Foxa2 Controls *Pdx1* Gene Expression in Pancreatic β-Cells In Vivo

Catherine S. Lee,[1,3] Newman J. Sund,[1,3] Marko Z. Vatamaniuk,[2,3] Franz M. Matschinsky,[2,3] Doris A. Stoffers,[3,4] and Klaus H. Kaestner[1,3]

Differentiation of early foregut endoderm into pancreatic endocrine and exocrine cells depends on a cascade of gene activation events controlled by various transcription factors. Prior in vitro analysis has suggested that the *forkhead*/winged helix transcription factor *Foxa2* (formerly HNF-3β) is a major upstream regulator of *Pdx1*, a homeobox gene essential for pancreatic development. *Pdx1* is also essential for the maintenance of glucose homeostasis, as its human orthologue, IPF-1, is mutated in a subset of patients with early-onset type 2 diabetes (MODY4). To analyze the *Foxa2/Pdx1* regulatory cascade during pancreatic β-cell differentiation, we used conditional gene ablation of *Foxa2* in mice. We demonstrated that the deletion of *Foxa2* in β-cell−specific knockout mice results in downregulation of *Pdx1* mRNA and subsequent reduction of PDX-1 protein levels in islets. These data represent the first in vivo demonstration that *Foxa2* acts upstream of *Pdx1* in the differentiated β-cell. *Diabetes* 51:2546–2551, 2002

The mammalian pancreas is comprised of exocrine, endocrine, and ductal cell types that are of endodermal origin. During mouse development, dorsal and ventral pancreatic primordia first appear as evaginations of the foregut endoderm on day 9 post coitum (p.c.). Prepancreatic endoderm is patterned in a stepwise manner, first by signals from mesoderm/ectoderm on day 7.5 p.c., and then by signals from the notochord during the next day (1,2). Shortly thereafter, endothelial cells provide additional signals to induce the prepatterned endoderm to differentiate into insulin-expressing β-cells (3). The exocrine pancreas is composed of a ductal system and a relatively homogenous population of acinar cells that secrete digestive enzymes. The endocrine compartment is organized into clusters of cells called islets of Langerhans that differentiate into four endocrine cell types—the α, β, δ, and pancreatic polypeptide (PP) cells—which express glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively (4,5).

The development of the endocrine compartment is controlled by a hierarchy of transcription factors that include *Isl1*, *Nkx2.2*, *Pax4*, *Pax6*, and *NeuroD*/BET**A2** (6–8). These factors are expressed during the early stages of pancreas development, and function at several levels of endocrine cell differentiation. However, the initial steps of pancreatic development are not perturbed in mice homozygous for null mutations in the corresponding genes (6–8).

In contrast, the homeodomain protein PDX-1 is not only important for the differentiation of β-cells, but is also essential for the initial development of the pancreas. Pancreatic differentiation is arrested at a very early stage in mice homozygous for a null mutation in the *Pdx1* gene, and pancreatic development is also severely affected in humans with homozygous mutation of the insulin promoter factor 1 (IPF-1) gene, the human orthologue of *Pdx1* (9–11). Despite the dramatic phenotype of *Pdx1* mutant mice, evagination of the foregut epithelium and formation of the dorsal pancreatic bud still occur. Likewise, the expression of early pancreatic markers is unaffected, suggesting the existence of additional genes upstream of *Pdx1* involved in the regulation of the earliest stages of pancreatic development (9–12). Therefore, *Pdx1* appears to act downstream of the initial specification of the gut endoderm to a pancreatic fate.

Recent evidence suggests that the *forkhead*/winged helix transcription factor *Foxa2* (formerly HNF-3β) is a key regulator of foregut development that may play an essential role in the cell type−specific transcription of the *Pdx1* gene in the pancreas (13,14). *Foxa2* is expressed in the foregut endoderm before and at the onset of pancreatic development and persists to adulthood, where it is expressed throughout the islet and in acinar cells (14–16). Conserved FOXA (HNF-3) binding sites were identified in the promoter/enhancer of the *Pdx1* gene in mice, rats, and humans, and the presence of FOXA-2 in the binding complexes was confirmed by EMSA (electrophoretic mobility shift assay) using nuclear extracts from multiple cell lines and primary pancreatic islet cell cultures (13–15,17,18). A transgene containing an enhancer fragment including the FOXA binding site fused to a β-galactosidase reporter directs islet-specific expression in transgenic mice (14). In addition, the FOXA binding site along with a NeuroD/BETA2 element form a composite enhancer in the rat *Pdx1* gene that mediates the synergis-

From the [1]Department of Genetics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; the [2]Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; the [3]Penn Diabetes Center, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; and the [4]Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania.

Address correspondence and reprint requests to Dr. Klaus H. Kaestner, Department of Genetics, University of Pennsylvania School of Medicine, 415 Curie Blvd., Philadelphia, PA 19104-6145. E-mail: kaestner@mail.med.upenn.edu.

Received for publication 16 January 2002 and accepted in revised form 6 May 2002.

C.S.L. and N.J.S. contributed equally to this article.

Hprt, hypoxanthine-phosphoribosyltransferase; IPF-1, insulin promoter factor 1; P*n*, postnatal day *n*; PBT, PBS containing 0.1% BSA and 0.2% Triton X-100; p.c., post coitum; PP, pancreatic polypeptide.

tic induction of *Pdx1* promoter activity by *Foxa2* and BETA2 in transfected HIT-T15 cells (13). When *Pdx1* promoter activity is repressed by glucocorticoids in HIT-T15 cells, this repression is reversed by overexpression of FOXA-2 (13).

Although the presence of a FOXA binding site in the *Pdx1* promoter in mice, rats, and humans suggests that FOXA-2 is a transcriptional activator of *Pdx1*, the contribution of *Foxa2* to the regulation of *Pdx1* in pancreatic β-cells in vivo is unknown. There are now several examples where predictions about regulatory circuits involving *Foxa* genes derived from in vitro studies were not borne out when examined in vivo (19–22). Analysis of the regulation of *Pdx1* by *Foxa2* in *Foxa2*-null mice is precluded by the fact that these mice die shortly after gastrulation because of the essential function of this gene in notochord and yolk sac development (23,24). To circumvent this limitation, mice lacking *Foxa2* specifically in pancreatic β-cells were generated using the Cre-loxP recombination system (25). We have previously shown that the FOXA-2 protein is deleted in 85% of β-cells on postnatal day 8 (P8) in these pancreatic β-cell−specific *Foxa2* knockout mice (*Foxa2*$^{loxP/loxP}$; Ins.Cre mice). In this study, we used these mice to investigate the regulation of *Pdx1* by *Foxa2* in β-cells in vivo.

RESEARCH DESIGN AND METHODS

Animals. The derivation of the *Foxa2*$^{loxP/loxP}$ mutant mouse and the Ins.Cre transgenic line has been previously reported (22,26,27). All mice were kept on a mixed outbred CD-1 background. Genotyping was performed by PCR analysis using genomic DNA isolated from the tail tip of newborn mice (22). We focused our studies on P8 mice, because mutant mice usually succumb to severe hypoglycemia 9–12 days after birth (25).

Immunofluorescence. Tissues were fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin, sectioned to 6 μm thickness, and applied to Probe-on Plus slides (Fisher Scientific). Deparaffinized and rehydrated slides were subjected to microwave antigen retrieval by being boiled for 6 min in a 10 mmol/l citric acid buffer (pH 6.0) and allowed to cool for 10 min at room temperature. Slides were washed in PBS and then blocked with protein blocking reagent (Immunotech) for 20 min at room temperature. The primary antibody was diluted in PBS containing 0.1% BSA and 0.2% Triton X-100 (PBT) and incubated with the sections overnight at 4°C. Slides were washed in PBS and then incubated with the appropriate secondary antibodies diluted in PBT for 2 h at room temperature. Slides were washed in PBS, mounted, and examined using confocal microscopy (Leica). The following antibodies were used at the indicated dilutions for immunofluorescence: rabbit anti-FOXA-2 (1:2,000; a gift from Dr. T.M. Jessell), guinea pig anti-insulin (1:1,000; Linco), rabbit anti-Pdx1 (IDX-1 253: 1:1,000), rabbit anti-GLUT2 (1:100; a gift from Dr. B. Thorens), Cy3-conjugated donkey anti-rabbit IgG (1:1,500; Jackson Immunoresearch), and Cy2-conjugated donkey anti-guinea pig IgG (1:400; Jackson).

Islet isolation and RNA analysis. Islets from two to five pancreata from P8 mice were isolated using the standard collagenase procedure and hand picked under a light microscope (28). Total RNA from islets was isolated in TRIzol (GIBCO) according to the manufacturer's instructions. The amount of total RNA recovered per islet was ~25 ng and did not differ between control and *Foxa2*$^{loxP/loxP}$;Ins.Cre mice. RT-PCR analysis was performed as described (29,30). Briefly, reverse transcription was performed using random hexamers and SuperScript II Reverse Transcriptase (GIBCO). PCR conditions were one cycle of 94°C for 3 min, followed by 28 cycles of 94°C for 45 s, 60°C for 45 s, 72°C for 90 s, and one cycle of 72°C for 5 min in a buffer containing 1.5 mmol/l $MgCl_2$, 10 μmol/l primers, 0.05 μCi ^{32}P-dATP, and 200 μmol/l dNTPs. PCR products were separated on 5% acrylamide gels that were dried and exposed to phosphorimager screens. The forward and reverse primers used were as follows:

Hprt (130bp): 5′-GGCCATCTGCCTAGTAAAGCT and 5′-GCTGGCCTATAGGCTCATAGT,

Pdx1 (553bp): 5′-TGGAGCTGGCAGTGATGTTGA and 5′-TCAGAGGCAGATCTGGCCAT,

Glut2 (193bp): 5′-CTGCTACTGCTCTTCTGTCCA and 5′-CATCCGTGAAGAGCTGGATCA.

Under the conditions used, determination of mRNA levels by RT-PCR was quantitative, as phosphorimager analysis (ImageQuant, Molecular Dynamics) of amplified products from serially diluted cDNAs confirmed the linearity of the assay for all primers used.

Protein isolation and Western blot analysis. Pancreatic islets from P8 mice were isolated on ice as described above. Protein was extracted in 50 mmol/l Tris (pH 7.5), 150 mmol/l NaCl, 1% NP40, 0.5% sodium deoxycholic acid and Complete Protease Inhibitor Cocktail Tablets (Roche) on ice. We obtained ~150–200 ng protein per islet, with yields comparable between control and mutant mice. Proteins were separated on 10% acrylamide Tris-HCl gels (Biorad), transferred to nitrocellulose membranes (Millipore), and blocked with 10% nonfat milk at room temperature for 1 h. Anti–IDX-1 253 was used at 1:10,000 in 1% milk for 1 h at room temperature (31). Goat α-rabbit conjugated to horseradish peroxidase was used at 1:10,000 (Biorad) for 1 h at room temperature. Then three 15-min washes were performed after each antibody incubation. The ECL-Plus detection system was used to detect the signal (Amersham Pharmacia). For quantification of band intensities, films were scanned and images imported into the ImageQuant (Molecular Dynamics) program. Intensities obtained for PDX-1 were normalized to those calculated for α-tubulin.

RESULTS AND DISCUSSION

***Foxa2* is a transcriptional activator of *Pdx1* in β-cells.** Because prior in vitro studies have suggested *Foxa2* as an important regulator of *Pdx1* (13,14,17,18), we examined *Pdx1* mRNA expression in 8-day-old *Foxa2*$^{loxP/loxP}$;Ins.Cre mice in which the *Foxa2* gene is deleted from 85% of all β-cells (25). When we measured *Pdx1* mRNA levels in whole pancreas using a RNAse Protection Assay, no differences were observed between *Foxa2*$^{loxP/loxP}$;Ins.Cre and control mice (data not shown). This result can be explained by the fact that *Pdx1* transcripts are present not only in islets but also in ducts and acinar cells. Consequently, because acinar and duct cells comprise >90% of pancreatic mass, normal *Pdx1* expression levels in these cells would overshadow any alteration in β-cell−specific *Pdx1* expression. Therefore, we isolated pancreatic islets from *Foxa2*$^{loxP/loxP}$;Ins.Cre mice and analyzed *Pdx1* mRNA levels by quantitative RT-PCR. Hypoxanthine-phosphoribosyltransferase (*Hprt*) was used as an internal control (Fig. 1*A*). We observed a 68% downregulation of *Pdx1* mRNA in *Foxa2*$^{loxP/loxP}$;Ins.Cre islets when compared to control islets (Fig. 1*B*). The observed effect is likely an underestimation of the true contribution of *Foxa2* to the regulation of *Pdx1* in β-cells, because *1*) islets also contain *Pdx1*-expressing δ-cells in which *Foxa2* was not deleted and *2*) *Foxa2* is still present in 15% of β cells in P8 islets (25). Because *Foxa2*$^{loxP/loxP}$;Ins.Cre mice are hypoglycemic (25), it is possible that the reduction of *Pdx1* expression observed in these mice might be the result of hypoglycemia rather than a direct effect of *Foxa2* deficiency. However, recent studies on the impact of glucose levels on *Pdx1* expression in pancreatic β-cells suggest that although persistent hyperglycemia (16 mmol/l glucose) extinguishes *Pdx1* mRNA and protein expression, hypoglycemia (0.8 mmol/l glucose) actually restores *Pdx1* levels (32). Therefore, it appears likely that the downregulation of *Pdx1* expression in *Foxa2*-deficient β-cells is not a consequence of the hypoglycemia observed in *Foxa2*$^{loxP/loxP}$;Ins.Cre mice, but rather a direct effect of *Foxa2* deficiency. Our data showed that *Foxa2* is an essential upstream factor that regulates *Pdx1* mRNA levels, directly or indirectly, in β-cells in vivo.

The level of PDX-1 protein is decreased in *Foxa2*$^{loxP/loxP}$;Ins.Cre mice. Next we examined PDX-1 protein levels in *Foxa2*$^{loxP/loxP}$;Ins.Cre mice by indirect

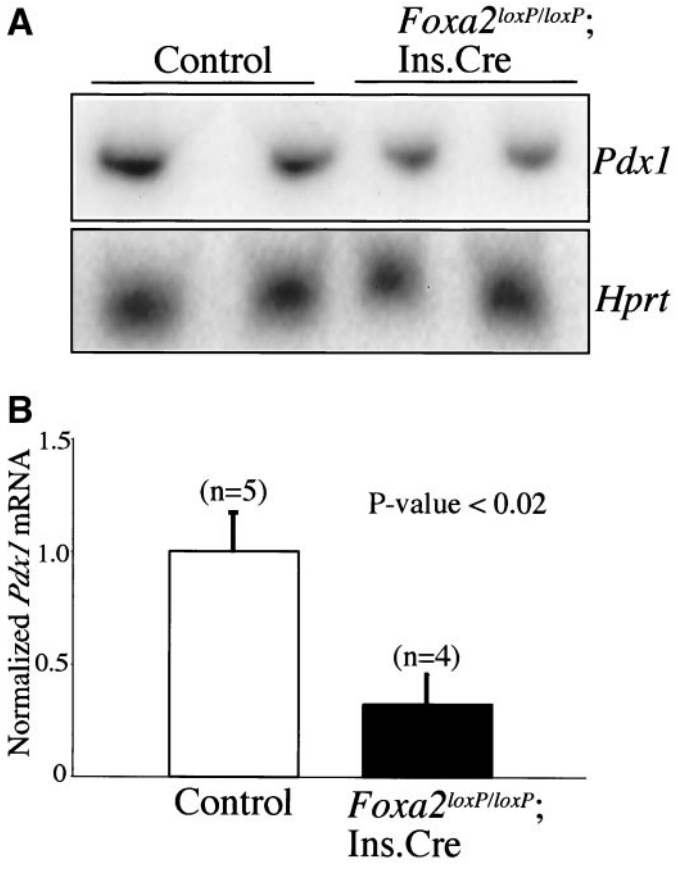

FIG. 1. FOXA-2 is required for the maintenance of *Pdx1* mRNA expression in β-cells. *A*: Representative phosphorimager image of RT analysis of total mRNA isolated from islets of P8 controls and *Foxa2*$^{loxP/loxP}$;Ins.Cre mice. For each sample, 150–170 islets from three animals were pooled. Then 10% of the synthesized cDNA was used for RT-PCR, equivalent to 15–17 islets per PCR. Total RNA was reverse transcribed, and cDNAs were amplified by PCR in the presence of [^{32}P]α-dATP with primers specific for the *Pdx1* and *HPRT* (hypoxanthine-phosphoribosyltransferase) genes. PCR products for *Pdx1* and *HPRT* are 553 and 130 bp, respectively. *HPRT* was used as internal standard. *B*: Quantitation of RT-PCR experiments exemplified in *A*. After separation on native polyacrylamide gels, bands were visualized and quantified using a phosphorimager. The *y*-axis of the graph represents the levels of *Pdx1* mRNA normalized to those of *HPRT*. *Pdx1* mRNA levels were reduced by 68% in the islets of P8 *Foxa2*$^{loxP/loxP}$;Ins.Cre animals (n = 4) when compared to controls (n = 5). Each bar represents the mean ± SE. P <0.02 by two-tailed Student's t test.

immunofluorescence (Fig. 2*A–D*). PDX-1 is normally expressed at high levels in β- and δ-cells and at a low level in acinar cells (Fig. 2*A* and *C*). In *Foxa2*$^{loxP/loxP}$;Ins.Cre animals, β-cells that lack *Foxa2* still express PDX-1 immunoreactivity similar to the extent seen in control islets (compare Fig. 2*B* and *D* with 2*A* and *C*). Because the determination of protein expression by immunofluorescence is at best semiquantitative, we next examined PDX-1 protein expression in islets by Western blot analysis (Fig. 2*E*). The level of PDX-1 protein was decreased by ~69% in *Foxa2*$^{loxP/loxP}$;Ins.Cre islets when compared to controls (Fig. 2*F*), which is similar to the reduction in *Pdx1* mRNA levels shown above. This reduction was not the result of a complete loss of PDX-1 protein in a subset of β-cells, as was demonstrated by the uniform distribution of PDX-1 protein in β-cells from *Foxa2*$^{loxP/loxP}$;Ins.Cre mice (Fig. 2*A–D*). Because the residual amount of PDX-1 protein found in *Foxa2*$^{loxP/loxP}$;Ins.Cre mice was comparable to the PDX-1 protein level (50%) in *Pdx1*/IPF-1 heterozygotes, it can be hypothesized that *Foxa2*$^{loxP/loxP}$;Ins.Cre mice would develop impaired glucose tolerance, similar to *Pdx1* heterozygous mice and MODY4 (IPF-1) patients (31,33–35). However, in part because of the requirement in *Foxa2* for the expression of both subunits of the ATP-sensitive K^+ channel, *Foxa2*$^{loxP/loxP}$;Ins.Cre mice succumb to hypoglycemia between P9 and P12 (25). Because the impaired glucose tolerance caused by *Pdx1*/IPF-1 heterozygocity is apparent only late in life (33,34), these long-term consequences of reduced *Pdx1* levels secondary to *Foxa2* deletion could not be evaluated in *Foxa2*$^{loxP/loxP}$;Ins.Cre animals.

***Glut2* expression is not altered in the Foxa2$^{loxP/loxP}$;Ins.Cre pancreas.** Targeted inactivation of the *Pdx1* gene in β-cells leads to disturbed glucose homeostasis (34). This is caused by the progressive loss of *Glut2* expression and gradual decrease of *insulin* expression (34). In addition, in *Pdx1* heterozygous animals, *Glut2* expression is markedly reduced at age 18 weeks (34). Given the reduction in *Pdx1* expression in *Foxa2*$^{loxP/loxP}$;Ins.Cre mice, we investigated whether the remaining PDX-1 protein in pancreatic islets of *Foxa2*$^{loxP/loxP}$;Ins.Cre mice is sufficient to activate downstream targets of *Pdx1* such as *Glut2* and insulin. In addition, overexpression of *Foxa2* in rat INS-1 insulinoma cells resulted in a dramatic (90%) decrease in *Glut2* expression, suggesting that *Foxa2* might be a negative regulator of *Glut2* gene expression in β-cells (36). To address this question, we determined *Glut2* protein and mRNA levels by immunofluorescence (Fig. 3*A* and *B*) and quantitative RT-PCR (Fig. 3*C*), respectively. *Glut2* mRNA levels were analyzed in total RNA samples from whole pancreas. Because *Glut2* expression is limited to β-cells, the dilution by exocrine cell mRNA did not affect the analysis. No significant differences were observed in *Glut2* mRNA level or protein expression between control and *Foxa2*$^{loxP/loxP}$;Ins.Cre islets (Fig. 3*A–C*). The discrepancy with the results obtained by overexpression of *Foxa2* in INS-1 cells (36) might be explained by the nonphysiological levels of *Foxa2* expression achieved in the INS-1 system. We have previously shown that another important target of *Pdx1* regulation, namely insulin, is not affected by the deletion of *Foxa2* from pancreatic β-cells (25). No differences were observed in insulin immunofluorescence, total pancreatic insulin content, or insulin mRNA levels between control and *Foxa2*$^{loxP/loxP}$;Ins.Cre mice (25). Thus, the residual amount of PDX-1 protein present in the β-cells of *Foxa2*$^{loxP/loxP}$;Ins.Cre mice is sufficient to maintain activation of the two *Pdx1* target genes, *Glut2* and insulin, at least until P8, after which time the *Foxa2*$^{loxP/loxP}$;Ins.Cre mice succumb to the severe hypoglycemia caused by the absence of *Foxa2* from β-cells (25).

In summary, the molecular analysis of *Foxa2*$^{loxP/loxP}$;Ins.Cre mice presented here has provided the first in vivo evidence of transcriptional regulation of *Pdx1* by *Foxa2* in β-cells, indicating that FOXA-2 acts as a transcriptional activator that is required for the maintenance of *Pdx1* transcription. Consistent with the finding of FOXA binding sites in the *Pdx1* promoter (13,14,17,18), we have shown downregulation of *Pdx1* mRNA and protein levels in β-cells from which *Foxa2* has been deleted. These in vitro and in vivo data have concurrently placed *Foxa2* upstream

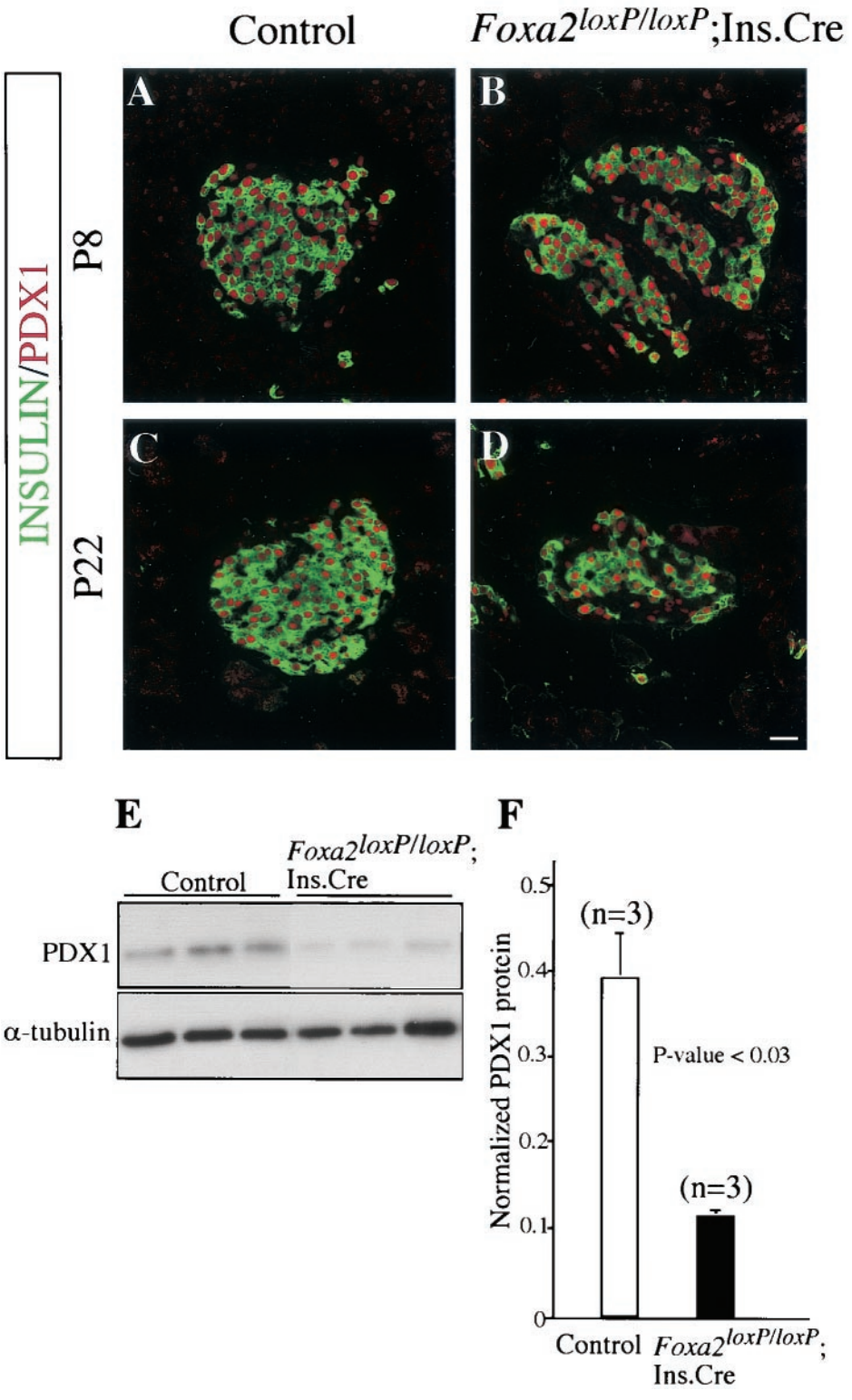

FIG. 2. *A*–*D*: Analysis of PDX-1 protein by immunofluorescence and Western blot on P8 pancreas. Double labeling of insulin (green) and PDX-1 (red) showed no apparent difference in the PDX-1 protein levels between control (*A* and *C*) and *Foxa2*$^{loxP/loxP}$;Ins.Cre pancreata (*B* and *D*) at P8 and P22. These images were captured using confocal microscopy (Leica; scale bar 20 μm; magnification 400×). *E*: Western blot analysis of PDX-1 protein level in islets from P8 control and *Foxa2*$^{loxP/loxP}$;Ins.Cre animals, showing a reduction of PDX-1 protein (38 kDa) in *Foxa2*$^{loxP/loxP}$;Ins.Cre islets when compared to control islets. α-Tubulin (55 kDa) was used to ensure equal protein loading. In all, ~80 islets were isolated from three control and three *Foxa2*$^{loxP/loxP}$;Ins.Cre pancreata. Protein extracted from 40 islet equivalents (7.5 μg) was used for both control and *Foxa2*$^{loxP/loxP}$;Ins.Cre animals. *F*: Quantitation by ImageQuant of Western blot experiment exemplified in *E*. The *y*-axis of the graph represents the levels of PDX-1 protein normalized to those of α-tubulin. PDX-1 protein levels were reduced by 69% in the islets of P8 *Foxa2*$^{loxP/loxP}$;Ins.Cre animals ($n = 3$) when compared to the controls ($n = 3$). Each bar represents the mean ± SE. $P < 0.03$ by two-tailed Student's t test.

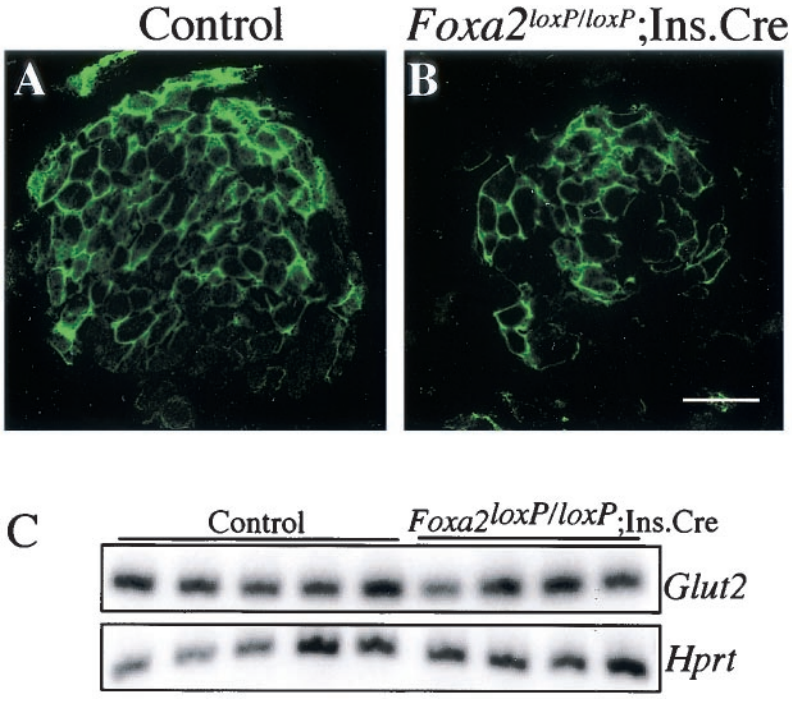

FIG. 3. Analysis of GLUT2 protein expression and *Glut2* mRNA level from P8 control and *Foxa2*$^{loxP/loxP}$;Ins.Cre animals by immunofluorescence and RT-PCR. *A* and *B*: GLUT2 protein is expressed at similar levels in the islets of *Foxa2*$^{loxP/loxP}$;Ins.Cre animals when compared to controls. Scale bar 20 μm. *C*: RT-PCR analysis of total RNA isolated from whole pancreata of P8 control and *Foxa2*$^{loxP/loxP}$;Ins.Cre mice. No significant differences in *Glut2* mRNA levels (193bp) were detected in whole pancreas RNA samples between control and *Foxa2*$^{loxP/loxP}$;Ins.Cre animals after quantification using phosphorimager analysis. *HPRT* served as an internal standard (130 bp). $n = 5$ for control and $n = 4$ for the *Foxa2*$^{loxP/loxP}$;Ins.Cre mice.

of *Pdx1* in the transcription factor hierarchy in the differentiated β-cell.

Future studies will be directed at developing a Cre-transgene that will allow for the deletion of *Foxa2* before the formation of the pancreatic primordia. This will allow the assessment of the role for *Foxa2* during the initial transcriptional activation of the *Pdx1* gene. We have shown that *Foxa2* is indeed required for the full activation of the *Pdx1* gene in pancreatic β-cells in vivo; however, *Foxa2* clearly is not the only activator of the *Pdx1* gene, as some *Pdx1* expression is maintained in cells devoid of *Foxa2*. Our findings support the notion expressed by others (37) that mutations in the human FOXA-2 gene might also contribute to abnormal glucose homeostasis and diabetes.

ACKNOWLEDGMENTS

This study was facilitated by the University of Pennsylvania Diabetes Center (P30-DK-19525) and the Penn Center for Molecular Studies in Digestive and Liver Disease (P30-DK-50306). This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases (RO1-DK-55342 to K.H.K.).

The authors are grateful to Drs. J. A. Blendy, N. Perreault, and N. R. May for their critical reading of the manuscript. The authors are thankful to Dr. B. Calvi for help in confocal microscopy and to K. A. O'Shea for maintaining the mouse colony and genotyping the animals.

REFERENCES

1. Kim SK, Hebrok M, Melton DA: Notochord to endoderm signaling is required for pancreas development. *Development* 124:4243–4252, 1997
2. Wells JM, Melton DA: Early mouse endoderm is patterned by soluble factors from adjacent germ layers. *Development* 127:1563–1572, 2000
3. Lammert E, Cleaver O, Melton D: Induction of pancreatic differentiation by signals from blood vessels. *Science* 294:564–567, 2001
4. Alpert S, Hanahan D, Teitelman G: Hybrid insulin genes reveal a developmental lineage for pancreatic endocrine cells and imply a relationship with neurons. *Cell* 53:295–308, 1988
5. Slack JM: Developmental biology of the pancreas. *Development* 121:1569–1580, 1995
6. Edlund H: Transcribing pancreas. *Diabetes* 47:1817–1823, 1998
7. Sander M, German MS: The beta cell transcription factors and development of the pancreas. *J Mol Med* 75:327–340, 1997
8. St-Onge L, Wehr R, Gruss P: Pancreas development and diabetes. *Curr Opin Genet Dev* 9:295, 1999
9. Jonsson J, Carlsson L, Edlund T, Edlund H: Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature* 371:606–609, 1994
10. Offield MF, Jetton TL, Labosky PA, Ray M, Stein RW, Magnuson MA, Hogan BL, Wright CV: PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development* 122:983–995, 1996
11. Stoffers DA, Zinkin NT, Stanojevic V, Clarke WL, Habener JF: Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence. *Nat Genet* 15:106–110, 1997
12. Ahlgren U, Jonsson J, Edlund H: The morphogenesis of the pancreatic mesenchyme is uncoupled from that of the pancreatic epithelium in IPF1/PDX1-deficient mice. *Development* 122:1409–1416, 1996
13. Sharma S, Jhala US, Johnson T, Ferreri K, Leonard J, Montminy M: Hormonal regulation of an islet-specific enhancer in the pancreatic homeobox gene STF-1. *Mol Cell Biol* 17:2598–2604, 1997
14. Wu KL, Gannon M, Peshavaria M, Offield MF, Henderson E, Ray M, Marks A, Gamer LW, Wright CV, Stein R: Hepatocyte nuclear factor 3beta is involved in pancreatic beta-cell-specific transcription of the pdx-1 gene. *Mol Cell Biol* 17:6002–6013, 1997
15. Cockell M, Stolarczyk D, Frutiger S, Hughes GJ, Hagenbüchle O, Wellauer PK: Binding sites for hepatocyte nuclear factor 3 beta or 3 gamma and pancreas transcription factor 1 are required for efficient expression of the gene encoding pancreatic alpha-amylase. *Mol Cell Biol* 15:1933–1941, 1995
16. Monaghan AP, Kaestner KH, Grau E, Schutz G: Postimplantation expression patterns indicate a role for the mouse forkhead/HNF-3 alpha, beta and

gamma genes in determination of the definitive endoderm, chordamesoderm and neuroectoderm. *Development* 119:567–578, 1993
17. Marshak S, Ben-Shushan E, Shoshkes M, Havin L, Cerasi E, Melloul D: Regulatory elements involved in human pdx-1 gene expression. *Diabetes* 50 (Suppl. 1):S37–S38, 2001
18. Ben-Shushan E, Marshak S, Shoshkes M, Cerasi E, Melloul D: A pancreatic beta -cell-specific enhancer in the human PDX-1 gene is regulated by hepatocyte nuclear factor 3beta (HNF-3beta), HNF-1alpha, and SPs transcription factors. *J Biol Chem* 276:17533–17540, 2001
19. Duncan SA, Navas MA, Dufort D, Rossant J, Stoffel M: Regulation of a transcription factor network required for differentiation and metabolism. *Science* 281:692–695, 1998
20. Kaestner KH, Katz J, Liu Y, Drucker DJ, Schütz G: Inactivation of the winged helix transcription factor HNF3alpha affects glucose homeostasis and islet glucagon gene expression in vivo. *Genes Dev* 13:495–504, 1999
21. Shen W, Scearce LM, Brestelli JE, Sund NJ, Kaestner KH: FoxA3 (hepetocyte nuclear factor 3 gamma) is required for regulation of hepatic GLUT2 expression and the maintenance of glucose homeostasis during a prolonged fast. *J Biol Chem* 276:42812–42817, 2001
22. Sund NJ, Ang SL, Sackett SD, Shen W, Daigle N, Magnuson MA, Kaestner KH: Hepatocyte nuclear factor 3beta (Foxa2) is dispensable for maintaining the differentiated state of the adult hepatocyte. *Mol Cell Biol* 20:5175–5183, 2000
23. Weinstein DC, Ruiz i Altaba A, Chen WS, Hoodless P, Prezioso VR, Jessell TM, Darnell JE Jr: The winged-helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo. *Cell* 78:575–588, 1994
24. Ang SL, Rossant J: HNF-3 beta is essential for node and notochord formation in mouse development. *Cell* 78:561–574, 1994
25. Sund NJ, Vatamaniuk MZ, Casey M, Ang SL, Magnuson MA, Stoffers DA, Matschinsky FM, Kaestner KH: Tissue-specific deletion of Foxa2 in pancreatic beta cells results in hyperinsulinemic hypoglycemia. *Genes Dev* 15:1706–1715, 2001
26. Kulkarni RN, Bruning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR: Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. *Cell* 96:329–339, 1999
27. Postic C, Shiota M, Niswender KD, Jetton TL, Chen Y, Moates JM, Shelton KD, Lindner J, Cherrington AD, Magnuson MA: Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. *J Biol Chem* 274: 305–315, 1999
28. Scharp DW, Kemp CB, Knight MJ, Ballinger WF, Lacy PE: The use of ficoll in the preparation of viable islets of Langerhans from the rat pancreas. *Transplantation* 16:686–689, 1973
29. Duncan SA, Nagy A, Chan W: Murine gastrulation requires HNF-4 regulated gene expression in the visceral endoderm: tetraploid rescue of Hnf-4(-/-) embryos. *Development* 124:279–287, 1997
30. Wilson PA, Melton DA: Mesodermal patterning by an inducer gradient depends on secondary cell-cell communication. *Curr Biol* 4:676–686, 1994
31. Stoffers DA, Stanojevic V, Habener JF: Insulin promoter factor-1 gene mutation linked to early-onset type 2 diabetes mellitus directs expression of a dominant negative isoprotein. *J Clin Invest* 102:232–241, 1998
32. Gleason CE, Gonzalez M, Harmon JS, Robertson RP: Determinants of glucose toxicity and its reversibility in the pancreatic islet beta-cell line, HIT-T15. *Am J Physiol Endocrinol Metab* 279:E997–E1002, 2000
33. Dutta S, Bonner-Weir S, Montminy M, Wright C: Regulatory factor linked to late-onset diabetes? *Nature* 392:560, 1998
34. Ahlgren U, Jonsson J, Jonsson L, Simu K, Edlund H: beta-cell-specific inactivation of the mouse Ipf1/Pdx1 gene results in loss of the beta-cell phenotype and maturity onset diabetes. *Genes Dev* 12:1763–1768, 1998
35. Stoffers DA, Ferrer J, Clarke WL, Habener JF: Early-onset type-II diabetes mellitus (MODY4) linked to IPF1 (Letter). *Nat Genet* 17:138–139, 1997
36. Wang H, Gauthier BR, Hagenfeldt KA, Iezzi M, Wollheim CB: Foxa2 (HNF3beta) controls multiple genes implicated in metabolism-secretion coupling of glucose-induced insulin release. *J Biol Chem* 277:17564–17570, 2002
37. Navas MA, Vaisse C, Boger S, Heimesaat M, Kollee LA, Stoffel M: The human HNF-3 genes: cloning, partial sequence and mutation screening in patients with impaired glucose homeostasis. *Hum Hered* 50:370–381, 2000