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Foxa2 (HNF3β) Controls Multiple Genes Implicated in Metabolism-Secretion Coupling of Glucose-induced Insulin Release*

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The transcription factor Foxa2 is implicated in blood glucose homeostasis. Conditional expression of Foxa2 or its dominant-negative mutant DN-Foxa2 in INS-1 cells reveals that Foxa2 regulates the expression of genes important for glucose sensing in pancreatic β-cells. Overexpression of Foxa2 results in blunted glucose-stimulated insulin secretion, whereas induction of DN-Foxa2 causes a left shift of glucose-induced insulin release. The mRNA levels of GLUT2 and glucokinase are drastically decreased after induction of Foxa2. In contrast, loss of Foxa2 function leads to up-regulation of hexokinase (HK) I and II and glucokinase (HK-IV) mRNA expression. The glucokinase and the low K_m hexokinase activities as well as glycolysis are increased proportionally. In addition, induction of DN-Foxa2 also reduces the expression of β-cell K_{ATP} channel subunits Sur1 and Kir6.2 by 70%. Furthermore, in contrast to previous reports, induction of Foxa2 causes pronounced decreases in the HNF4α and HNF1α mRNA levels. Foxa2 fails to regulate the expression of Pdx1 transcripts. The expression of insulin and islet amyloid polypeptide is markedly suppressed after induction of Foxa2, while the glucagon mRNA levels are significantly increased. Conversely, Foxa2 is required for glucagon expression in these INS-1-derived cells. These results suggest that Foxa2 is a vital transcription factor evolved to control the expression of genes essential for maintaining β-cell glucose sensing and glucose homeostasis.

The forkhead/winged-helix Foxa family of transcription factors, encoded by three genes *Foxa1* (*Hnf3α*), *Foxa2* (*Hnf3β*), and *Foxa3* (*Hnf3γ*), regulate hepatic and/or pancreatic gene expression (1–9). Foxa1 and Foxa3 are required for maintaining glucose homeostasis by activation, respectively, of pancreatic glucagon and hepatic gluconeogenic enzymes (2, 3, 5). Targeted disruption of *Foxa2* resulted in embryonic lethality with defective development of the foregut endoderm, from which the liver and pancreas arise (10). Foxa2, which is expressed in islets, has been suggested as the upstream transactivator of Hnf4α, Hnf1α, Pdx1, and Hnf1β in the transcriptional hierarchy (1, 9, 11). Mutations in the genes encoding these pancreatic transcription factors are linked to four monogenic forms of MODY[1] (maturity-onset diabetes of the young): MODY1/*HNF4α*, MODY3/*HNF1α*, MODY4/*IPF1(PDX1)*, and MODY5/*HNF1β* (12, 13). However, the search for the association of *FOXA2* mutations with MODY patients has not been successful (14, 15). Most recently, Sund *et al.* (16) have suggested that *FOXA2* rather might be a candidate gene for familial hyperinsulinism. Pancreatic β-cell-specific deletion of *Foxa2* resulted in postnatal death due to severe hyperinsulinemic hypoglycemia, and the down-regulation of ATP-sensitive K^+ (K_{ATP}) channel subunits Sur1 and Kir6.2 has been demonstrated in these mutant mice (16).

To assess whether Foxa2 indeed controls the expression of the transcription factors associated with MODY, we have established INS-1-derived stable cell lines, which allow conditional expression of the wild type Foxa2 or its dominant-negative mutant DN-Foxa2 under tight control of the reverse tetracycline-dependent transactivator (17). DN-Foxa2 is a Myc-tagged truncated Foxa2 mutant protein that possesses the intact DNA-binding domain but lacks the transactivation domain (7). DN-Foxa2 exerts its dominant-negative function by competing with the endogenous Foxa2 for cognate DNA binding (7). The impact of altered Foxa2 function on glucose metabolism and insulin secretion was assessed in these stable clones. The gene expression profile before and after induction of the Foxa2 or DN-Foxa2 was quantified.

EXPERIMENTAL PROCEDURES

Establishment of Stable Cell Lines—Rat insulinoma INS-1 cell line-derived stable clones were cultured in RPMI 1640 in 11.2 mM glucose (18), unless otherwise indicated. The first step stable clone INSrαβ, which expresses the reverse tetracycline-dependent transactivator, was described previously (17, 18). Plasmids used in the secondary stable transfection were constructed by subcloning the cDNAs encoding the mouse Foxa2 (kindly supplied by Prof. G. Schütz) and its dominant-negative mutant (DN-Foxa2) into the expression vector PUHD10–3 (a kind gift from Prof. H. Bujard). DN-Foxa2 (truncated mutation lacking the transactivation domain but containing the intact DNA-binding domain) (7) was PCR-amplified from Foxa2 cDNA using the following primers: 5′-gcaggatccgtaatggtgctcgggcttcaggtg-3′ and 5′-gcaggatccggcgccatggcgggcatgagcggctca3-′. The PCR fragment was subcloned into modified pcDNA3.1myc (Invitrogen, Groningen, The Netherlands) and sequenced. The stable transfection and the clone selection and screening procedures were described previously (17).

Immunoblot and Immunofluorescence—Immunoblotting procedures were performed as described previously using enhanced chemiluminescence (Pierce) for detection (18). The dilutions for antibodies against Foxa2 C terminus (Santa Cruz Biotechnology, Heidelberg, Germany)

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[1] The abbreviations used are: MODY, maturity-onset diabetes of the young; DN, dominant-negative; PBS, phosphate-buffered saline; BSA, bovine serum albumin; IAPP, islet amyloid polypeptide; GDH, glutamate dehydrogenase; ANT, adenine nucleotide translocator; GLP-1R, glucagon-like peptide-1 receptor; UCP, uncoupling protein.

and Myc-tag (19) were 1:5,000 and 1:10, respectively. Nuclear extracts were isolated from the cells cultured with or without 500 ng/ml doxycycline for 24 h.

For immunofluorescence, cells grown on polyornithine-treated glass coverslips were cultured for 24 h with or without 500 ng/ml doxycycline. The cells were then washed, fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline containing 1% BSA (PBS-BSA). The preparation was then blocked with PBS-BSA before incubating with the first antibodies, anti-Foxa2 (1:500 dilution) and mouse monoclonal anti-Myc-tag, (1:2 dilution), followed by the second antibody labeling.

Nuclear Extract Preparation—Nuclear extracts from INS-1 cells grown in culture medium with or without 500 ng/ml doxycycline for 24 h were prepared according to Schreiber *et al.* (20).

Measurements of Insulin Secretion and Cellular Insulin Content—Cells in 12-well plates were cultured in 11.2 mM glucose medium with or without 500 ng/ml doxycycline for 19 h, followed by an additional 5 h equilibration in 2.5 mM glucose medium. Insulin secretion was measured over a period of 30 min, in Krebs-Ringer-Bicarbonate-HEPES buffer (KRBH, 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 2 mM $NaHCO_3$, 10 mM HEPES, 0.1% BSA) containing the indicated concentrations of glucose. Insulin content was determined after extraction with acid ethanol following the procedures of Wang *et al.* (21). Insulin was detected by radioimmunoassay using rat insulin as standard (21).

Assay of Glucokinase and High Affinity Hexokinase Activities—Cytosolic proteins were extracted, according to Wang and Iynedjian (17), from cells cultured in 11.2 mM glucose medium in the presence or absence of 500 ng/ml doxycycline for 24 h. Total hexokinase activity was measured at 30 °C by a glucose-6-phosphate dehydrogenase-coupled assay in a fluorometer (Lambda Bio20, PerkinElmer Life Sciences) estimation of NADH production (17). Glucokinase activity and high affinity hexokinase activity were calculated, respectively, as the differences in NADH produced at 100, 0.5, and 0 mM glucose and expressed in nmol/min (=milliunits) per mg of protein.

Measurement of Glucose Utilization—Cells in 24-well dishes were cultured in 2.5 mM glucose medium with or without 500 ng/ml doxycycline for 24 h. The rate of glycolysis was estimated from the production of [^{3}H]water from D-[5-^{3}H]glucose according to Wang and Iynedjian (17).

Total RNA Isolation and Northern Blotting—Cells in 10-cm diameter dishes were cultured in 2.5 mM glucose medium with or without 500 ng/ml doxycycline for 16 h, followed by an additional 8 h in culture medium with 2.5, 6, 12, and 24 mM glucose. Total RNA was extracted and blotted to nylon membranes as described previously (17). The membrane was prehybridized and then hybridized to ^{32}P-labeled random primer cDNA probes according to Wang and Iynedijian (17). To ensure equal RNA loading and even transfer, all membranes were stripped and re-hybridized with a "housekeeping gene" probe cyclophilin. cDNA fragments used as probes for Foxa2, Hnf1α, Hnf4α, glucokinase, hexokinase I, Glut2, L-pyruvate kinase, insulin, Sur1, Kir6.2, and Pdx1 mRNA detection were digested from the corresponding plasmids. cDNA probes for rat islet amyloid polypeptide (IAPP), glucagon, Nkx6.1, Nkx2.2, Isl-1, β2/NeuroD, aldolase B, adenine nucleotide translocators 1 and 2 (ANT1, ANT2), mitochondrial uncoupling protein 2 (UCP2), mitochondrial glutamate dehydrogenase (GDH), citrate synthase, glyceraldehydes-3 phosphate dehydrogenase (GAPDH), hexokinase II and glucagon-like peptide-1 receptor (GLP-1R) were prepared by RT-PCR and confirmed by sequencing.

Statistics—Results are expressed as mean ± S.E., and statistical analyses were performed by Student's *t* test for unpaired data.

RESULTS

Foxa2 and DN-Foxa2 Were Induced in an All-or-None Manner—We have established over 10 clones positively expressing Foxa2 and DN-Foxa2, respectively, using the parental INS-r$\alpha\beta$ (INS-r3) cells (17, 18). The clones designated as Foxa2$^{\#}$51 and DN-Foxa2$^{\#}$45, which displayed the highest inducible protein levels without leakage under noninduced state, were selected for the present study. As illustrated in Fig. 1, *A* and *C*, the INS-1-derived cells express endogenous Foxa2 in the nucleus. Foxa2 protein was overexpressed in all of the cells treated with 500 ng/ml doxycycline for 24 h. As predicted, the antibody against the carboxyl terminus of Foxa2 did not detect DN-Foxa2 with the COOH-terminal deletion (7) (Fig. 1*B*). As shown in the Western blotting (Fig. 1*B*) and immunostaining (Fig. 1*D*)

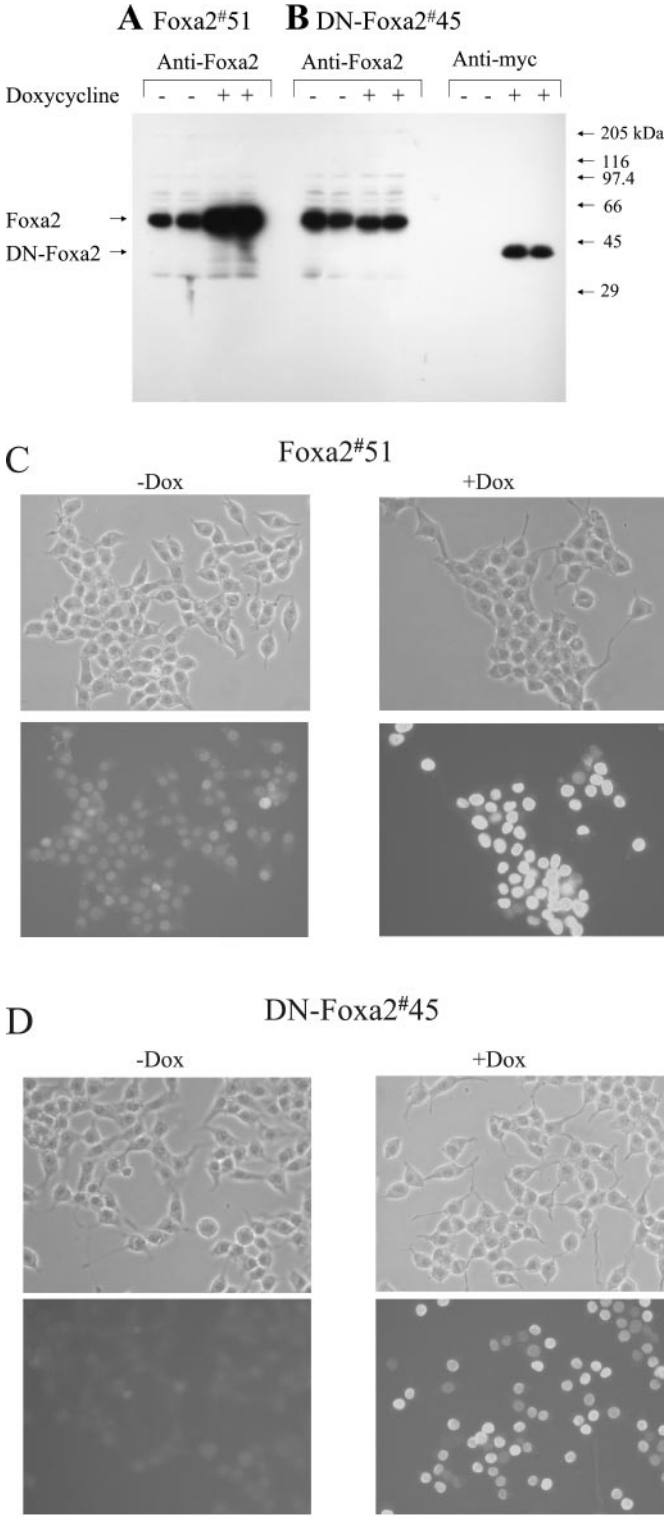

FIG. 1. **Foxa2 and DN-Foxa2 are induced in gene-manipulated INS-1 cells in an all-or-none manner.** Cells were cultured in 11.2 mM glucose medium with (+*Dox*) or without (−*Dox*) 500 ng/ml of doxycycline for 24 h. *A,* Western blotting of nuclear extracts from Foxa2$^{\#}$51 cells with antibody against the COOH terminus of Foxa2. *B*, immunoblotting of nuclear extracts from DN-Foxa2$^{\#}$45 cells with antibodies against, respectively, the Foxa2 COOH terminus and the Myc-tag. 10 μg of nuclear extract protein was resolved in 11% SDS-PAGE and transferred to nitrocellulose. *C*, the endogenous and induced Foxa2 proteins are shown by immunofluorescence staining with antibody against the Foxa2 COOH terminus. *D*, the nonspecific background and induced DN-Foxa2 protein were investigated by immunofluorescence using anti-Myc-tag antibody. The microscopic phase contrast images are shown in the *upper panel*.

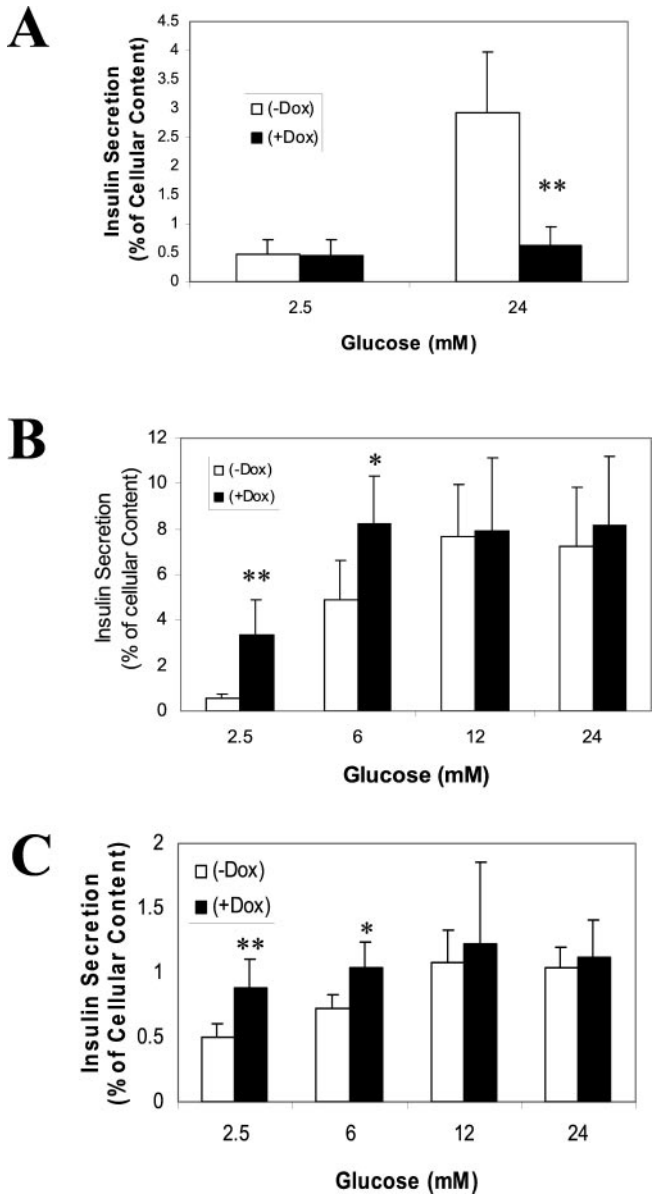

FIG. 2. **Foxa2 regulates the glucose responsiveness of insulin secretion.** Cells were cultured in 11.2 mM glucose medium with or without 500 ng/ml doxycycline for 19 h, followed by an additional 5 h equilibration in 2.5 mM glucose medium. *A*, insulin secretion from Foxa2$^{\#}$51 cells stimulated by 24 mM glucose was quantified by radioimmunoassay and normalized by cellular insulin content. *B*, glucose dose-dependent insulin release from DN-Foxa2$^{\#}$45 cells was expressed as a percentage of cellular insulin content. *C*, glucose dose-dependent insulin release from DN-Foxa2$^{\#}$2 cells was expressed as a percentage of cellular insulin content. Data represent mean ± S.E. of six to seven independent experiments. *, $p < 0.01$ and **, $p < 0.0001$.

with a monoclonal anti-Myc antibody, this Myc-tagged DN-Foxa2 protein was induced in a doxycycline-dependent and an all-or-none manner. Induction of DN-Foxa2 did not interrupt the endogenous Foxa2 expression (Fig. 1*B*), and the induced DN-Foxa2 protein was localized in the nucleus of DN-Foxa2$^{\#}$45 cells (Fig. 1, *B* and *D*). We also performed an electrophoretic mobility shift assay (data not shown) using the Foxa2-binding site containing glucagon G2 element as a probe (22). Induction of Foxa2 led to a 10-fold increase in the signal density of Foxa2 binding, whereas induction of DN-Foxa2 almost completely abolished the binding activity of endogenous Foxa2 (data not shown).

Foxa2 Regulates the Glucose Responsiveness of Insulin Secretion—As demonstrated in Fig. 2*A*, overexpression of Foxa2

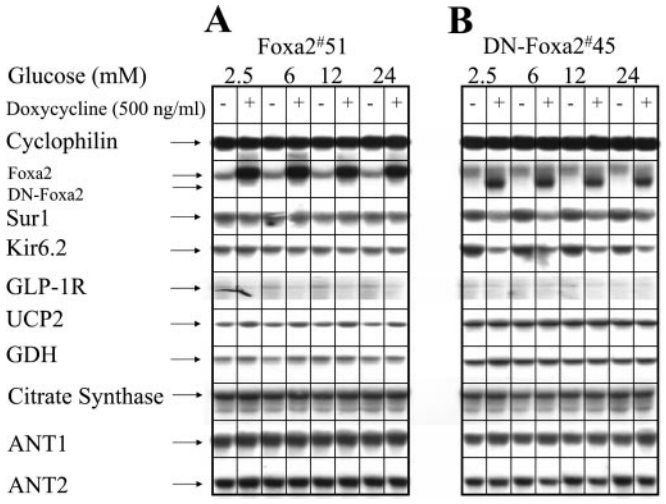

FIG. 3. **Foxa2 is required for maintaining the expression of K_{ATP} channel subunits Sur1 and Kir6.2.** Cells were cultured in 2.5 mM glucose medium with or without 500 ng/ml doxycycline for 16 h and then incubated further for 8 h at the indicated glucose concentrations. The gene expression profile in Foxa2$^{\#}$51 (*A*) and DN-Foxa2$^{\#}$45 (*B*) cells was quantified by Northern blotting. 20 μg of total RNA samples were analyzed by hybridizing with indicated cDNA probes.

almost completely blunted glucose-stimulated insulin release. The cellular insulin content was reduced by 46.3 ± 5.1% ($p < 0.001$) after induction of Foxa2 for 24 h (see also Fig. 6 for the decrease in insulin mRNA levels). Secretion data were therefore normalized for cellular insulin content. In contrast, induction of DN-Foxa2 resulted in a left shift of the dose-response curve of glucose-stimulated insulin release (Fig. 2*B*) without altering insulin content. To verify the clonal variability, we randomly chose another clone DN-Foxa2$^{\#}$ 2 and studied the effects of DN-Foxa2 induction on glucose-stimulated insulin secretion. As shown in Fig. 2*C*, induction of DN-Foxa2 in this clone also led to a typical left-shift of glucose-dependent insulin release, suggesting a common phenomenon rather than a clonal peculiarity. Next, we examined the gene expression patterns in these cell lines to elucidate the mechanisms underlying the changes in insulin secretion.

Foxa2 Is Required for Maintaining the Expression of K_{ATP} Channel Subunits Sur1 and Kir6.2—Northern blot analysis of the gene expression pattern in Foxa2$^{\#}$51 and DN-Foxa2$^{\#}$45 cells cultured in indicated concentrations of glucose and treated with or without 500 ng/ml doxycycline for 24 h is described in the legend to Fig. 3. Consistent with the immunoblotting (Fig. 1*B*), DN-Foxa2 mRNA was induced in an all-or-none manner, and such induction did not alter endogenous Foxa2 mRNA expression (Fig. 3*B*). The mRNA levels of the K_{ATP} channel subunits Sur1 and Kir6.2 were reduced by 60 and 70%, respectively, after dominant-negative suppression of Foxa2 function (Fig. 3*B*). However, overexpression of Foxa2 alone was not sufficient to promote the expression of Sur1 and Kir6.2 (Fig. 3*A*). The mRNA levels of mitochondrial GDH, citrate synthase, and adenine nucleotide translocators 1 and 2 (ANT1 and ANT2) were not modulated by Foxa2 (Fig. 3, *A* and *B*). On the other hand, overexpression of Foxa2 caused up-regulation of UCP2 (Fig. 3*A*), whereas induction of DN-Foxa2 did not affect the expression of UCP2 mRNA (Fig. 3*B*). Furthermore, overexpression of Foxa2 resulted in down-regulation of glucagon-like peptide-1 receptor (GLP-1R) (Fig. 3*A*).

Persistent hyperinsulinemic hypoglycemia of infancy has been linked to mutations in the genes encoding Sur1, Kir6.2, glucokinase, and GDH (23–28). Increased glucose-dependent insulin release was also observed in the UCP2-deleted mouse (29), whereas decreased insulin secretion was reported after

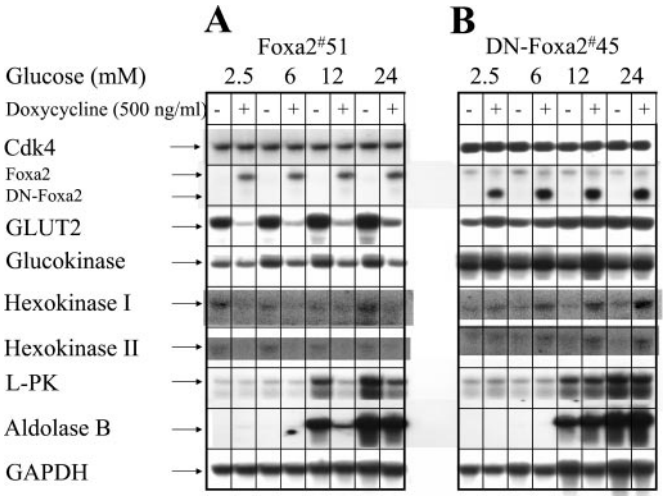

FIG. 4. **Foxa2 regulates genes implicated in glucose sensing.** Cells were cultured in 2.5 mM glucose medium with or without 500 ng/ml doxycycline for 16 h and then incubated further for 8 h at the indicated glucose concentrations. The gene expression profile in Foxa2#51 (*A*) and DN-Foxa2#45 (*B*) cells was quantitatively evaluated by Northern blotting. 20 μg of total RNA samples were analyzed by hybridizing with indicated cDNA probes. *L-PK*, L-pyruvate kinase; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *Cdk4*, cyclin-dependent kinase 4; *Glut2*, glucose transporter 2.

overexpression of UCP2 in islets (30) and INS-1 cells (31). We could rule out the possible involvement of GDH and UCP2 in the enhanced glucose-stimulated insulin secretion observed in β-cells deficient in Foxa2 function, since their expression was not altered by induction of DN-Foxa2.

Foxa2 Targets Genes Essential for β-Cell Glucose Sensing—The rodent pancreatic β-cell expresses high levels of the glucose transporter Glut2, which allows rapid equilibration of glucose across the plasma membrane (32, 33). This is associated with extremely low levels of high affinity hexokinase isoforms (hexokinases I, II, and III) to optimize glucose sensing in the physiological blood glucose range. A β-cell-specific promoter in the glucokinase (hexokinase IV) gene maintains a precise expression level of this rate-limiting enzyme for glucose metabolism, which determines the glucose sensing in pancreatic β-cells (reviewed in Refs. 33–35). Alterations of glucokinase activity by gene manipulation or pharmacological inhibition, or by naturally occurring genetic mutations, have been demonstrated to change the physiological threshold of β-cell glucose sensing (reviewed in Refs. 33–35).

As shown in Fig. 4, overexpression of Foxa2 in Foxa2#51 cells reduced the glucokinase mRNA level by 60%, whereas induction of DN-Foxa2 in DN-Foxa2#45 raised the glucokinase expression by 2-fold. The increased glucokinase expression after induction of DN-Foxa2 was also demonstrated in another clone, DN-Foxa2#2 (Fig. 7). The INS-1-derived clones expressed hexokinases I and II (but not III) mRNAs at barely detectable levels, and induction of Foxa2 and DN-Foxa2 resulted in, respectively, down- and up-regulation of these mRNA levels (Fig. 4). Overexpression of Foxa2 also caused a 90% reduction of Glut2 mRNA expression, while induction of DN-Foxa2 left-shifted the glucose dose-dependent increase in Glut2 transcript level (Fig. 4). The suppressive effects of Foxa2 on glucose sensing were also reflected by the blunted glucose responsiveness of L-pyruvate kinase and aldolase B mRNA expression (Fig. 4).

To confirm the Northern blot analysis, we also measured the activities of glucokinase and high affinity hexokinase. As seen in Table I, the glucokinase activity was reduced by 60% following overexpression of Foxa2 and was increased 2.5-fold by dominant-negative suppression of Foxa2 function. Similarly, the high affinity hexokinase activity was down-regulated by 50% and up-regulated by 3-fold, respectively, by induction of Foxa2 and DN-Foxa2. Thus, Foxa2 is essential for the transcriptional regulation of enzymes controlling the β-cell glucose phosphorylation. This conclusion was corroborated by the measurements of glycolytic flux, which was decreased by 60% after overexpression of Foxa2 and increased by 2-fold after induction of DN-Foxa2 (Fig. 5).

Foxa2 Promotes Glucagon Level and Suppresses β-Cell Gene Expression—Foxa2 has been previously suggested as a master transactivator of the pancreatic transcription factors, Hnf4α, Hnf1α, Hnf1β, and Pdx1, in the transcriptional hierarchy (1, 9, 11). The results we obtained were unexpected and in disagreement with previous reports (1, 9). We found that Pdx1 expression was not significantly affected by induction of Foxa2 or DN-Foxa2 (Fig. 6). Isl-1 is the only pancreatic transcription factor, the expression of which requires Foxa2 function (Fig. 6*B*). Overexpression of Foxa2 suppressed rather than enhanced the expression of Hnf4α and Hnf1α mRNAs (Fig. 6*A*). To verify whether this is due to a clonal variability or a paradoxical effect of high level overexpression, we also studied the effect of graded overexpression of Foxa2 on mRNA levels of Hnf4α and Hnf1α in another randomly selected clone, Foxa2#39 (Fig. 7). Titrated overexpression of Foxa2 by 3.5-, 10-, and 20-fold at 75, 150, and 500 ng/ml of doxycycline all caused significant inhibition of Hnf4α and Hnf1α expression (Fig. 7).

The Foxa2#51 and DN-Foxa2#45 clones were derived from a parental INS-r$\alpha\beta$ cell line that expresses not only insulin but also detectable levels of glucagon (18). These clones enable us to assess the function of Foxa2 in the regulation of both insulin and glucagon expression. The mRNA levels of β-cell-specific genes, insulin and IAPP, were reduced by 50 and 60%, respectively, after overexpression of Foxa2 for 24 h, whereas the glucagon expression was increased by 2-fold (Fig. 6*A*). Foxa2 is apparently required for maintaining the α-cell phenotype, since induction of DN-Foxa2 almost completely eliminated the glucagon expression (Fig. 6*B*).

DISCUSSION

Establishment of a Cellular Model for Studying Foxa2 Function in Pancreatic Endocrine Cells—Foxa2 has been proposed to be the master regulator of pancreatic transcription factors Pdx1 (9, 36), Hnf4α, Hnf1α, and Hnf1β (1, 11). Naturally occurring mutations in the human HNF4α, HNF1α, PDX1, and HNF1β genes have been associated with four monogenic forms of MODY caused by impaired glucose-induced insulin secretion (12, 13, 37). However, genetic analysis has failed to link the FOXA2 mutations to MODY pedigrees (14, 15). In addition, the phenotype of the mouse with β-cell specific deletion of Foxa2 does not support the notion that this transcription factor is the master regulator of Hnf4α, Hnf1α, Pdx1, and Hnf1β in the transcriptional hierarchy (16). Pancreatic β-cell-specific deletion of Foxa2 resulted in severe hyperinsulinemic hypoglycemia that led to postnatal death (16). The defective expression of K_{ATP} channel subunits Sur1 and Kir6.2 in the β-cells of the Foxa2-null mouse could not fully explain the severe phenotype, because the transgenic mouse lines deficient in K_{ATP} channel function showed milder and transient hyperinsulinemic hypoglycemia (38–40).

The implementation of the doxycycline-inducible system (Tet-on) (41) in INS-1-derived INSr$\alpha\beta$ cells permitted the induction of Foxa2 and DN-Foxa2 in an all-or-none manner. The INS-$\alpha\beta$ cells not only exhibit the normal β-cell phenotype of parental INS-1 cells but also express glucagon (17, 18). Glucose-stimulated insulin secretion and glucose metabolism in INS-$\alpha\beta$-derived clones were indistinguishable from those in the parental INS-1 cells (17, 18). The Foxa2# 51 and DN-Foxa2# 45

TABLE I

Effects of induction of Foxa2 and DN-Foxa2 on the activities of glucokinase and high affinity hexokinase

Enzyme activities were measured using cytosolic proteins isolated from cells cultured with or without 500 ng/ml doxycycline for 24 h and expressed as milliunits/mg of protein. Data represent means ± S.E. of seven to nine separate experiments.

	Foxa2#51		DN-Foxa2#45	
	−Dox	+Dox	−Dox	+Dox
Glucokinase	10.18 ± 0.97	3.94 ± 1.24[a]	9.53 ± 1.70	24.60 ± 3.78[a]
High affinity hexokinase	1.30 ± 0.36	0.56 ± 0.35[b]	1.32 ± 0.46	4.20 ± 1.53[a]

[a] $p < 0.0001$.
[b] $p < 0.005$.

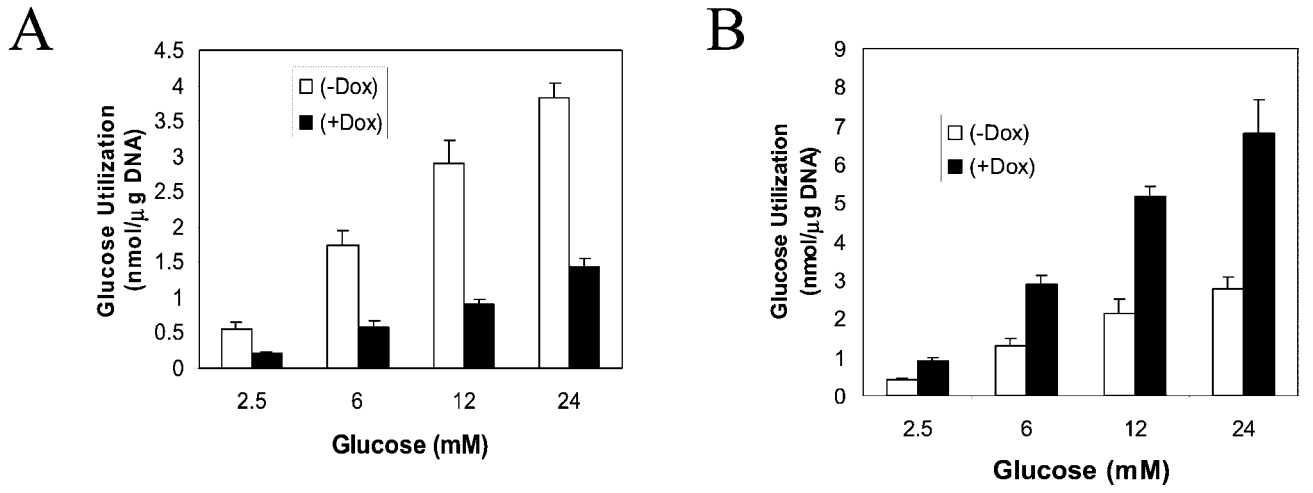

FIG. 5. **Foxa2 expression affects glycolytic flux.** Foxa2#51 (*A*) and DN-Foxa2#45 (*B*) cells were cultured in 2.5 mM glucose medium with or without 500 ng/ml doxycycline for 24 h. Cells were then incubated with the indicated concentrations of glucose and D-[5-^{3}H]glucose for 60 min. Data are expressed per μg of cellular DNA and represent means + S.E. from six separate experiments. Differences between induced and noninduced conditions at all glucose concentrations are statistically significant ($p < 0.001$).

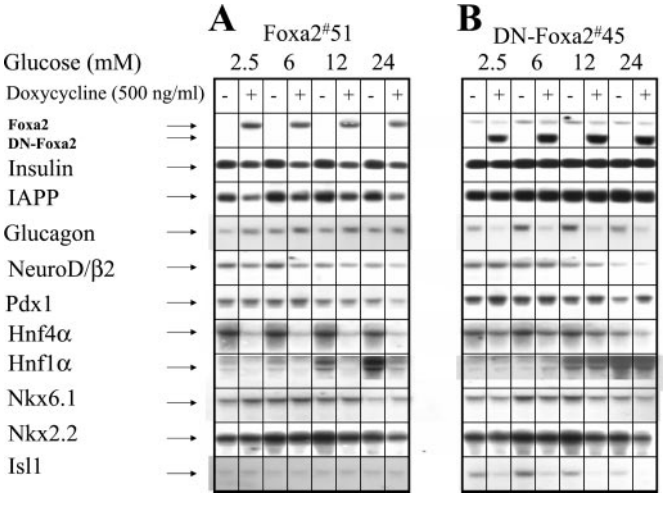

FIG. 6. **Foxa2 suppresses β-cell gene expression and promotes glucagon levels.** Cells were cultured in 2.5 mM glucose medium with or without 500 ng/ml doxycycline for 16 h and were then further incubated for 8 h at the indicated glucose concentrations. The gene expression profile in Foxa2#51 (*A*) and DN-Foxa2#45 (*B*) cells was quantified by Northern blotting. 20 μg of total RNA samples were analyzed by hybridizing with indicated cDNA probes.

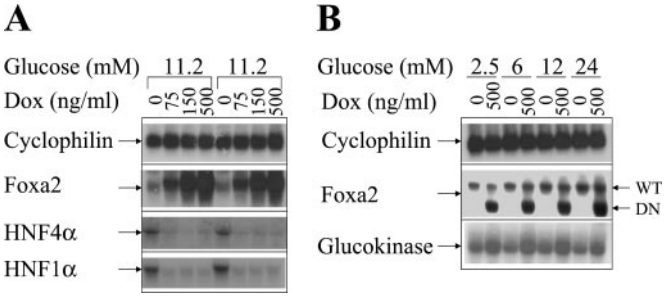

FIG. 7. **Graded overexpression of Foxa2 suppresses HNF4α and HNF1α expression and induction of DN-Foxa2 increases glucokinse expression.** *A*, Foxa2#39 cells were cultured for 24 h in normal (11.2 mM) glucose medium containing, respectively, 0, 75, 150, and 500 ng/ml doxycycline. Samples from two independent experiments were demonstrated in parallel. *B*, DN-Foxa2#2 cells were cultured in 2.5 mM glucose medium with or without 500 ng/ml doxycycline for 16 h and were then further incubated for 8 h at the indicated glucose concentrations. The gene expression was quantified by Northern blotting. 20 μg of total RNA samples were analyzed by hybridizing with indicated cDNA probes.

cell lines allowed us to study how up- and down-regulation of Foxa2 function affected the glucose metabolism and insulin secretion. These cell lines also provided us with a unique cell model and an unlimited source of RNA for the identification of Foxa2 target genes by quantitative analysis of the gene expression profile in these stable clones under noninduced and induced conditions.

The Molecular Mechanism Underlying the Foxa2-regulated Glucose Responsiveness of Insulin Secretion—The present study demonstrates that Foxa2 plays an important role in maintaining suppression of high affinity hexokinase expression. Overexpression of hexokinase I in isolated islets using recombinant adenovirus has been shown to elevate the basal (3 mM glucose) insulin release (42). Therefore, the increased high affinity hexokinase activity observed in the induced DN-Foxa2#45 cells should contribute in part to the higher basal (2.5 mM glucose) insulin secretion.

We found that another important function of Foxa2 is to regulate the precise level of high K_m glucokinase expression, which sets the threshold of β-cell glucose sensing (35). Graded overexpression of glucokinase within a limit of 4-fold has been shown to enhance glycolysis proportionally and left shift the

β-cell secretory response to glucose (17), whereas dose-dependent inhibition of glucokinase activity by mannoheptulose causes a stepwise right shift of β-cell glucose sensitivity (34). The pancreatic β-cell-specific deletion of glucokinase resulted in impaired glucose-stimulated insulin secretion (43). The concept of glucokinase as the β-cell glucose sensor is further established by genetic linkage to loss- and gain-of-function mutations in the human glucokinase gene, respectively, to MODY2 and hyperinsulinemic hypoglycemia (35). In the present study, we demonstrate that the mRNA level and the enzyme activity of glucokinase were down- and up-regulated, respectively, by induction of Foxa2 and DN-Foxa2. The glycolytic flux was altered in a similar way. Dominant-negative suppression of Foxa2 function resulted in a left shift of glucose-induced insulin secretion. A similar left shift of glucose dose-dependent insulin release has also been reported in perifused pancreatic fragments dissected from the mouse with β-cell-specific deletion of Foxa2. We therefore conclude that the increased glucokinase expression should be responsible, at least in part, for the left shift of glucose-stimulated insulin secretion in the induced DN-Foxa2$^{\#}$45 and DN-Foxa2$^{\#}$2 cells. However, a modest elevation in the glucokinase mRNA level may not be detected by reverse transcription PCR analysis as performed in the previous study (16). On the other hand, the defective glucose-induced insulin secretion observed in the Foxa2 overexpressing cells could be caused by both the down-regulation of glucokinase and the up-regulation of UCP2. The up-regulation of UCP2 has indeed been associated with attenuated insulin secretion (30, 31).

We confirm that Foxa2 is required for maintaining the expression of K_{ATP} channel subunits Sur1 and Kir6.2. Heteroctameric K_{ATP} channels are formed by association of the pore-forming kir6.2 subunit and the sulfonylurea receptor Sur, an ATP binding cassette protein harboring intrinsic ATPase activity (44, 45). Mutations in the *SUR1* and *KIR6.2* genes are associated with persistent hyperinsulinemic hypoglycemia of infancy (reviewed in Ref. 44). However, the left shift of glucose responsiveness in insulin secretion is not demonstrated in any of the mouse lines deficient in K_{ATP} channel activity (38–40). Induction of DN-Foxa2 resulted in 60% reduction of the transcript levels. The failure of Foxa2 overexpression to alter Kir6.2 and Sur1 mRNA suggests that these genes are already fully induced. Our results are in full agreement with the previous report showing decreased mRNA levels of these K_{ATP} channel subunits in β-cell Foxa2-deficient islets (16).

Foxa2 Transactivates Glucagon but Suppresses Insulin Gene Expression—We show that overexpression of Foxa2 suppresses the expression of the β-cell-specific genes insulin and IAPP. This is consistent with the decrease in cellular insulin content. In contrast, overexpression of Foxa2 raises the mRNA level of glucagon. In addition, Foxa2 was required for maintaining the glucagon expression. This is in good agreement with observations by Gauthier *et al.* (46) who demonstrated that Foxa2 transactivates the glucagon promoter activity by binding to the G1 and G2 elements.

However, our results contradict previous reports suggesting that Foxa2 is the upstream transactivator of Pdx1, Hnf4α, and Hnf1α (1) (36). Foxa2 may indeed transactivate these genes in early embryonic development as the authors performed the experiments in embryoid bodies differentiated from embryonic stem cells (1, 36). Sund *et al.* (16) have shown that Hnf1α and Hnf1β mRNA levels are unchanged, whereas Hnf4α mRNA expression is slightly elevated in the liver-specific *Foxa2* knockout mice. Most recently, Tan *et al.* (47) have demonstrated that adenovirus-mediated overexpression of Foxa2 in mouse liver led to drastic decrease in the mRNA levels of Hnf4α and Hnf1α. The phenotype of mice with β-cell-specific deletion of Foxa2 is severe hypoglycemia rather than diabetes as seen in the MODY1, MODY3, and MODY4 patients (12, 13) and the transgenic mouse lines with targeted disruption of *Hnf1α* (48) or β-cell-specific deletion of *Pdx1* (49). We found that the Pdx1 mRNA expression in INS-1 cells was not regulated by Foxa2. Overexpression of Foxa2 suppressed rather than transactivated the Hnf4α and Hnf1α mRNA levels in both Foxa2$^{\#}$39 and Foxa2$^{\#}$51 cells. We therefore suggest that Foxa2 is dispensable for maintaining the expression of Pdx1, Hnf4α, and Hnf1α in INS-1 cells and probably in islet cells.

Conclusion—Foxa2 exerts the following functions in pancreatic endocrine cells: 1) maintaining the high affinity hexokinase expression at a minimal level to limit insulin secretion at fasting blood glucose concentrations, 2) maintaining the precise level of glucokinase to set the threshold of β-cell glucose sensing, 3) maintaining the expression of K_{ATP} channel subunits Sur1 and Kir6.2 to regulate the nutrient-stimulated insulin secretion and glucose homeostasis, 4) maintaining glucagon expression. We therefore conclude that Foxa2 has evolved as a transcription factor to control the expression of genes essential for maintaining fasting blood glucose levels, a biological mechanism for adaptation to starvation. Foxa2 also regulates genes essential for β-cell glucose sensing, thereby ensuring normal nutrient-induced insulin secretion and glucose homeostasis.

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