Defining a Novel Role for the Pdx1 Transcription Factor in Islet β-Cell Maturation and Proliferation During Weaning

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The transcription factor encoded by the *Pdx1* gene is a critical transcriptional regulator, as it has fundamental actions in the formation of all pancreatic cell types, islet β-cell development, and adult islet β-cell function. Transgenic- and cell line–based experiments have identified 5′-flanking conserved sequences that control pancreatic and β-cell type–specific transcription, which are found within areas I (bp −2694 to −2561), II (bp −2139 to −1958), III (bp −1879 to −1799), and IV (bp −6200 to −5670). Because of the presence in area IV of binding sites for transcription factors associated with pancreas development and islet cell function, we analyzed how an endogenous deletion mutant affected *Pdx1* expression embryonically and postnatally. The most striking result was observed in male *Pdx1*$^{\Delta IV}$ mutant mice after 3 weeks of birth (i.e., the onset of weaning), with only a small effect on pancreas organogenesis and no deficiencies in their female counterparts. Compromised Pdx1 mRNA and protein levels in weaned male mutant β-cells were tightly linked with hyperglycemia, decreased β-cell proliferation, reduced β-cell area, and altered expression of Pdx1-bound genes that are important in β-cell replication, endoplasmic reticulum function, and mitochondrial activity. We discuss the impact of these novel findings to *Pdx1* gene regulation and islet β-cell maturation postnatally.

Pdx1 is the earliest tissue-selective transcription factor expressed in the developing primordium and is essential to formation of all pancreatic cell types and the activity of adult islet β-cells. Thus, mice and humans who completely lack Pdx1 function are apancreatic (1,2), whereas haploinsufficiency primarily affects islet β-cells after birth (3,4). Moreover, β-cell–specific inactivation of Pdx1 in the adult mouse causes severe hyperglycemia and loss of cell identity, with these cells transdifferentiating to an islet α-like cell capable of secreting the glucagon hormone (5). The wide-ranging importance of Pdx1 in the pancreas reflects a dynamic expression pattern, with production found throughout the earliest multipotent pancreatic progenitor cell pool, and then in a more restricted manner within all developing and adult islet insulin^{+} β-cells (6), as well as a small proportion of islet somatostatin^{+} δ-cells (7).

Extensive transgenic and cell line reporter-based experimentation in animal models strongly suggests that pancreatic cell-type–specific transcription of *Pdx1* is primarily controlled by four conserved 5′-flanking enhancer–like domains, referred to as areas I, II, III, and IV (8,9). For example, a transgene driven by areas I to II (bp 2917 to –1918) recapitulates in mice the islet β-cell–enriched expression pattern of the endogenous gene (10), whereas early embryonic removal of areas I–III from the mouse genome compromises *Pdx1* mRNA levels and pancreas development in vivo (11). Moreover, a Pdx1 coding region containing transgene driven by 5′-flanking region areas I–III and a portion of area IV rescues pancreatic organogenesis in *Pdx1*$^{-/-}$ mice (12). Areas I, II, III, and IV are also highly

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See accompanying article, p. 2738.

conserved in all *Pdx1*-expressing species, although area II is only found in mammals (8).

A variety of transcription factors involved in pancreas specification, differentiation, and islet β-cell activity appear to regulate *Pdx1* expression by directly binding within areas I, II, III, and/or IV enhancer sequences. Thus, Ptf1a, which, like Pdx1, is a transcription factor essential for pancreas exocrine and endocrine cell formation (13), binds in early pancreatic progenitor cells to areas III and IV in chromatin immunoprecipitation (ChIP) assays (14). Moreover, the apancreatic phenotype produced upon conditional ablation of the FoxA1 and FoxA2 transcription factors in the pancreatic primordium results from loss of *Pdx1* expression because of their necessity in stimulating area I, II, and/or IV activity (15). These Pdx1 control regions also seem to be regulated by transcription factors specifically involved in later islet cell formation and function, including neurogenin 3 (Ngn3) (16), Pax6 (17), Nkx2.2 (9), and Hnf1α (18). In addition, Pdx1 binding to areas I and IV creates a potential autoregulatory network (18).

However, what remains to be understood is exactly how each of these enhancer-like domains control *Pdx1* expression, appreciating that unique, independent control properties have been found for distal control regions in other cellular contexts (e.g., the globin genes [19]). Notably, recent analysis of an endogenous *Pdx1* area II deletion mutant in a Pdx1 protein null background (i.e., $Pdx1^{\Delta AII/-}$) resulted in a profound and specific reduction in pancreatic endocrine progenitor cell formation that caused the loss in hormone-producing cells and early postnatal cell death from hyperglycemia (20), thus revealing a requirement for area II in controlling *Pdx1* transcription during pancreas cell development. In this study, we focused on defining how area IV effected *Pdx1* expression. Consequently, we generated a new mouse deletion allele, termed $Pdx1^{\Delta AIV}$, in which endogenous area IV was precisely removed genetically. In contrast to the area II control region mutant mice (i.e., $Pdx1^{\Delta AII/-}$ [20]), there was only a very modest influence on pancreas cell formation developmentally and no impact on viability in an area IV mutant that also lacked a functional *Pdx1* allele (i.e., $Pdx1^{\Delta AIV/-}$). However, blood glucose levels were significantly elevated at weaning in male $Pdx1^{\Delta AIV/-}$ mice (i.e., after 3 weeks) and not age-matched female $Pdx1^{\Delta AIV/-}$ or control $Pdx1^{+/-}$ mice. This change in glucose homeostasis was associated with reduced expression of islet *Pdx1* mRNA, Pdx1 protein, and Pdx1-regulated genes, which resulted in decreased $Pdx1^{\Delta AIV/-}$ islet β-cell activity, β-cell proliferation, and β-cell area. In addition, Pdx1 binding to endogenous area IV (and not areas I–III) was specifically induced after weaning, suggesting temporal autoregulation of this enhancer. These studies not only provide insight into the distinct functional properties of the *Pdx1* area II and IV regulatory regions over the life span of the animal, but also reveal sexual dichotomy in area IV function during a crucial period of islet β-cell maturation.

RESEARCH DESIGN AND METHODS

Generation of the $Pdx1^{\Delta AIV}$ Allele

Recombinase-mediated cassette exchange was performed to insert *Pdx1* gene sequences lacking area IV sequences into $Pdx1^{LCA}$ embryonic stem cells (21). The exchange vector contained a *Pgk-hygromycin* resistance ($Hygro^R$) cassette, flanked by tandem FLP recombinase target sites, enabling use of a staggered positive-negative selection strategy (22). Mouse embryonic stem cells surviving hygromycin and then ganciclovir selection were screened by PCR for successful recombinase-mediated cassette exchange; chimeric mice were generated via blastocyst microinjection of a successfully exchanged clone. After breeding with C57BL/6J mice to obtain germline transmission, $Pdx1^{\Delta AIV(+HygroR)}$ mice were bred with FLPe-expressing transgenic mice (23) to remove the FLP recombinase target–flanked $Hygro^R$ cassette, thereby generating the $Pdx1^{\Delta AIV}$ allele. PCR primers were used to detect the presence of the *Pdx1* null allele (forward: 5′-CAAGCCAGGGAGTTAAATC-3′; and reverse: 5′-AGGTGAGATGACAGGAGATC-3′) and area IV deletion (forward: 5′-GCAGTCAGGATCCAGGTTTAG-3′; and reverse: 5′-AAAGTGTCTACGGTGGGAACA-3′). Noon of the day of the vaginal plug discovery was designated embryonic day (E) 0.5. The Vanderbilt University Institutional Animal Care and Use Committee approved all animal procedures and husbandry protocols.

Immunohistochemistry

Pancreata from staged embryonic and postnatal littermate controls were fixed in 4% (volume for volume) paraformaldehyde, paraffin-embedded, and cut to 6 μm. Sections were blocked with 5% (volume for volume) normal donkey serum in 1% (weight for volume) BSA/PBS and incubated with primary antibodies overnight at 4°C. Cyanine dye (Cy)2-, Cy3-, or Cy5-conjugated secondary antibodies (1:2,000; Jackson ImmunoResearch Laboratories, West Grove, PA) were used for fluorescent detection. Peroxidase staining was performed using the DAB substrate kit (Vector Laboratories, Burlingame, CA) and counterstained with eosin to obtain islet β-cell area. Images were collected on an Axioimager M2 (Carl Zeiss, Jena, Germany) or an Aperio ScanScope (Leica Microsystems, Buffalo Grove, IL) whole-slide scanner. DAPI dye (Southern Biotechnology Associates) was used to detect nuclei in immunofluorescent images. The following primary antibodies were used: insulin-guinea pig (1:1,000; A056401–2; DakoCytomation, Carpinteria, CA); glucagon-mouse (1:2,000; G2654; Sigma-Aldrich, St. Louis, MO); somatostatin-goat (1:1,000; sc-7819; Santa Cruz Biotechnology, Dallas, TX); Ki67-mouse (1:1,000; 550609; BD Pharmingen, San Jose, CA); MafB (1:1,000; IHC-00351; Bethyl Laboratories, Montgomery, TX); MafB (1:500; NB600–266; Novus Biologicals, Littleton, CO); MafA (1:1,000; IHC-00352; Bethyl Laboratories); MafA (1:500; NBP1–00121; Novus Biologicals); Nkx6.1 (1:500; NBP1–49672; Novus Biologicals); Glut2 (1:500; sc-7580; Santa Cruz Biotechnology); and Pdx1-goat (1:20,000; derived by C. Wright, Vanderbilt University).

Hormone Cell Quantification

The insulin$^+$ β-cell area was determined from random sections (eight total) taken throughout the pancreas of 3- and 5-week-old *Pdx1*$^{+/-}$ and *Pdx1*$^{\Delta AIV/-}$ mice. Embryonic islet cell quantification was analyzed in serial sections collected every 30 (E15.5) or 90 μm (E18.5) that were immunostained with primary antibodies and counterstained with DAPI (Invitrogen) for nuclei labeling. The effect of *Pdx1*$^{\Delta AIV/-}$ on total hormone$^+$ cell numbers was calculated from the ratio of *Pdx1*$^{\Delta AIV/-}$ hormone$^+$ cells to *Pdx1*$^{+/-}$ hormone$^+$ cells. Islet β-cell proliferation was determined using E18.5, 3-week-, and 5-week-old *Pdx1*$^{+/-}$ and *Pdx1*$^{\Delta AIV/-}$ pancreas sections costained with insulin and Ki67. The number of insulin$^+$ and Ki67$^+$ copositive cells were counted and divided by the total number of insulin$^+$ cells. At least three independently derived mouse samples were used in each analysis.

Islet Isolation, Immunoblotting, and Quantitative Real-time PCR

Islets were isolated from mice that were first anesthetized and then euthanized via cervical dislocation. The abdominal cavity was exposed, and collagenase P (Roche) in PBS was injected into the common bile duct with the end connecting to the duodenum blocked. The pancreas was then isolated and digested further with collagenase P and islets isolated by handpicking under ×10 magnification. Pancreatic islet protein was prepared from 5-week-old *Pdx1*$^{+/-}$ and *Pdx1*$^{\Delta AIV/-}$ mice by homogenizing in 50 mmol/L Tris (pH 7.5), 420 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholic acid, and Complete Protease Inhibitor Cocktail Tablets (Roche). Cleared extracts (5 μg) were separated on 10% acrylamide Tris-HCl gels, transferred to polyvinylidene fluoride membranes, blocked with 10% nonfat milk, and then analyzed with goat anti-Pdx1 (1:20,000) antibody and donkey anti-goat conjugated to horseradish peroxidase (1:2,000). β-Actin was used as a loading control. Horseradish peroxidase was detected by ECL (PerkinElmer) and quantification performed using ImageJ software (National Institutes of Health). Islet RNA was isolated using TRIzol (Invitrogen) and further treated with the DNA-Free RNA Kit (Zymo Research). RNA quality was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies) in the Vanderbilt Functional Genomics Shared Resource Core Laboratory; RNA was only used if the integrity value was ≥7. Quantitative real-time PCR (qPCR) was conducted as previously described on a Light Cycler 480 II (Roche) (24); primer sequences are available upon request. Gene expression changes between *Pdx1*$^{+/-}$ and *Pdx1*$^{\Delta AIV/-}$ islet RNA were normalized to 18s RNA levels and relative fold changes calculated using the ΔΔ threshold cycle method.

ChIP

Islets isolated from either 3- or 5-week-old male wild-type mice were formaldehyde cross-linked and the sonicated protein–DNA complexes obtained under conditions described previously (25). Sonicated chromatin was incubated for 12–14 h at 4°C with anti-Pdx1 antibody or IgG, and the complexes were isolated with protein A/G-agarose (Santa Cruz Biotechnology). qPCR was performed on the immunoprecipitated DNA using SYBR Green PCR Master Mix (Applied Biosciences) in a LightCycler 480 II Real-Time PCR System. Binding enrichment of Pdx1 is presented relative to the IgG control. Each ChIP experiment was repeated at least three times using independent islet chromatin preparations. Primers used to amplify *Pdx1* areas I, II, III, or IV are available upon request.

Pdx1 ChIP Sequencing Data Mining

Pdx1 target genes were determined by annotating the 8,504 peaks from Pdx1 ChIP–DNA sequencing of mouse βTC-6 cells (26) (accession number GSE70960 [http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM1824087]) to the nearest reference sequencing gene of the 1,597 produced differentially in weaning mouse islets (27) using Homer version 4.7.2 (28). Overlaying genes bound by Pdx1 with genes differentially regulated during weaning (27) identified 547 Pdx1-bound genes in this category.

Intraperitoneal Glucose Tolerance Test

Mice ($n \geq 7$) were fasted from 0800 to 1400 h, and blood glucose levels from the sampled saphenous vein were determined using a FreeStyle glucometer (Abbott Diabetes Care). The mice were then weighed, and 1 mg dextrose/g body weight (Fisher Scientific) in sterile PBS was injected intraperitoneally. Blood glucose levels were measured at 15, 30, 60, and 120 min postinjection and fed levels established prior to fasting.

Statistical Analysis

All data are expressed as mean ± SEM and were tested for statistical significance using the Student two-tailed *t* test.

Study Approval

All studies involving the use of animals were approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

RESULTS

Islet Endocrine Cell Development Is Only Modestly Affected in *Pdx1*$^{\Delta AIV/-}$ Mice

There were no overt developmental or postnatal deficiencies found in homozygous endogenous area IV deletion mutants (i.e., *Pdx1*$^{\Delta AIV/\Delta AIV}$) upon comparison with *Pdx1*$^{\Delta AIV/+}$, *Pdx1*$^{+/-}$ or *Pdx1*$^{+/+}$ mice, which were euglycemic, glucose tolerant, and produced control-like amounts of Pdx1 as well as Pdx1 activated gene targets, like Nkx6.1, MafA, and Glut2 (Supplementary Fig. 1). This is likely because of compensation by area I, II, and III, which is sufficient to direct transgenic reporter expression to exocrine and endocrine cells (14).

Pdx1$^{\Delta AIV}$ mice were then crossed with the heterozygous *Pdx1*$^{+/-}$ mutant to generate *Pdx1*$^{\Delta AIV/-}$ mice to determine if reducing Pdx1 protein levels revealed a stronger effect on the area IV deletion (Fig. 1*A*). *Pdx1*$^{+/-}$ served as the control in our analysis, because the heterozygous mutant is not only associated with type 2 diabetes (T2D) in humans (3,4) but also causes reduced islet β-cell function and mass

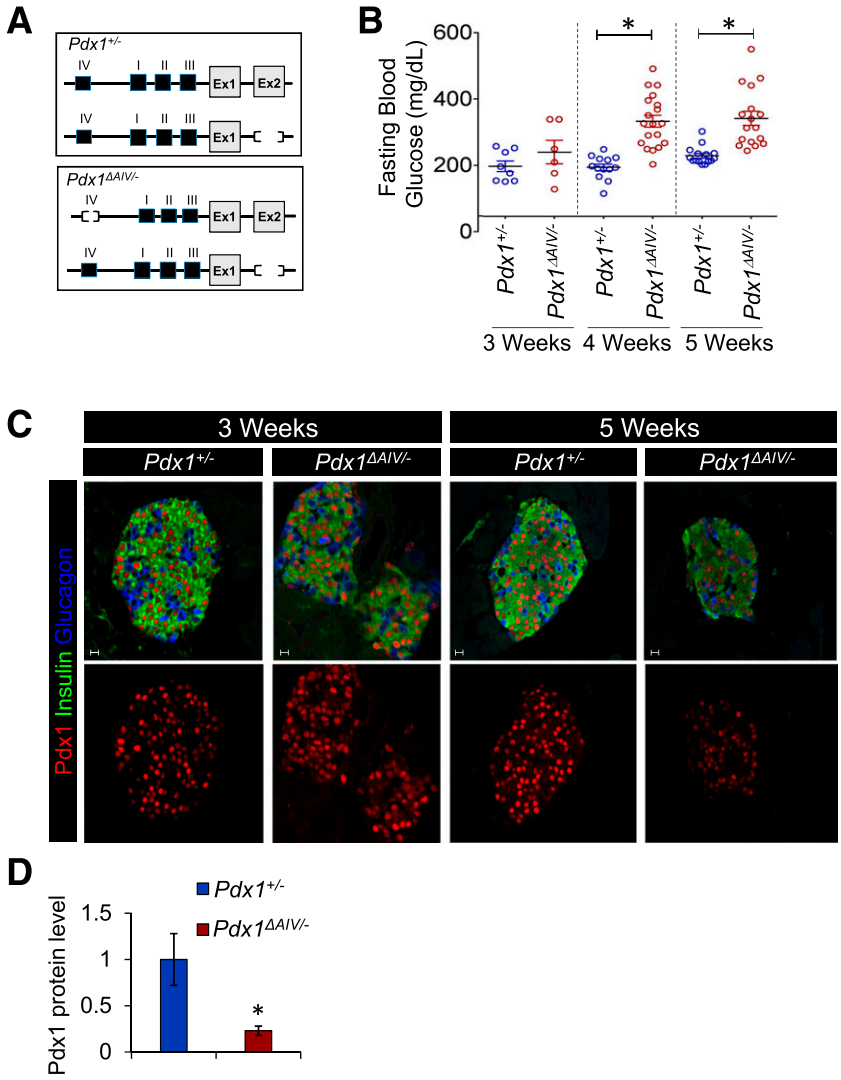

Figure 1—Islet β-cell dysfunction in *Pdx1*$^{\Delta AIV/-}$ mice coincides with reduced Pdx1 expression at weaning. *A*: Schematic depiction of the *Pdx1*$^{\Delta AIV/-}$ and *Pdx1*$^{+/-}$ genotypes. The *Pdx1* null (−) allele lacks exon (Ex) 2, which spans the homeodomain and a majority of the protein. *Pdx1*$^{\Delta AIV}$ is missing the 5′-flanking area IV transcriptional control domain. *B*: Fasting blood glucose levels in male *Pdx1*$^{\Delta AIV/-}$ mice were elevated significantly in relation to *Pdx1*$^{+/-}$ after 3 weeks of birth. *C*: Representative images illustrating the change in islet β-cell Pdx1 and insulin staining between 3- and 5-week-old *Pdx1*$^{\Delta AIV/-}$ mice. Scale bars, 10 μm. *D*: The change in Pdx1 amount in 5-week-old *Pdx1*$^{\Delta AIV/-}$ and *Pdx1*$^{+/-}$ islets. Pdx1 levels were normalized to β-actin using ImageJ software. $n = 3$. *$P < 0.05$, based on two-tailed Student *t* test.

in mice (29). *Pdx1*$^{\Delta AIV/-}$ mice were born in the expected ratios and had normal body weights (Supplementary Fig. 2*A*). However, there was an ∼50% decrease in *Pdx1*$^{\Delta AIV/-}$ endocrine islet Ngn3–positive (+) progenitor cell numbers at E15.5 (Supplementary Fig. 2*B*), which resulted in an ∼25% reduction in insulin and somatostatin (i.e., islet δ) hormone+ cell numbers in the E18.5 fetal pancreas (Supplementary Fig. 2*C*). In contrast, the E18.5 pancreas showed no change in either glucagon+ (α) cell numbers or the rate of β-cell proliferation and apoptosis between *Pdx1*$^{\Delta AIV/-}$ and *Pdx1*$^{+/-}$ mice (Supplementary Fig. 2*D* and *E*). Notably, endogenous *Pdx1*$^{\Delta AII/-}$ mutant mice produced a much more penetrant developmental phenotype, resulting in a profound loss in α- and β-cell numbers and early postnatal lethality (20).

The slight reduction in developmentally produced *Pdx1*$^{\Delta AIV/-}$ β- and δ-cells did not affect blood glucose levels at postnatal day (P) 3 (Supplementary Fig. 3*A*), nor the immunodetection pattern of various β-cell identity markers in relation to *Pdx1*$^{+/-}$ mice (e.g., MafA, MafB [i.e., expressed in both α and β at this time point] [30]), Glut2, Nkx6.1, and Pdx1 (Supplementary Fig. 3*B*). The absence of any phenotypic change during this time period was expected because *Pdx1*$^{\Delta AIV/-}$ E18.5 insulin+ cells produced the high level of Pdx1 protein associated with this committed islet cell population (Supplementary Fig. 2*F*) (31). These data not only showed that the modest reduction in endocrine cell numbers did not create any obvious physiological phenotype within newborn *Pdx1*$^{\Delta AIV/-}$ animals, but also that the change (if any) in Pdx1 expression in developing β-cells did

not significantly affect the distribution, abundance, or activity of key regulatory proteins.

Male *Pdx1*$^{\Delta AIV/-}$ Mice Develop Diabetes at Weaning

Upon monitoring blood glucose levels in *Pdx1*$^{\Delta AIV/-}$ *and* *Pdx1*$^{+/-}$ mice after birth, a significant elevation was observed at weaning (i.e., >3 weeks) in male *Pdx1*$^{\Delta AIV/-}$ mice (Fig. 1*B*). In contrast, glucose tolerance as well as ad libitum fed and fasted blood glucose levels were unchanged in 5-week-old female *Pdx1*$^{\Delta AIV/-}$ mice (Supplementary Fig. 4*A*–*C*). The induction of hyperglycemia in male *Pdx1*$^{\Delta AIV/-}$ mice correlated with reduced islet β-cell Pdx1 protein levels in immunostaining (Fig. 1*C*) and immunoblotting assays (Fig. 1*D*), whereas normoglycemic female *Pdx1*$^{\Delta AIV/-}$ islets had indistinguishable levels of *Pdx1* mRNA and protein levels in comparison with 5-week-old *Pdx1*$^{+/-}$ controls (Supplementary Figs. 4*D* and 5). Consequently, all subsequent analyses were performed with male *Pdx1*$^{\Delta AIV/-}$ and *Pdx1*$^{+/-}$ mice.

Although glucose tolerance was similar between 3-week-old *Pdx1*$^{\Delta AIV/-}$ and *Pdx1*$^{+/-}$ mice (Fig. 2*A*), there was a small reduction in β-cell area in 3-week-old *Pdx1*$^{\Delta AIV/-}$ mice (Fig. 2*E*), presumably reflecting the fewer number of β-cells produced developmentally (Supplementary Fig. 2*C*). However, the apparent change in β-cell activity was not observed until weaning, with 4- and 5-week-old *Pdx1*$^{\Delta AIV/-}$ mice becoming hyperglycemic (Figs. 1*B* and 2*B*) and producing comparatively lower serum insulin levels (Fig. 2*C*). In addition, islet β-cell proliferation and area were reduced significantly in 5-week-old *Pdx1*$^{\Delta AIV/-}$ mice (Fig. 2*D* and *E*). As Pdx1 regulates the expression of genes essential to islet β-cell activity and replication (5,32,33), these results imply that area IV–driven Pdx1 expression is required for the transcription of genes involved in these functions within the postweaning period.

Reduced Pdx1 Levels in *Pdx1*$^{\Delta AIV/-}$ Islets Are Associated With Changes in Expression of Genes Involved in Controlling the Functional and Proliferative State of Islet β-Cells

To further define the direct consequence on β-cells of the reduced Pdx1 levels produced in *Pdx1*$^{\Delta AIV/-}$ mice, we analyzed the mRNA and protein levels of several Pdx1 regulated genes essential to cell identity and function, including β-cell–enriched transcription factors required in glucose-responsive insulin secretion (i.e., MafA [24,25] and Nkx6.1 [34], the principal glucose transporter [Glut2] of rodent β-cells [35], and insulin). Transcript levels of each were significantly reduced in 5-week-old male *Pdx1*$^{\Delta AIV/-}$ islets (Fig. 3*A*). In contrast, no expression changes were observed in 3-week-old male *Pdx1*$^{\Delta AIV/-}$ islets (Fig. 3*A*) or 5-week-old female *Pdx1*$^{\Delta AIV/-}$ islets (Supplementary Fig. 4*D*), with the latter result predicted from the sexual dichotomy of the *Pdx1*$^{\Delta AIV/-}$ phenotype (Fig. 2 and Supplementary Fig. 4). Immunofluorescence analysis supported that these observed Pdx1 target gene transcript changes influenced MafA, Glut2, Nkx6.1, and insulin protein levels in only 5-week-old male *Pdx1*$^{\Delta AIV/-}$ β-cells (Supplementary Figs. 5 and 6).

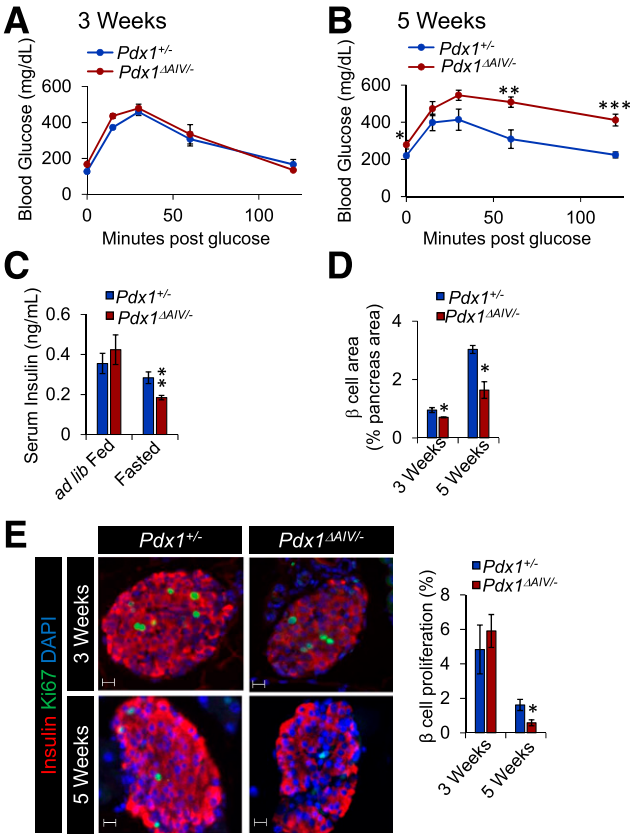

Figure 2—Glucose clearance, islet insulin levels, and β-cell area is decreased in 5-week-old male *Pdx1*$^{\Delta AIV/-}$ mice. *A* and *B*: The ability to reduce blood glucose levels was compromised in 5-week-old *Pdx1*$^{\Delta AIV/-}$ mice in glucose tolerance tests in comparison with *Pdx1*$^{+/-}$ mice or 3-week-old *Pdx1*$^{\Delta AIV/-}$ mice. n = 4–6. *C*: Serum insulin levels from fed and fasted 5-week-old *Pdx1*$^{\Delta AIV/-}$ and *Pdx1*$^{+/-}$ mice. *D*: The modest reduction at 3 weeks in islet β-cell area was increased by 5 weeks in *Pdx1*$^{\Delta AIV/-}$ mice. n = 3 to 4. *E*: Representative image illustrating the specific decrease in Ki67^{+} proliferation levels in 5-week-old *Pdx1*$^{\Delta AIV/-}$ β-cells. n = 3. Scale bars, 10 μm. The number of asterisks depict the statistical significance of the changes. *P < 0.05; **P < 0.01; ***P < 0.001.

The reduced levels of Pdx1 in weaning *Pdx1*$^{\Delta AIV/-}$ islet β-cells were also found to affect a variety of genes important to endoplasmic reticulum (ER) function, including *Bip* (an ER chaperone), *Wfs1* (a mediator of the unfolded protein response pathway), and neuronatin (*Nnat*; an ER-resident protein that regulates insulin secretion) (Fig. 3*A*). Moreover, the same pattern of Pdx1 dependence was found upon maintaining adult *Pdx1*$^{+/-}$ mice on a high-fat diet (36), with the decreased expression of ER genes concluded to compromise islet β-cell function. Moreover, a candidate screen performed on a variety of cell cycle regulators demonstrated that an activator of this process was decreased in 5-week-old male *Pdx1*$^{\Delta AIV/-}$ islets (i.e., *CyclinB1*), whereas an inhibitor (i.e., *p19*) was increased (Supplementary Fig. 7*B*), presumably effects that contribute to reduced *Pdx1*$^{\Delta AIV/-}$ islet β-cell area. These data support an essential role for Pdx1 in regulating β-cell activity upon

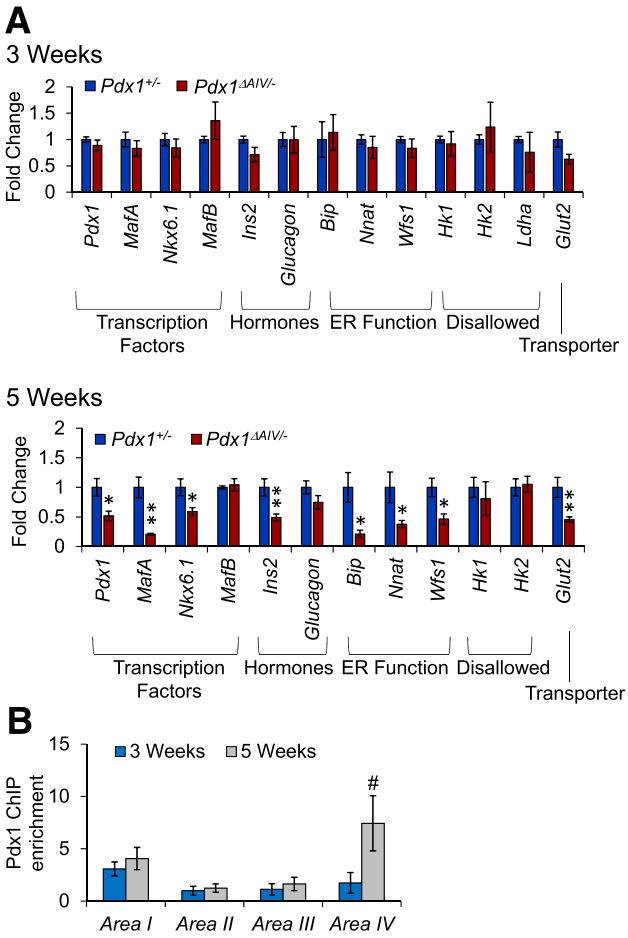

Figure 3—*Pdx1*$^{\Delta AIV/-}$ mice have reduced levels of gene products important to islet β-cell function. *A*: Expression levels of various genes associated with adult β-cell function are decreased in 5-week-old *Pdx1*$^{\Delta AIV/-}$ islets, although there was no change in the disallowed genes produced in neonatal cells. n = 4–6. *B*: Pdx1 binding to area IV was enriched at 5 weeks in wild-type islet ChIP assays. Binding was normalized to the IgG immunoprecipitation. n = 3. *P < 0.05; **P < 0.01; #P < 0.09.

weaning, which coincides with the progression to enhanced glucose-responsive insulin release (27).

In contrast to the importance of Pdx1 in regulating, for example, *insulin*, *Nkx6.1*, and *MafA* transcription in islet β-cells of weaning male *Pdx1*$^{\Delta AIV/-}$ mice, there was no change in *MafB* or *glucagon* levels (Fig. 3*A*), for which expression is repressed by Pdx1 in adult islet β-cells (5). Furthermore, expression of many other essential islet-enriched transcriptional regulators were unaltered in 5-week-old male *Pdx1*$^{\Delta AIV/-}$ islets (i.e., *Pax4*, *Pax6*, *Foxa1*, *FoxA2*, and *Hnf4α*; Supplementary Fig. 7*A*), as were *Hk1*, *Hk2*, and *Ldha* (Fig. 3), housekeeping genes that enable neonatal mouse islet β-cells to secrete insulin in a manner irrespective of blood glucose levels (37). In fact, and as described previously (38), *Ldha* transcripts were undetectable in 5-week-old islets. The silencing in β-cells of *Hk1*, *Hk2*, and *Ldha*, which are termed disallowed genes, occurs immediately after birth and is linked to the acquisition of glucose-responsive insulin secretion (38). These studies indicated that Pdx1 was regulating a distinct later stage of β-cell maturation than the disallowed genes through its influence on factors like MafA (39) and Nkx6.1 (40).

Area IV May Be Autoregulated by Pdx1 During Weaning

Because the change in postweaning *Pdx1* expression and islet β-cell function was observed in *Pdx1*$^{\Delta AIV/-}$ (Fig. 1*B*) and not *Pdx1*$^{\Delta AIV/\Delta AIV}$ mice (Supplementary Fig. 1 and data not shown), we considered that Pdx1 self-regulates transcription through area IV postnatally. Differential binding of this transcription factor to areas I, II, III, or IV was analyzed in 3- and 5 week-old male wild-type islets. Notably, although Pdx1 is capable of binding within areas I and IV in ChIP assays (9,18), elevated binding levels were only observed for area IV (Fig. 3*B*) in 5-week-old (and not 3-week-old) wild-type islets. These results suggest that autoregulation through area IV is involved in potentiating the increased *Pdx1* levels found in β-cells at weaning (27).

Pdx1-Directed Transcription of Genes Crucial to Islet β-Cell Replication and Oxidative Phosphorylation Are Compromised in Weaning *Pdx1*$^{\Delta AIV/-}$ Mice

The process of weaning has significant consequences on the metabolic demands of an animal. For example, the switch from high-fat–enriched maternal milk to high-carbohydrate chow diet requires the β-cell to adapt to changes in nutrient uptake, glucose metabolism, insulin secretion levels, and proliferation state (27). Analysis of the islet β-cell transcriptome from weaned and earlier suckling mice led to the identification of ~1,600 differentially expressed genes, including many potentially involved in glucose-stimulated replication and increased mitochondrial oxygen consumption (27).

To identify genes regulated by Pdx1 during weaning, we first aligned the islet transcriptome data set of differentially expressed genes produced during weaning with endogenous Pdx1 generated ChIP binding data from murine β-cells. Interestingly, Pdx1 binding sites were found within 547 of the 1,597 annotated genes differentially expressed in weaned islets (Fig. 4*A* and Supplementary Table 1). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis led to the identification of functional networks associated with these Pdx1-bound genes. The most highly enriched genes were related to functional terms including metabolism, maturity-onset diabetes of the young, and protein-processing pathways (Fig. 4*B* and Supplementary Table 2). Significantly, Pdx1 levels even influenced the less-enriched KEGG-identified pathways in weaning mice. Thus, many Pdx1-bound genes required for replication (i.e., *Nasp*, *Bard1*, *Mnx1*, and *Mcm7*) and oxidative phosphorylation/mitochondrial function (*NdufA4L2*, *Etfa*, and *Uqcrc2*) were only reduced in 5-week-old *Pdx1*$^{\Delta AIV/-}$ islets, with little to no change in 3-week-old *Pdx1*$^{\Delta AIV/-}$ or *Pdx1*$^{+/-}$islets (Fig. 4*C*). Significantly, the importance of Pdx1 in controlling expression of genes essential to mitochondrial function had also been demonstrated by infecting islets with an adenovirus encoding a dominant-negative form of this transcription factor

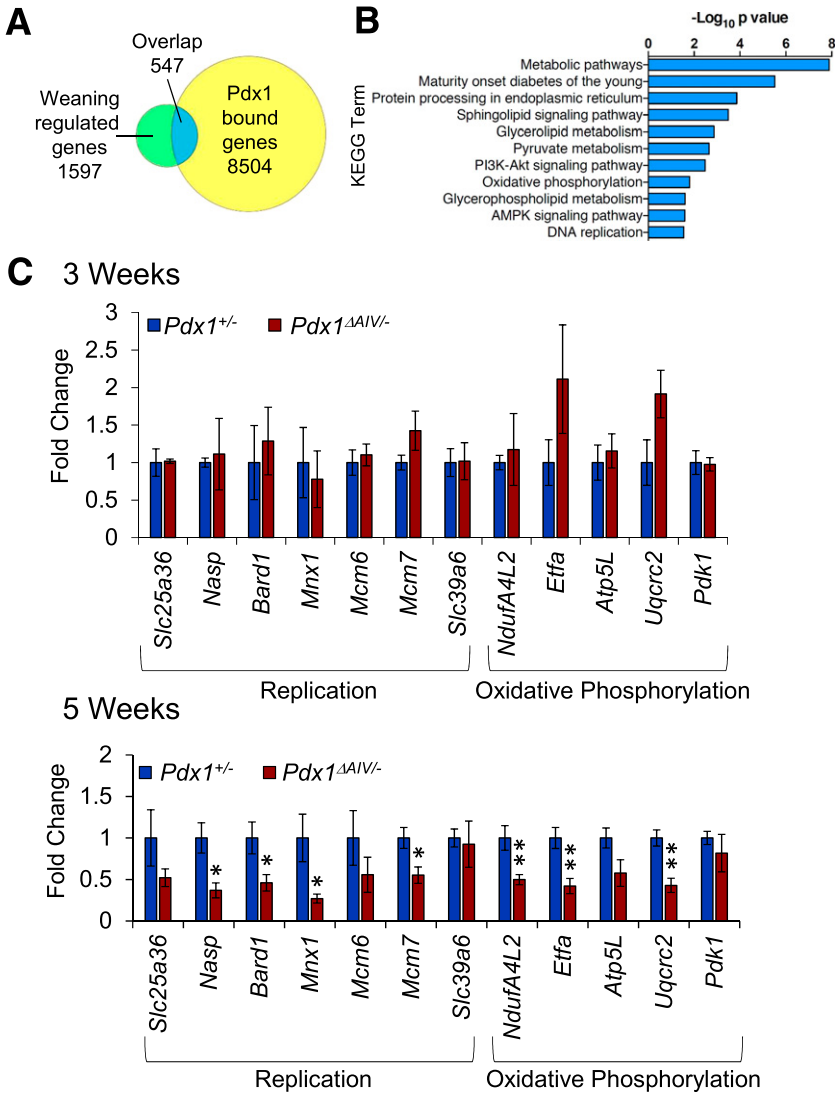

Figure 4—Pdx1 binding regulates pathways essential to β-cells during weaning. Pdx1 target genes were determined by annotating the 8,504 peaks from Pdx1 ChIP sequencing of mouse βTC-6 cells (26) (accession number GSE70960 [http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM1824087]) to the nearest reference sequencing gene of the 1,597 expressed differentially in weaning mouse islets (27) using Homer version 4.7.2 (28). *A*: Venn diagram illustrating the number of genes regulated during weaning that containing Pdx1 binding sites; the 547 Pdx1-bound genes in this category is provided in Supplementary Table 1. *B*: KEGG ontology pathway mapping of Pdx1-bound genes enriched in weaning mouse islets; these genes are listed in Supplementary Table 2. *C*: Many Pdx1-bound genes involved in β-cell replication and oxidative phosphorylation is uniquely decreased in 5 week-old $Pdx1^{\Delta AIV/-}$ islets. $n = 4$ to 5. $^{*}P < 0.05$; $^{**}P < 0.01$.

(41). Collectively, these studies revealed the critical nature of Pdx1 actions in controlling β-cell area and maturation during weaning.

DISCUSSION

The Pdx1 transcription factor is often viewed as a master transcriptional regulator within the pancreas because of its critical role in organ formation and many aspects of mature islet β-cell function, including insulin secretion, mitochondrial metabolism, cell proliferation, and survival (1,5,41,42). Consequently, understanding how Pdx1 levels and activity are controlled is of importance to efforts aimed at developing cell therapeutic reagents for diabetes treatment as well as for understanding disease ontogeny, which correlates with low PDX1 levels in T2D (43). In this study, we examined the influence of the conserved 5′-flanking area IV transcriptional control domain on endogenous Pdx1 expression and function during pancreas development and in islet β-cells. Endocrine cell formation was only marginally impacted in $Pdx1^{\Delta AIV/-}$ embryos, in stark contrast to $Pdx1^{\Delta AII/-}$ mutants, which had severe developmental deficiencies. We found the most profound effect on $Pdx1^{\Delta AIV/-}$ *Pdx1* expression was observed at weaning, but only in the males and never in male $Pdx1^{\Delta AIV/\Delta AIV}$ or female $Pdx1^{\Delta AIV/-}$ mutant mice. The reduction in Pdx1 mRNA and protein levels was accompanied by deficiencies in islet β-cell function, β-cell proliferation, and whole-body glucose homeostasis, effects directly related to the influence of this transcription factor

on weaning-regulated gene expression. These results reveal not only a novel period of postnatal Pdx1 significance, but also mechanistic distinctions in how transcription is controlled by the 5′-flanking area II and IV enhancers.

Our studies on area IV were originally motivated by the observed binding in vivo of many transcription factors that govern the earliest steps in pancreas development (14,15) and the distinct epigenetic marking of this region in relation to the *Pdx1* area I, II, and III enhancers in very early undifferentiated E8.25 endoderm progenitor cells (44). For example, the chromatin opening pioneering transcription factors FoxA1 and FoxA2 preferentially bind to area IV in relation to areas I or II in the embryonic pancreas, with conditional deletion of both in pancreatic primordia causing severe pancreatic hypoplasia because of deficiencies in *Pdx1* expression (15). However, we observed only modest defects in the number of embryonic Ngn3$^+$ cells or later produced endocrine β- or δ-cells in the *Pdx1*$^{\Delta AIV/-}$ mutant. Presumably, the specific effect on these islet cell types reflects a relatively late impact on embryonic *Ngn3* expression (45). The reduction in Ngn3$^+$ cell numbers also translated into a lower islet β-cell area in 3-week-old *Pdx1*$^{\Delta AIV/-}$ mice, which, notably, did not influence expression of key islet β-cell regulators or β-cell function. This observation differs significantly from either the early embryonic deletion of area I, II, and III in vivo (11) or the *Pdx1*$^{\Delta AII/-}$ mutant (20). Thus, severe defects in endocrine progenitor cell formation and insulin$^+$- and glucagon$^+$-coexpressing cells are found in *Pdx1*$^{\Delta AII/-}$ animals postnatally (20), phenotypes not observed in *Pdx1*$^{\Delta AIV/-}$ mutants. Collectively, our embryonic results strongly suggest that area IV has only a very limited impact on developmental regulation imposed by areas I, II, and III.

Interestingly, distinct differences in *Pdx1* control were observed between male and female *Pdx1*$^{\Delta AIV/-}$ mice, as only male mutant mice developed any abnormalities in glucose homeostasis, Pdx1 levels, or Pdx1 target gene expression. It is possible that female *Pdx1*$^{\Delta AIV/-}$ mice can also effectively autoregulate *Pdx1* transcription independently of area IV during the weaning transition. Presumably, this is through the promoter proximal area I enhancer (9,18), although a far distal upstream region found roughly −70- to −50-kilobase pairs from the *Pdx1* transcriptional start is also bound by Pdx1 and other islet cell–enriched transcription factors (46). Although the precise regulatory properties of this distal region are ill defined, *Pdx1* expression in β-cells appears to be controlled by a long noncoding RNA (i.e., PLUTO) binding within these sequences and areas I–IV (47).

The sexual dimorphism of the *Pdx1*$^{\Delta AIV/-}$ mutants could also reflect a differential responsiveness to circulating sex steroid testosterone and estrogen hormone levels. For example, testosterone signaling through the androgen receptor preferentially enhances glucose-stimulated insulin secretion in male mouse and human islets (48). In addition, the positive effects of estrogen signaling on β-cell function and survival in diabetes has been well established in both humans with diabetes and diabetic mouse models (49,50). In this regard, females have been shown to have greater insulin sensitivity and less incidence of T2D than men during ages with relatively high serum estrogen concentrations (e.g., between puberty and menopause) (51). Although neither androgen nor estrogen response elements were found within areas I–IV by in silico analysis, it will be of interest to determine how these sex hormones differentially effect Pdx1 expression at weaning.

Area IV activity could also be influenced by glucocorticoids produced during and upon changing the maternal environment (52). In addition, other distinct metabolic effectors expressed during this transition period may mediate the induced and selective binding of Pdx1 to area IV (Fig. 3*B*). For example, the biological activity of Pdx1 is tightly regulated at the posttranslational level by phosphorylation (53,54), glycosylation (55), and sumoylation (56). In particular, the switch from fat-enriched maternal milk to carbohydrate-rich chow may be affecting Pdx1 nuclear localization (57), DNA binding activity (58), and/or transcriptional cofactor recruitment (59).

Islet β-cell maturation can be separated into at least two distinct temporal stages. Within ∼2 weeks immediately following birth, β-cells acquire the ability to secrete insulin in response to glucose, which involves preventing the expression of a series of disallowed housekeeping genes that interfere with glucose sensing, such as *Hk1*, *Hk2*, and *Ldha* (37). The de novo DNA methyltransferase 3A is essential in silencing expression of these disallowed genes (38). Notably, there is no difference in *Hk1*, *Hk2* or *Ldha* regulation within 3- and 5-week-old *Pdx1*$^{\Delta AIV/-}$ islets (Fig. 3*A*). Further maturation of β-cells occurs at weaning and involves enhancement of glucose-stimulated oxidative phosphorylation and insulin secretion (27). Our results in this study demonstrate that Pdx1 is essential to this second phase, because many of the genes encoding proteins mediating these processes were significantly impacted in 5-week-old *Pdx1*$^{\Delta AIV/-}$ islets (Fig. 4). This included transcription factors essential for mature β-cell function (MafA [24,25] and Nkx6.1 [40]), as well as others necessary for glucose sensing (Glut2), mitochondrial function, and cell proliferation (Figs. 3 and 4). Presently, we have been unable to determine if there are temporal differences on expression of these effected genes in *Pdx1*$^{\Delta AIV/-}$ β-cells postnatally. However, *Pdk1* expression was unaffected under these conditions, a proposed negative regulator of mitochondrial function in weaning mice (27). Moreover, Pdx1 can also prevent transcription of genes within β-cell progenitors (11) and adult islet β-cells (5) that are essential to islet α activity, like *MafB* and *glucagon* (5). Significantly, the expression levels of these genes were unchanged in *Pdx1*$^{\Delta AIV/-}$ islets, demonstrating that different thresholds of Pdx1 are required for sustaining β-cell function and maintaining β-cell identity.

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Author Contributions. J.M.S., M.Gup., and R.S. developed the concept, designed and performed experiments, and performed data analysis. J.M.S. and R.S. wrote the manuscript. M.P., Y.-P.Y., H.C., S.G., J.-H.L., and M. Guo contributed to data acquisition and analysis. J.B. and C.W. performed manuscript editing. M.A.M. derived the *Pdx1*$^{\Delta A/V}$ allele and edited the manuscript. R.S. 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.

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