high glucose treatment caused a significant increase in basal insulin secretion (BIS), dramatic decreases in GSIS, and intracellular insulin content and insulin mRNA levels (Fig. 1d); silencing PRMT1 expression significantly reduced high glucose-mediated β cell dysfunction (Fig. 1d; Table 1).

siPRMT1 regulates the intracellular localization of PDX-1 and FOXO1 under high glucose conditions

A growing body of evidence suggests that FOXO1 is an important regulator of pancreatic β cell function [40]. The major target of FOXO1 in β cells is PDX-1, which is a pivotal regulator of pancreatic development and normal β cell function. FOXO1 exerts effects on PDX-1 by repressing its expression and/or modulating its subcellular localization [33, 41]. To study the effects of siPRMT1 on the expression of PRMT1, PDX-1, FOXO1, and the nucleo-cytoplasmic trafficking of FOXO1 and PDX-1, INS-1 cells were transfected with siPRMT1, and then incubated with 25 mM glucose for 48 h. Cells were then harvested for total, cytoplasmic, and nuclear protein extraction and western blotting analysis. As shown in Fig. 2, PDX-1 levels were decreased by ~50 % in the 25 G group, which was accompanied by a twofold increase in

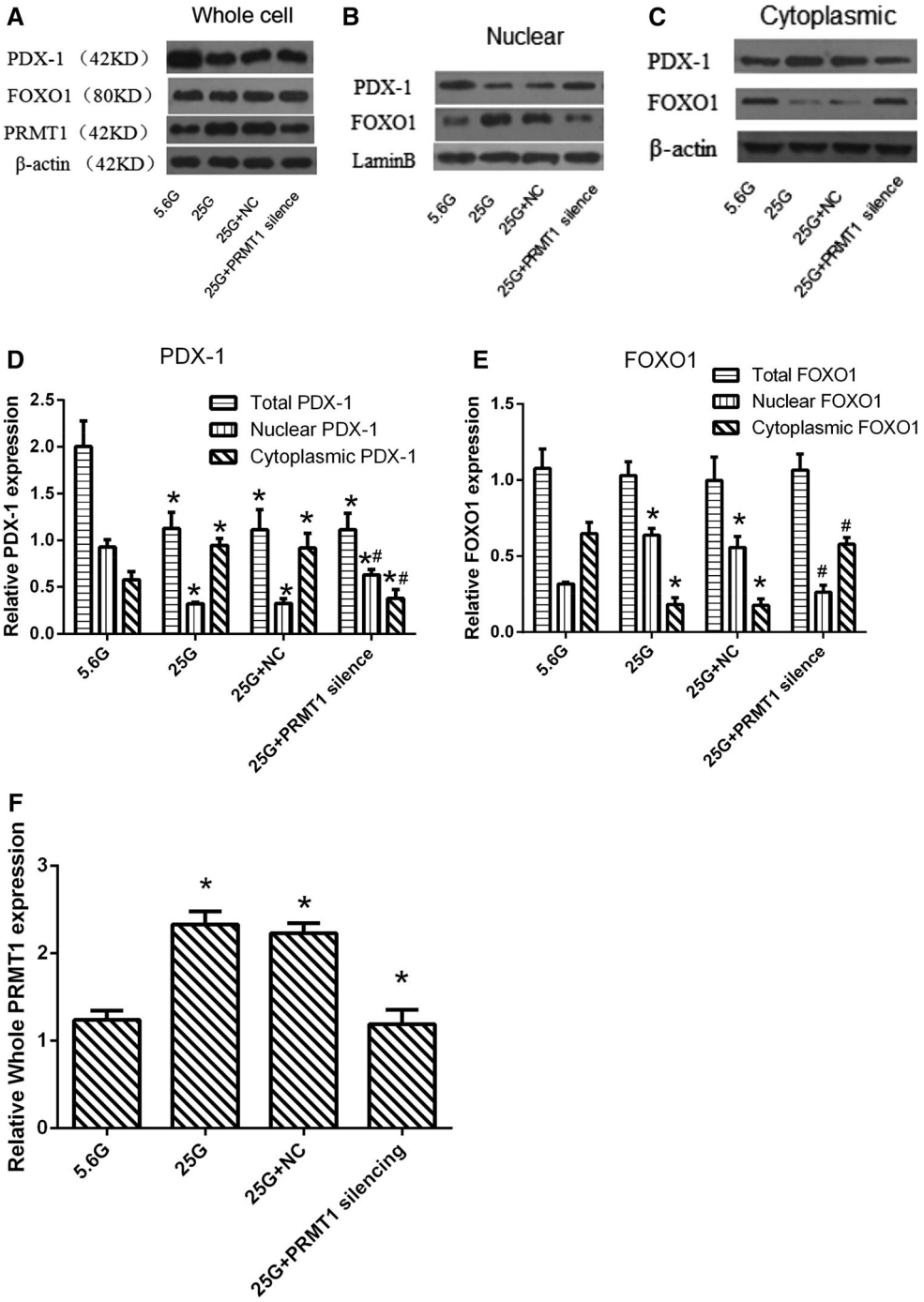


Fig. 2 The effects of high glucose and siPRMT1 on the expression of PRMT1, PDX-1, FOXO1, and the reciprocal translocations of FOXO1 and PDX-1. Whole cell (**a**), nuclear (**b**), and cytoplasmic (**c**) protein extracts were harvested, and western blotting was performed to assess the levels of nuclear and cytoplasmic PDX-1, FOXO1, and the total levels of PDX-1, FOXO1, and PRMT1. β-Actin and lamin B were used as loading controls for total or cytoplasmic and nuclear fractions, respectively. The immunoblots were representative of three independent experiments. The expression of PDX-1 (**d**), FOXO1 (**e**), AND PRMT1 (**f**) relative to the corresponding loading control was quantified by densitometric scanning and expressed as mean ± standard error of the mean (SEM) of three independent experiments. $^{*}P < 0.05$ versus 5.6 G, $^{\#}P < 0.05$ versus 25 G

PRMT1 expression (Fig. 2a, d, f). Although glucose had no obvious effect on the total levels of FOXO1, the levels of FOXO1 in the 25 G group were reduced in the cytoplasm and increased in the nucleus compared with the 5.6 G group (Fig. 2b, c, e), suggesting that high glucose stimulated the translocation of FOXO1 from cytoplasm to nucleus. In contrast, high glucose treatment caused the translocation of PDX-1 from the nucleus to the cytoplasm (Fig. 2b, c, d). Silencing PRMT1 expression reversed the high glucose-mediated translocations of FOXO1 and PDX-1 inside INS-1 cells (Fig. 2b–e), without affecting total PDX-1 and FOXO1 expression (Fig. 2a, d, e). Taken together, these data suggest that the impaired β cell function caused by high glucose concentrations might involve PRMT1-mediated changes in the reciprocal intracellular translocations of FOXO1 and PDX-1.

siPRMT1 regulates FOXO1 phosphorylation and methylation under high glucose conditions

Previously, PRMT1 was shown to catalyzes the asymmetric dimethylation of FOXO1 at arginines 248 and 250, thus interfering with Akt-dependent phosphorylation at adjacent serine 253 in mammalian cells [42]. We wanted to test a potential role of PRMT1 on FOXO1 in INS-1 cells. Indeed, we observed a strong association of endogenous PRMT1 and FOXO1 in INS-1 cells by co-immunoprecipitation assay (Fig. 3a). The nonselective arginine methylation

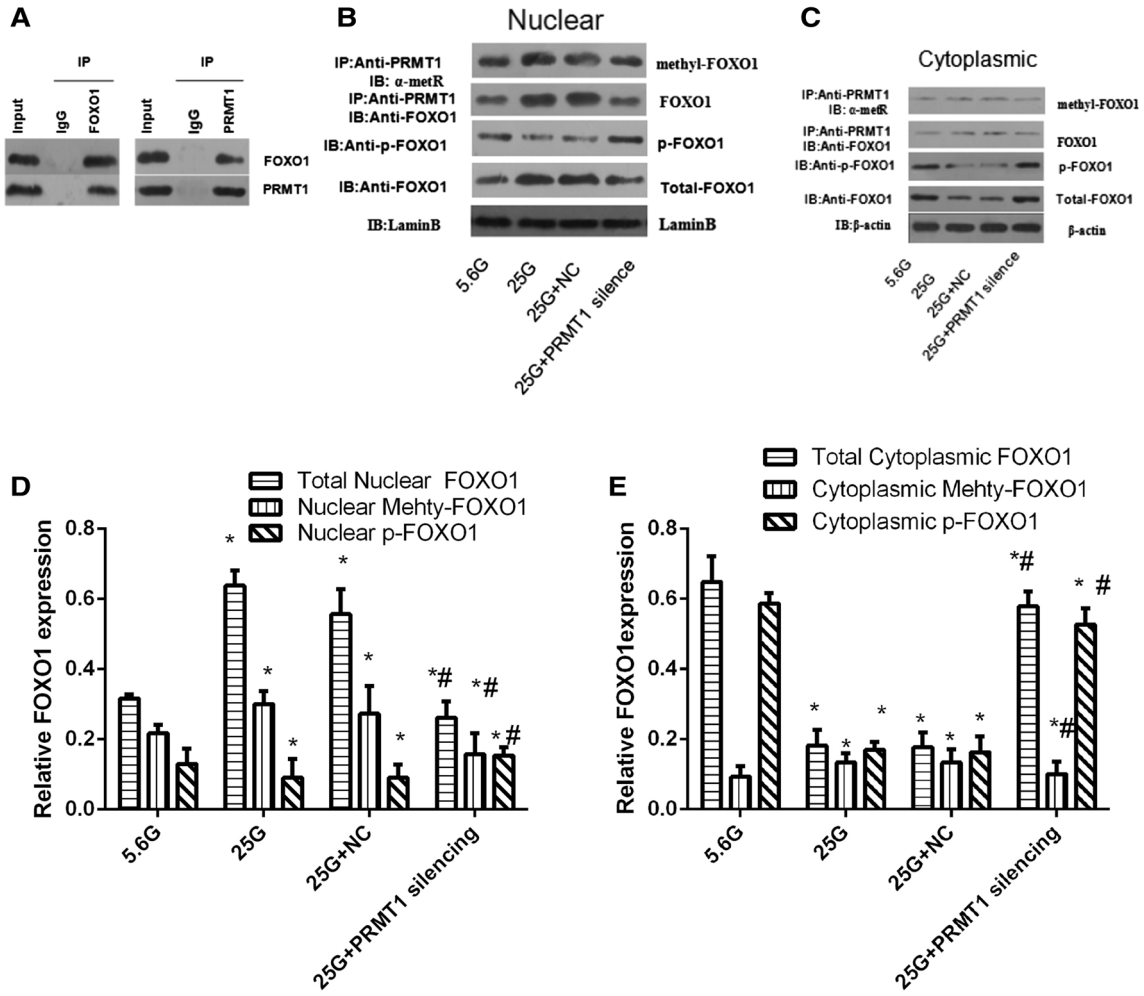


Fig. 3 *si*PRMT1 regulates FOXO1 phosphorylation and methylation under high glucose conditions. **a** Western blotting analysis showing endogenous interaction of FOXO1 and PRMT1. Cell lysates were immunoprecipitated with anti-FOXO1 antibody or anti-PRMT1 antibody and were immunoblotted with anti-PRMT1 antibody or anti-FOXO1 antibody. Representative data from at least three independent experiments are shown. **b**, **c** Western blotting analysis showing effects of PRMT1 silence on FOXO1 methylation and phosphorylation in the nucleus and cytoplasm. Nuclear (**b**) and cytoplasmic (**c**) protein extracts were harvested. Half of cells were lysed and then immunoprecipitated with anti-PRMT1 antibody, followed by immunoblotting with anti-FOXO1 antibody or α-metR and the other half were analyzed with anti-FOXO1 or anti-phospho-FOXO1 antibodies. Representative data from at least three independent experiments are shown

protein α-metR has been used to recognize a nonselective arginine methylation protein [43]. Using α-metR to detect an arginine methylation of immunoprecipitated FOXO1 from INS-1 cells and measurement of FOXO1 phosphorylation in cytoplasmic, and nuclear protein extraction by Western blotting, we were able to confirm high glucose treatment caused increased FOXO1 methylation and decreased FOXO1 phosphorylation in both nucleus and cytoplasm (Fig. 3b–e). In contrast, silencing PRMT1 expression reduced FOXO1 methylation and increased FOXO1 phosphorylation in both nucleus and cytoplasm (Fig. 3b–e). These data suggest that an reduction of PRMT1 expression reduced the arginine dimethylation and enhanced the phosphorylation of FOXO1 in INS-1 cells.

FOXO1 overexpression damages INS-1 cell function by reversing the reduced $[Ca^{2+}]$i and cAMP content caused by AMI-1

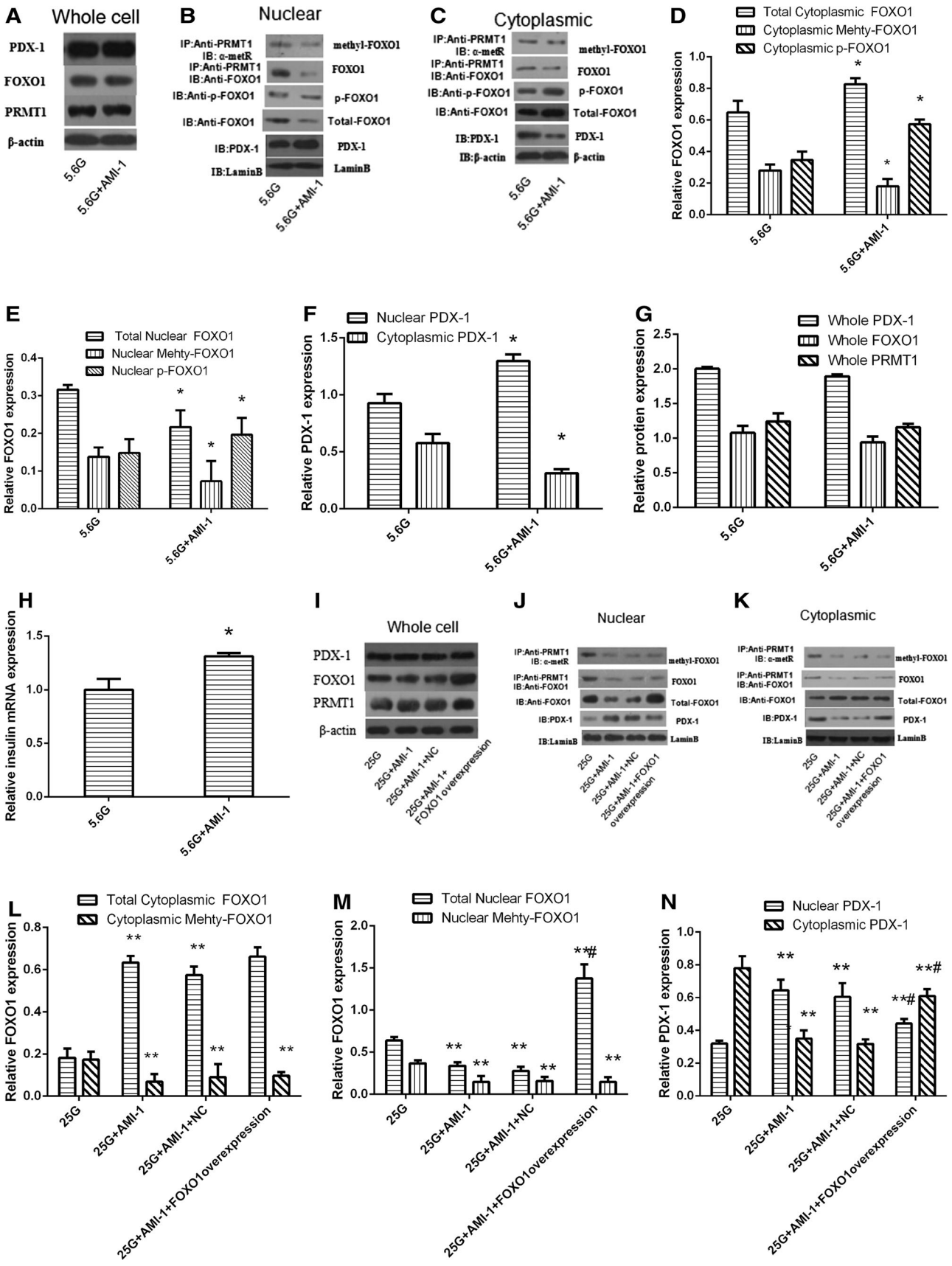
AMI-1 is a small molecule PRMT inhibitor that was first described by Cheng et al. [44]. We determined the effect of AMI-1 on insulin secretion and the nucleo-cytoplasmic trafficking of FOXO1 and PDX1 in INS-1 cells. Cells were incubated with 5.6 mM glucose without (control) or with AMI-1 (100 μM) for 48 h [44, 45], then harvested for western blotting analysis, GSIS assay, and insulin mRNA level. As shown in Fig. 4, AMI-1 significantly down-regulated methylation of FOXO1 and up-regulated the phosphorylation levels of FOXO1 in both nucleus and cytoplasm (Fig. 4b–e). In addition, AMI-1 caused the accumulation of PDX-1 in nucleus and translocation of FOXO1 from nucleus to cytoplasm (Fig. 4b–f) although having no obvious effect on the total protein levels of PDX-1, FOXO1, and PRMT1 proteins (Fig. 4a, g). These effects were further confirmed by increased insulin mRNA levels (Fig. 4h) and improved β cell function observed in INS-1 cells (Table 2). These data suggest that AMI-1 improves INS-1 cell function and mediates translocations of FOXO1 and PDX-1 intracellularly through regulates FOXO1 phosphorylation and methylation.

Although the nuclear accumulation of FOXO1 modulates the nucleo-cytoplasmic trafficking of PDX-1 [41], the role of this effect in the regulation of pancreatic β cell function remains controversial. The plasmid pALTER-FOXO1 contains a nuclearly localized constitutively active form of FOXO1, which mimics the effects of high glucose-induced FOXO1 intracellular location. This plasmid was used in this experiment to assess the importance of FOXO1-mediated PDX-1 translocation in high glucose-induced β cell dysfunction. In this experiment, cells were transfected with pALTER-FOXO1, and then incubated with 25 mM glucose in the presence of 100 μM AMI-1 for 48 h. The successful overexpression of the plasmid was first confirmed by western blotting (Fig. 4i, j, m, o). Importantly, overexpressing FOXO1 reversed the AMI-1-mediated translocations of FOXO1 and PDX-1 intracellularly, but did not alter FOXO1 methylation (Fig. 3j–n). These effects were further confirmed by reduction of insulin mRNA (Fig. 4p) and the increase in $[Ca^{2+}]$i and cAMP content (Fig. 3q, r) and reduced β cell function observed in FOXO1-overexpressing cells (Table 3). These data suggest that FOXO1 translocation, which mediates the nucleo-cytoplasmic shuttling of PDX-1, is essential cause of β cell dysfunction by high glucose culture conditions.

Fig. 4 FOXO1 overexpression reversed AMI-1-mediated reciprocal translocation of FOXO1 and PDX-1, and increased $[Ca^{2+}]$i and cAMP content. **a–g** AMI-1 induces reciprocal translocation of FOXO1 and PDX1 in INS-1 cells. INS-1 cells were incubated with 5.6 mM glucose without (control) or with AMI-1 (100 μM) for 48 h, followed by whole cell (**a**), nuclear (**b**), and cytoplasmic (**c**) protein extraction. Half of cells were lysed and then immunoprecipitated with anti-PRMT1 antibody, followed by immunoblotting with anti-FOXO1 antibody or α-metR and the other half were analyzed with anti-FOXO1 or anti-phospho-FOXO1 antibodies. **h** INS-1 cells were incubated with 5.6 mM glucose without (control) or with AMI-1 (100 μM) for 48 h, followed by cell total RNA was then harvested and analyzed by RQ-PCR. **i–o** AMI-1-induced intracellular translocation of FOXO1 and PDX1 is reversed by FOXO1 overexpression. INS-1 cells were transfected by pALTER-FOXO1 and pALTER-NC (scrambled negative control) plasmids as described in "INS-1 cell culture" section. Cells were then cultured for an additional 48 h in 25 mM glucose and AMI-1 (100 μM); and whole cell (**i**), nuclear (**j**), and cytoplasmic (**k**) protein extracts were harvested. The immunoblots in the figure were representative of three independent experiments. **p** Transfected or untransfected cell total RNA was then harvested and analyzed by RQ-PCR. The $[Ca^{2+}]$i (**q**) and cAMP content (**r**) were representative of four independent experiments. $^{*}P < 0.05$ versus 5.6 G; $^{**}P < 0.05$ versus 25 G; $^{\#}P < 0.05$ versus 25 G + AMI-1

Discussion

In the present study, we demonstrated that chronic exposure to high glucose increased basal insulin secretion, decreased glucose-stimulated insulin secretion and intracellular insulin content, insulin mRNA levels, increased basal $[Ca^{2+}]$i and cAMP levels, but abolishes both $[Ca^{2+}]$i and cAMP responses to glucose. This was caused by the significant decrease in PDX-1 expression, the disturbance of intracellular translocation of FOXO1 and PDX-1, and the increase in PRMT1 protein levels. In addition, blocking PRMT1 by either the specific inhibitor AMI-1 or the use of siRNA inhibited the high glucose-induced intracellular translocation of FOXO1 and PDX-1, resulting in a decreased basal intracellular $[Ca^{2+}]$i and cAMP content, increased insulin mRNA levels and *restored* both $[Ca^{2+}]$i and cAMP responses to glucose, improved β cell function, and insulin secretion in response to high level of glucose.


A
Whole cell
PDX-1
FOXO1
PRMT1
β-actin
5.6G
5.6G+AMI-1
B
Nuclear
IP:Anti-PRMT1
IB: α-metR
methyl-FOXO1
IP:Anti-PRMT1
IB:Anti-FOXO1
FOXO1
IB:Anti-p-FOXO1
p-FOXO1
IB:Anti-FOXO1
Total-FOXO1
IB:PDX-1
PDX-1
IB:LaminB
LaminB
C
Cytoplasmic
IB:β-actin
D
Total Cytoplasmic FOXO1
Cytoplasmic Mehty-FOXO1
Cytoplasmic p-FOXO1
Relative FOXO1 expression
E
Total Nuclear FOXO1
Nuclear Mehty-FOXO1
Nuclear p-FOXO1
F
Nuclear PDX-1
Cytoplasmic PDX-1
Relative PDX-1 expression
G
Whole PDX-1
Whole FOXO1
Whole PRMT1
Relative protien expression
H
Relative insulin mRNA expression
I
J
K
25G
25G+AMI-1
25G+AMI-1+NC
25G+AMI-1+FOXO1overexpression
L
M
N

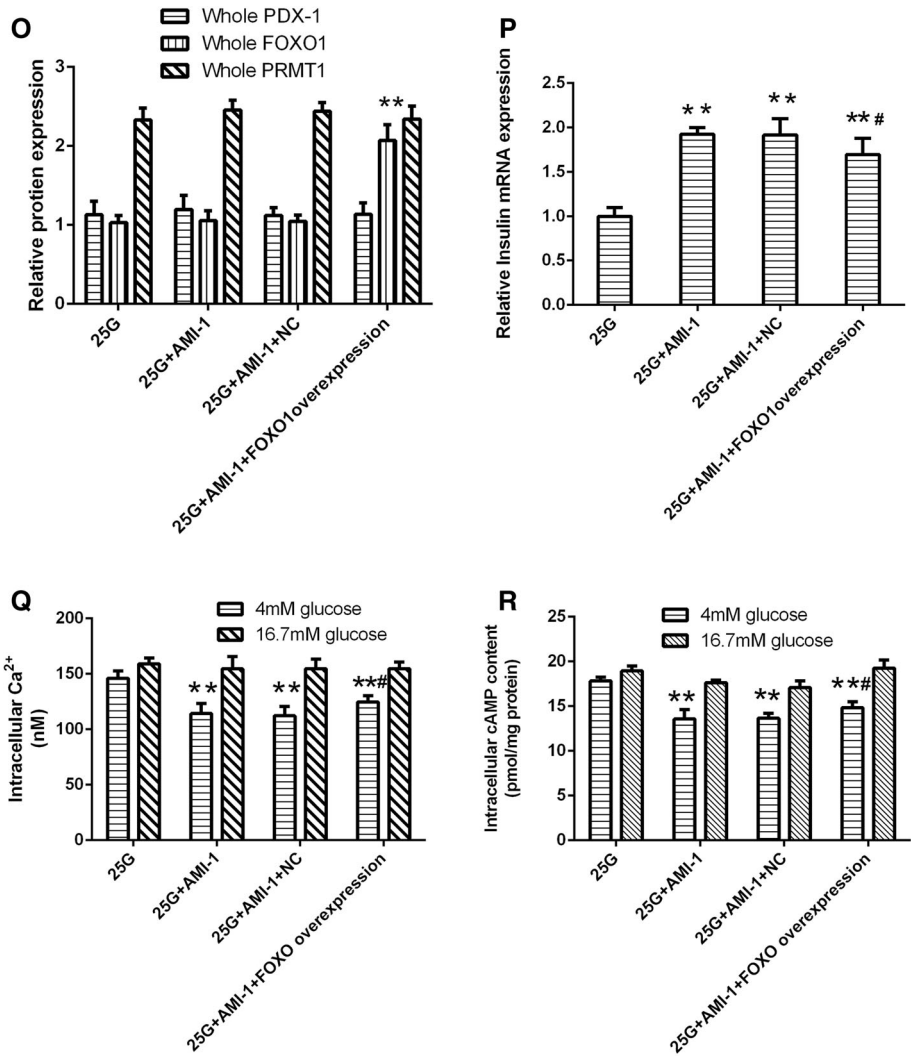


Fig. 4 continued

Table 2 Effects of AMI-1 on the GSIS and insulin content of INS-1 cells ($\bar{x} \pm s$)

Groups	BIS (4 mM glucose) (ng/mg protein/h)	GSIS (16.7 mM glucose) (ng/mg protein/h)	Insulin content (ng/mg protein)
5.6 G	44.8 ± 5.6	207.3 ± 17.7	1,391.5 ± 160.9
5.6 G + AMI-1	29.5 ± 2.9*	204.5 ± 20.0	1,799.5 ± 123.4*

Experiments were performed Four times. 5.6 G, cells cultured in media containing 5.6 mM glucose; 5.6 G + AMI-1, cells cultured in media containing 25 mM glucose with AMI-1 (100 μM)

* $P < 0.05$ versus the 5.6 G group

The export of PDX-1 from the nucleus to the cytoplasm was linked to the nuclear accumulation of FOXO1. FOXO1 overexpression increased basal intracellular Ca^{2+} and cAMP levels, reversed improved both $[Ca^{2+}]i$ and cAMP responses to glucose, which impaired β cell function. Moreover, the AMI-1-stimulated nuclear accumulation of PDX-1 was reversed by the overexpression of FOXO1, which caused β cell dysfunction.

Glucose homeostasis in mammals requires the appropriate insulin secretion from the β cells in pancreatic islets. The primary signal that activates insulin secretion is elevated blood glucose concentrations, which enhances the release of stored insulin [46]. Although exposing β cells to high concentrations of glucose for relatively short periods of time stimulates insulin secretion, chronic exposure has adverse effects on β cell function. For example, a previous

Table 3 Effects of FOXO1 overexpression on the GSIS and insulin content of INS-1 cells ($\bar{x} \pm s$)

Groups	BIS (4 mM glucose) (ng/mg protein/h)	GSIS (16.7 mM glucose) (ng/mg protein/h)	Insulin content (ng/mg protein)
25G	100.8 ± 7.3	107.5 ± 16.1	609.5 ± 104.3
25G + AMI-1	66.3 ± 8.4*	144.0 ± 18*	1,004.8 ± 78.2*
25G + AMI-1 + NC	64.0 ± 7.0*	143.8 ± 14.5*	1,003.0 ± 132.0*
25G + AMI-1 + FOXO1 overexpression	84.0 ± 7.7*,#	118.0 ± 12.6*,#	793.0 ± 76.8*,#

Experiments were performed four times

* $P < 0.05$ versus 25 G; # $P < 0.05$ versus the 25 G + AMI-1 group

study demonstrated that the prolonged exposure of INS-1 cells to high glucose diminished insulin secretion in response to glucose and intracellular insulin content [47, 48]. Consistent with this, our study revealed impaired basal and glucose-stimulated insulin secretion and intracellular insulin content after chronic exposure to high glucose. The reduction in insulin content may result from reduced insulin synthesis and hypersecretion during the culture at elevated glucose levels [49], we therefore tested the expression of insulin mRNA. Indeed, consistent with our data using qPCR, a large reduction in mRNA has been reported for INS-1 cells incubated for 48 h at 16.7 mm of glucose [24]. Thus, both increased basal insulin secretion and reduced insulin synthesis contribute to reduction in insulin content.

The levels of endoplasmic reticulum Ca^{2+} stores are regulated by cAMP. The intracellular cAMP level is determined by a balance between cAMP production by adenylate cyclases (ACs) and degradation by cyclic nucleotide phosphodiesterases. Glucose-stimulated AC activation in β cells increased cAMP levels, leading to increase levels of intracellular $[Ca^{2+}]i$ [50]. Similarly, the current study revealed that the exposure of INS-1 cells to chronic high glucose increased $[Ca^{2+}]i$ and cAMP concentrations. In addition, BIS was increased, whereas GSIS and intracellular insulin content were decreased. Previous studies revealed that high glucose toxicity increased intracellular $[Ca^{2+}]i$ and cAMP levels, which in turn impaired GSIS [47]. On the one hand, the increase in cAMP levels stimulated the release of Ca^{2+} stores, resulting in increased intracellular $[Ca^{2+}]i$ levels [6]; on the other hand, increase in cytosolic $[Ca^{2+}]i$ in turn promoted cAMP production by activating ACs [51]. The release of Ca^{2+} could then further increase $[Ca^{2+}]i$ levels via Ca^{2+}-mediated calcium release, thereby enhancing BIS in β cells. Previous studies revealed loss of a glucose-induced rise in cAMP and $[Ca^{2+}]i$ [52, 53]. Consistent with this, the current study revealed that chronic high glucose abolished both $[Ca^{2+}]i$ and cAMP responses to glucose in INS-1 cells. Some elevation of $[Ca^{2+}]i$ is necessary for insulin secretion in response to physiological stimuli [54]. Thus, the loss of stimulation of $[Ca^{2+}]i$ by glucose is a potential cause of the failure to insulin response. In our study, basal $[Ca^{2+}]i$ was markedly elevated by the high glucose culture, thus questioning the need for further $[Ca^{2+}]i$ elevation to induce insulin secretion after culture with glucose stimulation. However, no significant increase was shown in all groups, this implies a near-maximal effect by $[Ca^{2+}]i$ before the introduction of high glucose. Thus, it could be speculated that the elevation of $[Ca^{2+}]i$ not the glucose-stimutated $[Ca^{2+}]i$ levels promotes GSIS. In addition, the increased $[Ca^{2+}]i$ led to the L-type Ca^{2+} channel trafficking from plasma membrane fraction to cytoplasmic-free fraction; this resulted in decreased glucose-stimulated calcium influx, and subsequently impaired GSIS [55]. A previous study [56] reported PRMT1 activated RyR/Ca^{2+} release channels to promote Ca^{2+} release from the sarcoplasmic reticulum in coronary arterial smooth muscle cells. Johnson et al. [57] recently identified an increase in basal cytosolic $[Ca^{2+}]i$ and reduced β cell ER calcium levels with Pdx-1 knockdown, which could reflect a reciprocal increase in cytosolic $[Ca^{2+}]i$ resulting directly from a ER $[Ca^{2+}]i$ leak. Indeed, our data showed a increased basal cytoplasmic $[Ca^{2+}]I$ accompanied by increased PRMT1 and decreased PDX-1 expression under high glucose; and inhibiting methylation by PRMT1 knockdown or treatment with AMI-1 reversed this augment, accompanied by accumulation of PDX-1 in nucleus. However, the exact mechanism by which PRMT1 regulates cytoplasmic $[Ca^{2+}]i$ homeostasis need to be further studied in INS-1 cells.

AMI-1 was a selective and cell-permeable PRMT inhibitor [44] that dislodges protein/peptide substrates from the enzyme-binding pocket [58]. We determined the effect of AMI-1 on insulin secretion and the nucleo-cytoplasmic trafficking of FOXO1 and PDX1 in INS-1 cells. The data suggested that AMI-1 down-regulated methylation of FOXO1 and up-regulated the phosphorylation levels of FOXO1, resulting in exclusion of FOXO1 from nucleus and accumulation of PDX-1. In addition, AMI-1 increased

insulin mRNA level, insulin content, decreased basal insulin secretion, but had no effect on GSIS. A previous study [42] revealed that inhibiting methylation by PRMT1 knockdown or treatment with MTA, a nonselective methylation inhibitor, increased the cytoplasmic localization of FOXO1 in HeLa cells. Consistent with this, the current study demonstrated that inhibiting PRMT1 by knockdown or treatment with AMI-1, enhanced GSIS and modulated the intracellular translocations of FOXO1 and PDX-1 through down-regulated methylation of FOXO1 and up-regulated the phosphorylation levels of FOXO1. Taken together, these data suggest that PRMT1 regulates β cell function by inducing the intracellular translocations of FOXO1 and PDX-1, without affecting their total expressions. Furthermore, the effects of PRMT1 on FOXO1 translocation were due to a decreased FOXO1 methylation as shown in this study.

Current study revealed that chronic exposure to high glucose significantly decreased PDX-1 expression. Consistent with this, a previous study [21] reported a decrease in PDX-1 expression after exposure to chronic high glucose, leading to impaired insulin secretion. It was reported that PDX-1 was shuttled from the cytoplasm to the nucleus with 15 min in response to acute stimulation by high concentrations of glucose [29]. In contrast, the current study revealed that PDX-1 translocated from the nucleus to the cytoplasm in response to chronic stimulation with high concentrations of glucose. Moreover, because the *PDX-1* gene has a self-activation mechanism [59, 60], this translocation may result in a feedback mechanism, whereby reduced nuclear PDX-1 expression further decreases the total cellular expression of PDX-1.

The current study also revealed that chronic exposure to high glucose caused the nuclear accumulation of FOXO1 and the nucleo-cytoplasmic shuttling of PDX-1 in INS-1 cells. Two mechanisms by which FOXO1 may regulate PDX-1 have been proposed. First, FOXO1 may suppress FOXA2-dependent activation of the PDX-1 promoter [41]. Second, FOXO1 could regulate the subcellular localization of PDX-1 [33]. To further explore how FOXO1 regulates PDX-1, and determine whether PRMT1 induces the intracellular shuttling of PDX-1 via FOXO1 translocation, we overexpressed FOXO1 in the nucleus in the presence of AMI-1. The data suggested that FOXO1-regulated PDX-1 by modulating its subcellular translocation, but not by inhibiting total PDX-1 expression. Consistent with this, a previous study [42] demonstrated that the methylation of FOXO1 via the PRMT1-dependent modification of arginine residues 248 and 250 was critical for blocking the Akt-induced phosphorylation of FOXO1 at Ser253. This increase in FOXO1 nuclear localization inhibited the subsequent degradation in HeLa cells.

Conclusion

This study demonstrated that the prolonged exposure of INS-1 cells to high concentrations of glucose impaired basal and glucose-stimulated insulin secretion, and increased basal intracellular Ca^{2+} and cAMP levels. Inhibition of PRMT1 improved β cell function through decreased basal intracellular Ca^{2+} and cAMP levels and reversed the high glucose-induced translocation of PDX-1 caused by the nucleo-cytoplasmic translocation of FOXO1 through down-regulated methylation of FOXO1. The overexpression of FOXO1 partially reversed the protective effect of AMI-1 on β cell function. These signaling changes uncover a novel mechanism that may contribute to the β cell dysfunction caused by hyperglycemia in diabetes.

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Conflict of interest All the authors have no conflict of interest to declare.

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