Title
Convergence of the Insulin and Serotonin Programs in the Pancreatic β-Cell

Journal
Diabetes, 60(12)

ISSN
0012-1797

Authors
Ohta, Yasuharu
Kosaka, Yasuhiro
Kishimoto, Nina
et al.

Publication Date
2011-12-01

DOI
10.2337/db10-1192

Peer reviewed

Convergence of the Insulin and Serotonin Programs in the Pancreatic β-Cell

Yasuharu Ohta,[1] Yasuhiro Kosaka,[1] Nina Kishimoto,[1] Juehu Wang,[1] Stuart B. Smith,[1] Gerard Honig,[2,3,4] Hail Kim,[1] Rosa M. Gasa,[1] Nicole Neubauer,[1] Angela Liou,[2,3] Laurence H. Tecott,[2,3] Evan S. Deneris,[5] and Michael S. German[1,6]

OBJECTIVE—Despite their origins in different germ layers, pancreatic islet cells share many common developmental features with neurons, especially serotonin-producing neurons in the hindbrain. Therefore, we tested whether these developmental parallels have functional consequences.

RESEARCH DESIGN AND METHODS—We used transcriptional profiling, immunohistochemistry, DNA-binding analyses, and mouse genetic models to assess the expression and function of key serotonergic genes in the pancreas.

RESULTS—We found that islet cells expressed the genes encoding all of the products necessary for synthesizing, packaging, and secreting serotonin, including both isoforms of the serotonin synthetic enzyme tryptophan hydroxylase and the archetypal serotonergic transcription factor Pet1. As in serotonergic neurons, Pet1 expression in islets required homeodomain transcription factor Nkx2.2 but not Nkx6.1. In β-cells, Pet1 bound to the serotonergic genes but also to a conserved insulin gene regulatory element. Mice lacking Pet1 displayed reduced insulin production and secretion and impaired glucose tolerance.

CONCLUSIONS—These studies demonstrate that a common transcriptional cascade drives the differentiation of β-cells and serotonergic neurons and imparts the shared ability to produce serotonin. The interrelated biology of these two cell types has important implications for the pathology and treatment of diabetes. ***Diabetes*** **60:3208–3216, 2011**

The shared ability of many neurons and endocrine cells, including the pancreatic islet cells, to import amine precursors, decarboxylate them, and concentrate the products, provided simple methods to stain these cells (1) and led some investigators to hypothesize that cells with this capacity (amine precursor uptake and decarboxylation [APUD]) shared a common embryonic origin (2). While the common origin hypothesis for APUD cells has since been disproved, our expanding knowledge of the function and global gene expression patterns of the neuroendocrine secretory cells has only further confirmed their remarkable similarities (3–5).

The similarities between neurons and β-cells have an evolutionary basis. The function of insulin has been conserved, but the cells producing insulin have evolved (6). Invertebrates express insulin predominantly in neurons, while in the chordates, insulin expression has shifted to visceral endocrine cells of endoderm origin—although even in mammals, low but detectable neural expression of insulin has persisted in the central nervous system (7). Furthermore, despite its migration to the pancreas, the vertebrate β-cell continues to communicate with the central nervous system, and the autonomic nervous system plays a critical role in the regulation of insulin secretion (8,9).

Not surprisingly, given their common functions and evolutionary connections, islet cells and neurons also share many similarities in their development (5). These parallels are particularly obvious for the serotonergic neurons of the brainstem and the pancreatic β-cells. The homeodomain transcription factors Lmx1b, Nkx2.2, and Nkx6.1 form part of transcriptional cascades required for the formation of both cell types (5,10). In these cascades, Nkx2.2 drives the expression of Nkx6.1 and the final differentiation of both cell types (11,12).

The characteristic products of these cells, serotonin and insulin, also play conserved and interrelated roles in energy metabolism (13). Because both cell types share the machinery required to sense changes in extracellular glucose concentration, glucose modulates the secretion of both products (14,15). Once secreted, both molecules serve as signals of energy sufficiency. In mammals, both serotonin and insulin activate the proopiomelanocortin-expressing neurons in the anorexogenic pathway in the hypothalamus (16–18). In addition, in worms, flies, and mice, central serotonin signaling regulates insulin secretion either directly or indirectly (19–21).

To determine how far the similarities of the pancreatic β-cells and the serotonergic neurons extend, we tested the two cell types for additional similarities in their gene expression program and function. We found that β-cells express all the key components of the serotonergic program and that the prototypical serotonergic transcription factor Pet1 is also involved in the synthesis of insulin in the β-cells.

From the [1]Diabetes Center, University of California, San Francisco, San Francisco, California; the [2]Department of Psychiatry, University of California, San Francisco, San Francisco, California; the [3]Center for Neurobiology and Psychiatry, University of California, San Francisco, San Francisco, California; the [4]Neuroscience Graduate Program, University of California, San Francisco, San Francisco, California; the [5]Department of Neurosciences, School of Medicine, Case Western Reserve University, Cleveland, Ohio; and the [6]Department of Medicine, University of California, San Francisco, San Francisco, California.

Corresponding author: Michael S. German, mgerman@diabetes.ucsf.edu.

Received 20 August 2010 and accepted 15 September 2011.

DOI: 10.2337/db10-1192

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db10-1192/-/DC1.

Y.O. is currently affiliated with the Division of Endocrinology, Metabolism, Hematological Sciences and Therapeutics, Department of Bio-Signal Analysis, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi, Japan. R.M.G. is currently affiliated with the Diabetes and Obesity Laboratory, Institut D'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS)-Hospital Clínic, University of Barcelona, Barcelona, Spain/CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM).

RESEARCH DESIGN AND METHODS

All studies involving mice were approved by the University of California, San Francisco, Institutional Animal Care and Use Committee. Mice were housed on a 12-h light-dark cycle in a controlled climate. Midday of the day of vaginal plug discovery was set as embryonic day 0.5.

The *Fev*$^{-/-}$ (22), *Neurog3*$^{-/-}$ (23), *Nkx2.2*$^{-/-}$ (24), *Nkx6.1*$^{-/-}$ (11), *ePet-cre* (25), *pdx1-cre* (26), *R26R* (27), and *Z/EG* (28) mice have previously been described. All mice were maintained in the C57BL6 background.

Glucose and insulin tolerance tests were performed on fasting male mice injected intraperitoneally with glucose (2 g/kg) or insulin (0.75 units/kg). Blood glucose levels were measured from tail vein blood using the FreeStyle glucometer (Abbott Diabetes Care, Alameda, CA). Insulin was assayed using the Ultra Sensitive Rat Insulin ELISA kit (Crystal Chem, Downers Grove, IL) in serum from blood obtained from retroorbital plexus at 0 and 30 min after glucose injection.

Cell culture and transfection. Culture and transfection of mouse pancreatic ductal carcinoma (mPAC) cells and mouse βTC3, αTC1.9, and NIH3T3 cells were performed as previously described (29). The Min6 β-cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 15% FBS, 100 units/mL penicillin, 100 g/mL streptomycin, and 71.5 μmol/L β-mercaptoethanol. For adenovirus experiments, mPAC cells were infected at a multiplicity of infection of 50:1 with adenoviruses encoding the basic helix-loop-helix (bHLH) transcription factors or control adenovirus encoding β-galactosidase and cultured for 2 days prior to assay for *Fev* mRNA.

Mouse pancreatic islets were isolated by collagenase digestion and handpicked (30). Isolated islets were incubated overnight in RPMI 1640 medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Prior to secretion studies, media were replaced with the additives indicated and collected after 1 hour for insulin assays using the Ultra Sensitive Rat Insulin ELISA kit. Protein measurement by Western blot was performed as previously described (29).

Histological analyses. Harvesting and processing of embryonic and adult mouse pancreatic tissues were performed as previously described (29). For adult brain sections, mice were perfused with saline and then 4% paraformaldehyde in PBS; brains were removed and cryoprotected in 30% sucrose in PBS and sectioned (50 μm) on a freezing microtome. Immunostaining was performed overnight at 4°C in PBS containing 1% goat serum (pancreatic tissues) or 4% goat serum, 2% BSA, and 0.1% Triton X-100 in PBS (brain tissues) (primary and secondary antisera listed in Supplementary Table 1). Slides were imaged on a Zeiss Axioskop Microscope or on a Leica TCS SL Confocal Microscope.

In situ hybridization. For RNA in situ hybridization analysis of paraffin sections (5 μm), adult mouse pancreas was processed, sectioned, and hybridized with digoxigenin-labeled riboprobes as previously described (31). Digoxigenin-labeled sense and antisense riboprobes were detected with alkaline phosphatase–coupled antidigoxigenin antibodies using BM purple (Boehringer Mannheim) as color substrate. For fluorescent in situ hybridization/immunohistochemistry analysis, the slides were incubated with fluorescein isothiocyanate–conjugated anti-digoxigenin antibodies (Roche) followed by immunohistochemistry. The *Fev* sense probe gave no significant signal on mouse pancreas or brain.

RT-PCR. Total RNA was isolated from tissues and cell lines with the RNeasy kit (Qiagen, Valencia, CA) and treated with Turbo DNase (Ambion, Austin, TX). cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). For conventional RT-PCR (Fig. 3*A*), 25 ng was used per PCR reaction for 35 cycles for *Fev* using standard conditions. Real-time PCR assays were performed as previously described (32). Levels of assayed mRNAs were normalized to expression levels of mouse β-glucuronidase or β-actin. All oligonucleotide sequences are available on request.

Electromobility shift assays. Single-stranded oligonucleotides were 5′-end labeled using (γ-^{32}P)ATP and T4 polynucleotide kinase, annealed to excess complementary strand, and column purified. Electromobility shift assay (EMSA) buffers and electrophoresis conditions were as previously described (29), using 500 ng poly(dIdC):poly(dIdC) per 10 μL binding mix. Protein was generated in vitro with the TNT Coupled Reticulocyte Lysate System (Promega) in 50 μL total volume from 1 μg DNA; 1 μL (~5 ng protein) of the reaction mix was then used per binding reaction. Sequences for the coding strands are shown in Fig. 5*A*.

Chromatin immunoprecipitation assays. βTC3 cells were transfected with the pBAT16.Pet1-FLAG vector or the control pBAT16 vector with no insert. pBAT16.Pet1-FLAG contains the mouse *Fev* cDNA linked in frame at the 3′end to the coding sequence for the FLAG peptide tag and inserted downstream of the cytomegalovirus immediate early promoter and the first intron of the human β-globin gene. Forty-eight hours after transfection, cells were formaldehyde fixed, washed, and lysed; DNA was purified and sheared; and Pet1-DNA complexes were precipitated as previously described (29) with anti-FLAG antibody. Approximately 10 ng immunoprecipitated DNA per reaction was assayed by PCR for specific promoter fragments.

RESULTS

Serotonergic genes in the islet. To test for the presence of the serotonergic program in the pancreatic islet, we first measured the levels of the mRNAs encoding tryptophan hydroxylases TPH1 and TPH2, the two isoforms of the TPH enzyme that catalyze the initial and committed step in serotonin synthesis. The brain uses both isoforms, while nonneuronal tissues such as the gut express predominantly TPH1 (33). We could detect both mRNAs in the embryonic mouse pancreas, with a peak in both at embryonic day 18.5 (E18.5), shortly before birth (Fig. 1*A*). Both mRNAs persisted in the adult islet and could be detected at similar levels in the β-cell line βTC3 (Supplementary Table 2).

Islet expression was confirmed and localized by immunohistochemistry, which detected serotonin as early as E9.5 in a few glucagon-positive cells in the dorsal bud but not in the ventral bud (Supplementary Fig. 1*A*–*G*). Serotonin expression was detected in most of the glucagon-expressing cells at E12.5 (Supplementary Fig. 1*H*–*O*). At E14.5, most of the glucagon-expressing cells coexpressed serotonin (Supplementary Fig. 2*A*–*D*), but few of the insulin-expressing cells (Supplementary Fig. 2*E*–*H*) and none of the neurogenin3-expressing islet progenitor cells (Supplementary Fig. 2*I*–*L*) stained for serotonin. By E18.5, however, all insulin-expressing cells robustly stained for TPH and serotonin (Fig. 1*B* and Supplementary Fig. 3), supporting the conclusion that the peripartum peak in *Tph1* mRNA levels (Fig. 1*A*) predominantly results from induction in β-cells. Isoform-specific antisera (34) detected both TPH1 and TPH2 by immunohistochemistry (Fig. 1*C*) and by Western blot (Fig. 1*D*) in adult mouse islets.

Embryonic pancreas, adult islets, and the islet cell lines also expressed the mRNAs encoding all of the remaining proteins required for completing the synthesis and packaging of serotonin: aromatic L-amino acid decarboxylase (dopamine decarboxylase, gene name *Ddc*), vesicular monoamine transporter (VMAT)2 (Slc18a2), and the serotonin reuptake transporter (Slc6a4) (Supplementary Table 2). These pancreatic cells also expressed mRNA encoding one of the serotonin autoreceptors found on serotonergic neurons, Htr1b, but not mRNA encoding a second closely related autoreceptor, Htr1a.

Cells in the pineal gland convert serotonin to melatonin via a two-step process in which the enzyme arylalkylamine *N*-acetyltransferase (AANAT) catalyzes the acetylation of serotonin to *N*-acetylserotonin, which is then converted to melatonin by acetylserotonin *O*-methytransferase. Because most inbred laboratory mouse strains carry a mutation in AANAT (35), we did not attempt to measure melatonin in mouse islets. Instead, we tested for *AANAT* mRNA in human islets but did not detect any significant amount, although *TPH1* and *TPH2* mRNA were readily detected (data not shown).

Expression of Pet1 in the pancreas. We next tested mice for the expression of the serotonergic E-twenty-six (ETS) transcription factor Pet1 (gene name *Fev*) (36), which is also expressed in some endocrine cells in the gut (37). Quantitative analysis by real-time RT-PCR revealed a peak at E14.5 in the expression of the mRNA encoding Pet1 in the embryonic pancreas and persistent robust expression in the adult islet (Fig. 2*A*). In situ hybridization localized *Pet1* mRNA in the central regions of the embryonic pancreas at E14.5 (Fig. 2*B*), as well as in the serotonergic nuclei in the brainstem at E12.5 (Fig. 2*C*).

In the absence of antisera useful for immunohistochemical analysis of Pet1 expression, we used a line of transgenic mice in which 40 kb of genomic DNA from the region upstream of the Pet1 coding sequence controls the expression of the *cre* recombinase (*ePet-cre* mice) (25).

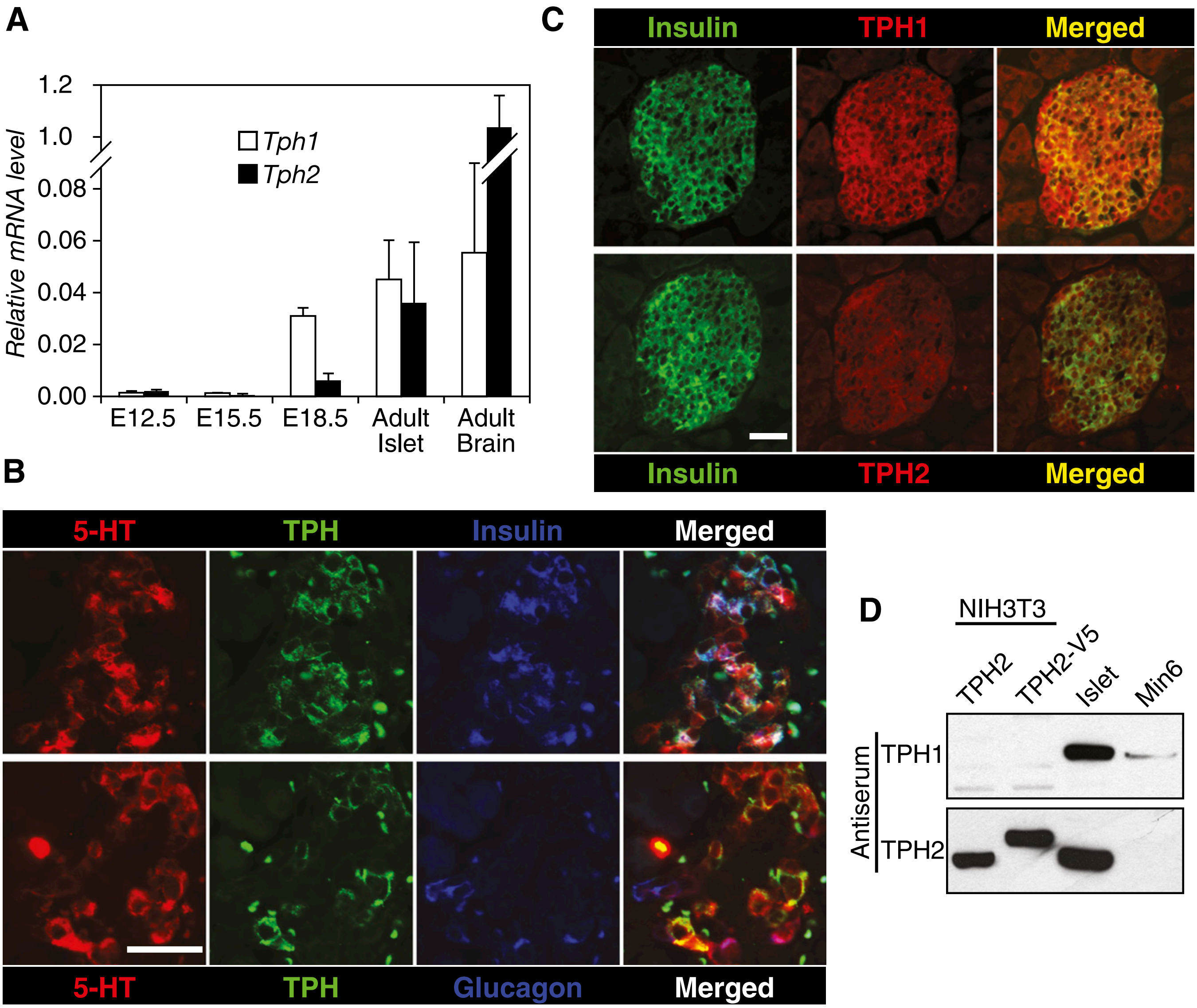

FIG. 1. Expression of TPH and serotonin in the pancreas. *A*: The relative expression levels of mRNA encoding the two TPHs, *Tph1* and *Tph2*, were quantified by real-time PCR from RNA isolated from the pancreas of mouse embryos at the ages shown and from adult islets and brain. *B*: Immunofluorescence staining was performed for serotonin (5-hydroxytryptamine [5-HT], red), TPH (using antiserum recognizing both TPH1 and TPH2 [green]) and insulin (blue) or glucagon (blue) in pancreas harvested from mouse embryos at E18.5. *C*: Antisera specific to TPH1 or TPH2 (red) were used to costain the adult pancreas along with insulin (green). *D*: The isoform-specific antisera to TPH1 and TPH2 were used in a Western blot of protein extracts of NIH3T3 cells expressing mouse TPH2 (*lane 1*) or TPH2 fused to the V5 tag from simian virus 5 (*lane 2*), adult mouse islets (*lane 3*), or Min6 mouse insulinoma cells (*lane 4*). All data points represent means ± SEM of at least three independent experiments. Scale bars, 25 μm. Additional staining is shown in Supplementary Fig. 1. (A high-quality digital representation of this figure is available in the online issue.)

When crossed with mice carrying the marker gene *ROSA26 loxP-stop-loxP lacZ* (*R26R*) (27), *cre* recombinase marks Pet-1–expressing cells and their descendents with β-galactosidase activity. We found that all insulin and most glucagon-expressing cells contained β-galactosidase in double transgenic embryos (Fig. 2*D–F*). At E14.5, all or almost all β-galactosidase$^+$ cells also expressed the homeodomain transcription factors Isl1 (Fig. 2*J*), Nkx2.2 (Fig. 2*K*), and Pax6 (Fig. 2*L*) and many also expressed pancreatic-duodenal homeobox (Pdx)1 (Fig. 2*G–I*), HB9, and Nkx6.1 (data not shown), but no cells coexpressed β-galactosidase and the proendocrine bHLH transcription factor neurogenin3 (Fig. 2*J–L*). In addition, fluorescent in situ hybridization at E14.5 in the pancreas also localized *Pet1* mRNA to the expression domain of Nkx2.2 (Supplementary Fig. 4*A–C*). In the adult pancreas, β-galactosidase was restricted to islet cells (Supplementary Fig. 5*A–H*). These data are consistent with the expression of Pet1 specifically in the endocrine lineage in the pancreas.

Interestingly, expression from the gene encoding the prototypical pancreatic transcription factor Pdx1 has also been reported in the brain (38). Using a strategy similar to that for tracing cells expressing the *ePet-cre* allele, we found that a Pdx1-cre allele (26) specifically marked serotonergic neurons in the brainstem (39) (Supplementary Fig. 5*I–N*) as well as pancreatic lineages.

Regulation of Pet1 expression in the pancreas. To identify determinants of Pet1 expression in the developing pancreas, we started by testing the role of the bHLH transcription factor neurogenin3, which initiates the differentiation of islet cells from pancreatic progenitor cells but is only expressed transiently. Consistent with that

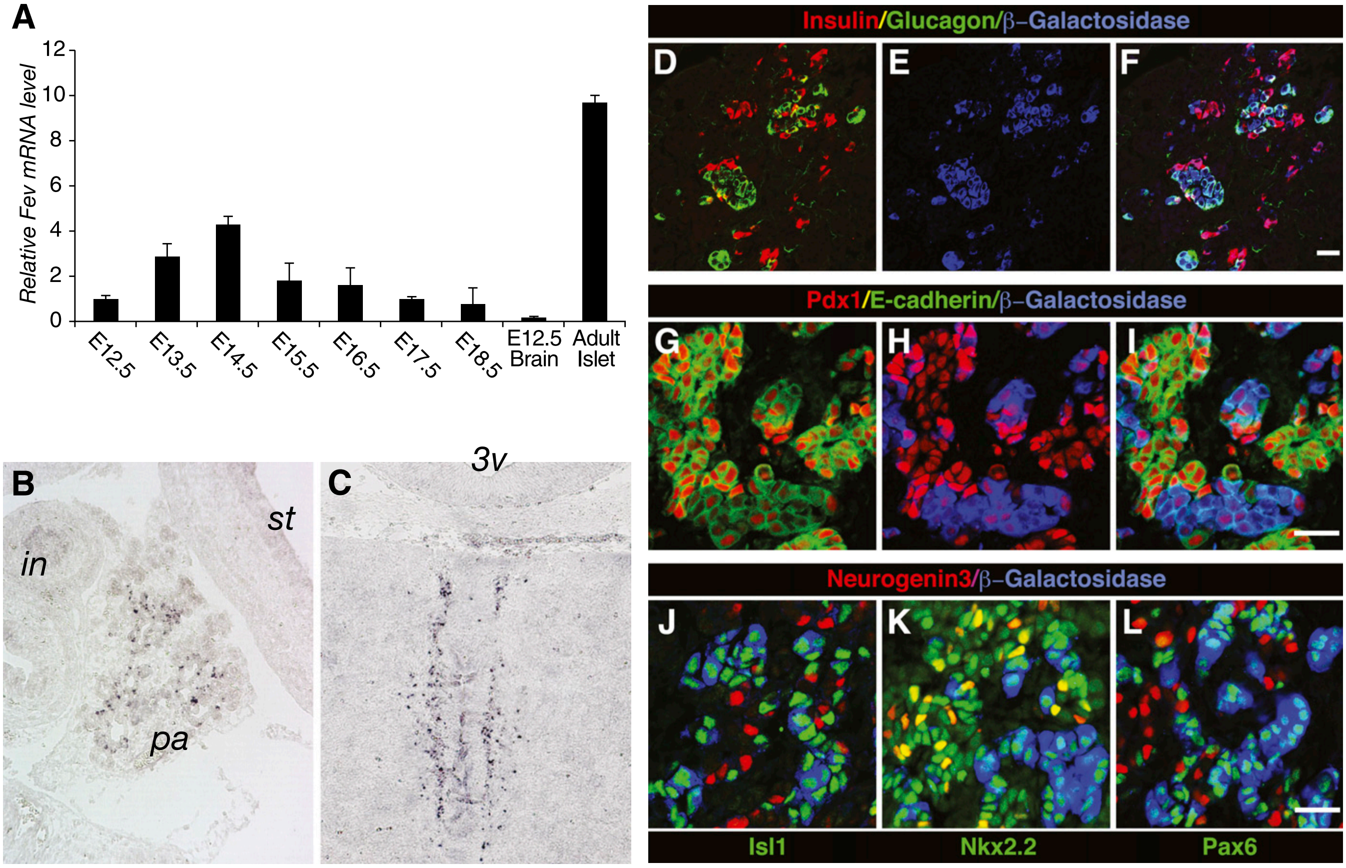

FIG. 2. Expression of serotonergic transcription factor Pet1 in the pancreas. *A*: Levels of *Fev* mRNA (encoding Pet1) were quantified by real-time PCR from RNA isolated from the pancreas and brain of mouse embryos at the ages shown and from adult islets. All data points represent means ± SEM of at least four independent experiments. *B* and *C*: In situ hybridization was performed for *Fev* in pancreas at E14.5 (*B*) and brainstem at E12.5 (*C*) from mouse embryos. *D–L*: Immunofluorescence staining was performed for β-galactosidase (blue) in pancreas from *ePet1-cre/R26R* mouse embryos at E14.5. Separate color channels are shown for red and green (*D* and *G*), red and blue (*H*), and blue (*E*). In *D* and *F*, most cells staining with insulin (red) and glucagon (green) costain for β-galactosidase. In *G–I*, a subset of cells staining for Pdx1 (red) and E-cadherin (green) costain for β-galactosidase. The β-galactosidase antiserum also costains cells with nuclear staining for Nkx2.2 (green [*K*]), Isl1 (green [*J*]), and Pax6 (green [*L*]) but not Neurog3 (red [*J–L*]). Scale bars, 25 μm. in, intestine; pa, pancreas; st, stomach; 3v, 3rd ventricle. Additional lineage tracing images are shown in Supplementary Fig. 5. (A high-quality digital representation of this figure is available in the online issue.)

transient expression, fluorescent in situ hybridization at E14.5 localized *Fev* mRNA in occasional cells expressing neurogenin3 (Supplementary Fig. 4*D–F*), while *ePet1-cre*–mediated recombination (which takes longer to yield detectable expression) did not (Fig. 2*J–L*).

Forced expression of neurogenin3 via adenovirus in the pancreatic progenitor cell line mPAC induced *Fev* mRNA transcription (Fig. 3*A* and *B*). Interestingly, *Fev* is the transcription factor gene most strongly induced in the neurogenin3-expressing progenitor cells in vivo (32). In addition, mouse embryos lacking neurogenin3 fail to express *Fev* mRNA in the developing pancreas (Fig. 3*C*). *Fev* mRNA similarly depends on Nkx2.2 (Fig. 3*D*) but not Nkx6.1 (Fig. 3*E*) or Lmx1b (K. Yang, Y.O., and M.S.G., unpublished data).

Fev$^{-/-}$ mice. To test the function of Pet1 in the islet, we examined mice homozygous for a targeted disruption of the *Fev* gene (22). At E18.5, pancreas size and both gross and histological appearances of the pancreata in the *Fev*$^{-/-}$ embryos were indistinguishable from their heterozygous and wild-type littermates. Staining for islet hormones did not detect any differences in the organization or size of the islets or in the numbers of the various islet cell types (Supplementary Fig. 6).

Levels of the mRNAs encoding glucagon, somatostatin, pancreatic polypeptide, and ghrelin were not significantly changed in the *Fev*$^{-/-}$ embryos, but the mRNAs encoding the β-cell hormones insulin (*Ins1* and *Ins2*) and islet amyloid polypeptide (*Iapp*) were significantly reduced (Fig. 4*A*). Consistent with these data, insulin content was also reduced in the pancreas of *Fev*$^{-/-}$ embryos (Fig. 4*B*).

Glucose metabolism in *Fev*$^{-/-}$ mice. As previously described, *Fev*$^{-/-}$ mice can reach adulthood without any apparent abnormalities except for behavioral phenotypes consistent with anxiety and depression (22). The weights of the Pet1-null animals and their wild-type littermates did not significantly differ throughout postnatal development and adulthood (Fig. 4*C* and data not shown). To assess glucose metabolism more closely, we performed intraperitoneal glucose tolerance tests at 12 weeks after birth. The adult Pet1-null animals cleared the glucose load significantly more slowly than their littermates (Fig. 4*D*). This defect was not due to decreased insulin sensitivity, since glucose levels fell as rapidly in mutant as in wild-type animals in response to injected insulin (insulin tolerance test [Fig. 4*E*]).

Instead, the Pet1-null animals displayed a defect in insulin secretion. At 30 min into the glucose tolerance test, serum insulin levels in the Pet1-null animals rose to approximately one-half the level in the wild-type littermate controls (Fig. 4*F*), despite the higher glucose levels (Fig. 4*D*). Islets isolated from the adult Fev$^{-/-}$ animals also

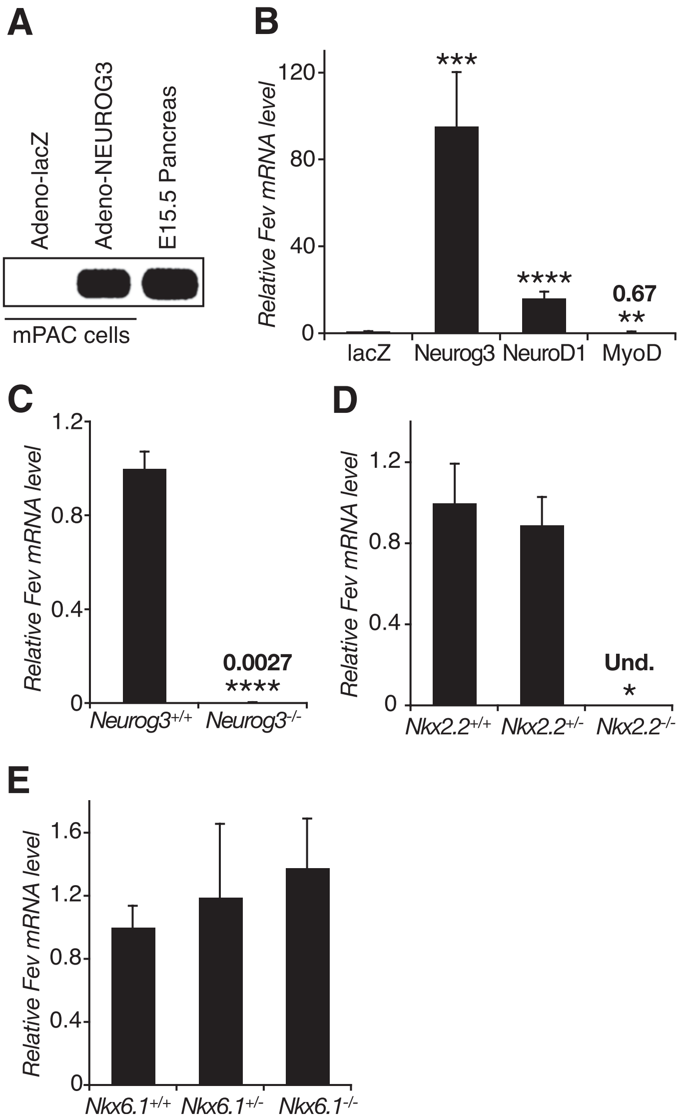

FIG. 3. Regulation of Pet1 in the pancreas. *A*: The mRNA encoding Pet1 (*Fev*) was amplified by RT-PCR from RNA isolated from mPAC cells infected with adenovirus expressing β-galactosidase (Adeno-LacZ) or human neurogenin3 (Adeno-NEUROG3) and from mouse E15.5 embryonic pancreas. *B*: The relative expression levels of *Fev* mRNA were quantified by real-time PCR from RNA isolated from mPAC cells infected with adenovirus expressing the proteins shown. *C–E*: The relative expression levels of *Fev* mRNA were quantified by real-time PCR with pancreas RNA isolated at E18.5 from mouse embryos with targeted deletions of *Neurog3* (*C*), *Nkx2.2* (*D*), and *Nkx6.1* (*E*). All data points represent means ± SEM of at least three independent experiments. *P < 0.05 vs. heterozygote and vs. wild type (*D*); **P < 0.01, ***P < 0.001, and ****P < 0.0001 vs. cells infected with adenovirus expressing β-galactosidase (*B*); and ****P < 0.0001 vs. wild type (*C*) by two-tailed Student t test. Numerical values of expression level relative to control are shown above lowest expressing samples. Und., undetected. In situ hybridization studies for *Fev* with immunofluorescent staining for Nkx2.2 and Neurog3 are shown in Supplementary Fig. 4.

demonstrated a defect in insulin secretion in vitro (Fig. 4*G*). These defects in insulin secretion correlated with a decrease in pancreatic insulin content (Fig. 4*H*) in the *Fev*-null animals but not with any deficit in β-cell mass, which was actually increased in the null animals tested (Fig. 4*I*), although the increase did not reach statistical significance. Therefore, insulin gene expression and insulin production per β-cell are substantially reduced in the absence of Pet1 in both fetal and adult mice.

To explore the possibility that Pet1 directly targets the insulin genes, we examined the insulin promoters. The sequences of the rodent and human insulin gene promoters contain several potential binding sites for Pet1 with the consensus core sequence GGAA (40) (Fig. 5*A*). In an EMSA, in vitro produced Pet1 protein bound to a labeled oligonucleotide containing the two most proximal GGAA elements (located at −143 and –132 bp) (Fig. 5*B–D*), and binding with mutant probes demonstrated that Pet1 bound to the more proximal of the two sites (−132 bp) with the highest affinity (Fig. 5*D*). This site, previously called GG-I (41), is conserved in the human and rodent genes and lies adjacent to binding sites for Nkx2.2 and v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA) (5). Pet1 binding to this region of the mouse insulin promoter was also confirmed in intact β-cells by chromatin immunoprecipitation (Fig. 5*E*). Finally, Pet1 activated the human insulin gene promoter linked to the luciferase reporter gene when expressed in the pancreatic ductal cell line mPAC L20 (Fig. 5*F*).

Gene expression in *Fev*$^{-/-}$ mice. To determine whether Pet1 targets other β-cell genes, we looked at the expression of the genes encoding key components in glucose sensing in the β-cell. Levels of the mRNAs encoding the sulfonyl receptor, ATP-sensitive K^+ channel and glucokinase (*Abcc8*, *Kcnj11*, and *Gck*, respectively), were not reduced in the *Fev*$^{-/-}$ embryos at E17.5, but *Slc2a2* mRNA encoding Glut2 was reduced (Fig. 6*A*). Pet1 bound to both the *Gck* and *Slc2a2* genes in β-cells as assessed by chromatin immunoprecipitation (Fig. 6*C*).

We also tested for changes in the expression of serotonergic genes in the pancreas of the *Fev*$^{-/-}$ mice at E18.5. Surprisingly, we saw no reduction in the levels of any of the serotonergic mRNAs at E18.5 (Fig. 6*B*), despite previous evidence of their regulation by Pet1 in the brainstem (22,36); this result was confirmed by the normal serotonin immunoreactivity in the *Fev*$^{-/-}$ pancreas at E18.5 (Supplementary Fig 7). However, in support of previous in vitro binding studies (36), Pet1 did bind to the 5′ flanking region of each of these genes in intact β-cells as assessed by chromatin immunoprecipitation (Fig. 6*C*). In contrast, the expression of *Tph2*, but not the other serotonergic genes, was reduced in *Nkx2.2*$^{-/-}$ and *Nkx6.1*$^{-/-}$ embryos (Fig. 6*E* and *F*).

Finally, we measured the expression of genes encoding multiple islet transcription factors, including many of those implicated in insulin gene expression. The only factor significantly reduced (albeit modestly) in expression in the pancreas at E18.5 in *Fev*$^{-/-}$ embryos was *Lmx1b* (Fig. 6*D*), which also plays a role in serotonergic neuron development downstream of Nkx2.2 (10). Expression of *Lmx1b* also depended on Nkx2.2 in the pancreas (Fig. 6*E*).

DISCUSSION

Similarities in the developmental programs that drive the differentiation of the serotonergic neurons and pancreatic β-cells led us to examine whether the β-cells can also produce serotonin. We found that the β-cells, as well as some other islet cell types, express all of the genes required to synthesize, package, and secrete serotonin. β-Cells express both isoforms of TPH, the enzyme that catalyzes the rate-limiting step in serotonin synthesis: hydroxylation of tryptophan to 5-hydroxytryptophan. Expression of TPH1 peaks in the neonatal period in the islet. In addition, β-cells express dopamine decarboxylase, the enzyme that catalyzes the next (final) step in serotonin synthesis: decarboxylation of 5-hydroxytryptophan to 5-hydroxytryptamine

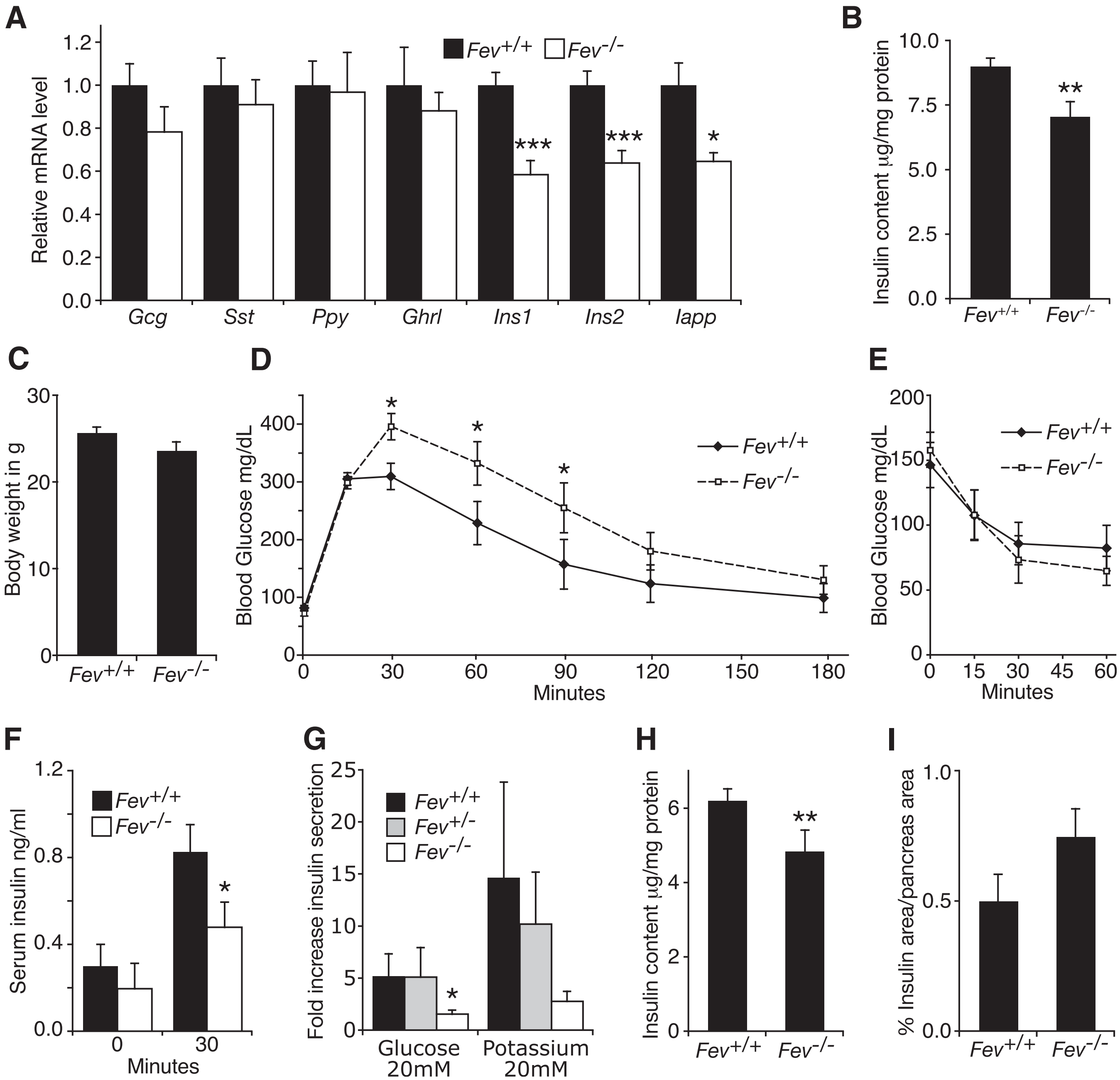

FIG. 4. Glucose metabolism in $Fev^{-/-}$ mice. *A*: The relative expression levels of the mRNA shown were quantified by real-time PCR from RNA isolated from pancreata from $Fev^{+/+}$ and $Fev^{-/-}$ mouse embryos at E18.5. *B*: Insulin content was measured in micrograms per milligram of total protein from pancreata harvested from mouse embryos with the genotypes shown at E18.5. *C*: Body weights are shown for 13-week-old adult male mice with the genotypes shown. *D* and *F*: Intraperitoneal glucose tolerance tests were performed by injecting 2 g/kg body wt glucose i.p. at time 0 followed by blood glucose measurements at the times shown in *D* in $Fev^{+/+}$ and $Fev^{-/-}$ mice and serum insulin levels at the times shown in *F* in 13-week-old male $Fev^{+/+}$ and $Fev^{-/-}$ mice. *E*: Insulin tolerance test was performed by injection of 0.75 units/kg body wt regular insulin i.p. at time 0 followed by blood glucose measurements at the times shown in *D* in 13-week-old male $Fev^{+/+}$ and $Fev^{-/-}$ mice. *G*: Insulin secretion over 1 h was measured from isolated islets from $Fev^{+/+}$, $Fev^{+/-}$, and $Fev^{-/-}$ mice. Data are expressed as the ratio between islets cultured at 2 and 20 mmol/L glucose or at 5 and 30 mmol/L KCl as indicated. *H*: Insulin content was measured in micrograms per milligram of total protein from pancreata harvested from mice with the genotypes shown. *I*: β-Cell area was measured as the percent of total pancreas area staining for insulin in pancreatic sections from 24-week-old male mice with the genotypes shown. All data points represent means ± SEM of at least three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. wild type in *A*, *B*, *D*, *F*, and *H*; **P* < 0.05 vs. combined wild type and heterozygotes in *G* by two-tailed Student *t* test. Immunofluorescent staining for islet hormones in $Fev^{+/+}$ and $Fev^{-/-}$ embryos at E18.5 is shown in Supplementary Fig. 6.

(serotonin). β-Cells also express VMAT2, the transporter that loads serotonin into secretory vesicles. Interestingly, islet cells express very low levels or none of the synapse-related serotonergic genes *SLC6A4* and *Htr1a*.

The expression of dopamine decarboxylase and the VMATs gives islet cells the ability to decarboxylate and store monoamine precursors and thus the APUD phenotype described almost 50 years ago (1,2). This capacity to take up and store serotonin has been exploited by using serotonin as a surrogate for insulin secretion (42) and by using ligands of VMAT2 for imaging β-cell mass in vivo (43). Monoamine uptake and storage are characteristics shared by many neuroendocrine cells, but since the islet cells also have TPH activity, they can specifically synthesize, store, and secrete serotonin.

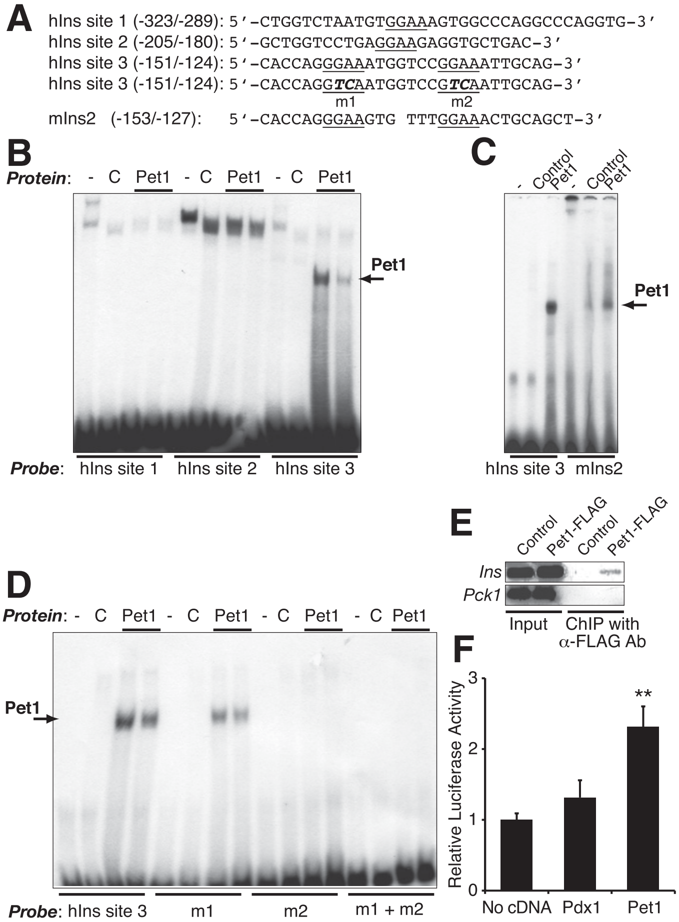

FIG. 5. Pet1 binds to and activates insulin gene promoters. ***A*****: The sequences of oligonucleotides from the human *INS* (hIns) and mouse *Ins2* (mIns) gene promoters are shown with potential Pet1 binding sites underlined. Sequences of mutations in two of these sites (m1 and m2) are shown in boldface. *B* and *D*: Double-stranded, radiolabeled oligonucleotides from *A* were used to test DNA binding of in vitro translated proteins by EMSA. The proteins used (in vitro translation extract alone [-], luciferase [C or control], or Pet1) are shown at the top of each lane, and the radiolabeled DNA probes used are shown at the bottom. *E*: DNA binding by Pet1 was tested in intact cells by chromatin immunoprecipitation (ChIP). βTC3 cells were transfected with an expression plasmid with no insert (control) or the Pet1 coding sequence fused to the FLAG epitope (Pet1-FLAG). Formaldehyde–cross-linked DNA was precipitated with α-FLAG antibody (Ab), and sequences from the phosphoenolpyruvate carboxykinase 1 *(Pck1)* and both of the Insulin1 and Insulin2 *(Ins)* gene promoters were amplified by PCR. *F*: Mouse pancreatic ductal mPAC L20 cells were cotransfected with a DNA plasmid containing the −362 bp human insulin promoter driving the expression of luciferase and another expressing the cDNAs shown, and luciferase activity was assayed and expressed relative to the activation of the promoterless luciferase vector. **P = 0.0015 vs. no cDNA by two-tailed Student t test.**

The peak in *Tph1* expression in islets that occurs during the perinatal period may provide one explanation for the variability in islet serotonin content seen in prior studies. We and others have also found that islet serotonin content is higher in females and during pregnancy (44–46). This variability in serotonin production by islets demonstrates a form of physiological regulation and suggests a function for islet serotonin. Given the much higher aggregate production and secretion of serotonin by the gut compared with that of the pancreatic islets, it seems unlikely that serotonin produced by the islet contributes substantially to systemic serotonin levels; but secretion by islet cells will primarily impact local concentrations and therefore could have autocrine or paracrine effects within the islet during the perinatal period and pregnancy analogous to the local effects of serotonin in the breast (47). During pregnancy, the high levels of serotonin drive β-cell proliferation (46). Since perinatal β-cells also rapidly proliferate (48,49), serotonin may have similar functions in pregnant and perinatal β-cells.

We also found that β-cells and other islet cells express the serotonergic transcription factor Pet1/Fev. In the pancreas, expression of *Fev* depends on the proendocrine transcription factor neurogenin3: neurogenin3 induced *Fev* expression in vitro, pancreatic *Fev* expression was lost in *Neurog3*$^{-/-}$ embryos, and as we have previously described, *Fev* expression is high in the transient neurogenin3-positive endocrine precursor cells during pancreatic development (32). This neurogenin3 dependence, together with the in situ hybridization and lineage tracing data, demonstrates that Pet1 is expressed specifically in the islet lineage. In addition, as in the serotonergic neurons (10), *Fev* expression in the pancreas requires the downstream target of neurogenin3, Nkx2.2, but not Nkx6.1 (itself regulated by Nkx2.2 [24]). Our data from the *Fev*$^{-/-}$ animals show, however, that none of these islet transcription factors depend on Pet1, thus placing Pet1 at the bottom of this cascade of transcription factors, as it is in the serotonergic neurons as well (10).

In serotonergic neurons, Pet1 drives the expression of the final differentiation products that characterize the mature cells, such as serotonergic genes *Tph2* and *Slc6a4* (22). Surprisingly, in the pancreas Pet1 was not necessary for *Tph2* expression, even though we found that it bound to *Tph2* and other serotonergic genes. In contrast, *Tph2* expression in the pancreas did depend on Nkx2.2 and Nkx6.1, as it does in serotonergic neurons (10).

Instead, our data demonstrate that in the pancreas Pet1 regulates the expression of genes encoding key differentiated β-cell products, including the glucose transporter gene *Slc2a2*, *Iapp*, and both insulin genes. As a result, the *Fev*$^{-/-}$ animals had defects in insulin production and secretion and impaired glucose clearance, despite compensatory increases in β-cell mass. Therefore, at the end of the transcription factor cascade, Pet1 guides the final differentiation and maturation of both serotonergic neurons and β-cells but does so by regulating overlapping but distinct sets of genes. It would be interesting to learn what role Pet1 may play in the expression of the β-cell glucose-sensing genes that are expressed in serotonergic neurons (14,15).

The developmental and functional parallels between the serotonergic neurons in the brain and the β-cells in the pancreas may have important practical implications. It must be kept in mind that pharmacological or genetic manipulations targeting the serotonergic neurons may inadvertently impact the β-cells as well—and vice versa. For example, transgenic strategies using regulatory elements from the *Fev* or *Pdx1* genes to target either cell type will likely target both cell types. Since both β-cells and serotonergic neurons regulate glucose metabolism, this genetic overlap may confound studies of energy homeostasis in mouse models using these genes for targeting. In addition, methods developed for generating these cells from stem cells or other sources must be assessed carefully, since the overlaps in gene expression profiles may lead to the misidentification of the generated cells.

Epidemiologists have long recognized an association between the risks of type 2 diabetes and depression (50). Manifold causes likely contribute to this clinical association,

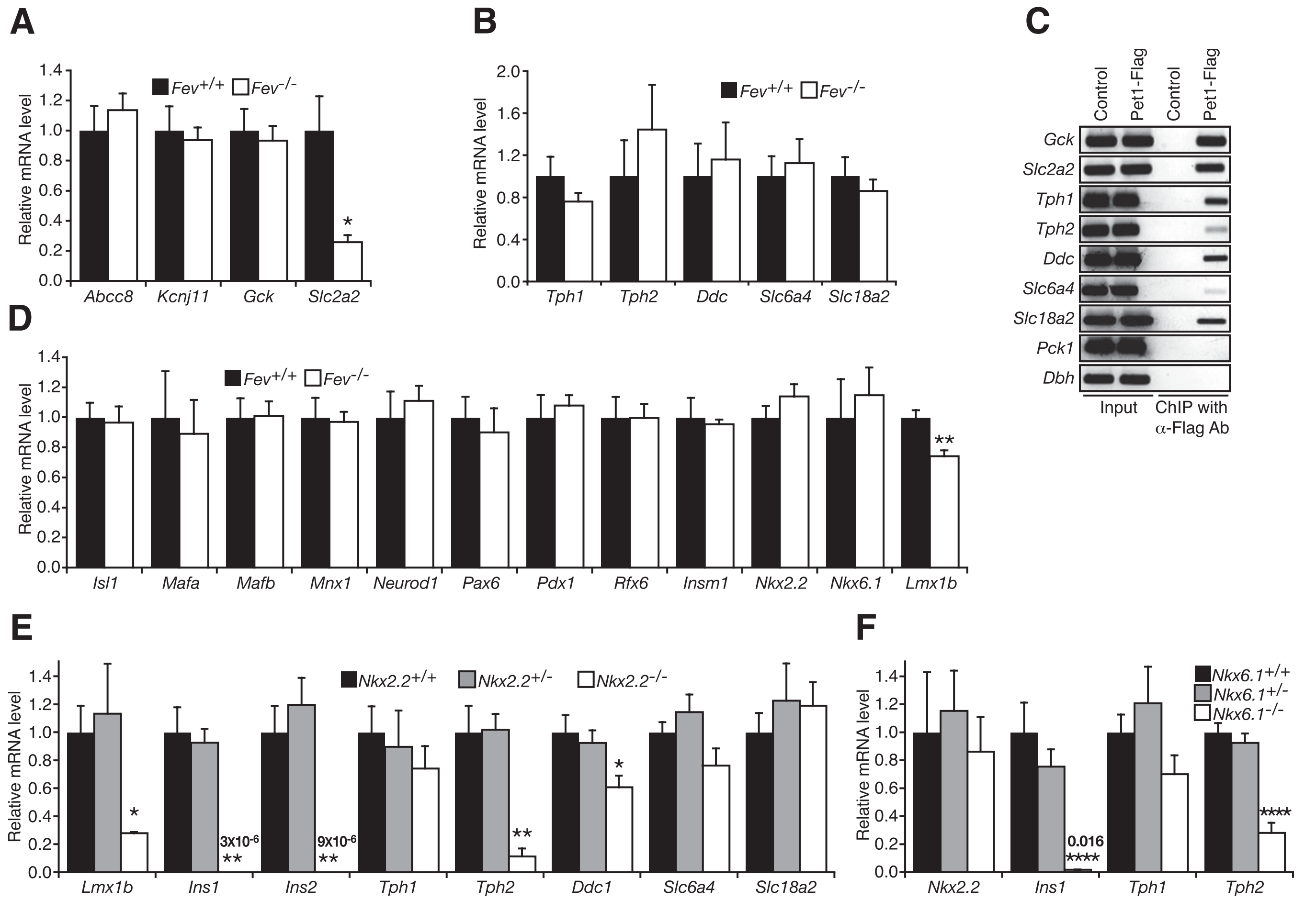

FIG. 6. Islet and serotonergic genes in *Fev*$^{-/-}$ mice. The relative expression levels of the mRNA shown were quantified by real-time PCR from RNA isolated from pancreata of mouse embryos at E18.5 with the following genotypes. *A*, *B*, and *D*: *Fev*$^{+/+}$ and *Fev*$^{-/-}$. *E*: *Nkx2.2*$^{+/+}$, *Nkx2.2*$^{+/-}$, and *Nkx2.2*$^{-/-}$. *F*: *Nkx6.1*$^{+/+}$, *Nkx6.1*$^{+/-}$, and *Nkx6.1*$^{-/-}$. All data points represent means ± SEM of at least four independent experiments. *C*: DNA binding by Pet1 was tested in intact cells by chromatin immunoprecipitation (ChIP). βTC3 cells were transfected with an expression plasmid with no insert (control) or the Pet1 coding sequence fused to the FLAG epitope (Pet1-FLAG). Formaldehyde–cross-linked DNA was precipitated with α-FLAG antibody, and sequences from the gene promoters shown were amplified by PCR. *$P < 0.05$ vs. wild type in *A*; **$P < 0.01$ vs. wild type in *D*; and *$P < 0.05$, **$P < 0.01$, and ****$P < 0.0001$ vs. wild type and vs. heterozygotes in *E* and *F* by two-tailed Student *t* test. Dbh, dopamine β-hydroxylase. Immunofluorescent staining for serotonin in pancreas of *Fev*$^{+/+}$ and *Fev*$^{-/-}$ embryos at E18.5 is shown in Supplementary Fig. 7.

but the genetic and functional similarities of the two key cell types involved in these diseases strongly suggest that some genetic or environmental insults may impair both serotonergic neurons and pancreatic β-cells and thus simultaneously increase the risk of both depression and type 2 diabetes. In addition, most drugs used to treat psychiatric disorders affect serotonergic signaling and may therefore also impact β-cells, especially during periods of high TPH activity in the islets, such as pregnancy and infancy.

Serotonin and insulin collaborate in an evolutionarily ancient partnership to regulate our response to changes in energy availability. Similarities in the function and development of the cells that produce serotonin and insulin reflect this evolutionary connection and have important implications for energy homeostasis and the pathology and treatment of diabetes.

ACKNOWLEDGMENTS

This work was supported by NIH grants R01 DK21344 and U19 DK61245, cores from P30 DK063720, Larry L. Hillblom Foundation grants 2002/1E and 2007/1B, the Nora Eccles Treadwell Foundation, American Diabetes Association Grant 7-07-MN-21, Juvenile Diabetes Research Foundation Grant 16-2007-428, fellowship award 33-2007-187 (to H.K.), and the University of California, San Francisco (UCSF) Sandler Program in Basic Sciences.

No potential conflicts of interest relevant to this article were reported.

Y.O. designed and performed research, analyzed data, and wrote the manuscript. Y.K. designed and performed research, analyzed data, and reviewed and edited the manuscript. N.K., G.H., and H.K. performed research, analyzed data, and reviewed and edited the manuscript. J.W., N.N., and A.L. performed research. S.B.S. and R.M.G. performed research and analyzed data. L.H.T. and E.S.D. provided reagents, analyzed data, and reviewed and edited the manuscript. M.S.G. designed research, analyzed data, and wrote the manuscript.

The authors thank members of the German laboratory and John Rubenstein, Gerold Grodsky, and William Rutter of UCSF for helpful advice and criticism.

Parts of this study were presented in abstract form at the 65th Scientific Sessions of the American Diabetes Association, San Diego, California, 10–14 June 2005.

REFERENCES

1. Falck B, Torp A. A fluorescence method for histochemical demonstration of noradrenalin in the adrenal medulla. Med Exp Int J Exp Med 1961;5:428–432

2. Pearse AG, Polak JM. Neural crest origin of the endocrine polypeptide (APUD) cells of the gastrointestinal tract and pancreas. Gut 1971;12: 783–788
3. Pictet RL, Rall LB, Phelps P, Rutter WJ. The neural crest and the origin of the insulin-producing and other gastrointestinal hormone-producing cells. Science 1976;191:191–192
4. Le Douarin NM. On the origin of pancreatic endocrine cells. Cell 1988;53: 169–171
5. Wilson ME, Scheel D, German MS. Gene expression cascades in pancreatic development. Mech Dev 2003;120:65–80
6. Falkmer S. Phylogeny and ontogeny of the neuroendocrine cells of the gastrointestinal tract. Endocrinol Metab Clin North Am 1993;22:731–752
7. Devaskar SU, Giddings SJ, Rajakumar PA, Carnaghi LR, Menon RK, Zahm DS. Insulin gene expression and insulin synthesis in mammalian neuronal cells. J Biol Chem 1994;269:8445–8454
8. Gelling RW, Morton GJ, Morrison CD, et al. Insulin action in the brain contributes to glucose lowering during insulin treatment of diabetes. Cell Metab 2006;3:67–73
9. Ahrén B. Autonomic regulation of islet hormone secretion—implications for health and disease. Diabetologia 2000;43:393–410
10. Cordes SP. Molecular genetics of the early development of hindbrain serotonergic neurons. Clin Genet 2005;68:487–494
11. Sander M, Sussel L, Conners J, et al. Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of beta-cell formation in the pancreas. Development 2000;127:5533–5540
12. Craven SE, Lim KC, Ye W, Engel JD, de Sauvage F, Rosenthal A. Gata2 specifies serotonergic neurons downstream of sonic hedgehog. Development 2004;131:1165–1173
13. Tecott LH. Serotonin and the orchestration of energy balance. Cell Metab 2007;6:352–361
14. Maekawa F, Toyoda Y, Torii N, et al. Localization of glucokinase-like immunoreactivity in the rat lower brain stem: for possible location of brain glucose-sensing mechanisms. Endocrinology 2000;141:375–384
15. Moriyama R, Tsukamura H, Kinoshita M, Okazaki H, Kato Y, Maeda K. In vitro increase in intracellular calcium concentrations induced by low or high extracellular glucose levels in ependymocytes and serotonergic neurons of the rat lower brainstem. Endocrinology 2004;145:2507–2515
16. Xu AW, Kaelin CB, Takeda K, Akira S, Schwartz MW, Barsh GS. PI3K integrates the action of insulin and leptin on hypothalamic neurons. J Clin Invest 2005;115:951–958
17. Lam DD, Przydzial MJ, Ridley SH, et al. Serotonin 5-HT2C receptor agonist promotes hypophagia via downstream activation of melanocortin 4 receptors. Endocrinology 2008;149:1323–1328
18. Xu Y, Jones JE, Kohno D, et al. 5-HT2CRs expressed by pro-opiomelanocortin neurons regulate energy homeostasis. Neuron 2008;60:582–589
19. Kaplan DD, Zimmermann G, Suyama K, Meyer T, Scott MP. A nucleostemin family GTPase, NS3, acts in serotonergic neurons to regulate insulin signaling and control body size. Genes Dev 2008;22:1877–1893
20. Murakami H, Murakami S. Serotonin receptors antagonistically modulate Caenorhabditis elegans longevity. Aging Cell 2007;6:483–488
21. Wade JM, Juneja P, MacKay AW, et al. Synergistic impairment of glucose homeostasis in ob/ob mice lacking functional serotonin 2C receptors. Endocrinology 2008;149:955–961
22. Hendricks TJ, Fyodorov DV, Wegman LJ, et al. Pet-1 ETS gene plays a critical role in 5-HT neuron development and is required for normal anxiety-like and aggressive behavior. Neuron 2003;37:233–247
23. 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
24. Sussel L, Kalamaras J, Hartigan-O'Connor DJ, et al. Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. Development 1998;125:2213–2221
25. Scott MM, Wylie CJ, Lerch JK, et al. A genetic approach to access serotonin neurons for in vivo and in vitro studies. Proc Natl Acad Sci USA 2005; 102:16472–16477
26. 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
27. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet 1999;21:70–71
28. Novak A, Guo C, Yang W, Nagy A, Lobe CG. Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. Genesis 2000;28:147–155
29. 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 USA 2007;104:10500–10505
30. Szot GL, Koudria P, Bluestone JA. Murine pancreatic islet isolation. J Vis Exp 2007;7:255
31. Schwitzgebel VM, Scheel DW, Conners JR, et al. Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. Development 2000;127:3533–3542
32. Miyatsuka T, Li Z, German MS. Chronology of islet differentiation revealed by temporal cell labeling. Diabetes 2009;58:1863–1868
33. Walther DJ, Peter JU, Bashammakh S, et al. Synthesis of serotonin by a second tryptophan hydroxylase isoform. Science 2003;299:76
34. Sakowski SA, Geddes TJ, Thomas DM, Levi E, Hatfield JS, Kuhn DM. Differential tissue distribution of tryptophan hydroxylase isoforms 1 and 2 as revealed with monospecific antibodies. Brain Res 2006;1085:11–18
35. Ebihara S, Marks T, Hudson DJ, Menaker M. Genetic control of melatonin synthesis in the pineal gland of the mouse. Science 1986;231:491–493
36. Hendricks T, Francis N, Fyodorov D, Deneris ES. The ETS domain factor Pet-1 is an early and precise marker of central serotonin neurons and interacts with a conserved element in serotonergic genes. J Neurosci 1999; 19:10348–10356
37. Wang YC, Zuraek MB, Kosaka Y, et al. The ETS oncogene family transcription factor FEV identifies serotonin-producing cells in normal and neoplastic small intestine. Endocr Relat Cancer 2010;17:283–291
38. Wicksteed B, Brissova M, Yan W, et al. Conditional gene targeting in mouse pancreatic ß-Cells: analysis of ectopic Cre transgene expression in the brain. Diabetes 2010;59:3090–3098
39. Honig G, Liou A, Berger M, German MS, Tecott LH. Precise pattern of recombination in serotonergic and hypothalamic neurons in a Pdx1-cre transgenic mouse line. J Biomed Sci 2010;17:82
40. Fyodorov D, Nelson T, Deneris E. Pet-1, a novel ETS domain factor that can activate neuronal nAchR gene transcription. J Neurobiol 1998;34: 151–163
41. Boam DS, Clark AR, Docherty K. Positive and negative regulation of the human insulin gene by multiple trans-acting factors. J Biol Chem 1990;265: 8285–8296
42. Smith PA, Duchen MR, Ashcroft FM. A fluorimetric and amperometric study of calcium and secretion in isolated mouse pancreatic beta-cells. Pflugers Arch 1995;430:808–818
43. Harris PE, Ferrara C, Barba P, Polito T, Freeby M, Maffei A. VMAT2 gene expression and function as it applies to imaging beta-cell mass. J Mol Med (Berl) 2008;86:5–16
44. Rieck S, White P, Schug J, et al. The transcriptional response of the islet to pregnancy in mice. Mol Endocrinol 2009;23:1702–1712
45. Schraenen A, Lemaire K, de Faudeur G, et al. Placental lactogens induce serotonin biosynthesis in a subset of mouse beta cells during pregnancy. Diabetologia 2010;53:2589–2599
46. Kim H, Toyofuku Y, Lynn FC, et al. Serotonin regulates pancreatic beta cell mass during pregnancy. Nat Med 2010;16:804–808
47. Matsuda M, Imaoka T, Vomachka AJ, et al. Serotonin regulates mammary gland development via an autocrine-paracrine loop. Dev Cell 2004;6:193–203
48. Finegood DT, Scaglia L, Bonner-Weir S. Dynamics of beta-cell mass in the growing rat pancreas. Estimation with a simple mathematical model. Diabetes 1995;44:249–256
49. Meier JJ, Butler AE, Saisho Y, et al. Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans. Diabetes 2008;57:1584–1594
50. Mezuk B, Eaton WW, Albrecht S, Golden SH. Depression and type 2 diabetes over the lifespan: a meta-analysis. Diabetes Care 2008;31:2383–2390