Legacy Effect Of Foxo1 In Pancreatic Endocrine Progenitors

On Adult β-cell Mass And Function

Shivatra Chutima Talchai1,2, Domenico Accili1

1Department of Medicine and Berrie Diabetes Center, Columbia University, New York, NY 10032,

2Faculty of Science, King Mongkut's University of Technology Thonburi, Bangkok, 10140, Thailand

Word count: 4,011

Figures: 7

Supplemental figures: 7

Correspondence: da230@columbia.edu
SUMMARY

β-cell dysfunction in diabetes results from abnormalities of insulin production, secretion, and cell number. These abnormalities may partly arise from altered developmental programming of β-cells. Foxo1 is important to maintain adult β-cells. But little is known about its role in pancreatic progenitor cells as determinants of future β-cell function. We addressed this question by generating an allelic series of somatic Foxo1 knockouts at different stages of pancreatic development in mice. Surprisingly, ablation of Foxo1 in pancreatic progenitors resulted in delayed appearance of Neurogenin3+ progenitors and their persistence into adulthood as a self-replicating pool, causing a 4-fold increase of β-cell mass. Similarly, Foxo1 ablation in endocrine progenitors increased their numbers, extended their survival, and expanded β-cell mass. In contrast, ablation of Foxo1 in terminally differentiated β-cells didn’t increase β-cell mass nor did it affect Neurogenin3 expression. Despite the increased β-cells mass, islets from mice lacking Foxo1 in pancreatic or endocrine progenitors responded poorly to glucose, resulting in glucose intolerance. We conclude that Foxo1 integrates cues that determine developmental timing, pool size, and functional features of endocrine progenitor cells, resulting in a legacy effect on adult β-cell mass and function. Our results illustrate how developmental programming predisposes to β-cell dysfunction in adults, and raise questions on the desirability of increasing β-cell mass for therapeutic purposes in type 2 diabetes.
INTRODUCTION

Environmental and nutritional cues can affect developmental programming and organ plasticity in utero, resulting in the metabolic syndrome and type 2 diabetes in adults (1). Examples of such gene/environment interactions include: (i) low concordance rate for type 2 diabetes among identical twins; (ii) increased risk of diabetes in children of diabetic mothers; (iii) fetal malnourishment as a risk factor for diabetes; (iv) limited success of adult lifestyle modifications in preventing type 2 diabetes (2). Although there are multiple explanations for these disparate observations, one unifying feature is that fetal programming of organ plasticity partly dictates adult predisposition to metabolic diseases.

There is evidence that developmental cues can result in acquired abnormalities of β-cell function. For example, intrauterine growth retardation in rodents and human results in reduced pancreas size that predisposes to diabetes (3). And obese adolescents that were exposed to gestational diabetes in utero exhibit a fourfold higher risk than controls of becoming glucose intolerant or diabetic (4).

Foxo1 integrates cell differentiation, proliferation, and response to metabolic and cellular stressors (5). In the adult pancreas, Foxo1 expression is restricted to β-cells (6; 7), where it modulates metabolic flexibility—i.e., the ability to switch between glucose and lipids as a source of acyl-CoA for mitochondrial oxidative phosphorylation (8)—and enforces the β-cell fate, allowing β-cells to retain terminally differentiated features (9). Disruption of these activities results in β-cell dysfunction (8-10).

In contrast to the adult pancreas, Foxo1 expression in the fetal pancreas parallels that of the master regulator of pancreas specification, Pdx1 (11). Like the latter, Foxo1 is expressed widely at
embryonic day (E)12.5-13.5, becomes restricted to Neurog3+ endocrine progenitors at E14.5, and to β-cells at birth (7). Here, we investigated whether Foxo1 determines number and function of pancreatic or endocrine progenitors, using stage-specific genetic inactivation strategies. The surprising results of this investigation indicate that Foxo1 controls timing and size of the endocrine progenitor cell pool, such that embryonic Foxo1 ablation delays formation of endocrine progenitors and causes their persistence into adulthood (12). Accordingly, β-cell mass continues to expand in mice lacking Foxo1 in endocrine progenitors, but cells don’t function properly, impairing insulin secretion. The notion that Foxo1 pre-programs adult β-cell mass and function has important implications for the hypothesis of fetal programming of adult β-cell function, and invites caution with regard to expanding β-cell mass as a treatment for diabetes.
RESEARCH DESIGN AND METHODS

Antibodies and immunohistochemistry

We fixed and processed tissues for immunohistochemistry as described (7; 9). We used the following antibodies: rabbit primary antibodies to FoxO1, amylase and glucokinase (Santa Cruz), Pdx1 (gift from C. Wright), Ki67 (Dako), H2B (Cell Signaling), glucagon (Sigma, Phoenix Peptide), Gfp (Invitrogen), Sox9 (Chemicon); guinea pig primary antibodies to insulin (Dako), somatostatin (Chemicon), pancreatic polypeptide (Linco), Gfp (Rockland), and goat primary antibodies to Neurog3, NeuroD1, Hnf6 (Santa Cruz), and Pdx1 (from C. Wright); mouse primary antibody to cytokeratin 19 (Sigma). We used FITC-, Cy3-, and Alexa-conjugated donkey secondary antibodies (Jackson Immunoresearch Laboratories, and Molecular Probes), peroxidase staining, and DAPI for nuclear counterstaining (7; 9). We used Apoptag kit for TUNEL assays (Millipore) and stained nuclei with DAPI or DRAQ5 (Cell Signaling). Image acquisition and morphometry were done as described (7; 9).

Flow cytometry

We digested and dissociated the pancreas as described (13) and incubated single cell suspensions with glucose prior to flow cytometry (14), or performed immunohistochemistry with antibodies to insulin, Neurog3, amylase, or Ki67 (13).

Physiological studies

Pdx1-Cre, Neurog3-Cre, Ins-Cre, Rosa26-eGfp, Neurog3-Gfp, Neurog3 Gfp/+, and Foxo1 flox/+ mice have been described (9; 15). We carried out intraperitoneal glucose tolerance tests in overnight-fasted 8-month-old male mice (16), and static incubations of collagenase-purified islets as described
We prepared acid-ethanol extracts from adult pancreas as described (9). We measured glucagon by radioimmunoassay and insulin, C-peptide, and proinsulin by ELISA (Millipore, and Alpco).

**RNA procedures**

We applied standard techniques for mRNA isolation and quantitative PCR (9). Primer sequences for Gck, Kir6.1, Sur1, Pdk1, Ucp, Pcl, Pcl2, Glut2, Glut4, Neurog3, Pdx1, MafA, Nkx6.1, NeuroD1, Nkx2.2, Tubulin2, Hprt, Foxo1, Insulin1, Insulin2 (9), Sox9 (18), Hnf6 (19), cyclin D1, D2, A2, CDK4, CDK6, p18, p21, CDK5, cyclin G1, and Cyclin H (RT2 profiler PCR array Qiagen, Mississauga, ON) have been described (9; 15). Tubulin2 and Hprt were used as standard.

**Statistical analysis**

We analyzed data using Student’s t-test and used the traditional threshold $P<0.05$ to declare statistical significance.
RESULTS

Developmental stage-specific pancreatic Foxo1 knockouts

Foxo1 is a negative regulator of β-cell mass (6; 20; 21) that is expressed in pancreatic and endocrine progenitors during fetal development, and becomes restricted to β-cells as the latter become terminally differentiated (7). We investigated the mechanism by which Foxo1 limits β-cell mass, and asked whether it does so by controlling β-cell or endocrine progenitor cell number, i.e., pre- or post-β-cell formation. To distinguish between these two possibilities, we inactivated Foxo1 at three distinct developmental stages: (i) in pancreatic precursors (using Pdx1-cre-mediated gene knockout) (22); (ii) in endocrine progenitors (using Neurog3-cre) (23); and (iii) in terminally differentiated β-cells (using Ins-cre) (9) (Figure 1A). For each cross, we generated two independent lines and line-specific littermates carrying Cre (denoted WT) to control for locus effects and ectopic recombination (24). Genotyping of DNA extracted from multiple organs, including collagenase-purified islets, confirmed the specificity of Cre-mediated excision (not shown).

We first compared mice with pan-pancreatic or β-cell-specific Foxo1 ablation (PKO and IKO, respectively). qRT-PCR showed that Foxo1 mRNA was reduced by ~90% in islets from PKO, and ~70% in islets from IKO mice, compared to WT (Figure S1A). Conversely, Foxo3 and Foxo4 transcripts increased 3- to 7-fold in PKO and IKO compared to controls (Figure S1A-C), as seen before (9). Indeed, triple Foxo-deficient mice generated using a similar approach phenocopy PKO mice (J.Y.Kim-Muller et al., unpublished observation). In both lines, we introduced a Rosa26-Gfp allele as marker of Cre activity to identify cells in which recombination had occurred. Immunohistochemistry demonstrated Gfp reactivity in all pancreatic cell types of PKO (Figure
S1D), and only in β−cells of IKO mice (9). PKO and IKO mice had similar body weight and glucose levels to WT controls up to 8 months of age (Figure S1E-F). Thus, Foxo1 ablation occurred specifically and efficiently, and did not result in overt metabolic abnormalities.

Altered β−cell mass following Foxo1 ablation in pancreatic precursors

Homeostatic β−cell mass is the product of β−cell differentiation, survival, and self-renewal (25), whereas pancreas and islet size are predetermined by the number of pancreatic precursors (26). Therefore, the predicted outcome of Foxo1 deletion in pancreatic precursors or β−cells was that: (i) if Foxo1 suppresses proliferation of differentiated β−cells, both IKO and PKO should show increased β−cell mass; (ii) if Foxo1 acts on β−cell precursor differentiation and/or proliferation, but not on proliferation of mature β−cells, increased β−cell mass should be found in PKO, but not IKO. Histomorphometry of the pancreas showed that PKO mice were born with slightly lower β−cell mass but caught up rapidly, exhibiting a ~twofold increase at 3 months, threefold at 7 months, and fivefold of WT at 12 months (Figure 1B-C and Figure S1G). Other endocrine cell types showed commensurate increases (not shown). Analysis by flow cytometry in mice carrying a Cre-dependent Rosa-Gfp reporter confirmed increased numbers of insulin-producing (Gfp+) cells (Figure 1D-E). In contrast, β−cell and pancreas mass in IKO mice remained unchanged throughout (Figure 1B-C). These findings suggest that Foxo1 regulates β−cell mass through actions that precede β−cell formation.

Interestingly, total pancreas weight in PKO mice increased by ~15% at 3 months, and by 50% at 8 months (Figure S1H). Analysis of exocrine cell proliferation by flow cytometry of
Ki67+/amylose+ cells and Ki67+ immunohistochemistry showed a ~15% increase in 5-month-old PKO mice that petered out by 7 months (Figure S1I-J).

**Adult expansion of endocrine progenitors in PKO pancreas**

The preservation of β-cell mass in IKO mice focused our investigations on Foxo1 action in endocrine progenitors. Pan-pancreatic precursors and endocrine progenitors develop at distinct temporal windows (27). Neurog3+ progenitors give rise to endocrine cells (12). Their numbers peak at E13.5-15.5, decline after E17.5 (28), and are exceptionally rare in the adult, except in a duct ligation model (29). Thus, we investigated whether Foxo1 ablation in pancreatic progenitors affected generation and properties of endocrine progenitors, including their contribution to adult β-cell mass.

We surveyed endocrine progenitor pool size during peak endocrine differentiation (E15.5). Surprisingly, in PKO pancreas, Neurog3 expression was hardly detectable by either qPCR or immunohistochemistry (Figure S2A-B). To increase the sensitivity of detecting Neurog3+ cells, we introduced a transgene encoding Gfp driven by the Neurog3 promoter in PKO mice, yet failed to find pancreatic Gfp+ cells at E15.5, while intestinal Gfp+ cells were present (Figure S2B). Endocrine lineage development appeared to stall at E15.5 in PKO, as additional coeval markers of β-cell differentiation such as NeuroD1 (30) were also undetectable (Figure S2B). In contrast, we observed a 50–fold increase of Neurog3 mRNA at E17.5 that persisted into adulthood, reaching 18-fold over WT at P14, and remaining over twofold higher thereafter (Figure S2A).

These data suggest that Foxo1 ablation delays appearance but promotes post-natal survival of endocrine progenitors. In adult animals, Neurog3 protein can be detected in hormone-producing cells using reporter genes, and its function is required for endocrine maturation (31). To detect Neurog3+ cells in adult mice, we introduced two different genetically modified alleles in PKO mice.
in two separate experiments: one allele encodes a Neurog3-Gfp transgene (12), and the other one a Neurog3-Gfp knock-in (32). We took advantage of the longer half-life of Gfp than endogenous Neurog3 (up to 1-2 days) (23)–to increase the likelihood of detecting Neurog3+ cells.

In 3-month-old PKO mice carrying transgenic or knock-in Neurog3 reporters, double immunohistochemistry with Gfp and insulin revealed Neurog3-Gfp+/insulin+ cells alongside with Neurog3-Gfp+/insulin− cells. Neurog3-Gfp+ cells resided within islets, or near ducts (Figure 2A), and differed by the levels of green fluorescence, consistent with the identification of distinct Neurog3hi and Neurog3low cell populations during development (27). We quantified Neurog3-Gfp+/insulin− cells by flow cytometry (Figure 2B), using glucose-induced autofluorescence to separate them from Neurog3-Gfp+/insulin+ cells (14). The latter increased their fluorescence following incubation with glucose, while Neurog3-Gfp+/insulin− cells did not. The population of Neurog3-Gfp+/insulin− cells was enriched 15-fold in PKO pancreas (Figure 2B). The results from both Gfp reporters were similar, confirming that there is an expanded Neurog3-Gfp+ pool in adult PKO mice. These data show that Foxo1 ablation in pancreatic precursors leads to delayed expansion of Neurog3+ endocrine progenitors and their persistence into adulthood.

**Juxta-ductal β-cells in PKO mice**

β-cells are occasionally found near pancreatic ducts. We investigated whether the ductal milieu contributes to the enlarged β-cell mass in PKO mice. In 3-month-old WT mice, juxta-ductal insulin+ cells were rare, but in PKO pancreas their frequency increased 100-fold (Figure S2C-D), with other hormone+ cells present at lower rates (Figure S2C) (7). Juxta-ductal insulin+ cells expressed Pdx1, confirming that they are bona fide β-cells (Figure S2E). We asked whether juxta-ductal insulin+ cells arise from Foxo1-deleted or -undeleted cells by comparing PKO and WT mice.
carrying Rosa26-Gfp. All surveyed juxta-ductal insulin+ cells were Gfp+ (Figure S2D). Thus, even considering the mosaicism of Pdx1-driven Rosa26-Gfp expression (22), these cells should be considered descendants of Foxo1-ablated cells, indicating that juxta-ductal hormone+ cells in PKO mice arise cell-autonomously (7).

A replicative pool of endocrine progenitors in adult PKO mice

The data above indicate that Foxo1 ablation in pancreatic progenitors increases progenitor pool size and β-cell mass. We further investigated whether increased β-cell mass was also due to altered β-cell turnover. We have previously shown that β-cell turnover is normal in IKO mice throughout life (9). We surveyed β-cell death by Tunel assay and found no difference between PKO and WT mice (Figure S3A-B). We assessed β-cell proliferation by immunohistochemistry or flow cytometry with cell cycle marker, Ki67. The percentage of Ki67+/insulin+ cells in PKO mice was slightly decreased at P7, but rose at 1 month, and remained elevated up to one year of age, a time when β-cell turnover was no longer detectable in WT mice (Figure 3A-B). Immunohistochemistry yielded similar data (Figure 3C-D). Next, we surveyed a panel of cell cycle genes (33). Quantitative mRNA measurements showed modest elevations of p18, p21, CDK5, and cyclin G1 and H in PKO, but not IKO islets (Figure 3E and S3B). The small extent of these mRNA changes is commensurate with the increase in β-cell proliferation rates, but neither is sufficient to account for the large increase of β-cell mass in PKO mice. We therefore tested if there was an increase in endocrine progenitor proliferation, resulting in β-cell neogenesis. Strikingly, we identified Neurog3hi cells that were also positive for H2B—a marker of S phase cell division—and Ki67 in islets of 3- and 15-month-old PKO mice (Figure 3F-G). These data indicate that Foxo1 ablation in β-cell precursors increases endocrine
progenitor replication in the adult pancreas, contributing to *bona fide* post-natal \( \beta \)-cell neogenesis, and providing an explanation for the lack of effects on \( \beta \)-cell mass in IKO mice.

**Abnormal insulin production and release in PKO mice**

We next investigated the function of PKO \( \beta \)-cells *in vivo*. The expectation was that PKO mice would be more glucose tolerant, owing to larger islet size. But to our surprise, 8-month-old PKO mice showed greater glucose excursions during glucose tolerance tests (Figure 4A and C), accompanied by impaired insulin release in response to glucose and arginine *in vivo* (Figure 4B and D). These data indicate that PKO \( \beta \)-cells are dysfunctional, consistent with findings that Foxo1 ablation in pancreatic progenitors exacerbates diabetes in \( db/db \) mice (10).

To determine whether PKO islet function was impaired due to cell-autonomous abnormalities of insulin secretion, we isolated islets and performed glucose-stimulated insulin secretion *ex vivo*. The results showed that insulin release from PKO islets was significantly reduced at 11.2 and 16.7 mM glucose, as well as in response to the membrane-depolarizing agent, KCl (Figure 4E). Consistent with these findings, serum insulin levels in the re-fed state fell by nearly 40%, accompanied by a 20-fold rise in proinsulin levels (Figure 4F). Pancreatic insulin content rose two- and threefold in 3- and 8-month-old PKO mice, respectively, as did glucagon content (Figure 4G and S4A). However, when we normalized insulin content by \( \beta \)-cell mass, we found a 40% decrease in PKO mice (Figure 4H), which might explain the reduced response to KCl. In contrast, IKO \( \beta \)-cell function appeared indistinguishable from controls at these ages (Figure S4B-D). The findings are suggestive of defects in insulin production, processing, and secretion in PKO mice.
Foxo1-deficient endocrine progenitors yield defective β-cells

The enlarged β-cell mass in PKO was accompanied by a 50% increase of pancreas weight in adult mice (Figure S1G), raising the question of whether it was simply a result of increased pancreas size. Thus, we wanted to determine whether the effect of Foxo1 ablation was exerted in pancreatic or endocrine progenitors. To analyze this point, we compared endocrine progenitor-specific Foxo1 knockouts (denoted NKO) with WT and PKO mice. To rule out the potential mosaicism of Neurog3-Cre transgene expression (23), we introduced a Rosa26-Gfp allele into NKO mice to verify the cell types in which recombination had occurred. Immunohistochemistry with Gfp and either insulin or a cocktail of Pp, glucagon, and somatostatin antibodies showed 98% co-localization. Assuming that Rosa26-Gfp is a faithful surrogate of Foxo1 deletion, NKO mice should be considered a model of endocrine cell-specific Foxo1 knockout (Figure S5A).

The prediction was that, if increased islet size and impaired β-cell function in PKO mice were due to an effect of Foxo1 in pancreatic progenitors, these phenotypes should not appear when inactivating Foxo1 at the endocrine progenitor stage (Figure 1A), whereas if they were due to effects in endocrine progenitors, NKO mice should phenocopy PKO mice. In islets isolated from 3-month-old NKO mice, Foxo1 expression decreased by 85%, confirming efficient deletion of Foxo1. Similar to PKO and IKO mice, Foxo3 and Foxo4 levels rose (Figure S5B).

β-cell mass increased as a function of age in NKO mice, peaking at ~fivefold of controls at 15 months (Figure 5A-B); and was ~18% larger than in age-matched PKO mice (Figure 1), while NKO pancreas weight was identical to WT (Figure 5C). Next, we examined β-cell replication by flow cytometry. We detected a threefold increase of Ki67+/insulin+ cells in islets from 8-month-old NKO mice (Figure 5D). Moreover, ~0.5% of insulin+ cells were still replicating in 12-month-old NKO mice, compared to none in WT. In addition, we observed Ki67+/Neurog3+ cells in 8- and 15-
month-old NKO mice (Figure 5E). *Neurog3* mRNA levels were consistently 2-3 fold higher in NKO mice compared to WT, regardless of age (Figure 5F). Moreover, quantitative measurements of mRNAs encoding cell cycle genes showed evidence of increased cell replication, with higher transcripts of *cyclin G1*, *cyclin H*, *Cdk5*, *p18*, and *p21* (Figure S5C).

We evaluated whether Neurog3+ progenitors were present in adult NKO pancreas, using NKO mice bearing a Neurog3-Gfp reporter. Similar to PKO pancreas, Gfp staining was detectable within and outside the islet, consistent with increased *Neurog3* mRNA (Figure 5F, and S5D). This result indicates that cell-autonomous changes in endocrine progenitors caused by Foxo1 ablation during embryonic development allow them to persist in adult mice. These data support a model in which the effects of Foxo1 on adult β-cell mass and function are exerted in endocrine progenitors, rather than (or in addition to) pancreatic precursors.

Like PKO, NKO mice exhibited decreased insulin release in response to glucose and KCl (Figure 6A), ~30% reduction of re-fed insulin levels, and 11-fold increase of proinsulin levels, compared to WT littermates (Figure 6B). Whereas total insulin (Figure 6C) and glucagon content (Figure S6A) were increased in NKO islets, insulin and c-peptide content per β-cell declined by 50% (Figure 6D-E). The latter was similarly reduced in PKO mice (Figure 6E). Thus, Foxo1-deficient endocrine progenitors recapitulate features of PKO mice, including increased β-cell mass, altered gene expression, and reduced in vivo function.

**β-cell markers in PKO and NKO**

We compared β-cell markers in 3-month-old PKO and NKO mice by immunohistochemistry. Expression of MafA, Pc1/3, and c-peptide was reduced (Figure 6F). qRT-PCR of islet mRNA from 3-month-old PKO and NKO mice showed that *Pdx1* and *Neurog3* mRNA were three- to fivefold
higher, while *Maf*A was reduced by ~60-70% in both PKO and NKO (Figure S6B), consistent with findings that Foxo1 suppresses Pdx1 (6) and Neurog3 (9), but activates MafA (20). Since Foxo1 ablation impairs Notch signaling (15; 34; 35), and the latter is required to activate Neurog3 (36), we measured mRNA transcripts of genes required for Notch signaling and observed a 30% reduction of *Hes1* in PKO and NKO islets, consistent with the increase in *Neurog3* (Figure S6B-C). We also saw increased expression of *Nkx6.1* and *Nkx2.2*, and reduced expression of *Ins2* and *PC1/3* (Figure S6D-F).

Transcription factor Sox9 is expressed in pancreatic progenitors but not in adult islets (37), and becomes reactivated in human diabetic islets (38), and in diabetic mice harboring Vhl mutations (39). Given the similarities between Sox9 gain-of-function and PKO mice, we asked whether Sox9 expression was altered. Indeed, immunohistochemistry showed similar numbers of Sox9⁺ progenitors in PKO and WT mice at E15.5 (Figure S2B), but a 10-fold increase at E17.5 (Figure 7A-B) –a time when Sox9⁺ cells become restricted to a subset of ductal cells in normal mice (Figure S7A) (37). These findings were associated with persistent expression of Hnf6—a marker of early pancreatic progenitors, whose suppression is required for endocrine differentiation (40)—and elevated transcripts of *Hnf6* at E17.5 and P14 (Figure 7C and S7B). Moreover, we detected Sox9⁺/insulin<sup>low</sup> cells in 3-month-old PKO but not WT mice, as well as increased Sox9 mRNA in 3- and 7-month-old PKO mice (Figure 7A, D).

If elevated Sox9 were responsible for the phenotype of PKO mice, we would expect a similar increase in NKO mice. But, Sox9 protein and mRNA levels were neither detected in islets nor increased in whole pancreas (Figure S7C-D). These data indicate that the temporal and spatial regulation of Sox9 expression during pancreatic development is altered by Foxo1 ablation in pancreatic, but not in endocrine progenitors, raising the possibility that the exocrine effect of PKO
can be accounted for by Sox9 activation, while regulation of endocrine mass and function is
dependent on Foxo1 action in endocrine progenitors.
DISCUSSION

The key conclusion of this work is that altered Foxo1 function during pancreas development causes a legacy effect on adult organ plasticity and maturation, predisposing to β-cell dysfunction. Given the role of Foxo1 as a sensor of the nutritional and proliferative status of the cell (5), it could be envisioned that a nutrient-rich, hyperinsulinemic environment, by causing Foxo1 nuclear exclusion, will mimic aspects of Foxo1 ablation, potentially providing an explanation for why offspring of hyperinsulinemic or insulin-resistant mothers tend to have larger pancreata and islets (25), and progress to diabetes as adults nonetheless (4).

Another novel finding of our study pinpoints the expanded β−cell mass of PKO mice as a result of Foxo1 action in endocrine progenitors, rather than β−cells proper. Thus, it appears that during pancreatogenesis Foxo1 inhibits differentiation and expansion of pancreatic and endocrine progenitors, allowing them to mature (7). The delayed differentiation of Foxo1-deficient pancreatic precursors allows the pancreas to retain cells with progenitor-like features and maintain its developmental plasticity into adulthood. PKO pancreata show sustained and ectopic Sox9 expression, consistent with the role of insulin receptor (43) and Akt (44) in the generation of Sox9⁺ progenitors. We should emphasize that Foxo1 regulation of progenitor cell number is both Sox9−dependent and −independent. Ablation of early Sox9⁺ precursors results in pancreatic hypoplasia and early Sox9⁺ cells give rise to Neurog3⁺ progenitors (45). However, while increasing the number of Foxo1−deficient Sox9⁺ progenitors increased exocrine mass, reduced numbers of Sox9⁺ cells fail to affect pancreas size (37).
Foxo1 activation sets timing of endocrine progenitor cell formation and determines their number. FOXO1 knockdown by siRNA increased NEUROG3⁺ cell number in human fetal pancreatic epithelium (46). The results in PKO mice do not allow us to establish whether Foxo1 controls Neurog3⁺ endocrine progenitors directly or indirectly (28). The expansion of Neurog3⁺ progenitors into adulthood in Foxo1 knockouts, independent of exocrine or ductal plasticity, phenocopies the developmental stage-specific effects of Notch inhibition during endocrine differentiation (36). These data indicate that there is a specific temporal window during which these signaling pathways play a role in lineage determination.

Transgenic gain-of-function of Foxo1 in pancreatic progenitors causes exocrine hypoplasia (7). Consistent with these data, we show that Foxo1 ablation increases exocrine mass. In the transition from pancreatic progenitor replication to differentiation, other temporal regulators include Wnt and Notch. Wnt is required to expand pancreatic epithelial progenitors: gain of β−catenin function induces exocrine hyperplasia, without affecting endocrine cell formation (47; 48). Notch activation allows pancreatic and endocrine progenitors to survive, but Notch inhibition blocks both exocrine and endocrine differentiation (36). Foxo1 inhibition in endocrine progenitors gives rise to cells arrested at a precursor-like stage (Pdx1⁺, Neurog3⁺, MafA⁻, Ins⁻) in adult life, indicating that changes in hormone sensitivity during the latter half of fetal development change the proliferative and functional capacity of adult β−cells.

Based on extensive data (46), we can integrate the findings into a general theory of Foxo function in the endocrine pancreas. The three isoforms play overlapping and redundant roles (8). During embryogenesis, Foxos suppress differentiation, similar to their roles in other cell types. In terminally differentiated β-cells, Foxos act as transcriptional sensors of nutrient and hormone signaling: Foxos are to β-cell transcription what glucokinase is to insulin secretion. In response to an
altered metabolic environment, Foxos translocate to the nucleus, and activate the Hnf4/Hnf1α networks, while suppressing nuclear receptors Pparα and γ (8). If the metabolic stress persists, Foxos are degraded via deacetylation, possibly caused by altered NAD/NADH ratios (20). The loss of Foxo function impairs the β-cell’s ability to sense glucose, and activates lipid oxidation, resulting in impaired mitochondrial function, and reduced insulin secretion. β-cells lose their terminally differentiated features, and revert to a “progenitor-like” state, possibly as an escape mechanism to prevent death. Thus, Foxos belong to a core network of β-cell transcription factors whose function is necessary to maintain “functional” β-cell mass (49; 50). Our report highlights that β-cell mass per se is not sufficient to preserve physiologic insulin secretion and prevent development of diabetes.
FUNDING SOURCES

Supported by NIH grants DK64819 and DK63608 (Columbia Diabetes Research Center), and by a grant from the JPB Foundation. We thank members of the Accili laboratory for discussion of the data.

AUTHORS’ CONTRIBUTION

S.C.T. designed, performed, analyzed experiments and wrote the manuscript. D.A. oversaw data acquisition and analysis, and wrote the manuscript.

CONFLICT OF INTEREST STATEMENT

We have no conflict of interest to disclose.

GUARANTOR INFORMATION

Dr. Domenico Accili is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
REFERENCES

1. Vaag AA, Grunnet LG, Arora GP, Brons C: The thrifty phenotype hypothesis revisited. Diabetologia 2012;55:2085-2088

2. International Diabetes Federation: Diabetes Atlas. Brussels, Belgium, International Diabetes Federation, 2007

3. Gatford KL, Simmons RA: Prenatal programming of insulin secretion in intrauterine growth restriction. Clinical obstetrics and gynecology 2013;56:520-528

4. Holder T, Giannini C, Santoro N, Pierpont B, Shaw M, Duran E, Caprio S, Weiss R: A low disposition index in adolescent offspring of mothers with gestational diabetes: a risk marker for the development of impaired glucose tolerance in youth. Diabetologia 2014;57:2413-2420

5. Accili D, Arden KC: FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. Cell 2004;117:421-426

6. Kitamura T, Nakae J, Kitamura Y, Kido Y, Biggs WH, 3rd, Wright CV, White MF, Arden KC, Accili D: The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. The Journal of clinical investigation 2002;110:1839-1847

7. Kitamura T, Kitamura YI, Kobayashi M, Kikuchi O, Sasaki T, Depinho RA, Accili D: Regulation of pancreatic juxtaductal endocrine cell formation by FoxO1. Molecular and cellular biology 2009;29:4417-4430

8. Kim-Muller JY, Zhao S, Srivastava S, Mugabo Y, Noh HL, Kim YR, Madiraju SR, Ferrante AW, Skolnik EY, Prentki M, Accili D: Metabolic Inflexibility Impairs Insulin Secretion and Results In MODY-like Diabetes in Triple FoxO-Deficient Mice. Cell Metab 2014;20:593-602
9. Talchai C, Xuan S, Lin HV, Sussel L, Accili D: Pancreatic beta cell dedifferentiation as a mechanism of diabetic beta cell failure. Cell 2012;150:1223-1234

10. Kobayashi M, Kikuchi O, Sasaki T, Kim HJ, Yokota-Hashimoto H, Lee YS, Amano K, Kitazumi T, Susanti VY, Kitamura YI, Kitamura T: FoxO1 as a double-edged sword in the pancreas: analysis of pancreas- and beta-cell-specific FoxO1 knockout mice. American journal of physiology Endocrinology and metabolism 2012;302:E603-613

11. Kaneto H, Miyatsuka T, Shiraiwa T, Yamamoto K, Kato K, Fujitani Y, Matsuoka TA: Crucial role of PDX-1 in pancreas development, beta-cell differentiation, and induction of surrogate beta-cells. Curr Med Chem 2007;14:1745-1752

12. Gu G, Dubauskaite J, Melton DA: Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development 2002;129:2447-2457

13. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA: In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 2008;455:627-632

14. Smelt MJ, Faas MM, de Haan BJ, de Vos P: Pancreatic beta-cell purification by altering FAD and NAD(P)H metabolism. Experimental diabetes research 2008;2008:165360

15. Talchai C, Xuan S, Kitamura T, DePinho RA, Accili D: Generation of functional insulin-producing cells in the gut by Foxo1 ablation. Nat Genet 2012;44:406-412, S401

16. Xuan S, Kitamura T, Nakae J, Politi K, Kido Y, Fisher PE, Morroni M, Cinti S, White MF, Herrera PL, Accili D, Efstratiadis A: Defective insulin secretion in pancreatic beta cells lacking type 1 IGF receptor. The Journal of clinical investigation 2002;110:1011-1019

17. Kido Y, Burks DJ, Withers D, Bruning JC, Kahn CR, White MF, Accili D: Tissue-specific insulin resistance in mice with mutations in the insulin receptor, IRS-1, and IRS-2. The Journal of clinical investigation 2000;105:199-205
18. Matoba S, Hiramatsu R, Kanai-Azuma M, Tsunekawa N, Harikae K, Kawakami H, Kurohmaru M, Kanai Y: Establishment of testis-specific SOX9 activation requires high-glucose metabolism in mouse sex differentiation. Dev Biol 2008;324:76-87

19. Pierreux CE, Poll AV, Kemp CR, Clotman F, Maestro MA, Cordi S, Ferrer J, Leyns L, Rousseau GG, Lemaigre FP: The transcription factor hepatocyte nuclear factor-6 controls the development of pancreatic ducts in the mouse. Gastroenterology 2006;130:532-541

20. Kitamura YI, Kitamura T, Kruse JP, Raum JC, Stein R, Gu W, Accili D: FoxO1 protects against pancreatic beta cell failure through NeuroD and MafA induction. Cell metabolism 2005;2:153-163

21. Okamoto H, Hribal ML, Lin HV, Bennett WR, Ward A, Accili D: Role of the forkhead protein FoxO1 in beta cell compensation to insulin resistance. The Journal of clinical investigation 2006;116:775-782

22. Hingorani SR, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA, Ross S, Conrads TP, Veenstra TD, Hitt BA, Kawaguchi Y, Johann D, Liotta LA, Crawford HC, Putt ME, Jacks T, Wright CV, Hruban RH, Lowy AM, Tuveson DA: Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer Cell 2003;4:437-450

23. Schonhoff SE, Giel-Moloney M, Leiter AB: Neurogenin 3-expressing progenitor cells in the gastrointestinal tract differentiate into both endocrine and non-endocrine cell types. Dev Biol 2004;270:443-454

24. Magnuson MA, Osipovich AB: Pancreas-specific Cre driver lines and considerations for their prudent use. Cell Metab 2013;18:9-20

25. Rieck S, Kaestner KH: Expansion of beta-cell mass in response to pregnancy. Trends Endocrinol Metab 2010;21:151-158

26. Stanger BZ, Tanaka AJ, Melton DA: Organ size is limited by the number of embryonic progenitor cells in the pancreas but not the liver. Nature 2007;445:886-891
27. Johansson KA, Dursun U, Jordan N, Gu G, Beermann F, Gradwohl G, Grapin-Botton A: Temporal control of neurogenin3 activity in pancreas progenitors reveals competence windows for the generation of different endocrine cell types. Dev Cell 2007;12:457-465

28. Schwitzgebel VM, Scheel DW, Conners JR, Kalamaras J, Lee JE, Anderson DJ, Sussel L, Johnson JD, German MS: Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. Development 2000;127:3533-3542

29. Xu X, D'Hoker J, Stange G, Bonne S, De Leu N, Xiao X, Van de Casteele M, Mellitzer G, Ling Z, Pipeleers D, Bouwens L, Scharffmann R, Gradwohl G, Heimberg H: Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. Cell 2008;132:197-207

30. Huang HP, Liu M, El-Hodiri HM, Chu K, Jamrich M, Tsai MJ: Regulation of the pancreatic islet-specific gene BETA2 (neuroD) by neurogenin 3. Mol Cell Biol 2000;20:3292-3307

31. Wang S, Jensen JN, Seymour PA, Hsu W, Dor Y, Sander M, Magnuson MA, Serup P, Gu G: Sustained Neurog3 expression in hormone-expressing islet cells is required for endocrine maturation and function. Proceedings of the National Academy of Sciences of the United States of America 2009;106:9715-9720

32. Lee CS, Perreault N, Brestelli JE, Kaestner KH: Neurogenin 3 is essential for the proper specification of gastric enteroendocrine cells and the maintenance of gastric epithelial cell identity. Genes Dev 2002;16:1488-1497

33. Fatrai S, Elghazi L, Balcazar N, Cras-Meneur C, Krits I, Kiyokawa H, Bernal-Mizrachi E: Akt induces beta-cell proliferation by regulating cyclin D1, cyclin D2, and p21 levels and cyclin-dependent kinase-4 activity. Diabetes 2006;55:318-325

34. Kitamura T, Kitamura YI, Funahashi Y, Shawber CJ, Castrillon DH, Kollipara R, DePinho RA, Kitajewski J, Accili D: A Foxo/Notch pathway controls myogenic differentiation and fiber type specification. The Journal of clinical investigation 2007;117:2477-2485
35. Pajvani UB, Shawber CJ, Samuel VT, Birkenfeld AL, Shulman GI, Kitajewski J, Accili D: Inhibition of Notch signaling ameliorates insulin resistance in a FoxO1-dependent manner. Nat Med 2011;17:961-967

36. Murtaugh LC, Stanger BZ, Kwan KM, Melton DA: Notch signaling controls multiple steps of pancreatic differentiation. Proc Natl Acad Sci U S A 2003;100:14920-14925

37. Seymour PA, Freude KK, Tran MN, Mayes EE, Jensen J, Kist R, Scherer G, Sander M: SOX9 is required for maintenance of the pancreatic progenitor cell pool. Proceedings of the National Academy of Sciences of the United States of America 2007;104:1865-1870

38. Marselli L, Thorne J, Dahiya S, Sgroi DC, Sharma A, Bonner-Weir S, Marchetti P, Weir GC: Gene expression profiles of Beta-cell enriched tissue obtained by laser capture microdissection from subjects with type 2 diabetes. PLoS One 2010;5:e11499

39. Puri S, Akiyama H, Hebrok M: VHL-mediated disruption of Sox9 activity compromises beta-cell identity and results in diabetes mellitus. Genes Dev 2013;27:2563-2575

40. Tweedie E, Artner I, Crawford L, Poffenberger G, Thorens B, Stein R, Powers AC, Gannon M: Maintenance of hepatic nuclear factor 6 in postnatal islets impairs terminal differentiation and function of beta-cells. Diabetes 2006;55:3264-3270

41. Carmody JS, Wan P, Accili D, Zeltser LM, Leibel RL: Respective contributions of maternal insulin resistance and diet to metabolic and hypothalamic phenotypes of progeny. Obesity (Silver Spring) 2011;19:492-499

42. Louvi A, Accili D, Efstratiadis A: Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. Dev Biol 1997;189:33-48

43. Nef S, Verma-Kurvari S, Merenmies J, Vassalli JD, Efstratiadis A, Accili D, Parada LF: Testis determination requires insulin receptor family function in mice. Nature 2003;426:291-295
44. Elghazi L, Weiss AJ, Barker DJ, Callaghan J, Staloch L, Sandgren EP, Gannon M, Adsay VN, Bernal-Mizrachi E: Regulation of pancreas plasticity and malignant transformation by Akt signaling. Gastroenterology 2009;136:1091-1103

45. Lynn FC, Smith SB, Wilson ME, Yang KY, Nekrep N, German MS: Sox9 coordinates a transcriptional network in pancreatic progenitor cells. Proc Natl Acad Sci U S A 2007;104:10500-10505

46. Al-Masri M, Krishnamurthy M, Li J, Fellows GF, Dong HH, Goodyer CG, Wang R: Effect of forkhead box O1 (FOXO1) on beta cell development in the human fetal pancreas. Diabetologia 2010;53:699-711

47. Murtaugh LC, Law AC, Dor Y, Melton DA: Beta-catenin is essential for pancreatic acinar but not islet development. Development 2005;132:4663-4674

48. Heiser PW, Lau J, Taketo MM, Herrera PL, Hebrok M: Stabilization of beta-catenin impacts pancreas growth. Development 2006;133:2023-2032

49. Mondal P, Song WJ, Li Y, Yang KS, Hussain MA: Increasing beta-cell mass requires additional stimulation for adaptation to secretory demand. Mol Endocrinol 2014:me20141265

50. Guo S, Dai C, Guo M, Taylor B, Harmon JS, Sander M, Robertson RP, Powers AC, Stein R: Inactivation of specific beta cell transcription factors in type 2 diabetes. J Clin Invest 2013;123:3305–3316
FIGURE LEGENDS

**Figure 1. Increased β-cell mass following pan-pancreatic Foxo1 ablation** (A) Strategy to delete Foxo1 in all pancreatic cell types, endocrine progenitors, and differentiated β-cells. (B) Analysis of β-cell mass by immunohistochemistry (n= 6 each genotype and each age). At each time point, β-cell mass in WT littermates was normalized to 1 for clarity. (C) Representative images of insulin immunohistochemistry (brown) of pancreatic sections from 3-month-old mice used for the analysis in panel B. Original magnification 100x. (D-E) Analysis of β-cell number by flow cytometry, showing a representative plot (D), and quantification of results (E). We digested pancreata into single cells, fixed and stained cells with anti-insulin antibodies. Insulin⁺ cells were normalized by DNA content (n=6 each genotype). *= P< 0.05; ** = P< 0.01 throughout the figure.

**Figure 2. Neurog3⁺ cells and β-cell neogenesis in adult PKO pancreas** (A) Left panels: immunohistochemistry with antibodies against insulin (red) and Neurog3 (green) in WT and PKO mice. The inset in the lower panel shows representative double-positive cells (yellow). Middle panels: immunohistochemistry with anti-insulin (red) and anti-Gfp antibodies (green) in WT and PKO carrying a Neurog3-Gfp transgenic reporter. Right panel: immunohistochemistry with anti-Gfp antibodies (green) in WT and PKO mice carrying a Neurog3-Gfp knock-in reporter. The inset shows a representative Neurog3-Gfp⁺ cell next to a Neurog3-Gfp⁻ cell. Original magnification 20X (n=4 each genotype). (B) Representative flow cytometry plots of dissociated live pancreatic cells treated with glucose to separate Neurog3-Gfp⁺/insulin⁻ cells (red arrow) from Neurog3-Gfp⁺/insulin⁺ cells that are found in the central bracket. (C) Quantification of the results of
multiple experiments to determine the relative percentage of Neurog3+/insulin− cells in 3-month-old WT (*Neurog3-Gfp;Foxo1fl/fl*) and PKO mice (*Neurog3-Gfp;Neurog3-cre Foxo1fl/fl*) (n=6 each). * = P<0.05.

**Figure 3. A self-replicating pool of endocrine progenitors in adult PKO mice (A)**
Quantification of insulin+/Ki67+ cells by flow cytometry in mice of the indicated ages and genotypes. We normalized the number of double-positive cells by the total number of insulin+ cells per pancreas (n=6 each). (B) Quantification of insulin+/Ki67+ cells by double immunohistochemistry in 12-month-old mice of the indicated genotype by manual counting, normalized by total number of insulin+ cells per pancreas (n=4 each). (C) Double immunohistochemistry with anti-Ki67 (dark brown), and anti-insulin antibodies (light brown) on pancreatic sections of 7-day-old mice (n=4). (D) Immunohistochemistry with anti-Ki67 antibody (dark brown) on pancreas sections of 15-month-old mice (n=4 each). Insets represent Ki67+ cells in islets. (E) qPCR analysis of a panel of cell cycle regulatory genes in purified islets from control and PKO mice (n=6 each). (F) Immunofluorescence with anti-Neurog3 (green) and anti-H2B (purple) in pancreas sections from 3-month-old control and PKO mice (n=4 each). Inset shows Neurog3+/H2B+ cells. (G) Immunofluorescence with anti-Neurog3 (green) and anti-Ki67 (red) in pancreas sections from 15-month-old mice (n=4 each). Insets show Neurog3+ and Neurog3+/Ki67+ cells. Data show means ± SEM. * = P<0.05, ** = P<0.01 by Student’s t-test.

**Figure 4. Metabolic analysis (A)** Intraperitoneal glucose tolerance tests (GTT) in 12-hr-fasted 8-month-old male mice (n=12 each genotype). (B) Acute insulin response to glucose (AIR<sub>glu</sub>) (3 mg/kg) (n=5 each genotype). Symbols are the same as in panel A. (C) Calculated area under the curve (AUC) from GTT (n=8 each genotype), (D) Arginine-stimulated insulin secretion (AIR<sub>arg</sub>) in
8-month-old mice (n=5 each). Symbols are the same as in panel A. (E) Glucose-stimulated insulin secretion from static incubations of islets isolated from 3-month-old mice (n=5). Three independent experiments were performed. Data show means ± SEM. * = P < 0.05 by Student’s t-test. (F) Serum insulin and proinsulin levels in fed 8-month-old mice (n=8). (G) Insulin content and (H) ratio of insulin content to β-cell mass in control and PKO pancreas, normalized by the content in WT mice (n=6). Data are presented as means ± SEM. * = P < 0.05, and ** = P < 0.01 by Student’s t-test.

**Figure 5. β-cell mass and endocrine progenitor proliferation in NKO mice** (A) Representative insulin immunohistochemistry (brown) of pancreatic sections from 15-month-old mice. Original magnification 40x. (B) Age-dependent increase in β-cell mass, measured as % insulin-immunoreactive area normalized by pancreas weight (n= 6 each genotype). β-cell mass in WT littermates at 3 months was normalized to 1. (C) Pancreas weight in mice of the indicated ages and genotypes (n= 6 each genotype). (D) Quantification of Ki67+/insulin+ cells normalized by total insulin+ cells per pancreas (n=4 to 6 each genotype). (E) Immunofluorescence with anti-Neurog3 (red) and anti-Ki67 antibodies (green) in pancreas sections from 8- and 15-month-old mice (n=4 each genotype). Insets show Neurog3+/Ki67+ cells. (F) qPCR measurements of Neurog3 mRNA from collagenase-purified islets (n=6 each genotype). *= P< 0.05, **= P < 0.01.

**Figure 6. β-cell characterization** (A) Insulin release from static incubations of islets isolated from 3-month-old mice normalized by islet DNA content (n=5 each genotype). Three independent experiments were performed. (B) Serum insulin and proinsulin in random fed 8-month-old mice (n=8 each genotype). (C) Pancreatic insulin content. (D) Ratio of insulin content to β-cell mass in
control and NKO pancreas (n=6 each genotype). (E) C-peptide-2 content in acid-ethanol extracts from 3- and 8-month-old mice. (F) Immunofluorescence with anti-insulin (green), anti-MafA (red), anti-Pc1/3 (red), and anti-C-peptide 2 in pancreatic sections from 8-month-old mice (n=4 each genotype). Data show means ± SEM. *= P < 0.05; ** = P < 0.01.

Figure 7. Altered Sox9 expression in PKO pancreas (A) Immunohistochemistry with anti-Sox9 (green), and anti-insulin antibodies (red) in pancreata of 3-month-old mice (n=4 each genotype). (B-C) Immunohistochemistry with anti-Sox9 (red), anti-Hnf6 (red), or anti-Ck19 antibodies (green) in sections from E17.5 embryos (n=4 each genotype). (D-E) qPCR analysis of RNA extracted from whole pancreas (D) or collagenase-purified islets (E) of mice of different ages (n=6 to 8 for each genotype). * = P < 0.05, ** = P < 0.01.
**Figure 1**

A. Diagram showing the development of pancreas with Pdx1-Cre, Ngn3-Cre, and Ins-Cre targeting.

B. Graph showing β-cell mass (AU) with different genotypes over age.

C. Images of insulin expression in WT, PKO, and IKO pancreases.

D. Flow cytometry histograms for WT and PKO for APC and % insulin+ pancreas.

E. Bar graph showing % insulin+ pancreas over age (months) for WT and PKO.
Figure 2

A

Neurog3 Insulin

Neurog3-Gfp Insulin

Neurog3 (+/Gfp)

WT

PKO

B

WT

PKO

C

Neurog3-Gfp/Ins (% of Neurog3)

WT

PKO

*
Figure 3

A. Graph showing the percentage of Ki67+ cells in insulin-producing cells (ins+) across different age groups (P7, 1M, 3M, 7M) for WT, IKO, and PKO mice. The bars are labeled with asterisks (*), indicating statistical significance.

B. Graph showing the percentage of Ki67+ cells across different age groups (WT, IKO, PKO) at 15M. The bar graph includes asterisks (***), indicating a significant difference.

C. Immunohistochemical staining of Ki67 in WT and PKO mice at P7. The images show insulin-producing cells stained for Ki67.

D. Immunohistochemical staining of Ki67 in WT and PKO mice at 15M. The images show insulin-producing cells stained for Ki67.

E. Bar graph showing mRNA expression (AU) for various genes in WT and PKO mice across different age groups (P7, 1M, 3M, 7M, 15M). The genes include Cyclin D1, Cyclin D2, Cyclin D3, Cyclin A2, Cyclin E1, Cyclin G1, Cyclin H, CDK4, CDK6, p18, p21, and p27. The bars are labeled with asterisks (*, **, ***), indicating statistical significance.
Figure 4

(A) Glucose levels in WT (PKO), PKO, WT (IKO), and IKO over time (min).

(B) Insulin levels in AIR_glu (ng/dl) for WT (PKO), PKO, WT (IKO), and IKO over time (min).

(C) IPGTT_AUC (AU) for WT and PKO over age (months).

(D) Insulin levels in AIR_arg (ng/dl) for WT (PKO), PKO, WT (IKO), and IKO over time (min).

(E) Insulin secretion in ng/hr/islet/DNA for WT and PKO with glucose concentrations of 2.8mM, 5.5mM, 11.2mM, 16.7mM, and KCl.

(F) Insulin and proinsulin levels in Insulin and Proinsulin for WT and PKO over time (min).

(G) Insulin and proinsulin levels in ng/mg pancreas for WT and PKO over age (3M and 8M).

(H) Insulin/β-cell mass for WT and PKO.
Figure 5

A. Immunohistochemical staining for insulin in control, PKO, and NKO pancreases.

B. β-cell mass (AU) at 3M, 8M, and 15M for WT and NKO groups.

C. Pancreas weight (mg) at 3M, 8M, and 15M for WT and NKO groups.

D. % Ki67/ins+ Neurog3 mRNA at 3M, 8M, and 15M for WT and NKO groups.

E. Immunohistochemical staining for Neurog3 and Ki67 at 8M and 15M for WT and NKO groups.

F. Neurog3 mRNA (AU) at 3M, 8M, and 15M for WT and NKO groups.
Figure 6

A

WT  
NKO

insulin (ng/hr/islet/DNA)

2.8mM  5.5mM  11.2mM  16.7mM  Glucose  KCl

B

WT  
NKO

Insulin (ng/dl)

Insulin  Proinsulin

C

WT  
NKO

insulin/β-cell mass

Age (months)

0  3  8

D

WT  
NKO

pg/mg pancreas

Age (months)

0  3  8

E

Control  PKO  NKO

Ins MaA

Ins Pi1 M

Ins C-pep M

*  **

Diabetes
Figure 7

A

WT

PKO

Ins Sox9

3M

E17.5

B

Sox9 Ck19

E17.5

C

Hnf6 Ck19

E17.5

D

pancreas Sox9 (AU)

WT

PKO

E17.5

P14

E

islet Sox9 (AU)

3M

7M

Age
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Characterization of PKO and IKO mice (A-C) mRNA levels of Foxo1, Foxo3, and Foxo4. (D) Immunohistochemistry with Rosa-Gfp (green), insulin (red) and merged (yellow) from pancreas sections of PKO mice and control Pdx-Cre; Foxo1+/+. D: duct; E: exocrine pancreas; Is: islet. (E-F) Body weight and fasting blood glucose in male WT, PKO, and IKO mice of the indicated ages. (G) β-cell mass calculated from flow cytometry (% of insulin+ cells normalized by pancreas weight). (H) Pancreas weight in WT, PKO, and IKO mice of the indicated ages. (I) Representative immunohistochemistry with anti-Ki67 (brown) of 5-month-old mice. Arrows point to proliferative cells. (J) Flow cytometry analysis of pancreatic amylase+/Ki67+ cells from 5- and 7-month-old IKO, PKO, and WT littermates (n=6 each) * = P<0.05 throughout.

Supplemental Figure 2. Pancreatic plasticity in PKO mice (A) qPCR analysis of age-dependent changes in Neurog3 expression in pancreas (n=6-8 per genotype and time point). WT=1 for fold change. ** = P< 0.01. (B) Immunohistochemistry with anti-Sox9 and anti-Gfp in pancreas and gut sections from E15.5 mice carrying Neurog3-Gfp (n= 4 each). (C) Quantification of juxta-ductal hormone+ cells in adult mice using immunohistochemistry with antibodies to the indicated pancreatic hormones in sections from 3-month-old mice (n=6 mice per genotype and 4 sections per mouse). Individual hormone-positive cells were seen in 1-5% of ducts scored in control animals. ** = P < 0.001. (D) Left: immunohistochemistry with anti-insulin (red) and anti-somatostatin antibodies (green) of representative juxta-ductal hormone+ cells in sections from 3-month-old mice (n=6 mice per genotype and 3 sections per mouse). Original magnification 20X; dotted lines outline ducts. Right: Lineage tracing of juxta-ductal hormone+ cells in 3-month-old mice of the indicated genotypes using immunohistochemistry with anti-Insulin (red), and anti-Gfp (green) antibodies.
Gfp-labeled cells are Foxo1-ablated cells. (E) Immunohistochemistry with anti-Pdx1 (green) and anti-insulin antibodies (red) antibodies in 3-month-old mice. Original magnification 20X (n=4 each).

**Supplemental Figure 3. Cell turnover studies in pancreas** (A) TUNEL assay (green) in insulin-immunoreactive cells (red) of WT and PKO mice. (B) Quantification of apoptotic nuclei as detected by TUNEL assays in WT, PKO, and IKO mice (n = 6 each). (C) qRT-PCR array of cell cycle gene expressions in islets from 3-month-old WT and IKO mice (n=6 each).

**Supplemental Figure 4. Metabolic analysis of PKO mice** (A) Pancreatic glucagon content measured by radioimmunoassay (n=6 each). (B) AUC from intraperitoneal GTT (n=8 each). (C) Glucose-stimulated insulin secretion from static incubations of islets isolated from 3-month-old mice normalized by DNA content (n=5 each). (D) Pancreatic insulin content measured by Elisa in acid-ethanol extracts (n=6 each).

**Supplemental Figure 5. Lineage tracing and cell cycle analysis in NKO mice** (A) Neurog3-Cre reporter activity, as detected by immunofluorescence with anti-Gfp (Green), co-localizes with insulin (red), glucagon (Gcg), pancreatic polypeptide (Pp), and somatostatin (Sms) (red) in pancreatic section of 3-month-old Neurog3-Cre:Rosa26-Gfp mice. Original magnification 200X (n=3 each). (B) qPCR measurement of Foxo1, 3, 4 mRNA in 3-month-old mice, relative to WT controls, normalized by Hprt (n=6 each). * = P< 0.05, ** = P< 0.01. (C) qRT-PCR analysis of cell cycle genes in pancreas extracts from 3-month-old mice (n=6 each). * = P< 0.05, ** = P< 0.01. (D) Neurog3-Gfp+ cells in 3-month-old mice detected by immunohistochemistry with anti-Gfp antibody (brown) (n=4 each), original magnification 200x.
Supplemental Figure 6. Comparison of NKO and PKO mice (A) Pancreatic glucagon content in 3-month-old mice. (B-F) qRT-PCR analysis of gene expression in islets from 3-month-old mice, including transcripts related to β-cell differentiation (B), Notch signaling (C) transcription factors (D), channels and transporters, and (E) metabolic sensing/insulin processing (F) (n=6 each). *= P < 0.05; ** = P < 0.01.

Supplemental Figure 7. Persistent ectopic Sox9 expression in adult PKO, but not NKO pancreas (A) Immunohistochemistry with anti-Sox9 (red) and anti-Neurog3 (green) pancreas sections from 3-month-old mice. (B) qRT-PCR analysis of Hnf6 transcripts from pancreas extracts of E17.5 and postnatal day (P)14 mice (n=5 for each genotype). (C) Immunohistochemistry with anti-Sox9 (green), and anti-insulin antibodies (red) in pancreata of 3-month-old mice (n=4 for each genotype). (D-E) qRT-PCR measurements of Sox9 mRNA in whole pancreas (D) and isolated islets (E) of mice of the indicated ages (n=5-6 for each time point and each genotype). *= P < 0.05, ** = P < 0.01.
Figure 7

A

WT

PKO

Ins Sox9

3M

B

Sox9 Ck19

E17.5

C

Hnf6 Ck19

E17.5

D

pancreas Sox9 (AU)

E17.5

P14

**

E

islet Sox9 (AU)

3M

7M

**

**

**

**
Supplemental figure 1

I. Control PKO

Ki67

J. 

* 

n.s.

% Ki67+ that are Amylase+

Control

IKO

PKO

P150

P210
Supplemental Figure 2

A

**Neurog3 mRNA (AU)**

- WT
- PKO

| Age (days) | e15.5 | e17.5 | P14 | P90 | P210 |
|-----------|-------|-------|-----|-----|------|
| WT        | **    | **    | **  | **  | **   |
| PKO       |       |       |     |     |      |

B

**Neurog3-Gfp Sox9**

- WT Pancreas
- PKO Gut

C

**Hormone+ cells (fold increase)**

- β
- α
- δ
- Pp

WT PKO

D

**Insulin Rosa-Gfp**

WT PKO

E

**Insulin Pdx1**

WT PKO
Supplemental figure 5

A

(Neurog3Cre) Rosa-Gfp

Gfp  Gfp insulin  Gfp Gcg/Sms/Pp

B

Relative mRNA levels

WT  NKO

Foxo1  Foxo3  Foxo4

C

mRNA (AU)

WT  NKO

D

Neurog3-Gfp

WT  NKO
Supplemental figure 7

Supplemental figure 7

A

WT

PKO

Sox9 Neurog3

B

mRNA (AU)

WT

PKO

**

**

e17.5

P14

C

WT

NKO

Sox9 Insulin

D

pancreas mRNA (AU)

islet mRNA (AU)

WT

PKO

Age (days)

P60

P210