PDX1 in Ducts Is Not Required for Postnatal Formation of β-Cells but Is Necessary for Their Subsequent Maturation

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Pancreatic duodenal homeobox-1 (*Pdx1*), a transcription factor required for pancreatic development and maintenance of β-cell function, was assessed for a possible role in postnatal β-cell formation from progenitors in the pancreatic ducts by selectively deleting *Pdx1* from the ducts. Carbonic anhydrase II $(CAII)^{Cre}$;$Pdx1^{Fl}$ mice were euglycemic for the first 2 postnatal weeks but showed moderate hyperglycemia from 3 to 7 weeks of age. By 10 weeks, they had near-normal morning fed glucose levels but showed severely impaired glucose tolerance and insulin secretion. Yet the loss of *Pdx1* did not result in decreased islet and β-cell mass at 4 and 10 weeks of age. Within the same pancreas, there was a mixed population of islets, with PDX1 and MAFA protein expression normal in some cells and severely diminished in others. Even at 10 weeks, islets expressed immaturity markers. Thus, we conclude that *Pdx1* is not necessary for the postnatal formation of β-cells but is essential for their full maturation to glucose-responsive β-cells. ***Diabetes* 62:3459–3468, 2013**

Diabetes results from an inadequate functional β-cell mass; therefore, the possible replenishment of β-cells receives much attention. Endogenous replenishment can occur by replication and by neogenesis or differentiation of β-cells from nonendocrine progenitors or precursors (1). Neogenesis occurs during specific periods of normal embryonic and postnatal growth, after some forms of pancreatic injury (2–6), and can be induced by growth factors and/or cytokines (7–10). For example, in rodents over the first month after birth, while β-cell replication continues, significant neogenesis has been documented (11–16).

The mechanisms responsible for neogenesis are still poorly understood. A potentially important contributor is pancreatic duodenal homeobox-1 (PDX1), a transcription factor necessary for pancreatic development and maintenance of β-cell function. Global deletion of *Pdx1* results in pancreatic agenesis (17,18). PDX1 function has been shown to be required for proliferation of β-cells at late gestation (19) and for maintaining the function of the mature β-cells (20,21). PDX1 is expressed in the embryonic pancreatic progenitors before becoming restricted to the β-cells and a small proportion of δ-cells. PDX1 protein is transiently expressed, however, in replicating ducts during regeneration (22–25).

We hypothesized that PDX1 was necessary for the neogenetic formation of β-cells from mature ducts and therefore generated duct-specific *Pdx1*-deficient mice using the Cre-lox system with Carbonic Anhydrase II $(CAII)^{Cre}$ (14) and $Pdx1^{floxed\ E2}$ mice (19) in which *Pdx1* expression should be specifically deleted from ducts only starting around birth. Here, we show that *Pdx1* is not necessary for formation of new β-cells from postnatal pancreatic ducts, unlike its required role for formation of all pancreatic cell types during embryonic organogenesis, but that *Pdx1* is essential for these newly formed cells to mature into fully functional β-cells.

RESEARCH DESIGN AND METHODS

Animals. Transgenic mice with *floxed Pdx1* ($Pdx1^{FL/FL}$) (19) and constitutive $CAII^{Cre}$ (14) were mated. In some experiments $CAII^{Cre}$ animals carried the reporter gene from being mated with B6.129X1-*Gt(ROSA)26Sortm1(EYFP)Cos*/J (ROSA26ReYFP) mice from The Jackson Laboratories. DNA extracted from tails at weaning was used for genotyping with primers recognizing the floxed *Pdx1* primer 5′-AGGGTTCCGGATCGATCCCC-3′ and 5′-AGCAGCTGGAGCTAGGC-3′, the wild-type (WT) *Pdx1* primers 5′-CCTTTGCGGATCCTT-3′ and 5′-GCCAACAACTGGCAGATTC, and *Cre* primers 5′-ACCTGAAGATGTTCGCGATTATCT-3′ and 5′-GATCATCAGCTACACCAGAGA-3′. PCR was used 40 cycles for *Cre*, 31 cycles for floxed *Pdx1*, and 37 cycles for WT *Pdx1* allele.

Mice were housed in the Joslin Animal Facility on a 12-h light/12-h dark cycle and with water and food ad libitum. $CAII^{Cre+}$;$Pdx1^{FL/+}$ mice were used for breeding to generate six genotypes: $CAII^{Cre+}$;$Pdx1^{Fl/Fl}$, $CAII^{Cre+}$;$Pdx1^{Fl/+}$, $CAII^{Cre+}$;$Pdx1^{+/+}$, $CAII^{Cre-}$;$Pdx1^{Fl/Fl}$, $CAII^{Cre-}$;$Pdx1^{Fl/+}$ and $CAII^{Cre-}$;$Pdx1^{+/+}$. The first two were considered bigenic experimental mice, and the others served as controls.

Body weight and morning fed glucose levels were measured weekly. Blood glucose values were measured using One-Touch glucometer (LifeScan, Milpitas, CA) on blood from tail snip. Samples for intraperitoneal glucose tolerance tests were collected from mice fasted overnight (15 h) at 0, 15, 30, 60, 90, and 120 min after an intraperitoneal injection of glucose (2 g/kg body weight). Plasma insulin was measured with a rat insulin ELISA kit (ALPCO, Salem, NH). For insulin tolerance tests, blood glucose was measured at 0, 15, 30, and 60 min after intraperitoneal insulin injection (Humulin R; Eli Lilly, Indianapolis, IN; 0.75 units/kg body weight) of fasted (9:00 A.M.–3:00 P.M.) mice.

Animals were killed under anesthesia, and the pancreas was excised for histology or islet isolation. For immunostaining, the excised pancreas was spread flat and fixed for 2 h in 4% paraformaldehyde for embedding in paraffin or for frozen blocks. For secretion studies or RNA analysis, islets were isolated by the collagenase method (26), with each mouse as a separate sample for islet studies. The Joslin Institutional Animal Care and Use Committee approved all animal procedures.

Immunochemistry. Sections were immunostained for immunoperoxidase using the ABC kit (Vector Laboratories, Burlingame, CA) or immunofluorescence.

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Antigen retrieval was performed in 10 mmol/L citric acid buffer by microwave or PickCell 2100 antigen retriever (BD Biosciences). Sections were incubated overnight at 4°C with primary antibodies, followed by species-appropriate secondary antibodies (Supplementary Table 1). The tyramide (TSA) system (PerkinElmer, Waltham, MA) was used for amplification of PDX1, MAFA, and MAFB, following the manufacturer's instruction. Images were taken in confocal mode on a Zeiss LSM 410 microscope. For comparison of the intensity of PDX1 and MAFA staining in mice of different genotypes, images were taken at the same settings on sections from littermates stained in parallel and handled identically in Adobe Photoshop. At least three animals per genotype were examined for each antigen.

Morphometric analysis of β- and non–β-cell mass. Paraffin sections of 4- or 10-week-old male mouse pancreas stained by immunoperoxidase with a cocktail of non–β-cell islet hormones (glucagon, somatostatin, and pancreatic polypeptide [PP]) were analyzed by point counting morphometry for islet mass (27). β-cell mass was similarly determined on adjacent sections stained for insulin. Intersections with a 90-point grid were counted systematically in nonoverlapping fields to obtain β- and non–β-cell relative volumes (% total tissue) as well as the percentage of pancreatic parenchyma of total tissue; at least 150 fields were counted for each full footprint of pancreas section. Absolute mass was determined by multiplying the relative volume by pancreatic weight.

Insulin secretion. After overnight culture in RPMI 1640 medium (11 mmol/L glucose and 10% FBS), triplicate samples of 10 equilibrated islets for each mouse placed in wells of a 24-well plate were sequentially incubated with 2.6 and 16.8 mmol/L glucose in Krebs-Ringer buffer (16 mmol/L HEPES and 0.1% BSA, pH 7.4) (28,29). Supernatant fractions and cell lysates were frozen until assayed for insulin, as above. DNA was measured on cell lysates using a Cyquant Cell Proliferation Kit (Molecular Probes, Grand Island, NY).

Quantitative real-time PCR. Islets in excess of those needed for secretion were extracted for RNA using an Arcturus Picopure RNA isolation kit (Arcturus, Carlsbad, CA). After RT-PCR using a RT-PCR kit (Promega, Madison, WI), quantitative RT-PCR with SYBR green detection was performed using the ABI7300 real-time PCR system (Applied Biosystem, Foster City, CA) with primers (Supplementary Table 2). Samples were normalized to ribosomal 18S, an internal control gene, and the $\Delta\Delta C_t$ method was used to calculate gene expression levels.

Statistical analysis. Data are shown as mean ± SEM. For statistical analysis, an unpaired Student t test was used to compare two groups, and one-way ANOVA, followed by Bonferroni post hoc test, was used for more than two groups. A P value < 0.05 was considered statistically significant.

RESULTS

***Pdx1* was efficiently deleted from ducts in bigenic mice.** To test if *Pdx1* expression in pancreatic ducts was necessary for islet neogenesis, we generated duct-specific *Pdx1*-deficient mice by mating $CAII^{Cre}$ mice and $Pdx1^{Fl/Fl}$ mice. Previously we showed the specificity of this promoter in that *1*) CAII protein starts to be expressed in mouse pancreatic ductal cells at about embryonic day 18.5 (30), *2*) lineage tracing showed the human *CAII* construct used in the transgenic mice followed a similar timing, *3*) neither CAII nor Cre mRNA was expressed in the β-cells of the $CAII^{Cre}$ mice, *4*) *hCAII*-driven reporter at birth and Cre protein were only detected in ducts and ganglia in the pancreas, and *5*) $CAII^{CreERT}$-marked β-galactosidase background expression was about 1% of β-cells in both WT and transgenic mice (14). PDX1 protein has expression that is very low to undetectable in normally quiescent adult ductal cells but has transient (3–5 days) expression after proliferation (22). Ductal cells of 4-week-old WT and $CAII^{Cre}$;$Pdx1^{Fl/Fl}$ mice had comparable proliferation (% $Ki67^+$) (Fig. 4*F*), but PDX1 protein was expressed in far fewer duct cells in $CAII^{Cre}$;$Pdx1^{Fl/Fl}$ mice than in WT mice (Fig. 1*A–D*), indicating efficient excision of *Pdx1* in the ducts. Because PDX1 is not expressed in pancreatic ganglia, expression of the transgene in the ganglia should have no effect on the phenotype.

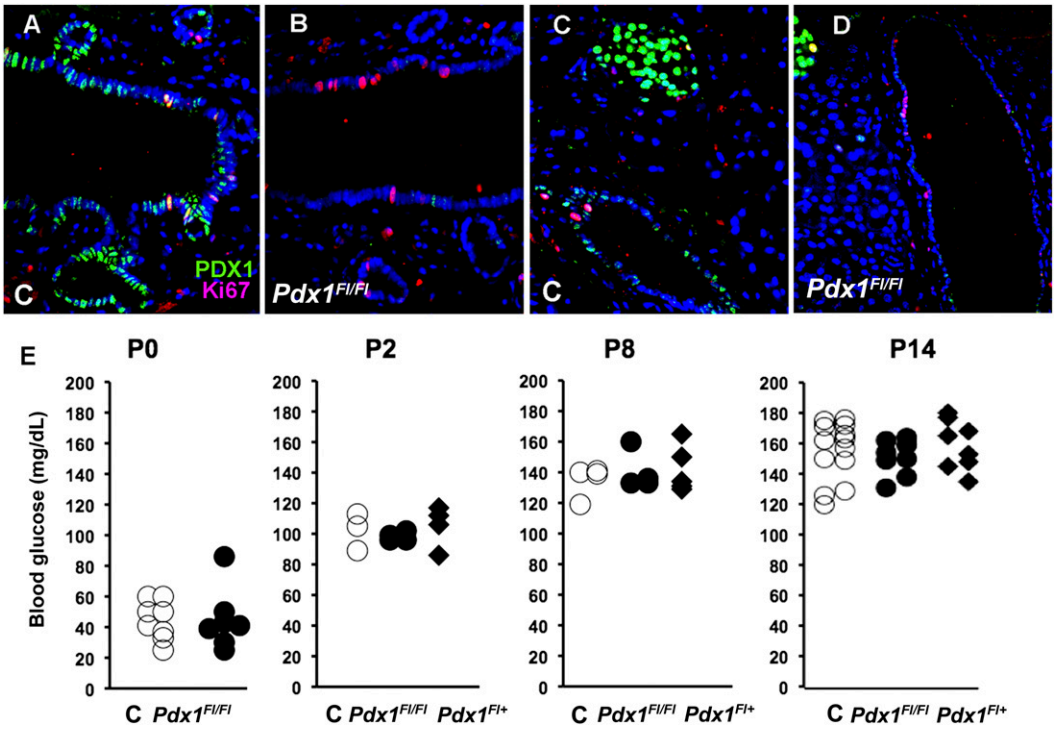

FIG. 1. Characterization of duct-specific deletion of *Pdx1* mice. *A–D*: Immunofluorescent evidence of effective *Pdx1* excision at 4 weeks of age in $CAII^{Cre}$;$Pdx1^{Fl/Fl}$ pancreas. PDX1 protein is normally expressed transiently after replication of pancreatic duct cells. The common pancreatic ducts (*A* and *B*) and main duct (*C* and *D*) of control (C) (*A* and *C*) and bigenic $CAII^{Cre}$;$Pdx1^{Fl/Fl}$ (*B* and *D*) mice had comparable proliferation seen as $Ki67^+$ (red). (Quantification given in Fig. 4*F*.) However, bigenic pancreas (*B* and *D*) had few $PDX1^+$ (green) duct cells. $PDX1^+$ islets are seen in upper left corner of both *C* and *D*. *E*: Blood glucose values over the first 2 postnatal (P) weeks did not differ between control (C) and bigenic mice (shown as $Pdx1^{Fl/Fl}$ and $Pdx1^{Fl/+}$). Values from individual littermates are shown.

CAII starts to be expressed in ductal cells only just before birth, so embryonic development was expected to be normal. The duct-specific *Pdx1*-deficient mice were normal in Mendelian proportion, in body weight, and morphology of the pancreas at birth (data not shown) and had nonfasting blood glucose levels within normal reference ranges over the first 2 postnatal weeks (Fig. 1*E*); pancreatic weight in 2-week-old littermates did not differ (control: 29.3 ± 1.0 mg, $n = 4$; bigenic: 31.9 ± 1.0 mg, $n = 10$; $P < 0.16$). Together these parameters indicate appropriate embryonic development.

We reasoned (Fig. 2) that if PDX1 expression in the ducts were necessary for postnatal neogenesis, neonatal formation of new β-cells from ductal precursors would be impaired in the $CAII^{Cre}$;$Pdx1^{Fl/Fl}$ mice, and thus, animals at 4 weeks should have an inadequate β-cell mass and be hyperglycemic (Fig. 2 option 1). By contrast, if PDX1 in the ducts were not necessary for postnatal β-cell formation, the population of β-cells at 4 weeks would include those formed before birth expressing PDX1 plus those formed from *CAII* promoter-driven Cre-expressing ducts after birth without PDX1 (Fig. 2 option 2).

Impaired glucose tolerance and reduced plasma insulin in duct-specific *Pdx1*-deficient mice. By weaning (Fig. 3*A*), the bigenic mice were moderately hyperglycemic (at 4 weeks $CAII^{Cre}$;$Pdx1^{Fl/Fl}$: 254 ± 12 mg/dL, $n = 23$; $CAII^{Cre}$;$Pdx1^{Fl/+}$: 224 ± 8 mg/dL, $n = 26$; control: 171 ± 5 mg/dL, $n = 52$). Yet by 10 weeks, they had near-normal morning fed blood glucose values ($CAII^{Cre}$;$Pdx1^{Fl/Fl}$: 188 ± 10 mg/dL, $n = 17$; $CAII^{Cre}$;$Pdx1^{Fl/+}$: 180 ± 5 mg/dL, $n = 27$; control: 153 ± 6 mg/dL, $n = 33$; $P < 0.05$ either bigenic compared with controls). Fed blood glucose values differed between $CAII^{Cre}$;$Pdx1^{Fl/Fl}$ and $CAII^{Cre}$;$Pdx1^{Fl/+}$ mice only at 3 and 4 weeks of age. Unless specified, data from these genotypes are presented together as bigenic mice because we did not find differences between them. Despite near-normal blood glucose levels at age 10–11 weeks, duct-specific *Pdx1*-deficient mice had severely impaired glucose tolerance, as seen in intraperitoneal glucose tolerance tests (Fig. 3*B*), with significantly decreased plasma insulin levels (Fig. 3*C*) compared with the control littermates. Their ability to clear glucose in response to insulin, however, as seen in insulin tolerance tests (data not shown), did not differ. In a cohort taken to age 22 weeks, the morning fed blood glucose values of control and bigenic mice did not statistically differ from age 13 weeks onward, but there were elevated fasting glucose levels and still some impairment of glucose tolerance (Supplementary Fig. 1).

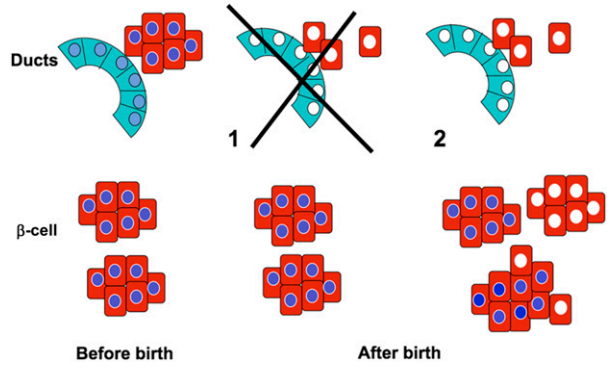

FIG. 2. Schema of possible outcomes of duct-specific *Pdx1* deletion. Before birth, all islets should be normal and homogeneously express PDX1 (blue nuclei). At 4 weeks, two findings are possible: *1*) if PDX1 is necessary for new β-cell formation from ducts, there should be fewer islets but all should have homogeneous PDX1 expression; *2*) if PDX1 is not necessary, there should be a mixed population of islets with those β-cells formed before birth with homogeneous PDX1 and those formed after birth from the *Pdx1*-depleted ducts, without PDX1 (white nuclei).

Impaired glucose-induced insulin secretion in isolated islets of duct-specific *Pdx1*-deficient mice. Islets from 11-week-old bigenic mice secreted less insulin than control islets in response to 16.8 mmol/L glucose (Fig. 3*D*). At high glucose, control islets secreted 0.15% of their total insulin, whereas islets from bigenic mice secreted only 0.06% of their total insulin (Fig. 3*E*), even though their islet insulin content was very similar (Fig. 3*F*). This impaired glucose responsiveness probably resulted from β-cell immaturity and a contribution from chronic mild hyperglycemia (this cohort of 11-week-old bigenic: 170 ± 6 vs. 144 ± 3 mg/dL in controls, $n = 10$ each group; $P < 0.001$), the latter known to be associated with reduced glucose-stimulated insulin secretion.

Islet and β-cell mass of duct-specific *Pdx1*-deficient mice were not reduced. These physiological data support the concept of a reduced β-cell mass at 4 weeks due to a lack of postnatal neogenesis in the absence of PDX1 in the ducts offset by some hyperglycemia-driven compensation by 10 weeks. However, we found, unexpectedly, that the islet and β-cell mass did not differ between bigenic and control male mice at age 4 or 10 weeks (Fig. 4*A* and *B*). Our technique uses a cocktail of antibodies against the non–β-cell hormones glucagon, somatostatin, and PP to allow quantification of non–β-cell and β-cell mass, so the islet peripheral mantle consisting of non–β-cells is clearly defined, and even partially degranulated β-cells are still counted. At 4 and 10 weeks, although many islets of bigenic mice had a well-defined mantle, as seen in controls, we noticed a population of islets in which core cells were immunostained with both insulin and hormone-cocktail antibodies. Immunostaining for individual non–β-cell hormones showed that the PP antibody accounted for the large number of cells coexpressing insulin and non–β-cell hormones, a notable coexpression rarely seen in postnatal control mice (Supplementary Fig. 2). We therefore quantified the β-cell mass directly on adjacent insulin-stained sections from 4-week-old male animals (Fig. 4*B*). Although the β-cell relative volume (% of pancreatic tissue) of bigenic mice was significantly decreased (Fig. 4*C*), their pancreatic weight (Fig. 4*D*) was increased although the animals had similar body weight (Fig. 4*E*). The result was that absolute β-cell mass was similar for bigenic and control animals (Fig. 4*B*). There was no difference in acinar or duct replication (Fig. 4*F*). In contrast, at age 2 weeks, although pancreatic weights did not differ among genotypes, the $CAII^{Cre}$;$Pdx1^{FlFl}$ mice had significantly increased ductal proliferation (Supplementary Fig. 3). However, at 4 weeks (Fig. 4*F*-H) but not at 10 weeks (data not shown), more $Ki67^{+}insulin^{+}$ cells were seen in islets of bigenic mice, and some of these $Ki67^{+}$ cells were $PDX1^{null}insulin^{+}$ (Fig. 4*I*), indicating that *Pdx1*-deficient β-cells can replicate.

Mixed population of islets in duct-specific *Pdx1*-deficient mice, some islets having loss of key β-cell markers. Although images for both $CAII^{Cre}$;$Pdx1^{Fl/Fl}$ and controls were taken with the same confocal settings on parallel-processed sections, there was remarkable variation in the PDX1-immunodetection signal in $insulin^{+}$ cells, even within the same section of pancreas, from 10- to 12-week-old $CAII^{Cre}$;$Pdx1^{Fl/Fl}$ mice compared with strong homogeneous staining in control pancreas (Fig. 5*A*). Within

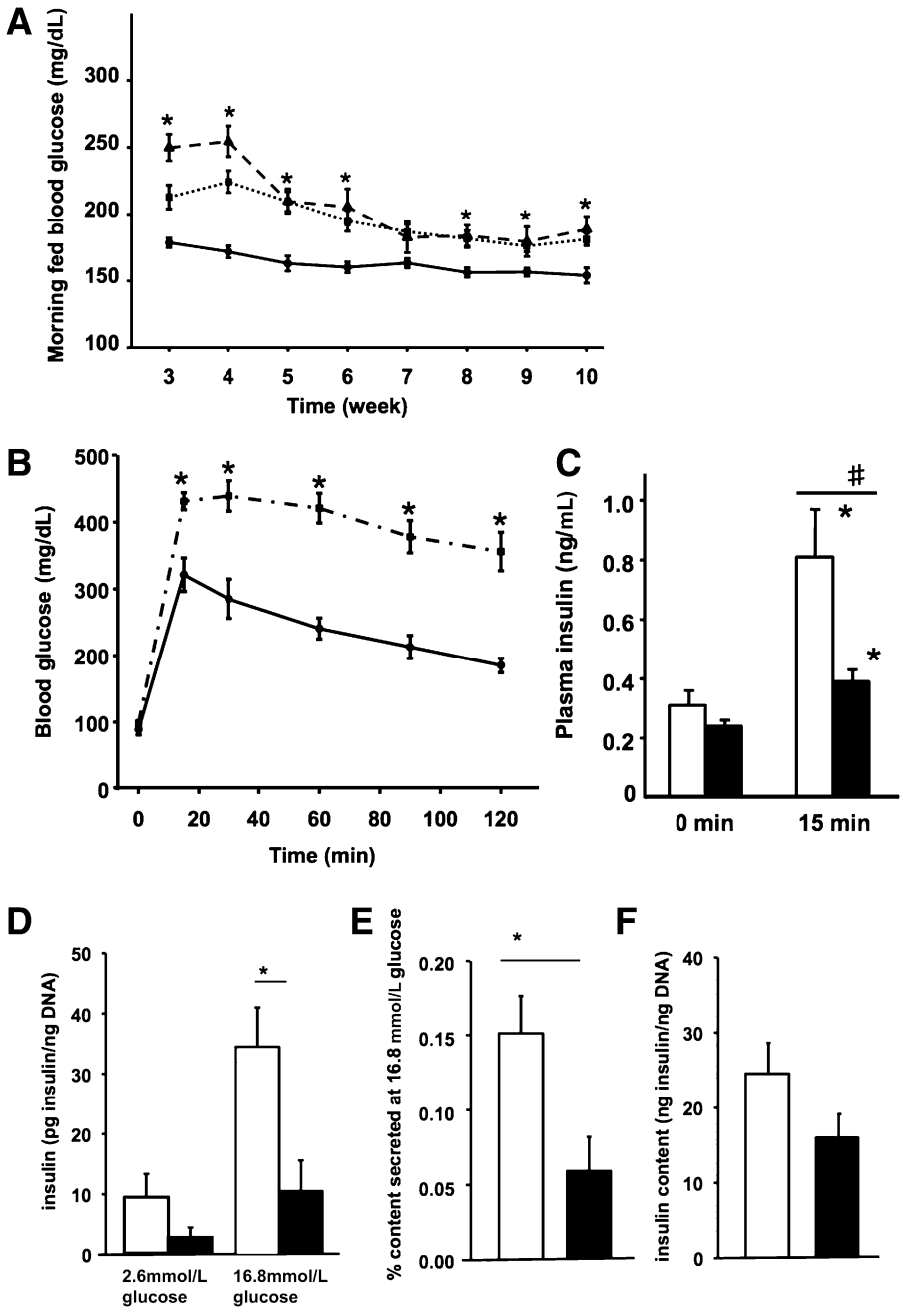

FIG. 3. Duct-specific *Pdx1*-deficient mice had impaired glucose tolerance and impaired insulin secretion. *A*: Time course of morning fed blood glucose values of the controls (solid line, $n = 33$), $CAII^{Cre};Pdx1^{Fl/Fl}$ (dashed line, $n = 17$), and $CAII^{Cre};Pdx1^{Fl/+}$ (dotted line, $n = 23$) littermates. Only at 3 and 4 weeks did the two bigenic genotypes differ from each other. *B*: Intraperitoneal glucose tolerance test (IPGTT) in 10-week-old animals comparing control (solid line) and bigenic mice ($CAII^{Cre};Pdx1^{Fl/Fl}$ and $CAII^{Cre};Pdx1^{Fl/+}$, dashed-dotted line; $n = 8$–16) showed impaired glucose tolerance. *C*: Plasma insulin levels from the IPGTT showed significant increases in both groups at 15 min after glucose injection compared with fasting at 0 min in controls (□, $n = 4$) and bigenic (■, $n = 9$). *$P < 0.025$ compared with 0 min. #$P < 0.004$ comparing groups at 15 min. *D–E*: Isolated islets from 11-week-old bigenic mice (both $CAII^{Cre};Pdx1^{Fl/Fl}$ and $CAII^{Cre};Pdx1^{Fl/+}$, ■, $n = 10$ animals) in sequential static incubation had impaired glucose-responsive insulin secretion compared with controls (□, $n = 10$ animals) (*D*) and lower percentage insulin content secreted (*E*) even though the islet insulin content was not significantly different (*F*). Data are mean ± SEM. *$P < 0.007$. Even if each islet aliquot with values for both glucose concentrations ($n = 23$ for bigenic and $n = 26$ for control) was used for the averaging, the basal levels and islet insulin content do not differ, but the bigenic islets showed a modest glucose-stimulated insulin release (2.6 mmol/L glucose: 3.6 ± 1.1 pg insulin/ng DNA; 16.8 mmol/L glucose: 12.5 ± 3.6 pg insulin/ng DNA; $P < 0.003$, paired t test).

a section of $CAII^{Cre};Pdx1^{Fl}$ pancreas, some islets (whether large, small or as smaller clusters) could be found containing cells with very low to undetectable PDX1 expression. Some islets had strongly homogeneous PDX1 staining, with a minority of cells displaying little or no PDX1 staining. The intensity of insulin staining also varied similarly. Thus, there was a mixed population of islets in the $CAII^{Cre};Pdx1^{Fl}$ mice (Fig. 5*B*): about 30% had homogeneously high or normal PDX1 expression, 20% had low to undetectable expression, and 50% displayed mixed-level expression. $PDX1^{null}insulin^{+}$ cells accounted for 31 ± 7.7% of all $insulin^{+}$ cells ($n = 3$ animals with at least 18 islet/aggregates, and 625 $insulin^{+}$ cells counted for each). The loss of PDX1 expression was similarly seen in the pancreas of 4-week-old

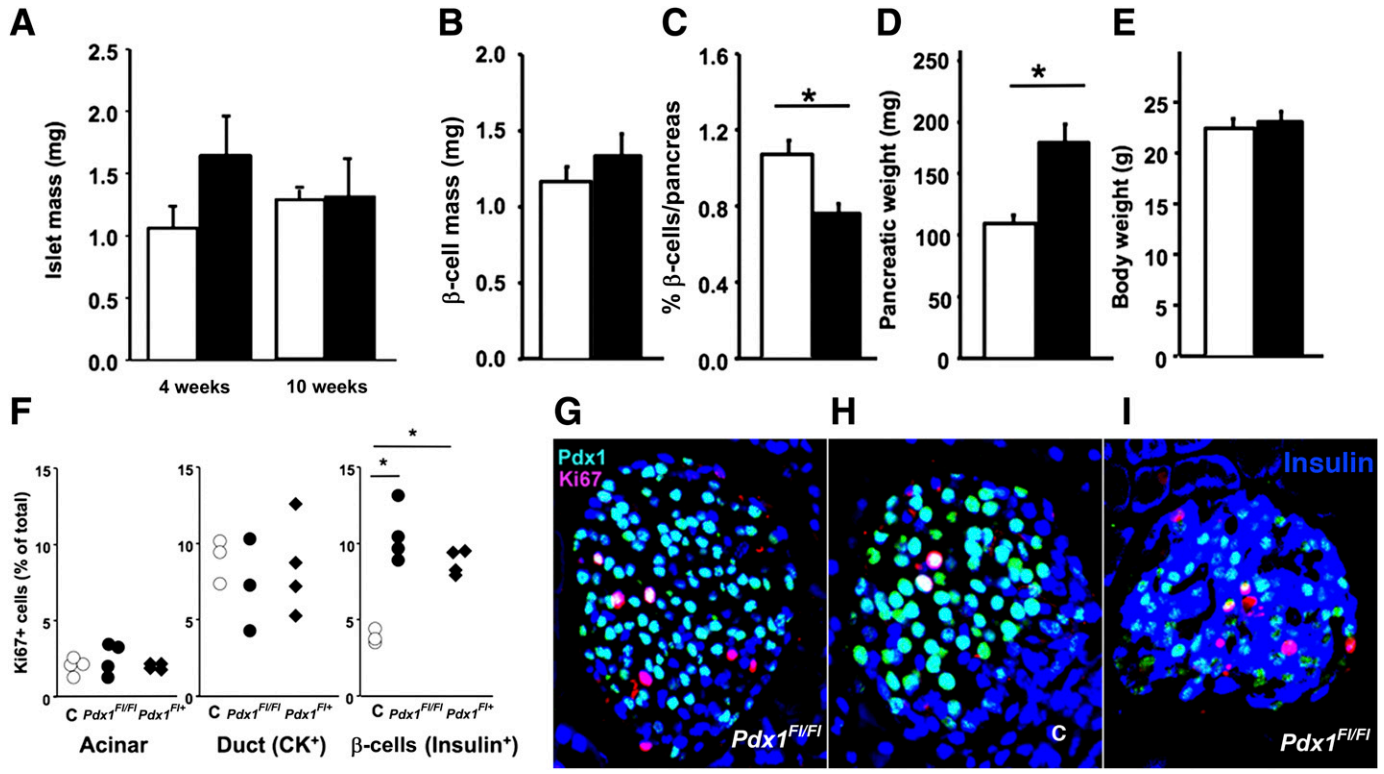

FIG. 4. Duct-specific *Pdx1*-deficient mice had similar islet and β-cell mass as controls. Islet mass at 4 and 10 weeks (*A*) and β-cell mass at 4 weeks (*B*) did not differ between control (□) and *CAII*^{Cre};*Pdx1*$^{Fl/Fl}$ (■) male mice (4 weeks: $n = 5$ control, $n = 6$ bigenic; 10 weeks: $n = 3$ both groups). At 4 weeks the relative density of β-cells (*C*) differed, but because the pancreatic weights (*D*) were increased in the bigenic (even though they had similar body weights) mice (*E*), the absolute β-cell mass was not reduced in the bigenic mice. *F*: At 4 weeks, although there was no difference in proliferation of acinar or duct (CK$^+$) cells between control and bigenic mice, proliferation in insulin$^+$ cells was increased in both bigenic groups (*G*) compared with controls (*H*) with Ki67$^+$ (red), PDX1 (green), and nuclei DAPI (blue). Data for individual animals are shown in *F*. *I*: Some Ki67$^+$insulin$^+$ (blue) cells were PDX1$^-$. Data are mean ± SEM. *$P < 0.05$.

CAII^{Cre};*Pdx1*$^{Fl/Fl}$ (Supplementary Fig. 4) and of *CAII*^{Cre};*Pdx1*$^{Fl/+}$ mice at both ages (data not shown). When the ROSA26R^{eYFP} reporter gene was introduced into the *CAII*^{Cre};*Pdx1* mice for lineage tracing, some lobes had YFP$^+$ acinar and islet cells (Fig. 6*A* and Supplementary Fig. 5). These YFP islets have some β-cells with low to undetectable PDX1 expression, and others cells had strong PDX1 expression.

In islets of 10- to 12-week-old mice, the β-cell transcription factor MAFA had a similarly mixed expression pattern to that of PDX1. Within the same section, some islets of the bigenic mice had little to no MAFA protein expression, in a highly heterogeneous pattern, whereas others had expression indistinguishable from controls (Fig. 6*B*); islets with MAFA$^{low/null}$ were also PDX1$^{low/null}$ (Supplementary Fig. 6). Because MAFA has been found to be important for the functional maturation of β-cells (29), we suspected that the β-cells with low to undetectable MAFA expression were functionally immature.

Increased neuropeptide Y and MAFB protein in β-cells of duct-specific *Pdx1*-deficient mice supports the concept of immaturity of some β-cells. Neonatal rodent β-cells lack glucose-stimulated insulin secretion (31), with a gene expression profile different from adult β-cells (32). During early development, insulin$^+$ cells express MAFB, followed by a switch to MAFA expression that can occur shortly after birth, but in adult mouse islets, the pattern resolves to MAFB expression restricted to glucagon$^+$ cells and MAFA to insulin$^+$ cells (33). Yet, in islets of 10-week-old bigenic mice, MAFB expression was detected in some insulin$^+$ cells (Fig. 7*A*) and in some glucagon$^-$ cells (Fig. 7*B*), strongly suggesting an early stage of β-cell development.

As mentioned above, the large number of cells copositive for PP and insulin were distributed throughout the pancreas. It is unlikely, however, that these cells were actually PP cells: *1*) authentic PP cells are mainly localized in the head of the pancreas, *2*) PP$^+$insulin$^+$ cells are rarely seen, even in normal early stages of pancreatic organogenesis (34), and *3*) importantly, most PP, peptide YY (PYY), and neuropeptide Y (NPY) antibodies cross-react (35–37). In fact, our PP antibody stained scattered cells within the colon, so it must be considered as cross-reacting with PYY (35,36). The limited selectivity of PP or NPY antibodies leads us to consider these cells as "NPY or PYY" (NPY/PYY) cells. When anti-NPY antibody was used, islets of 4- and 10-week-old bigenic mice had many insulin$^+$NPY/PYY$^+$ and glucagon$^-$ NPY/PYY$^+$ (Fig. 7*C*) cells in contrast to those of control mice (Fig. 7*D*). Bigenic mice were clearly hyperglycemic at 4 weeks, so we questioned whether the coexpression of insulin and NPY/PYY resulted from hyperglycemia. Pancreatic sections from adult rats 4 weeks after partial pancreatectomy, which showed chronic moderate hyperglycemia, had no cells with insulin-NPY/PYY copositivity (Supplementary Fig. 7), indicating that induction of NPY/PYY expression in β-cells was not caused by hyperglycemia. Recently, NPY expression was reported in adult insulin$^+$ cells after embryonic-stage β-cell–specific deletion of *NeuroD1*, and these cells were characterized as immature β-cells based on expression of NPY and lactate dehydrogenase A

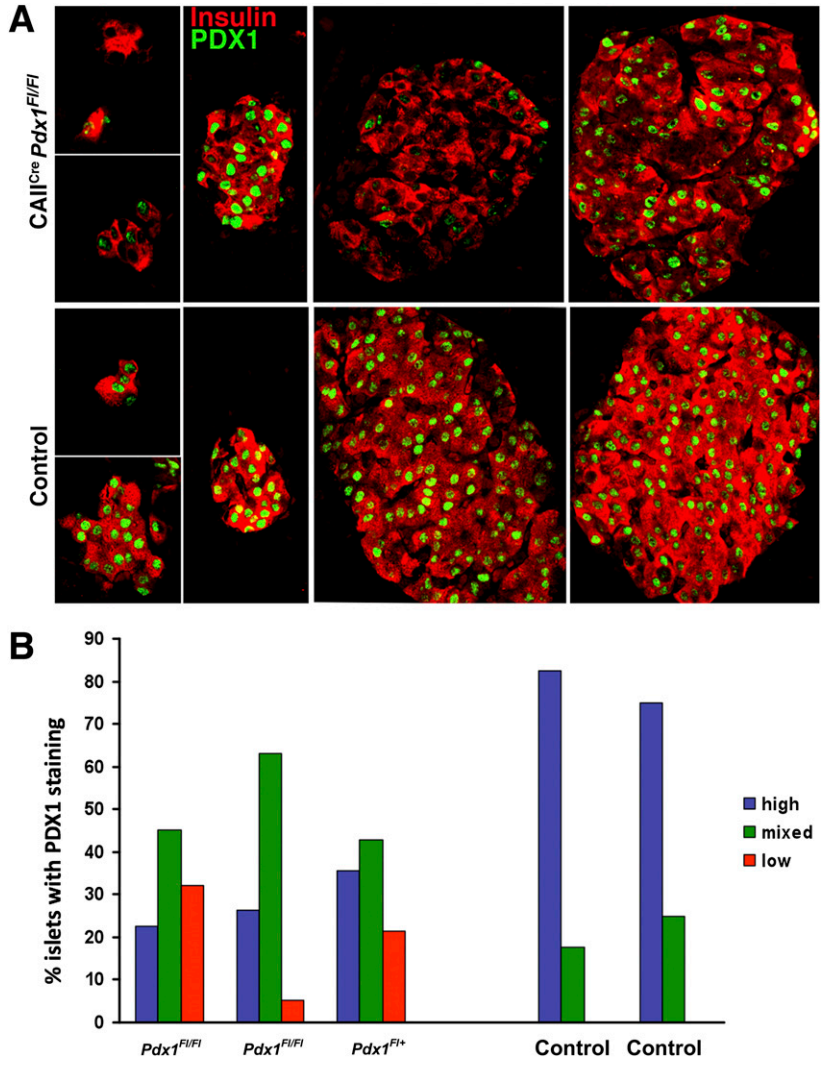

FIG. 5. A mixed population of PDX1-expressing islets was seen in adult duct-specific *Pdx1*-deficient mice. *A*: Islets from same section of *CAII*^{Cre}; *Pdx1*$^{Fl/Fl}$ pancreas (12 weeks old, blood glucose at 4 weeks: 363 mg/dL, 12 weeks: 120 mg/dL) (*top panel*) showed variation in intensity of PDX1 (green) and insulin (red) immunostaining in contrast to those of control pancreas (12 weeks old, blood glucose at 4 weeks: 173 mg/dL, 12 weeks: 179 mg/dL) (*bottom panel*). *B*: On the basis of PDX1 immunostaining (in graph as blue: homogenous high intensity; green: mixed; red: low to undetectable intensity), bigenic mice had decreased proportion of islets with high, homogenous PDX1 expression and, importantly, the appearance of islets without PDX1 immunostaining. Data are shown for individual animals.

(LDHA), plus their lack of glucose responsiveness (38). In our study, insulin$^+$ cells with low levels of PDX1 and MAFA expression, coexpressing MAFB and NPY/PYY seen in duct-specific *Pdx1*-deficient pancreas, strongly suggest that the β-cells formed postnatally remained immature, even at 10 weeks of age.

Decreased expression of β-cell functional genes and increased expression of immature β-cell markers in islets of duct-specific *Pdx1*-deficient mice. Consistent with our immunostaining findings, insulin, *Pdx1*, and *mafa* mRNA levels were significantly lower in islets of 11-week-old duct-specific *Pdx1*-deficient mice than in controls (Fig. 7*E*). Increased gene expression of both *mafb* and *LDHA*, the latter not expressed in adult β-cells but expressed (in rat islets) up to about 1 week postnatally (39), is consistent with our conclusion of the functional immaturity of these islets. Importantly, *PYY* mRNA was elevated in islets of duct-specific *Pdx1*-deficient mice compared with controls, in contrast to *PP* and *NPY* mRNA.

DISCUSSION

By specifically deleting *Pdx1* from pancreatic ducts using duct-specific Cre-lox methods, we showed that β-cell development occurs even in the postnatal absence of PDX1 in ducts but that the resultant neogenetic insulin$^+$PDX1^{null} cells have characteristics of immature β-cells. Thus, we are able to arrive at the significant conclusion that *Pdx1* is not necessary postnatally for formation of β-cells but is necessary for their full maturation to glucose-responsive β-cells. It is especially interesting that some islets, even within the same section, showed strong heterogeneity, with most β-cells PDX1-deficient, yet other islets showed uniformly strong PDX1 staining. These extremes probably represent, respectively, populations of newer postnatal islets and older prenatally formed islets. Importantly, we speculate that the presence of some islets with mostly strong uniform PDX1 staining, with small numbers of cells showing little or no PDX1 signal, could represent newly formed β-cells migrating to and coalescing with older islets.

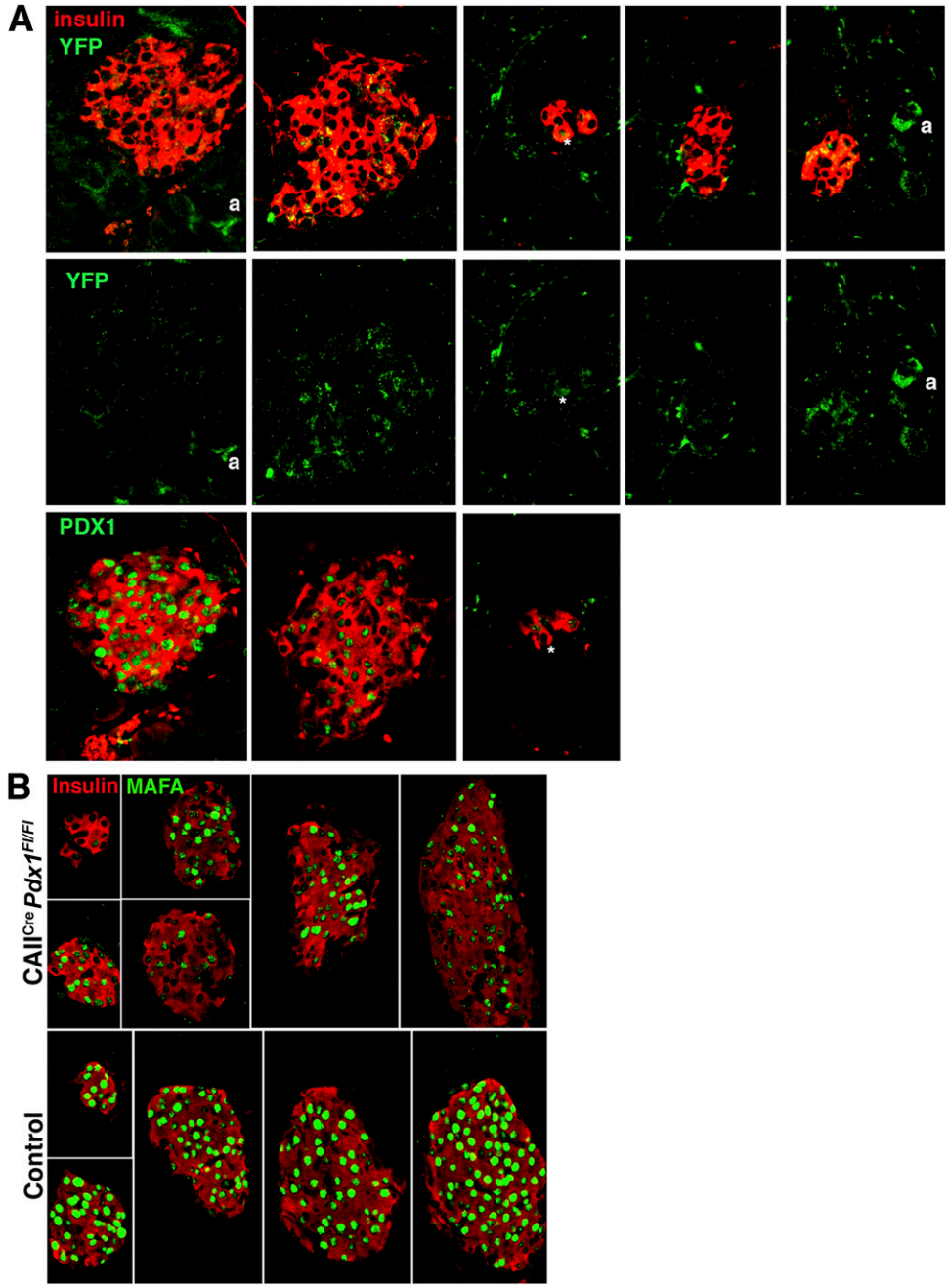

FIG. 6. Islets with PDX1^{null} β-cells show lineage tracing marker and low to undetectable MAFA expression. *A*: The variation of PDX1 immunostaining corresponded with the expression of lineage marker YFP in islets from a 4-week-old *CAII^{Cre};Pdx1$^{Fl/Fl}$* (blood glucose: 278 mg/dL) mouse. The *middle panel* shows YFP expression as split green channel of images shown in the *top panel* (insulin, red; YFP, green). The *bottom panel* shows same islets on adjacent section (due to antibody compatibility issues) with PDX1 (green) and insulin (red). a, lineage-marked acinar cell. *Identifies the same cell in different images. *B*: MAFA expression (green) showed similar variation from high intensity to low/undetectable in insulin^{+} (red) islets from same section of a 10-week-old *CAII^{Cre};Pdx1$^{Fl/Fl}$* mouse (blood glucose at 4 weeks: 272 mg/dL, 10 weeks: 189 mg/dL) compared with homogeneous high intensity of control littermate (blood glucose at 4 weeks: 172 mg/dL, 10 weeks: 178 mg/dL).

Contrary to our initial hypothesis that duct-specific deletion of *Pdx1* would limit postnatal islet neogenesis and result in lower islet mass at 4 weeks, with a possible "compensatory rebound" resulting from increased replication by 10 weeks, our data show that islet and β-cell mass were normal in the duct-specific *Pdx1*-deficient mice, with at least 30% of the β-cells lacking PDX1 protein. The lineage of such cells was verified by eYFP expression of the lineage marker. Thus, we conclude that new β-cells are able to form, in true neogenetic fashion, from postnatal ducts in which *Pdx1* function is prevented. The finding that pancreatic weights were increased in bigenic mice at age 4 weeks but not at age 2 weeks was puzzling. In control mice, this 2-week period is one of an extensive expansion of the pancreas (three- to fourfold increase, from 29.3 to 110.2 mg). In bigenic mice at 2 weeks, ductal proliferation was increased above the already high level of controls, whereas at 4 weeks, the proliferation of the exocrine pancreas (acinar and duct) was similar to the controls. Analyses of *Pdx1* tet-off inducible mouse model (40,41)

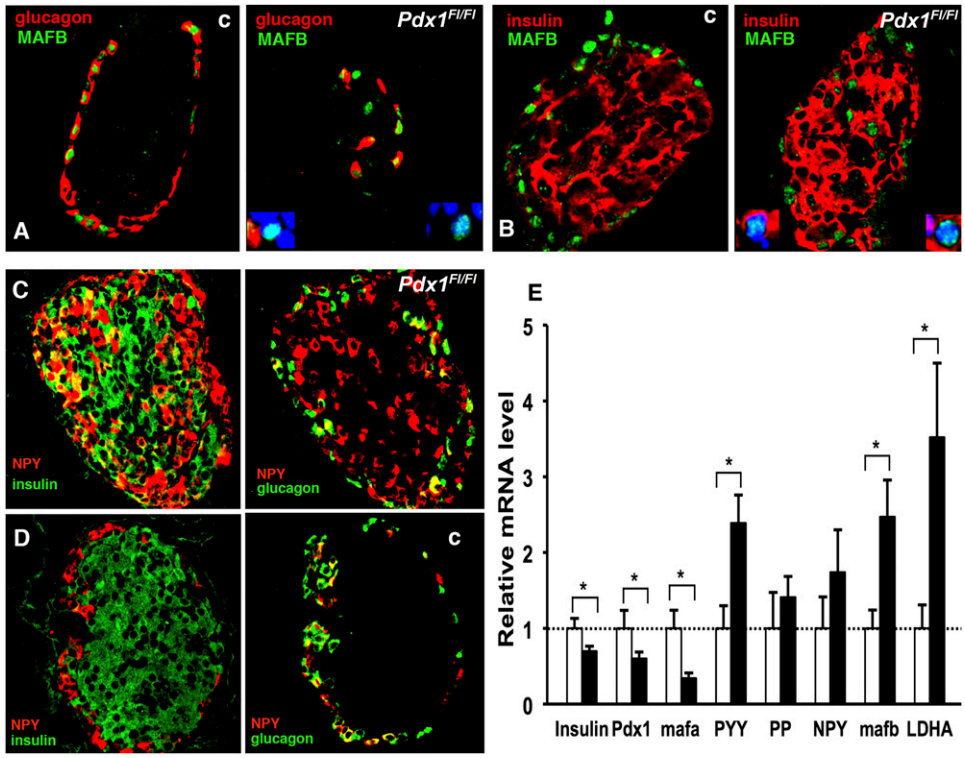

FIG. 7. Islets of 10- to 11-week-old bigenic mice expressed markers of immature β-cells. *A* and *B*: MAFB protein (green) was restricted to glucagon$^+$ cells (red) in adult control (c) islets, but in bigenic (*Pdx1$^{fl/fl}$*) there were both glucagon$^-$ cells (red) and insulin$^+$ cells (red) that were MAFB$^+$. The *insets* in the bigenic images show higher magnification of positive cells with DAPI-stained nuclei. In bigenic mice (*C*) (here blood glucose at 4 weeks: 254 mg/dL, 10 weeks: 145 mg/dL), many insulin$^+$ cells (green) and some glucagon$^+$ cells (green) coexpressed NPY/PYY (red), whereas in controls (*D*) (here blood glucose at 4 weeks: 162 mg/dL, 10 weeks: 156 mg/dL), only some glucagon$^+$ cells coexpressed NPY/PYY (red). The same islets from adjacent sections are shown for insulin/NPY and glucagon/NPY immunostaining for bigenic and controls. *E*: Quantitative PCR for selected genes on RNA from islets of the same 11-week-old animals as used for insulin secretion (Fig. 3*D–F*) showed significant decreased expression of insulin, *pdx1,* and *mafa* mRNA and significant increased expression of *PYY*, *mafb,* and *LDHA* mRNA in bigenic mice (■), shown normalized to controls (□, n = 7–9). Data are mean ± SEM. *$P < 0.05$.

showed that repression of *Pdx1* had very different results dependent on its timing. If *Pdx1* repression were initiated in mid-embryonic stage, acinar differentiation was impeded, but if initiated in the adult, exocrine (acinar and duct) proliferation was stimulated. Our data indicate that during the neonatal period of rapid pancreatic expansion, the lack of *Pdx1* in the ducts resulted in a greater proliferation of duct cells that gave rise to more acinar cells and greater pancreatic weights.

With the current strong controversy over whether pancreatic ducts can give rise to new islet cells or even acinar cells postnatally (1), it is relevant to consider alternative explanations to our current findings. Could there be misexpression of carbonic anhydrase II, and thus Cre recombinase expression, in β-cells? CAII is normally expressed in rodent glucagon-expressing α-cells but not β-cells (30). In the experiments reported here, we used the human *CAII* promoter because CAII is limited to ductal expression in humans, and Cre immunostaining in the *CAII^{Cre}* pancreas was only seen in ducts and ganglia (14). With no injury involved in the current study, any misexpression would have to be significant to result in 30% labeled β-cells. Previously, however, we reported that even 40 cycles of RT-PCR failed to detect *Cre* or *CAII* mRNA in fluorescence-activated cell sorted β-cells from day 1, 2, 4, or 8-week-old *CAII^{Cre};MIP^{GFP}* mice but was easily detected in the kidneys from the same animals (14). The isolated islets used in the current study had no detectable Cre mRNA expression by quantitative PCR.

The glucose intolerance of the bigenic mice showing 70% of the β-cells as "immunofluorescently normal" was unexpected because rodents with 60% partial pancreatectomy maintain normal glucose homeostasis. Regeneration and adaptation have been found in mice and rats after 60% partial pancreatectomy, seen as the 40% β-cell mass of the remnant increasing to about 55% of sham controls (42,43) with an accompanying increase in function of individual β-cells (44,45). One must consider that the reduced glucose responsiveness partly results from glucotoxicity because chronic mild hyperglycemia was present from at least 3 weeks of age in these mice. Even slightly increased (15–20 mg/dL) blood glucose levels for at least 6 weeks can result in impaired glucose-responsive insulin secretion (42) and large alterations in gene expression (46). In our case, it is still unclear why hyperglycemia began at between 2 and 3 weeks of age. Lineage tracing experiments have suggested substantial de novo β-cell formation during this period (47). Moreover, studies of β-cell maturation in neonatal rats (13,31,32,48) show that 3-week-old pups are transiently insulin-resistant and that their β-cells are not functionally mature. In this context, a large functional impairment in 30% of the β-cells may result in modest hyperglycemia.

The presence of several markers of immature β-cells suggests that functional immaturity is partly responsible for the lack of glucose responsiveness of the isolated bigenic islets. In islets from duct-specific *Pdx1*-deficient mice, *mafa* mRNA and protein had lower than normal

expression for adult β-cells, being similar to those in neonatal β-cells (29). We previously showed that although *mafa* overexpression could induce the maturation of glucose-responsiveness in neonatal islets, *Pdx1* overexpression could not within the experiment's timeframe (29). However, PDX1^{high} is expressed before MAFA in insulin^{+} cells during development (33), suggesting that *Pdx1* is an upstream regulator of *mafa*; thus, we expect that with longer incubation, *Pdx1*-infected P2 islets would have induced *mafa* expression and subsequently acquire glucose responsiveness. Furthermore, *mafb*, *LDHA*, and *PYY* mRNA were more highly expressed in bigenic islets compared with control. We conclude that the increased *mafb* mRNA did not reflect an increased proportion of glucagon-expressing cells, because the islet and β-cell mass were unaltered. The continued coexpression of MAFB (which is normally extinguished in mouse β-cells) and insulin in adult bigenic mice suggests that those cells remained in an early stage of β-cell development (33). Isolated islets of adult *Pdx1*-deficient mice also had elevated *LDHA* mRNA, another gene highly expressed in immature islets (39) but hardly expressed in normal adult β-cells (39,49) and induced by chronic hyperglycemia (50). Taken together, the increased expression of *NPY/PYY*, *mafb*, and *LDHA* and low *mafa* in β-cells suggest that PDX1 is necessary for the full maturation of β-cells.

We conclude that PYY is likely the specific member of the NPY/PYY/PP family that is aberrantly expressed in the duct-specific *Pdx1*-deficient β-cells. The cross-reactivity of most PP, PYY, and NPY antibodies has probably contributed to several previously apparently discordant conclusions. PYY and NPY were reported as markers of immature β-cells when coexpressed with insulin (34,36,38,51) and PYY as a marker of early islet precursors (35,36). After birth, NPY expression in pancreatic islets was reported as restricted to neonatal β-cells and absent from adult β-cells (52). Recently, however, NPY was reported in adult-stage insulin^{+} cells after embryonic β-cell–specific deletion of *NeuroD1*, and these cells were classified as immature based on expression of NPY protein/mRNA, LDHA, and lack of glucose-responsiveness (38). In our bigenic genetic manipulation, a large number of insulin^{+}NPY^{+}/PYY^{+} cells were detected in islets, but mRNA for only PYY, not NPY nor PP, was increased in islets from 11-week-old bigenic mice compared with controls. The discrepancy of NPY mRNA between the analyses of islets from *NeuroD1*-deficient mice and our *Pdx1* duct-deleted mice possibly resulted from inclusion of NPY-expressing intrapancreatic ganglia in others' islet preparations.

At 4 weeks, *Pdx1*-deficient mice had a higher percentage of proliferating β-cells, at least some of which were Pdx1^{null}. This increase was likely a compensatory mechanism in response to hyperglycemia, because glucose stimulates β-cell proliferation in vivo (53–55) and in vitro (56,57). The increase was only transient, however, and by 10 weeks, there was no difference between bigenic and control mice. The finding that significant numbers of PDX1^{null}insulin^{+} cells were proliferative indicates that PDX1 is obligatory for proliferation only under some contexts; other studies reported that *Pdx1* was required for replication of β-cells at late gestation (19) or in adults (58).

Another striking finding in *CAII^{Cre};Pdx1^{FL}* mice was the mixed population of islets with varying immunofluorescent signals for PDX1, such that some islets had homogeneously normal levels, others uniformly almost none, with most consisting of a mixture of deficient and normal PDX1-expressing β-cells. The variation of PDX1 expression within and among islets is unlikely to result from hyperglycemia, because animals had only mild hyperglycemia from 7 to 8 weeks of age onward, and many β-cells had a normal PDX1 immunodetection signal that should be associated with good functional status. The variation in islet types, even within the same tissue section, suggests that besides the number of normal-level PDX1^{+} islets that likely represent those formed before birth, PDX1-deficient β-cells derived by neogenesis in the postnatal period from the *Pdx1*-depleted ducts can produce new homogeneously PDX1-depleted islets or can coalesce with older preexisting (strongly PDX1^{+}) islets to yield "chimeric islets." It is unclear whether such a migration would require long-range movement or a behavior distinct from that seen in normal embryonic phases of endocrine/islet ontogeny, but the proximity of many islets to ducts does render this idea plausible.

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L.G., A.I., C.A.-M., J.H.-L., and S.B.-W. collected data. L.G. and S.B.-W. wrote the manuscript. A.I. and Y.F. conceived the project. G.C.W., C.V.E.W., and A.S. provided critical discussions during study design and interpretation. C.V.E.W. provided mice. All authors edited and approved the manuscript. S.B.-W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the accuracy of the data analysis.

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