Dynamic Regulation of PDX-1 and FoxO1 Expression by FoxA2 in Dexamethasone-Induced Pancreatic β-cells Dysfunction

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Transcription factors forkhead box (Fox)O1 and pancreatic and duodenal homeobox-1 (PDX-1) are involved in dexamethasone (DEX)-induced dysfunction in pancreatic β-cells. However, the molecular mechanism underlying the regulation of FoxO1 and PDX-1 expression in β-cells treated with DEX is not fully understood. In this study, we found that DEX markedly increased FoxO1 mRNA and protein expression, whereas it decreased PDX-1 mRNA and protein expression in a dose- and time-dependent manner. Further study showed that FoxA2 was involved in regulation of FoxO1 and PDX-1 expression in DEX-induced pancreatic β-cells dysfunction. Interestingly, we demonstrated for the first time that FoxA2 could bind to the *FoxO1* gene promoter and positively regulate FoxO1 expression. Moreover, we found that DEX increased the activity of FoxA2 binding to the *FoxO1* promoter but decreased the activity of FoxA2 binding to the *PDX-1* promoter of RINm5F cells. Knockdown of FoxA2 by RNA interference inhibited FoxO1 expression and restored PDX-1 expression in pancreatic β-cells treated with DEX. However, DEX had no effect on the expression of FoxA2. Together, the results of the present study demonstrated that FoxA2 could dynamically regulate FoxO1 and PDX-1 expression in pancreatic β-cells treated with DEX, which provides new important information on the transcriptional regulation of FoxO1 and PDX-1 in DEX-induced pancreatic β-cells. Inhibition of FoxA2 can effectively protect β-cells against DEX-induced dysfunction. **(*Endocrinology* 152: 1779–1788, 2011)**

Steroid diabetes mellitus is a well-known severe side effect of long-term and high-dose glucocorticoid treatment (1, 2). Besides peripheral insulin resistance and excessive hepatic glucose production, glucocorticoid excess can directly impair pancreatic β-cell function (3–7). Due to the importance of pancreatic β-cell function in diabetes mellitus, more and more attention is paid to understanding the β-cell destruction under treatment with glucocorticoids in recent years. The molecular mechanisms involved are not fully understood, but evidence has been accumulating to show that treatment with glucocorticoids could down-regulate expression of the pancreatic and duodenal homeobox-1 (PDX-1) in pancreatic β-cells (8, 9).

PDX-1, which is expressed in β-cells and the few δ-cells of the adult islet of Langerhans, is one of the well-studied transcription factors that are critical to both β-cell development and function (10, 11). Previous studies showed that the transcription factor, forkhead box (Fox)A2 [hepatocyte nuclear factor (HNF)3β], could bind to the PDX-1 promoter and positively regulate PDX-1 gene expression both *in vitro* and *in vivo* (12–15). On the other hand, PDX-1 was reported to be negatively regulated by another forkhead transcription factor FoxO1 in pancreatic β-cells (16). Kitamura *et al.* (17) reported that FoxO1 and FoxA2 shared common DNA-binding sites in the PDX-1 promoter (PDX-1 homology region 2), which in-

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2010-1048 Received September 7, 2010. Accepted February 14, 2011.
First Published Online March 8, 2011

Abbreviations: ChIP, Chromatin immunoprecipitation; Ct, threshold cycle; DC, Detergent Compatible; DEX, dexamethasone; FBS, fetal bovine serum; Fox, forkhead box; GSIS, glucose-stimulated insulin secretion; HNF, hepatocyte nuclear factor; KRB, Krebs-Ringer bicarbonate; PDX-1, pancreatic and duodenal homeobox-1; qPCR, quantitative real-time PCR; siRNA, small interfering RNA.

dicated that both FoxO1 and FoxA2 could regulate PDX-1 expression through competition at the same binding sites.

It has been reported that overexpression of FoxA2 could restore inhibition of PDX-1 expression mediated by dexamethasone (DEX), a synthetic glucocorticoid (18). In our previous study, we found that DEX could stimulate the expression of FoxO1, which resulted in the inhibition of PDX-1 and impairment of glucose-stimulated insulin secretion (GSIS) function in pancreatic islet β-cells (19). However, silencing of FoxO1 expression could only partially restored PDX-1 expression levels when RINm5F cells were exposed to DEX. To further explore the mechanism of DEX suppression of PDX-1 expression, we investigated the effect of FoxA2 on regulation of FoxO1 and PDX-1 expression in RINm5F cells under DEX treatment. Our results demonstrated that the binding activity of FoxA2 on the *PDX-1* promoter was inversely related to that of FoxA2 on the *FoxO1* promoter. Thus, we tested in this study the hypothesis that FoxA2 is involved in DEX-induced dynamic regulation of FoxO1 and PDX-1 expression.

Materials and Methods

Reagents

RPMI 1640 and Lipofectamine 2000 transfection reagent were obtained from Invitrogen Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was purchased from GIBCO (Burlington, Ontario, Canada). DEX, antibody against β-actin, and type V collagenase were from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal antibody against PDX-1 was from Upstate Cell Signaling Solutions (Lake Placid, NY). Mouse polyclonal antibodies against α-tubulin, rabbit polyclonal antibodies against FoxO1 as well as goat polyclonal antibodies against insulin and FoxA2 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated antirabbit and antigoat IgG were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Prestained protein markers and restriction enzymes were from New England Biolabs (Beverly, MA). The Detergent Compatible (DC) Protein Assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). The Luciferase Assay System was obtained from Promega (Madison, WI). The TaqMan One-step PCR Master Mix Reagents kit and Assays-on-Demand gene expression products were purchased from ABI (Applied Biosystems, Foster City, CA).

Cell culture

RINm5F, a rat insulinoma cell line, was obtained from American Type Culture Collection (Manassas, VA). The cells were grown in RPMI 1640 medium supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 1 mM pyruvate sodium, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 C in a humidified atmosphere containing 95% air and 5% CO_2. Cultured cells were treated with DEX (dissolved in 100% ethanol) or transfected with FoxA2 expression plasmid in complete RPMI 1640 medium. To obtain reliable results, the final concentration of ethanol in the culture medium was kept less than 0.1%, and the total plasmid was equal in each well.

Islet isolation and culture

Male 8-wk-old Sprague Dawley rats were purchased from Nanjing Medical University Laboratory Animal Centre. Islet isolation and culturing techniques were described previously (20). Freshly isolated islets were transferred to sterile six-well dishes and cultured in RPMI 1640 plus 10% FBS at 37 C and 5% CO_2. The islets were allowed to equilibrate for 3 h, after which they were counted and repacked into six-well plates (400 islets per well for RNA or protein extraction) and cultured overnight for further studies.

Real-time PCR assay

The total RNA of RINm5F cells and rat islets were extracted by TRIzol reagent (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. After spectrophotometric quantification, 1 μg total RNA was used for RT in a 20 μl final volume with avian myeloblastosis virus reverse transcriptase (Promega) according to the manufacturer's instructions. cDNA aliquots corresponding to equal amounts of RNA were used for quantification of mRNA by real-time PCR using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The reaction system (20 μl) contained the corresponding cDNA, forward and reverse primers, and SYBR GREEN PCR Master Mix (Applied Biosystems). The specific primers were as follows: 1) FoxO1, 5′-AAGAGGCTCACCCTGTCGC-3′ (forward) and 5′-GCATCCACCAAGAACTTTTCC-3′ (reverse); 2) PDX-1, 5′-GGATGAAATCCACCAAAGCTC-3′ (forward) and 5′-TTCCACTTCATGCGACGGT-3′ (reverse); and 3) FoxA2, 5′-GTATGCTGGGAGCCGTGAAG-3′ (forward) and 5′-AGCCTGCGCTCATGTTGC-3′ (reverse). All data were analyzed using β-actin gene expression as an internal standard.

Western blot analysis

RINm5F cells and isolated rat islets were lysed with ice-cold lysis buffer containing: 50 mmol/liter Tris-HCl (pH 7.4), 1% Nonidet P-40, 150 mmol/liter NaCl, 1 mmol/liter EDTA, 1 mmol/liter phenylmethylsulfonyl fluoride, and complete proteinase inhibitor mixture (one tablet per 10 ml; Roche Molecular Biochemicals, Indianapolis, IN). Nuclear and cytoplasmic extracts were prepared using the nuclear extraction kit (Pierce, Rockford, IL). Protein concentration in the cell lysate was quantified using the DC Protein Assay kit (Bio-Rad Laboratories). After protein content determination using a DC Protein Assay kit, Western blot analysis was performed as described (21).

Construction of reporter gene plasmids

Luciferase reporter constructs containing the rat FoxO1 promoter (−1137/+5) were prepared by using the pGL3-promoter vector. The rat FoxO1 promoter region was amplified by PCR from the rat genomic DNA with appropriate primers and using standard PCR conditions. The following primers (including the sites of restriction enzymes) were synthesized: forward, 5′-GGGCCTCGAGATCTTCAATTCTAAGGTGTC-3′ and reverse, 5′-AAAAAGCTTCCCTTGACTGACAGGCTGC-3′. The PCR amplifications were performed using a thermocycler (Stratagene, La Jolla, CA) under the following conditions: 98 C for 5 min (1 cycle); 95 C for 10 sec, 58 C for 15 sec, and 72 C for 2 min (30 cycles); and final extension of 10 min at 72 C. The PCR products were resolved on an agarose gel, and the correct sized fragments were recovered using a DNA extraction kit. The recovered fragments and pGL3-promoter vector were digested with the corresponding restriction enzymes to construct the

pGL3-FoxO1 promoter luciferase reporter plasmid. All sequences were confirmed by automated DNA sequencing.

Construction of expression plasmids

To construct the FoxA2 expression plasmid, the coding sequences for these factors were amplified by PCR from the rat full-length cDNA based on the GenBank sequences. The following primers (including the sites of restriction enzymes) were synthesized: forward, 5′-AATTCTCGAGATGCTGGGAGCCGTGAAGATG-3 and reverse, 5′-GCGCAAGCTTTTAGGACGAGTTCATAATAGGC-3′. The PCR amplifications were performed under the following conditions: 94 C for 5 min (1 cycle); 95 C for 10 sec, 58 C for 15 sec, and 72 C for 2 min (30 cycles); and final extension of 10 min at 72 C. The PCR products were resolved on an agarose gel, and the correct sized fragments were recovered using a DNA extraction kit. The recovered fragments and pAdTrack-CMV vector were digested with the corresponding restriction enzymes to construct the FoxA2 expression plasmids. All sequences were confirmed by automated DNA sequencing.

Generation of recombinant adenoviruses overexpressing FoxA2

The coding sequence of FoxA2 was cloned into pAdTrack-CMV using *Kpn*I and *Hin*dIII restriction enzymes. DNA encoding the FoxA2 was subcloned from pCMV3.0b-myc-FoxA2 into pAdTrack-CMV. All sequences were confirmed by automated DNA sequencing. These shuttle plasmids were then recombined with the backbone vector pAdEasy-1 in BJ5183 bacteria. Adenovirus generation, amplification, and titration were performed as described (19). Viral particles were purified using the ViraBind Adenovirus Purification kit (Cell Biolabs, Inc., San Diego, CA). Isolated rat islets were infected with adenovirus at a multiplicity of infection of 100 at 37 C for 2 h, the islets were then cultured in fresh medium for 24 or 48 h.

Transient transfection and luciferase reporter assay

RINm5F cells were transiently cotransfected with three plasmids (0.8 μg of luciferase reporter plasmid containing FoxO1 promoter, several doses of expression plasmid or PCMV3.0b vector, and β-galactosidase as an internal control for transfection efficiency) using the Lipofectamine Plus regent. RINm5F cells were passaged on 12-well plates the day before transfection to achieve 90–95% confluence on the day after. Thirty-six hours after transfection, the cells were washed with PBS and lysed using 1× passive lysis buffer. Luciferase activity was determined as previously described (22).

Knockdown of FoxA2 by RNA interference

FoxA2-specific small interfering RNA (siRNA) and control siRNA were designed and synthesized by RIBOBIO (Ribobio Co., Ltd., Guangzhou, China). The sequences of the three designed FoxA2 siRNAs were as follows: FoxA2 siRNA-1, 5′-GGUCUCGGGUCUGAUUUAAtt-3′; siRNA-2, 5′-GGACCUCAAGACCUACGAAtt-3′; and siRNA-3, 5′-GCCAAUAUGAACUCCAUGAtt-3′. RINm5F cells were transiently transfected with siRNA using the Lipofectamine 2000 reagent according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were treated with 100 nM DEX. After 24 or 48 h of treatment, the cells were harvested for real-time PCR or Western blot analysis as described above.

We also used siRNA to knockdown FoxA2 expression in isolated rat islets. One hour before transfection, islets (400 islets per well of six-well plate) were cultured in 2 ml of antibiotic-free medium. For each transfection sample, FoxA2 siRNA or control siRNA was diluted in 200 μl serum-free medium, and final concentration adding to islets was 100 nM; 4 μl Lipofectamine 2000 was also diluted in 200 μl serum-free medium and incubated for 5 min at room temperature. Then, the mixture of the diluted siRNA and the diluted Lipofectamine 2000, which was incubated for 20 min at room temperature, was added to each well containing islets and medium. The medium was changed to RPMI 1640 plus 10% FBS after islets were incubated at 37 C and 5% CO_2 for 6 h. The islets were cultured in fresh medium for another 18 h before treating with DEX (100 nM) for 48 h. Transfection efficiency was monitored using 100 nM of Cy3-NControl (fluorescence labeled siRNA) for 24 h.

GSIS assay

Isolated rat islets (eight islets per well) were seeded into 500 μl RPMI 1640 medium with standard glucose concentration (11.1 mM) in a 48-well plate and treated with corresponding drugs for 48 h as described above. After incubation for 1 h in glucose-free Krebs-Ringer bicarbonate (KRB) buffer (115 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM KH_2PO_4, 20 mM $NaHCO_3$, 16 mM HEPES, 2.56 mM $CaCl_2$, and 0.2% BSA) and drug solutions, the cells were treated for 1 h in KRB buffer and drug solutions with low (3.3 mM) and high (16.7 mM) glucose. The supernatants were obtained for insulin concentration determination using RIA as described (23).

Chromatin immunoprecipitation (ChIP) assay

RINm5F cells were seeded in 10-cm plates and transfected with 12 μg of FoxA2 expression plasmid for 16 h. Then the chromatin in the cells was fixed and immunoprecipitated using the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) following the manufacturer's protocol. Briefly, cells were harvested and fixed in 1% (vol/vol) formaldehyde for 10 min at room temperature. After washing with cold PBS, cells were lysed with sodium dodecyl sulfate lysis buffer. The lysates were sonicated to shear the genomic DNA to 200-1000 bp in size using Sonicator VCX130 (Sonics & Materials, Newtown, CT). Chromatin solution was precleared with A-agarose beads for 1 h at 4 C, and the supernatant was collected. A portion of the chromatin solution (1%) was reserved as input sample. The remaining chromatin was incubated overnight at 4 C with anti-FoxA2 antibody (Santa Cruz Biotechnology, Inc.) and normal goat serum (IgG) as a negative control. The chromatin/antibody complexes captured on the beads were extensively washed and then eluted with 200 μl of elution buffer. The immunoprecipitated and input samples were incubated for 5 h at 65 C to reverse the cross-links. After treatment with ribonuclease and proteinase K for 1 h, the DNA was purified by phenol-chloroform extraction. The presence of the selected DNA sequence was assessed by PCR using primers framing the rat FoxO1 promoter region of interest (−1219 to −1006 bp, 214 bp): forward primer, 5′-TCAATTCTAAGGTGTCCCTAGTC-3′ and reverse primer, 5′-TGGGGCACAGCTCGTCTC-3′ [these primers were also used for ChIP-quantitative real-time PCR (qPCR)]. PCR conditions were as follows: 94 C, 3 min; 30 cycles at 94 C for 30 sec, 56 C for 30 sec, and

72 C for 1 min; final elongation at 72 C for 10 min. The PCR products were analyzed on a 1.5% agarose gel.

ChIP-qPCR assay was used to examine whether DEX could affect the binding activity of FoxA2 on the *PDX-1* and *FoxO1* gene promoters. RINm5F cells were seeded in 10-cm plates and treated with DEX for 1, 3, or 24 h. ChIP assay was performed as described above. The relative proportions of coimmunoprecipitated promoter fragments were determined based on the threshold cycle (Ct) value for each PCR (24). For every promoter studied, a ΔCt value was calculated for each sample: ΔCt = Ct (sample) − Ct (input). Next, a ΔΔCt value was calculated: ΔΔCt = ΔCt (sample immunoprecipitated with FoxA2 antibody) − ΔCt (sample immunoprecipitated with IgG). The fold difference between specific antibody-immunoprecipitated samples and those immunoprecipitated with IgG was thus calculated using the formula: $2^{-\Delta\Delta Ct}$. Due to FoxO1 and FoxA2 sharing common DNA-binding sites in the *PDX-1* promoter, we used the same primers to determine the activity of FoxA2 on the *PDX-1* promoter and that of FoxO1 on the *PDX-1* promoter. Sequences of the primers were 5′-TGTGAAAGGCAGCGAGTT-3′ (forward) and 5′-ACCTGCCTAACCCACACC-3′ (reverse).

Statistical analysis

Comparisons were performed using Student's *t* test for two groups. Data were presented as mean ± SEM. *P* values of less than 0.05 were considered statistically significant and are provided in the figures.

Results

DEX increases FoxO1 expression and decreases PDX-1 expression in a dose- and time-dependent manner

We previously reported that inhibition of FoxO1 could protect pancreatic β-cells against DEX-induced dysfunction (19). In this study, we further investigated the change of both FoxO1 and PDX-1 expression under DEX treatment. As shown in Fig. 1, FoxO1 mRNA levels increased (Fig. 1A), whereas PDX-1 mRNA levels decreased (Fig. 1B) in a dose-dependent manner in RINm5F cells after the treatment of DEX for 3 h. Moreover, the results also showed a time-dependent increase in FoxO1 mRNA levels, but a decrease in PDX-1 mRNA levels was observed under treatment with 100 nM DEX (Fig. 1, C and D). Consistent with changes in mRNA levels, FoxO1 protein levels increased as PDX-1 protein levels decreased in a dose- and time-dependent manner when cells were treated with DEX (Fig. 1, E and F). Thus, these results indicated that DEX strongly induced FoxO1 expression and inhibited PDX-1 expression in pancreatic β-cells.

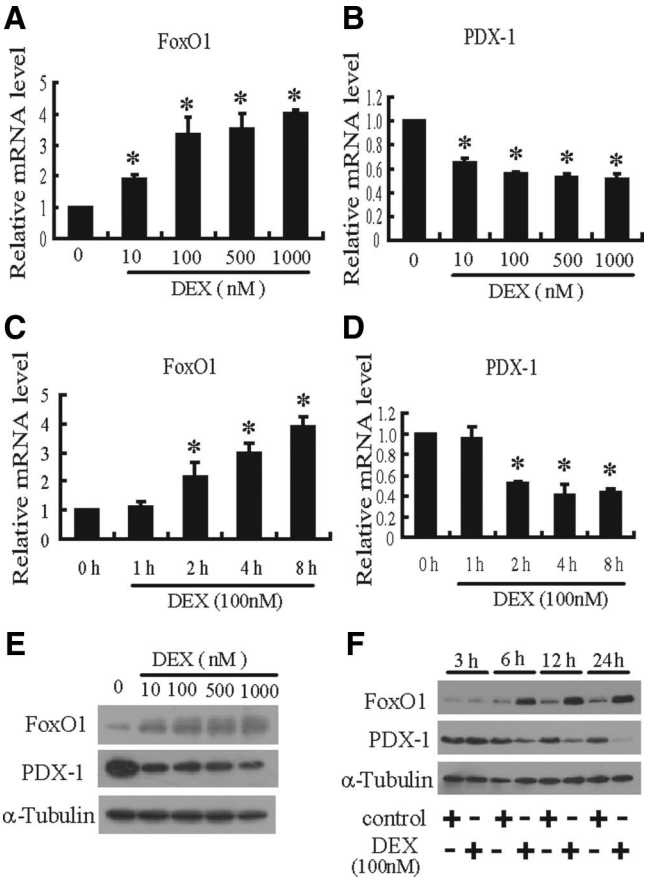

FIG. 1. DEX increases FoxO1 expression and decreases PDX-1 expression in a dose- and time-dependent manner. A, RINm5F cells were treated without (control) or with varying concentrations of DEX for 3 h and then harvested for real-time PCR analysis to determine FoxO1 mRNA levels. B, RINm5F cells were treated without (control) or with varying concentrations of DEX for 3 h and then harvested for real-time PCR analysis to determine PDX-1 mRNA levels. C, RINm5F cells treated with DEX (100 nM) were harvested at indicated times for real-time PCR to determine FoxO1 mRNA levels. D, RINm5F cells treated with DEX (100 nM) were harvested at indicated times for real-time PCR to determine PDX-1 mRNA levels. *, $P < 0.05$ *vs.* control. E, RINm5F cells were treated with varying concentrations of DEX for 24 h, and FoxO1 and PDX-1 protein levels were determined by Western blot analysis. F, FoxO1 and PDX-1 protein levels in RINm5F cells treated with DEX (100 nM) for the indicated times were determined by Western blot analysis.

FoxA2 exerts a positive effect on expression of FoxO1

The FoxA2 transcription factor was reported to directly enhance the expression of PDX-1 by binding to the promoter of the *PDX-1* gene (25). However, it was not known whether FoxA2 could regulate the expression of FoxO1. Therefore, we used RINm5F cells and rat islets transfected with plasmid and infected with adenovirus, respectively, to investigate the effects of FoxA2 on expression of FoxO1 in detail. Here, we found that overexpression of FoxA2 in RINm5F cells led to a dose-dependent increase in *FoxO1* promoter activity (Fig. 2A) and a time-dependent increase in FoxO1 mRNA levels (Fig. 2B).

Consistent with alteration of the mRNA level, FoxO1 proteins also increased in the cells in a dose- and time-dependent manner after transfection with FoxA2 expression plasmid (Fig. 2, C and D). The effect of FoxA2 on FoxO1 expression in primary cultures of rat islets was also

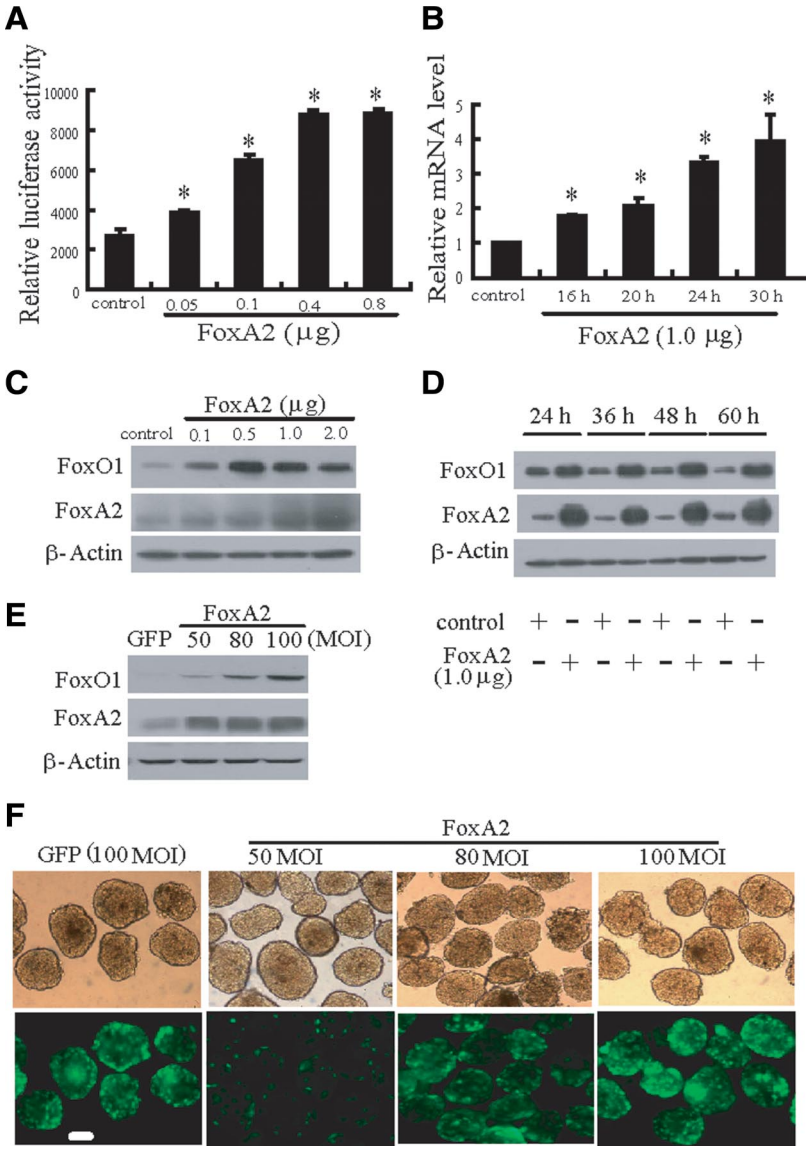

FIG. 2. FoxA2 positively affected FoxO1 expression in RINm5F cells and rat islets. A, RINm5F cells were transiently transfected with various amounts of pcDNA3.0-wild-type FoxA2 expression plasmid (or control pcDNA3.0 plasmid) and a pGL3-FoxO1 promoter luciferase reporter plasmid. β-Galactosidase was cotransfected with the reporter construct to normalize the luciferase activity. Luciferase activity was assayed 24 h after transfection. B, RINm5F cells were transiently transfected with the FoxA2 expression plasmid and the control plasmid at indicated times. Twenty-four hours after transfection, the cells were harvested for real-time PCR to determine FoxO1 mRNA levels. *, $P < 0.05$ *vs.* control. C, RINm5F cells were transiently transfected with various amounts of FoxA2 expression plasmid and the control plasmid for 48 h, and then FoxO1 protein levels were assayed by Western blot analysis. D, RINm5F cells were transiently transfected with 1.0 μg of FoxA2 expression plasmid and the control plasmid for the indicated times, and then FoxO1 protein levels were assayed by Western blot analysis. E, The rat islets were infected with FoxA2 expression adenovirus and control adenovirus for 48 h, and then FoxO1 protein levels were determined by Western blot analysis. F, Representative images of islets infected with indicated adenoviruses for 48 h. *Scale bar*, 100 μm.

evaluated and confirmed (Fig. 2E). Together, our data demonstrated that FoxA2 could up-regulate the expression of FoxO1 at both mRNA and protein levels.

FoxA2 binds to the *FoxO1* gene promoter

After the observations above, a key question that remains unanswered was how FoxA2 regulates the expression of FoxO1. By using the MATCH and PATCH programs of Transfac Professional 7.0, we predicted that FoxA2 may bind to the *FoxO1* gene promoter and at two possible FoxA2-binding sites, located within the rat *FoxO1* promoter −1145 from the transcriptional start site. When using primers framing the rat *FoxO1* promoter region of interest (−1219 to −1006 bp, 214 bp), an obvious PCR product was amplified from the cross-linked chromatin immunoprecipitated with anti-FoxA2 antibody. By contrast, little PCR-amplified product was observed after the immunoprecipitation using a control goat IgG (Fig. 3A). Furthermore, the PCR amplification product was not observed from immunoprecipitation with either the anti-FoxA2 antibody or the goat IgG when using the other primers (−985/−761 bp, 225 bp) (Fig. 3B).

DEX treatment causes FoxA2 to dissociate from the *PDX-1* promoter to bind to the *FoxO1* promoter

To explore whether DEX could affect FoxA2 transcriptional activity on the *PDX-1* and *FoxO1* promoters, ChIP-qPCR assay was performed by using specific primers. The results showed that DNA fragments pulled down by the anti-FoxA2 antibody in the DEX treatment group for 1, 3, and 24 h were reduced to approximately 0.37-, 0.56-, and 0.69-fold, respectively, for the *PDX-1* promoter site (Fig. 4A). However, the effect of DEX on FoxA2 binding to *FoxO1* promoter activity was elevated up to 1.8-, 2.9-, and 3-fold, respectively, for the *FoxO1* promoter site (Fig. 4B). Additionally, in the control groups, DNA fragments pulled down by anti-FoxA2 antibody were five times higher than those pulled down by IgG for the *PDX-1* promoter site. As for the *FoxO1* promoter site, DNA fragments pulled down by anti-FoxA2 antibody were almost equal to those pulled down by the control IgG.

DEX treatment enhances FoxO1 binding to the *PDX-1* promoter

It has been established that FoxO1 inhibits PDX-1 gene transcription by binding to the PDX-1 homology region 2 of the *PDX-1* promoter, which contains a FoxO1-binding site (17). It is also known that the PDX-1 expression levels

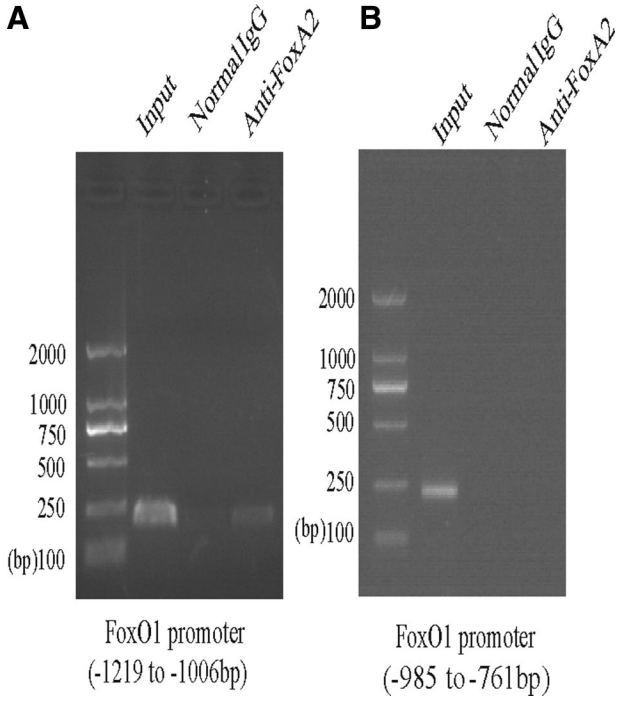

FIG. 3. FoxA2 could bind to *FoxO1* promoters. A, FoxA2 bound directly to the *FoxO1* promoter (−1219/−1006 bp, 214 bp) in RINm5F cells in a ChIP analysis. B, FoxA2 did not bind to another *FoxO1* promoter (−985/−761 bp, 225 bp) in RINm5F cells in the ChIP assay.

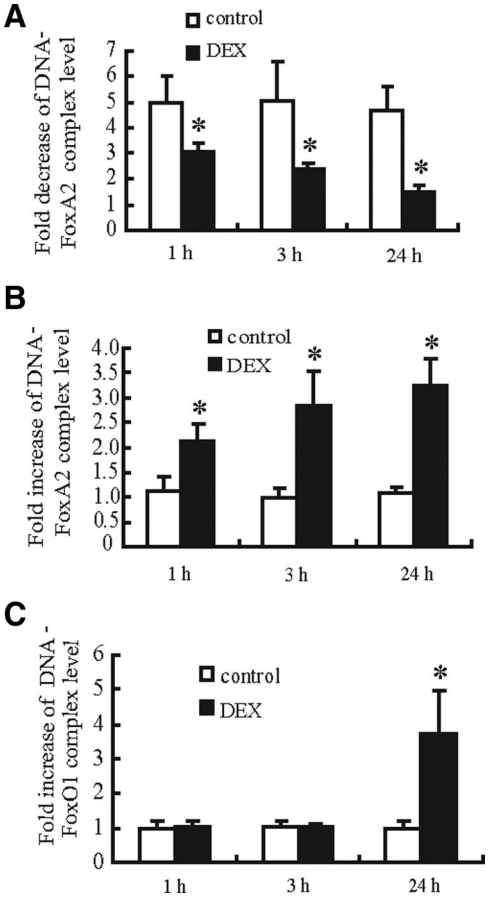

FIG. 4. DEX treatment resulted in alteration of FoxA2 and FoxO1 transcriptional activity. A, The capacity of FoxA2 binding to the *PDX-1* promoter was determined using ChIP-qPCR analysis in RINm5F cells treated with DEX (100 nM) at indicated times. B, The capacity of FoxA2 binding to the *FoxO1* promoter was determined using ChIP-qPCR analysis in RINm5F cells treated with DEX (100 nM) at indicated times. C, The capacity of FoxO1 binding to the *PDX-1* promoter was determined using ChIP-qPCR analysis in RINm5F cells treated with DEX (100 nM) at indicated times.

could be restored by silencing of FoxO1 when RINm5F cells were exposed to DEX (19). Therefore, we wanted to determine the time course of FoxO1 inhibition of PDX-1 transcription in RINm5F cells under DEX treatment. DNA fragments pulled down by the anti-FoxO1 antibody in a ChIP-qPCR assay were approximately equal to those pulled down by IgG in the groups treated with DEX for 1 and 3 h. However, the activity of FoxO1 binding to the *PDX-1* promoter was elevated up to 4-fold in RINm5F cells treated with DEX for 24 h (Fig. 4C). The DNA fragments pulled down by the anti-FoxO1 antibody was not higher than those pulled down by IgG in the control groups, which suggests that FoxO1 does not bind to *PDX-1* promoter under basal conditions.

Inhibition of FoxA2 reverses DEX-induced impairment on GSIS in rat islets

RINm5F cells were transfected either with FoxA2 siRNAs (including three siRNAs) or control siRNA. FoxA2 mRNA and protein levels were measured to determine which siRNAs that can effectively silence FoxA2 expression. As shown in Fig. 5, A and B, FoxA2 siRNA-1 effectively silenced FoxA2 gene expression. Therefore, we chose to use FoxA2 siRNA-1 in the subsequent experiments.

We silenced FoxA2 expression in RINm5F cells and then measured FoxO1 and PDX-1 mRNA expression with or without DEX treatment. FoxA2 mRNA level was significantly silenced without being influenced by DEX treatment (Fig. 5C). When RINm5F cells were exposed to DEX (100 nM), silence of FoxA2 could decrease the FoxO1 mRNA level but increase PDX-1 mRNA level (Fig. 5, D and E). However, without DEX treatment, inhibition of FoxA2 could only decrease the PDX-1 mRNA levels and had no effect on FoxO1 mRNA levels.

To observe the transfection efficiency of isolated rat islets, we transfected islets with 100 nM Cy3-NControl (fluorescence labeled siRNA) for 24 h (Fig. 5F). FoxA2 expression was effectively inhibited when the islets infected with FoxA2 siRNA-1 (Fig. 5G). This inhibition on FoxA2 exhibited decrease on FoxO1 protein levels but increase on PDX-1 protein levels when exposed to 100 nM DEX, compared with that in control-siRNA transfected islets (Fig. 5G). Without DEX treatment, inhibition of FoxA2 could only decrease the PDX-1 protein levels and had no effect on FoxO1 protein levels in islets (Fig. 5G).

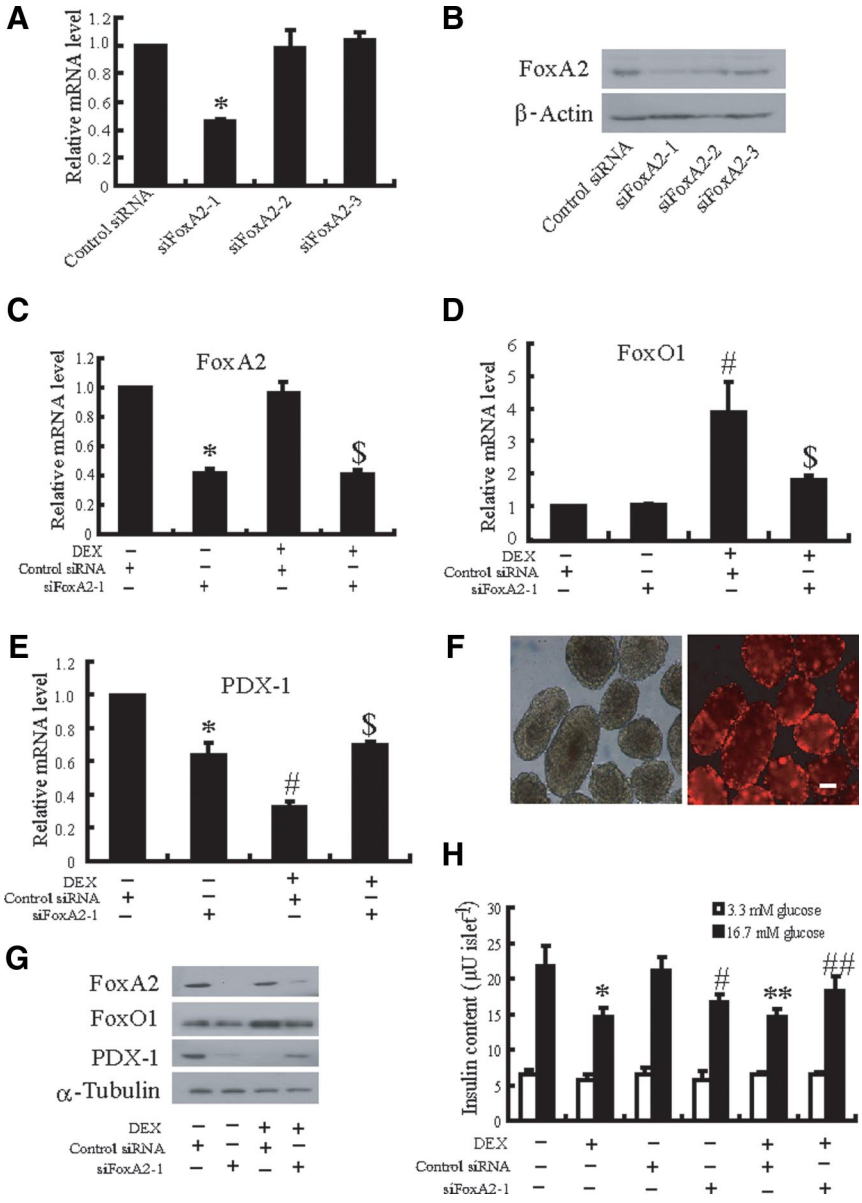

FIG. 5. Inhibition of FoxA2 reverses DEX-induced impairment on GSIS in rat islets. A, RINm5F cells were transiently transfected with control siRNA and FoxA2 siRNAs (1–3) for 24 h, respectively. FoxA2 mRNA levels were determined by real-time PCR and are shown as mean ± SEM; *, $P < 0.05$ *vs.* control siRNA-transfected group. B, RINm5F cells were transiently transfected with control siRNA and FoxA2 siRNAs (1–3) for 48 h, respectively. FoxA2 protein levels were determined by Western blot analysis. C, RINm5F cells were transiently transfected with control siRNA and FoxA2 siRNA-1 (siFoxA2-1), respectively. Twenty-four hours after transfection, the cells were treated with or without DEX (100 nM) for 24 h. FoxA2 mRNA levels were determined by real-time PCR. D, FoxO1 mRNA levels under the treatment as described in C. E, PDX-1 mRNA levels under the treatment as described in C. *, $P < 0.05$ *vs.* control siRNA-transfected group; #, $P < 0.05$ *vs.* control siRNA-transfected group; $, $P < 0.05$ *vs.* control siRNA-transfected and DEX-treated group. F, Isolated rat islets were infected with 100 nM of Cy3-NControl (fluorescence-labeled siRNA) for 24 h. *Scale bar*, 100 μm. G, FoxA2, FoxO1, and PDX-1 protein levels were determined by Western blot analysis in rat islets transfected siFoxA2–1. H, Isolated rat islets (eight islets per well) were treated as above. After incubation for 1 h in glucose-free KRB buffer with vehicle (ethanol) or with DEX (100 nM), the islets were treated for 1 h in KRB buffer with low (3.3 mM) or high (16.7 mM) concentrations of glucose, and then the supernatant fractions were collected for insulin concentration determination as described in *Materials and Methods*. *, $P < 0.01$ *vs.* control; #, $P < 0.01$ *vs.* control siRNA-transfected group; **, $P < 0.01$ *vs.* control siRNA-transfected group; ##, $P < 0.01$ *vs.* control siRNA-transfected and DEX-treated group.

We also evaluated the effects of FoxA2 siRNA on DEX-induced GSIS dysfunction in rat islets. The result showed that inhibition of FoxA2 exhibited a markedly improvement of GSIS when exposed to DEX in rat islets. Moreover, without DEX stimulation, silencing FoxA2 also showed the inhibition of GSIS (Fig. 5H).

DEX had no effect on FoxA2 expression and nuclear localization

It has been reported that overexpression of FoxA2 suppresses DEX-mediated inhibition of the PDX-1 gene expression (18). To further explore whether DEX could affect FoxA2 expression, we examined FoxA2 mRNA and protein levels in RINm5F cells treated with DEX. The results showed that the FoxA2 mRNA levels did not change in cells treated with different doses of DEX or in those treated with DEX (100 nM) for different times (Fig. 6, A and B). Consistent with the changes in mRNA levels, FoxA2 protein levels also did not change under different dose of DEX treatment or treatment for different times (Fig. 6, C and D).

To examine the effect DEX treatment on FoxA2 nuclear localization, RINm5F cells were treated with DEX followed by Western blot analysis of extracted nuclear and cytoplasmic proteins. We found that DEX treatment had no apparent effect on the nuclear localization of FoxA2 (Fig. 6E).

Discussion

In the present study, we demonstrated that the transcription factor FoxA2 played an essential role in enhancement of FoxO1 expression and inhibition of PDX-1 expression in pancreatic β-cells treated with DEX. DEX increased the activity of FoxA2 binding to the *FoxO1* promoter but decreased its binding to the *PDX-1* promoter in RINm5F cells. In addition, we demonstrated for the first time that FoxA2 could bind to the

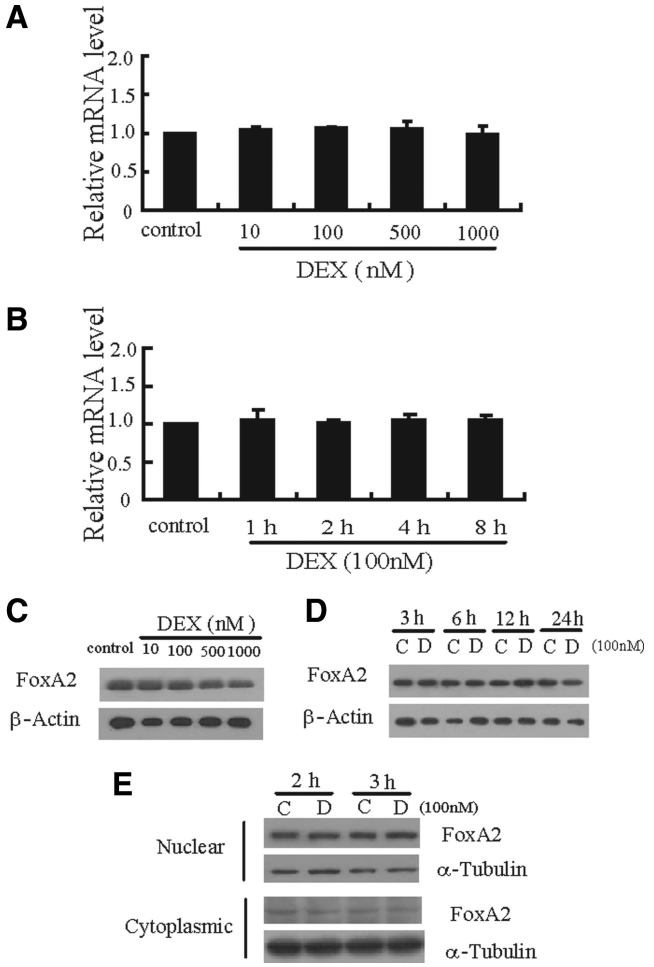

FIG. 6. DEX had no effect on FoxA2 expression and nuclear localization. Panel A, RINm5F cells were treated without (control) or with varying concentrations of DEX for 3 h and then harvested for real-time PCR to determine FoxA2 mRNA levels. Panel B, RINm5F cells treated with DEX (100 nM) were harvested at indicated times for real-time PCR to determine FoxA2 mRNA levels. Panel C, RINm5F cells were treated with varying concentrations of DEX for 24 h, and FoxA2 protein levels were determined by Western blot analysis. Panel D, FoxA2 protein levels in RINm5F cells treated with DEX (100 nM) for the indicated times determined by Western blot analysis. Panel E, RINm5F cells were treated without (control) or with DEX (100 nM) at indicated times. Cytoplasmic FoxA2 and nuclear FoxA2 levels were determined by Western blot analysis. C, Control; D, DEX.

promoter of *FoxO1* and positively regulate FoxO1 expression. Knockdown of FoxA2 could inhibit FoxO1 expression and enhance PDX-1 expression; moreover, it protects pancreatic β-cells against GSIS dysfunction induced by DEX. However, DEX had no effect on FoxA2 expression and nuclear inclusion or exclusion of FoxA2.

In the latest decade, there have been many studies demonstrating that FoxA2 plays an important role in organ development and function (26–28). As the upstream transactivator of HNF4α, HNF1α, PDX-1, and HNF1β, the transcription factor FoxA2 has been shown to be involved in the progression of diabetes (14, 29, 30). In mature β-cells, FoxA2 has been established as an essential activator of genes that regulate multiple pathways in insulin secretion (31, 32). Gao *et al.* (33) reported that FoxA2, in collaboration with FoxA1, could bind to a regulatory domain in the PDX-1 gene, and their ablation resulted in complete loss of PDX-1 expression and severe pancreatic hypoplasia. It is known that PDX-1 plays a pivotal role in pancreatic β-cell development and maintenance of normal function (34–36). However, Wang *et al.* (37) demonstrated that overexpression of FoxA2 resulted in blunted GSIS and a decrease in cellular insulin content. In this study, we identified for the first time that FoxA2 could bind to the *FoxO1* gene promoter (−1219/−1006 bp) and positively regulate FoxO1 expression. Accumulating evidence indicates that FoxO1 may account for impaired GSIS in diabetes (16, 38).

Here, we showed that under DEX treatment, the decrease of PDX-1 expression level was accompanied by the reduction of activity of FoxA2 binding to the *PDX-1* promoter, which is consistent with the previous report by Sharma *et al.* (18). Meanwhile, the increase of FoxO1 expression was accompanied by the enhancement of FoxA2 binding to the *FoxO1* promoter. We also revealed that DEX had no effect on FoxA2 expression and nuclear inclusion or exclusion of FoxA2. Based on our results, we concluded that FoxA2 dissociated from the *PDX-1* promoter while increasing its binding to the *FoxO1* promoter, resulting in a simultaneous change of PDX-1 and FoxO1 expression in RINm5F cells with short-term treatment with DEX. With the increase of FoxO1 expression, FoxO1 occupied the area vacant left vacant by FoxA2 on the *PDX-1* promoter; thus, FoxO1 competed with FoxA2 for binding to the *PDX-1* promoter. As a result, the activity of FoxO1 binding to the *PDX-1* promoter was notably enhanced, whereas that of FoxA2 on the *PDX-1* promoter was further reduced. Through FoxA2 stimulation of PDX-1 expression and FoxO1 inhibition of PDX-1 expression, the level of PDX-1 was gradually and remarkably reduced in pancreatic β-cells after treatment with DEX.

It should be noticed that FoxA2 bound to the *PDX-1* promoter but did not bind to the *FoxO1* promoter in the control group, and interference of basal FoxA2 gene transcription decreased PDX-1 expression but did not affected FoxO1 expression (Fig. 5, D and G), which indicated that FoxA2 only induces PDX-1 expression under certain physiological situations. As a result, silencing FoxA2 could make a decrease in PDX-1 expression as well as GSIS function without DEX treatment (Fig. 5, E, G, and H). When β-cells were treated with DEX or FoxA2 was overexpressed, we found that FoxA2 could bind to the *FoxO1* promoter and stimulate FoxO1 expression. Similarly, the FoxO1 transcription factor demonstrated almost no binding to the *PDX-1* gene promoter without DEX treatment (Fig. 4C). Although in our previous study silencing FoxO1

could not increase PDX-1 expression under basal situation, we found here that DEX could induce FoxO1 binding to *PDX-1* promoter, which further confirmed our previous results (19).

FoxA2 seems to exert a more complicated effect in DEX-induced pancreatic β-cell failure. In this study, silence of FoxA2 could decrease FoxO1 expression and increase PDX-1 expression, which resulted in prevention DEX-induced dysfunction of GSIS in rat islet (Fig. 5). However, Sharma *et al.* (18) reported that overexpression of FoxA2 could restore inhibition of PDX-1 expression mediated by DEX. We thought there was no conflict that the improved expression of PDX-1 in DEX-treated pancreatic β-cell can be caused by both up-regulation or down-regulation of FoxA2. On one hand, DEX had no effect on FoxA2 expression but decreased the activity of FoxA2 binding to the *PDX-1* promoter. With FoxA2 expression increased, more and more FoxA2 bound to the *PDX-1* promoter and stimulated PDX-1 expression. On the other hand, DEX increased the activity of FoxA2 binding to the *FoxO1* promoter and enhanced FoxO1 expression. Through suppression of FoxO1 expression, inhibition of FoxA2 expression could lead to improvement of PDX-1 expression. Therefore, no matter enhancement or repression of FoxA2 expression could restore DEX-induced pancreatic β-cell failure.

Taken together, this study demonstrated that short-term treatment with DEX led to FoxA2 dissociation from the *PDX-1* promoter to binding to the *FoxO1* promoter, resulting in inhibition of PDX-1 expression and enhancement of FoxO1 expression. With extended DEX treatment, FoxO1 was also involved in the down-regulation of PDX-1 expression. These findings provide insight into the molecular mechanisms underlying steroid diabetes.

Acknowledgments

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This work was supported by National Basic Research Program of China Grants (973 Program, no. 2011CB504003) (to X.H.).

Disclosure Summary: The authors have nothing to disclose.

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