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Persistent expression of HNF6 in islet endocrine cells causes disrupted islet architecture and loss of beta cell function

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SUMMARY

We used transgenesis to explore the requirement for downregulation of *hepatocyte nuclear factor 6* (*HNF6*) expression in the assembly, differentiation, and function of pancreatic islets. In vivo, *HNF6* expression becomes downregulated in pancreatic endocrine cells at 18.5 days post coitum (d.p.c.), when definitive islets first begin to organize. We used an islet-specific regulatory element (*pdx1^{PB}*) from *pancreatic/duodenal homeobox* (*pdx1*) gene to maintain *HNF6* expression in endocrine cells beyond 18.5 d.p.c. Transgenic animals were diabetic. HNF6-overexpressing islets were hyperplastic and remained very close to the pancreatic ducts. Strikingly, α, δ, and PP cells were increased in number and abnormally intermingled with islet β cells. Although several mature β cell markers were expressed in β cells of transgenic islets, the glucose transporter GLUT2 was absent or severely reduced. As glucose uptake/metabolism is essential for insulin secretion, decreased GLUT2 may contribute to the etiology of diabetes in *pdx1^{PB}-HNF6* transgenics. Concordantly, blood insulin was not raised by glucose challenge, suggesting profound β cell dysfunction. Thus, we have shown that *HNF6* downregulation during islet ontogeny is critical to normal pancreas formation and function: continued expression impairs the clustering of endocrine cells and their separation from the ductal epithelium, disrupts the spatial organization of endocrine cell types within the islet, and severely compromises β cell physiology, leading to overt diabetes.

Key words: Pancreas, Islets, Organogenesis, Transgenics, HNF6, Diabetes, Mouse

INTRODUCTION

The murine pancreas derives from dorsal and ventral buds of the foregut endoderm that later merge into a single organ precursor (reviewed in Gannon and Wright, 1999). The pancreatic primordia contain precursor cells for both the exocrine (acinar cells that synthesize, store and secrete digestive enzymes) and endocrine (β cells: insulin; α cells: glucagon; δ cells: somatostatin; PP cells: pancreatic polypeptide) cell lineages. Even at early pancreatic bud stages (approx. 9.5 d.p.c.), cells expressing endocrine markers such as glucagon and insulin are detected, preceding the formation of a proper ductal epithelium, while exocrine-specific gene transcription commences at 14.5 d.p.c. (Gittes and Rutter, 1992; Teitelman et al., 1993). There is good evidence that later in embryogenesis, and into postnatal life, the ductal epithelium contains precursors of both the exocrine and endocrine lineages (Gu and Sarvetnick, 1993; Herrera et al., 1991). At 16.5 d.p.c., islet precursors start to organize into clusters initially located close to ducts. At 18.5 d.p.c., these clusters begin to lose their proximity to the ductal epithelium, become surrounded by exocrine tissue, and form mature islets of Langerhans (Herrera et al., 1991). As islets form, the endocrine cells segregate, such that the mature islet has insulin-producing β cells at the core, and glucagon-, somatostatin- and PP-producing cells at the islet periphery, or mantle.

Recently, several homeodomain-containing transcription factors have been identified that are expressed in developing and/or mature islet endocrine cell populations (Edlund, 1998; Gannon and Wright, 1999). Some were identified on the basis of their ability to bind and transactivate the insulin promoter, and reverse genetic analysis in mouse has subsequently revealed critical roles for these factors in the development of endocrine cell lineages, and/or proper islet morphology. Our laboratory has focused on the *pdx1* homeobox gene, which is expressed embryonically in a restricted domain within the posterior foregut endoderm and throughout the pancreatic epithelium, eventually becoming restricted mainly to β cells in the adult (Gannon and Wright, 1999). *pdx1* is essential for pancreatic development beyond the initial bud outgrowth stage, and differentiation of specific cell types in the antral stomach and rostral duodenum (Jonsson et al., 1994; Larsson et al., 1996; Offield et al., 1996). Mice and humans homozygous for inactivating mutations of *pdx1* are apancreatic, and

heterozygous humans develop non-insulin-dependent diabetes (NIDDM; Stoffers et al., 1998; Stoffers et al., 1997b). Mutations in *pdx1* were recently found to cause maturity-onset diabetes of the young type 4 (MODY-4; Stoffers et al., 1997a).

Several additional homeobox genes, including *Isl1, Pax4* and *Pax6*, are also required for normal pancreatic/islet development. *Isl1*, a member of the LIM class of homeodomain proteins, is expressed in all islet cell types. In $Isl^{-/-}$ embryos, the dorsal pancreatic bud does not form and, while the ventral bud develops, differentiated endocrine cells are not found within it (Ahlgren et al., 1997). *Pax4* and *Pax6*, which encode related paired homeodomain proteins, are required in different subsets of endocrine cells. *Pax4* is expressed in β cells and $Pax4^{-/-}$ animals have increased numbers of α cells at the expense of both β and δ cells (Sosa-Pineda et al., 1997). *Pax6* is expressed in all four islet cell types. Mice homozygous for a null allele of *Pax6* lack α cells and the remaining cell types fail to organize into normal islets (St-Onge et al., 1997); a truncating mutation in *Pax6* also affects endocrine cell differentiation (Sander et al., 1997).

Similar studies implicate other transcription factor families, e.g. basic helix-loop-helix (bHLH) proteins, in pancreas organogenesis. Loss of *Beta2/NeuroD* function results in a reduced number of differentiated islet cell types, disorganized islets and elevated blood glucose (Naya et al., 1997; Naya et al., 1995). In addition to a direct role for endocrine cell types in maintaining euglycemia, there is evidence that the exocrine compartment of the pancreas provides an appropriate tissue environment for islet morphogenesis and endocrine function. For example, a null mutation of $PTF1^{p48}$, which encodes part of the heterotrimeric complex activating exocrine gene transcription (Cockell et al., 1989; Krapp et al., 1996), leads to a complete lack of acinar tissue (Krapp et al., 1998). In $PTF1^{p48}$ mutants, endocrine cells differentiate and take up residence as isolated cells in the spleen, where they produce low levels of islet hormones.

Information on the mechanisms controlling the separation of endocrine cell precursors from the ductal epithelium, and their subsequent clustering into islets, is now beginning to emerge. Differential cell adhesion may be linked to the apparent migration of islet clusters away from the ductal epithelium, and the sorting out of the different endocrine cell types within the islet (Cirulli et al., 1994, 1998; Dahl et al., 1996; Esni et al., 1999). Studies of *HNF6* suggest that it is involved in islet morphogenesis and maturation. HNF6 is a liver-enriched cut-homeodomain factor expressed in the endoderm of the developing liver, intestine and pancreas, and has been implicated in the regulation of *HNF3β* (Landry et al., 1997; Lemaigre et al., 1996; Rausa et al., 1997; Samadani and Costa, 1996). During early embryonic development, in situ hybridization of pancreatic tissue revealed *HNF6* expression in pancreatic ducts, developing acinar cells and differentiating endocrine cells (Landry et al., 1997; Rausa et al., 1997). *HNF6* expression is then downregulated in pancreatic endocrine cells at late gestation, coincident with the initiation of islet morphogenesis and separation from the ductal epithelium. These observations suggested to us that continued *HNF6* expression is incompatible with islet maturation. Consistent with this idea, *HNF6* continues to be absent from islets in the adult (Rausa et al., 1997).

Here we tested the hypothesis that programmed downregulation of *HNF6* during development is required for normal islet morphogenesis. *HNF6* expression was maintained in islets using a 1 kb islet-specific enhancer element, $pdx1^{PB}$, from the *pdx1* gene (Wu et al., 1997). This manipulation lead to profound diabetes. The $pdx1^{PB}$-*HNF6* transgenic pancreata showed intermixed endocrine cell types, and failed to release insulin in response to glucose, a dysfunction associated with reduced levels of the β cell-specific GLUT2 glucose transporter. In contrast to the critical role for transcription factors in pancreas differentiation as determined by gene inactivation studies, we have shown that downregulation of *HNF6* during early islet morphogenesis is essential for the proper formation and function of islets. These observations suggest that inappropriate reactivation of this transcriptional regulator may contribute to the etiology of diabetes in some humans.

MATERIALS AND METHODS

Transgene construction and generation of transgenic mice

A 1 kb *Pst*I to *Bst*EII genomic fragment within the 5′ region of *pdx1* was previously described (Wu et al., 1997). The $pdx1^{PB}$-*HNF6* transgene was generated using a modified form of the FPEhsplacZ plasmid (a gift from Y. Furuta and B. Hogan; Sasaki and Hogan, 1996), which contains the floorplate enhancer (FPE) from *HNF3β*, upstream of the *hsp68* minimal promoter, a *lacZ* cDNA, and intron 2 and polyadenylation sequences from the rabbit *β globin* gene. The *lacZ* cassette was replaced by a 1.6 kb *HNF6* cDNA, and the FPE was replaced with the 1 kb Pst-Bst (PB) *pdx1* islet enhancer. DNA was *Not*I-digested, and the entire $pdx1^{PB}$-HNF6 transgene isolated from low-melt agarose gels using Gelase (Epicentre Technologies). DNA (3 ng/μl) was injected into pronuclei of one-cell embryos from B6D2 females, and embryos transplanted into pseudopregnant ICR females. Some F_0 founder embryos were analyzed at different stages of gestation; others were used to establish transgenic lines. $pdx1^{PB}$-*HNF6* transgenics were identified by Southern blotting of *Eco*RI-digested tail or embryonic brain DNA using an *Eco*RI-*Pst*I *HNF6* partial cDNA probe. Transgene copy number was estimated from copy number controls of each parent plasmid. Animals were given water and Lab Diet #5015 mouse pellets ad lib, in a 12 hour light/dark cycle.

Tissue preparation and histology

Embryonic pancreata or digestive organs were dissected in PBS and fixed immediately in ice-cold 4% paraformaldehyde (4°C; 45 minutes). Adult pancreata, livers and eyes were fixed for 1 hour. Tissues were dehydrated in an increasing ethanol series followed by two xylene washes, infiltrated with xylene:paraffin (1:1, v/v) and two changes of paraffin under vacuum at 56°C and embedded for sectioning. Serial 7 μm sections mounted on glass slides with Sta-on (Surgipath) were used for histology, immunohistochemistry and immunofluorescence analyses. Hematoxylin (Sigma) and/or eosin (Surgipath) were used as counterstains. Periodic acid-Schiff (PAS) staining was performed using a kit according to the manufacturer's instructions (Sigma).

β-galactosidase detection

Following fixation, tissues were washed twice for 30 minutes in permeabilization solution (2 mM MgCl, 0.01% sodium deoxycholate, 0.02% Nonidet P-40 in PBS). β-galactosidase activity was detected using X-gal as previously described (Wu et al., 1997). Tissues were post-fixed (4% paraformaldehyde in PBS; 1 hour at 4°C) and then dehydrated for embedding as above, with isopropanol replacing xylene to minimize leaching of the blue precipitate.

Immunohistochemistry and immunofluorescence

Sections were xylene deparaffinized and rehydrated in a decreasing ethanol series to distilled water. Protein localization was determined using immunoperoxidase labelling kits: Histomouse SP (Zymed) for insulin or the appropriate Vectastain ABC kit (Vector Labs) for all other primary antibodies, according to the maufacturers' instructions. Endogenous peroxidase was quenched with 0.6% H_2O_2/methanol for 30 minutes. Primary antibodies were used at the following dilutions: guinea pig anti-bovine insulin (Linco), 1:2000; rabbit anti-glucagon (Linco), 1:1000; guinea pig anti-glucagon (Linco), 1:500; rabbit anti-human somatostatin (Dako), 1:500; guinea pig anti-pancreatic polypeptide (Linco), 1:500; rabbit anti-XlHbox8, 1:25 (Peshavaria et al., 1994); rabbit anti-HNF6, 1:100 (Landry et al., 1997); rabbit anti-mouse HNF3β (a gift from B. Hogan), 1:50; rabbit anti-NKX6.1 (a gift from M. German, UCSF), 1:6000; rabbit anti-rat GLUT2 (a gift from M. Magnuson), 1:500; and mouse anti-human PECAM (Sigma) 1:400. All primary antibody incubations were overnight in a humid chamber; 4°C for antibodies against islet hormones, room temperature for antibodies against nuclear or cell surface proteins. Detection of Pdx1, NKX6.1 and GLUT2 required 'antigen retrieval' prior to quenching of endogenous peroxidase: sections were microwaved (1000 W, 2× 3 minutes in 10 mM citrate buffer) with brief cooling between each heating, then cooled to room temperature before continuing. The color reaction was performed according to the manufacturer's instructions using the DAB Peroxidase substrate kit (Vector Labs) for a maximum of 10 minutes. Samples were viewed under bright-field illumination and photographed with Kodak Ektachrome 64T film.

For immunofluorescence, donkey anti-guinea pig CY2 (insulin) and donkey anti-rabbit CY3 (glucagon and GLUT2) were used as secondary antibodies. CY2 was excited at 543 nm and CY3 at 488 nm using an LSM 410 confocal microscope (Zeiss). There was no significant cross-talk between the two channels. TIFF images were processed in Adobe photoshop.

Glucose tolerance tests (GTT)

Following a 14-16 hour fast, baseline blood glucose levels (mg/dl) were measured in tail vein blood from mildly anesthetized mice using the Accu-Chek Advantage glucose meter and Accu-Chek test strips (Roche). Glucose (2 mg dextrose/g body weight) in sterile PBS was injected intraperitoneally and blood glucose measured 15, 30, 60, 90 and 120 minutes after injection. GTT were performed every 2 weeks for 2-4 months beginning at weaning. In cases where plasma insulin levels were measured simultaneously, blood was obtained via puncture of the retro-orbital plexus. In some cases, L-arginine (3 mg/g body weight; Sigma) was co-injected with glucose.

Radioimmunoassay

Plasma insulin and glucagon levels were measured by radioimmunoassay (RIA; Linco) using a modification of the manufacturer's protocol allowing quantitation of insulin from small sample volumes.

RESULTS

A *pdx1* enhancer with islet endocrine cell specificity

The 1 kb *Pst*I-*Bst*EII (*pdx1*^{PB}) fragment of the *pdx1* 5′ upstream region directs expression of β-galactosidase (β-gal) to pancreatic islets of transgenic (Tg) animals (Wu et al., 1997). To date, nine founder transgenics have been obtained. β-gal expression was detected in five of the nine founders and was islet-specific in all five; transgenic lines were established from four of these founders. The other four founders showed no expression of the reporter transgene, presumably due to integration position effects. Further whole-mount analysis of *pdx1*^{PB}*-lacZ* lines detected transgene expression as early as 11.5 d.p.c. in isolated insulin- or glucagon-positive cells within the pancreatic bud epithelium, without evidence for expression elsewhere within the embryo. Expression continued at 16.5 d.p.c. in 86% of insulin$^+$ cells, 75% of glucagon$^+$ cells, and 85% of somatostatin$^+$ cells (M. G. and C. V. E. W., unpublished observations). One of the four transgenic lines was analyzed by sectioning at 16.5 d.p.c., which revealed very low numbers of β-gal$^+$ cells scattered in the rostral duodenum. Duodenal expression was not observed at postnatal day one in the same line. At postnatal day two (P2), 85% of insulin-producing β

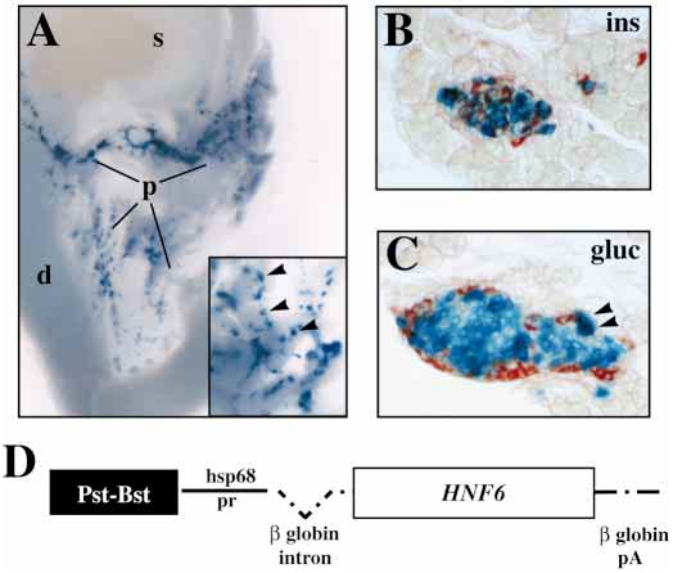

Fig. 1. The 1 kb *Pst*I-*Bst*EII (*pdx1*^{PB}) region of the *pdx1* promoter directs islet-specific expression. (A) Pancreas from 2-day-old neonatal mouse stained with X-gal for *pdx1*^{PB}*-lacZ* (blue). (Inset: higher magnification; arrowheads indicate islets). (B,C) Islet sections immunostained for insulin or glucagon, (brown) respectively. Arrowheads indicate glucagon$^+$/β-gal$^+$ cells. Acinar cells do not express the transgene. (D) Diagram representing the transgene construct (see Materials and Methods). p, pancreas; d, duodenum; s, stomach.

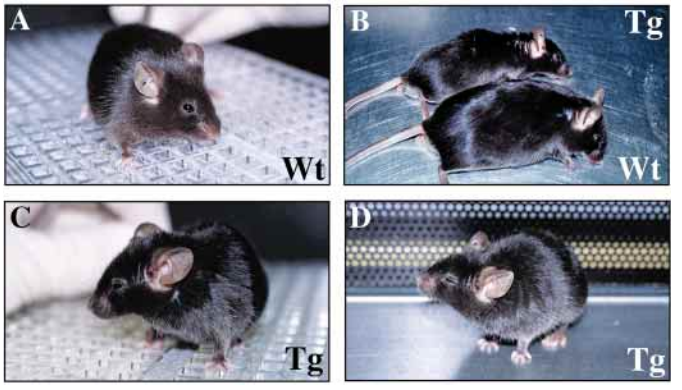

Fig. 2. External phenotype of *pdx1*^{PB}*-HNF6* transgenics. (A) Wild-type mouse, 6-months old. (B) Comparison of a wild-type mouse and transgenic littermate. (C,D) Transgenics have dry, unhealthy fur (C) and are photosensitive (D). Wt, wild type; Tg, transgenic.

cells express the *lacZ* reporter (Fig. 1A-C), although the number of β-gal-positive cells per islet ranged from 62% to 95%. At P2, 22% of glucagon$^+$ cells and 57% of somatostatin$^+$ cells continue to express *pdx1^{PB}-lacZ* (Fig. 1C and data not shown). Thus, this 1 kb islet-specific *pdx1* enhancer represents a strong cassette for driving expression of genes of interest to most β cells and significant numbers of α and δ cells, beginning at very early embryonic stages, continuing through the postnatal period, and into adulthood.

Maintaining *HNF6* expression in pancreatic islets

We tested the hypothesis that downregulation of *HNF6* in islets is necessary for their normal morphogenesis and function. The *pdx1* islet enhancer was used to maintain *HNF6* expression in islet endocrine cells past 18.5 d.p.c and into adulthood (Fig. 1D). Eight founder animals were obtained, four males and four females, with various transgene copy numbers. Three males (I7, 2 copies; F5, 10 copies; and H1, 50 copies) were used to generate transgenic lines. All three of these lines presented with a similar diabetic phenotype (discussed below); H1 was the most severe, F5 the weakest and I7 intermediate. Although some founders (one male and all four females) failed to show any externally obvious signs of diabetes, histological analysis of pancreata from all transgenics analyzed revealed some degree of islet dysmorphogenesis (data not shown) consistent with the *pdx1^{PB}-HNF6* transgenic phenotype described below. The variability in phenotype in some founders is likely connected to transgene insertion position effects. With the exception noted (Fig. 4G), the data presented below are from lines I7 and H1, hereafter referred to as L (low copy number) and H (high copy number) for brevity.

pdx1^{PB}-HNF6 transgenics are diabetic

pdx1^{PB}-HNF6 transgenics appeared identical to wild-type littermates until 3 weeks after birth. At this age, when mice were weaned to mouse chow, H line Tg animals began to display several characteristics consistent with diabetes in humans; most notably, transgenics had polydipsia and polyuria (frequent and excessive water intake and urination, respectively). In addition, they were highly touch-sensitive, their fur appeared dry and unhealthy, and they squinted continuously, suggesting photosensitivity (Fig. 2C,D). Histological examination of the eyes from H transgenics suggested an increase in intraocular volume associated with an oblong shape along the longitudinal axis, as well as thickening of the neural retina in some areas (data not shown). Beginning at 8 weeks, both H and L transgenics were noticeably smaller than wild-type littermates and gained weight more slowly. *pdx1^{PB}-HNF6* transgenics consistently weighed 20-40% less than age- and sex- matched wild-type mice (Fig. 2B). In addition, Tg males showed reduced fecundity, producing litters of 2-6 pups instead of 8-12, the normal number expected for the B6D2 hybrid strain.

Since some of the aforementioned characteristics (polydipsia, polyuria, eye and skin defects) are reminiscent of secondary complications of untreated diabetes in humans, we tested for abnormal glucose homeostasis by measuring fasting blood glucose and performing glucose tolerance tests (GTT) in wild-type and *pdx1^{PB}-HNF6* transgenics. In wild-type mice, blood glucose measurements between 70 and 150 mg/dl are consistent with normoglycemia, while 150-250 mg/dl is indicative of mild diabetes, and >250 mg/dl indicates severe diabetes. Fasting blood glucose was, in general, higher in *pdx1^{PB}-HNF6* transgenics than in wild type (122-214 mg/dl; *n*=7 versus 98-139; *n*=6, respectively, at four months of age) and increased as the animals aged (Fig. 3 and data not shown). Thus, even after 16 hours of fasting, severely affected *pdx1^{PB}-HNF6* transgenics were still diabetic. In addition to their increased basal blood glucose levels, transgenics showed a decreased ability to clear glucose from the blood following intraperitoneal glucose injection (Fig. 3). Wild-type mice peaked at levels between 150 and 240 mg/dl 15 minutes after injection, and reached baseline, or below, by the end of the 2-hour testing period. In contrast, Tg animals had dramatically elevated blood glucose levels (350 to >600 mg/dl) over controls within 15 minutes of injection. With increasing age, there was a progressive increase in the inability of blood glucose levels in Tg animals to return to baseline levels – they remained within the diabetic range during the entire GTT. Both the external phenotype and glucose intolerance were consistently more severe in H than L transgenics.

The diabetic phenotype became exacerbated in the F_1 generation from all three founder males (Fig. 3B and data not shown), manifesting as an earlier age of onset and a more dramatic impairment in glucose clearance. Even at an age where founder transgenics were able to return to baseline, F_1 transgenic females from line H remained in the diabetic range 2 hours after glucose injection (Fig. 3B). As these animals aged, blood glucose measurements following injection exceeded the glucometer detection limit (>600 mg/dl). One 2-month-old female began the GTT with a fasting blood glucose level greater than 600 mg/dl. This animal subsequently displayed seizure activity consistent with osmotic coma, and died during the test period.

pdx1^{PB}-HNF6 transgenics have disrupted islet morphology

To ensure that the Tg phenotype correlated with expression of the *pdx1^{PB}-HNF6* transgene in islets, we analyzed HNF6 expression in pancreata. While *HNF6* transcripts are no longer detectable in islets at 18.5 d.p.c. (Landry et al., 1997; Rausa et al., 1997), immunohisochemical analysis of wild-type nuclei at this stage reveals low levels of residual protein (Fig. 4A). Tg islet nuclei showed much increased HNF6 immunostaining at 18.5 d.p.c. (Fig. 4B). HNF6 expression within acinar tissue was unchanged (data not shown), consistent with the cell type-specific expression from the *pdx1^{PB}* regulatory element.

The overall appearance of the pancreas was similar in control and Tg animals; Tg pancreata displayed no evidence of necrosis or fibrosis. *HNF6* misexpression caused no alterations in gastric, duodenal, acinar or ductal development. Any observed effects were specific to the expected expression domain of this transgene in islets. Pancreata from Tg and age-matched controls were then analyzed histologically to determine if *pdx1^{PB}-HNF6* transgenics had obvious defects in pancreatic islet morphogenesis. Lymphocyte infiltration, a characteristic of Type I autoimmune diabetes, was not observed.

Islet size was estimated by measuring the longest axis of 30 wild-type and 30 Tg randomly selected sectioned islets that were at least 10 cells wide. The majority (approx. 70%) of islets in Tg animals were 1.5 to 2-fold larger than wild-type islets (compare Fig. 4E and F); although some (3%) (Fig. 5H) could be up to 3-fold larger. This linear difference represents

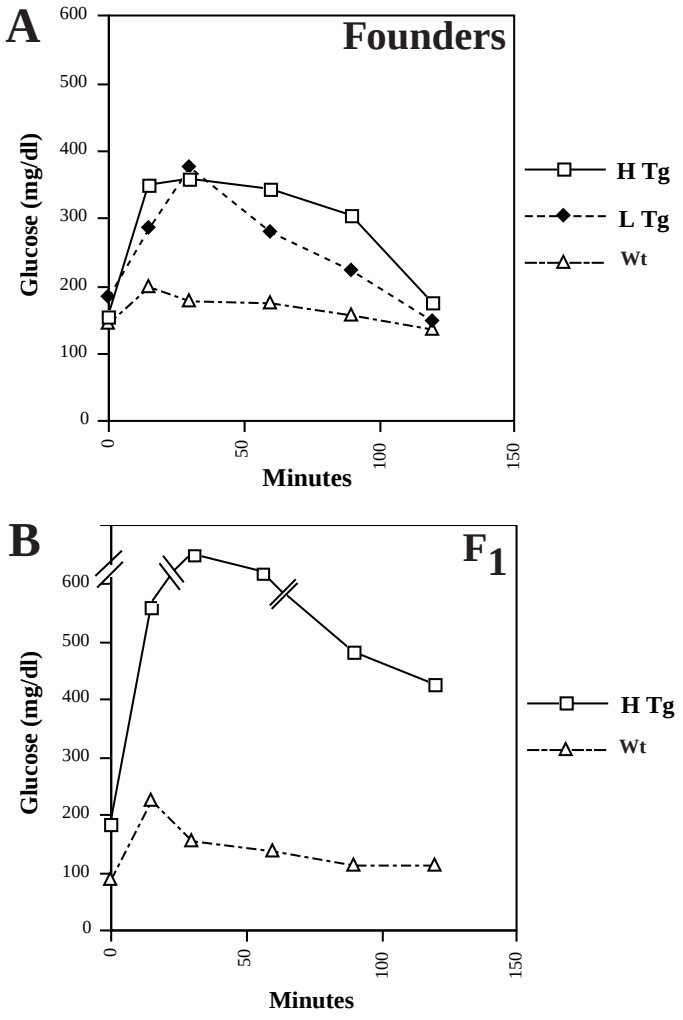

Fig. 3. *pdx1*^{PB}*-HNF6* transgenic mice have impaired glucose tolerance and are diabetic. Blood glucose levels were measured after 16 hours fasting (time 0) and after glucose injection. (A) Founders. (B) F_1 generation. >250 mg/dl = diabetic. Blood glucose levels beyond the meter detection limit (30 and 60 minute data points) are indicated by broken lines. H, 50 copy transgenic line; L, 2 copy transgenic line; Wt, wild type; Tg, transgenic.

a much larger increase in islet volume. In many cases, Tg islets were irregularly formed, (e.g. dumbbell shaped), or appeared to be composed of multiple, fused islets (Figs 4D,G, 5C,D,H), in contrast to the roughly spherical and uniformly sized islets in wild-type pancreatic tissue (Fig. 4A,C,E). In addition, Tg islets were more often found adjacent to and partially enfolding the pancreatic ducts (Fig. 4D,G), whereas wild-type islets were well separated from ducts and embedded within acinar tissue. Regardless of their shape and size, Tg islets had well-defined borders; endocrine cells were not scattered throughout the pancreas but organized into islet-like clusters.

A striking difference between control and Tg islets was an increased number of islet hormone-positive cells within the pancreatic ductal epithelium. Examination of 7 μm sections through ductal epithelium revealed that 20.3% of ducts (*n*=79) had at least one positive cell, and 5.7% had two or more positive cells (e.g. Fig. 4H). Islet hormone-expressing cells are rarely found within ducts in wild-type adult pancreata (7.5% of ducts contained single positive cells, *n*=67; multiple positive cells were not observed). In addition, Tg islets appeared hypervascularized compared to control islets; in some cases this was evident as gaps in the islet core filled with red blood cells (Fig. 4F). Analysis with an endothelial cell marker, PECAM, confirmed the increased vascularization (data not shown).

Abnormal islet architecture in transgenic animals

Since diabetes is caused by insulin deficiency, we assayed for the presence of insulin in Tg islets. In both wild-type and Tg islets, high levels of insulin were detected within cells of the islet core (Fig. 4E,F), with no obvious differences in levels of expression between wild-type and Tg animals. Thus, despite a decreased ability to clear blood glucose, the level of intracellular insulin per β cell appears unchanged in Tg animals.

We tested for the presence of peripheral endocrine cell types within transgenic pancreata by immunostaining for glucagon (α cells), somatostatin (δ cells), and pancreatic polypeptide (PP cells). Islets from wild-type pancreata had the expected peripheral distribution (Fig. 5A,E,G), with the occasional cell lying more internally than the outer two cell layers. In examining over twenty sections of similarly sized islets, wild-type islets contained, on average, 13 glucagon cells per islet section, 10% (range: 0-27%) of which were within the islet core (ie., deeper than the two outermost cell layers). Similar distributions were observed for somatostatin- (14%) and PP-producing (5%) cells within the islet core (Fig. 5; Table 1).

In contrast, α cells in Tg islets were highly scattered and more than tripled in number (Fig. 5B-D; Table 1). Consistent with the GTT data, the degree of disturbance in islet architecture correlated with transgene copy number (compare L line in Fig. 5B with H line in C and D). The average number of glucagon-positive cells in 11 randomly selected islet sections from H line mice was 47, 27% (range: 26-40%) of which were located within the islet core (Table 1). Islets selected for the highest degree of scattering contained greater than 50% of α cells within the islet core, including one example where 67% of the glucagon$^+$ cells were scattered. Similar to the effects of HNF6 overexpression on glucagon$^+$ cells, PP cells were also scattered and increased in number, while somatostatin cells showed increased core invasion, but no increase in cell number in transgenic islets (Fig. 5F,H; Table 1). The increased number of peripheral cell types is larger than the 1.5- to 2-fold increase in islet size: α and PP cells were increased 3.5 and 3 fold, respectively. Intermingling of α and β cells within the islet core has been observed in several transgenic and mutant mouse models, and been termed a ‘mixed islet’ phenotype (Esni et al., 1999; also see Discussion).

In agreement with the increased blood glucose seen in the F_1 generation of all Tg lines, endocrine cell clustering and organization was more profoundly affected in offspring from the founders, and was disrupted in offsrping from the H line. The pancreas from the 2 month-old F_1 H line female, which had fasting blood glucose levels greater than 600 mg/dl and died during GTT, did not contain well-organized endocrine cell clusters resembling islets. Very low numbers of glucagon- or insulin-positive cells were observed, and these were dispersed among exocrine cells, or located in small clusters at the

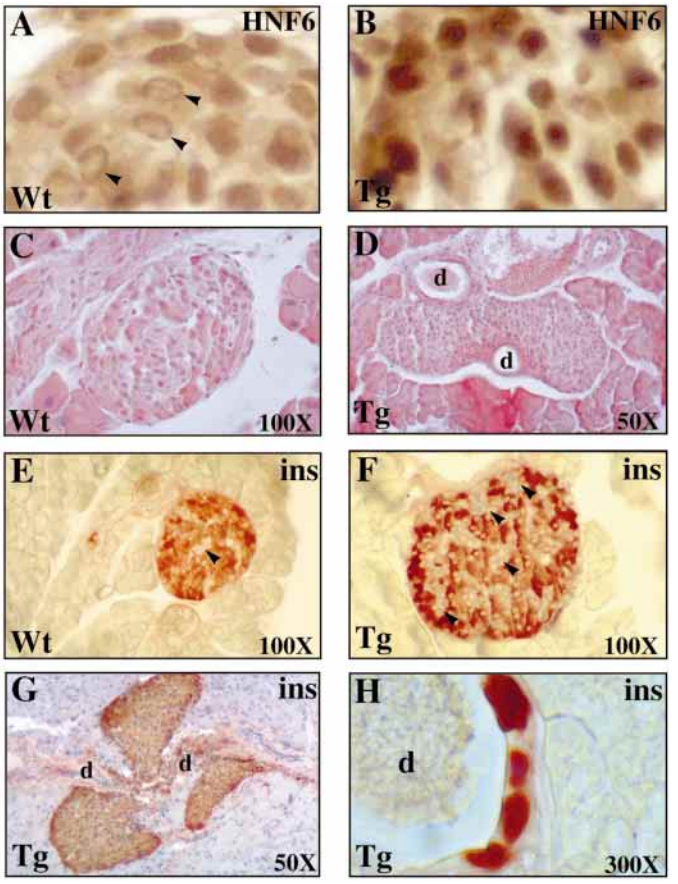

Fig. 4. Abnormal islet morphology in *pdx1*^{PB}*-HNF6* transgenics. (A,B) HNF6 expression (dark brown) in L line at 18.5 d.p.c. (A) Wild-type islet nuclei (arrowheads: nuclei with background levels of HNF6 labeling). (B) Tg islet nuclei. (D,F,G) Transgenic islets are larger, show altered morphology and are closely associated with the ductal epithelium compared to (C,E) typical wild-type islets. (E) Wild-type (Wt) and (F,G) transgenic (Tg), insulin (brown). (F) Transgenic islets were also more highly vascularized (arrowheads) than in control animals (E). (H) Insulin in Tg ducts. ins, insulin; d, duct. (C,D) Hematoxylin stained, (G) hematoxylin counterstained.

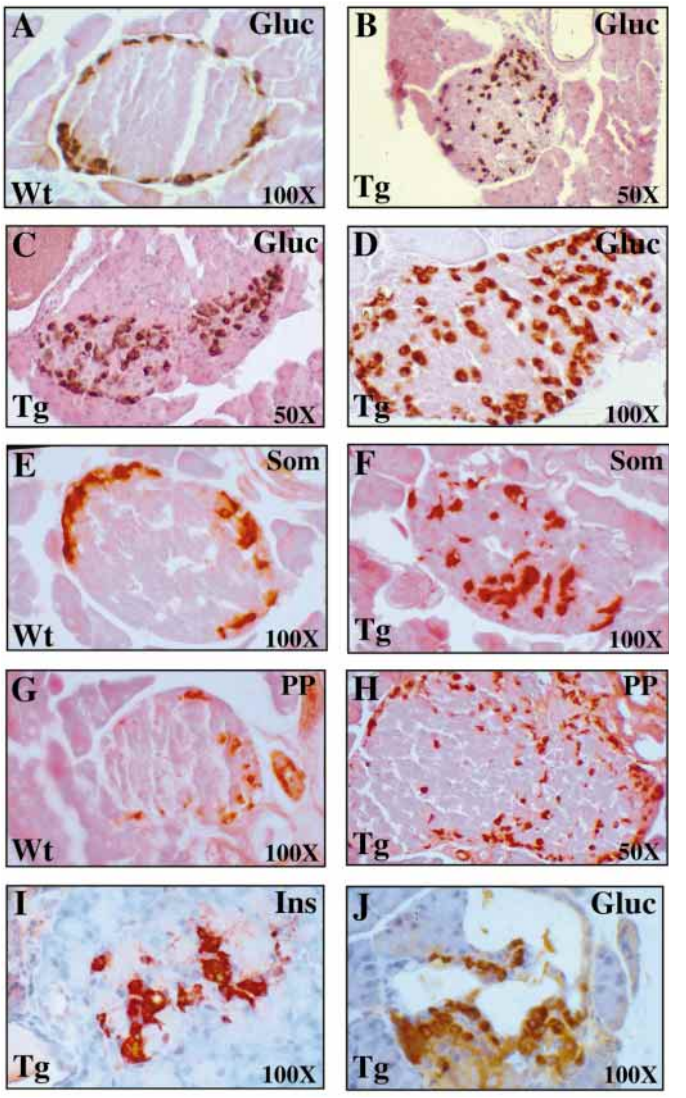

Fig. 5. Disrupted islet architecture in *pdx1*^{PB}*-HNF6* mice. (A,E,G) Wild-type (Wt) islets. (B-D,F,H-J) Transgenic (Tg) islets. Immunolabelling of islet hormones shown in brown. (A-D) Glucagon. (E,F) Somatostatin. (G,H) PP. (B) 2 copy L line. (C,D, F,H-J) 50 copy H line. (I,J) Islets in H line F_1 female. (I) Insulin-positive cells scattered among acinar cells. (J) Glucagon cells closely associated with ductal structures. gluc, glucagon; som, somatostatin; PP, pancreatic polypeptide. Sections were counterstained with eosin (A-H) or hematoxylin (I,J).

periphery of 'holes' lined with epithelial cells (Fig. 5I,J). It is currently unclear whether these holes represent an overproliferation of duct-like structures or spaces previously occupied by islets that degenerated due to the severe diabetes. They do, however, resemble the ductal hyperplasia and reversed budding of islets seen in transgenic mice overexpressing either IFNγ or TGFα (Gu and Sarvetnick, 1993; Song et al., 1999).

Given the large increase in peripheral islet cell types within the islet core, we tested whether core cells might co-express insulin and a peripheral islet cell hormone, e.g. glucagon. Precedent for this comes from mice specifically lacking *pdx1* in β cells, in which adult onset diabetes was associated with the appearance of many islet cells co-expressing these two hormones (Ahlgren et al., 1998). Confocal fluorescence microscopy detected insulin/glucagon co-expression in only one cell within 12 wild-type islet sections examined, and in only a single cell among 23 islet sections analyzed from the L and H lines (data not shown). Glucagon-positive cells in Tg animals are therefore scattered among insulin-expressing cells.

We next analyzed late gestation embryos to determine if alterations in early stages of islet morphogenesis could be seen that prefigured the severe islet defects and diabetes seen in adults. As mentioned above, propagating the Tg lines has been challenging due to reduced fecundity. We committed two litters of embryos from the L Tg line, and three litters containing F_0 transient transgenics to analysis at 18.5 d.p.c., the time at which *HNF6* expression is normally downregulated in islets. In seven wild-type embryos at 18.5 d.p.c., the majority of endocrine cell clusters were observed close to pancreatic ducts (Fig. 6A-E). Some clusters were recognizable as developing islet-like

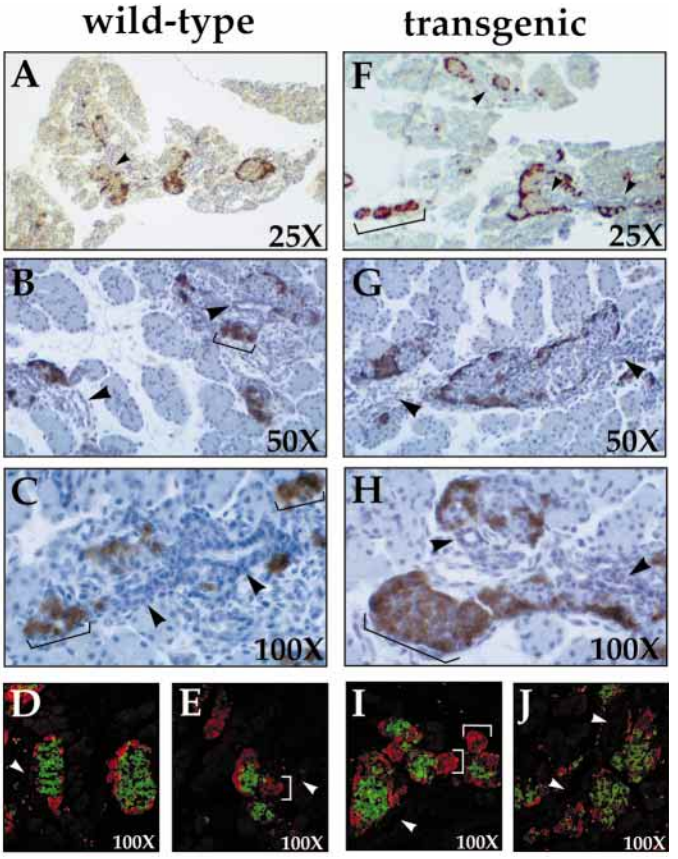

Fig. 6. HNF6 overexpression affects early islet morphogenesis. Insulin (green) and glucagon expression (brown or red) in wild type (Wt; A-E) and transgenic (Tg; F-J) islets at 18.5 d.p.c. Differentiating endocrine cells and forming islets are found close to ducts (arrowheads). (C) Small clusters of glucagon-positive cells are seen. (G,I,J) Transgenic embryos have larger islet-like structures and larger clusters of α cells (H,I) compared to wild-type. Brackets indicate clusters of glucagon cells containing ten or more cells. Sections in A-C and F-H were hematoxylin counterstained.

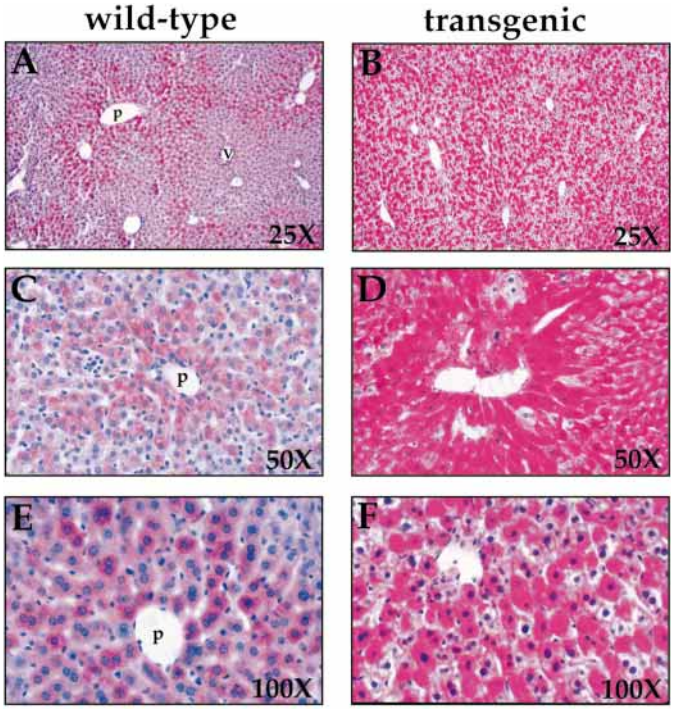

Fig. 7. Transgenic livers accumulate large glycogen stores. PAS staining of stored liver glycogen (magenta). (A,C,E) Wild-type. (B,D,F) Transgenic livers. (B,F) H line, (D) L line. v, central vein; p, portal system. Sections were hematoxylin counterstained.

Table 1. Increased numbers and scattering of peripheral islet cell types in *pdx-1*^{HNF6}transgenics

	Islet sections (*n*)	Avg. total/section	Avg. within core	(%)
Wild-type				
α cells	20	12.9	1.3	(10)
δ cells	12	18.4	2.5	(14)
PP cells	9	7.4	0.4	(5)
Transgenic				
α cells	11	47.4	13.0	(27)
δ cells	5	23.8	10.0	(42)
PP cells	5	22.2	5.8	(26)

Islets were selected at random and the total number of cells expressing glucagon (α), somatostatin (δ), or pancreatic polypeptide (PP) were counted. Cells were considered to lie within the core of the islet if they were located more internally than the outer two cell layers. All wild-type islets were approximately the same size.

structures (Fig. 6A,C,D), while others consisted of only a few cells (Fig. 6B,C). Glucagon-producing cells were detected at the periphery in all forming wild-type islets. Small groups of approximately 5-10 glucagon$^+$ cells were observed as isolated clusters (Fig. 6C) or adjacent to a subset (35%) of forming wild-type islets (Fig. 6B,E). The six Tg embryos obtained had two characteristics distinguishing them from wild type. First, aggregating endocrine cells formed larger islet-like structures running along the ducts, with some α cells located internally (Fig. 6F,G,I,J). Second, clusters of glucagon-positive cells were larger, containing up to 30 cells (Fig. 6F,H). 75% of Tg islets exhibited one or both of these characteristics; only 25% could be classified as similar in appearance to wild-type islets. Thus, *pdx1*^{PB}*-HNF6* transgenics exhibit alterations in islet development, including an abnormal number and distribution of α cells that are evident even during early stages of islet ontogeny.

Excessive glycogen storage in *pdx1*^{PB}*-HNF6* transgenics

The elevated blood glucose in Tg mice could be due to several factors including defective insulin production and/or secretion, and increased gluconeogenesis (liver glycogen breakdown). Since gluconeogenesis is stimulated by increased plasma glucagon levels, the increased number of glucagon$^+$ cells within Tg islets led us to ask whether increased gluconeogenesis could contribute to the observed increase in fasting blood glucose levels. We analyzed both fasting (n=2 experiments) and ad lib feeding (n=1 experiment) plasma glucagon levels in several animals and observed no significant difference between wild type and Tg (data not shown).

Rather than increased glycogen breakdown in Tg animals, histological analysis detected increased glycogen storage in Tg livers. On gross examination, Tg livers were lighter in color than controls (data not shown), reminiscent of glycogen

storage disease. PAS staining of wild-type livers revealed a characteristic gradient of glycogen deposition, predominantly in hepatocytes near the periportal region and declining toward the pericentral zone, reflecting the normal preferential uptake of glucose by periportal hepatocytes (Fig. 7A). In contrast, Tg livers reproducibly contained abundant glycogen-containing hepatocytes throughout the entire organ (Fig. 7B,D,F). The excess storage of glycogen by *pdx1^{PB}-HNF6* livers further argues that the increased blood glucose levels in transgenics are not due to increased gluconeogenesis.

pdx1^{PB}-HNF6 islets have impaired physiological response to glucose

The severe glucose intolerance and diabetes in *pdx1^{PB}-HNF6* transgenic animals indicated alterations in the differentiated state or function of the β cells. We therefore examined β cell function and expression of additional β cell markers to analyze more fully the defects in these cells.

As discussed above, defects in insulin production and/or secretion would be expected to result in diabetes. Our immunostaining data indicated that intracellular insulin production is relatively normal in *pdx1^{PB}-HNF6* mice (Fig. 4F). We therefore tested whether Tg animals could release insulin into the bloodstream in response to glucose challenge, since glucose-stimulated insulin release (GSIR) is a primary physiological indicator of normal β cell function. While basal insulin levels were similar in wild-type and Tg mice, transgenics did not secrete additional insulin in response to glucose (Fig. 8). Wild-type animals showed the typical first phase response of 2.5- to 3-fold increase in plasma insulin levels within 15 minutes of glucose administration; as a result, within 30 minutes, blood glucose levels had already begun to decline in these mice (data not shown). The continued increase in plasma insulin concentration in wild type is most likely due to the stress caused by the bleeding procedure. In striking contrast, the H line showed plasma insulin levels remaining at basal levels throughout the 2-hour test period (Fig. 8). L transgenics also failed to show a first phase insulin response to glucose within 15 minutes, but did show a moderate but variable elevation (605 or 720 pg/ml) in insulin levels towards the end of the 2-hour test. The continued increase in blood glucose levels in both Tg lines (from approx. 170 mg/dl to >600 mg/dl in the H line) demonstrates that the level of insulin released from Tg islets is insufficient to eliminate the excess glucose load in the bloodstream.

In preliminary experiments to test whether Tg β cells could secrete insulin in response to a more aggressive stimulus, GTTs were performed on animals co-injected with glucose and L-arginine. Transport of arginine into islet endocrine cells causes rapid membrane depolarization and endocrine hormone release, and glucose specifically potentiates the effect of arginine on insulin secretion (Henquin, 1987). Arginine/glucose co-injection failed to raise insulin levels above baseline in transgenics (data not shown), and blood glucose remained in the diabetic range. Thus, we conclude that there is a fundamental defect in the ability of *pdx1^{PB}-HNF6* Tg islets to release stored insulin.

Loss of GLUT2 in transgenic β cells

To test whether the abnormal physiological response of islets to secretagogs indicated a partial loss of β cell characteristics,

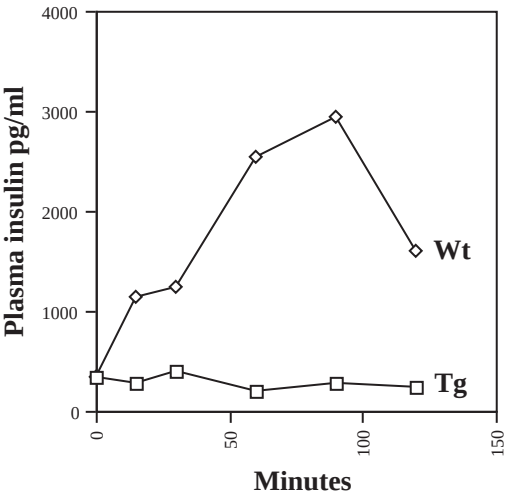

Fig. 8. Glucose fails to stimulate insulin secretion in transgenic islets. Plasma insulin (pg/ml) was measured by RIA. Wt, wild type; Tg, transgenic.

we analyzed three markers associated with mature functional β cells: the transcription factors Pdx1 and Nkx6.1, and the glucose transporter GLUT2. Expression of Pdx1 in β cell nuclei of *pdx1^{PB}-HNF6* transgenics was indistinguishable from control animals (Fig. 9A,B). The Nkx6.1 homeodomain protein is a marker of certain neurons and mature β cells (Madsen et al., 1997; Oster et al., 1998), and is genetically downstream of *pdx1* in pancreatic development (Ahlgren et al., 1998). In islets in which *pdx1* has been inactivated in a β cell-specific manner, Nkx6.1 is almost completely absent, and it has been suggested that the many insulin$^+$/glucagon$^+$ cells in these islets indicate that Nkx6.1 normally represses *glucagon* transcription in β cells (Ahlgren et al., 1998). Nkx6.1 was expressed in β cells of *pdx1^{PB}-HNF6* Tg islets (Fig. 9C,D), consistent with the lack of insulin/glucagon co-expression in these cells (see above). Thus, we conclude that core cells in transgenic islets have retained at least three markers characteristic of true β cells: insulin, Pdx1, and Nkx6.1.

GLUT2 is the high K_m glucose transporter expressed within β cells (Orci et al., 1989; Thorens et al., 1988). Intracellular glucose transport is the first step in the glucose-sensing mechanism of the β cell, and is critical for insulin secretion. In wild-type islets, GLUT2 is localized to the β cell membrane (Fig. 9E,F). In striking contrast, GLUT2 expression was undetectable in 58% (n=20) of H and L Tg islet sections (Fig. 9G), while 42% contained small clusters of core cells expressing GLUT2 at low levels. The H line Tg islet shown in Fig. 9H contained the greatest number of GLUT2$^+$ cells detected; the remaining GLUT2$^+$ islets contained many fewer expressing cells (data not shown). If, as in several animal models of diabetes, the loss of GLUT2 in *pdx1^{PB}-HNF6* transgenics was secondary to prolonged hyperglycemia, then Tg islets would be expected to contain large glycogen stores (Jansson et al., 1995; Leahy et al., 1992; Marynissen et al., 1990). Analysis by PAS staining, however, revealed little to no intracellular glycogen in islets from two wild-type and three Tg animals (data not shown), indicating that loss of GLUT2 is probably not dependent on longstanding prior hyperglycemia. To determine whether loss of GLUT2 was an early response

to the *HNF6* misexpression during embryonic pancreas formation, we also examined wild-type and Tg embryos at 18.5 d.p.c. In wild-type embryos, GLUT2 was readily detected in developing islets (Fig. 9I). The majority of Tg islet clusters (81%) contained undetectable levels of GLUT2 (e.g. Fig. 9J). In the remaining 19% of forming Tg islets, low levels of GLUT2 expression were observed (data not shown).

DISCUSSION

The timing of *HNF6* downregulation at the onset of islet ontogeny lead us to propose that transient *HNF6* expression in endocrine cells is crucial for normal islet morphogenetic processes. We analyzed the significance of *HNF6* downregulation during islet development by using a *pdx1* gene enhancer to extend the normal time frame of HNF6 expression in pancreatic endocrine cells. *pdx1^{PB}-HNF6* transgenic mice are diabetic, and show dramatic alterations in islet morphogenesis, final architecture, and function. While the phenotype of Tg animals is ultimately diabetes, our studies trace this 'disease state' back to a chain of events initiated during embryogenesis, at the time when HNF6 is normally silenced in developing islets. In general, our results suggest that downregulation of *HNF6* expression at late gestation is essential for the establishment of appropriate numbers of the different endocrine cell types, their proper sorting out and subsequent function.

The diabetic phenotype of *pdx1^{PB}-HNF6* transgenics most closely resembles human maturity-onset diabetes of the young (MODY): a Type II (adult onset) form of diabetes characterized by a generally younger age of onset (<25 years) and an autosomal dominant mode of inheritance (Tattersall, 1998, 1974). The *pdx1^{PB}-HNF6* transgenics show diabetic symptoms soon after weaning, prior to the onset of sexual maturity, and like MODY diabetics, show a decreased or absent GSIR. To date, most of the genetic lesions resulting in MODY are associated with mutations in transcription factors expressed in β cells, including *HNF1α, HNF1β, HNF4α,* and *pdx1*. Our studies suggest that abnormal upregulation of *HNF6* in islet endocrine cells in humans could also contribute to the MODY phenotype. This transgenic model provides a system for dissecting early events of islet morphogenesis, as well as studying secondary complications of prolonged, untreated diabetes on organs such as the eye and kidney.

Maintenance of islet *HNF6* expression

The 1 kb *pdx1* promoter/enhancer fragment used here directs expression of reporter genes specifically to islet cell lineages both in vitro and in vivo (Wu et al., 1997; this study). The detection of pancreatic endocrine-specific expression of the *pdx1^{PB}-lacZ* reporter as early as 11.5 d.p.c. (M. G. and C. V. E. W., unpublished observations), suggests strongly that expression of the *pdx1^{PB}-HNF6* transgene is likely to initiate at the time when the first endocrine cells are emerging from the pancreatic epithelium. The reduced litter sizes from Tg animals limit our ability to perform embryonic analyses that address issues such as the early proliferation of peripheral endocrine cell types. Nevertheless, analysis of pancreata from litters obtained at early stages of endocrine cell clustering (18.5 d.p.c.) showed obvious dysmorphogenesis (larger coalescing islets and increased numbers of glucagon-positive cell clusters) and reduced GLUT2 expression. Although we interpret these results cautiously in light of the currently small sample size, they suggest that *HNF6* overexpression in the embryo begins to affect morphogenesis and gene expression at the earliest stages of islet ontogeny.

Etiology of the diabetic phenotype in *pdx1^{PB}-HNF6* transgenics

How might HNF6 overexpression disrupt islet morphology and cause diabetes? It is unlikely that misexpression of HNF6 affects the expression of only a single gene, resulting in the observed transgenic phenotype. HNF6 may act as a transcriptional modulator of gene pathways regulating islet morphogenesis. In the liver, HNF6 activates transcription of *HNF3β* (Landry et al., 1997; Samadani and Costa, 1996), a factor previously linked to endoderm/pancreatic differentiation and function (Ang and Rossant, 1994; Weinstein et al., 1994; Wu et al., 1997). It is unlikely, however, that the effect in *pdx1^{PB}-HNF6* transgenics is mediated by HNF3β, since its expression appears grossly unaffected in Tg islets (M. G. and C. V. E. W., unpublished observations). At present, the target genes for HNF6 in endocrine development are not known. We are, however, investigating the possibility that HNF6 alters expression of cell adhesion molecules in pancreatic endocrine cells (see discussion below).

Prior to its downregulation, *HNF6* is expressed in cells that have already committed to a specific endocrine cell fate and are hormone monopositive (Rausa et al., 1997). One possibility is that the precise level of HNF6, in combination with other factors, plays an instructive role in peripheral (α, δ, PP) cell fate determination. A potential outcome of maintaining *HNF6* expression beyond 18.5 d.p.c. would be the activation of α, δ, or PP markers within cells already committed to the β cell fate, thereby leading to the establishment of cells with intermediate or indeterminate fates. The lack of glucagon expression in Tg insulin$^+$ cells, however, suggests that HNF6 does not simply activate the glucagon gene in β cells. Alternatively, maintaining *HNF6* within fully committed islet endocrine cells might specifically stimulate overproliferation of peripheral endocrine cell types relative to β cells. Testing for increased peripheral cell-type proliferation in *pdx1^{PB}-HNF6* transgenics awaits the availability of embryos for BrdU incorporation studies.

At the current level of analysis, we cannot eliminate the possibility that *pdx1^{PB}-HNF6* results in *HNF6* overexpression within early, uncommitted or multipotential endocrine precursors. Increased HNF6 in such cells might favor the development of a peripheral cell type, altering the balance of differentiated endocrine cell types. Regardless of the mechanism, abnormal proportions of islet cell types might affect their ability to organize into proper islets.

GLUT2 levels may affect glucose homeostasis in *pdx1^{PB}-HNF6* transgenics

The lack of GLUT2 expression in Tg β cells might result directly from cell-autonomous transcriptional repression by overexpressed HNF6. HNF6 has been shown in some cases to act as a repressor of transcription (Pierreux et al., 1999), and the GLUT2 promoter contains a potential HNF6 binding site (R. H. C., unpublished observations). Alternatively, a partial loss of β cell identity resulting from *HNF6* misexpression in these cells could secondarily affect GLUT2 expression.

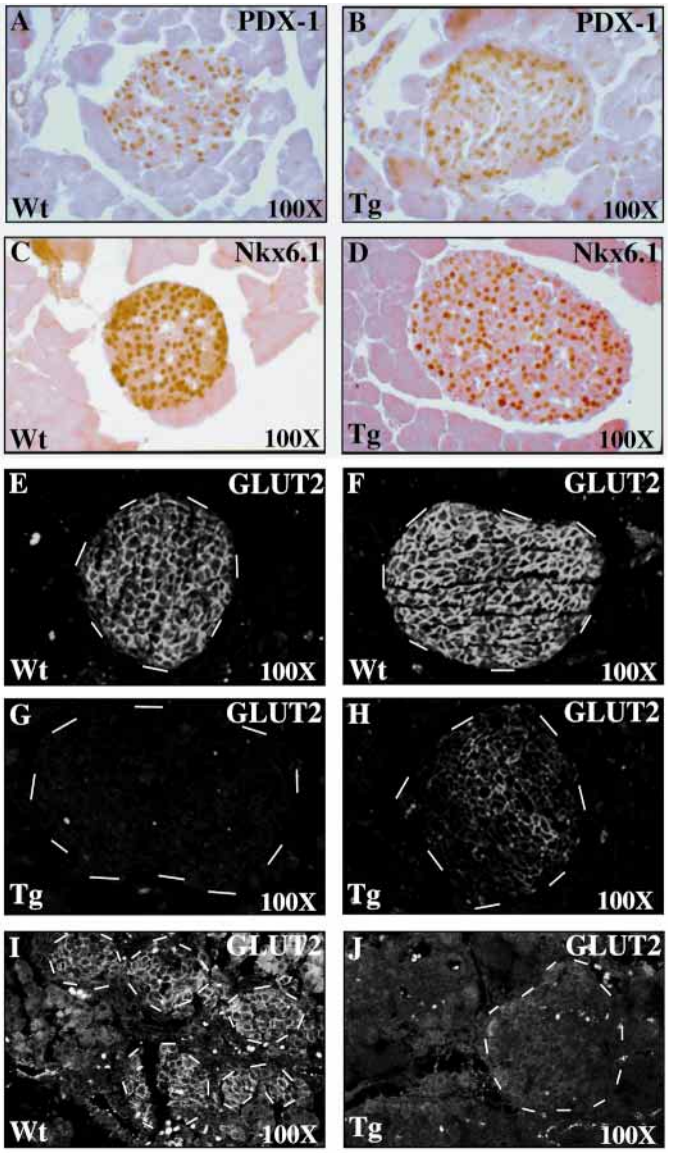

Fig. 9. Partial loss of the β cell phenotype in transgenic islets. (A,C,E,F) Adult wild type (Wt). (B,D,G,H) Adult transgenics (Tg). (A,B) Pdx1 expression (brown). (C,D) Nkx6.1 expression (brown). (E-J) GLUT2 expression outlines β cells. (G,J) L line. (H) H line. (I) 18.5 d.p.c Wt. (J) 18.5 d.p.c Tg. Sections in A-D were counterstained with eosin. Dotted lines outline islets.

Another factor potentially contributing to GLUT2 downregulation in Tg islets may be the abrogation of β cell-β cell contacts in the mixed islets. In normal islets, GLUT2 is found on microvilli at the site of β cell-β cell contacts, and is absent from surfaces facing peripheral islet endocrine cells, blood vessels, or spaces between cells (Orci et al., 1989). As discussed below, the intercalation of peripheral islet cell types and increased vascularization in Tg islets would effectively reduce the sites of β cell-β cell contact that would normally express GLUT2.

In animal models of both Type I and Type II diabetes, reduced GLUT2 expression is correlated with hyperglycemia and loss of glucose-stimulated insulin release (GSIR). For example, in mice treated with the β cell toxin streptozotocin, loss of GLUT2 expression is observed prior to overt effects of the toxin, i.e. hyperglycemia and islet inflammation (Wang and Gleichmann, 1995), suggesting that reduced GLUT2 expression is a primary defect leading to diabetes. This idea is supported by the hyperglycemia and inability to clear blood glucose in transgenic mice expressing antisense GLUT2 in β cells (Valera et al., 1994), and the diabetes and reduced insulin secretion observed in mice homozygous for a null mutation in GLUT2 (Guillam et al., 1997). The role of reductions in GLUT2 levels as a causative factor in diabetes remains controversial, however, as even dramatic reductions in GLUT2 may not be rate-limiting for glucose transport and induction of β cell-specific physiological responses (Tal et al., 1992; Zangen et al., 1997). Thus, observations of decreased GLUT2 do not necessarily account entirely for a decreased GSIR.

In some glucose toxicity animal models, intracellular accumulations of glycogen in affected islets have been interpreted as evidence for a massive upregulation of glucose uptake prior to the loss of GLUT2, in an attempt to compensate for the overabundant circulating glucose (Gepts and Lecompte, 1981; Inoue et al., 1994; Jansson et al., 1995). The loss of GLUT2 expression in Tg islets at 18.5 d.p.c. and the absence of significant glycogen storage in adult Tg islet supports our hypothesis that loss of GLUT2 is a causative factor in the diabetes seen in these animals. Nevertheless, the dramatic reductions in GLUT2 expression and failure to release insulin in response to glucose, indicate a partial loss of the β cell phenotype in Tg islets, and suggest that defects in glucose sensing contribute to the observed diabetic phenotype.

Islet morphogenesis and endocrine cell function

In addition to cell-autonomous alterations in endocrine cell function, inappropriate physiological interactions of core β cells with intermingled peripheral cell types could severely perturb islet physiology and contribute to the diabetes in *pdx1^{PB}-HNF6* transgenics. The intimate association of β cells with one another in normal islets is highlighted by gap junctional communication and electrical coupling between β cells (Bennett and Goodenough, 1978; Meissner, 1976). In addition to affecting the expression of GLUT2 (discussed above), minimal alterations in β cell-β cell contact markedly affect insulin output; in vitro, basal insulin secretion in single β cells from dispersed islets is normal, but GSIR is absent (Bosco et al., 1989). Basal insulin secretion is synergistically enhanced and GSIR is re-established when β cells contact each other, but not non-β cells (Bosco et al., 1989; Halban et al., 1982). Both the mixing of islet cell types and increased vascularization in Tg islets may contribute to decreased β cell contacts in *pdx1^{PB}-HNF6* transgenics. The basal insulin secretion and lack of GSIR in *pdx1^{PB}-HNF6* transgenics indicates that Tg β cells behave similarly to physically isolated β cells.

The vascular organization and directional blood flow within islets from the core to the mantle (i.e. β→α →δ) is also critical for normal islet cell interactions and regulated hormone secretion (Samols et al., 1988). The lack of a distinct core/mantle structure, and the increased vascularization in *pdx1^{PB}-HNF6* transgenics might therefore disrupt hormonal communication between islet cell types. A net reduction in functional β cell-β cell interactions would abrogate the ability of β cells to respond to external glucose in a concerted manner.

The importance of local environment on islet architecture and the correlation of morphology with function was underscored in the analysis of p48 homozygous mutants, where loss of the endocrine pancreaas resulted in individual islet endocrine cells scattered throughout the spleen. Consistent with results from cultured, dispersed islets, the isolated β cells within the spleen secrete very low levels of insulin (Krapp et al., 1998).

Endocrine cell adhesion and islet morphogenesis

Altered cell-cell or cell-substratum interactions in *pdx1^{PB}-HNF6* transgenics could explain many of the aspects of the transgenic phenotype: the lack of migration of endocrine cells away from the ductal epithelium, the failure of these cells to separate into islets of typical size and shape, the scattering of peripheral cell types within the islet, and, as mentioned above, the reduction in GLUT2. For example, there is some evidence that regulated expression of CAM and cadherin families of cell adhesion molecules is critical for budding of endocrine precursors from the pancreatic ducts and islet morphogenesis (Cirulli et al., 1994, 1998; Dahl et al., 1996; Esni et al., 1999; Rouiller et al., 1990). During islet development in humans, epithelial cell adhesion molecule (EpCAM) is downregulated in forming islet clusters and mature islets compared to the ductal epithelium and newly budding endocrine cells (Cirulli et al., 1998). The increase in islet hormone-positive cells within the ducts in *pdx1^{PB}-HNF6* transgenics suggests that the budding of these cells from the ductal epithelium is delayed by HNF6 expression, possibly due to persistent expression of a ductal cell adhesion molecule related to EpCAM, and/or delayed or reduced activation of a specific islet cell adhesion molecule. An intriguing possibility is that these cells are 'captured' in what would normally be a very transient state, suggesting that *pdx1^{PB}* may be initiated in new endocrine cells as they begin to leave the ductal epithelium. The fact that Tg embryos show islet defects at 18.5 d.p.c., further supports the idea that early stages of islet formation are impacted by HNF6 overexpression in endocrine cells.

Subsequent to the budding of endocrine cells from the duct and the initial clustering into islet structures, differential expression of cell adhesion molecules such as NCAM, may regulate sorting of endocrine cells within the islet. For example, NCAM levels are approximately 2-fold higher on non-β cells than β cells (Rouiller et al., 1990), and α cells in NCAM heterozygotes and null mutant mice infiltrate the islet core (Esni et al., 1999) similar to the dispersal of α cells in *pdx1^{PB}-HNF6* transgenics. In contrast to *pdx1^{PB}-HNF6* transgenics, however, δ and PP cells are localized normally in NCAM mutant animals, there is no increase in the number of α, δ, or PP cells, and NCAM mutants maintain normoglycemia (Esni et al., 1999). The more severely disrupted islet morphogenesis in *pdx1^{PB}-HNF6* transgenics (impaired budding from ducts, larger misshapen islet clusters, and lack of endocrine cell sorting) suggests that HNF6 overexpression impacts the expression of multiple cell adhesion molecules, both at early and late stages of islet morphogenesis.

Islet morphogenesis requires programmed downregulation of *HNF6*

Reverse genetic approaches have recently begun to uncover the genetic networks regulating the generation, proliferation and differentiation of the various pancreatic lineages (reviewed in Edlund, 1998; Gannon and Wright, 1999). These studies have particularly focussed on transcription factors expressed in mature endocrine or exocrine cells that are known to activate cell-type-specific genes. The emerging paradigm is that temporally regulated expression of different combinations of these factors controls the differentiation pathway of islet or acinar precursors. In such a model, not only the loss but the reduction of a critical component can alter the balance of pancreatic lineages. For example, mice with only a single functional *pdx1* allele have twice as many α cells as wild-type mice, although the core/mantle organization is not disturbed (Dutta et al., 1998). In addition, complete loss of *Pax4* results in loss of both β and δ cell compartments, and an associated increase in the number of α cells. In contrast to studies demonstrating that the presence of a factor is required for normal pancreatic development, here we show that the programmed downregulation of a particular factor, HNF6, is essential for normal cell fate allocation, gene expression, morphogenesis and function of pancreatic islets.

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