TISSUE-SPECIFIC REGULATION AND FUNCTION OF *PANCREATIC AND*

DUODENAL HOMEOBOX 1

By

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CHAPTER I

INTRODUCTION

The mammalian pancreas arises from separate ventral and dorsal evaginations of

foregut endoderm that develop posterior to the stomach anlage and subsequently fuse to form

the mature pancreatic organ (Fig. 1.1; reviewed by Slack, 1995). Endoderm-derived

precursors in the early pancreatic epithelium differentiate to give rise to acinar cell clusters,

which produce digestive enzymes, a ductal network that transports the enzymes to the

duodenum, and the endocrine islets of Langerhans, spherical clusters of hormone-producing

cells interspersed amongst the exocrine pancreatic tissue (i.e., the acinar cells and ducts).

The pancreatic islets are derived from common endocrine progenitor cells (Gu et al.,

2002) that differentiate to produce the four major pancreatic endocrine cell lineages -- the

insulin-producing β cells, glucagon-producing α cells, somatostatin-producing δ cells, and

pancreatic-polypeptide-producing PP cells -- in a ratio of approximately 75:15:6:4 (Cabrera

et al., 2006; Brissova et al., 2005). In mice, the pancreatic islets are stereotypically organized

with the β cells in the core of the sphere and the other three cell types around the periphery

(Fig. 1.1E). Because the islets are a mammal's major source of insulin and glucagon

production, they are essential for maintenance of glucose homeostasis. The pathways that

regulate pancreas development have attracted clinical interest because mutations in

components of these pathways have been linked to congenital disorders resulting in heritable

forms of diabetes mellitus (reviewed by Bell and Polonsky, 2001). More recently,

understanding the embryonic regulation of pancreas development has come to be viewed as
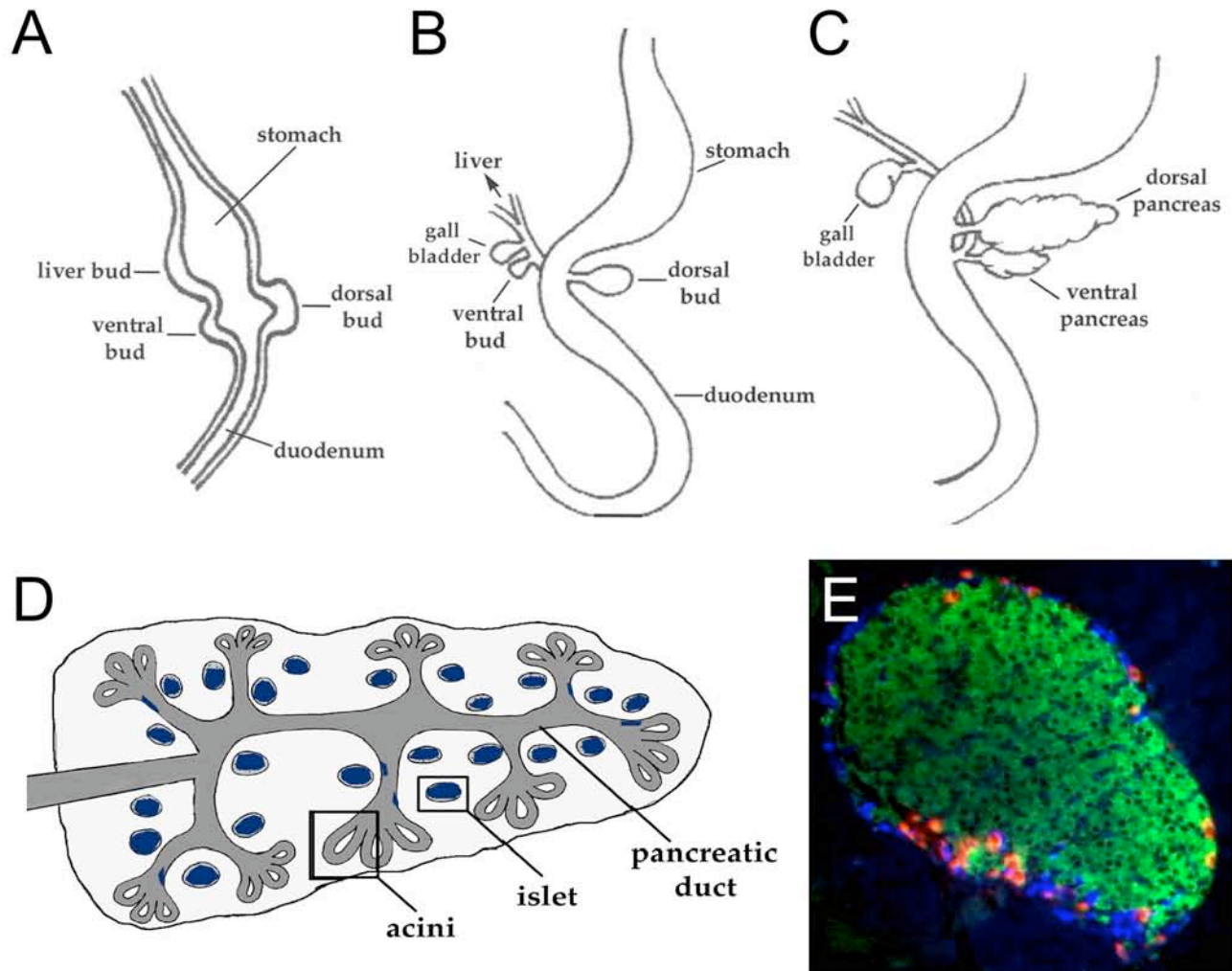
Fig. 1.1 Schematic overview of murine pancreas morphogenesis and differentiation.
(A) The morphological ventral and dorsal pancreatic buds are distinguishable by E9.5 immediately posterior to the liver and stomach anlagen. (B, C) Over the course of the next 2 days of development the pancreatic buds come to lie on the left side of the primitive duodenum, and will eventually fuse to form a single organ. (D) The mature pancreas comprises exocrine acini that secrete digestive enzymes, exocrine ducts that transport the enzymes to the duodenum, and endocrine islets that secrete hormones in response to vascular and neural input. (E) Mature murine pancreatic islet with a central core of β cells surrounded by α, δ and PP cells. Stained for insulin (green), glucagon (blue), somatostatin (red) and PP (red). Panels (A – D) reprinted from Gannon and Wright, 1999. Panel (E) downloaded from http://seungkimlab.stanford.edu

essential for developing techniques to generate functional, transplantable β cell replacements from cultured progenitor cells (reviewed by Keller, 2005).

The homeodomain transcription factor Pancreatic and Duodenal Homeobox 1 (Pdx1), a member of the Parahox family of Hox-like transcription factors (Brooke et al., 1998), has emerged as a central node in the genetic network governing ontogenesis of the pancreas and neighboring tissues of the posterior foregut (e.g., Fig. 1.2; reviewed by McKinnon and Docherty, 2001; Shih and Stoffel, 2001; Servitja and Ferrer, 2004). The diversity of inputs regulating Pdx1 expression and the pleitropic roles of Pdx1 during foregut development make it an attractive model for elucidating the principles governing tissue-specific gene expression and cell fate differentiation.

Recent large-scale efforts to characterize the *cis*-regulatory motifs that regulate tissue-specific gene expression have revealed a general trend that *cis*-elements governing expression in mature tissues are often much shorter and less complex than the sequences that regulate expression during embryogenesis (reviewed by Ochoa-Espinoza and Small, 2006). The main reason for this disparity is probably that mature tissues typically express a core set of transcription factors that activate many of the genes involved in the adult function of that tissue, whereas embryological transcriptional regulation requires interpretation of morphogen gradients and fluctuating levels of broadly expressed and overlapping transcription factors to specify distinct cell and tissue fates. This model of *cis*-regulatory complexity aptly describes the regulation of *Pdx1*. Sequences of as little as 200 bp from the 5' non-coding region of *Pdx1* have been shown to be sufficient to promote reporter gene expression specifically in β cells of adult mice (Samaras et al., 2002). By contrast, there are more than 2 kb of non-coding sequence in the *Pdx1* locus showing greater than 70% identity between mouse and
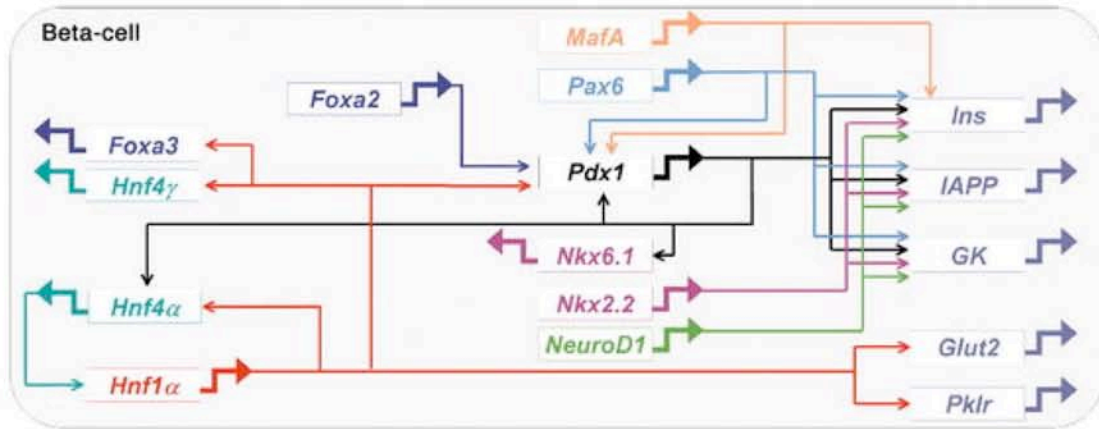
Fig. 1.2 Pdx1 is a central node in the genetic regulatory network of pancreatic β cells. Known transcriptional regulatory interactions in mature β cells are summarized as a network diagram. The central position of Pdx1 has made it a focus of efforts to differentiate cultured progenitor cells to β cell fate. Reprinted from Servitja and Ferrer, 2004.

human sequences (see Fig. 1.6), a level of conservation suggesting potential regulatory function (e.g., Gerrish et al., 2000). Diverse subsets of these conserved sequences have been shown to recapitulate aspects of the expression pattern of Pdx1 in developing endoderm, suggesting that the developmental expression of Pdx1 is regulated cooperatively by multiple spatially segregated elements rather than through a single core enhancer element.

The main focus of my thesis research was to characterize further the *cis*-regulatory regions required for tissue-specific expression of Pdx1 and to identify additional roles of Pdx1 in the diverse developing and mature tissues where it is expressed. Subsequent sections of this introduction review (1) the early patterning events that regulate expression of Pdx1 in the posterior foregut and pancreatic buds, (2) the *cis*-elements of *Pdx1* that regulate its transactivation, (3) the developmental and physiological functions of Pdx1, and (4) the specific aims of my thesis research.

Part I -- Early patterning of the endoderm and induction of Pdx1 expression

Initiation of Pdx1 expression

Pdx1 is the earliest factor known to be expressed specifically in the nascent pancreatic buds, although its domain of expression soon expands to include the endodermal progenitors of the caudal stomach, rostral duodenum and common bile duct (reviewed by Gannon and Wright, 1999). The mechanism by which Pdx1 expression is initiated and restricted to foregut endoderm remains largely unknown. The first endodermal precursors to migrate through the primitive streak give rise to anterior endoderm and later migrating precursors give rise to posterior endoderm (reviewed by Wells and Melton, 1999). By the

end of gastrulation, intrinsic anterior-posterior (AP) polarity of the definitive endoderm is already apparent, based on the regionalized expression of *Cerl* (Bouwmeester et al., 1996), *Otx1* (Rhinn et al., 1998), *Hex* (Thomas and Beddington, 1996), IFABP (Green et al., 1992) and *Cdx2* (Beck et al., 1995) in E7.5 mouse embryos. Pdx1 is first detected in ventral endoderm at the lip of the anterior intestinal portal at the 7 somite stage (~E8.0) (Fig. 1.3; Gannon and Wright, 1999). Deutsch et al. (2001) isolated this region of endoderm at the 2 – 6 somite stage and demonstrated that it autonomously activated Pdx1 after 2 days in culture. A similar result was obtained from cultures of chick ventral endoderm, and it was shown that only endoderm from the appropriate AP level would activate Pdx1 expression in culture, indicating that regional patterning of ventral pancreatic fate occurs prior to the onset of Pdx1 expression (Kumar et al., 2003). Deutsch et al. also showed that the expression of Pdx1 could be abrogated in favor of liver gene activation by co-culture with cardiogenic mesoderm, which suggested that the anterior extent of the ventral Pdx1 expression domain may be limited by the heart-forming region. The effect of cardiogenic mesoderm on ventral endoderm culture could be mimicked by FGF2, which had been previously shown to inhibit Pdx1 expression in frog embryos (Gamer and Wright, 1995).

Unlike the ventral pre-pancreatic endoderm, the dorsal pre-pancreatic endoderm did not activate Pdx1 expression in culture (Deutsch et al., 2001). This difference appeared to correlate with the induction of Shh expression in cultured dorsal endoderm but not in ventral pre-pancreatic endoderm. It had previously been shown that inhibition of expression of Shh in dorsal endoderm is required for induction of dorsal Pdx1 expression in chick (Hebrok et al., 1998). Repression of Shh is normally provided by signals from the notochord (Hebrok et
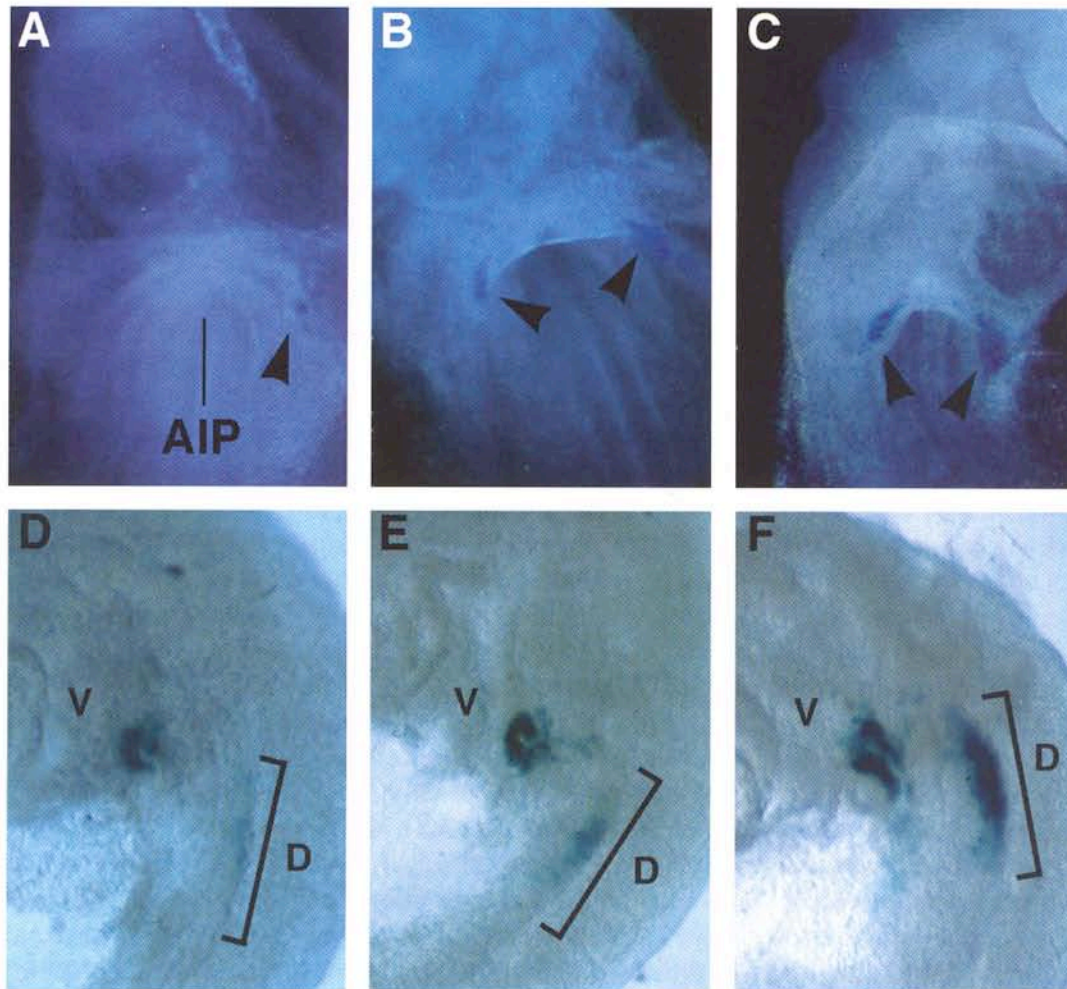
Fig. 1.3 Expression of Pdx1 during early stages of posterior foregut development. Whole-mount XGal staining of E8.0 – E9.5 *Pdx1*^{lacZ/+} mouse embryos. (A – C) Ventral views of 7, 8 and 9 somite stage embryos. Arrowheads indicate Pdx1 expression. (D – F) Lateral views of E9.0 – E9.5 embryos. AIP, anterior intestinal portal; D, dorsal; V, ventral. Reprinted from Gannon and Wright, 1999.

al., 1998, Kim et al., 1997), which could be mimicked by activin or low levels of FGF2. High levels of FGF2 inhibited Pdx1 expression and enhanced Shh expression (Hebrok et al., 1998). *Ptc*$^{-/-}$ mice do not activate Pdx1 expression, which implies that overactive Hedgehog signaling inhibits initiation of Pdx1 expression in mice as in chickens (Hebrok et al., 2000). Although the Hedgehog inhibitor cyclopamine induced formation of ectopic pancreatic tissue in the chick stomach and duodenum, the Pdx1 expression domain was not notably expanded after cyclopamine treatment (Kim and Melton, 1998) nor in *Shh*$^{-/-}$ mice (Hebrok et al., 2000). Along with the finding that notochord could not induce Pdx1 expression in non-pancreatic endoderm (Kim et al., 1997), these results indicate that repression of Shh is permissive but not instructive for Pdx1 expression (Fig. 1.4).

Inductive signals from mesoderm

Studies in frog (Horb and Slack, 2001) and chick (Kumar et al., 2003) have shown that lateral plate mesoderm (LPM) from the AP level of the pancreatic buds can induce Pdx1 expression in anterior regions, but not posterior regions, of early endoderm. Futhermore, hindgut LPM was shown to inhibit pancreas differentiation and induce expression of the intestinal transcription factor Cdx in the pancreatic endoderm (Kumar et al., 2003). These studies suggest that regional patterning of the mesoderm instructs the fate of underlying endoderm, a model that is reminiscent of previous reports of the nested pattern of Hox gene expression in the hindgut mesoderm imparting regional patterning on the endoderm (Fig. 1.4; reviewed in Roberts, 2000). More recently, mesodermal expression of Sox9 and Nkx2.5 has been shown to be instructive for differentiation of pyloric sphincter endoderm (Theodosiou and Tabin, 2005), and expression of Barx1 in mesoderm of the developing stomach is
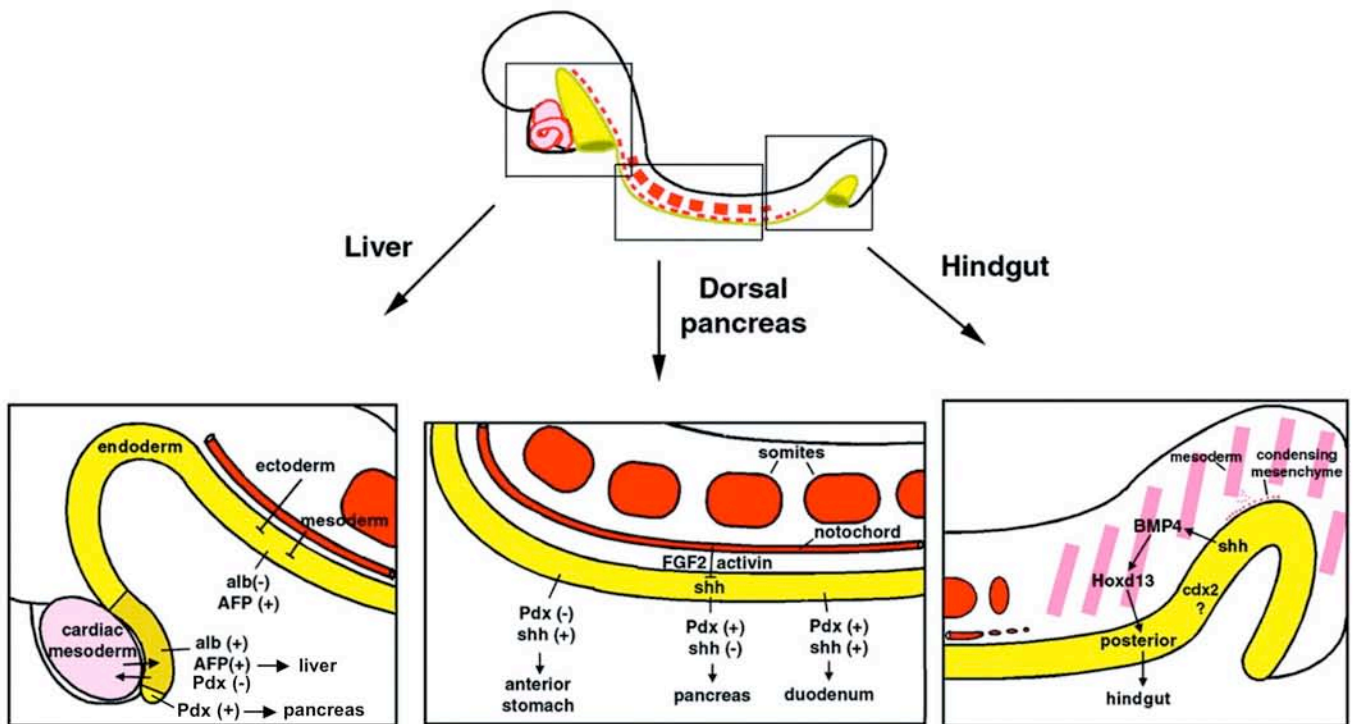
Fig. 1.4 Interactions between endoderm and mesoderm pattern the gut tube.
Schematic of ~E8.5 mouse embryo focusing on nascent liver, dorsal pancreas, and hindgut regions. *Left panel*, signals from cardiac mesoderm induce liver gene expression and repress ventral pancreatic differentiation. Signals from mesoderm and ectoderm repress liver differentiation in dorsoanterior endoderm. *Middle panel*, signals from notochord repress Shh expression in prospective dorsal pancreatic bud, thereby permitting dorsal bud outgrowth and Pdx1 expression. *Right panel*, crosstalk between endoderm and splanchnic mesoderm patterns the hindgut. Shh from endoderm induces BMP4 in neighboring mesoderm. BMP4 induces mesodermal Hoxd13 expression, which activates signals inducing region-appropriate differentiation of the underlying endoderm. Endoderm, yellow; notochord and somites, red; cardiac mesoderm and posterior mesoderm, pink. Modified from Wells and Melton, 1999.

required for differentiation of stomach endoderm (Kim et al., 2005). Thus, crosstalk between mesoderm and endoderm seems to be a general mechanism for patterning of the gut and is probably instructive for positioning the pancreatic buds and inducing expression of Pdx1.

One candidate proposed to be a critical mesodermal signal promoting Pdx1 expression is retinoic acid (RA). RA signaling from mesoderm to endoderm has been shown to be necessary and sufficient for formation of insulin-producing cells in zebrafish (Stafford et al., 2006). Ectopic RA can induce Pdx1 expression in anterior endoderm in zebrafish (Stafford and Prince, 2002) and chick (Kumar et al., 2003), although it requires the presence of additional permissive mesodermal factors. *Raldh2$^{-/-}$* mice are deficient in production of RA and fail to initiate dorsal Pdx1 expression, although expression in the ventral pancreatic bud appears to be unimpaired (Martin et al., 2005; Molotkov et al., 2005). These results indicate that RA signaling is an important component for establishment of the Pdx1 expression domain, but given the broad expression of RA in posterior tissues of the embryo, it is unlikely that RA alone can account for the precise positioning of the pancreatic buds.

After initiation of Pdx1 expression, signals from neighboring mesodermal structures continue to be important for maintenance of Pdx1 expression and pancreatic outgrowth. The LIM homeodomain transcription factor Isl1 begins to be expressed in the dorsal pancreatic bud and dorsal pancreatic mesenchyme shortly after onset of Pdx1 expression. In *Isl1$^{-/-}$* mice, the dorsal pancreatic mesenchyme is absent, resulting in loss of Pdx1 expression and aborted outgrowth of the dorsal bud (Ahlgren et al., 1997). The supportive role of the dorsal mesenchyme appears to be mediated at least in part by FGF10 (Bhushan et al., 2001). Signals from the endothelial cells of the dorsal aorta are also required for maintenance of Pdx1 expression and dorsal bud outgrowth (Yoshitomi et al., 2004; Lammert et al., 2001),

although endothelial cells do not appear to be required for early stages of ventral bud development (Yoshitomi et al., 2004).

Maturation of the Pdx1 expression domain

Pdx1 is expressed in all epithelial cells of the pancreatic bud except for the early differentiating glucagon and insulin cells (Ahlgren et al., 1996). The early hormone-positive cells do not appear to be the progenitors of the mature islets, as genetic lineage tracing experiments indicate that Pdx1 is expressed by progenitors of all of the endoderm-derived components of the mature pancreas (Gu et al., 2002). Between E9.5 and E12, expression of Pdx1 spreads to include the epithelial progenitor cells of the antropyloric stomach, the rostral duodenum and the common bile duct (Fig. 1.5; Offield et al., 1996). As embryogenesis progresses, Pdx1 is upregulated in differentiating β cells and downregulated to lower levels of expression in the exocrine pancreas and gut epithelia (Fig. 1.5; Guz et al., 1995; Offield et al., 1996). In adult mice, Pdx1 is highly expressed in all pancreatic β cells and some δ (somatostatin-producing) cells. It is expressed at a low level in pancreatic acinar cells, some pancreatic duct cells, the antropyloric mucosa of the stomach, rostral duodenal epithelium, duodenal Brunner's glands and the epithelial lining of the common bile duct.
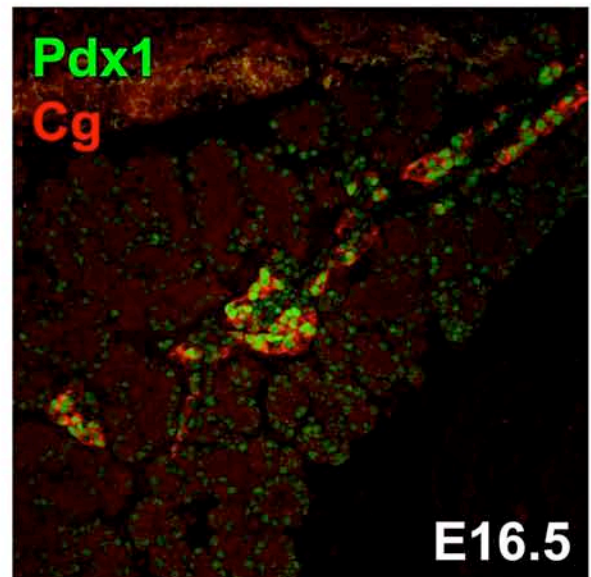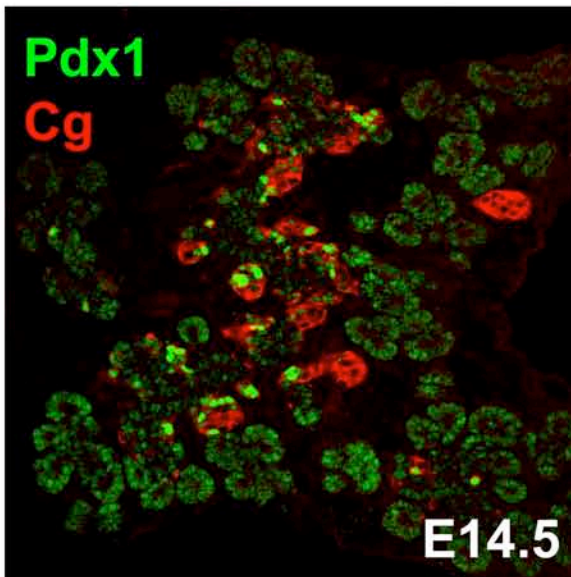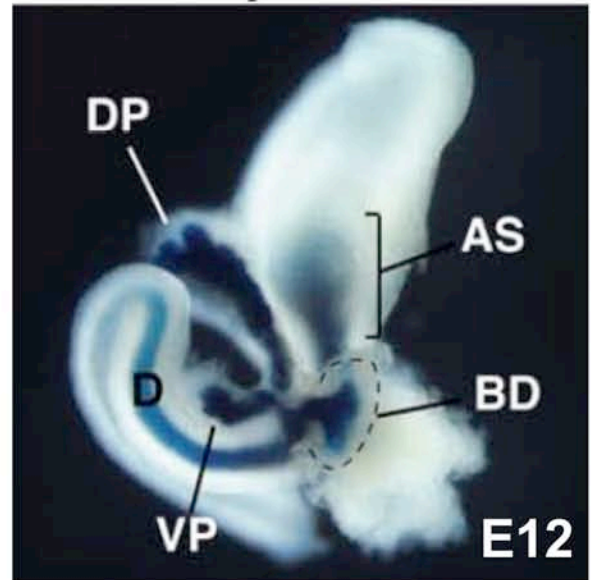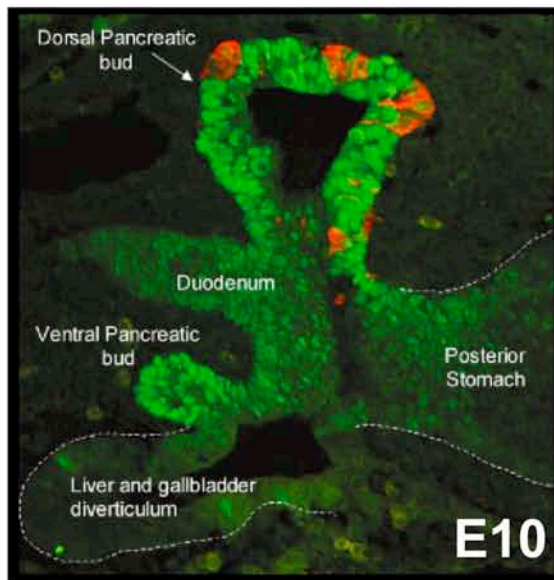
Fig. 1.5 Developmentally dynamic expression of Pdx1. At E10, Pdx1 is strongly expressed in the dorsal and ventral pancreatic buds and weakly expressed in neighboring endoderm. By E12, expression of Pdx1 has spread to include the antral stomach, common bile duct, and rostral duodenum. At E14.5, Pdx1 is strongly expressed in a subset of pancreatic endocrine cells and at a lower level in the rest of the pancreatic endoderm. By E16.5, the population of Pdx1 high-expressing endocrine cells has increased, and the level of expression of Pdx1 in acinar cells has decreased. Top right panel shows whole-mount XGal staining of $Pdx1^{lacZ/+}$ embryo. Other panels depict immunofluorescence staining for Pdx1 (green), glucagon (top left panel, red) and chromogranin (bottom panels, red). DP, dorsal pancreas; VP, ventral pancreas; AS, antral stomach; BD, bile duct; D, duodenum. Top left panel reprinted from Jensen, 2004. Top right panel reprinted from Fujitani et al., 2006.

Part II -- Regulation of Pdx1 transcription

The literature reviewed above provides compelling evidence that intercellular

signaling pathways are critical for the establishment and maintenance of Pdx1 expression.

Surprisingly little is known about regulation of the *Pdx1* promoter by the intracellular

effectors of these signaling pathways. Instead, the bulk of known regulatory interactions on

Pdx1 are mediated by transactivators expressed intrinsically in the endoderm. Whether the

activity of these transactivators on the *Pdx1* locus is modulated by intercellular signaling

pathways remains to be demonstrated.

Early studies of the *Pdx1* promoter

Initial characterization of transactivation of *Pdx1* was accomplished by *in vitro*

promoter deletion and DNase footprinting analysis of the rat *Pdx1* (*STF1*) 5' region (Sharma

et al., 1996, 1997). These studies revealed that a proximal E box consensus sequence located

-104 bp from the transcription start site is important for transcription of *Pdx1* and is bound by

the ubiquitous basic helix-loop-helix (bHLH) factor USF in β cell lines and in HeLa cells,

suggesting that it is essential for basal transcription of *Pdx1* but not for specific activity in β

cells (Sharma et al., 1996). A more distal region of the rat *Pdx1* promoter (-6200 to -5670

bp) was found to confer β-cell-specific activity. Examination of the sequence of this distal

region revealed the presence of FoxA and E box consensus sequences (Sharma et al., 1997).

DNase I protection and gel shift assays confirmed that the FoxA element was bound by FoxA

factors and that the E box was bound by a heterodimer of NeuroD/Beta2 and E47.

Furthermore, the ability of FoxA2 to activate this region could be inhibited by treatment with

the glucocorticoid dexamethasone. A more recent study compared the -6200 to -5670 bp region of rat *Pdx1* to the human, mouse and chicken *Pdx1* sequences and revealed that this region, referred to as Area IV, is evolutionarily conserved, and that the mouse and human sequences have β-cell-specific enhancer activity (Gerrish et al., 2004). Gel shift and chromatin immunoprecipitation (ChIP) assays confirmed conservation of the FoxA2 binding site originally identified in rat Area IV and also demonstrated binding of Nkx2.2 and Pdx1 itself to Area IV. The terminal G of the E box consensus (CANNTG) is changed to an A in the mouse Area IV sequence, suggesting that NeuroD/Beta2 may not be able to bind to mouse Area IV.

Independent efforts identified a more proximal region of the *Pdx1* promoter that also promotes β-cell-specific transcriptional activation. In the first study to characterize this region, three DNase hypersensitive sites were identified in *Pdx1* genomic DNA isolated from β cell lines but not NIH3T3 cells, suggesting that nucleosomes are displaced from these regions by transcription factor binding in β cells (Wu et al., 1997). One of these hypersensitive sites (-2560 to -1880 bp) promoted reporter gene activity specifically in β cells. Furthermore, a 1 kb restriction fragment overlapping this region (*PstI-BstEII*, aka PB; -2917 to -1918 bp) promoted *lacZ* reporter activity in the pancreatic islets of transgenic mice. A FoxA2 binding element was identified in this region and mutation of the FoxA2 element reduced transcriptional activity of the PB region.

Identification of evolutionarily conserved sequences

Two groups aligned mouse and human *Pdx1* sequences including approximately 4.6 kb 5' of the *Pdx1* coding region (Fig. 1.6; Gerrish et al., 2000; Marshak et al., 2000). Both
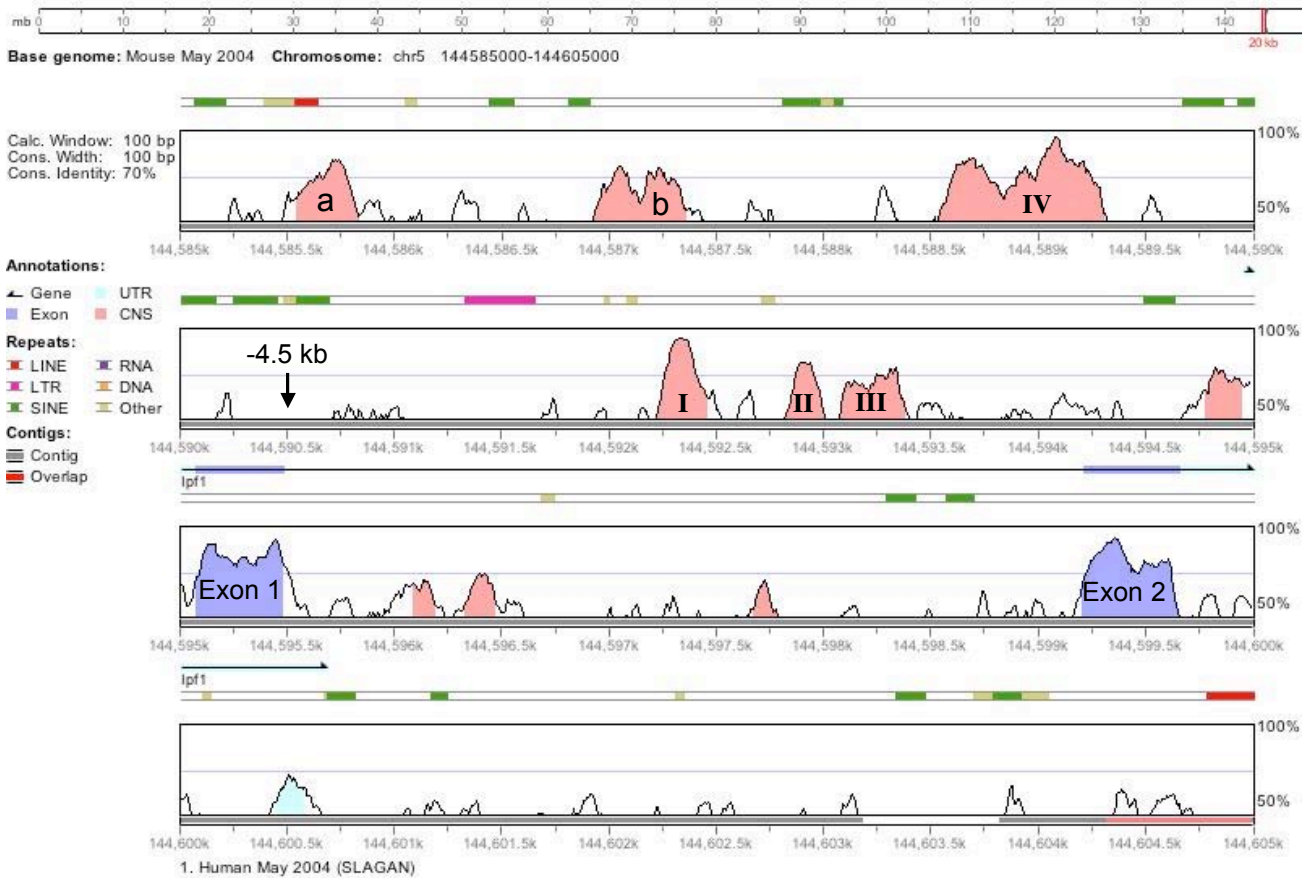
Fig. 1.6 Vista alignment of mouse and human *Pdx1* genomic sequences. Non-coding regions containing at least 100 bp of >70% identity are shaded pink, exons are shaded purple, and untranslated regions are shaded light blue. Previously characterized conserved regions are labeled with roman numerals. Two uncharacterized distal conserved regions are labeled "a" and "b". Black arrow indicates approximate position of the XbaI site at -4.5 kb that has often been used as the 5' end of *Pdx1*-promoter-driven transgenes. Alignment was generated using the Lawrence Berkeley Laboratories' online Vista browser (http://pipeline.lbl.gov).
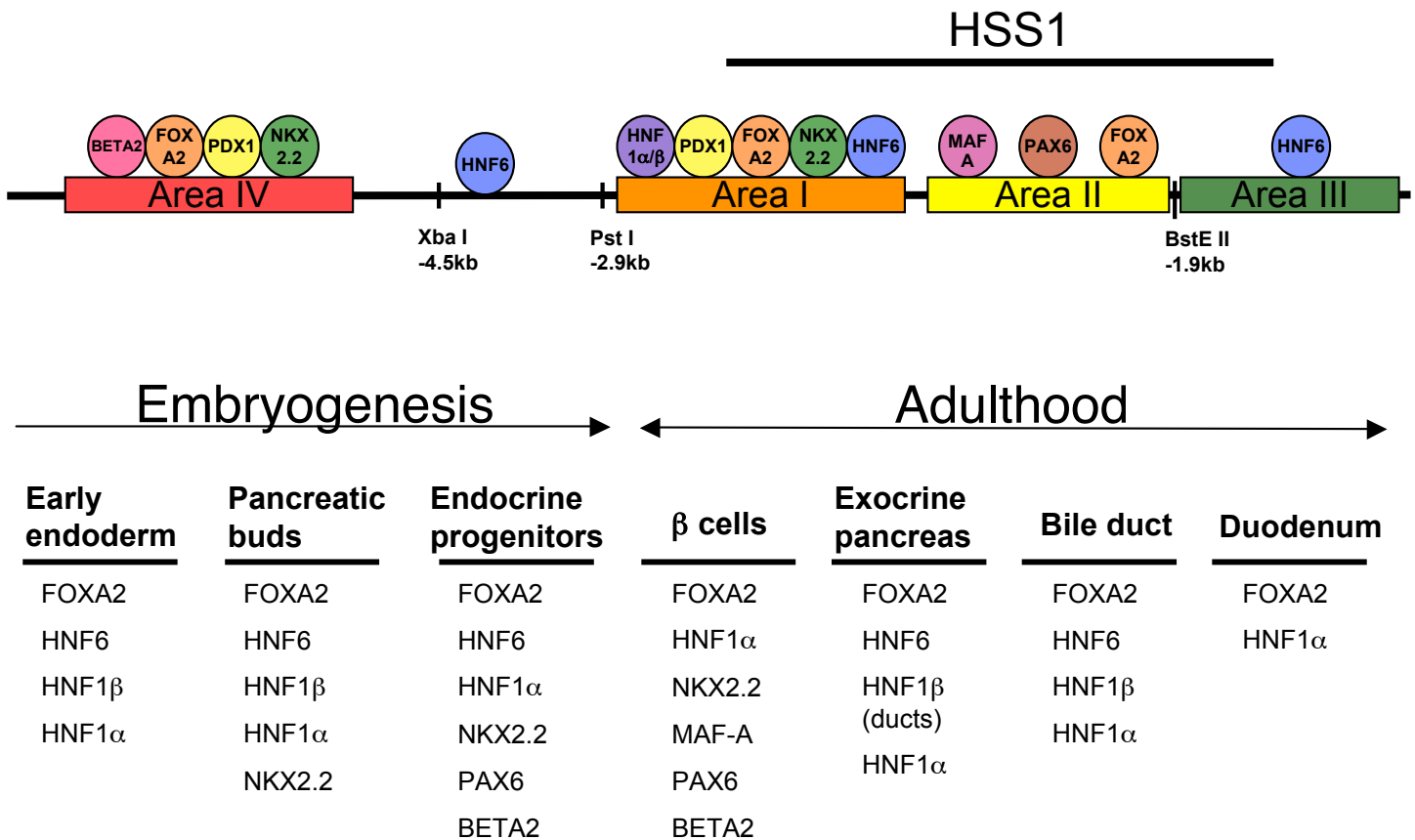
Fig. 1.7 Summary of transactivator binding to *Pdx1* promoter. *Top*, previously reported sites of protein-DNA interactions in relation to phylogenetically conserved *cis*-elements. HSS1 indicates approximate span of DNase hypersensitive site 1 (Wu et al., 1997). The PB region is delimited by the indicated PstI and BstEII restriction sites. *Bottom*, chart indicates spatiotemporal overlap of expression of transactivators of *Pdx1* with the sites where Pdx1 is expressed in mice.

groups identified four regions of greater than 75% identity, which included the sequence

around the transcription start site and three areas clustered in a 1.2 kb region overlapping the

DNase hypersensitive site characterized by Wu et al. These three areas, in order from 5' to

3', are typically referred to as Areas I, II, and III in mice and PH1, 2 and 3 in humans. Areas

I and II are contained in the PB fragment (Fig. 1.7) and each has β-cell-specific activity in

isolation (Gerrish et al., 2000; Marshak et al., 2000) and synergistic activity in combination

(Van Velkinburgh et al., 2005). FoxA2 was found to bind and activate Areas I and II

(Gerrish et al., 2000; Marshak et al., 2000). Also, Pdx1 itself was shown to bind and activate

Area I cooperatively with FoxA2, which was the first demonstration of Pdx1 autoregulation

(Marshak et al., 2000).

Subsequent block mutational analysis of subregions of Areas I and II revealed

additional sites for binding and activation of Area I by Hnf1α (Gerrish et al., 2001) and

Nkx2.2 (Van Velkinburgh et al., 2005), and of Area II by Pax6 (Samaras et al., 2002) and

MafA (Samaras et al., 2003). Binding of Hnf6 has been demonstrated by gel shift analysis of

sites in Areas I and III and also to a site midway between Areas I and IV (Jacquemin et al.,

2003). This intermediate region of the human *Pdx1* (*IPF1)* promoter was shown to bind the

transcription factors Sp1, Sp3, FoxA2 and Hnf1α, although these sites do not appear to be

conserved in the mouse sequence (Ben-Shushan et al., 2001). Interestingly, all of the binding

activities characterized on the *Pdx1* promoter are positive regulatory interactions. Promoter

deletion and mutation experiments have provided evidence of repressor elements (Sharma et

al., 1997; Samaras et al., 2002), but protein binding to these elements has not been

characterized.

Analysis of *lacZ* reporter transgenes

Activities of some of the *cis*-regulatory elements described above have been characterized in transgenic and gene-targeted mice. The shortest sequence shown to promote transgenic reporter activity selectively in β cells is the ~200 bp comprising evolutionarily conserved Area II, although reporter activity was detected in less than 50% of β cells (Samaras et al., 2002). Combination of Area II with Area I sequences either by direct juxtaposition of these elements (Van Velkinburgh et al., 2005) or in the context of the PB region (Wu et al., 1997) promoted reporter expression in nearly all β cells. Of the factors shown to bind to Area II, FoxA2 and Pax6 are expressed in endocrine progenitors and mature β cells, whereas MafA (Nishimura et al., 2006; Matsuoka et al., 2003, 2004) is restricted to the mature β cells that begin to appear during the so-called "secondary transition" period of pancreas development between E13.5 and E15.5 in mouse. Onset of expression of an AreaI+II-*lacZ* transgene was observed at E14.5 in insulin-positive cells, consistent with the timing and location of MafA expression (Van Velkinburgh et al., 2005). Also, Van Velkinburgh et al. proposed that binding of Nkx2.2 to Area I may be important to boost the β-cell-specific activity of Area II to a sufficient level for reporter expression to be detectable in most β cells.

While the highly conserved elements of Areas I and II promote transcription primarily in differentiating and mature β cells, broader activity within the Pdx1 expression domain has been demonstrated for longer regions of the 5' *Pdx1* promoter. Stoffers et al. (1999) analyzed the developmental expression of a *lacZ* reporter transgene driven by the proximal 4.6 kb of *Pdx1* promoter sequence (from the Xba I site at -4.5 kb to the start codon at +92 bp), demonstrating that it recapitulated the endogenous ontogenesis of Pdx1

expression. Gannon et al. (2001) extended this analysis by examining transgenic *lacZ* expression using several fragments from the 4.6 kb promoter. The general trend to emerge was that shorter sequences that overlapped portions of Areas I, II and III were expressed in islet cells, while longer sequences that included portions of Areas I, II and III along with less conserved flanking sequences recapitulated aspects of the developmental expression of Pdx1 in foregut endoderm. In addition, Gannon et al. showed that a transgene spanning 14.5 kb of the *Pdx1* locus, which included the coding sequence and Areas I-IV, rescued pancreas development in *Pdx1*$^{-/-}$ mice. This result suggested that most, if not all, of the critical *cis*-regulatory elements are contained within this region (Gannon et al., 2001).

Transactivators of *Pdx1*

The transactivators known to bind the *Pdx1* promoter (Fig. 1.7) can be broadly grouped into two categories: factors that are expressed primarily in endocrine and neural tissue (Nkx2.2, Pax6, MafA and NeuroD) and factors that are widely expressed in embryonic endoderm (FoxA2, Hnf1α/β, and Hnf6). While the former can account for the high level of expression of Pdx1 in pancreatic β cells and the latter could explain the presence of Pdx1 in early foregut endoderm, it remains unknown why expression of Pdx1 is much more restricted spatially and temporally than its FoxA/Hnf activators. It is very likely that more *trans*-acting factors than the handful listed above are involved in regulating *Pdx1*, including repressors that may restrict the domain of Pdx1 expression within FoxA/Hnf-expressing endoderm. It is also possible that the ability of FoxA/Hnf factors to bind and activate *Pdx1* is modulated by intercellular signaling pathways. As mentioned above, the ability of FoxA2 to activate Area IV in vitro was shown to be repressible by glucocorticoids (Sharma et al., 1997). Also,

binding of MafA to *Pdx1* appears to be inhibited by tyrosine phosphatase activity (Samaras et al., 2003), suggesting that transactivation of *Pdx1* may be subject to extrinsic regulation.

Part III -- Roles of Pdx1 in pancreas and gut development and function

Transactivator function of Pdx1

While the *Pdx1* gene is a substrate for diverse regulatory interactions, the Pdx1 protein is itself a critical transcriptional regulator involved in several developmental and physiological processes in the posterior foregut. The first functions of Pdx1 to be elucidated were its ability to bind and transactivate the promoters of genes encoding the peptide hormones insulin and somatostatin (Ohlsson et al., 1993; Peers et al., 1994; Peshavaria et al., 1994; Leonard et al., 1993; Miller et al., 1994). Pdx1 was later shown to promote transcription of *Glut2* and *Glucokinase* (Waeber et al., 1996; Watada et al., 1996), which encode components of the glucose-sensing machinery in pancreatic β cells. Targeted inactivation of *Pdx1* in mice led to the remarkable finding that, in addition to its role in transactivating genes in the pancreatic islets, Pdx1 is essential for formation of the pancreas (Jonsson et al., 1994). Subsequent identification of a human family carrying an inactivating mutation in *IPF1* (the human homolog of *Pdx1*) revealed that heterozygosity for the mutant allele resulted in a heritable form of early-onset type II diabetes known as maturity onset diabetes of the young type 4 (MODY4) (Stoffers et al., 1997a). In humans as in mice, homozygosity for the mutant allele was found to result in pancreatic agenesis (Stoffers et al., 1997b).

Characterization of *Pdx1^{-/-}* phenotypes

Further studies of *Pdx1^{-/-}* mice have revealed that the dorsal and ventral pancreatic buds form in the absence of Pdx1, but the ventral bud regresses and the dorsal bud fails to differentiate properly. The rudimentary dorsal bud arrests as a duct-like cyst whose epithelium contains a few immature insulin- and glucagon-expressing cells, but no other markers of mature pancreatic cell types (Ahlgren et al., 1996; Offield et al., 1996). These defects reflect an autonomous requirement for Pdx1 in the pancreatic epithelium, since recombination of wild-type mesenchyme with *Pdx1^{-/-}* epithelium did not rescue pancreatic development, while *Pdx1^{-/-}* mesenchyme could support the development of wild-type pancreatic epithelium (Ahlgren et al., 1996).

Pdx1^{-/-} mutants display a variety of defects in non-pancreatic tissues of the posterior foregut. The rostral duodenum fails to mature properly, exhibiting a flattened cuboidal epithelium, very few submucosal Brunner's glands, reduced numbers of enteroendocrine cells, and a malformed, stenotic gastroduodenal junction (Offield et al., 1996). Antropyloric gastrin cells and duodenal GIP cells are virtually absent in *Pdx1^{-/-}* mice (Larsson et al., 1996; Jepeal et al., 2005). It was recently shown that the common bile duct develops abnormally in *Pdx1^{-/-}* mutants, as characterized by malformation of the major duodenal papilla, lack of peribilliary glands, absence of mucin-producing cells, and formation of bile stones secondary to reflux of duodenal contents (Fukuda et al., 2006b).

Haploinsufficieny for *Pdx1* has also been shown to have deleterious consequences. *Pdx1^{+/-}* mice are glucose intolerant due to impaired glucose-stimulated insulin secretion and have increased susceptibility to β-cell apoptosis (Brissova et al., 2002; Johnson et al., 2003).

Conditional inactivation of *Pdx1*

To explore the roles of Pdx1 after the early patterning events for which it is required, two strategies have been employed to conditionally inactivate or repress expression of *Pdx1*. The first strategy was to flank the second exon with loxP sites, generating a null allele subsequent to "loop-out" excision of the loxP-flanked ("floxed") sequence by Cre recombinase (Ahlgren et al., 1998; method reviewed by Nagy, 2000; Branda and Dymecki, 2004). Ahlgren et al. (1998) used a transgene to express Cre in differentiated β cells, showing that loss of Pdx1 from mature β cells resulted in fewer insulin-expressing cells and more glucagon-expressing cells per islet. The remaining insulin-expressing cells had decreased levels of proteins associated with β cell maturation and function.

The second strategy involved replacing the *Pdx1* coding sequence with a gene encoding the tetracycline-repressible transcriptional activator tTA (Holland et al., 2002). Homozygosity for the knock-in allele resulted in a Pdx1 null condition that could be rescued by the presence of a transgene encoding *Pdx1* cDNA under the regulation of a promoter that depends on tTA for activation. With this elegant system in place, Pdx1 was expressed normally in the absence of tetracycline, but the level of Pdx1 could be rapidly reduced by treatment with the tetracycline analog doxycycline during embryonic development (Hale et al., 2005) or in adult mice (Holland et al., 2002, 2005). These experiments revealed that suppression of Pdx1 production in adult mice resulted in a reversible loss of insulin expression in β cells and decreased expression of elastase and amylase in the exocrine pancreas (Holland et al., 2002, 2005). When doxycycline was administered during embryogenesis, differentiation and proliferation of pancreatic endocrine and acinar cells were

arrested shortly after the onset of doxycycline treatment, and could recover somewhat after discontinuation of treatment (Hale et al., 2005).

Targeted deletion of *cis*-regulatory regions of *Pdx1*

The wealth of knowledge about the transcriptional regulation of *Pdx1* and the various phenotypic consequences of reduced or absent Pdx1 protein has made the *Pdx1* locus an attractive model for studying how manipulation of *cis*-regulatory regions affects gene regulatory networks in vivo. The first study of this type involved targeted removal of a 1.2 kb region encompassing the evolutionarily conserved Areas I, II and III (described above) from the endogenous *Pdx1* gene (Fujitani et al., 2006). As expected for deletion of a region known to have strong β-cell-specific enhancer activity, the most severely affected population in the enhancer-deletion mutants was the β cells. β cells in mice heterozygous for the enhancer deletion ($Pdx1^{\Delta I-II-III/+}$) had dramatically decreased expression of Glut2 and were deficient in glucose-stimulated insulin secretion. While β cells are the most abundant pancreatic endocrine cell (~75%) in wild-type mice, $Pdx1^{\Delta I-II-III/\Delta I-II-III}$ mice formed very few insulin-expressing cells. In contrast, clusters of glucagon- and somatostatin-expressing cells were formed along with acinar and ductal structures that appeared to be well differentiated.

In addition to abrogated production of β cells, $Pdx1^{\Delta I-II-III/\Delta I-II-III}$ mice had a variety of defects indicating that Pdx1-dependent developmental processes require different threshold levels of Pdx1. For instance, a low level of Pdx1 in the early pancreatic buds was sufficient for outgrowth and differentiation of the dorsal bud but not the ventral bud. The mildly reduced level of Pdx1 in the developing duodenum was sufficient for the normal

differentiation of the submucosal Brunner's glands, but resulted in an approximately 50%

reduction in the number of GIP cells.

The finding that the dorsal pancreatic bud of $Pdx1^{ΔI\text{-}II\text{-}III/ΔI\text{-}II\text{-}III}$ mice was selectively

deficient for the production of β cells is of particular interest in light of a recent report

showing a correlation between the timing of upregulation of Pdx1 in differentiating β cells

and the onset of expression of MafA and other markers of β-cell maturity (Nishimura et al.,

2006). Considering that MafA is a strong activator of the PB region of the *Pdx1* promoter

(Samaras et al., 2003), the results of Nishimura et al. suggested that MafA might trigger

upregulation of Pdx1 (or perhaps vice versa) during differentiation of β cells from endocrine

cell precursors. These data led us to hypothesize that lack of the PB region in the $Pdx1^{ΔI\text{-}II\text{-}III/ΔI\text{-}II\text{-}III}$ mice blocks the Pdx1 upregulation step of β cell differentiation, thereby altering the

proportion of endocrine progenitors that develop into insulin-producing cells.

Part IV -- Aims of the dissertation research

Based on the results of the past studies described above, we designed experiments to

(1) test for an influence of *cis*-regulatory elements outside of Areas I, II and III on the in vivo

expression and function of Pdx1, and (2) to assess the role of Pdx1 in cell fate determination

of pancreatic endocrine progenitor cells. Our strategy for the first project was to genetically

complement $Pdx1^{-/-}$ mice with a transgene including Areas I, II and III and the *Pdx1* coding

region but lacking putative *cis*-regulatory sequences upstream of -4.5 kb. We hoped that this

experiment would resolve the apparent discrepancy between in vitro experiments showing

that the distal promoter elements in the Area IV region strongly promote β-cell-specific

expression (Sharma et al., 1996; Sharma et al., 1997; Gerrish et al., 2004) and the in vivo reporter experiments (Wu et al., 1997; Stoffers et al., 1999; Gannon et al., 2001) indicating that all the *cis*-elements required to establish the Pdx1 expression domain are contained within the proximal 4.6 kb promoter region. Because Gerrish et al. (2004) had recently shown that Area IV synergizes with Areas I and II, we hypothesized that mice expressing Pdx1 from the 4.6 kb promoter would have a normal pattern of Pdx1 expression but abnormally low levels of expression, particularly in mature β cells. Another clue as to a potential role of sequences upstream of -4.5 kb was that expression of a *lacZ* reporter transgene driven by the 4.6 kb promoter resulted in surprisingly weak XGal staining in adult duodenal mucosa (Stoffers et al., 1999). The results of the transgene complementation experiments are presented in Chapter 3.

For the second project, we used mice expressing Cre recombinase from regulatory elements of *Ngn3* to recombine floxed alleles of *Pdx1* in common progenitors of all four pancreatic endocrine lineages. Lineage-tracing experiments and analysis of mice with mutations in genes encoding endocrine-specific transcription factors have led to a model of pancreatic endocrine differentiation where the α cell lineage is the first to lose the potential to give rise to the other endocrine cell types (reviewed by Wilson et al., 2003), possibly due to mutual inhibition between the Pax4 and Arx transcription factors (Collombat et al., 2003). The β, δ and PP lineages subsequently diverge through a poorly understood mechanism that may involve Pax4 (Collombat et al., 2005). Because the highest level of Pdx1 expression is observed in β cells, a lower level is expressed in some δ cells, and there is no detectable expression in mature α and PP cells (Guz et al., 1995), we hypothesized that complete loss of Pdx1 from endocrine progenitors would impair allocation to the β and δ cell lineages,

whereas reduced expression of Pdx1 would inhibit generation of β cells while sparing production of δ cells. Preliminary results of these experiments are described in Chapter 4.

Shortly before I joined the lab, a transcription factor known as Mist1 was reported to be expressed in the early pancreatic buds and then subsequently restricted to developing and mature pancreatic acinar cells (Pin et al., 2001). This pattern of expression was strikingly similar to that of Ptf1a, an essential regulator of pancreatic acinar cell development that other members of the lab were studying at the time (e.g., Kawaguchi et al., 2002). Despite the intriguing expression pattern of Mist1, $Mist1^{-/-}$ mutants had very mild defects in pancreas development (Pin et al., 2001). We postulated that Ptf1a and Mist1 might have partially redundant developmental roles, and I embarked on a side project to determine if $Ptf1a^{-};Mist1^{-}$ compound mutants have synthetic pancreatic phenotypes. During the course of these experiments, I serendipitously discovered a $Ptf1a$ haploinsufficiency phenotype affecting the balance of endocrine versus exocrine pancreatic differentiation in the early pancreatic buds. These results are described in Chapter 5.

CHAPTER II

MATERIALS AND METHODS

Mouse strains

The studies described in the following chapters used several transgenic and gene-targeted

mouse strains that have been previously described. The null alleles of *Pdx1*, *Ptf1a*, and *Mist1*

are the previously characterized *Pdx1^XBko^* (Offield et al., 1996), *Ptf1a^cre^* (Kawaguchi et al.,

2002), and *Mist1^lacZ^* (Pin et al., 2001) alleles. Details of the *Pdx1^floxE2^* allele will be provided

in an upcoming publication from Maureen Gannon, VUMC Department of Medicine, but it is

in most respects equivalent to the *Ipf1/Pdx1-loxP* allele described by Ahlgren et al. (1998).

The other floxed alleles are *Pdx1^floxI-II-III^* (Fujitani et al., 2006) and *ROSA26^R^* (Soriano, 1999).

Transgenic Cre-expressing lines used in Chapter 4 are *Ngn3Cre* (Gu et al., 2002),

Ngn3CreBAC (Schonhoff et al., 2004b), *RIPCre* (Postic et al., 1999) and *ElastaseCre*

(Grippo et al., 2002). Generation of the 12.5 kb *Pdx1* rescue transgene used in Chapter 3 is

described below. Mice were genotyped by PCR amplification of genomic DNA purified

from tail snip digests, except for the transgene-rescue mice, which were genotyped by a

combination of PCR and Southern hybridization strategies (described below). All mice used

for these experiments were maintained on a B6D2 (C57BL/6:DBA/2J) mixed-inbred

background. Experiments were performed in accordance with the guidelines of the

Vanderbilt University Institutional Animal Care and Use Committee.

Generation of 12.5 kb rescue transgene

To make the 12.5 kb *Pdx1* transgene, the 14.5 kb genomic clone described in Offield *et al*. (1996) was digested with XbaI and BamHI to yield a fragment extending from the XbaI site at -4.5 kb to the first BamHI site in the intron. The genomic clone was also digested with BamHI and SalI to yield a fragment extending from the second BamHI site in the intron to the SalI site in the 3' λ linker. These two fragments were ligated between the XbaI and SalI sites in the MCS of pBluescript KSII (Stratagene). The resulting plasmid was digested with NotI and SalI and electrophoresed in a low melting temperature agarose gel to isolate the linear 12.5 kb transgene. The transgene DNA was recovered by GELase (Epicentre) enzymatic treatment and purified by phenol/chloroform and chloroform extraction. Transgenic founders were generated by the Vanderbilt ES Cell/Transgenics core by injection of the transgene into pronuclei of fertilized B6D2 oocytes, which were then transferred to pseudopregnant ICR females. F1 offspring were screened by genomic Southern blot to identify germline transmission of the transgene (Fig. 2.1). Subsequent generations were genotyped for presence of the transgene using the same Southern hybridization strategy. $Pdx1^{-/-;12.5kbTg}$ (transgene-rescue) mice could be identified based on the lack of a 5 nt sequence between the paired BamHI sites in the intron of *Pdx1*. This sequence is absent from the $Pdx1^{XBko}$ allele and from the rescue transgene. Therefore, an oligonucleotide primer designed to match the genomic sequence spanning the paired BamHI sites annealed to the wild-type allele but not to the transgene or XBko. Absence of PCR product when using this primer was diagnostic for mice that were homozygous for the XBko null allele regardless of presence of the transgene.

Fig. 2.1 Genotyping rescue transgene by Southern blot. Mouse genomic DNA was digested overnight with EcoRI and probed with a 1.25 kb XbaI-EcoRI fragment from the 5' end of the rescue transgene sequence. The probe labels a 4.6 kb band from the endogenous *Pdx1* locus. The size of band labeled by the probe for the transgenic alleles depends on the distance to the next EcoRI site 5' of the transgene insertion site. For b3Tg the next EcoRI site is approx. 750 bp away, 750 bp + 1.25 kb = 2.0 kb. For b1Tg the next EcoRI site is approx. 2.45 kb distant, 2.45 kb + 1.25 kb = 3.6 kb. The band of approx. 1.5 kb that is labeled for both transgenic alleles most likely represents head-to-tail insertion of the transgene. The most 3' located EcoRI site in the transgene is 320 bp from the 3' end. If the transgene is inserted head to tail, a band of 320 bp + 1.25 kb = 1.57 kb would be expected. Densitometric analysis of band intensity yielded average intensities of approx. 2 for the 4.6kb band in all lanes, 1 for the 3.6 kb band, 1 for the 2.0 kb band, 1 for the 1.5 kb band in the b3Tg lane and 4 for the 1.5 kb band in the b1Tg lane. Therefore we conclude that the b3Tg allele consists of two copies of the rescue transgene integrated head to tail, and the b1Tg allele comprises 5 copies head to tail in a concatemer.

Rescue transgene

RI   RV   X   RI   P   B Xh   Bg Sc   Sm P Sp   RI X S
        IV   I II III   Ex1   Exon 2   320bp
    ——— 1 kb   Probe
                     1.25kb

    4.6kb

WT b3Tg b1Tg

4.6kb
3.6kb

2.0kb
1.5kb
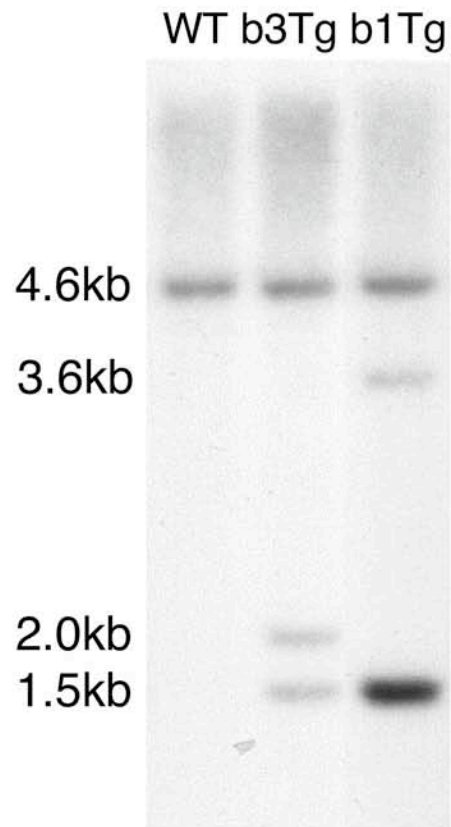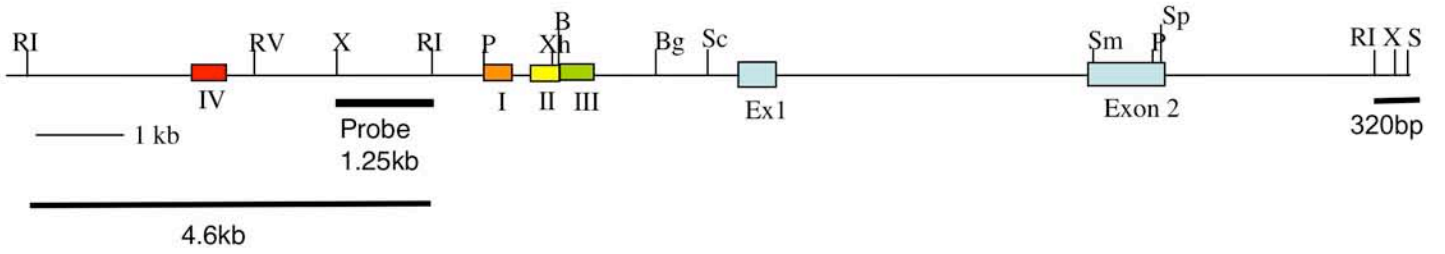
Whole-mount XGal staining

LacZ reporter expression in Chapters 4 and 5 was visualized by incubation of dissected tissues with 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside (XGal). Tissues were fixed in ice-cold 4% paraformaldehyde/PBS for 1 h at 4°C, then washed in permeabilization solution (2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40 in PBS) for 1 h at room temperature, and then incubated for 12-48 h at 4°C in staining solution (2 mM $MgCl_2$, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 20 mM Tris pH 7.4, 1 mg/ml XGal in PBS). After the stain had developed to the desired intensity, samples were post-fixed in 4% paraformaldehyde/PBS for 3-5 h at 4°C, and then embedded and sectioned as described below.

Whole-mount IHC

Dissected tissues were fixed overnight at 4°C in 4:1 CH_3OH/DMSO, rehydrated in 1:1 CH_3OH:PBS followed by PBS, and then blocked with two 1 hour incubations in PBSMT (2% dried nonfat milk, 0.5% Triton X-100 in PBS). Samples were incubated overnight at 4°C with the first primary antibody, guinea-pig anti-glucagon (Linco), diluted 1/2000 in PBSMT. The next day, samples were washed in PBST (0.5% Triton X-100 in PBS) three times for 1 h each and then incubated overnight with the next primary antibody, rabbit anti-Mist1 (gift of S. Konieczny, Purdue University), diluted 1/1000 in PBSMT. The wash and incubation steps were repeated for the secondary antibodies, Cy2-conjugated donkey anti-guinea pig (1/500) and Cy3-conjugated donkey anti-rabbit (1/500). After the final secondary antibody incubation and subsequent washes in PBST, the samples were dehydrates through a series of 50%, 80% and 100% CH_3OH. Prior to visualization, the stained samples were

cleared in BABB (1:2 benzyl alcohol:benzyl benzoate) for 5 min. 3D projections of confocal z-stacks of the stained tissues were obtained using a Zeiss LSM510 confocal microscope and the included software.

Histology

Dissected tissues were fixed in 4% paraformaldehyde/PBS for 3-5 h at 4°C (1 h for E12.5 embryos). After fixation, tissues were dehydrated in increasing concentrations of ethanol, washed into Histo-Clear (National Diagnostics) and embedded in paraffin. 5-μm sections were cut using a Leica RM2135 microtome. PAS staining was performed using reagents and protocol from Sigma. After staining with Schiff's reagent, samples were washed briefly in 10% sodium metabisulfite to reduce background. Hematoxylin (Zymed) counterstain was applied for 30-90 seconds depending on desired intensity of stain, followed by thorough rinsing in deionized tap water.

Immunoperoxidase staining used the Vectastain ABC and DAB kits (Vector Laboratories). The provided secondary antibodies were applied at 1/1000 dilution for 1 h at room temperature. For immunoperoxidase-DAB staining, primary antibodies were incubated overnight at 4°C at the indicated dilutions: rabbit anti-Gastrin (1/1000, Research Diagnostics), rabbit anti-GIP (1/5000, Research Diagnostics), rabbit anti-CCK (1/2000, Peninsula/Bachem), rabbit anti-Somatostatin (1/2000, Dako), rabbit anti-ChormograninA/B (1/500, Abcam), guinea-pig anti-Insulin (1/5000, Linco), rabbit anti-glucagon (1/20,000, Linco) and guinea pig anti-Pdx1 (1/7500, generated by Strategic Biosolutions from a fusion protein made in our lab, which consisted of GST plus the N-terminal 75 amino acids of mouse Pdx1). Dilutions were made in 5% normal donkey serum/PBS, which was also used

as a blocking reagent. Prior to addition of anti-Pdx1 antibody, antigen retrieval was performed by heating slides to 95°C for 10 min in 10 mM sodium citrate, pH 6.0.

Immunofluorescence staining used the following primary antibodies and dilutions: rabbit anti-Glucagon (1/2000, Linco), guinea pig anti-Insulin (1/1000, Linco), rabbit anti-Somatostatin (1/1000, Dako), guinea pig anti-PP (1/750, Linco), goat anti-Pdx1 (1/2000, generated by Strategic Biosolutions from our GST-Pdx1 fusion protein) guinea pig anti-Pdx1 (1:5000), goat anti-Villin (1/100, Santa Cruz), rabbit anti-GIP (1/500, Research Diagnostics), rabbit anti-ChromograninA/B (1/200, Abcam), goat anti-Insulin C-peptide (1/5000, Linco), rabbit anti-human Amylase (1/500, Sigma), rabbit anti-Mist1 (1/10,000, gift of S. Konieczny, Purdue University), and mouse anti-Ngn3 (1/10,000, gift of Palle Serup, Hagedorn Research Institute, Denmark). Secondary antibodies were incubated for 2 h at room temperature at the indicated dilutions: donkey anti-goat-Cy5 (1/500, Jackson Immuno), donkey anti-rabbit-Cy3 (1/500, Jackson Immuno), donkey anti-guinea-pig-Cy2 (1/250, Jackson Immuno). 5% normal donkey serum/PBS was used to dilute antibodies and for blocking. Secondary detection of Villin, Ngn3, Mist1 and guinea pig anti-Pdx1 was by Vectastain ABC kit (Vector) followed by TSA-Fluoroscein reagent (1/100, Perkin-Elmer) or TSA-Cyanine reagent (1/200, Perkin-Elmer) for 20 min at room temperature. When TSA was used for amplification, blocking and primary antibody dilutions utilized Perkin-Elmer blocking reagent. Secondary detection of GIP and chromogranins was by goat anti-rabbit-biotin (1/1000, Vector) for 1 h followed by Cy3-Streptavidin (1/500, Jackson Immuno) for 2 h at room temperature. Villin-stained sections were counterstained with YoPro-1 (1/1000, Molecular Probes) for 20 min to label nuclei. Pdx1 and Mist1 antigens were retrieved by heating slides to 95°C for 10 min in 10 mM sodium citrate pH 6.0 (for Pdx1) or 100 mM Tris

pH 10.0 (for Mist1). All fluorescently labeled slides were mounted with ProLong Anti-fade

Gold reagent (Molecular Probes/Invitrogen).

Western blotting and quantification

Whole-pancreas nuclear extract was recovered as previously described (Gannon *et al.*, 2001).

10 mg of sample per lane was run on pre-cast NuPAGE 10% Bis-Tris gels (Invitrogen) in

MES buffer and transferred to Immobilon –P PVDF membranes (Millipore). Membranes

were blocked for 1 h at room temperature in TBSMT (Tris-buffered saline, 5% dried nonfat

milk, 0.1% Tween-20) and incubated overnight at 4°C with primary antibody solution

(1/2000 rabbit anti-Pdx1 (Peshavaria *et al.*, 1994), 1/10,000 rabbit anti-Histone 2B (Sigma)

in TBSMT). Membranes were washed 5 times in TBST and then incubated in secondary

antibody solution (1/2000 goat anti-rabbit-HRP, Santa Cruz Biotech) for 1 h at room

temperature, followed by 5 more TBST washes. Membranes were incubated in SuperSignal

West Pico Chemiluminescent Substrate (Pierce) for 5 min and then visualized using the

BioRad Chemidoc imaging system and Quantity One software (BioRad). Images were

captured at 30 sec intervals for 20 min to determine optimum exposure. Integrated intensity

of bands was measured and corrected for background using Quantity One software.

Integrated intensity of the Pdx1 band was divided by the integrated intensity of the Histone

2B band for each lane. One of the *Pdx1*^{+/-} samples was selected to be loaded on every gel to

ensure that relative signal intensies were equivalent from blot to blot.

Intraperitoneal glucose tolerance test

Following a 16-h fast, dextrose (2 mg/g body weight) was injected intraperitoneally in the right lower quadrant. Blood glucose was measured at 0, 15, 30, 60, 90 and 120 min after injection using a BD Logic glucose monitor (Becton Dickinson).

Quantification of duodenal enteroendocrine cells

P7 mice were sacrificed and the gut tube was cut through the body of the stomach superiorly and at the junction of the duodenum and jejunum inferiorly. The dissected tissue was fixed and embedded as described above. 5-μm sections were cut parallel to the long axis of the duodenum. For each sample, 5 groups of 3 adjacent sections were chosen based on roughly equal spacing across the diameter of the duodenum, and were immunostained for GIP, gastrin and CCK. Stained sections were photographed at 20X magnification resulting in images of 450 x 340 μm microscopic fields. For each section, five fields evenly spaced between the gastroduodenal junction and the base of the first loop of the duodenum were selected for quantification. DAB-stained cells were counted manually. Hematoxylin-stained nuclei were counted using Metamorph software (Molecular Devices) with manually set boundaries to restrict the count to villus epithelium. For each mouse, the total number of hormone-positive cells was divided by the total number of villus epithelial cell nuclei to determine the frequency of GIP-, gastrin- and CCK-expressing cells.

Microscopy

Brightfield images were photographed using an Olympus DP-70 digital camera and Olympus BH-2 compound microscope. Fluorescent images were captured using a Zeiss LSM 510 Meta confocal microscope.

Statistics

Sample means for pancreatic nuclear Pdx1 level and enteroendocrine cell frequency were compared by two-tailed, unpaired Student's t-test assuming unequal variance. The null hypothesis was rejected for $P < 0.05$.

COMPLEMENTATION RESCUE OF *PDX1* NULL PHENOTYPE DEMONSTRATES DISTINCT ROLES OF PROXIMAL AND DISTAL *CIS*-REGULATORY SEQUENCES IN PANCREATIC AND DUODENAL EXPRESSION

Introduction

The homeodomain transcription factor P̲ancreatic and d̲uodenal homeobo̲x factor 1̲ (Pdx1) has been a focus of research in the fields of developmental biology and endocrinology for more than a decade because of its essential role in pancreas morphogenesis and involvement in heritable Type II diabetes (reviewed by McKinnon and Docherty, 2001; Melloul, 2004; Melloul et al., 2002). Pdx1 is first detectable only in the nascent pancreatic buds, but its expression very rapidly expands to include the epithelial progenitors of the antrum and pylorus of the stomach, the rostral duodenum, and the common bile duct (Guz et al., 1995; Offield et al., 1996). Pdx1 expression persists in all of these organs in the adult, although at lower levels than during embryogenesis. While it is also expressed at low levels in mature pancreatic acini and a subset of the duct cells, Pdx1 continues to be highly expressed in pancreatic β cells and is required for their function and maintenance in adult animals (Ahlgren et al., 1998; Holland et al., 2005; Holland et al., 2002).

Pdx1$^{-/-}$ mice do not form any mature pancreatic tissue, although a few glucagon-expressing cells can be found in a poorly differentiated cyst-like rudiment that evaginates from the duodenum (Ahlgren et al., 1996; Jonsson et al., 1994; Offield et al., 1996). The pancreas is the most severely affected organ in *Pdx1*$^{-/-}$ mice, but abnormalities are present in all of the organs that normally express Pdx1. Defects in the stomach include drastic reduction in the number of gastrin-producing enteroendocrine cells (Larsson et al., 1996) and

malformation of the gastroduodenal junction, which is characterized by an abnormal transition between gastric and duodenal epithelia and malformation of the pyloric sphincter, often leading to pyloric stenosis and stomach dilation (Offield et al., 1996). The rostral duodenum forms very few, if any, submucosal Brunner's glands, contains reduced numbers of glucose-dependent insulinotropic polypeptide (GIP)-, secretin- and cholecystokinin (CCK)-expressing enteroendocrine cells, has shorter and sparser villus structures, and lacks a functional sphincter of Oddi, resulting in reflux from the duodenum into the common bile duct (Fukuda et al., 2006; Jepeal et al., 2005; Offield et al., 1996). The common bile duct is deficient in formation of peribiliary glands and mucin-producing cells (Fukuda et al., 2006).

$Pdx1^{+/-}$ mice have abrogated insulin secretion in response to glucose, presumably because of the reduced expression of Pdx1 target genes including *Glucokinase*, *Glucose transporter 2 (Glut2)* and *Insulin* (Brissova et al., 2002). Humans who are heterozygous for inactivating mutations in *IPF1* (*Insulin Promoter Factor* 1, the human homolog of *Pdx1)* develop a heritable form of strong early-onset Type II diabetes known as Maturity Onset Diabetes of the Young (MODY) Type 4 (Stoffers et al., 1998; Stoffers et al., 1997a). In addition to *IPF1/Pdx1* itself, mutations in MODY families have been mapped to genes encoding transactivators of the *Pdx1* gene, *Hnf1α*, *Hnf1β*, and *NeuroD1/Beta2*, as well as to *Glucokinase* and *Hnf4α,* which are transcriptional targets of Pdx1 (reviewed by Bell and Polonsky, 2001; Shih and Stoffel, 2001).

The essential roles of *Pdx1* in glucose homeostasis and endoderm patterning have prompted great interest in understanding the regulation of *Pdx1* transcription. There is still a very poor understanding of the interplay of inter- and intra-cellular factors in driving pancreatic differentiation. *Pdx1* provides a valuable entry point into dissecting these

processes because of its early, specific expression in the pancreas and its critical roles in promoting pancreatic differentiation. *Pdx1* has a TATA-less promoter and transcription can initiate from multiple sites ranging from approximately 90 to 130 bp upstream of the translational start codon (Sharma et al., 1996). In keeping with precedent, we refer to 91 bp upstream of the translational start codon as the +1 position. Promoter-deletion studies and DNase hypersensitivity analysis led to the identification of proximal (-2.6kb — -1.9kb in mouse) and distal (-5.67kb — -6.2kb in rat) enhancer sequences in the 5′ region of the rodent *Pdx1* locus (Sharma et al., 1997; Sharma et al., 1996; Wu et al., 1997). Sequence alignment between mouse and human revealed three highly conserved areas in the proximal enhancer region, referred to as Areas I, II and III (Gerrish et al., 2000; Marshak et al., 2000), as well as a more distal block of conservation (Area IV) (Gerrish et al., 2004). Electrophoretic Mobility Shift Assays (EMSA) and Chromatin Immunoprecipitation (ChIP) have confirmed binding of several endoderm or endocrine cell enriched transactivating factors to these areas: Foxa2 (Areas I, II and IV), Hnf6 (I and III), Hnf1 (I), L-Maf (II), Nkx2.2 (I and IV), Pax6 (II) and Pdx1 itself (I and IV) (Ben-Shushan et al., 2001; Gerrish et al., 2001; Gerrish et al., 2000; Gerrish et al., 2004; Jacquemin et al., 2003; Marshak et al., 2000; Samaras et al., 2002; Samaras et al., 2003; Sharma et al., 1997; Van Velkinburgh et al., 2005; Wu et al., 1997).

Reporter gene expression in transgenic mice has shown that Area II can promote expression specifically in β cells, and that this effect is more robust in combination with Area I (Samaras et al., 2002; Van Velkinburgh et al., 2005). Longer genomic sequences overlapping Areas I, II and III promote expression in the pancreas and duodenum (Gannon et al., 2001; Stoffers et al., 1999), although expression in the duodenum appears to be limited to the crypts (Stoffers et al., 1999), which contain progenitors for all of the duodenal epithelial

cell types. Area IV has not yet been shown to be sufficient to promote tissue-specific gene

expression *in vivo*, but *in vitro* evidence in cell lines suggests that it promotes β-cell-specific

transcriptional activation synergistically with Areas I and II (Gerrish et al., 2004).

The collective influence of Areas I, II and III was recently evaluated *in vivo* by

targeted deletion of the 1.2 kb of sequence encompassing these three areas (referred to as

Area I-II-III) (Fujitani et al., 2006). Deletion of Area I-II-III from both alleles of *Pdx1*

severely disrupted pancreas morphogenesis, but the only extra-pancreatic abnormality that

was observed was a 50% reduction in stomach gastrin cells and duodenal GIP cells.

Correspondingly, reduction in Pdx1 protein expression was more extreme in the pancreas

than in the stomach and duodenum. These results demonstrated the necessity of Area I-II-III

for pancreas development and robust expression of *Pdx1*, but did not test if Area I-II-III is

sufficient to establish the correct spatiotemporal expression of *Pdx1*.

To test the sufficiency of proximal *cis*-elements for correct regulation of *Pdx1*

transcription, we used a transgene containing the *Pdx1* coding region and proximal

regulatory regions to attempt to rescue the pancreatic, stomach and duodenal phenotypes in

Pdx1$^{-/-}$ mice. A previous study demonstrated rescue of the pancreatic phenotype of *Pdx1*$^{-/-}$

mice using a transgene that included sequences extending 5′ through Area IV, but the extra-

pancreatic expression and activity of Pdx1 was not examined in that study (Gannon et al.,

2001). For the current study, Area IV sequence was excluded from the rescue transgene to

test for distinct roles of proximal and distal promoter elements, and the ability of the

transgene to rescue pancreatic and extra-pancreatic Pdx1 expression and activity was

evaluated. We carried out a detailed analysis of Pdx1 distribution and levels from E12.5

through 8 months of age, and examined the influence of a spectrum of *Pdx1* gene dosages on

glucose tolerance and enteroendocrine cell number. We find that expression of Pdx1

encoded by the transgene is sufficient to rescue pancreas morphogenesis and function, but

that the transgene does not support normal levels of extra-pancreatic Pdx1 expression. Pdx1

expression from the transgene was particularly weak in postnatal duodenum, and was

associated with a 90% reduction in the number of GIP-expressing cells without other evident

defects in morphology or differentiation of the duodenum.

Results

Construction of *Pdx1* transgene lacking distal regulatory elements

A 14.5 kb region of the *Pdx1* locus was previously isolated from a lambda phage

mouse genomic library (Fig. 3.1A; Offield et al., 1996). The fragment includes 6.4 kb of

sequence 5′ of the transcription start site and 3 kb extending 3′ of the stop codon. Breeding

of transgenic mice carrying this 14.5 kb region showed its sufficiency in rescuing pancreas

formation and glucose homeostasis in mice that were homozygous null at the endogenous

Pdx1 locus (Gannon et al., 2001). To investigate the regulatory influence of Area I-II-III

independent of Area IV, we tested if excluding Area IV from the transgene would affect its

ability to rescue the foregut organogenesis defects in $Pdx1^{-/-}$ mice. We removed sequences 5′

of an XbaI site located approximately 4.5 kb upstream of the transcription start site,

producing a transgene that extended from 1.7 kb 5′ of Area I-II-III to 3 kb 3′ of the stop

codon (Fig. 3.1A). Three F1 mice tested positive for this 12.5 kb transgene by genomic

Southern analysis. Independent transgenic lines were established and bred onto a $Pdx1^{-/-}$

background. Two of the three lines rescued pancreas formation. The third line generated

Fig. 3.1 12.5 kb transgene rescues pancreas formation in *Pdx1*^{-/-} mice.
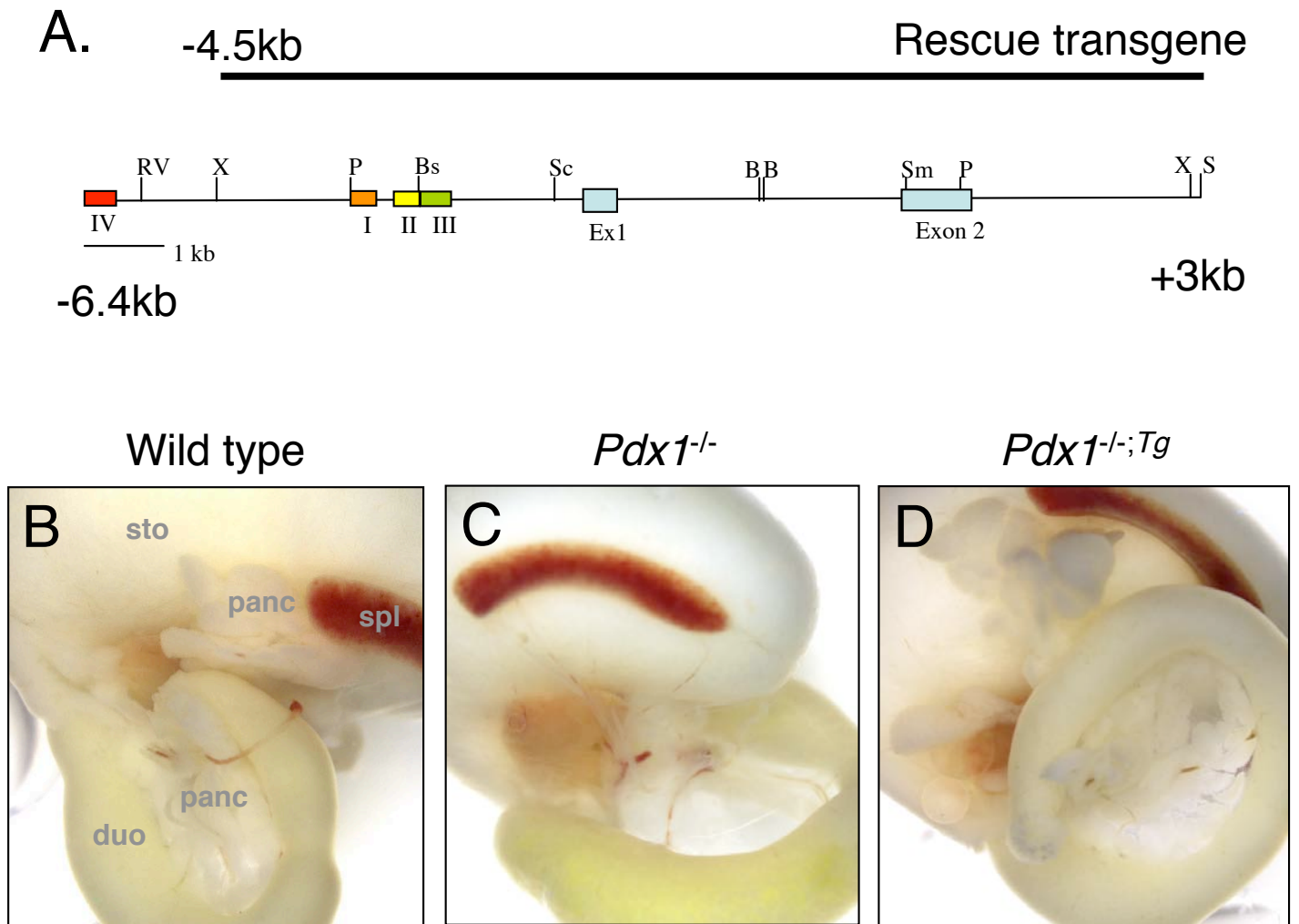
Fig. 3.1 12.5 kb transgene rescues pancreas formation in *Pdx1*-/- mice. (A) Schematic of *Pdx1* genomic region. Non-coding sequences >100 bp with >75% human-to-mouse conservation are indicated by colored boxes (I-IV) after Gerrish *et al*. (2000). The transgene used for genetic complementation extended from an XbaI site 4.5 kb 5' of the transcription start site to a SalI site introduced 3 kb 3' of the stop codon. Restriction sites shown are EcoRV (RV), XbaI (X), PstI (P), BstEII (Bs), SacI (Sc), BamHI (B), SmaI (Sm), SalI (S). (B – D) Dissected and fixed stomach (sto), spleen (spl), pancreas (panc) and first duodenal loop (duo) of newborn (P0) mice. (C) No mature pancreatic tissue was present in *Pdx1*-/- pups. (D) Complementation of *Pdx1*-/- with 12.5 kb transgene rescued the formation of pancreas grossly indistinguishable from wild type.

a slightly thicker pancreatic rudiment than was seen in *Pdx1*$^{-/-}$ littermates, but Pdx1 was not detectable by immunohistochemistry at postnatal day 6 (P6) in this line (data not shown). Southern analysis indicated that both of the rescuing lines contained single-site insertions of the transgene. One line (referred to hereafter as b1Tg) contained approximately 5 copies of the transgene in a concatemer, while the other (b3Tg) had 2 copies inserted head-to-tail (see Fig. 2.1).

Transgene complementation of *Pdx1*$^{-/-}$ rescues pancreas morphology and function

For both active transgenic lines, the rescued pancreas (*Pdx1*$^{-/-;Tg}$) was equivalent in size and gross morphology to wild type (Fig. 3.1B,D). Histological analysis indicated that the organization and differentiation of acinar, duct and endocrine pancreatic tissues were very similar between wild-type and transgene-rescue mice (Fig. 3.2A-F). Immunohistochemical staining for Pdx1 revealed that its expression was higher than normal in acinar cell nuclei of both transgenic lines, although the intensity of staining was still much higher in islets than in acinar cells (Fig. 3.2G-I). Anti-Pdx1 staining appeared to be slightly weaker than wild type in the islets of *Pdx1*$^{-/-;b3Tg}$ mice and slightly stronger than wild type in *Pdx1*$^{-/-;b1Tg}$.

Because previous studies have shown that perturbation of Pdx1 expression affects the composition and architecture of the islets (Ahlgren et al., 1998; Fujitani et al., 2006; Johnson et al., 2003), we examined the expression of Pdx1 and the islet hormones at 3 months (Fig. 3.3) and 8 months (not shown) of age. Some islets in *Pdx1*$^{+/-}$ mice showed modest infiltration of glucagon-producing cells into the interior (Fig. 3.3B) and co-expression of somatostatin and pancreatic polypeptide (PP) (yellow cells, Fig. 3.3E). These abnormalities
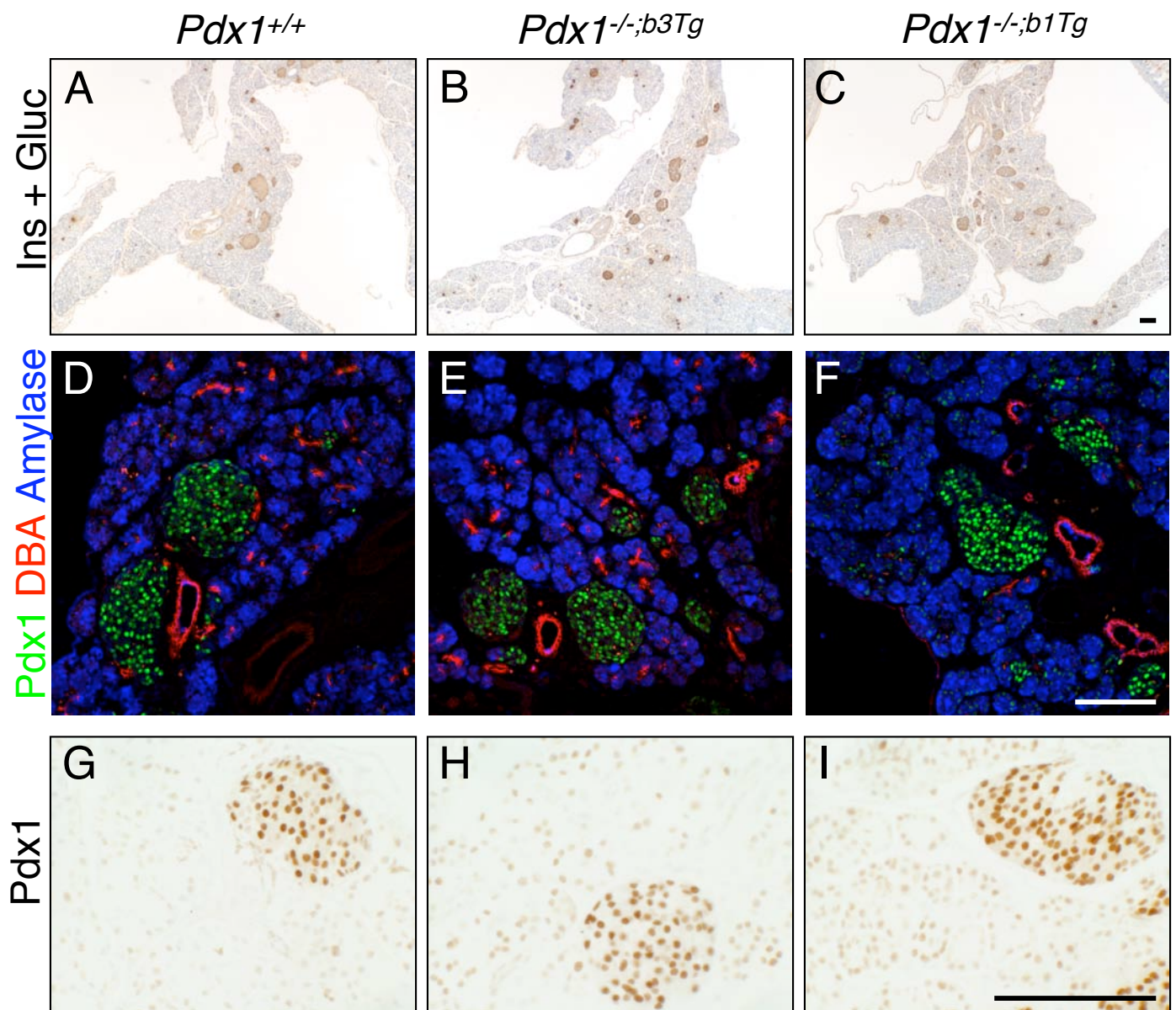
Fig. 3.2 Pancreatic tissue of transgene-rescue mice is well differentiated. (A – C) Sections of splenic lobe of pancreas were stained for insulin and glucagon (brown) and counterstained with hematoxylin (purple), showing similar structure and distribution of endocrine and exocrine pancreatic components in transgene-rescue and wild-type mice. (D – F) Labeling of acinar tissue for amylase (blue) and ducts by DBA-lectin (red) indicated that these structures were properly differentiated in transgene-rescue mice. Islets were visualized by anti-Pdx1 labeling (green). (G – I) Staining for Pdx1 (brown) indicated similar expression in the islets of wild-type and transgene-rescue mice, but abnormally high expression in acinar cell nuclei in both transgenic lines. Tissues from 1-week-old mice. Scale bars 100 μm.
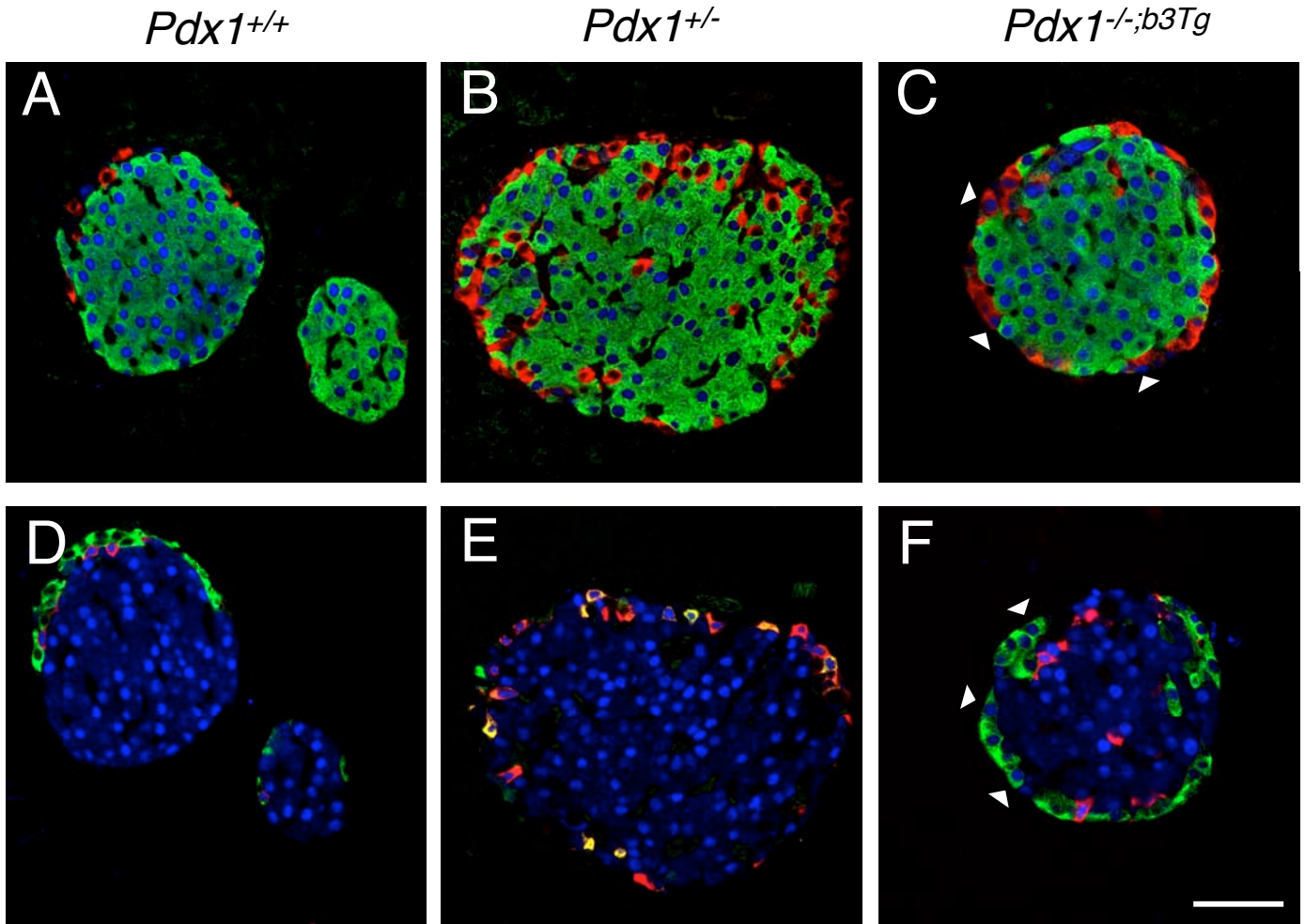
Fig. 3.3 Normal islet morphology in transgene-rescue mice. (A – C) Confocal immunofluorescence analysis for Insulin (green), Glucagon (red) and Pdx1 (blue). (D – F) Sections adjacent to (A-C) were analyzed for PP (green), Somatostatin (red) and Pdx1 (blue). (A,D) Stereotypical wild-type islet structure: core β cells surrounded by mantle of α, δ and PP cells. Nuclear Pdx1 was observed in all β cells and most δ cells, but very rarely in α or PP cells. (B,E) Islets from *Pdx1+/-* mice tended to contain more α cells, which were often located more centrally in the islets. (C,F) Islets from transgene rescue mice contained normal numbers and spatial organization of endocrine cells. A few α and PP cells showed weak nuclear Pdx1 immunoreactivity (arrowheads). Although the islet sections shown in (C,F) have more α, δ, and PP cells than (A,D), there was no consistent difference in islet composition between transgene-rescue and wild-type mice. Tissues from 3-month-old mice. Scale bar in (F) is 50 μm. All panels 250X magnification.

were not found in *Pdx1*^{-/-;Tg} mice (Fig. 3.3C,F), nor in *Pdx1*^{+/-;Tg} mice (not shown). The only apparent difference between wild-type and transgenic islets was that we observed co-expression of Pdx1 with glucagon or PP in a few cells of each transgenic islet examined (Fig. 3.3C,F, white arrowheads), while co-expressing cells were not evident in most wild-type islets. Co-expression of Pdx1 with glucagon and PP was also seen in *Pdx1*^{+/+;Tg} and *Pdx1*^{+/-;Tg} mice (not shown). These results suggest that regulatory elements missing from the transgene may help to repress the expression of Pdx1 in mature glucagon-producing (α) and PP cells. Induction of insulin expression was not observed in the α cells that ectopically expressed Pdx1.

To assess the function of the rescued islets, mice were subjected to intraperitoneal glucose tolerance testing (IPGTT) using wild-type littermates as controls. In agreement with previous reports (Brissova et al., 2002; Dutta et al., 1998; Johnson et al., 2003), *Pdx1*^{+/-} mice at 5-6 weeks and 5 months of age experienced higher peak blood glucose levels and slower glucose clearance than wild-type littermates (Fig. 3.4A,B). Presence of b3Tg (Fig. 3.4A) or b1Tg (Fig. 3.4B) restored wild-type glucose tolerance to *Pdx1*^{+/-} mice, but *Pdx1*^{-/-;Tg} mice displayed intermediate glucose clearance curves between wild type and *Pdx1*^{+/-}.

Pdx1^{-/-;b1Tg} mice are glucose intolerant despite abundant Pdx1 protein

Several studies have shown a correlation between reduced Pdx1 expression and glucose intolerance (*e.g.,* Ahlgren et al., 1998; Brissova et al., 2002; Gannon et al., 2001; Holland et al., 2005; Holland et al., 2002). We postulated, therefore, that the intermediate IPGTT performance of *Pdx1*^{-/-;Tg} animals corresponded to an intermediate level of Pdx1 protein in the pancreas. Based on immunohistochemical analysis (Fig. 3.2G-I), the level of

Fig. 3.4 Comparison of IPGTT and pancreatic Pdx1 content among offspring of *Pdx1$^{+/-}$;^{Tg}* parents. (A) Offspring of *Pdx1$^{+/-;b3Tg}$* mice; 5 litters of 5-6 weeks of age. Glucose tolerance of *Pdx1$^{+/-}$* mice (white) was impaired compared to wild type (black). *Pdx1$^{-/-;b3Tg}$* (yellow) had a mild impairment, while glucose tolerance of *Pdx1$^{+/-;b3Tg}$* (orange) and *Pdx1$^{+/+;b3Tg}$* (red) was normal. Number of each genotype: wild type (5), *Pdx1$^{+/-}$* (10), *Pdx1$^{+/+;b3Tg}$* (2), *Pdx1$^{+/-;b3Tg}$* (10), *Pdx1$^{-/-;b3Tg}$* (8). (B) Offspring of *Pdx1$^{+/-;b1Tg}$* mice; 4 litters, 5 months of age. Glucose tolerance of *Pdx1$^{-/-;b1Tg}$* mice (green) was intermediate between wild type (black) and *Pdx1$^{+/-}$* (white). *Pdx1$^{+/-;b1Tg}$* (purple) and *Pdx1$^{+/+;b1Tg}$* (blue) were similar to wild type. Glucose tolerance for mice in (B) was generally worse than in (A) because of age. Number of each genotype: wild type (2), *Pdx1$^{+/-}$* (11), *Pdx1$^{+/+;b1Tg}$* (4), *Pdx1$^{+/-;b1Tg}$* (9), *Pdx1$^{-/-;b1Tg}$* (4). (C) Nuclear proteins from whole pancreas of 3-month-old mice (genotype indicated; color coding as for A and B). Relative Pdx1 level was determined based on western blot signal intensity with the average wild-type value set as "1". Anti-histone 2B immunoreactivity, loading control. $P < 0.05$ for *Pdx1$^{+/-}$* versus all other genotypes. $P < 0.05$ for wild type versus all genotypes except *Pdx1$^{+/+;b3Tg}$*. Number of each genotype: wild type (8), *Pdx1$^{+/-}$* (9), *Pdx1$^{+/+;b3Tg}$* (3), *Pdx1$^{+/-;b3Tg}$* (6), *Pdx1$^{-/-;b3Tg}$* (6), *Pdx1$^{+/+;b1Tg}$* (5), *Pdx1$^{+/-;b1Tg}$* (6), *Pdx1$^{-/-;b1Tg}$* (4). Error bars in (A-C) indicate +/- SEM.
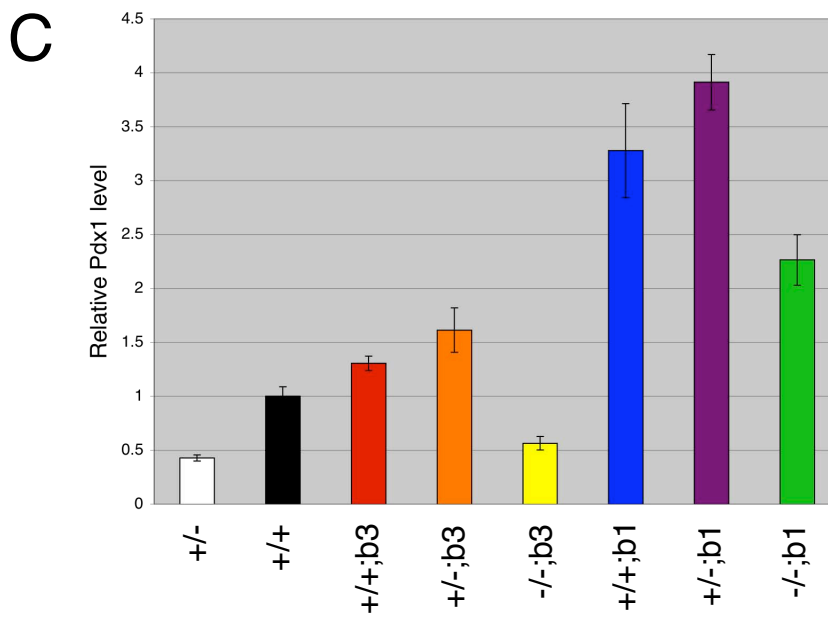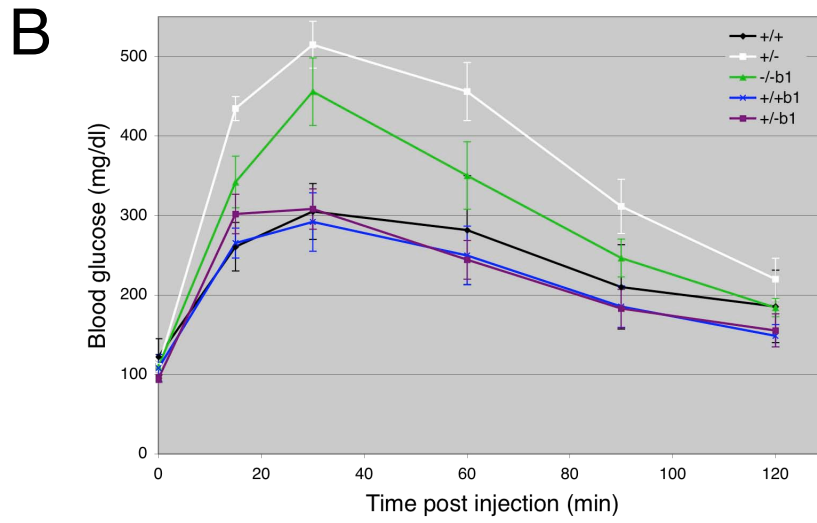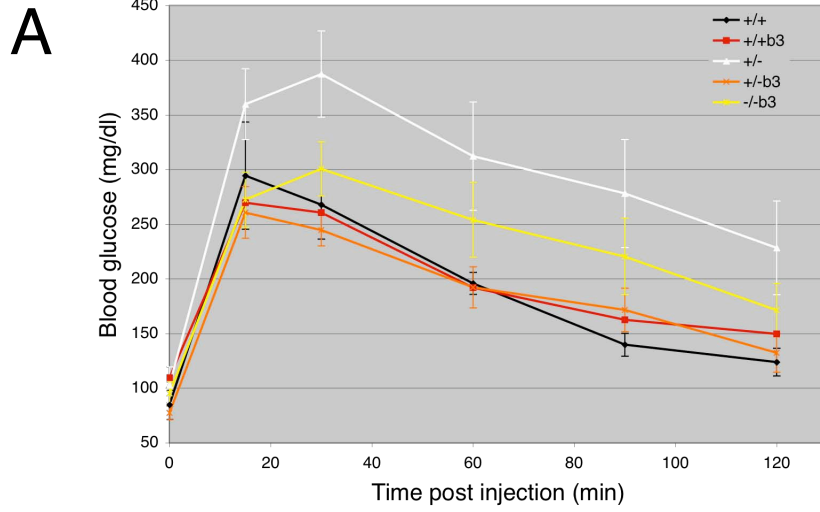
Pdx1 protein in islets of $Pdx1^{-/-;b3Tg}$ mice appeared to be slightly lower than wild type, while that in $Pdx1^{-/-;b1Tg}$ mice was slightly higher than wild type. To obtain a more quantitative assessment of the level of Pdx1 expression in the transgene-rescue mice, we measured the amount of Pdx1 protein in pancreatic extracts by densitometric analysis of western blots. By normalizing band intensity to that of a constitutively expressed nuclear protein, histone 2B, we calculated the amount of nuclear Pdx1 in heterozygotes to be approximately 43% of wild-type levels in 3-month-old littermates (Fig. 3.4C). Western blot analysis of b3Tg yielded results consistent with our prediction: the $Pdx1^{-/-;b3Tg}$ mice had protein levels between $Pdx1^{+/-}$ and wild type (~56% of wild type). However, the level of Pdx1 was at least twice that of wild type for all mice carrying b1Tg (Fig. 3.4C). Overexpression in acinar tissue may have contributed to the high level of Pdx1 in b1Tg mice, but the level of Pdx1 in the islets of $Pdx1^{-/-;b1Tg}$ was also higher than in wild-type mice (Fig. 3.2). It seems unlikely, therefore, that glucose intolerance in $Pdx1^{-/-;b1Tg}$ mice resulted from an insufficient level of Pdx1 in mature β cells. In addition, the lack of glucose intolerance in $Pdx1^{+/-;b1Tg}$ mice suggested that overexpression of Pdx1 in acinar, α, and PP cells did not significantly impair islet function. We have yet to decipher the underlying cause of glucose intolerance in the transgene-rescue mice, but reduced production of duodenal incretins, or minor perturbation of islet development, may be contributing factors (see Discussion).

Pdx1 expression selectively reduced in postnatal duodenum and stomach

We next characterized the developmental time-course of Pdx1 expression from b1Tg and b3Tg to determine whether proximal regulatory regions were sufficient for normal spatiotemporal expression of Pdx1 during development. Breeding the 12.5 kb transgene onto

a *Pdx1*^{-/-} background (the "XBko" allele (Offield et al., 1996) from which no protein product is detectable, *e.g.*, Fig. 3.7D) ensured that the only Pdx1 protein detected was transgene-encoded. No consistent differences in Pdx1 expression were observed after side-by-side preparation and staining of tissues from wild-type and *Pdx1*[-/-;Tg] embryos from E12.5 and E16.5 (Fig. 3.5A,B,D,E), indicating that relatively proximal sequences are indeed sufficient to mediate the proper timing and distribution of embryonic Pdx1 expression. In addition, immunostaining for glucagon, insulin and amylase indicated that pancreatic tissues were properly differentiated in transgene-rescue embryos at these stages (Fig. 3.6).

In contrast to the normal spatiotemporal expression observed during embryogenesis, the 12.5 kb transgene was not sufficient for normal levels of expression in postnatal duodenum. In neonatal (P0) mice, expression of Pdx1 was much weaker in the duodenal epithelium of *Pdx1*[-/-;Tg] mice as compared to wild-type littermates, but pancreatic expression was not appreciably different (Fig. 3.5C,F). Reduced duodenal Pdx1 expression was observed in both transgenic lines and persisted throughout adulthood (Fig. 3.5G-N), although the level of transgenic Pdx1 expression was consistently lower in b3Tg than in b1Tg mice (*cf.* Fig. 3.5J,M). Pdx1 expression was not substantially reduced in the submucosal Brunner's glands that cuff the rostral-most portion of the duodenum (Fig. 3.5H,K).

Pdx1 immunoreactivity was substantially weaker than wild-type or *Pdx1*^{+/-} levels in the mucosal epithelium of all *Pdx1*[-/-;Tg] duodena examined from both transgenic lines. There was, however, some variability in the level and pattern of duodenal Pdx1 expression among transgene-rescue mice of identical genotype. Also, most of the *Pdx1*[-/-;Tg] mice from both lines showed a variable intensity of Pdx1 staining along the duodenal epithelium. In general, Pdx1 was more strongly expressed in crypts than in villi (*e.g.*, Fig. 3.5J), although

Fig. 3.5 Developmental profile of Pdx1 expression in *Pdx1$^{-/-;Tg}$* compared to wild type.
(A,B,D,E) Pdx1 expression in *Pdx1$^{-/-;Tg}$* was similar to wild type during embryonic stages. The extent of expression in the stomach appeared to be reduced in *Pdx1$^{-/-;Tg}$* at E12.5, but this may be an artifact due to the plane of section. No difference in expression in the stomach was seen at E16.5. Representative sections, *Pdx1$^{-/-;b1Tg}$* at e12.5 (D) and *Pdx1$^{-/-;b3Tg}$* at e16.5 (E). VP, ventral pancreatic bud; DP, dorsal pancreatic bud; Du, duodenum; St, stomach; Pa, pancreas. (C,F) After birth, reduced Pdx1 immunoreactivity was observed in *Pdx1$^{-/-;Tg}$* duodena (*Pdx1$^{-/-;b3Tg}$* shown in F). (G – M) Duodenal Pdx1 expression continued to be substantially lower in *Pdx1$^{-/-;Tg}$* postnatally. Representative sections: *Pdx1$^{-/-;b1Tg}$* (J) and *Pdx1$^{-/-;b3Tg}$* (K,L,M). (G, J, M, N) One week of age. Duodenal Pdx1 expression was weaker in *Pdx1$^{-/-;b3Tg}$* (M) than in *Pdx1$^{-/-;b1Tg}$* (J). Both were much weaker than *Pdx1$^{+/-}$* (N) or wild type (G). The intensity of Pdx1 staining in pancreatic tissue was similar among the different genotypes. (H,K) 3 months of age, sections of duodenum near the gastroduodenal junction showed Pdx1 expression in *Pdx1$^{-/-;Tg}$* that was relatively well-preserved in Brunner's glands (Br), variable in crypts (Cr) and weakest in villi (Vi). Insets in (H,K), higher magnification of Pdx1+ nuclei in Brunner's glands. All tissues from each stage were processed, stained and photographed side-by-side under the same conditions. Dotted outlines in (F,G,J,N) indicate islet tissue. Scale bars 200 μm. Panels (B,E) 20X magnification; all others 55X.

E12.5   E16.5   P0

1 week   3 months   8 months
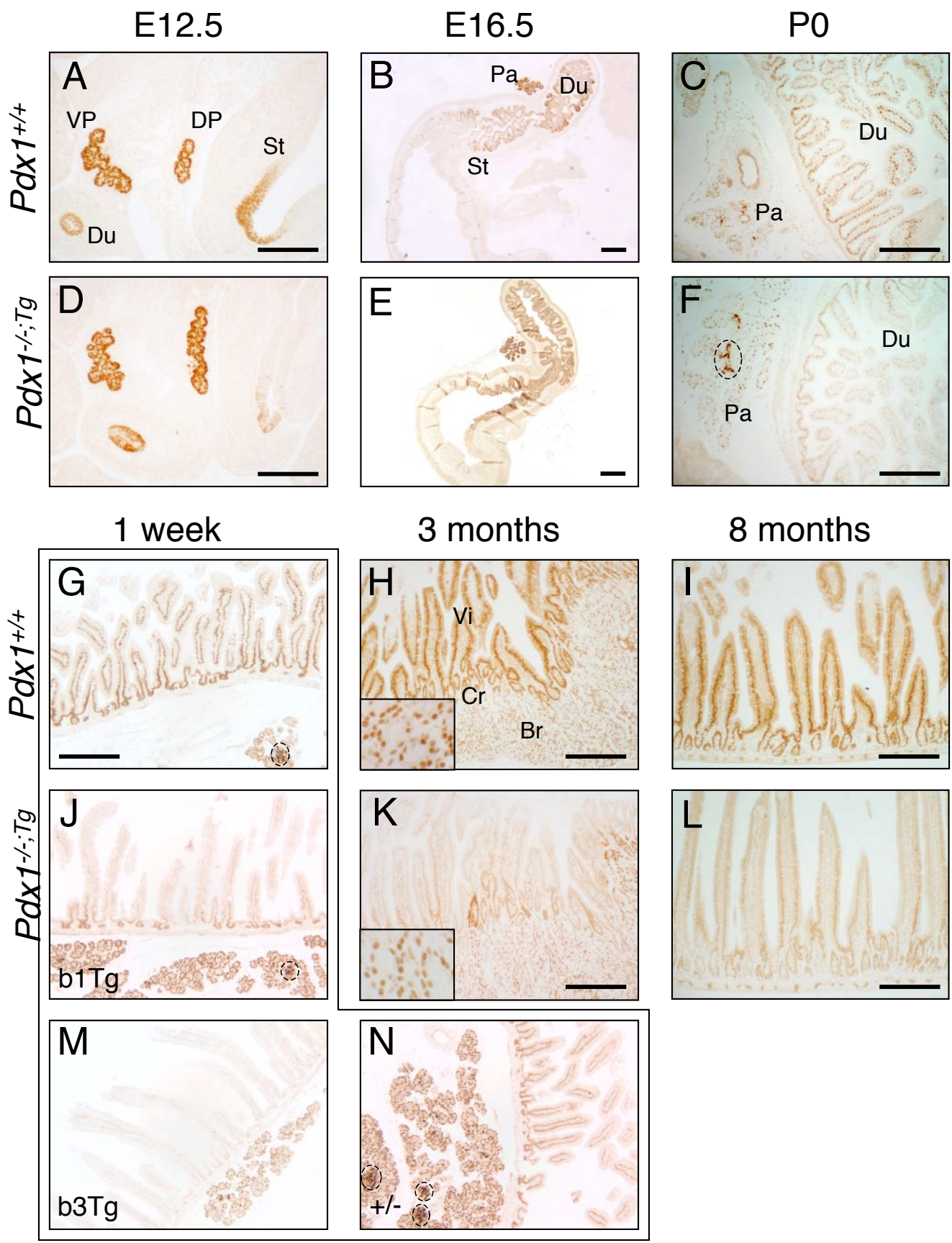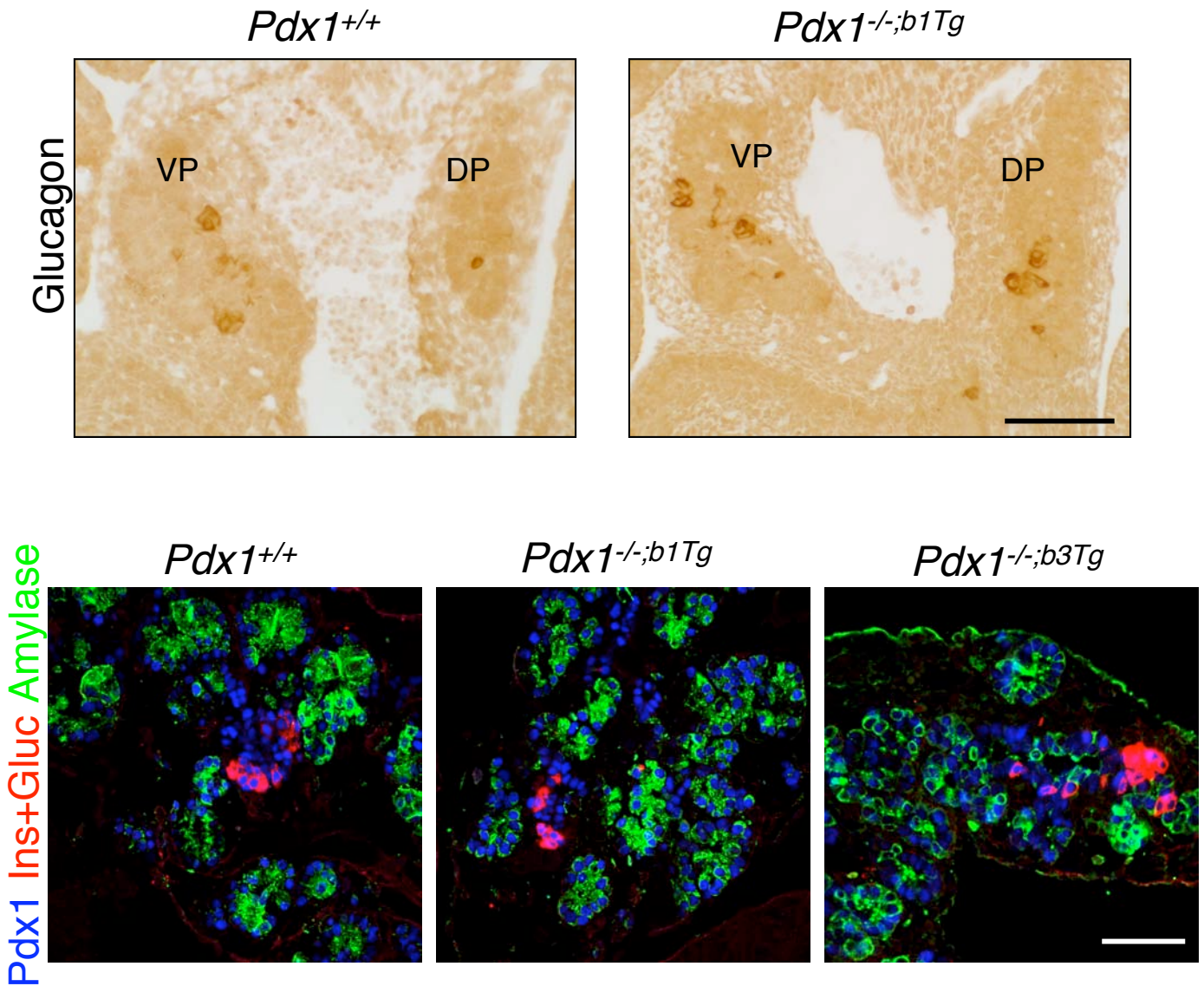
Fig. 3.6 Pancreatic buds of transgene-rescue embryos were properly differentiated at E12.5 and E16.5. *Upper panels*, similar numbers of glucagon-expressing cells were detected in the ventral (VP) and dorsal (DP) pancreatic buds at E12.5 in wild-type and transgene-rescue mice. *Lower panels*, the pancreatic buds of transgene-rescue mice appeared to be appropriately differentiated at E16.5 based on expression of amylase (green) and endocrine hormones (red, insulin and glucagon). Nuclei of pancreatic epithelial cells were labeled for Pdx1 (blue). Scale bars 50 μm.

the level of expression varied among neighboring crypts (*e.g.*, Fig. 3.5K, Fig. 3.12C).

Ribbons of relatively high-expressing cells were frequently observed extending up the

proximal villi associated with the more strongly expressing crypts, consistent with the

progenitor-daughter relationship between the surrounding crypts and their derived villus

epithelial cells. In wild type and $Pdx1^{+/-}$ mice, Pdx1 appeared to be expressed at equal levels

in all crypts and villi of the rostral portion of the duodenum, although the intensity of

immunostaining often appeared stronger at the bases of the villi than at their tips. We

hypothesize that a variegated expression pattern is observed in $Pdx1^{-/-;Tg}$ duodenal epithelium

because an abnormally low level of *Pdx1* transcription in postnatal crypt stem cells

exacerbates the influence of stochastic transcriptional activation (*i.e.*, "transcriptional

noise"). This leads to the production of a low, but variable, level of Pdx1 protein in basal

enterocytes that is gradually lost as they ascend the villi.

The 12.5 kb transgene also failed to provide full expression of Pdx1 in the

antropyloric mucosa of the postnatal stomach (Fig. 3.7A-D). Detection of Pdx1 was rather

weak even in wild-type stomach at P7 but was more readily detected at 3 months of age, at

which time the difference in immunoreactivity between wild type and $Pdx1^{-/-;b3Tg}$ was more

pronounced (Fig. 3.8A,B). In wild-type P7 mice, Pdx1 was detectable in all antropyloric

epithelial cells with scattered cells staining much more intensely for Pdx1 (Fig. 3.7A). In

$Pdx1^{-/-;b1Tg}$ mice, the scattered high-expressing cells were still readily detected, but the

overall level of Pdx1 appeared to be lower (Fig. 3.7B). As in the pancreas and duodenum,

the level of expression from b3Tg was less than from b1Tg. Although scattered cells in

$Pdx1^{-/-;b3Tg}$ stomach had higher levels of Pdx1 expression, their immunostaining intensity

Fig. 3.7 Concordant reduction in Pdx1 and gastrin in stomach at P7. (A – D) Immunoperoxidase staining for Pdx1 in the antropyloric mucosa showed progressive reduction in intensity from wild type to $Pdx1^{-/-;b1Tg}$ to $Pdx1^{-/-;b3Tg}$, and no staining in $Pdx1^{-/-}$. (E – H) Frequency of gastrin-immunopositive cells (brown) showed a similar correlation to genotype. Immunoperoxidase-DAB, hematoxylin counterstain. Scale bars 100 μm. All panels 150X magnification.

A *Pdx1+/+*

B *Pdx1-/-;b1Tg*

C *Pdx1-/-;b3Tg*

D *Pdx1-/-*

E *Pdx1+/+*

F *Pdx1-/-;b1Tg*

G *Pdx1-/-;b3Tg*

H *Pdx1-/-*
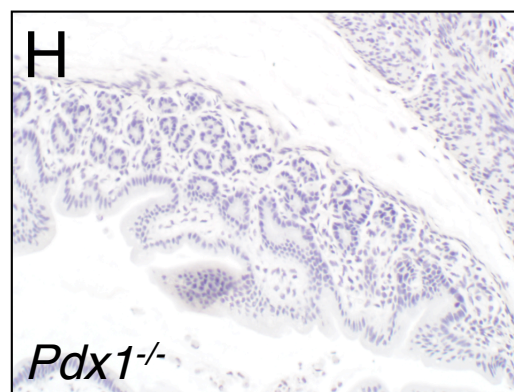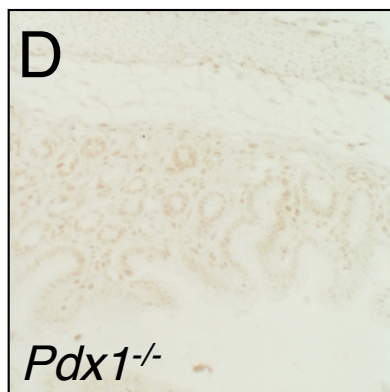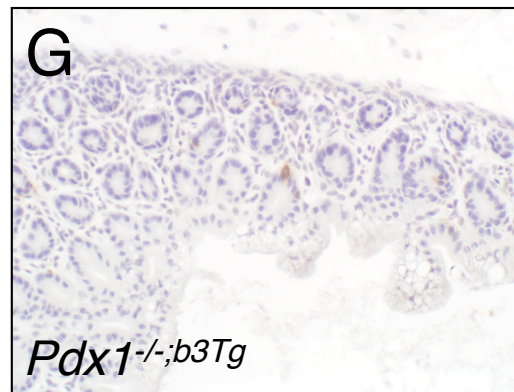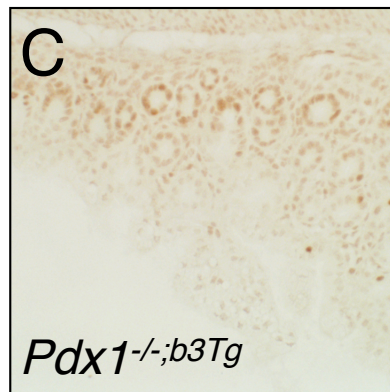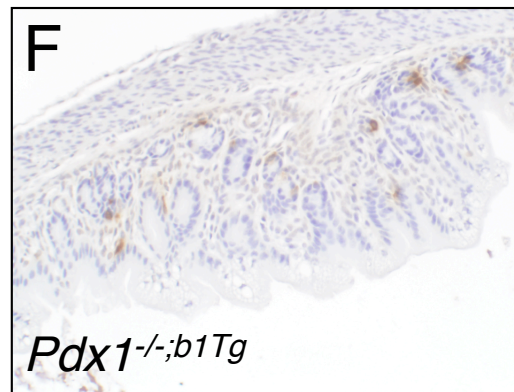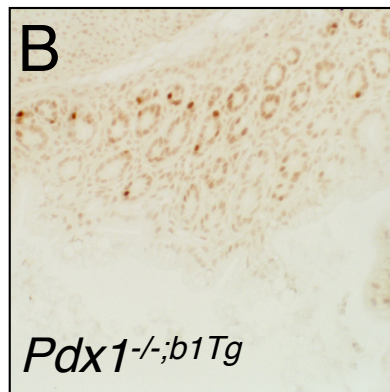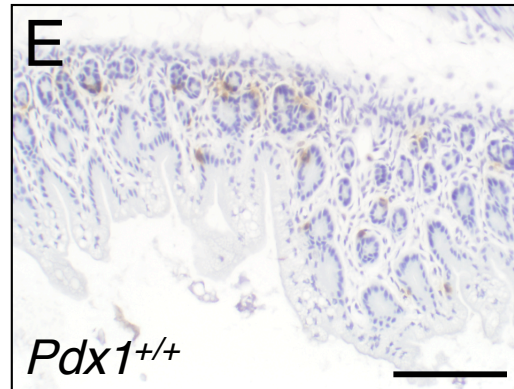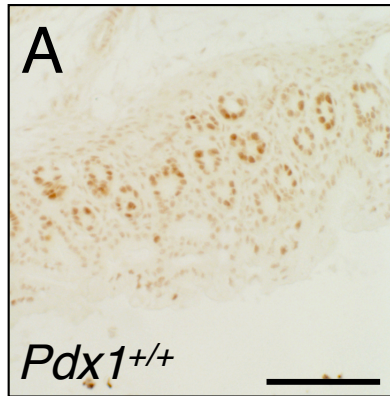
Fig. 3.8 Antral mucosa of stomach was labeled for Pdx1 at 3 mo and somatostatin at P7. (A) In antral mucosa of wild-type mice, Pdx1 staining was most intense in the pits and was detectable in all epithelial cell nuclei. (B) In *Pdx1*$^{-/-;b3Tg}$ mice at 3-months-of-age, Pdx1 staining of variable intensity was detected in the antral pits, and Pdx1 staining was not evident in the upper portion of the glands. (C,F) As previously reported, somatostatin-expressing cells (dark brown cytoplasmic staining) were detectable in the antropyloric mucosa of P7 *Pdx1*$^{-/-}$ mice at a similar frequency as in wild type. (D,E) Somatostatin-expressing cells were also detected in transgene-rescue mice at P7.

A *Pdx1$^{+/+}$*

B *Pdx1$^{-/-;b3Tg}$*

C *Pdx1$^{+/+}$*

D *Pdx1$^{-/-;b1Tg}$*

E *Pdx1$^{-/-;b3Tg}$*

F *Pdx1$^{-/-}$*
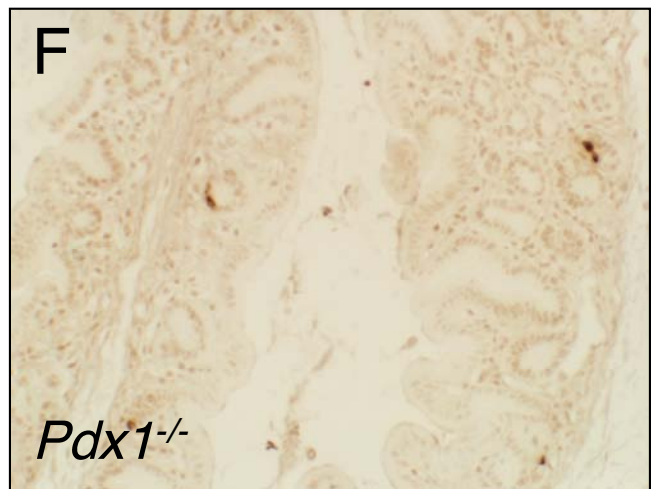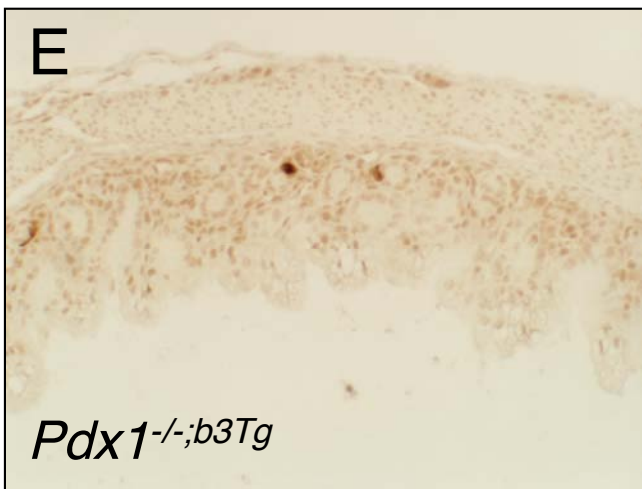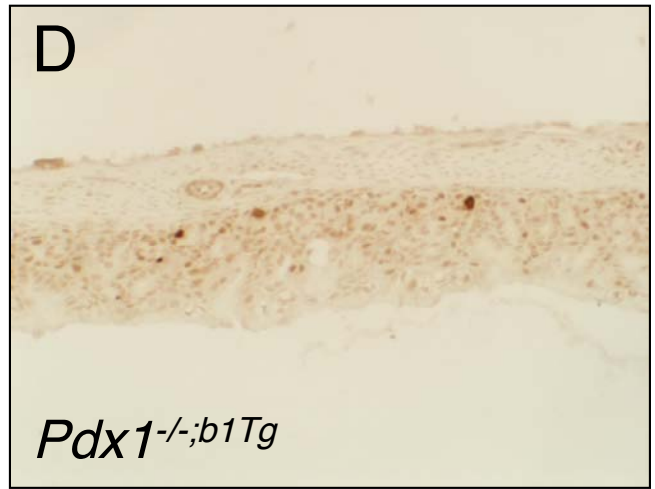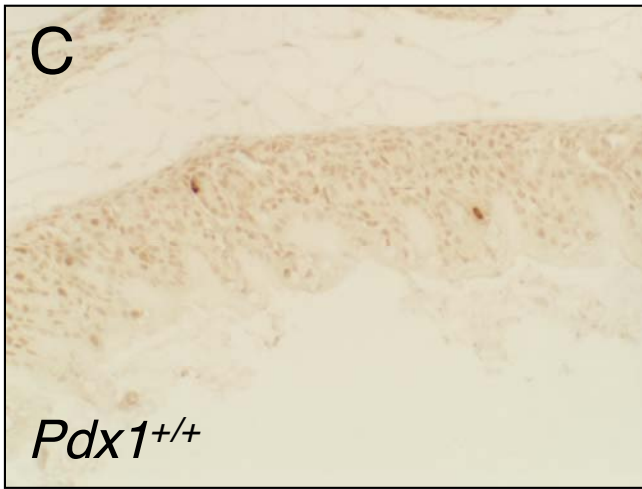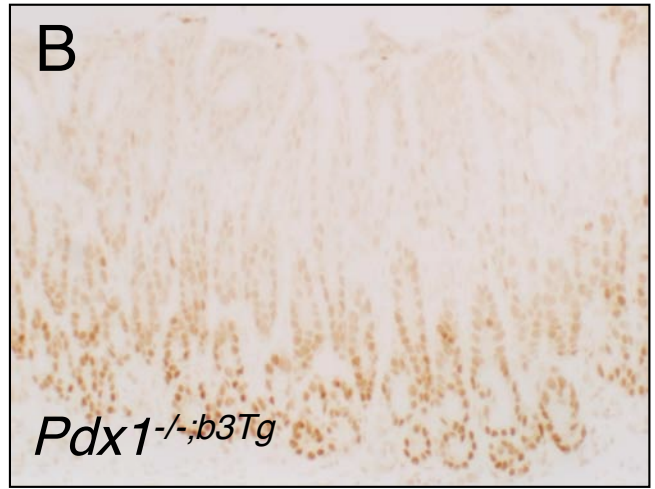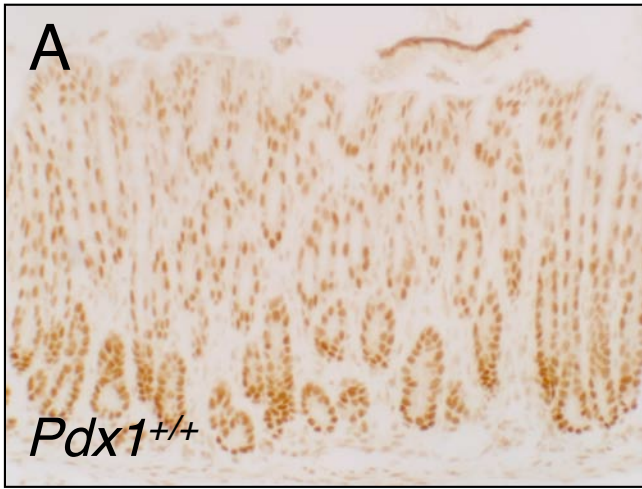
was still weaker than wild type (Fig. 3.7C). Consistent with previous reports (Fujitani et al., 2006; Larsson et al., 1996), expression of gastrin in the antropyloric mucosa was undetectable in P7 $Pdx1^{-/-}$ mice (Fig. 3.7H). The number of gastrin cells appeared to be slightly fewer than wild type in $Pdx1^{-/-;b1Tg}$ and was substantially reduced in $Pdx1^{-/-;b3Tg}$ at P7 (Fig. 3.7E-G). The number of somatostatin cells in the stomach was not reduced in $Pdx1^{-/-}$ or in transgene-rescue mice (Fig. 3.8C-F).

Well-differentiated duodenum in $Pdx1^{-/-;Tg}$ mice

To assess the phenotypic consequences of reduced Pdx1 expression in the duodenum, we compared morphology and marker expression in wild-type, $Pdx1^{+/-}$, $Pdx1^{-/-}$ and $Pdx1^{-/-;Tg}$ mice. $Pdx1^{+/-}$ mice were indistinguishable from wild type for all of the criteria examined, and for simplicity are therefore omitted from Fig. 3.9. While the transformation from a pseudostratified epithelium to the initial crypt-villus architecture occurs over a relatively short period towards the end of gestation, the maturation of the duodenum continues significantly after birth. Over the first few weeks of life there is a thickening of the muscular wall of the duodenum, increased crypt depth, increased numbers of enteroendocrine cells, and proliferation of Brunner's glands. Our analysis focused on P7 mice because it was the latest stage at which $Pdx1^{-/-}$ pups could consistently be recovered. The major anatomical features of the duodenum, *i.e.,* the crypts, villi and submucosal Brunner's glands, were all present in $Pdx1^{-/-}$ pups, but the villi tended to be much shorter and sparser than wild type, with a crumpled appearance (Fig. 3.9B,D). Brunner's glands were greatly reduced in number and were poorly differentiated based on morphology and PAS staining (Fig. 3.9H). These defects were not found in $Pdx1^{-/-;Tg}$ mice (Fig. 3.9C,F,I) representing either the b1 or b3 lines.
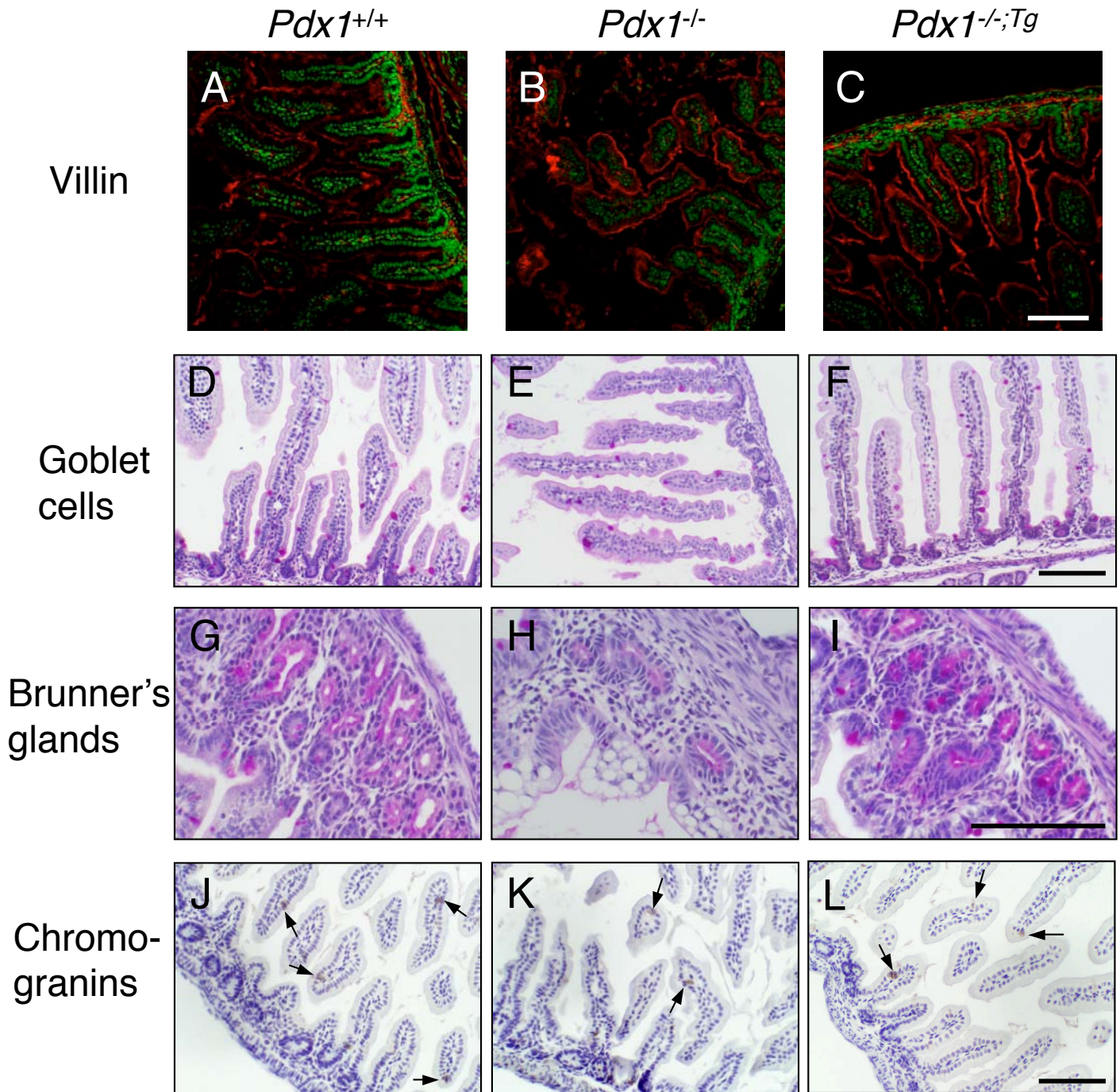
Fig. 3.9 Well-differentiated duodenal epithelium in *Pdx1*$^{-/-;Tg}$ mice at P7. (A – C) Apical villin expression (red), characteristic of differentiated enterocytes in wild-type, *Pdx1*$^{-/-}$ and *Pdx1*$^{-/-;Tg}$ mice (green: nuclei detected with YoPro-1). (D – F) PAS-stained goblet cells (magenta; hematoxylin counterstain) were present at similar frequencies in wild-type, *Pdx1*$^{-/-}$ and *Pdx1*$^{-/-;Tg}$ mice. Villi in *Pdx1*$^{-/-}$ had a ragged appearance not observed in *Pdx1*$^{-/-;Tg}$. (G – I) Number, elaboration and mucin content of Brunner's glands was greatly reduced in *Pdx1*$^{-/-}$, but normal in *Pdx1*$^{-/-;Tg}$. Staining as in (D – F). (J – L) Immunoperoxidase-DAB staining of Chromogranins (brown; hematoxylin counterstain) indicated enteroendocrine cells (arrows) in wild type, *Pdx1*$^{-/-}$ and *Pdx1*$^{-/-;Tg}$ duodena, although fewer in *Pdx1*$^{-/-}$. Scale bars are 100 μm.

The predominant duodenal epithelial cell is the enterocyte, which was confirmed to be present in wild-type and mutant animals by its apical villin (Fig. 3.9A-C). The villus epithelium also contains mucous-secreting goblet cells, stained by the PAS method (Fig. 3.9D-F), and hormone-producing enteroendocrine cells, identified by production of chromogranins (Fig. 3.9J-L). While the frequency of goblet cells appeared unchanged in the $Pdx1^{-/-}$ mutants, the number of epithelial cells expressing chromogranins appeared lower, consistent with previous reports of reduced enteroendocrine cell populations in these mutants (Jepeal et al., 2005; Larsson et al., 1996; Offield et al., 1996). The number of chromogranin-expressing cells was equivalent between $Pdx1^{-/-;Tg}$ and wild-type pups. Because the presence of specific enteroendocrine hormone-producing cells is differentially sensitive to the loss of $Pdx1$ (Jepeal et al., 2005; Larsson et al., 1996; Offield et al., 1996), we analyzed our transgene-rescue mice for cells producing gastrin, GIP, and CCK.

GIP cell number is exquisitely sensitive to the level of Pdx1

The number of GIP cells in the rostral duodenum is profoundly reduced in $Pdx1^{-/-}$ mice (Jepeal et al., 2005), and deletion of Area I-II-III revealed that low levels of Pdx1 during embryogenesis resulted in dose-dependent reduction in the number of GIP cells at E18.5 (Fujitani et al., 2006). In contrast to mice with Area I-II-III deletion, the duodenum of transgene-rescue mice showed normal levels of Pdx1 during embryogenesis, but markedly reduced postnatal expression. We quantified the number of GIP-expressing cells to evaluate whether postnatal maintenance of the GIP cell population required sustained expression of Pdx1. Gastrin- and CCK-producing cells were included in the quantitative analysis because

they have been shown to be decreased in $Pdx1^{-/-}$ mice (Larsson et al., 1996; Offield et al., 1996).

Compared to wild type, the frequency of GIP cells was reduced by approximately 50% in $Pdx1^{-/-;b1Tg}$, and 90% in $Pdx1^{-/-;b3Tg}$ mice (Fig. 3.10A). No GIP-expressing cells were identified in extensive sampling of rostral duodena from three $Pdx1^{-/-}$ mice. A slight, but statistically insignificant, reduction in GIP cell frequency was found in $Pdx1^{+/-}$ mice compared to wild type. The trend of GIP cell frequency among the five genotypes correlated well with the intensity of Pdx1 immunostaining on duodenal sections processed side-by-side under identical conditions (Fig. 3.5G,J,M,N), suggesting that Pdx1 has a dose-dependent influence on the postnatal generation of GIP-producing cells. The frequencies of gastrin- and CCK-expressing cells were reduced in $Pdx1^{-/-}$ mice by approximately 57% and 60% respectively, but were not significantly affected in mice of the other genotypes (Fig. 3.10B,C). The 25% reduction in CCK cell frequency observed in $Pdx1^{-/-;b3Tg}$ mice compared to wild type had a P value between 0.1 and 0.05 by t-test analysis, suggesting that the reduction was correlated with the transgene-rescue genotype. In summary, duodenal GIP, gastrin and CCK cells were reduced in $Pdx1^{-/-}$ mice at P7, but a strong dose-dependent effect of Pdx1 was found for GIP only. We also observed a dose-dependent influence of Pdx1 on antropyloric gastrin cells (Fig. 3.7). The specific effect of postnatal Pdx1 dosage on duodenal GIP and antropyloric gastrin cells is consistent with our previous analysis of Pdx1 dosage effects (Fujitani et al., 2006).

Fig. 3.10 Frequency of enteroendocrine cells in duodenum at P7. The number of GIP, gastrin and CCK cells relative to the total number of duodenal epithelial cell nuclei was determined in longitudinal sections of duodenum from wild-type (n=5), $Pdx1^{+/-}$ (n=5), $Pdx1^{-/-;b1Tg}$ (n=4), $Pdx1^{-/-;b3Tg}$ (n=4) and $Pdx1^{-/-}$ (n=3) mice (see Methods for details). Crosses indicate values for individual mice, bars the mean of each group. (A) The frequency of GIP cells varied by genotype in accordance with the relative level of Pdx1 expression observed (Fig. 3.5). $P < 0.05$ for $Pdx1^{-/-;b1Tg}$, $Pdx1^{-/-;b3Tg}$ and $Pdx1^{-/-}$ compared to wild type. (B) Gastrin cell frequency in the duodenum was reduced to varying degrees in $Pdx1^{-/-}$ mice but was similar among mice of the other genotypes examined. $P < 0.05$ for $Pdx1^{-/-}$ compared to wild type. (C) CCK cell frequency was significantly reduced only in $Pdx1^{-/-}$ ($P < 0.05$) but appeared to follow a slight downward trend paralleling the relative levels of Pdx1 shown in Fig. 3.5.
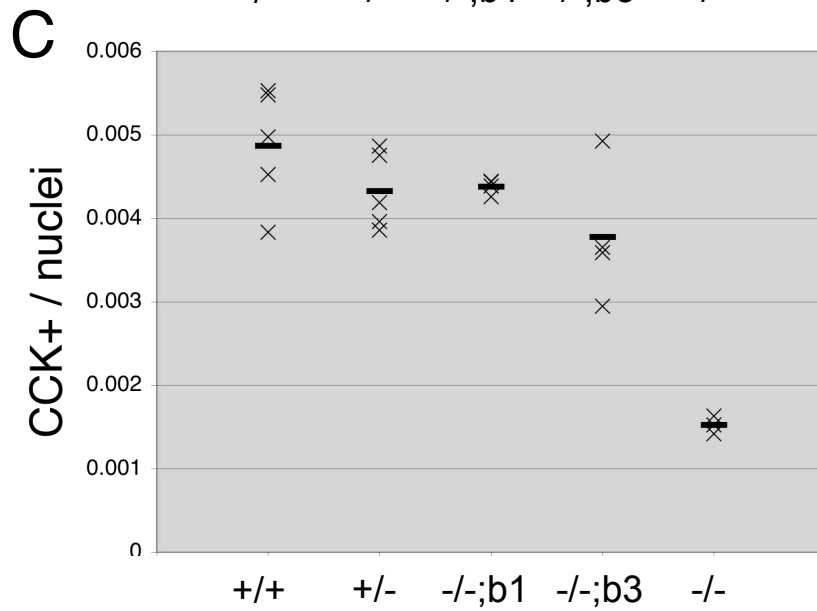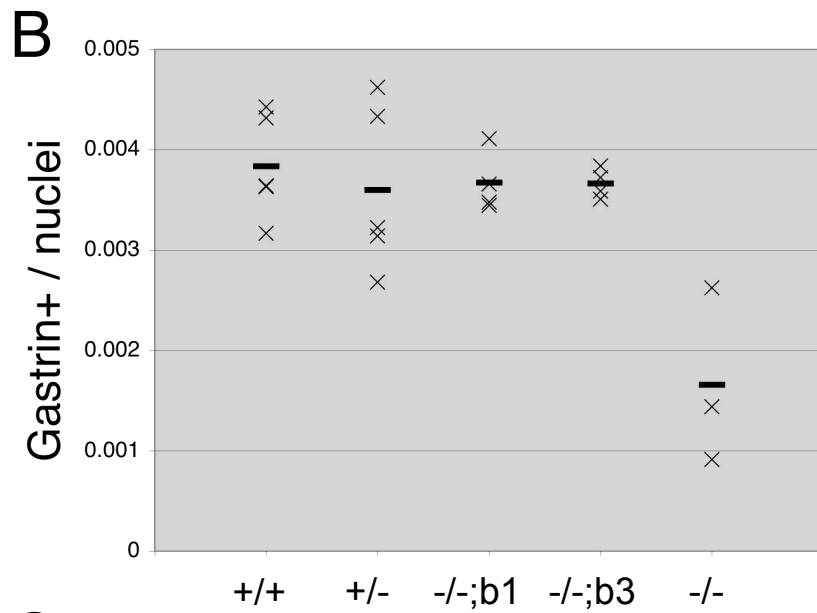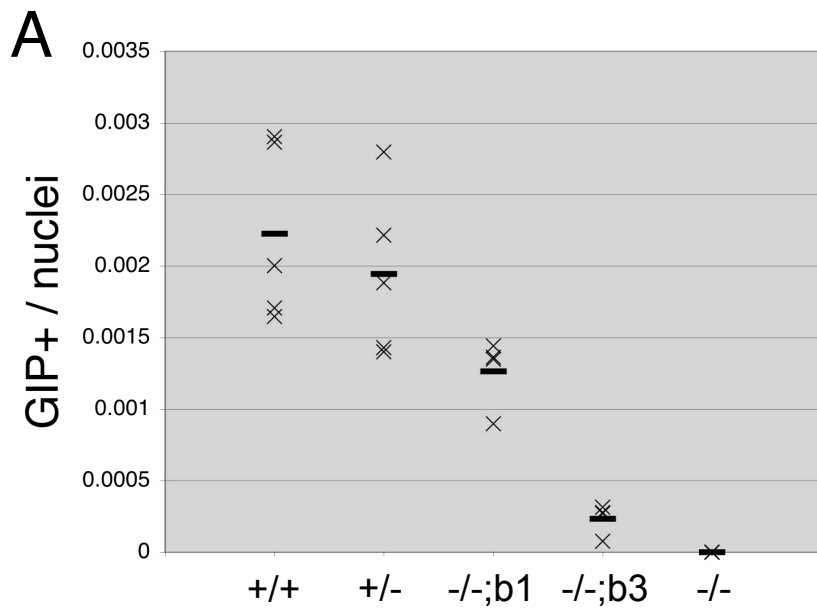
Temporal and spatial correlation of Pdx1 and enteroendocrine hormone expression

To determine if the reduction in duodenal GIP and stomach gastrin cells was temporally synchronized to the postnatal reduction in Pdx1 expression in the transgene-rescue mice, GIP and gastrin expression were examined at E16.5 and P0 (Fig. 3.11). At E16.5, gastrin expression was detected in both stomach and duodenum in wild-type, $Pdx1^{-/-;b1Tg}$ and $Pdx1^{-/-;b3Tg}$ mice. The same distribution was observed at P0 for wild-type and b3Tg mice. In $Pdx1^{-/-}$ mice, gastrin was detected in the duodenum but not stomach at E16.5 or P0. These results indicate that antropyloric gastrin cells form normally in $Pdx1^{-/-;Tg}$ mice during late embryogenesis, when Pdx1 is still robustly expressed in the posterior foregut, but the number of gastrin cells decreases during the first week of life, subsequent to the reduction of Pdx1 expression. GIP-expressing cells were not detected in the rostral duodena of any of the various genotypes at E16.5, and were only found in wild type at P0 (note: b1Tg rescue mice were not evaluated at P0). These data suggest that the initial formation of GIP cells was impaired in $Pdx1^{-/-;b3Tg}$ mice, because the time of appearance of GIP-expressing cells in wild-type mice is similar to the stage when transgene-rescue mice exhibit decreased duodenal Pdx1 expression.

Previous studies showed that Pdx1 is expressed throughout the rostral duodenal epithelium, declining caudally to a punctate expression pattern (Offield et al., 1996). These sparse caudal cells have been postulated to be enteroendocrine cells, but to our knowledge this has not yet been directly tested. Considering the evidence from the current and previous studies for strong dependence of GIP cells on Pdx1, we tested for co-expression of Pdx1 and

Fig. 3.11 Development of gastrin and GIP cells in *Pdx1* mutants. (A) DAB-labeling of gastrin-expressing cells in the stomach and duodenum of E16.5 embryos. Gastrin-positive cells could be found in both stomach and duodenum of wild-type, *Pdx1*$^{-/-;b1Tg}$ and *Pdx1*$^{-/-;b3Tg}$ embryos. In *Pdx1*$^{-/-}$ embryos, gastrin was detectable in the duodenum but not in the stomach. Although there are more gastrin-producing cells in the *Pdx1*$^{-/-;b1Tg}$ section than in the wild-type section, this was not consistently observed. (B) Gastrin-expressing cells were present in stomach and duodenum of newborn wild-type and *Pdx1*$^{-/-;b3Tg}$ pups. Gastrin cells were not found in the stomach of *Pdx1*$^{-/-}$ newborns, but were present in the duodenum (arrows) in reduced numbers as compared to wild type. (C) GIP expressing cells were detected in the rostral duodenum of newborn wild-type mice but not in *Pdx1*$^{-/-;b3Tg}$ or *Pdx1*$^{-/-}$ mice. For all images in this figure, the compound microscope iris was narrowed to aid visualization of unstained tissues.

Fig. 3.11A Gastrin E16.5

Stomach   Duodenum

Pdx1⁺ᐟ⁺

Pdx1⁻ᐟ⁻;b1Tg

Pdx1⁻ᐟ⁻;b3Tg

Pdx1⁻ᐟ⁻
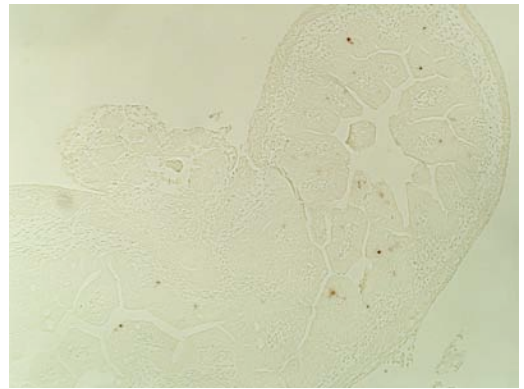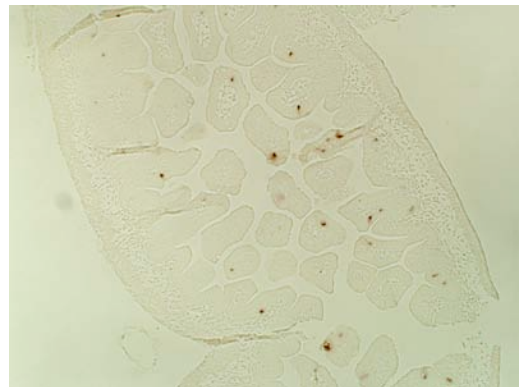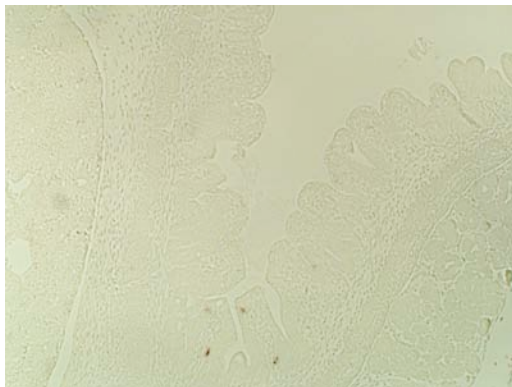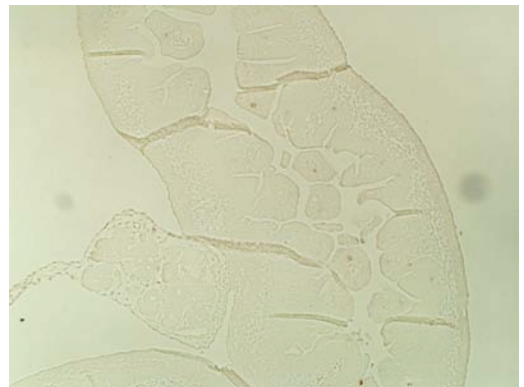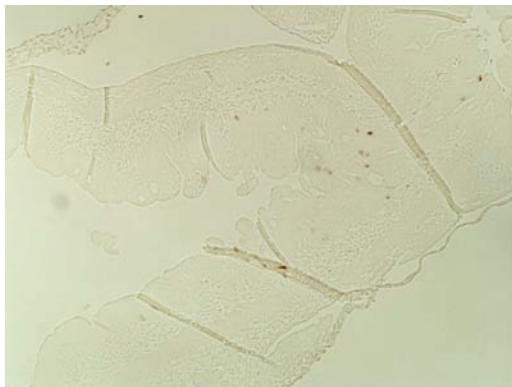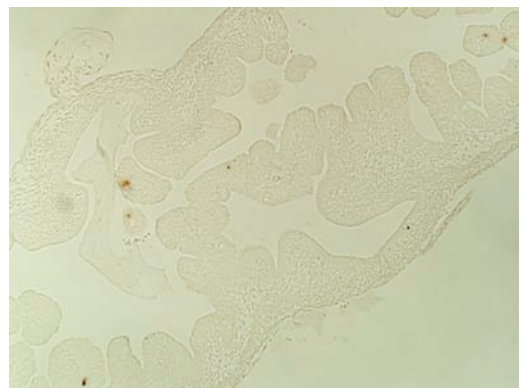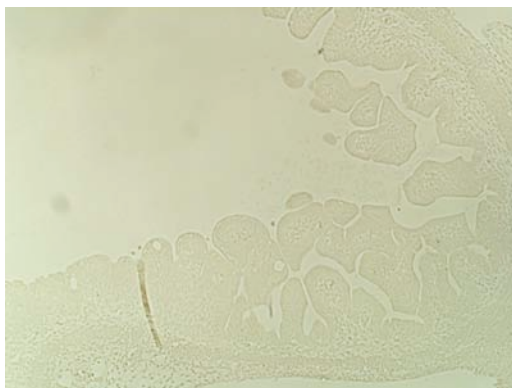
Fig. 3.11B   Gastrin P0

Stomach   Duodenum
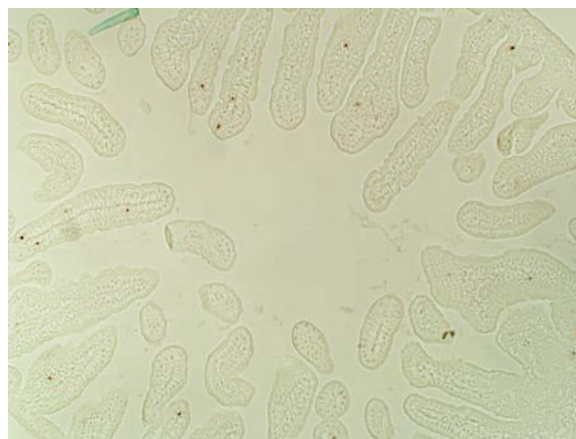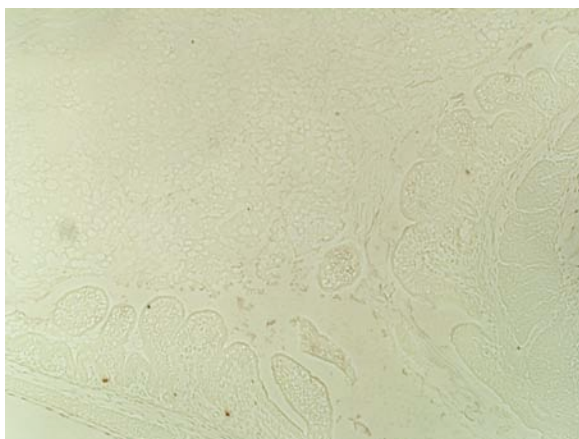
Pdx1+/+

Pdx1-/-;b3Tg
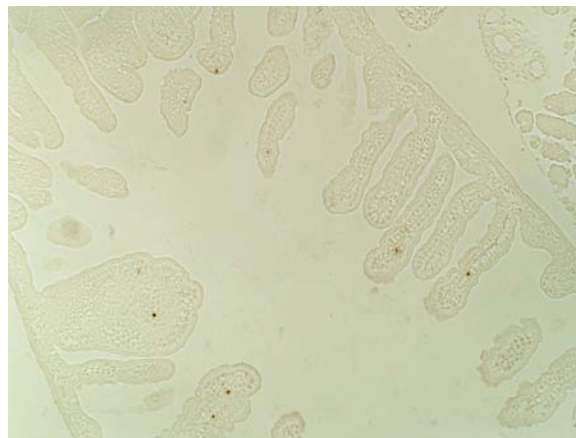
Pdx1-/-
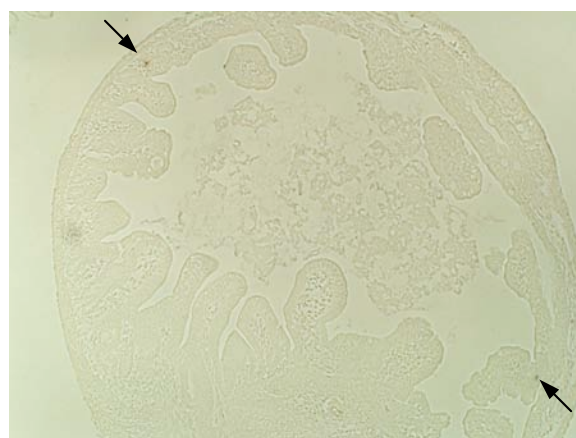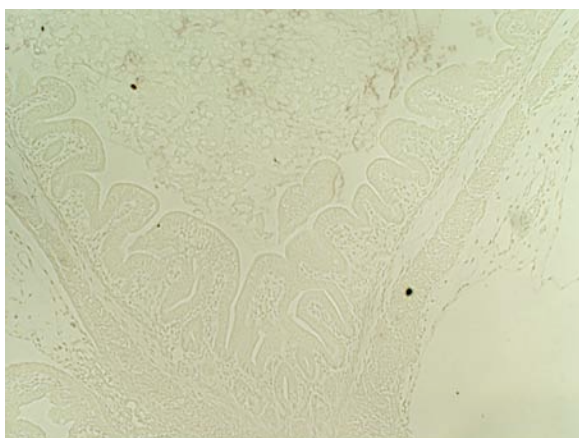
Fig. 3.11C GIP P0

Pdx1$^{+/+}$
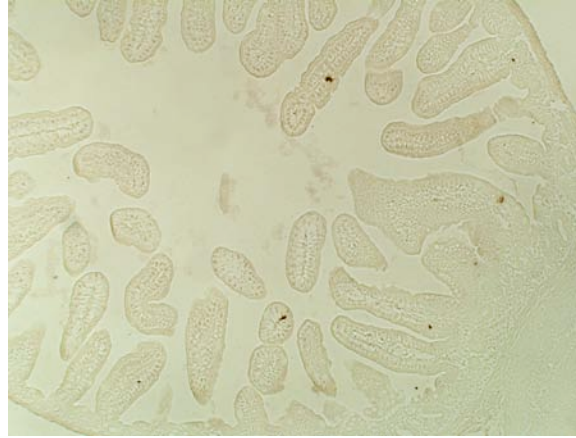
Pdx1$^{-/-;b3Tg}$
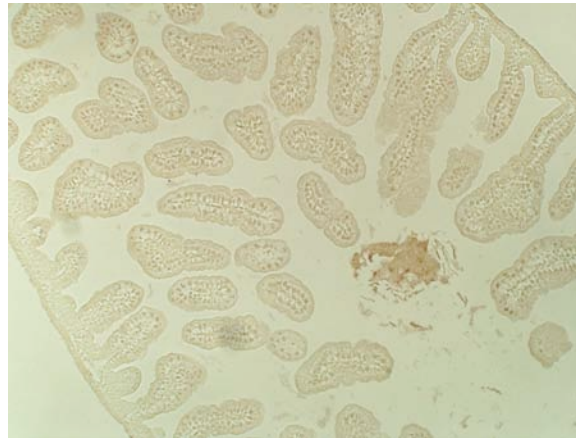
Pdx1$^{-/-}$
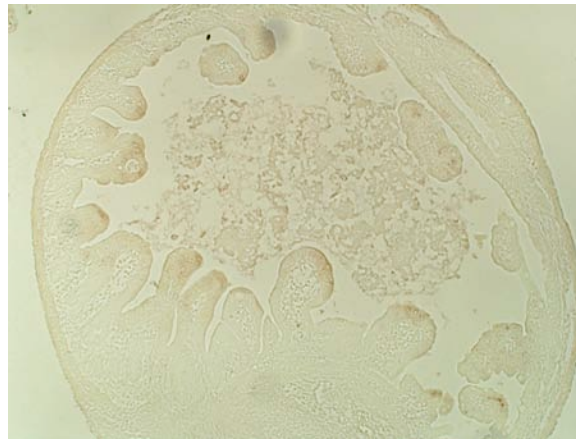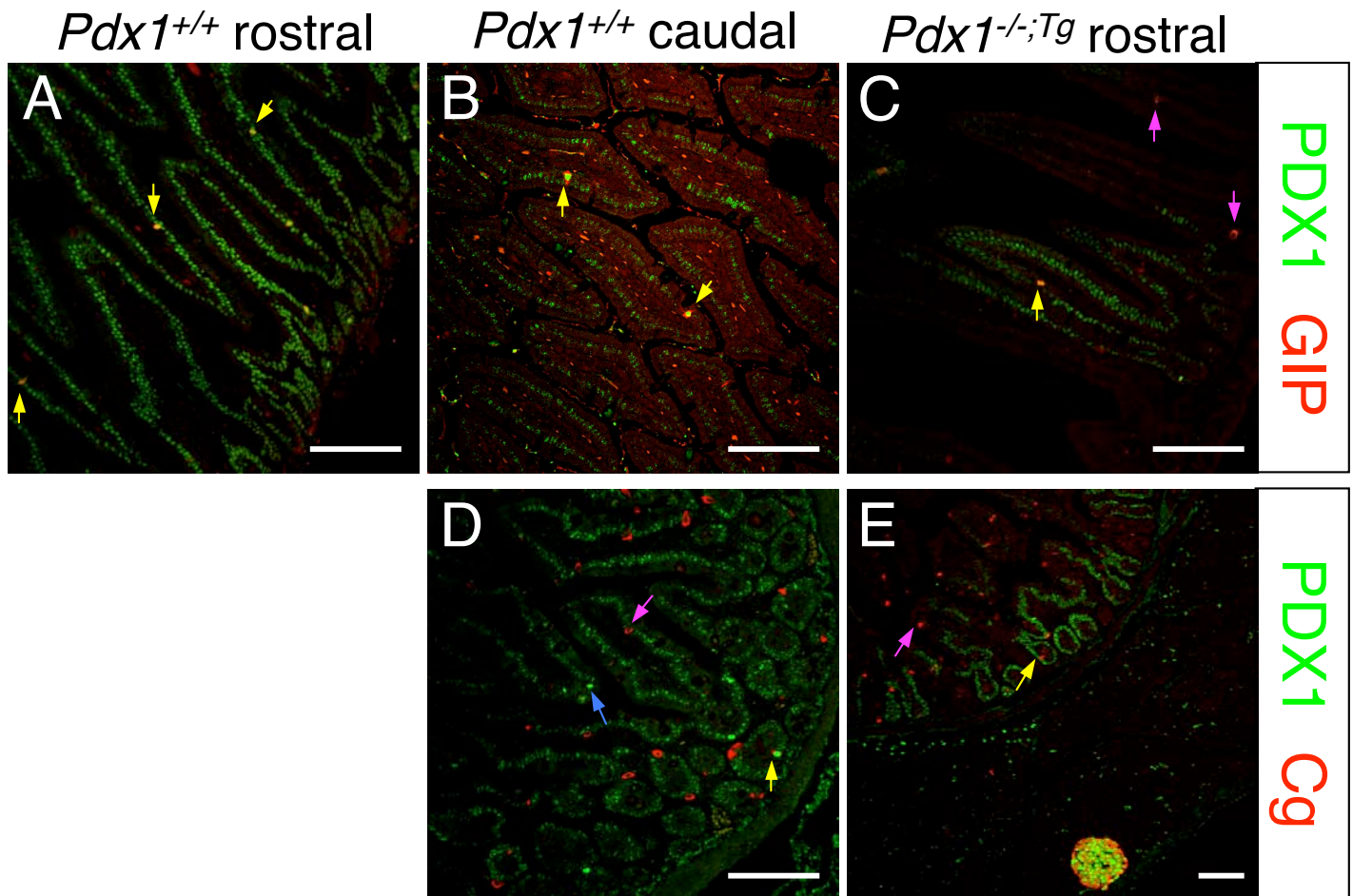
Fig. 3.12 Comparison of Pdx1 expression and enteroendocrine cell distribution in mature duodenum. Immunofluorescence analysis for (A – C) Pdx1 (green) and GIP (red), or (D,E) Pdx1 (green) and Chromogranins (red). (A) In rostral wild-type duodenum, all epithelial cells robustly expressed Pdx1. (B) In the caudal portion of wild-type duodenum, Pdx1 expression was weaker with isolated strongly immunopositive epithelial cells. All of the GIP-expressing cells observed in the caudal region had relatively strong Pdx1 expression. Background in the red channel (including RBC autofluorescence in the stromal tissue) is rather high in this panel because the gain was increased to detect weak GIP labeling. (D) Some Chromogranin-expressing cells in caudal wild-type duodenum strongly expressed Pdx1 (yellow arrow), but many did not. Some Pdx1 high-expressers did not co-label for Chromogranins (blue arrow). (C,E) The variegated pattern of Pdx1 expression in mature $Pdx1^{-/-;b3Tg}$ duodenum did not appear to influence the distribution of GIP cells (C) or of enteroendocrine cells in general (E). Pancreatic islet at lower edge of panel E is intensely Pdx1- and Chromogranin-positive. All sections from 8-month-old mice. Yellow arrows, double-labeled cells. Purple arrows indicate enteroendocrine cells with low/undetectable Pdx1 expression. Scale bars are 100 μm.

GIP in the caudal duodenum. All caudal GIP-producing cells had strong Pdx1 immunoreactivity (*e.g.,* Fig. 3.12B), while co-labeling for Pdx1 and the general endocrine marker chromogranin A/B revealed that the majority of endocrine cells in the caudal duodenum expressed relatively low levels of Pdx1 (Fig. 3.12D). These results are consistent with greater dependence of GIP expression on Pdx1 as compared to other duodenal enteroendocrine hormones. Because the duodenal epithelium of $Pdx1^{-/-;Tg}$ mutants showed a low level of Pdx1 expression with considerable regional variability (*e.g.,* Fig. 3.12C), we hypothesized that the remaining GIP cells in these mice would be found in regions with relatively high Pdx1 expression. In contrast, however, GIP-producing cells with little or no Pdx1 immunoreactivity were often observed in $Pdx1^{-/-;b3Tg}$ mice, and adjacent Pdx1 high- and low-expressing regions showed no noticeable difference in the frequency of GIP-expressing cells (Fig. 3.12C). Chromogranin-positive cells also appeared equally abundant regardless of the level of Pdx1 expression (Fig. 3.12E). We conclude that while wild-type GIP cells express a relatively high level of Pdx1, and wild-type levels of Pdx1 are required to produce the normal number of GIP cells, GIP hormone can still be produced by cells expressing a low level of Pdx1.

Discussion

Proximal sequences sufficient for pancreatic Pdx1 expression

The studies described above indicate that *cis*-acting elements within 12.5 kb surrounding and including the *Pdx1* coding sequence are sufficient to regulate most aspects of *Pdx1* gene expression in the pancreas. Expression of Pdx1 from the 12.5 kb transgene recapitulated the normal spatiotemporal expression profile of Pdx1 and supported full

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pancreatic morphogenesis, specification of all endocrine islet cells in properly organized islets, and glucose-stimulated insulin secretion from mature β cells. One possibility to explain the increased expression of Pdx1 in most pancreatic acinar cells and some α and PP cells is that the transgene lacks regulatory elements that suppress expression of Pdx1 in non-β cells in the mature pancreas.

The only non-coding regions in the transgene with at least 100 bp of greater than 75% conservation between mice and humans are the previously identified Areas I, II and III, which reside within the 4.6 kb of 5′ non-coding sequence (Fig. 3.1A). The sufficiency of this region to regulate most aspects of Pdx1 expression is consistent with *lacZ*-reporter expression profiling experiments (Gannon et al., 2001; Stoffers et al., 1999), which showed that the proximal 4.6 kb sequence (from the XbaI site at -4.5 kb to the translational start codon) mediated expression throughout the endogenous Pdx1 expression domain. The pancreatic buds of our transgene-rescue mice were indistinguishable from wild type at E12.5, suggesting that the 4.6 kb promoter region is sufficient to initiate expression in the full pancreatic progenitor population from early stages of pancreas development. Although the rescue transgene was sufficient to mediate the proper spatiotemporal expression of Pdx1 in embryonic gut epithelium, it did not support robust expression of Pdx1 in the postnatal stomach and duodenum.

Complementation phenotype consistent with enhancer deletion results

Our recent findings (Fujitani et al., 2006) demonstrated that mice homozygous for a targeted deletion of the Area I-II-III enhancer region had severely impaired pancreas development, but only mild defects in the duodenum and stomach. The current findings are

largely consistent with those results, showing that complementation rescue of $Pdx1^{-/-}$ with a transgene covering Area I-II-III is sufficient to support pancreas development but only weakly promotes Pdx1 expression in postnatal duodenum and stomach. One caveat to the enhancer deletion study was that deletion of an enhancer region from the $Pdx1$ locus might affect expression of neighboring genes, such as $Gsh1$ and $Cdx2$ in the evolutionarily conserved Parahox gene cluster (Brooke et al., 1998). By evaluating expression of Pdx1 from randomly integrated transgenes, we confirmed that regulation of $Pdx1$ by proximal elements is sufficient for pancreas morphogenesis, independent of other gene regulatory events that may occur in the endogenous locus. Because both lines that expressed detectable Pdx1 showed identical spatiotemporal expression, it seems reasonable to conclude that the expression pattern was specific to the regulatory elements contained in the transgene sequence, and was not significantly influenced by the site of transgene integration.

Transgene rescues glucose tolerance fully in $Pdx1^{+/-}$, partially in $Pdx1^{-/-}$

Both transgenic lines were able to rescue the glucose intolerance that results from heterozygosity at the endogenous $Pdx1$ locus, and complementation of $Pdx1^{-/-}$ mice resulted in glucose clearance that was intermediate between wild type and $Pdx1^{+/-}$. We were surprised to discover that the $Pdx1^{-/-;b1Tg}$ mice were glucose intolerant despite producing higher levels of Pdx1 than wild type. We have not yet been able to determine the cause of glucose intolerance in these mice, but one possibility is that the transgene-rescue mice have a subtle defect in islet development that results in a mildly abnormal glucose response even when their β cells contain abundant Pdx1. Gap junction connectivity between β cells and interactions between β cells and the islet capillary network are important for islet function

(Rocheleau et al., 2006; Lammert et al., 2003). Subtle abnormalities in the expression of

Pdx1 in developing or maturing islets might disrupt the proper establishment of these

interactions, resulting in impaired glucose tolerance. Another possibility is that the impaired

islet function is secondary to the reduced number of GIP cells in the transgene-rescue mice.

GIP has a well-characterized incretin effect enhancing the response of β cells to glucose

(reviewed by Drucker, 2006), such that decreased GIP production in the transgene-rescue

mice may limit the responsiveness of their β cells.

Mice that had both wild-type alleles of *Pdx1* plus either b1Tg or b3Tg (*Pdx1*$^{+/+;Tg}$)

produced supraphysiological levels of pancreatic Pdx1. Neither of these genotypes had

deleterious effects on pancreatic development or function, nor was there accelerated glucose

clearance in the presence of excess Pdx1. We conclude that the wild-type level of Pdx1 is

required for normal glucose clearance, but that excess Pdx1 does not enhance the response of

β cells to glucose challenge.

Proximal *Pdx1* promoter elements express poorly in duodenum

Although both transgenic alleles produced more Pdx1 in the pancreas than a single

wild-type allele (*i.e., Pdx1*$^{+/-}$), rescue of *Pdx1*$^{-/-}$ with either transgenic allele resulted in much

weaker expression of Pdx1 in the duodenum than was observed for *Pdx1*$^{+/-}$ mice (*cf.* Fig.

3.5J,M,N). The transgene-rescue mice also had reduced Pdx1 expression in the stomach, but

the reduction compared to the wild-type level was not as dramatic as in the duodenum.

Stoffers *et al*. (1999) examined expression of a *lacZ* reporter driven by the 4.6 kb 5′ *Pdx1*

promoter region and reported weak X-gal staining in the duodenal epithelium, even though

Pdx1 protein was readily detected by immunohistochemistry. They speculated that weak X-

gal staining in the duodenum resulted from poor perdurance of β-galactosidase after

production in the crypts. Arguing against this reasoning, however, mice heterozygous for a

knock-in of *lacZ* into the *Pdx1* locus are X-gal positive throughout the epithelium of the

rostral duodenum (Offield et al., 1996). The difference in duodenal expression between the

knock-in and transgenic β-galactosidase reporter alleles is consistent with the reduced level

of Pdx1 expressed from our 12.5 kb rescue transgene as compared to the endogenous locus.

These results suggest that sequences upstream of -4.5 kb are required for full duodenal

expression.

Dose-dependent influence of Pdx1 on GIP and antral stomach gastrin cells

The intensity of Pdx1 immunoreactivity indicated that b1Tg produced more Pdx1

protein in the duodenum than b3Tg, but less than $Pdx1^{+/-}$. We were therefore able to analyze

the influence of a spectrum of Pdx1 protein levels in the duodenum: $Pdx1^{+/+} > Pdx1^{+/-} >$

$Pdx1^{-/-;b1Tg} > Pdx1^{-/-;b3Tg} > Pdx1^{-/-}$. Interestingly, the frequency of GIP-expressing cells at one

week of age followed the same trend as the level of Pdx1 protein. GIP has recently been

shown to be a direct transcriptional target of Pdx1, and expression of GIP is virtually absent

from $Pdx1^{-/-}$ duodenal epithelium (Jepeal et al., 2005). If Pdx1 was simply a direct

transactivator of the *GIP* gene, then reduced Pdx1 might have resulted in the same number of

GIP-expressing cells, but each expressing less GIP. We observed, however, a 90% reduction

in the proportion of duodenal epithelial cells that expressed GIP in the $Pdx1^{-/-;b3Tg}$ mice,

suggesting that Pdx1 affects the frequency at which enteroendocrine progenitors become

allocated to the GIP cell fate. In contrast, the proportion of duodenal epithelial cells

producing gastrin and CCK in the transgene-rescue mice was not significantly reduced

compared to wild type, although the number of these cell types is substantially reduced in *Pdx1^-/-* mutants. This suggests that a relatively small amount of Pdx1 is sufficient for formation of CCK and duodenal gastrin cells.

The number of gastrin cells in the stomach appeared to be greatly reduced in *Pdx1^-/-;b3Tg* mice despite a lack of significant change in duodenal gastrin cell frequency. While some gastrin cells were present in the duodena of *Pdx1^-/-* mice, stomach gastrin cells were undetectable at E16.5, P0 and P7 in *Pdx1^-/-* mice. The stricter dependence of antropyloric gastrin cells on Pdx1 may reflect region-specific differences in the progenitor cell populations in the stomach and duodenum.

Potential *in vivo* roles of Area IV

Among the previously characterized evolutionarily conserved regions of *Pdx1*, Area IV is the only one not contained in our transgene, making it an obvious candidate for the *cis*-regulatory activity required for full expression of Pdx1 in the duodenum and stomach. However, the factors that likely bind within Area IV also bind within Areas I, II and III, and are primarily expressed in endocrine pancreas (Fig. 3.13). Furthermore, *in vitro* analysis of Area IV activity suggested that it functions in synergy with Areas I and II to promote β-cell-specific transcription (Gerrish et al., 2004). The current findings raise the possibility that Area IV may contain additional binding sites for factors that are particularly important for transcription in foregut crypt stem cells. Although the transgene provided near-to or greater-than wild-type levels of Pdx1 protein in the pancreas, these data do not rule out an *in vivo* role for Area IV in enhancing pancreatic *Pdx1* expression, or that the activity of Area IV may

Fig. 3.13 Overview of transactivator interactions on *Pdx1* 5′ non-coding region. *Top*, previously reported sites of protein-DNA interaction in relation to regions of phylogenetic sequence conservation (Ben-Shushan et al., 2001; Gerrish et al., 2001; Gerrish et al., 2000; Gerrish et al., 2004; Jacquemin et al., 2003; Marshak et al., 2000; Samaras et al., 2002; Samaras et al., 2003; Sharma et al., 1997; Van Velkinburgh et al., 2005; Wu et al., 1997). "?", hypothetical transactivator promoting expression in postnatal foregut. *Middle*, text boxes indicate tissues where proximal or distal *cis*-regulatory regions seem to be of particular importance for Pdx1 expression. "β cells" in bold or grey for the proximal or distal region, respectively, indicate the stronger influence of proximal regions on β-cell-specific expression, from *in vitro* and *in vivo* evidence. *Bottom*, embryonic and adult locations of Pdx1 expression and its transactivators that are reported as expressed in those tissues (Coffinier et al., 1999; Maestro et al., 2003; Matsuoka et al., 2004; Naya et al., 1997; Rausa et al., 1997; Sander et al., 1997; St-Onge et al., 1997; Sussel et al., 1998).

be required under specific physiological demands. For instance, the presence of tandem copies of the transgene in the b1 and b3 lines may circumvent the requirement for synergy between Area IV and Areas I and II. Another possibility is that Area IV may contribute to regulation of *Pdx1* during islet development, and the absence of that regulatory influence in the *Pdx1$^{-/-;Tg}$* mice might explain the impairment of glucose tolerance despite abundant Pdx1 in mature *Pdx1$^{-/-;b1Tg}$* pancreas. In support of this potential role of Area IV, previous analysis of rescue of *Pdx1$^{-/-}$* using a transgene that included Area IV (Gannon et al., 2001) showed GTT performance that appears to be closer to wild type than the current results using the 12.5 kb transgene. We were unable to directly compare the 14.5 kb and 12.5 kb transgenes because the 14.5 kb transgenic lines were not kept, and in any case we would be unable to rule out position of transgene integration effects. We plan to delete distal enhancer elements from the endogenous *Pdx1* locus, which will hopefully resolve these uncertainties.

Our recent examination of mouse and human *Pdx1/IPF1* genomic sequence alignment using the Lawrence Berkeley Laboratories online Vista browser (http://pipeline.lbl.gov) revealed two additional regions of greater than 75% sequence conservation within 5 kb upstream of Area IV and additional conserved regions between *Pdx1* and its nearest 5′ located neighbor, the transcriptional regulator-encoding gene, *Gsh1*. These as-yet uncharacterized conserved sequences may also contribute to *cis*-activation of *Pdx1* in the duodenum and stomach.

Shared and distinct roles of proximal and distal promoter elements

Cis-regulatory sequences included in the 12.5 kb transgene were sufficient for expression in embryonic duodenal and stomach endoderm but not in the mature organs.

Deletion of Area I-II-III, the only highly conserved non-coding sequence present in the 12.5 kb transgene, mildly reduced expression in both embryonic and mature gut tissues (Fujitani et al., 2006). These results suggest that proximal and distal promoter elements cooperatively promote expression of Pdx1 in the posterior foregut. In the embryonic duodenum and stomach, lack of either proximal or distal *cis*-regulatory elements can be compensated by the remaining regulatory regions. In the postnatal gut, however, distal regulatory regions appear to be more important than proximal elements for maintenance of Pdx1 expression. Review of previously characterized transactivator-binding motifs in the 5′ *Pdx1* locus (Fig. 3.13) reveals several binding sites for the Foxa and Hnf factors, which are broadly expressed in early endoderm, in both the proximal and distal promoter regions. The presence of multiple binding sites for these factors may explain the maintenance of at least basal levels of Pdx1 expression throughout the embryonic posterior foregut when either proximal or distal 5′ sequences are removed.

Several binding sites for factors enriched in pancreatic endocrine cells are found within Areas I and II, which may account for the strong pancreatic phenotype of Area I-II-III deletion mutants (Fujitani et al., 2006). Pax6 and MafA binding in Area II may be particularly important for expression of Pdx1 in β cells (Samaras et al., 2002; Samaras et al., 2003). Based on the results of the current study, it seems likely that there is a *cis*-regulatory activity in the distal 5′ region that is important for *Pdx1* transcription in duodenal crypt stem cells, an activity that cannot readily be accounted for by previously characterized transactivator binding interactions.

CHAPTER IV

TISSUE-SPECIFIC RECOMBINATION OF *PDX1* FLOXED ALLELES

Introduction

Onset of Pdx1 expression at E8.0-8.5 is specific to endoderm that will give rise to the

pancreatic buds (Gu et al., 2002). Pdx1 is expressed homogeneously in the early pancreatic

buds and the level of expression subsequently becomes upregulated in differentiating and

mature β cells and downregulated in other differentiated pancreatic tissues (Guz et al., 1995).

A recent report showed that upregulation of Pdx1 in differentiating β cells occurs after the

initiation of insulin and Nkx6.1 expression but prior to the switch from MafB to MafA

expression (Nishimura et al., 2006), indicating that upregulation of Pdx1 is associated with

maturation of specified β cells.

Targeted inactivation of *Pdx1* resulted in arrest of pancreatic outgrowth and

differentiation soon after the initial evagination of the pancreatic buds, resulting in the

replacement of the pancreas by a duct-like rudiment that lacked mature pancreatic tissue

except for a few glucagon-expressing cells (Jonsson et al., 1994; Offield et al., 1996). Two

approaches have been employed to circumvent the early arrest of pancreas development in

Pdx1^{-/-} mice and study later roles of Pdx1 in pancreatic tissue. One approach was to

inactivate the *Pdx1* gene specifically in β cells. These experiments revealed that Pdx1 is

required for expansion (Gannon et al., in preparation) and maintenance of the β cell

population (Ahlgren et al., 1998). Conditional deletion of *Pdx1* in postnatal β cells resulted

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first in loss of Glut2 expression, followed by loss of insulin expression and eventually a decrease in the number of β cells (Ahlgren et al., 1998).

The other approach that has been used to study the requirement for Pdx1 beyond the early stages of pancreas development was to make expression of Pdx1 repressible by treatment with doxycycline (Dox). In adult mice, β cells lost expression of insulin after onset of Dox treatment, but insulin expression was restored after removal of Dox (Holland et al., 2002, 2005). Expression of the acinar cell markers amylase and elastase was also downregulated after repression of Pdx1 expression (Holland et al., 2002). It remains unclear if prolonged suppression of Pdx1 would result in failure to maintain the viability of endocrine or exocrine tissue, or if the small amount of Pdx1 that is still expressed in the presence of Dox is sufficient for maintenance of pancreatic endocrine and exocrine mass. When Pdx1 was repressed by Dox during embryogenesis, proliferation and maturation of endocrine and exocrine pancreatic cells were arrested shortly after Dox addition, but the arrested cells survived until the experiment was terminated at birth (Hale et al., 2005). It is unclear whether the developmental arrest results primarily because precursor cells fail to differentiate or because differentiated cells fail to proliferate.

Targeted deletion of a conserved enhancer region (Area I-II-III) of *Pdx1* that promotes β-cell-specific gene expression resulted in a hypomorphic allele that impaired initial pancreatic outgrowth and severely impaired β cell development (Fujitani et al., 2006). The amount of acinar tissue formed in the mutants was substantially less than in wild-type mice, perhaps due to the early bud outgrowth defect. The acinar tissue that formed appeared to be well-differentiated but expressed less Pdx1. These data indicated that early stages of pancreas development and generation of β cells from endocrine precursors require a high

level of Pdx1 expression that is dependent on the Area I-II-III enhancer, but that a lower

level of Pdx1 may be sufficient for acinar-cell specification and maturation. The four major

pancreatic endocrine cell types arise from common Ngn3-expressing progenitors (Gu et al.,

2002). Because production of β cells was more severely affected than the other endocrine

cell types in mice homozygous for the enhancer deletion, one interpretation could be that the

level of Pdx1 expression influences the cell-fate choice of endocrine progenitors.

Mice that were heterozygous for deletion of Area I-II-III had worse defects than

$Pdx1^{+/-}$ mice in terms of glucose intolerance, decreased Glut2 expression, and disrupted islet

architecture (including infiltration of peripheral cell types into the core and a significant

increase in PP cells). Because other experiments suggested that the $Pdx1^{\Delta I-II-III}$ allele had

intermediate activity between a wild-type and null allele, it was surprising that $Pdx1^{+/\Delta I-II-III}$

mice had a stronger islet phenotype than $Pdx1^{+/-}$ mice. One explanation could be that lack of

the enhancer, even when absent on one of two alleles, interferes with the upregulation of

Pdx1 during β-cell maturation, which may impair β-cell differentiation and result in

improper patterning of the islets.

To test whether the dominant negative influence of lack of the Area I-II-III enhancer

on islet function results from a role for the enhancer during islet development or a role of the

enhancer in mature β cells, we used the Cre/loxP system to delete the Area I-II-III enhancer

in pancreatic endocrine progenitors and in differentiated β cells. A conditional null allele of

Pdx1 was tested side-by-side with the floxed Area I-II-III allele to compare the effects of

varying levels of Pdx1 reduction at different stages of endocrine development. Specifically,

we wanted to test if removing Pdx1 would prevent specification of endocrine progenitors to

the β-cell lineage, and if this effect would be manifest as arrested development of immature β

cells or conversion of β-cell precursors to other differentiated cell fates. In addition, we used a pancreatic acinar cell specific Cre transgene to recombine the conditional Area I-II-III and conditional null alleles of *Pdx1* to determine if differentiated acinar cells require Pdx1, and if so whether a low level of Pdx1 would suffice for acinar cell maturation and maintenance.

Results

These studies employed two Cre-sensitive alleles of *Pdx1* generated previously in our laboratory (Fig. 4.1). In one allele, the second exon of *Pdx1* is flanked by loxP sites resulting in a null allele after Cre-mediated recombination (*floxE2;* M. Gannon, manuscript in preparation). The other allele contains loxP sites flanking the 1.2 kb region encompassing the phylogenetically conserved enhancer regions known as Areas I, II and III (*flox123;* Fujitani et al., 2006). Mice were bred to generate offspring of the genotype *Pdx1$^{floxE2/flox123}$;ROSA26$^{R/R}$*, where *ROSA26^R* is a reporter allele that expresses the *lacZ* gene after it is recombined by Cre (Soriano, 1999). Crosses between *Pdx1$^{floxE2/flox123}$* mice and Cre-expressing mice resulted in offspring with conditional recombination of either floxed allele. This strategy allowed us to control for potential genetic background effects by comparing conditional null versus conditional hypomorphic phenotypes among littermates. Lineage-specific activity of Cre was confirmed based on the pattern of expression of the *ROSA26^R* reporter allele.

Fig. 4.1 Gene targeting strategies for generation of floxed *Pdx1* alleles.
(A) Targeting construct used to generate the *Pdx1*^{floxE2} allele. The PGK-neo^R/HSV-TK
selection cassette was removed prior to using this strain for conditional deletion experiments.
Recombination of the remaining two loxP sites deletes Exon 2, resulting in a null allele.
Detailed characterization of this allele will be reported in an upcoming publication from
Maureen Gannon, VUMC Department of Medicine. (B) Strategy for generating the
Pdx1^{flox123} allele. Detailed description of the targeting construct can be found in Fujitani et
al., 2006.
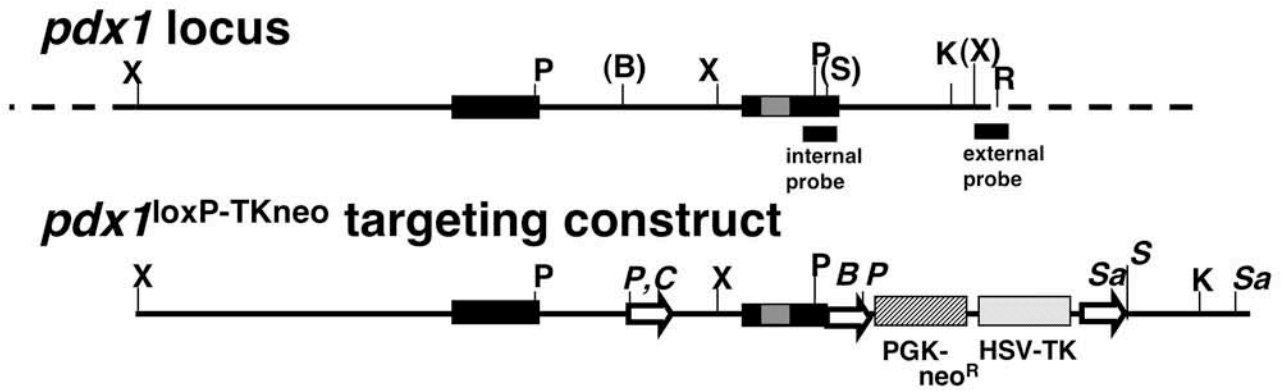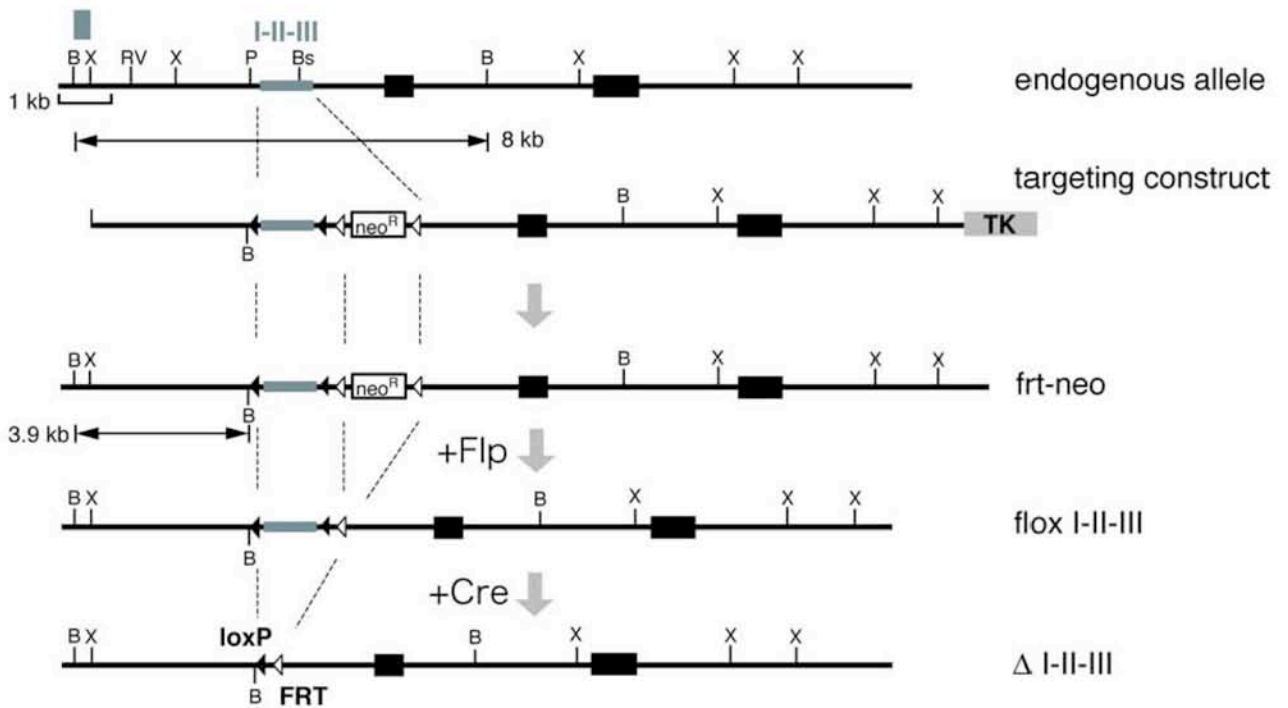
Mice expressing Cre transgenes were bred to heterozygosity for a null allele of *Pdx1* (*Pdx1^{XBko}*; Offield et al., 1996). We reasoned that offspring with one floxed allele of *Pdx1* and one null allele would be convenient for detecting conditional-deletion phenotypes because only one allele of *Pdx1* would need to be recombined, hopefully providing greater sensitivity to transient or low-level activity of Cre. In addition, because *Pdx1$^{+/-}$* mice have impaired pancreatic islet function (Dutta et al., 1998; Ahlgren et al.,1998; Brissova et al., 2002; Johnson et al., 2003) and develop worsening islet disorganization with age (Johnson et al., 2003), we thought it likely that *Pdx1$^{flox/-}$* mice would be sensitized to further reduction in Pdx1 expression even if the efficiency of Cre-mediated recombination was fairly low.

Ngn3Cre-mediated deletion

To examine the role of Pdx1 in pancreatic endocrine progenitors, we used a transgene expressing Cre recombinase from *Ngn3* regulatory sequences (Gu et al., 2002) to recombine the *floxE2* and *flox123* alleles. Ngn3 is transiently expressed in the precursors of all pancreatic endocrine cells, and Ngn3Cre mediates recombination in a high percentage of endocrine cells of the four major pancreatic endocrine lineages (Gu et al., 2002). Previous characterization of the Ngn3Cre mice by Gu et al. utilized the Z/AP transgenic reporter for Cre activity and showed that recombination in the pancreas was limited to islets. When we crossed the Ngn3Cre mice to *ROSA26$^{R/R}$* mice we found that virtually all islet tissue was lineage-labeled, but there was also significant recombination in non-endocrine tissue of the pancreas and duodenum (Fig. 4.2A,C,E).

Fig. 4.2 Pattern of recombination mediated by Ngn3Cre transgenes. Recombination of the ROSA26^R reporter was evaluated by XGal staining of posterior foregut tissues in mice expressing the Ngn3Cre (A, C, E) and Ngn3CreBAC (B, D, F) transgenes. The Ngn3Cre transgene consistently activated the reporter in pancreatic endocrine tissue (dashed outlines in C), but also recombined the reporter in most smooth muscle (A, E) and duodenal crypts (E), as well as substantial patches of pancreatic exocrine tissue (C). (B) Reporter activation in Ngn3CreBAC transgenic mice was consistently restricted to the pancreas, as well as gut enteroendocrine cells (faintly visible in duodenal whole-mount). (D) In most *Ngn3CreBAC;ROSA26^R* pancreata, the majority of endocrine islets were labeled (dashed outlines) and scattered acinar clusters were also recombined (arrow). (F) In a few *Ngn3CreBAC;ROSA26^R* animals extensive recombination was seen in pancreatic acinar tissue. Arrow in (F) indicates an acinar lobule where all of the cells are labeled by XGal. Sections in (C-F) were counterstained with hematoxylin.

Ngn3Cre; R26^R
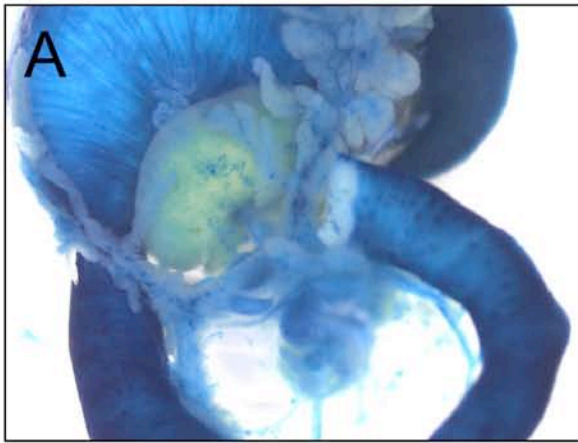
Ngn3CreBAC; R26^R
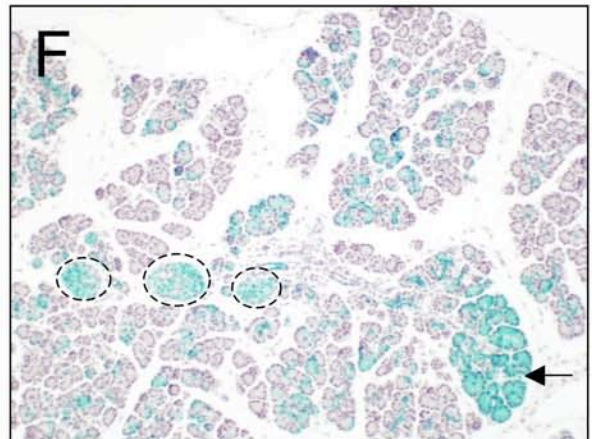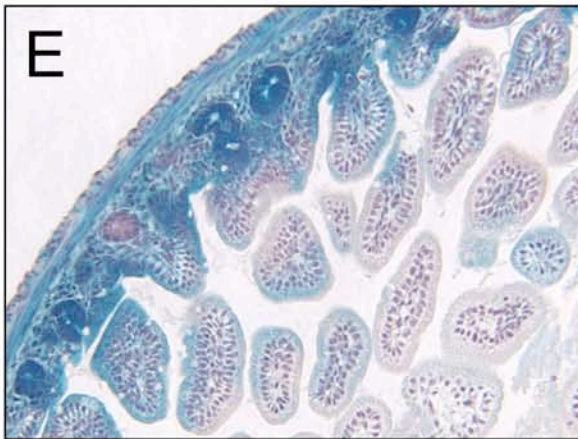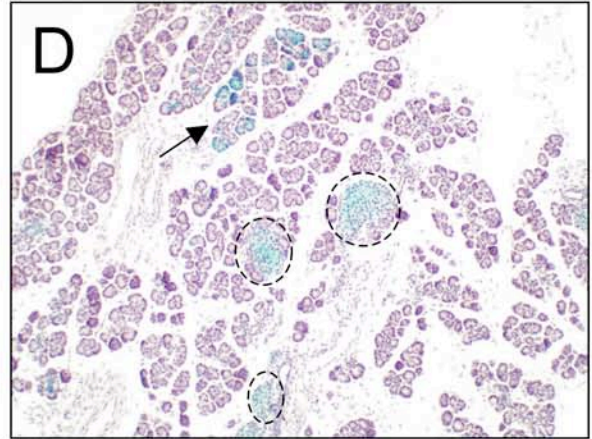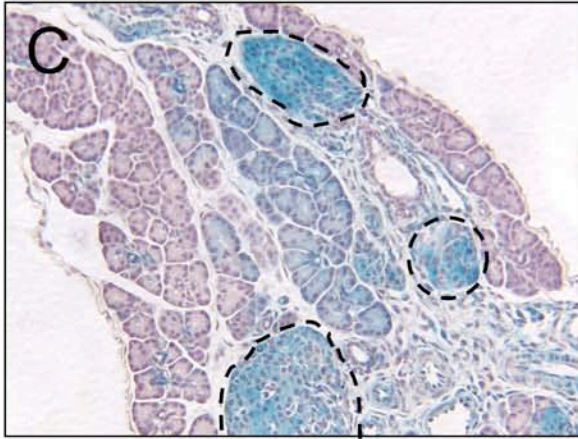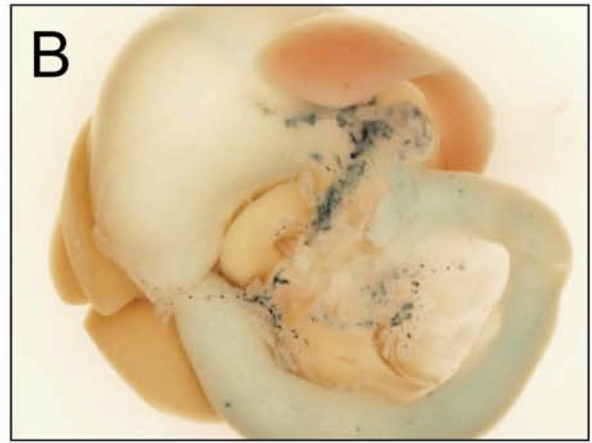
Genetic background may have contributed to the discrepancy between our results and the previous report. The transgene was on a mostly ICR background when we received the mice, but we subsequently bred them onto a C57BL/6:DBA/2J (B6D2) hybrid background for at least three generations before using them in these experiments. Also, we have heard anecdotal evidence that the ROSA26 reporter is more sensitive to Cre activity than the Z/AP reporter, perhaps because the distance between the loxP sites is significantly greater in the Z/AP reporter (~4.5kb compared to ~2.3kb). Thus, there may be low-level activity of the Ngn3Cre transgene in early progenitors that is sufficient to recombine $ROSA26^R$ but not enough to recombine *Z/AP*.

Regardless, the Ngn3Cre transgene is adequte for our study because it mediates Cre activity in all endocrine progenitors, so we were able to evaluate the role of Pdx1 in endocrine differentiation independent of the general requirement for Pdx1 for growth and maturation of the pancreas. In addition, we recently obtained the Ngn3CreBAC transgenic mice reported by Schonhoff et al. (2004b). These mice provided more precisely restricted recombination in endocrine progenitors (Fig. 4.2B,D), although some mice showed substantial $ROSA26^R$ activation in pancreatic acinar tissue (Fig. 4.2F). We have begun to use the Ngn3CreBAC transgene to corroborate and extend our findings from Ngn3Cre-mediated deletion of the floxed *Pdx1* alleles, as described below.

Ngn3Cre;Pdx1$^{floxE2/-}$;ROSA26$^{R/+}$ mice were examined at one week of age (P7). The amount of pancreatic tissue in the conditional mutants was substantially reduced compared to littermates (data not shown), likely resulting from deletion of *Pdx1* in some of the early pancreatic progenitors, consistent with the pattern of recombination shown in Fig. 4.2.

Acinar and ductal structures appeared to be properly organized within the reduced mass of pancreatic tissue and the number of islets seemed consistent with the reduction in total organ size, though the individual islets were significantly smaller than in littermates (e.g. Fig. 4.4).

Co-labeling with XGal and IHC for Pdx1 or insulin indicated that Cre had been activated in progenitors of all of the islet cells (Fig. 4.3). Pdx1 expression was retained in a minority of the islet cells and insulin was expressed primarily in the Pdx1-positive cells. This result suggested that the *floxE2* allele was less efficiently recombined than *ROSA26^R* but was recombined in most endocrine progenitors. The finding that the reduction in the number of insulin expressing cells paralleled the efficiency of conditional deletion of *Pdx1* was consistent with previous reports of loss of β cells after conditional deletion of *Pdx1* (Ahlgren et al., 1998, Gannon et al., in preparation).

Immunostaining for the other islet hormones, i.e. glucagon, somatostatin, and pancreatic polypeptide (PP), revealed a relative increase in the proportion of islet cells expressing these hormones (Fig. 4.4). The increase in non-β cells may have resulted from crowding together of the peripheral islet cells due to a reduction in the number of β cells in the islet core. However, the increase in the proportion of islet cells expressing PP appeared to be consistently greater than the increase in glucagon and somatostatin cells. This observation led us to consider the possibility that a common precursor of β and PP cells was being shunted to PP cell fate after deletion of *Pdx1* from endocrine progenitors.

The hypothesis that loss of *Pdx1* resulted in shunting of common precursors to PP cell fate was supported by the phenotype of *Ngn3Cre;Pdx1$^{flox123/-}$* conditional mutants. Unlike the *floxE2* conditional mutants, there was not an appreciable decrease in the size of the pancreas in P7 *Ngn3Cre;Pdx1$^{flox123/-}$* pups (data not shown). Islets in the *flox123* conditional
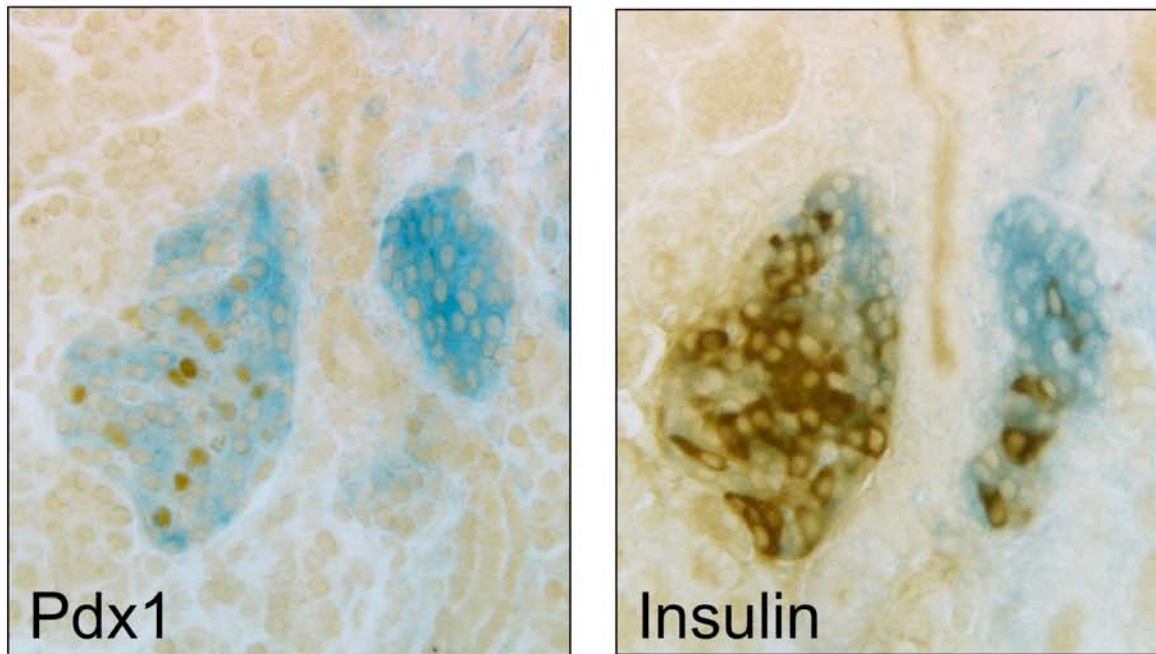
Fig. 4.3 Ngn3Cre recombines *ROSA26^R* more efficiently than *Pdx1^{floxE2}*. P7 *Ngn3Cre;Pdx1$^{floxE2/-}$;ROSA26^R* mice were dissected and pancreata were stained with XGal, then sectioned and stained by IHC for insulin and Pdx1. *Left panel*, some of the blue XGal-stained cells express Pdx1, indicating that *Pdx1^{floxE2}* was not recombined in some cells where *ROSA26^R* was recombined. *Right panel*, examination of insulin expression on an adjacent 5-μm section suggests that insulin is expressed mainly in the cells that retain Pdx1.
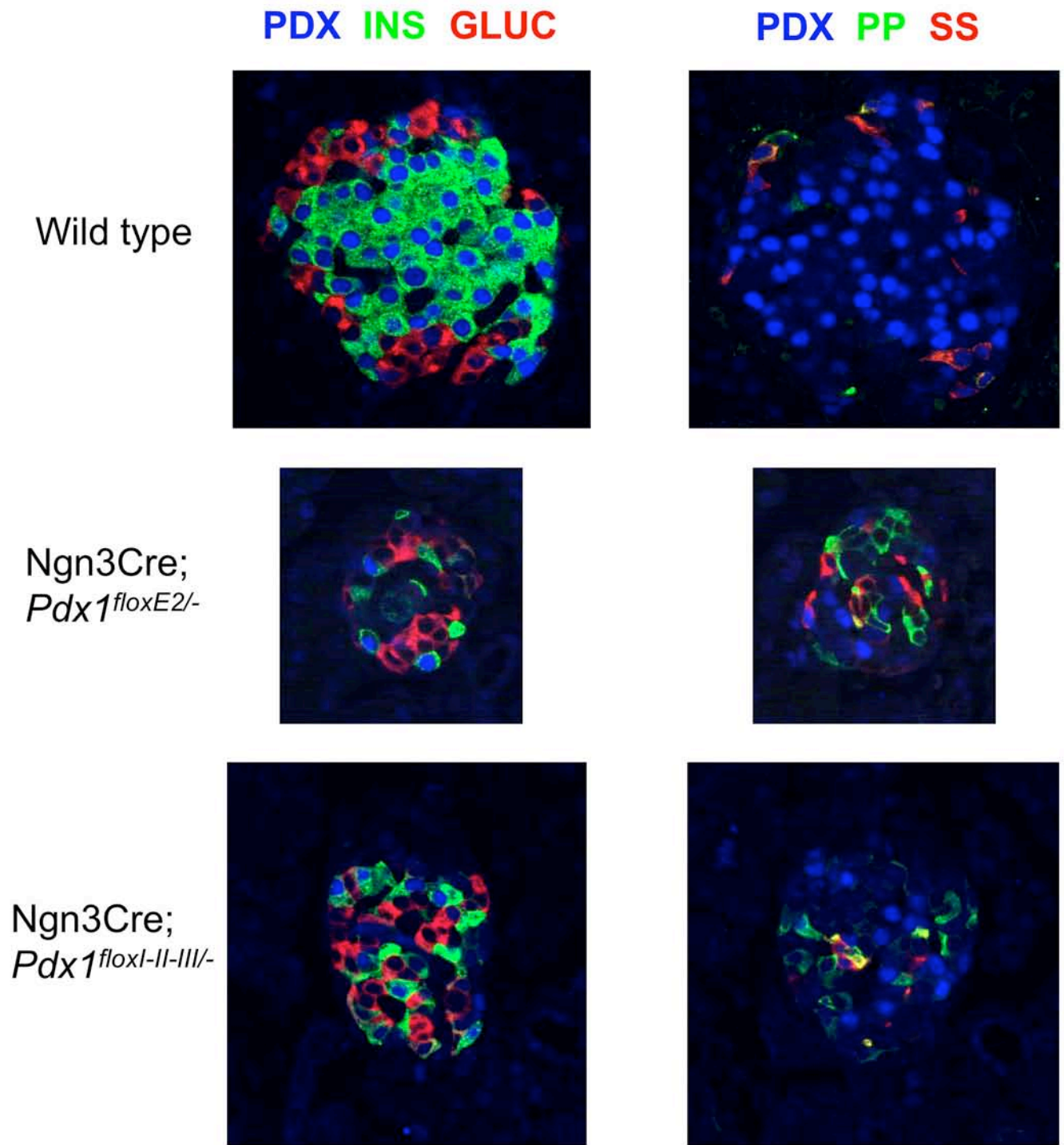
PDX INS GLUC   **PDX PP SS**

Wild type

Ngn3Cre;
Pdx1^{floxE2/-}

Ngn3Cre;
Pdx1^{floxI-II-III/-}

Fig. 4.4 Islet morphology perturbed by Ngn3Cre-mediated recombination of *Pdx1*^{flox} alleles. *Left panels*, representative islets from P7 mice of the indicated genotypes were stained for Pdx1 (blue), insulin (green) and glucagon (red). *Right panels*, adjacent 5-μm sections were stained for Pdx1 (blue), PP (green) and somatostatin (red).

mutants were significantly larger than in the *Ngn3Cre;Pdx1^{floxE2/-}* mutants but tended to be

slightly smaller than wild type. Although the size and number of islets did not appear to be

severely affected in *Ngn3Cre;Pdx1^{flox123/-}* mice, the islets showed considerable mixing of the

peripheral cell types into the core and increased numbers of PP-expressing cells (Fig. 4.4).

The shift in the proportion of islet cells expressing insulin and PP was more obvious at P14,

by which time the number and size of islets had grown in both wild-type and

Ngn3Cre;Pdx1^{flox123/-} mice (Fig. 4.5).

We recently examined *Ngn3CreBAC;Pdx1^{flox123/-}* mice at P2 and found that they

exhibited a similar reduction in insulin-expressing cells and increase in glucagon- and PP-

expressing cells as described above for *Ngn3Cre;Pdx1^{floxE2/-}* and *Ngn3Cre;Pdx1^{flox123/-}* mice

(Fig. 4.6). The reduction in the size of islets in *Ngn3CreBAC;Pdx1^{flox123/-}* mice as compared

to wild-type littermates was more substantial than was observed for *Ngn3Cre;Pdx1^{flox123/-}*,

and there appeared to be a greater reduction in expression of insulin and Pdx1. These results

suggest that Ngn3CreBAC-mediated recombination of *flox123* might be more efficient than

Ngn3Cre-mediated deletion.

RIPCre-mediated deletion

To determine if expansion of the PP cell population was a specific result of deleting

the floxed *Pdx1* alleles from endocrine progenitors, we used a transgene expressing Cre

under the regulation of sequences from the rat insulin promoter (RIPCre; Postic et al., 1999)

to recombine the *floxE2* and *flox123* alleles in differentiating and mature β cells. RIPCre-

mediated deletion of the *flox123* allele did not appear to have an effect on the number of PP

cells (Fig. 4.7F), but we have only examined one mouse of this genotype, so additional
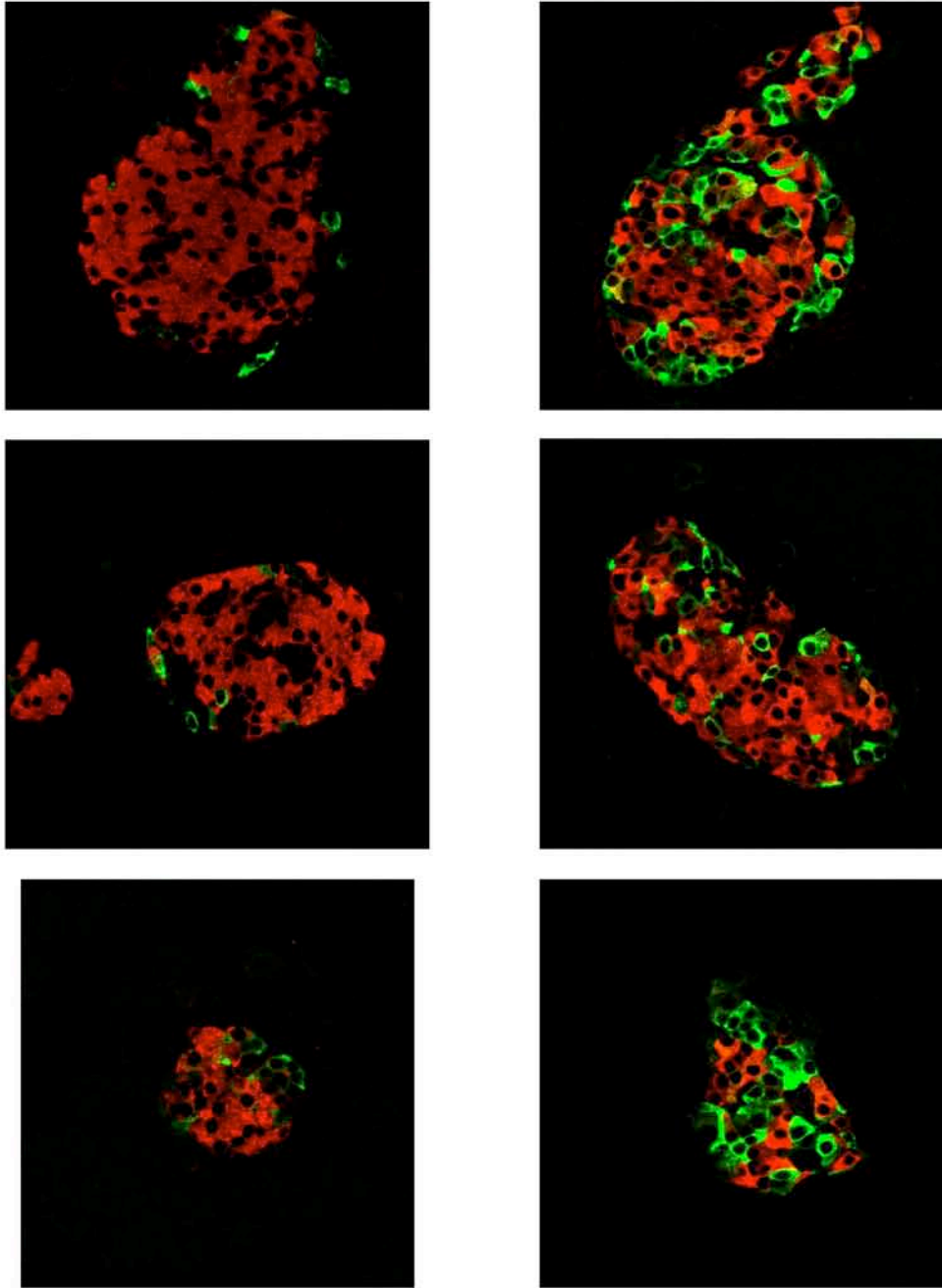
Fig. 4.5 Ngn3Cre-mediated deletion of Area I-II-III increases the PP/β cell ratio.
Representative islets of equivalent size from P14 wild-type (left panels) and
Ngn3Cre;Pdx1^{flox123/-} mice (right panels) were compared for PP/insulin ratio by
staining for insulin C-peptide (red) and PP (green).

PDX INS GLUC　　　　**PDX PP SS**

Wild type

Ngn3CreBAC; Pdx1$^{flox123/-}$
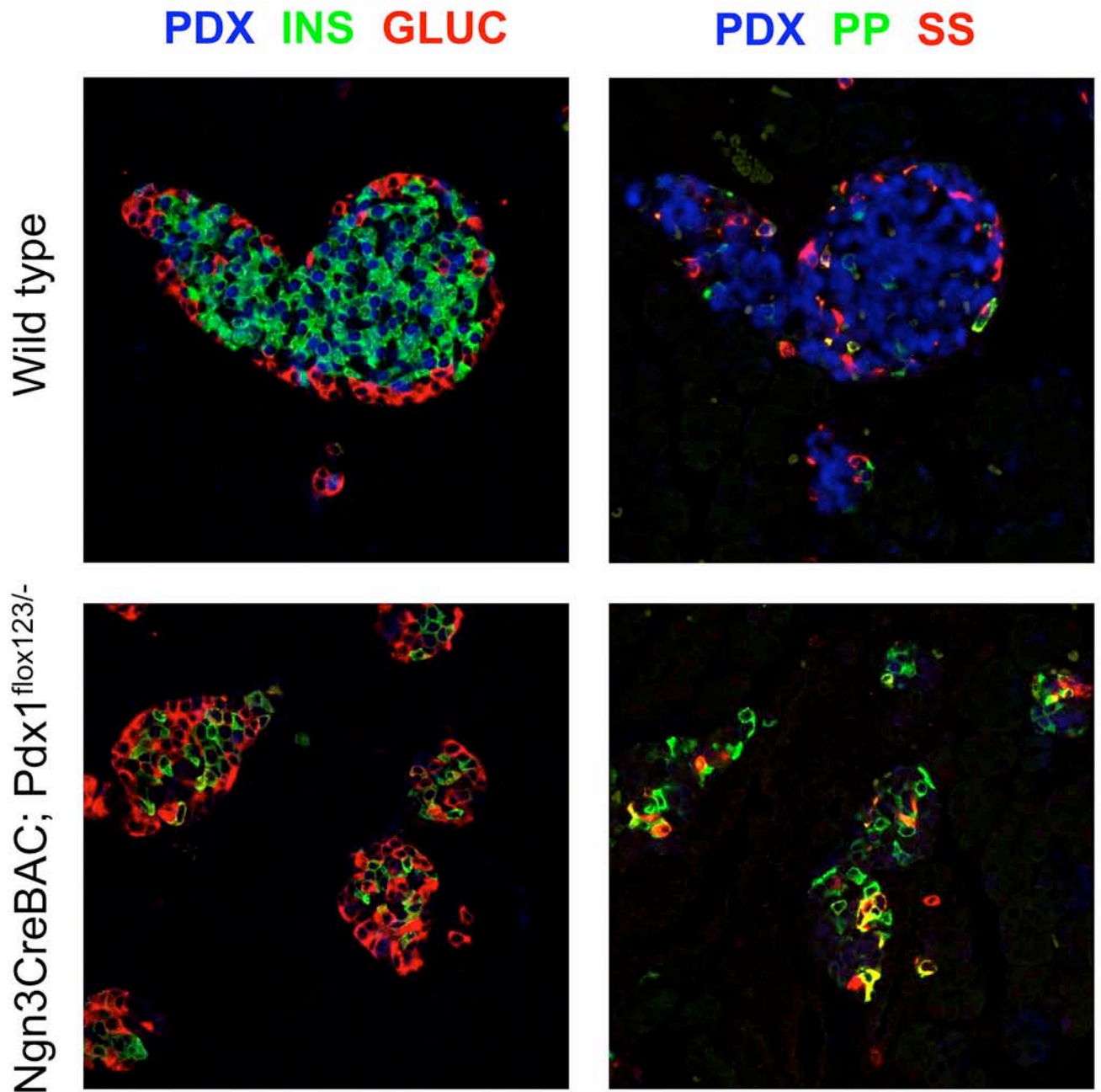
Fig. 4.6 *Ngn3CreBAC;Pdx1$^{flox123/-}$* **islets also show increased PP/β cell ratio.** *Left panels*, splenic lobes of pancreata from P2 littermates were stained for Pdx1 (blue), insulin (green) and glucagon (red). *Right panels*, adjacent 5-μm sections were stained for Pdx1 (blue), PP (green) and somatostatin (red). All panels have the same magnification and gain settings.

experiments will be required for a definitive assessment of PP cell number. We also plan to examine PP cell number in *RIPCre;Pdx1$^{floxE2/-}$* mice, as the expression of this hormone was not described in previous reports of β-cell specific deletion of *Pdx1*.

The number of β cells appeared to be decreased in the *RIPCre;Pdx1$^{flox123/-}$* mouse and there was a concomitant increase in the number of glucagon-expressing cells, which were found distributed throughout the islets rather than restricted to the periphery as in wild-type mice (Fig. 4.7E). This phenotype was similar to previous reports of RIPCre-mediated deletion of the *floxE2* allele (Ahlgren et al., 1998; Gannon et al., in preparation), which is not suprising considering that germ-line deletion of Area I-II-III severely compromised β-cell development (Fujitani et al., 2006).

Although decreased β cells and increased glucagon-positive cells were seen in both *floxE2* and *flox123* conditional mutants, the loss of β cells after conditional deletion of *flox123* appeared to be less severe than was reported for the *floxE2* conditional mutant. Also, the *RIPCre;Pdx1$^{flox123/+}$* mouse had less severely impaired glucose tolerance than *RIPCre;Pdx1$^{floxE2/+}$* mice, which showed the same glucose intolerance as *Pdx1$^{+/-}$* mice (Fig. 4.8). GTT analysis of more mice will be required to determine if this is a consistent phenotype, but based on these preliminary results, the more severe phenotype of *floxE2* deletion suggests that *floxE2* is acting as a conditional null allele while *flox123* acts as a conditional hypomorphic allele in differentiated β cells. Indeed, Pdx1 was detectable in most nuclei of *RIPCre;Pdx1$^{flox123/-}$* islets, although at lower levels than in wild-type and *RIPCre;Pdx1$^{flox123/+}$* animals (Fig. 4.7). In contrast, Pdx1 was not detectable in most islet nuclei after recombination of *flox123* by Ngn3Cre or Ngn3CreBAC (cf. Fig. 4.4, 4.6).

Fig. 4.7 No apparent alteration of PP/β cell ratio in *RIPCre;Pdx1$^{flox123/-}$* mice. Islets of 4-wk-old wild-type (A, B), *RIPCre;Pdx1$^{flox123/+}$* (C, D) and *RIPCre;Pdx1$^{flox123/-}$* (E, F) mice were stained for Pdx1 (blue), insulin (green) and glucagon (red) (left panels) or Pdx1 (blue), PP (green) and somatostatin (red) (right panels). *RIPCre;Pdx1$^{flox123/-}$* islets showed substantial infiltration of α cells into the islet core (E) but no apparent increase in the number of PP or somatostatin cells (F). Same magnification and gain settings for all panels.

PDX INS GLUC   PDX PP SS

A   B
Wild type

C   D
RIPCre;
Pdx1^flox123/+

E   F
RIPCre;
Pdx1^flox123/-

IPGTT 4wk-old mice
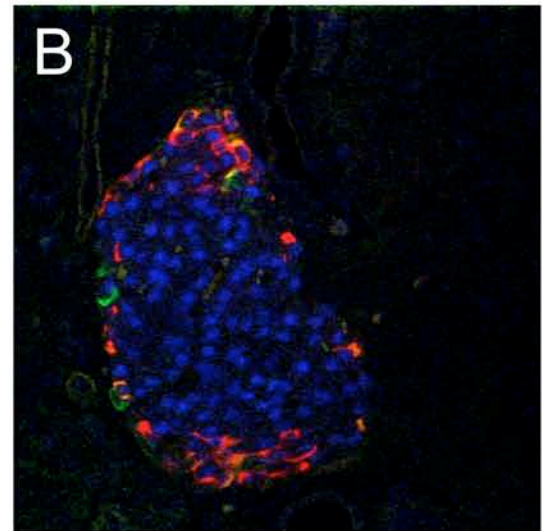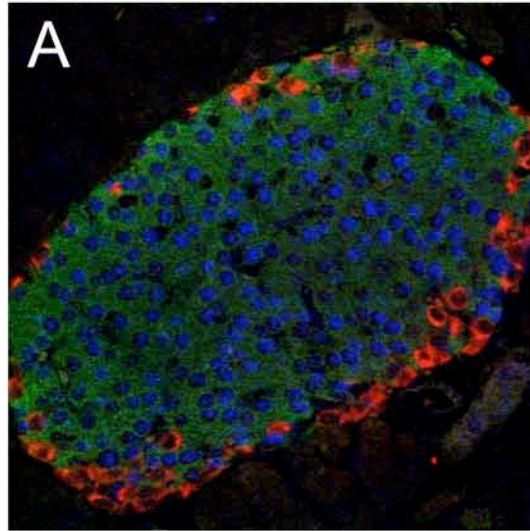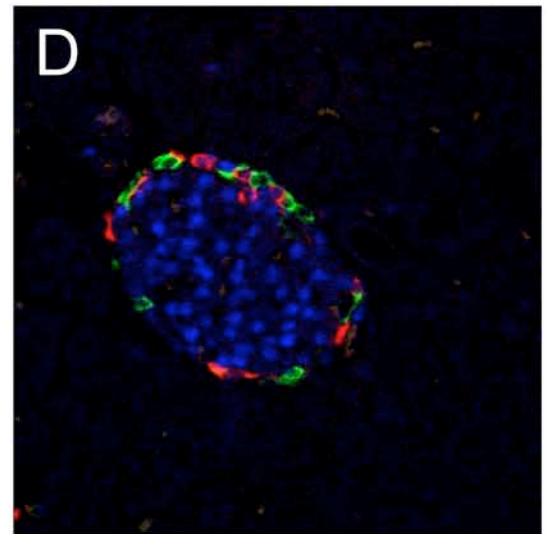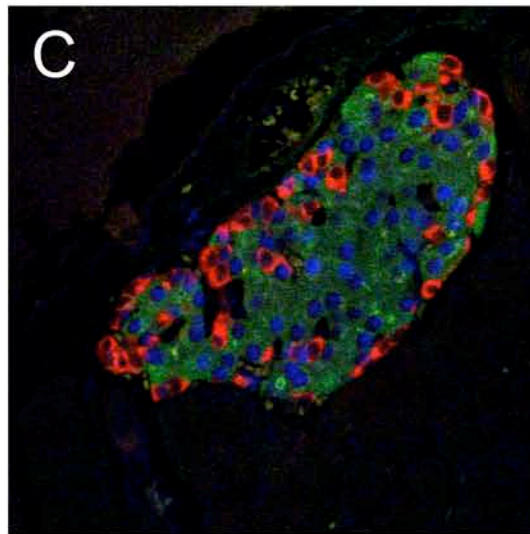
Legend:
- wt
- het
- cond123/+
- condE2/+
- cond123/-
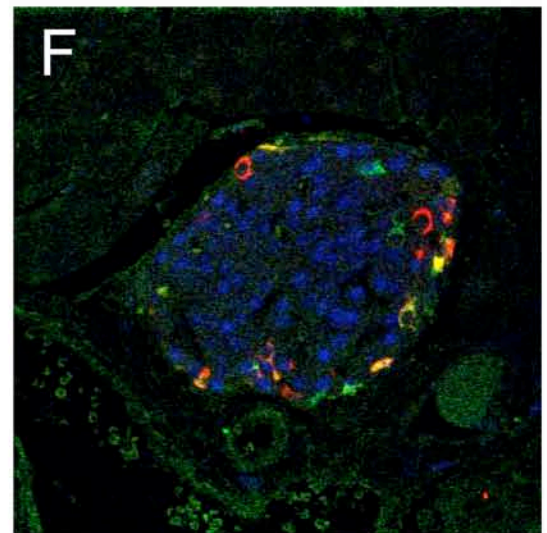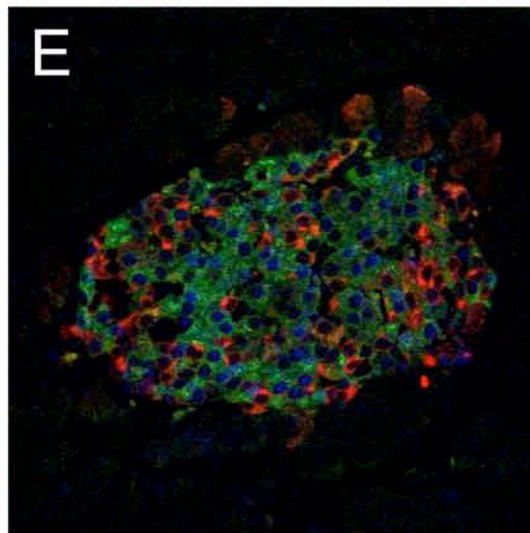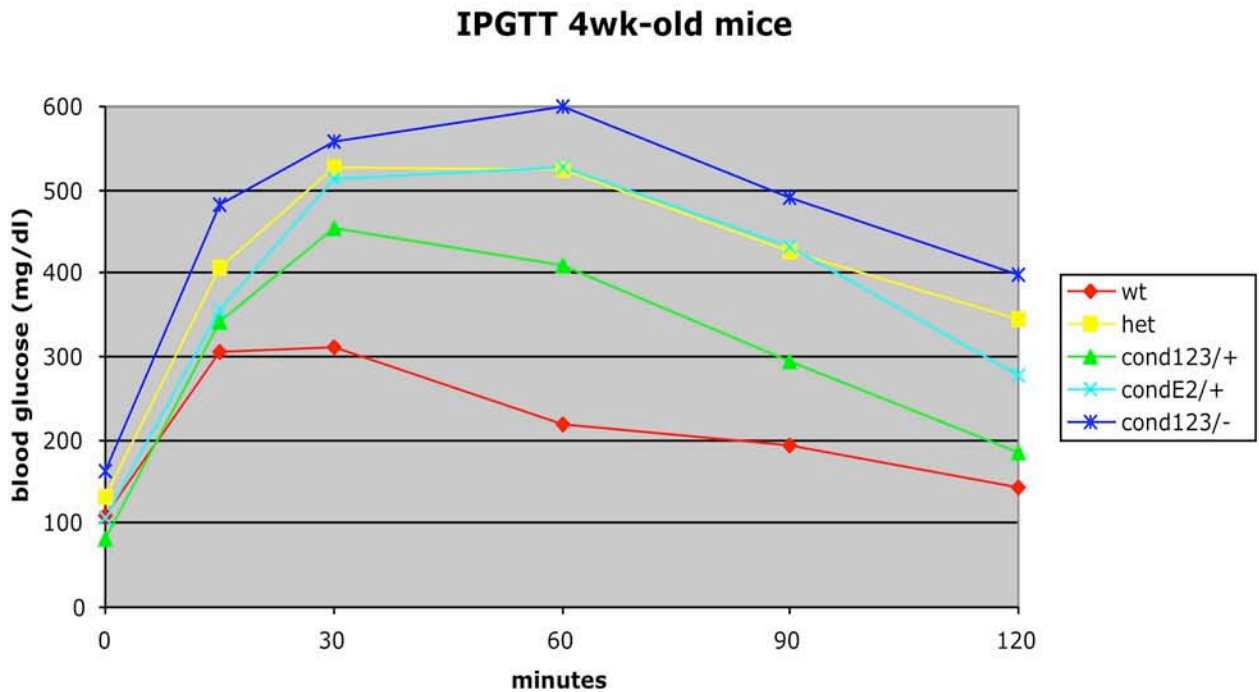
Fig. 4.8 Glucose intolerance less severe in *RIPCre;Pdx1^{flox123/+}* than *RIPCre;Pdx1^{floxE2/-}* mice. 4-week-old mice were evaluated by IPGTT. Consistent with previous reports, *Pdx1^{+/-}* mice (yellow line) had worse glucose tolerance than wild type (red line). *RIPCre;Pdx1^{floxE2/+}* (light blue) had similar glucose clearance to *Pdx1^{+/-}*, indicating that the floxed allele was efficiently recombined. *RIPCre;Pdx1^{flox123/+}* (green) had intermediate glucose tolerance between wild-type and *RIPCre;Pdx1^{floxE2/+}* mice, consistent with function of *Pdx1^{flox123}* as a conditional hypomorphic allele. *RIPCre;Pdx1^{flox123/-}* mice (dark blue) were severely glucose intolerant, consistent with a low level of expression of Pdx1 in these mice (cf. Pdx1 IHC in Fig. 4.7). Number of each genotype: wild type (2), *Pdx1^{+/-}* (5), *RIPCre;Pdx1^{flox123/+}* (1), *RIPCre;Pdx1^{floxE2/+}* (2), *RIPCre;Pdx1^{flox123/-}* (1).

If glucose tolerance of *RIPCre;Pdx1$^{flox123/+}$* mice is found to be consistently better than *RIPCre;Pdx1$^{floxE2/+}$* mice, it will support the hypothesis that the dominant islet phenotype of the *Pdx1$^{ΔI-II-III}$* allele results from a role for Area I-II-III during an early phase of islet development and not from lack of the enhancer in mature β cells. We have yet to test glucose tolerance after Ngn3Cre-mediated deletion, but if deletion of Area I-II-III truly has an early dominant-negative effect then we predict that the *Ngn3Cre;Pdx1$^{flox123/+}$* mice will have worse glucose intolerance than *Pdx1$^{+/-}$* mice.

ElastaseCre-mediated deletion

To test if Pdx1 is required cell-autonomously in acinar cells and if a lower level of Pdx1 expression suffices for acinar cell maintenance, we used the acinar-cell-specific ElastaseCre transgene (Grippo et al., 2002) to conditionally delete the *floxE2* and *flox123* alleles. Examination of P7 *ElastaseCre;Pdx1$^{flox/-}$;ROSA26$^{R/+}$* mice revealed that *ElastaseCre;Pdx1$^{floxE2/-}$;ROSA26$^{R/+}$* pancreata were much smaller than wild type, while the size of *ElastaseCre;Pdx1$^{flox123/-}$;ROSA26$^{R/+}$* pancreata was only mildly decreased (Fig. 4.9).

Histological analysis of XGal-stained *ElastaseCre;Pdx1^{flox};ROSA26$^{R/+}$* pancreata revealed that activity of ElastaseCre was limited to the exocrine pancreas, and that most of the remaining exocrine tissue in the *ElastaseCre;Pdx1$^{floxE2/-}$;ROSA26$^{R/+}$* mice had not recombined *ROSA26^R* (Fig. 4.9). This result suggested that acinar tissue with low Cre activity had a selective growth advantage in *ElastaseCre;Pdx1$^{floxE2/-}$* mice.

The total endocrine mass appeared to be greatly reduced in the *ElastaseCre;Pdx1$^{floxE2/-}$* mice and slightly reduced in the *ElastaseCre;Pdx1$^{flox123/-}$* mice. Because the endocrine tissue was not lineage labeled by Cre activity, the reduction in

Fig. 4.9 Cell-autonomous requirement for Pdx1 in pancreatic acinar cells. *Left panels*, whole-mount XGal staining of P7 posterior foregut tissues from mice of indicated genotypes. ElastaseCre-mediated deletion of Area I-II-III resulted in slightly decreased size of pancreas, while deletion of the conditional null allele dramatically reduced the size of pancreas. *Right panels*, histological sections of pancreata from left panels were stained for XGal (blue/green) and Pdx1 (brown). Most of the acinar tissue present in *ElastaseCre;Pdx1$^{floxE2/-}$;ROSA26^R* mice was not lineage-labeled, indicating selection for non-recombined cells. Acinar tissue of *ElastaseCre;Pdx1$^{flox123/-}$;ROSA26^R* mice was lineage-labeled, indicating that a low level of Pdx1 is sufficient for development of acinar tissue to P7.

ElastaseCre
Pdx1flox123/+
R26R
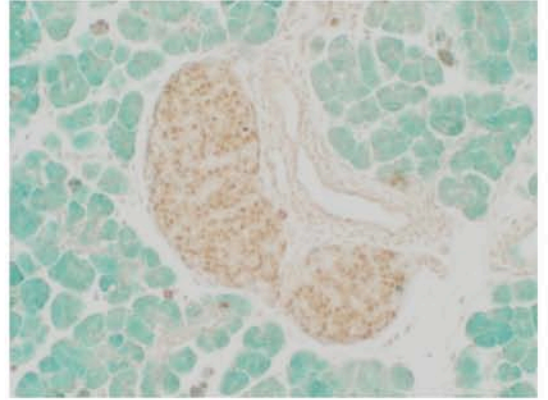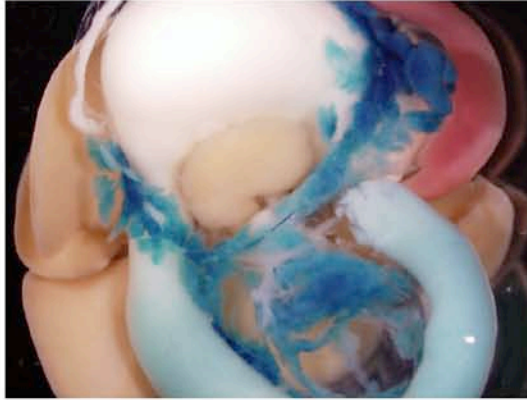
ElastaseCre
Pdx1flox123/-
R26R
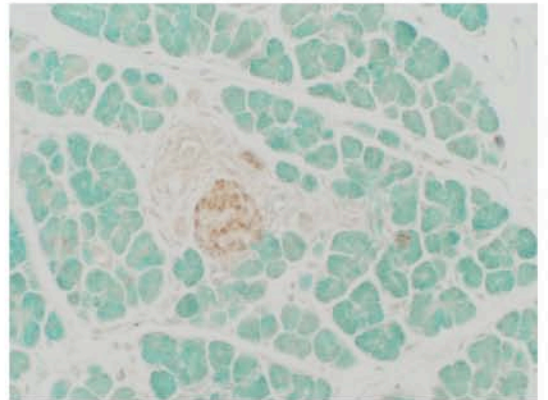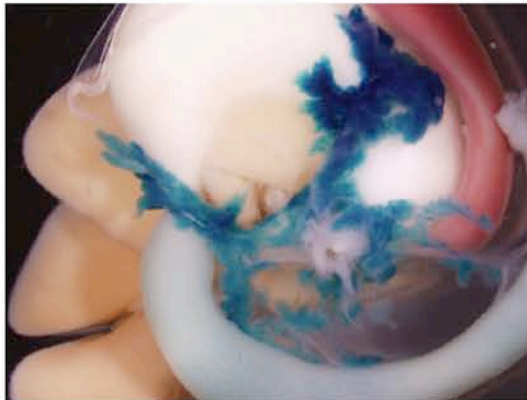
ElastaseCre
Pdx1floxE2/-
R26R
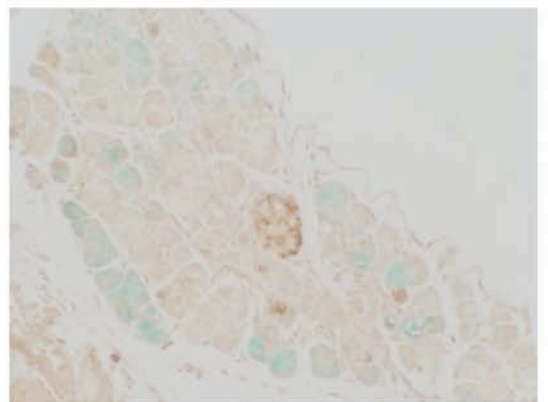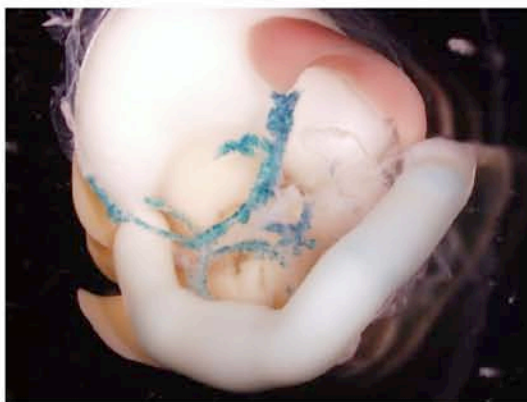
endocrine mass was probably secondary to reduction in the size of the pancreas resulting from deletion of *Pdx1* in the exocrine tissue.

Discussion

Conditional deletion of floxed *Pdx1* alleles results in supernumerary PP cells

Pdx1 is required for the differentiation of early pancreatic progenitors and for the maintenance of glucose responsiveness, insulin production, and proliferative capacity in differentiated β cells. The selective impairment of β cell development in mice homozygous for a hypomorphic allele of *Pdx1* (*Pdx1$^{\Delta I\text{-}II\text{-}III/\Delta I\text{-}II\text{-}III}$*; Fujitani et al., 2006) suggested that Pdx1 may play a role in lineage allocation of endocrine progenitors. To investigate the influence of reduced Pdx1 dosage in endocrine progenitors, we used combinations of a null allele (*Pdx^{XBko}*), conditional null allele (*floxE2*), and conditional hypomorphic allele (*flox123*). Conditional alleles were recombined using the Ngn3Cre and RIPCre transgenic lines. We assumed that phenotypes seen after recombination with Ngn3Cre but not after recombination with RIPCre would be indicative of specific functions of Pdx1 in endocrine progenitors.

Deletion with either RIPCre or Ngn3Cre reduced the number of insulin-expressing cells per islet, and there was an increase in glucagon-, somatostatin- and PP-expressing cells per islet section. To some extent, the observed increase in non-β endocrine cells probably resulted from concentration of cells that are normally on the periphery of the islet into the center of the islets. The increased proliferation of glucagon cells observed previously in *RIPCre;Pdx1$^{floxE2/-}$* mice (Gannon et al., in preparation) may also have contributed to the increased number of non-β islet cells. Even taking these factors into account, the number of

PP cells appeared to be substantially increased in *Ngn3Cre;Pdx1^{floxE2/-}* and

Ngn3Cre;Pdx1^{flox123/-} mice, but only slightly increased (if at all) in *RIPCre;Pdx1^{flox123/-}* mice.

Although more detailed analysis is required, our tentative conclusion is that impaired

expression of Pdx1 in endocrine progenitors diverts a common β/PP progenitor to mostly

make PP-expressing cells. This hypothesis is consistent with lineage tracing results

indicating that the PP promoter is active in progenitors of all PP and β cells of the adult

pancreas, and probably also in precursors of the pancreatic somatostatin cells (Herrera,

2002).

There have been concerns about the specificity of the promoter in the PPCre

transgene because it labels endocrine cells as early as E16.5 although PP protein is not

typically detected until ~E18.5 (Myrsen-Axcrone et al., 1997). The discrepancy between

promoter activity and detection of PP protein may depend on the sensitivity of the method of

protein detection and may also be influenced by post-transcriptional regulation of PP

production. The level of PP production is known to be regulated by parasympathetic

signaling (Havel et al., 1993). In Arx/Pax4 double null mutants, the islets of newborn mice

are composed primarily of somatostatin-expressing cells, but after the pups eat their first

meal many of the somatostatin-expressing cells begin to co-express PP (Collombat et al.,

2005). It seems likely that these cells are related to the common PP/β/δ progenitor and retain

the capacity to express PP, but do not express levels of PP that are detectable by IHC until

stimulated by parasympathetic signaling in response to feeding. Thus the early activity of

PPCre is probably indicative of endogenous promoter activity in common precursor cells,

which reflects competence to express PP even though PP protein does not accumulate to

detectable levels until PP production is upregulated by later signals.

Role of Pdx1 in differentiation of endocrine progenitors

The increase in PP-expressing cells seen in *Ngn3Cre;Pdx1*$^{flox/-}$ but not in *RIPCre;Pdx1*$^{flox123/-}$ may occur because common PP/β progenitors lose the ability to activate β-specific genes and repress PP-specific genes when *Pdx1* is deleted. These findings suggest that there is a series of stage-specific requirements for Pdx1 in endocrine pancreas development. Firstly, expression of Pdx1 during early pancreas development is required to produce endocrine progenitors. Pdx1 is subsequently required in specified endocrine progenitors for selection of β- over PP-cell fate by common β/PP progenitors. In differentiated endocrine cells, Pdx1 is required for maintenance and function of β cells. The role of Pdx1 in the differentiation of β/PP progenitors may involve upregulation of Pdx1 during β-cell differentiation. Nishimura et al. (2006) showed that the timing of upregulation of Pdx1 correlated with a switch from MafB to MafA expression, suggesting that Pdx1 upregulation may be a critical event in β-cell maturation. We are planning to evaluate whether lack of the Area I-II-III enhancer inhibits this upregulation of Pdx1.

Dominant phenotype of *Pdx1*$^{ΔI-II-III}$ probably results from role in endocrine progenitors

An interesting observation during characterization of the *Pdx1*$^{ΔI-II-III}$ allele was that *Pdx1*$^{+/ΔI-II-III}$ mice had more severely impaired glucose tolerance and more severely reduced Glut2 expression than *Pdx1*$^{+/-}$ mice (Fujitani et al., 2006). This was surprising because the *Pdx1*$^{ΔI-II-III}$ allele appeared to be able to produce significantly more Pdx1 than the null allele (which does not make any), as evidenced by IHC for Pdx1 and the production of some relatively normal-looking pancreatic tissue in *Pdx1*$^{ΔI-II-III/ΔI-II-III}$ mice.

From our preliminary results, it appears that deletion of *flox123* from differentiated β cells (mediated by RIPCre or RIPCreER) has a milder effect on glucose tolerance than deletion of *floxE2*, which yielded GTT results similar to $Pdx1^{+/-}$. These results are consistent with the $Pdx1^{\Delta I\text{-}II\text{-}III}$ allele being hypomorphic in terms of level of Pdx1 production. However, the lack of (perhaps even one copy of) Area I-II-III may interfere with upregulation of Pdx1 in precursors that are making the transition from common progenitors to β cells, thereby interfering with β-cell maturation and possibly also generating more PP-expressing cells. This scenario could explain why deletion of Area I-II-III from endocrine progenitors has a stronger phenotype than $Pdx1^{+/-}$, but a weaker effect when it is deleted in differentiated β cells. In support of this putative role of Area I-II-III in endocrine progenitors, many islets in $Pdx1^{+/\Delta I\text{-}II\text{-}III}$ mice were found to have unusually large numbers of PP cells (Fujitani et al., 2006).

Pdx1 promotes expansion and maintenance of differentiated acinar tissue

Results from ElastaseCre-mediated deletion of the *floxE2* allele indicate that there is a continuing requirement for Pdx1 in outgrowth of acinar tissue, in agreement with the previous analysis of Dox-repressible Pdx1 (Hale et al., 2005). Furthermore, Pdx1 appears to be required in cells that express markers of differentiated acinar tissue (i.e., elastase), so it is not merely required for expansion of an undifferentiated progenitor population that subsequently undergoes acinar differentiation.

Previous characterization of *ElastaseCre;Pdx1^{floxE2/-}* mice in our lab indicated that exocrine mass was similar to wild type during embryogenesis but decreased progressively as the mice aged (Y. Kawaguchi and C.V.E. Wright, unpublished observations). Together with

our current results, these findings suggest that acinar expansion is slowed when Pdx1 levels are very low (*ElastaseCre;Pdx1$^{flox123/-}$*), and that acinar tissue actually regresses when Pdx1 is completely removed (*ElastaseCre;Pdx1$^{floxE2/-}$*). The reason that loss of acinar tissue was not seen after Dox-mediated repression of Pdx1 (Holland et al., 2002, Hale et al., 2005) may have been that the period of analysis was too short or there was still sufficient Pdx1 for acinar tissue maintenance even with repression by Dox. We plan to use a tamoxifen-inducible acinar-specific Cre driver to delete *Pdx1* from mature acinar cells to determine whether Pdx1 continues to be required for maintenance and function of acinar tissue in the mature organ or if it is only required during the initial expansion of differentiating acinar tissue.

Assessing efficiency of recombination of floxed alleles

It is difficult to reliably assess the efficiency of recombination of the floxed *Pdx1* alleles by the Cre driver lines used in this study. We used the *ROSA26^{R} lacZ* reporter to determine the typical pattern of lineage labeling by the Cre transgenes, but it was clear from co-staining *Ngn3Cre;Pdx1$^{floxE2/-}$* tissues with XGal and Pdx1 IHC that Pdx1 was not absent from all of the cells with activated *ROSA26^{R}* (Fig. 4.3). However, judging by the small number of Pdx1+XGal+ cells, it appeared that Pdx1 was absent from most of the cells that had expressed Cre. GTT analysis of *RIPCre;Pdx1$^{floxE2/+}$* mice revealed equivalent glucose intolerance to *Pdx1$^{+/-}$* mice, providing additional evidence that *floxE2* was efficiently recombined.

Evaluating the efficiency of recombination of *flox123* was more difficult because Pdx1 can be produced from the recombined allele and there was not a clear precedent for

predicting the severity of glucose intolerance after 100% recombination of *flox123*. Reduced intensity of Pdx1 IHC in the islets of *Ngn3Cre;Pdx1$^{flox123/-}$*, *Ngn3CreBAC;Pdx1$^{flox123/-}$*, and *RIPCre;Pdx1$^{flox123/-}$* mice (Fig. 4.4, 4.6, 4.7) indicated that *flox123* was fairly efficiently recombined, although some cells with strong Pdx1 IHC were observed (e.g., Fig. 4.4F).

Recombination efficiency varies significantly among floxed loci, making it difficult to use independent reporter alleles to trace the lineage of cells that have recombined a conditional allele. For future experiments it may be beneficial to design conditional alleles that activate a reporter gene after deleting the intervening sequence. For example, a conditional allele could be designed such that Cre-mediated deletion of an exon creates a null allele and simultaneously brings a reporter gene embedded in one intron under the regulation of a cryptic promoter inserted into another intron.

CHAPTER V

DELAYED ACINAR DIFFERENTIATION AND ACCELERATED PRODUCTION OF
ENDOCRINE CELLS IN EARLY PANCREATIC BUDS OF *PTF1A+/-* MICE

Introduction

Progenitor cells residing in the endodermal component of the early pancreatic buds
give rise to the exocrine, endocrine and ductal tissues of the mature pancreas (Fontaine and
LeDouarin, 1977; Gu et al., 2002), but the mechanism by which the proper number of
progenitors is specified to each of the three main pancreatic lineages remains elusive.
Experiments utilizing pancreatic bud explant cultures have shown that signals from
mesenchyme promote outgrowth of exocrine tissue and inhibit differentiation of endocrine
tissue (reviewed by Kim and Hebrok, 2001; Scharfmann, 2000). Several molecules have
been proposed to mediate these effects, including follistatin (Miralles et al., 1998), laminin
(Li et al., 2004), and Notch signaling (Duvillie et al., 2006), perhaps through expression of
Jagged ligands in the mesenchyme (Norgaard et al., 2003). In addition, the TGFβ-related
ligand Gdf11 is expressed in the epithelium of the developing pancreas and appears to inhibit
proliferation of endocrine progenitors while promoting expansion of acinar tissue (Harmon et
al., 2004). In contrast, previous studies had implicated TGFβ and activin signaling in
promoting endocrine differentiation (Sanvito et al., 1994; Yamaoka et al., 1998; Shiozaki et
al., 1999; Kim et al., 2000; Ogihara et al., 2003) and inhibiting exocrine differentiation
(Ritvos et al., 1995). The intracellular factors that are responsible for the differential
response of developing exocrine and endocrine pancreas to these signals have yet to be
elucidated.

Two basic helix-loop-helix (bHLH) transcription factors, Pancreatic transcription factor 1a (Ptf1a) and Neurogenin 3 (Ngn3), are critical for generation of pancreatic exocrine and endocrine tissue, respectively (Krapp et al., 1998; Gradwohl et al., 2000). *Ptf1a* is expressed in the pancreatic buds from as early as E9.0 (Krapp et al., 1998; Yoshitomi et al., 2004) and lineage-labeling experiments showed that it is present in progenitors of the three main pancreatic lineages (Kawaguchi et al., 2002), although in adult pancreas its expression is restricted to acinar cells (Krapp et al., 1996). Ngn3 is also present in the early pancreatic buds (Jensen et al., 2000a), but it is expressed exclusively in cells committed to endocrine cell fate (Gu et al., 2002). Consistent with the restricted expression of Ngn3 in endocrine cells, *Ngn3* null mice do not appear to have defects in development of exocrine pancreatic tissue (Gradwohl et al., 2000). On the other hand, *Ptf1a* null mice suffer a complete failure of exocrine differentiation, but also produce many fewer pancreatic endocrine cells than wild-type mice. The remaining endocrine cells fail to aggregate properly during development (Krapp et al., 1998; Kawaguchi et al., 2002). It is not clear if the endocrine defects are secondary to defects in the exocrine pancreas, or if they reflect a role of Ptf1a in endocrine progenitors. Common pancreatic progenitors are dominantly converted to endocrine cell fate by overexpression of Ngn3 (Apelqvist et al., 1999). It is not yet known if overexpression of Ptf1a can alter the cell fate of early pancreatic progenitors.

Notch signaling inhibits differentiation of pancreatic precursors resulting in greatly reduced expression of both Ngn3 and Ptf1a (Hald et al., 2003; Murtaugh et al., 2003). These effects are mediated, at least in part, through induction of Hairy/Enhancer of Split 1 (Hes1), an inhibitory bHLH factor that is a direct repressor of *Ngn3* (Lee et al., 2001) and probably also of *Ptf1a* (Fukuda et al., 2006a). Targeted inactivation of *Hes1*, or of genes encoding the

Notch ligand Delta-like 1 (Dll1) or the Notch cofactor Recombining Binding Protein

Suppressor of Hairless J (RBPSUH-J), results in precocious activation of Ngn3 in the

pancreatic buds, converting early pancreatic progenitors to glucagon-expressing cells (Jensen

et al., 2000b; Apelqvist et al., 1999). *Hes1* null mice show ectopic activation of both Ptf1a

and Ngn3 in the common bile duct, resulting in homeotic transformation of the bile duct into

well-differentiated pancreatic tissue (Sumazaki et al., 2004; Fukuda et al., 2006a). Although

Hes1 appears to negatively regulate transcription of *Ptf1a*, there is some evidence that

Notch1 and Ptf1a can cooperatively enhance transcription of target genes (Obata et al.,

2001). Furthermore, RBPSUH-J can act as a coactivator for both Notch1 and Ptf1a (Beres et

al., 2006). Thus, it appears that Ptf1a activity is both positively and negatively regulated by

components of the Notch signaling pathway.

In addition to Ptf1a, a second bHLH factor, Mist1, is specifically expressed in the

acinar cells of the adult pancreas (Pin et al., 2000, 2001). Like Ptf1a, Mist1 is initially

expressed in the pancreatic buds and its expression subsequently becomes restricted to

developing and mature acinar cells, where it promotes transcription of factors involved in

acinar cell function (Pin et al., 2001). While Ptf1a regulates expression of digestive

hormones (Cockell et al., 1989; Petrucco et al., 1990; Rose et al., 2001), Mist1 regulates

expression of factors involved in vesicular transport and exocytosis (Rukstalis et al., 2003;

Johnson et al., 2004; Luo et al., 2005). In contrast to the severe disruption of pancreas

development in *Ptf1a*$^{-/-}$ mutants, *Mist1*$^{-/-}$ mutants have no discernable defects in pancreas

development (Pin et al., 2001), although they may be predisposed to ductal metaplasia (Zhu

et al., 2004).

Because Ptf1a and Mist1 are bHLH family members that share very similar patterns of expression in developing and mature pancreas, we hypothesized that they might have redundant functions during development. We generated $Ptf1a^{-/-};Mist1^{-/-}$ and $Ptf1a^{+/-};Mist1^{-/-}$ compound mutants to look for synthetic developmental phenotypes, but did not find any. In the course of these experiments, however, we discovered that $Ptf1a^{+/-}$ mice exhibit a greatly decreased ratio of acinar/endocrine tissue production during early development. Further examination revealed that this alteration resulted from simultaneous delay in acinar tissue differentiation and acceleration of endocrine tissue production.

Results

Lack of genetic interaction between *Ptf1a* and *Mist1*

XGal staining for a *lacZ* reporter knocked into the *Mist1* locus was used to visualize pancreatic acinar tissue, revealing that heterozygosity or homozygosity for the $Mist1^{lacZ}$ null allele did not affect the extent of acinar tissue or the organization of pancreatic exocrine and endocrine structures that developed in newborn $Ptf1a^{+/+}$ or $Ptf1a^{+/-}$ animals (Fig. 5.1). In $Ptf1a^{-/-}$ animals, $Mist1^{lacZ}$ was not active in the pancreatic epithelial rudiment at E13.5 or P0 suggesting that acinar cell differentiation is not initiated in the absence of Ptf1a (Fig. 5.2). Interestingly, the endocrine clusters that are found in the vicinity of the pancreatic rudiment in $Ptf1a^{-/-}$ mice (Krapp et al., 1998; Kawaguchi et al., 2002) were labeled by XGal in $Ptf1a^{-/-};Mist1^{+/-}$ and $Ptf1a^{-/-};Mist1^{-/-}$ mice (Fig. 5.2). One possibility is that these cells activated *Mist1* transcription because they were originally specified as acinar cells and then converted to endocrine cell fate. However, we also observed faint staining for $Mist1^{lacZ}$ in $Ptf1a^{+/+}$ and

Fig. 5.1 Disruption of *Mist1* does not impair pancreas morphogenesis in combination with *Ptf1a*$^{+/-}$. Offspring of *Ptf1a*$^{+/-}$*;Mist1*$^{+/-}$ parents were dissected at P0 and posterior foregut tissues were stained with XGal to visualize nuclear *lacZ* reporter knocked into the *Mist1* locus. Whole-mount and histological analysis showed no difference in the extent and morphology of pancreatic exocrine and endocrine tissue in *Mist1* homozygous or heterozygous null mutants in combination with *Ptf1a*$^{+/-}$. (A, B) *Ptf1a*$^{+/-}$*;Mist1*$^{+/-}$. (C, D) *Ptf1a*$^{+/-}$*;Mist1*$^{-/-}$. (E, F) *Ptf1a*$^{+/+}$*;Mist1*$^{-/-}$. Blue/green nuclear stain in (B, D, F) is XGal staining of *Mist1*^{lacZ} allele. Brown stain in (B) is IHC for Pdx1. Brown stain in (D, F) is IHC for glucagon. Arrows indicate weakly XGal-stained nuclei in endocrine tissue.

Ptf1a$^{+/-}$; Mist1$^{+/-}$
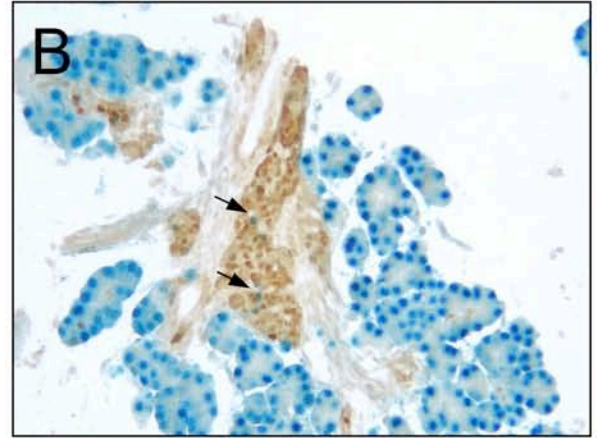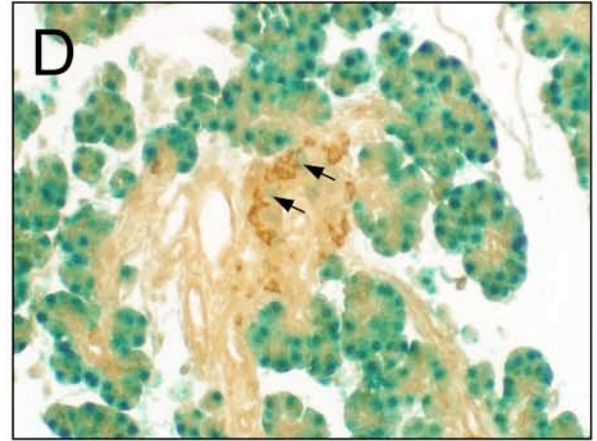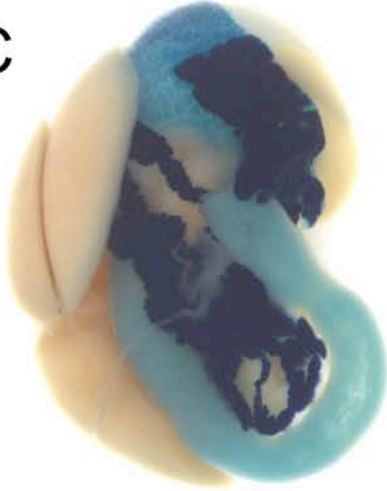
Ptf1a$^{+/-}$; Mist1$^{-/-}$

Ptf1a$^{+/+}$; Mist1$^{-/-}$
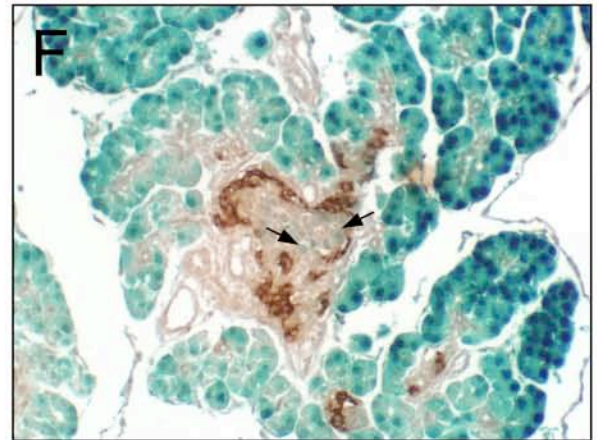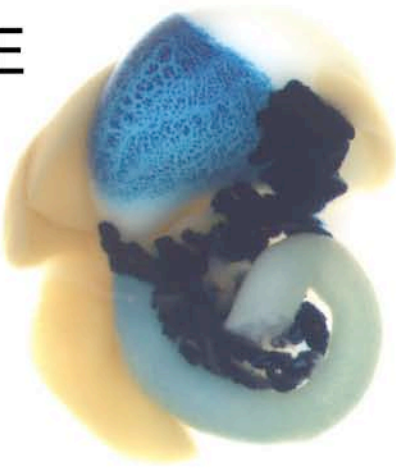
Fig. 5.2 *Mist1^{lacZ}* **expressed in endocrine clusters but not epithelial rudiment of *Ptf1a$^{-/-}$***
mice. (A, B) *Ptf1a$^{-/-}$;Mist1$^{+/-}$* P0. (C, D) *Ptf1a$^{-/-}$;Mist1$^{-/-}$* P0. (E, F) *Ptf1a$^{-/-}$;Mist1$^{-/-}$* E13.5.
Mist1 nullizygosity did not appear to affect morphology of pancreatic rudiment or endocrine
clusters in *Ptf1a$^{-/-}$* mice. (B, D, F) XGal (*Mist1^{lacZ}*) and glucagon IHC staining of histological
sections revealed that *Mist1* was very rarely expressed in the duct-like rudiment (arrow in B),
but was expressed in many of the endocrine clusters that bud off of the duct during
embryogenesis. (F) Glucagon-expressing cells bud from E13.5 ductal rudiment. H&E
counterstain. *Inset in (F)*, E13.5 endocrine cluster contains XGal-stained cells.

Ptf1a−/−; Mist1+/−

Ptf1a−/−; Mist1−/−

Ptf1a−/−; Mist1−/−
E13.5

A

B

C

D

E

F
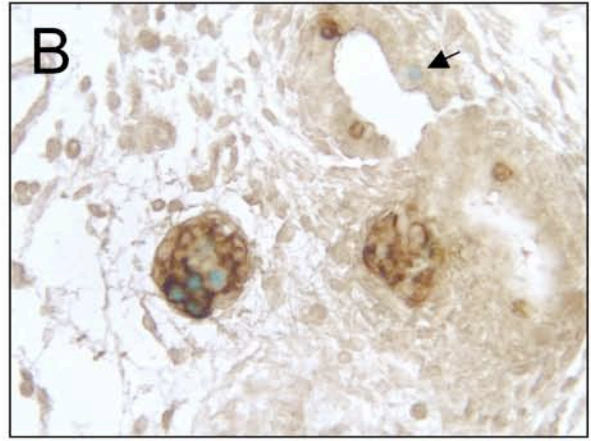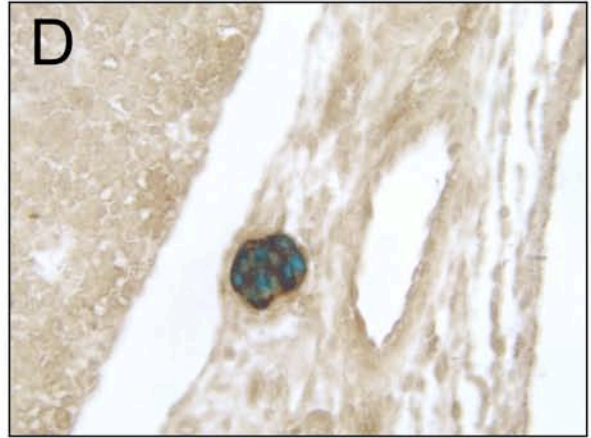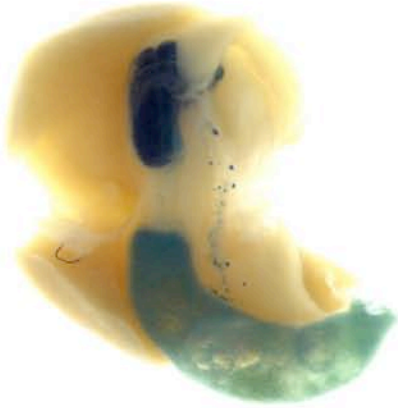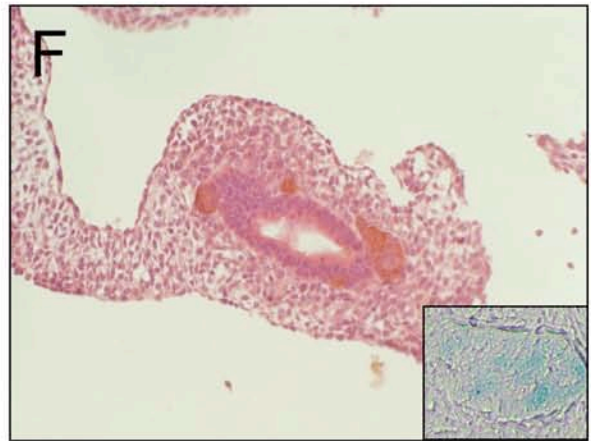
Ptf1a$^{+/-}$ endocrine tissue (e.g. Fig. 5.1B,D,F, Fig. 5.3C), so it is possible that the XGal staining simply appears more intense in the *Ptf1a*$^{-/-}$ endocrine cells because of the lack of surrounding pancreatic tissue. Overall, we did not see evidence for synthetic defects in pancreas development in compound mutants of *Ptf1a* and *Mist1*.

Delayed acinar cell differentiation in *Ptf1a*$^{+/-}$

Using *Mist1*^{lacZ} as a reporter to assess acinar cell differentiation, we observed reduced XGal staining in *Ptf1a*$^{+/-}$ as compared to *Ptf1a*$^{+/+}$ littermates at E13.5 (Fig. 5.3). XGal staining on histological sections of E13.5 *Ptf1a*$^{+/+}$;*Mist1*^{lacZ} pancreas was localized to the tips of developing acini. Similar acinar structures were identifiable in *Ptf1a*$^{+/-}$;*Mist1*^{lacZ} based on morphology, but the developing acini appeared to be smaller, fewer in number and lacked XGal staining. Whole-mount IHC of E14.5 dorsal pancreatic buds revealed that Mist1 was detectable in both *Ptf1a*$^{+/-}$ and wild-type mice, but substantially more acinar tissue was present in wild type, particularly in regions proximal to the junction with the duodenum (Fig. 5.4A,B). Note that rotation of the tissue positioned the *Ptf1a*$^{+/-}$ pancreatic bud closer to the microscope objective, resulting in a relatively higher magnification than the wild-type bud in Fig. 5.4A. Nonetheless, it is evident that the wild-type tissue contains more anti-Mist1-stained acinar clusters. Furthermore, because the size of individual acinar clusters does not differ between wild type and *Ptf1a*$^{+/-}$, it can be inferred that the wild-type pancreatic bud in Fig. 5.4A is slightly larger at this stage, and is significantly thicker at the base of the bud. In addition, the *Ptf1a*$^{+/-}$ bud appeared to contain more glucagon-expressing cells, which occupied a larger volume of the pancreatic bud than in wild type (as described in detail below).

Fig. 5.3 E13.5 *Ptf1a*$^{+/-}$ embryos show retarded acinar development and accelerated endocrine development. (A, C, E) XGal staining for *Mist1*^{lacZ} labeled tips of epithelial branches in dorsal (DP) and ventral (VP) pancreatic buds of *Ptf1a*$^{+/+}$*;Mist1*$^{-/-}$ embryos. (B, D, F) *Ptf1a*$^{+/-}$*;Mist1*$^{-/-}$ embryos had less extensive epithelial branching with very little XGal staining of branch tips. (C, D) Glucagon IHC showed substantially larger endocrine clusters in *Ptf1a*$^{+/-}$. (E, F) Insulin IHC indicated accelerated differentiation of insulin-producing cells in *Ptf1a*$^{+/-}$. Arrows in (C) and (F) indicate XGal-stained nuclei in endocrine clusters. (C – F) Purple nuclei result from hematoxylin counterstain. Turquoise nuclei result from XGal staining of *Mist1*^{lacZ}.

Ptf1a$^{+/+}$; Mist1$^{-/-}$ Ptf1a$^{+/-}$; Mist1$^{-/-}$

A   stomach   DP   VP   duodenum

B

Glucagon

C   D

Insulin

E   F
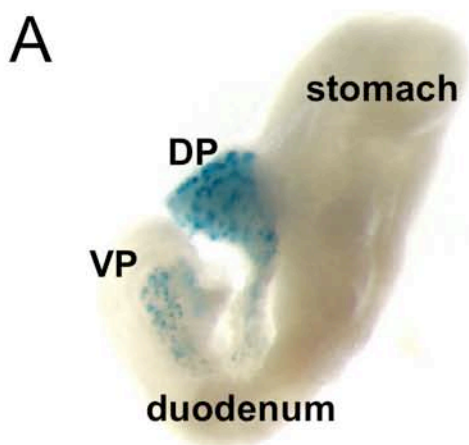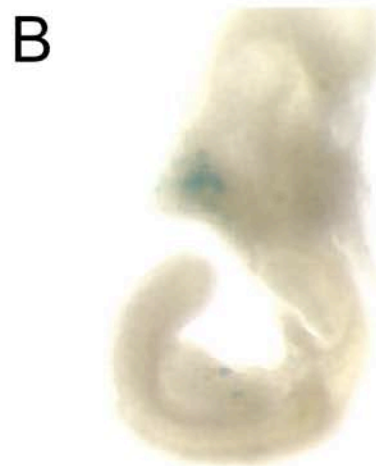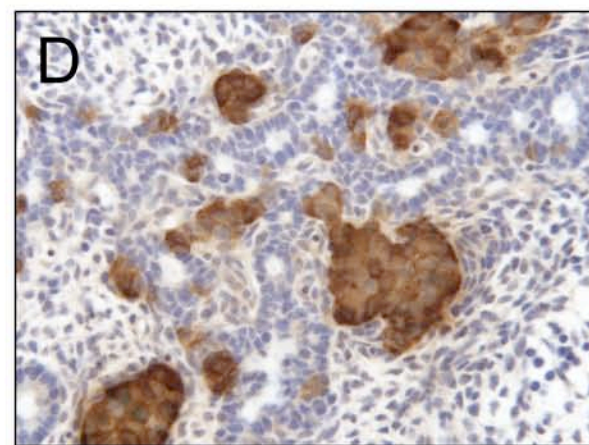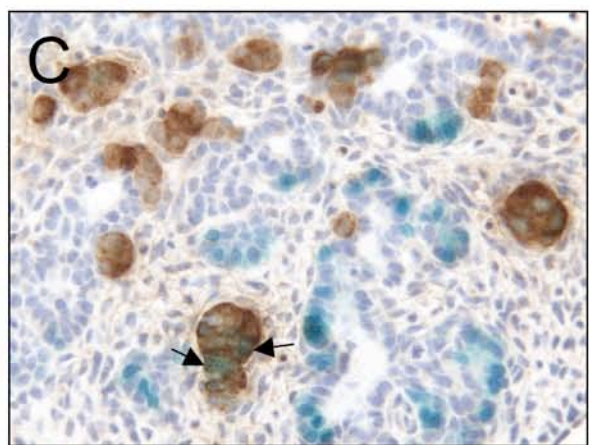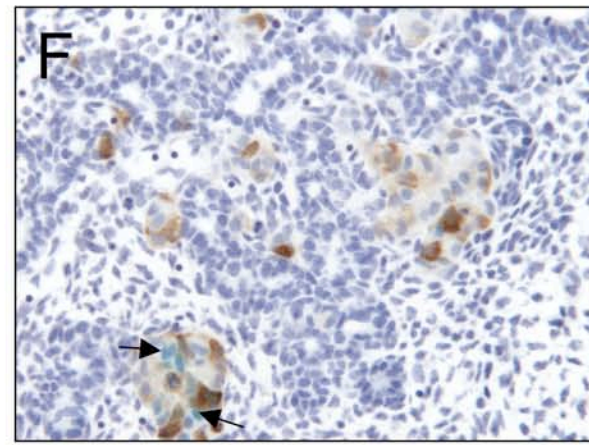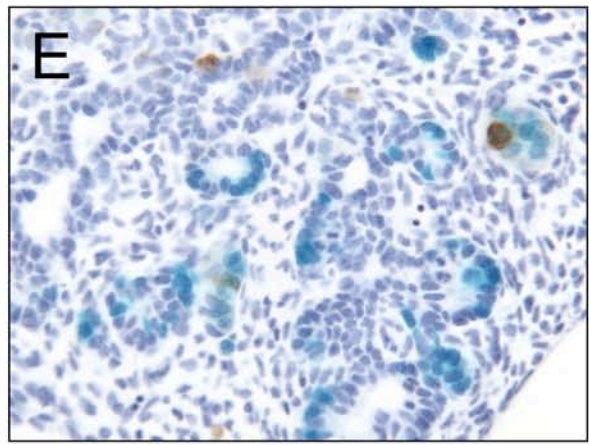
Fig. 5.4 Delayed acinar cell differentiation in *Ptf1a*$^{+/-}$ embryos at E14.5. (A, B) Whole-mount IHC for Mist1 (red) and glucagon (green) revealed decreased acinar/endocrine ratio in *Ptf1a*$^{+/-}$. (C, D) Fewer Mist1-positive (red) acinar cells were detectable per section in *Ptf1a*$^{+/-}$ and the intensity of staining appeared to be slightly weaker. Nuclei are counterstained with TOTO-3 (blue). (E, F) Less expression of amylase (red) in developing exocrine tissue of *Ptf1a*$^{+/-}$ embryos. (C – F) The number of Ngn3-positive (green) cells was not appreciably different between wild type (C, E) and *Ptf1a*$^{+/-}$ (D, F).
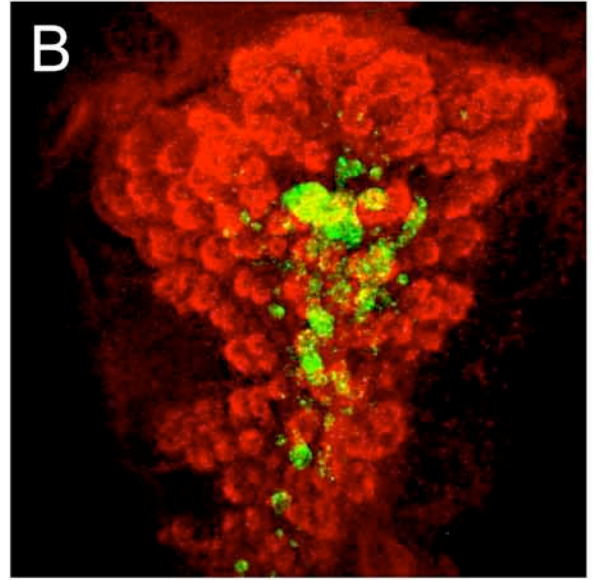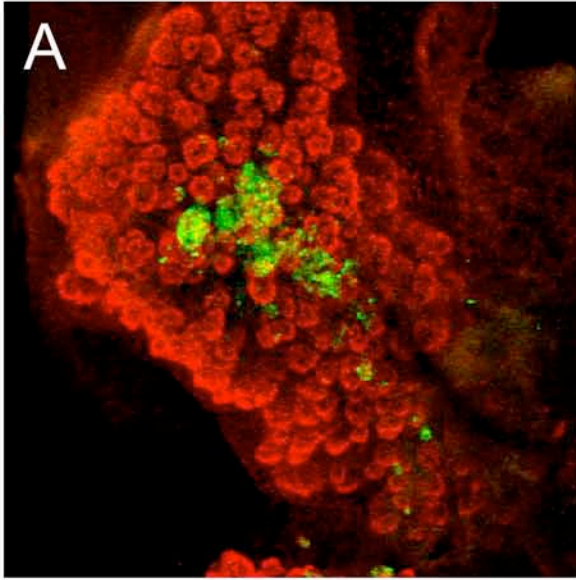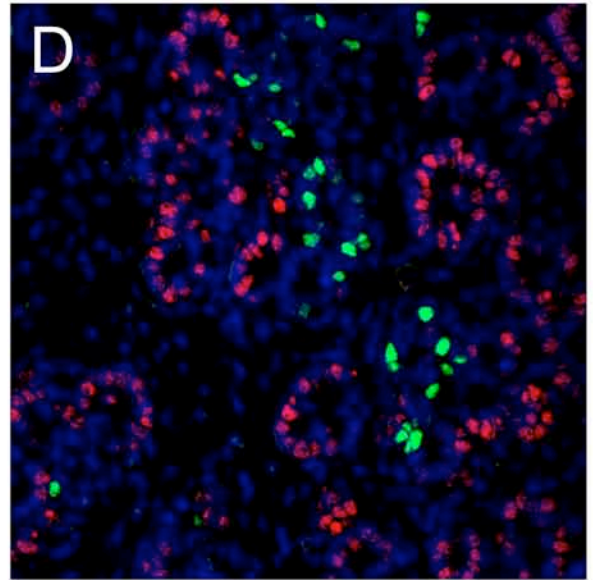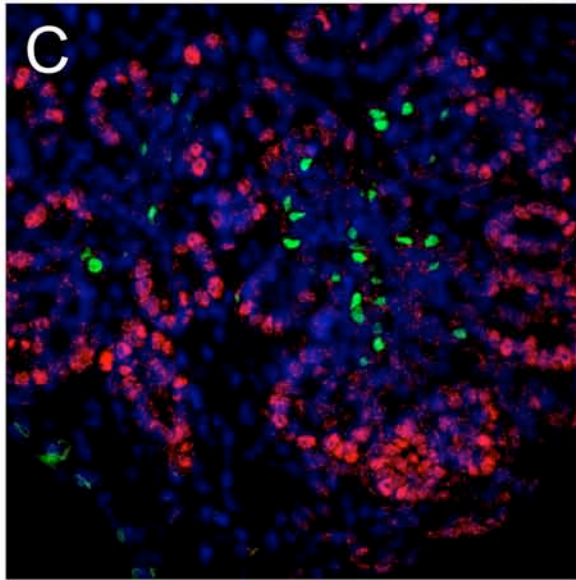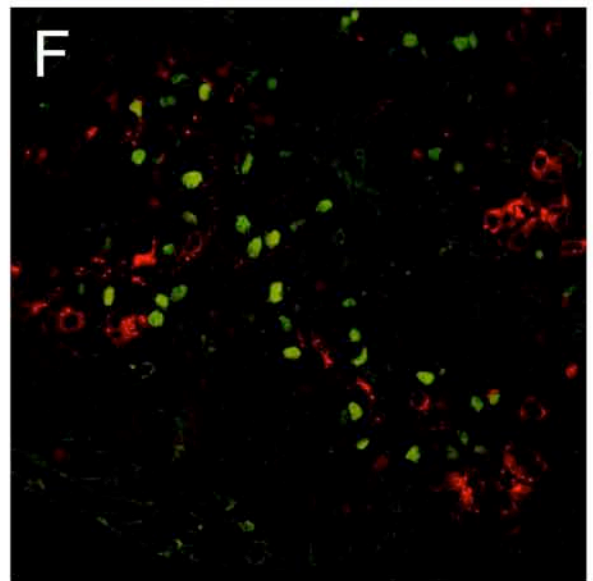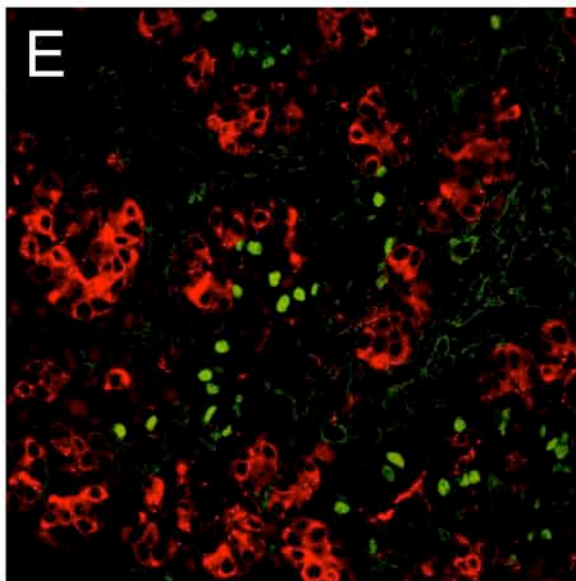
IHC staining for Mist1 on histological sections of E14.5 pancreata indicated that fewer acinar cell nuclei expressed high levels of Mist1 in $Ptf1a^{+/-}$ embryos as compared to wild-type littermates (Fig. 5.4C,D). The digestive enzyme amylase was abundantly expressed in wild-type pancreas at E14.5, but was more weakly expressed and present in substantially fewer cells in $Ptf1a^{+/-}$ littermates (Fig. 5.4E,F).

These results indicate that production of acinar tissue is delayed in $Ptf1a^{+/-}$ mice at mid-embryogenesis, and the decreased mass of acinar tissue at these stages is accompanied by a delay in the initiation of expression of early (Mist1) and late (amylase) markers of acinar cell differentiation. Decreased acinar tissue volume was not observed in $Ptf1a^{+/-}$ newborns (e.g. Fig. 5.1), suggesting that acinar tissue production "catches up" later during embryogenesis. We still need to examine later embryonic stages to evaluate this hypothetical "catch-up" process.

Accelerated endocrine cell differentiation in *Ptf1a+/-*

At the same stages when production of acinar tissue lagged in $Ptf1a^{+/-}$ embryos, there appeared to be an increased amount of tissue expressing the endocrine hormones glucagon and insulin. As a result of the delayed production of acinar tissue, it is possible that endocrine tissue appeared to be increased simply due to the same amount of endocrine tissue being concentrated into a smaller pancreatic organ. If this were the case, we might expect to see more endocrine clusters per section but not necessarily larger individual clusters. On the contrary, anti-glucagon IHC indicated that the endocrine clusters in $Ptf1a^{+/-}$ contained more cells, suggesting that there was increased proliferation of endocrine cells in $Ptf1a^{+/-}$ embryos

rather than crowding of the wild-type number of clusters (Fig. 5.3C,D). Whole-mount IHC

analysis also indicated that the overall volume of glucagon clusters was increased in $Ptf1a^{+/-}$

embryos (Fig. 5.4A,B). In wild-type embryos at E13.5, insulin-positive cells were rare and

scattered, whereas they appeared to be more abundant and clustered in $Ptf1a^{+/-}$ embryos (Fig.

5.3E,F). These results suggest that endocrine cell proliferation and differentiation may be

accelerated in $Ptf1a^{+/-}$ embryos.

Increased production of endocrine cells was also apparent at earlier stages of

development. The size of glucagon-positive endocrine cords appeared to be increased at

E12.5 in $Ptf1a^{+/-}$, and the number of Ngn3-expressing cells also appeared to be increased at

this stage (Fig. 5.5C,D). At E10.5 the proportion of pancreatic bud cells that expressed

glucagon was greater in $Ptf1a^{+/-}$ embryos. The number of Ngn3-expressing cells appeared to

be slightly increased in $Ptf1a^{+/-}$, but we are not yet certain if this is a consistent trend (Fig.

5.5A,B, Fig. 5.6). Overall, the increase in hormone-expressing cells appeared to be greater

than the increase (if any) in Ngn3-expressing cells, suggesting that increased endocrine mass

is primarily attributable to increased proliferation of endocrine cells rather than increased

specification of common pancreatic progenitors to endocrine cell fate. Quantitative analysis

of a larger number of embryos will be required to determine if the number of Ngn3-positive

cells is increased. We also plan to examine markers of proliferation in hormone-positive and

Pax6-positive endocrine tissue at these stages.
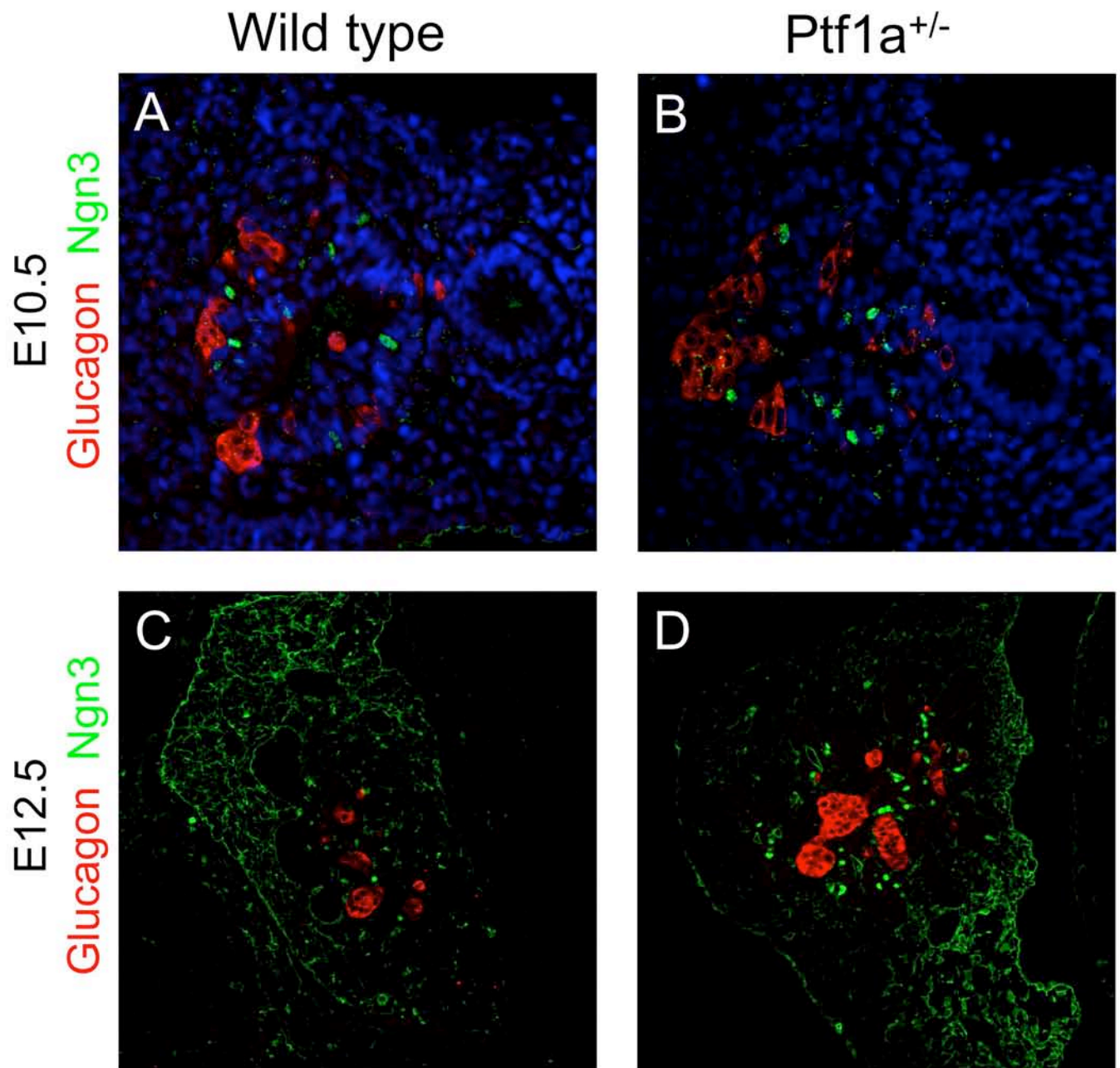
Fig. 5.5 Expanded endocrine tissue in early pancreatic buds of *Ptf1a*^{+/-} embryos.
A greater proportion of pancreatic bud epithelium expressed the endocrine markers glucagon (red) and Ngn3 (green) in *Ptf1a*^{+/-} embryos (B, D) than in wild-type littermates (A, C) at both E10.5 (A, B) and E12.5 (C, D). Nuclei in (A, B) are counterstained with TOTO-3 (blue).
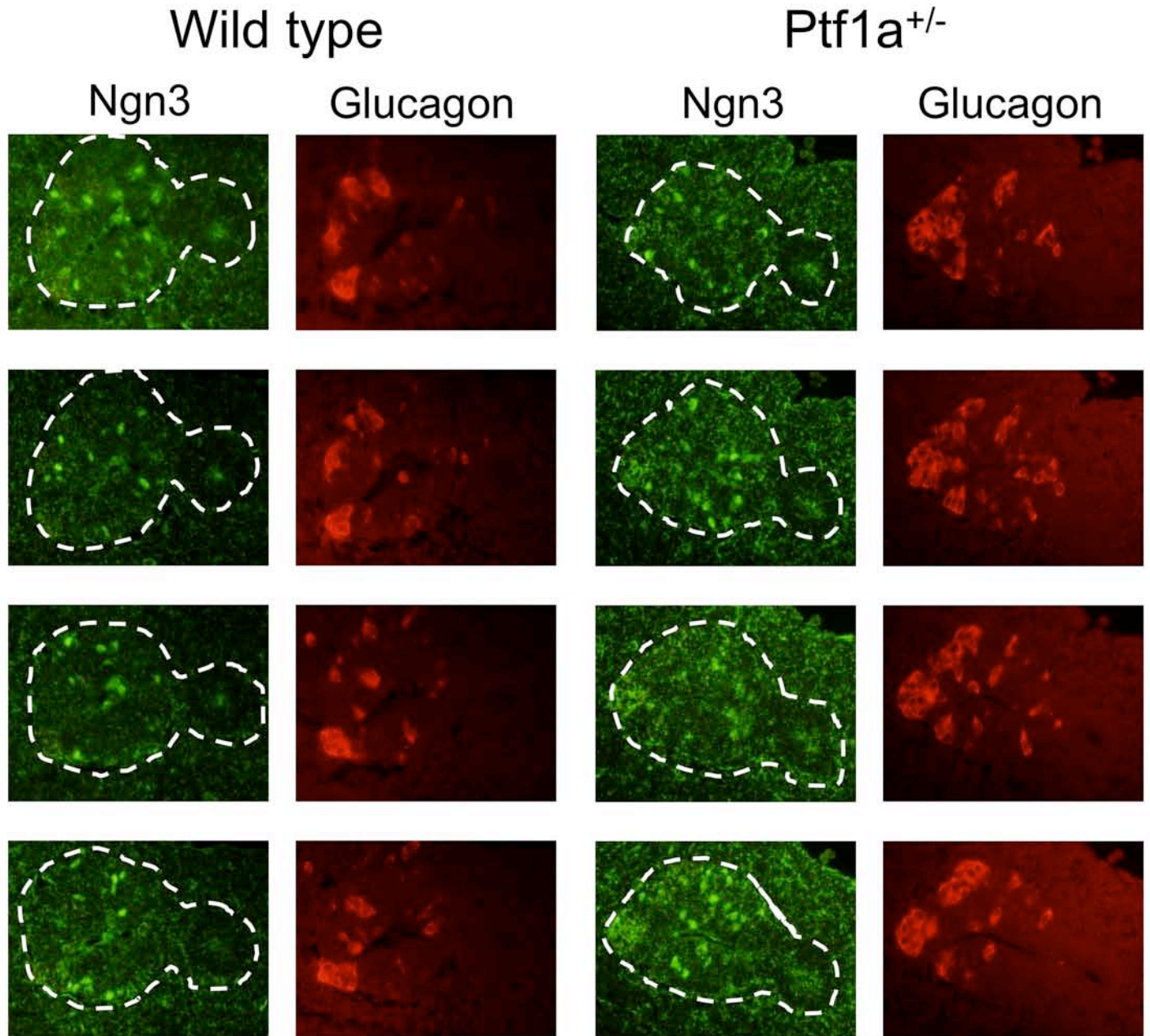
Fig. 5.6 Serial section analysis of Ngn3 and glucagon in E10.5 dorsal pancreatic bud. Epifluorescence imaging of immunostaining for Ngn3 (green) and glucagon (red) on serial sections of wild-type (left panels) and *Ptf1a*^{+/-} (right panels) embryos. There appeared to be a slight increase in Ngn3 and marked increase in glucagon-expressing cells in *Ptf1a*^{+/-}. Dashed outlines demarcate dorsal pancreatic bud and duodenum.

Clusters of Xgal-stained cells in rostral duodenal epithelium of *Pdx1⁻/⁻;Mist1⁺/lacZ*

One interpretation of the finding that endocrine clusters in *Ptf1a⁻/⁻;Mist1^lacZ* mice were labeled by XGal is that progenitors that were originally specified as acinar cells were reassigned to endocrine fate in the absence of Ptf1a. If this hypothesis is true, then the transdifferentiated cells were able to retain the nuclear-localized β-galactosidase protein that was generated when they initially activated *Mist1* transcription. In light of the possibility that *Mist1^lacZ* might serve as a lineage label for acinar progenitors, we examined newborn *Pdx1⁻/⁻;Mist1⁺/lacZ* mice by whole-mount XGal staining to determine if any of the rudimentary pancreatic tissue remaining in *Pdx1⁻/⁻* mutants may have undergone early steps of acinar differentiation.

XGal labeling was not detected in the dorsal pancreatic rudiment, but patches of XGal-stained cells were found in the epithelium of the rostral duodenum (Fig. 5.7). Scattered individual Mist1-positive cells were present in wild-type duodenum (Fig. 5.7E), but clusters of strongly stained cells were only seen in *Pdx1⁻/⁻* duodena. The pattern of duodenal Xgal labeling was reminiscent of the location of lineage-labeled cells that take residence in the duodenum of *Ptf1a^cre/cre;ROSA26R* mice (Kawaguchi et al., 2002). This finding raises the possibility that early acinar specification occurs in the ventral bud of *Pdx1⁻/⁻* embryos and the cells that produced β-galactosidase subsequently come to reside in the duodenal epithelium after the ventral bud is reincorporated into the duodenum. This possibility could be tested more rigorously using a mouse expressing Cre under the control of *Mist1* regulatory elements.

An alternative explanation is that improper patterning of the gastroduodenal junction in *Pdx1⁻/⁻* mutants results in ectopic expression of *Mist1* in the rostral duodeum in a pattern

Fig. 5.7 Poorly differentiated duodenal epithelium of *Pdx1$^{-/-}$* mice ectopically expresses *Mist1^{lacZ}*. Many patches of *Mist1^{lacZ}*-expressing cells (blue) were found on the dorsoposterior aspect of the rostral duodenum (B), and a few on the ventroanterior aspect (A), in newborn *Pdx1$^{-/-}$;Mist1$^{+/-}$* mice. Arrow in (B) indicates pancreatic rudiment. (C, D) Histological anaylsis indicated that the XGal-stained cells (blue) were localized to clusters of epithelial cells in dysmorphic regions of the rostral duodenum. (E) In *Pdx1$^{+/+}$* duodenum, *Mist1^{lacZ}* expression (blue) was found in scattered epithelial cells. (F) *Mist1^{lacZ}* was expressed in secretory glands of the stomach in *Pdx1$^{+/+}$;Mist1$^{-/-}$* mice. Sections in (C, D) are counterstained with eosin. Pdx1 IHC (brown) is shown in (E).
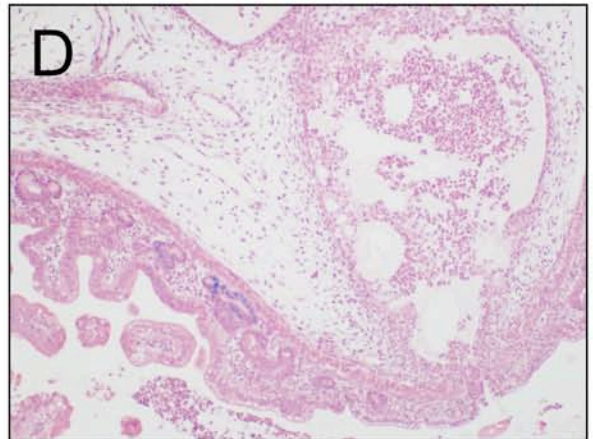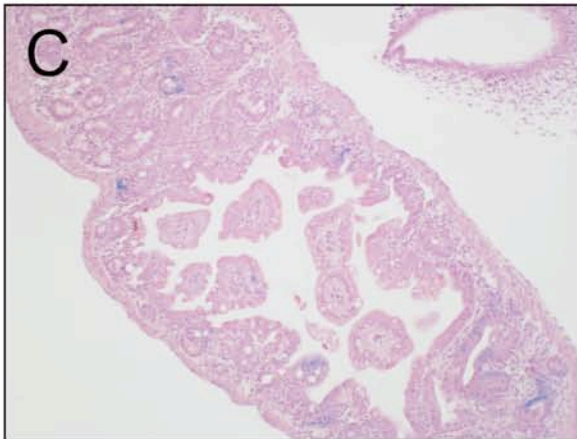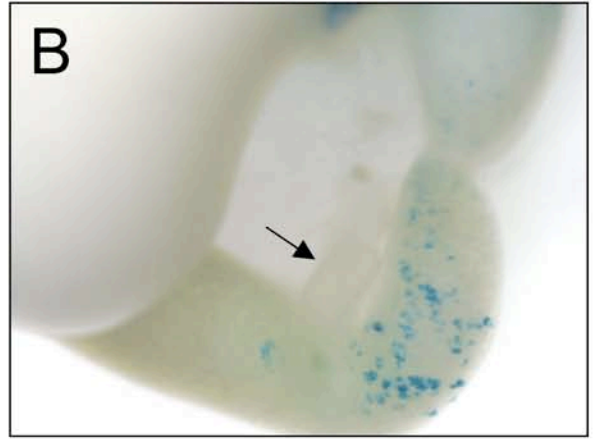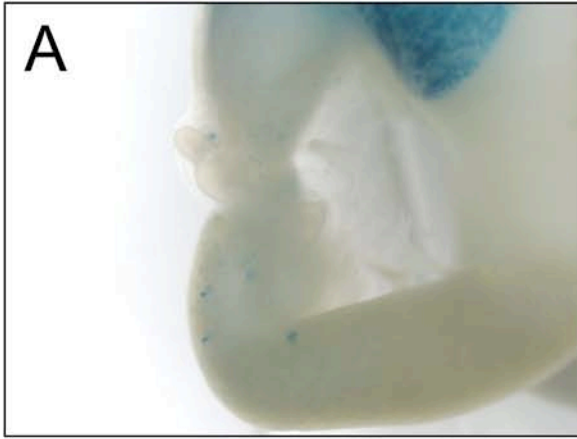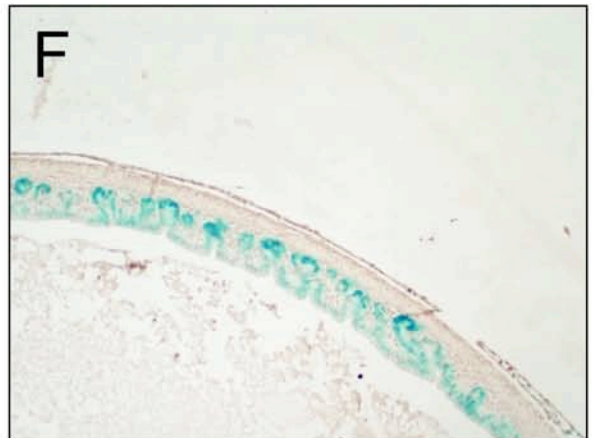
similar to that normally seen in the glandular tissues of the stomach (e.g., Fig. 7F). Further evidence for the latter hypothesis could be obtained by examining embryonic stages to determine if the onset of Mist1 expression in the rostral duodenum of $Pdx1^{-/-}$ embryos matches the timing of Mist1 induction in stomach glands. We also plan to use IHC analysis to determine if Mist1-postive patches of $Pdx1^{-/-}$ duodenum ectopically express stomach markers or downregulate duodenal markers.

Discussion

Due to their similar developmental expression patterns and roles in promoting acinar-cell-specific gene expression, we predicted that Mist1 and Ptf1a might act in the same pathways during acinar cell development, or perhaps even in early pancreatic bud development, and that a synthetic defect might be seen in $Ptf1a^{+/-};Mist1^{-/-}$ embryos. We did not, however, detect any synthetic developmental defects in mice with various combinations of $Ptf1a$ and $Mist1$ null alleles. We conclude that although Mist1 and Ptf1a are involved in the related but distinct processes of enzyme production and transport in mature acinar cells, they do not appear to contribute to the same pathways in acinar cell development.

$Mist1^{lacZ}$ proved to be a useful marker for the early stages of acinar cell development, and during our analysis of $Ptf1a^{cre};Mist1^{lacZ}$ compound mutants we noticed that acinar cell development is delayed in $Ptf1a^{+/-}$ embryos. Further characterization of differential marker expression between $Ptf1a^{+/-}$ and wild-type embryos revealed that along with a delay in the activation of acinar cell markers and outgrowth of acinar tissue, there appears to be increased production of pancreatic endocrine cells in mid-gestation $Ptf1a^{+/-}$ embryos. Much work remains to be done in analyzing the nature of the increase in endocrine tissue, such as

examining whether the increase results primarily from enhanced proliferation of specified endocrine cells or from increased specification of common progenitors to endocrine cell fate.

Because the relative endocrine and exocrine mass does not appear to be abnormal in newborn $Ptf1a^{+/-}$ mice, we hypothesize that there is a catch-up process during late gestation. We plan to characterize this catch-up process in terms of whether we can observe increased proliferation of acinar tissue and decreased proliferation of endocrine tissue during late embryogenesis to compensate for the delayed acinar differentiation and increased generation of endocrine tissue during early pancreatic development. Characterization of the catch-up process will hopefully provide insight into the mechanism regulating final organ size during normal pancreatic organogenesis.

Based on the absolute requirement for Ptf1a in the development of the pancreatic exocrine lineages (Krapp et al., 1998), a likely reason for delayed acinar development in $Ptf1a^{+/-}$ embryos is that retarded accumulation of Ptf1a protein results in slower triggering of acinar cell specification. Because Ptf1a has been shown to be expressed in progenitors of most pancreatic endocrine cells (Kawaguchi et al., 2002), the expansion of early pancreatic endocrine development may be a direct effect of a decreased level of Ptf1a in endocrine progenitors. Another possibility is that accelerated endocrine development is secondary to the delayed differentiation of acinar tissue.

A candidate mechanism for indirectly linking the rates of acinar and endocrine cell differentiation is secretion of the TGFβ-family molecule Gdf11 by developing acinar tissue. Gdf11 is expressed in the early pancreatic bud epithelium and becomes restricted to acinar cells during development (Harmon et al., 2004). $Gdf11^{-/-}$ mutants have increased proliferation of endocrine progenitors and decreased production of acinar tissue, making it a

good candidate for a putative signal from developing acinar cells to limit expansion of the endocrine compartment. We plan to test if Gdf11 localizes to specified acinar progenitors during early pancreatic development based on co-localization of Gdf11 and Mist1 expression, and whether pancreatic Gdf11 expression is delayed in $Ptf1a^{+/-}$ embryos.

Another potential mechanism for accelerated endocrine development in $Ptf1a^{+/-}$ mutants is cooperation between Ptf1a and the Notch signaling pathway to repress Ngn3 expression. To test if accelerated endocrine development in $Ptf1a^{+/-}$ embryos results from enhanced Ngn3 expression, we plan to generate $Ptf1a^{+/-};Ngn3^{+/-}$ double heterozygous mice to see if equizygosity of the two genes restores balance to the relative amount of acinar and endocrine cells in early embryos.

A third mechanism to be evaluated is perturbed communication between pancreatic epithelium and mesenchyme in $Ptf1a$ heterozygotes. For instance, if Ptf1a promotes the secretion of a signal from the epithelium that enhances the release of follistatin from the mesenchyme, then a decreased level of Ptf1a could indirectly increase TGFβ/Activin signaling, which would in turn stimulate endocrine differentiation. Another possibility is that higher doses of proliferative signals from the mesenchyme, such as FGFs, reach the endocrine progenitors when the mass of surrounding acinar tissue is decreased due to insufficient levels of Ptf1a. In summary, we have uncovered a novel, dosage-dependent role of Ptf1a in regulating the relative rates of endocrine and acinar tissue production from pancreatic progenitors, but much work remains to be done to characterize the mechanism underlying this phenotype.

CHAPTER VI

CONCLUSIONS AND FUTURE AIMS

Conclusions

Roles of distal and proximal *cis*-regulatory regions of *Pdx1*

In the *Pdx1* locus, almost all of the non-coding sequences that show significant

conservation between mice and humans reside within 10 kb 5' of the transcription start site.

The proximal 4.6 kb of this region was shown to be sufficient to recapitulate the expression

pattern of Pdx1 in developing and mature mice (Stoffers et al., 1999) despite the fact that

Area IV, the largest conserved region (Fig. 1.6) and first shown to mediate β-cell-specific

activity (Sharma et al., 1996), is not included in the 4.6 kb region. Our transgene

complementation experiment (Chapter 3) revealed that *cis*-elements outside of the proximal

4.6 kb region are required for normal levels of Pdx1 expression in mature duodenal and

stomach epithelia. In addition, mice carrying the transgene produced more Pdx1 in mature

pancreatic acinar, α, and PP cells than did wild-type controls, indicating that distal *cis*-

elements may have a repressive influence on *Pdx1* transcription in these tissues.

Interestingly, the embryonic expression domain of Pdx1 was not affected by the lack

of distal regulatory elements (Chapter 3) nor was it substantially altered by absence of the

proximal enhancer region Area I-II-III (Fujitani et al., 2006). These results suggest

functional redundancy between proximal and distal *cis*-regulatory elements for mediating

transcriptional activation of *Pdx1* in response to the development cues that dictate the early

expression pattern of Pdx1. Redundancy between the proximal and distal regions during embryogenesis is consistent with the presence of binding sites for FoxA and Hnf factors in both regions (Sharma et al., 1996; Wu et al., 1997; Gerrish et al., 2000; Marshak et al., 2000; Gerrish et al., 2001; Jacquemin et al., 2003). Expression of the three FoxA factors (Ang et al., 1993; Monaghan et al., 1993; Sasaki and Hogan, 1993), Hnf1β (Barbacci et al., 1999), Hnf4 (Duncan et al., 1994) and Hnf6 (Jacquemin et al., 2003) precedes expression of Pdx1 in posterior foregut endoderm, and it has been proposed that they act as a core endodermal genetic network by reinforcing and stabilizing their own expression and promoting the expression of other endoderm-specific genes (Shih and Stoffel, 2001; Jensen, 2004; Odom et al., 2004). It appears that this core endodermal network can interact with multiple regions of the *Pdx1* promoter and as long as some of those regions are intact, then there is sufficient recruitment of the core endodermal factors to activate *Pdx1* in its normal expression domain. Because the embryonic domain of Pdx1 expression was not expanded in either the transgene-rescue or *Pdx1*$^{ΔI-II-III}$ mutants, it can be inferred that the binding of repressors to proximal or distal 5' regions is not critical for restricting the early Pdx1 expression domain. These findings suggest that the early Pdx1 expression domain is dictated primarily by the distribution and level of activity of the transactivators of *Pdx1*.

Redundant roles for Area IV and Area II may also contribute to functional redundancy between proximal and distal promoter regions. Areas I, II and IV have β-cell-specific transcriptional activity in isolation, and the level of activity increases synergistically when these areas are combined (Gerrish et al., 2004; Van Velkinburgh et al., 2005). Although Area II appears to be the most important for expression in murine β cells (Samaras et al., 2002), only Areas I, III and IV are present in chickens (Gerrish et al., 2004). When

considered together with our findings that proximal (including Areas I, II, and III) and distal (including Area IV) regions of the *Pdx1* promoter have redundant functions in promoting embryonic expression of Pdx1, the phylogenetic analysis suggests that perhaps ancient functions of Area IV have been subsumed by Area II in mammals, but mammalian Area IV retains the capacity to partially compensate for deletion of Area II.

In situ hybridization for *Pdx1* message in the duodenum indicated that it is transcribed mainly in the crypts (Stoffers et al., 1999), where the adult intestinal stem cells are located. Our analysis of the consequences of reduced duodenal Pdx1 expression in the transgene-rescue mice revealed a dose-dependent influence of Pdx1 on the postnatal production of GIP enteroendocrine cells. While Pdx1 had previously been shown to activate transcription of *GIP* (Jepeal et al., 2005), our results demonstrated that Pdx1 also determines the rate of production of GIP cells from crypt progenitors. Lineage analysis of intestinal epithelial cells (reviewed by Schonhoff et al., 2004a) has shown that Notch signaling inhibits expression of Math1, which promotes differentiation of common progenitors to secretory rather than absorptive cell lineages. A subset of the Math1-expressing cells activate expression of Ngn3 and subsequently differentiate to produce enteroendocrine cells. Very little is known about the mechanisms that allocate Ngn3-expressing progenitors to the various enteroendocrine lineages, but our results indicate that Pdx1 plays a role in this process by promoting GIP cell fate.

Pdx1 regulates ratio of β/PP cells

In addition to demonstrating a role of Pdx1 in regulating the cell fate of enteroendocrine precursors, we have uncovered a similar role of Pdx1 in the lineage

allocation of pancreatic endocrine progenitors. It had been difficult to assess the role of Pdx1 in endocrine progenitors because germline inactivation of *Pdx1* disrupts development of the pancreatic buds and global repression of Pdx1 during mid-embryogenesis arrests all aspects of pancreas development (Jonsson et al., 1994; Hale et al., 2005). By using Ngn3Cre transgenes to recombine floxed alleles of *Pdx1*, we have demonstrated that the level of Pdx1 expression in endocrine progenitors regulates the relative number of β and PP cells that are produced (Chapter 4). This result is consistent with previous studies indicating that β and PP cells derive from a common progenitor cell that is distinct from the α cell lineage (Herrera, 1994; Herrera, 2000).

The mechanism by which PP expression is increased in *Ngn3Cre;Pdx1$^{flox/-}$* mice remains to be elucidated. Our immunohistochemical (IHC) analysis of PP expression indicates that low levels of PP expression are detectable in many β cells of neonatal wild-type mice. More thorough analysis is required, but it appears that the low-level co-expression of PP diminishes as the mice get older, suggesting that PP expression is a remnant of the bipotential state of β/PP precursors that is progressively lost as the β cells mature. Thus, one explanation for the simultaneous decrease in insulin-expression and increase in PP-expression in *Ngn3Cre;Pdx1$^{flox/-}$* mice could be that Pdx1 directly represses PP expression in specified β-cell precursors. In this case, common β/PP progenitors might be correctly specified to β-cell fate after conditional deletion of *Pdx1*, but the lack of Pdx1 results in decreased transcription of *Insulin* and failure to repress expression of PP. This hypothesis could be tested by evaluating if the supernumerary PP-expressing cells in *Ngn3Cre;Pdx1$^{flox/-}$* animals have the characteristic catecholamine responsiveness (Havel et al., 1993) and electron micrographic characteristics (Larsson et al., 1976) of proper PP cells, or if they

inappropriately express factors typically found in β cells but not in PP cells, such as MafA, Nkx6.1, and Nkx2.2.

If the extra PP-expressing cells are found to be properly differentiated PP cells, it would support the alternative hypothesis that conditional deletion of *Pdx1* tips the balance of common β/PP progenitors toward production of PP cells. A caveat to our observation of decreasing levels of PP in young, wild-type β cells is that the closely related peptide NPY is known to be expressed in immature β cells (Myrsen-Axcrona et al., 1997). It will be important to confirm the specificity of detection of PP versus NPY.

Pdx1 influences cell fate decisions downstream of Ngn3

Our experiments fill a gap in the previous understanding of the role of Pdx1 in endocrine cell development in the posterior foregut. Previous analysis of $Pdx1^{-/-}$ mutant mice had revealed gross morphological defects of the pancreas, rostral duodenum, pylorus and common bile duct (Jonsson et al., 1994; Offield et al., 1996; Fukuda et al., 2006b), and several in vitro and in vivo studies demonstrated that Pdx1 is a positive transcriptional regulator of genes encoding the hormones insulin, somatostatin and GIP, as well as factors involved in β-cell function (Ohlsson et al., 1993; Peers et al., 1994; Peshavaria et al., 1994; Leonard et al., 1993; Miller et al., 1994; Waeber et al., 1996; Watada et al., 1996; Jepeal et al., 2005). We have now shown that, in addition to regulating the function of foregut endocrine cells and patterning the organs in which they reside, Pdx1 acts downstream of Ngn3 in both the pancreas and duodenum to influence the lineage choice of common endocrine progenitors.

Our hypothesis that the level of expression of Pdx1 in progenitors influences cell fate choice has important ramifications for efforts to produce well-differentiated tissues from stem cells in vitro. Current protocols for guiding the differentiation of stem cells typically use RT-PCR to check for the presence of transcripts that are thought to be important markers for intermediate stages of differentiation. Our findings indicate that the efficient production of β cells requires a higher level of Pdx1 than is needed for the production of endocrine precursors. This suggests that simply assessing the presence of established markers for progenitor cells may not be adequate to insure that stem cells are following the desired pathway of differentiation. In order to refine multi-step protocols for differentiation of stem cells into β cells or other mature cell types, it may be important to monitor the level of expression of key transcriptional regulators at intermediate steps, e.g. using real-time PCR, to make sure that they are sufficient to support differentiation to the desired mature cell fate.

Future directions

Ptf1a haploinsufficiency phenotype

Many studies have shown that intercellular signaling influences the balance between exocrine and endocrine differentiation of the pancreatic buds (reviewed by Scharfmann, 2000), but our characterization of $Ptf1a^{+/-}$ embryos may be the first example of a transcription factor having opposite effects on the rates of exocrine and endocrine differentiation without arresting either process. Because *Ptf1a* is transcribed in common progenitors of endocrine and exocrine pancreatic tissue, one hypothesis is that the level of Ptf1a protein influences the response of common progenitors to extracellular cues that favor

exocrine over endocrine differentiation. An alternative hypothesis, considering that Ptf1a is required for acinar cell differentiation, is that reduced expression of Ptf1a in $Ptf1a^{+/-}$ embryos results in a cell-autonomous retardation of acinar differentiation, which leads to an indirect stimulation of endocrine cell proliferation.

In either case, further analysis of the *Ptf1a* haploinsufficiency phenotype should help to elucidate the mechanisms that regulate production of the correct proportion of endocrine and exocrine pancreatic tissue. The first step will be to determine the mechanism of expansion of endocrine tissue in early $Ptf1a^{+/-}$ pancreatic buds. For instance, are more of the early bud cells being specified to endocrine fate by ectopic activation of Ngn3, or are cells that have already been specified to endocrine fate undergoing extra cycles of proliferation? The next question to be addressed is whether the $Ptf1a^{+/-}$ pancreatic buds have an altered response to soluble factors that have been previously shown to influence exocrine and endocrine differentiation of cultured pancreatic buds.

Conditional deletion of floxed *Pdx1* alleles

We have shown that recombination of conditional null and hypomorphic alleles of *Pdx1* in endocrine progenitors (via Ngn3Cre transgenes) results in increased PP expression in the islets of young mice. An important next step will be to examine the embryonic endocrine progenitors in the conditional mutants to determine if Pdx1 expression decreases rapidly after expression of Ngn3, and if alteration in expression of insulin and PP is apparent during early stages of endocrine development. As discussed above, the phenotype of the supernumerary PP-expressing cells needs to be characterized to determine if they are properly differentiated PP cells or poorly differentiated β cells. It would also be interesting to test whether Pdx1 can

directly repress the PP promoter in cultured cells, and conversely whether knockdown of Pdx1 expression in cultured β cells results in upregulation of endogenous PP expression. To test the limits of the hypothesis that the level of Pdx1 in endocrine progenitors determines the relative proportion of β and PP cells, the recently described Cre-activatable Pdx1 transgene (Miyatsuka et al., 2003) could be used to overexpress Pdx1 in endocrine progenitors and potentially drive more progenitors to β cell fate.

Regulation of *Pdx1* promoter activity

Now that we have discovered that regulatory regions outside of the proximal 4.6 kb promoter sequence of *Pdx1* play a critical role in promoting expression in adult duodenum and stomach, the first outstanding task is to map the distal enhancer elements that promote expression in mature gut mucosa. Based on the hypothesis that phylogenetically conserved sequences regulate conserved expression patterns, the top candidates to examine are Area IV and the two uncharacterized conserved regions that lie between -7 and -10 kb (Fig. 1.6). If a Pdx1-expressing duodenal epithelial cell line can be obtained, it should be a valuable tool for mapping duodenal enhancers in the *Pdx1* promoter. In addition, we are developing a cassette-acceptor locus for the 5' region of *Pdx1* to facilitate rapid, iterative mutagenesis of *Pdx1* promoter sequences. This tool will be used to delete Area IV from the endogenous *Pdx1* locus, and to make compound mutations in proximal and distal elements to further characterize the redundant developmental roles described above.

An important future direction in characterizing the developmental regulation of Pdx1 expression will be to determine how intercellular signaling influences transcription of *Pdx1*. Because the overall extent of the embryonic Pdx1 expression domain did not appear to be

altered by loss of either distal or proximal regulatory elements, it seems likely that the initial Pdx1 expression domain is determined primarily by regulation of *Pdx1* transactivators via extracellular cues. Studies of *Pdx1* promoter occupancy and chromatin structure in response to soluble factors or ectopic activation of signaling pathways might provide insight into these developmental gene regulatory mechanisms.

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