Conditional inactivation of Pax6 in the pancreas causes early onset of diabetes

Ruth Ashery-Padan, a,* Xunlei Zhou, b Till Marquardt, c Pedro Herrera, d
Leanne Toube, a Asher Berry, a and Peter Gruss b,*

a Department of Human Genetics and Molecular Medicine, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978, Israel
b Department of Molecular Cell Biology, Max-Planck-Institute of Biophysical Chemistry, D-37077 Göttingen, Germany
c The Salk Institute for Biological Studies, Gene Expression Laboratory, La Jolla, CA 92037, USA
d Department of Morphology, University of Geneva Medical School, CH-1211 Genève 4, Switzerland

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Abstract

Pax6 transcription factor is required for islet cell number, morphology, and hormone gene expression. The perinatal lethality of Pax6 null mutants has restricted investigation of the role of Pax6 in normal endocrine cell function. Therefore, we devised the conditional inactivation of Pax6 using the Pdx1 and Pax6 regulatory domains to activate Cre in cells of either the entire pancreatic bud or only in endocrine cell lineages, respectively. Mutant pups died few days after birth, suffering from an overt diabetic phenotype that includes hyperglycemia, hypoinsulinemia, weight loss, and ketosis, indicating an essential role for Pax6 in beta cell function. Glucose-transporter type-2 expression was downregulated, but expression of several transcription factors essential for endocrine development was maintained. Our findings support a role for Pax6 activity in maintaining normal beta cell function after birth, but not for beta cell neogenesis during late embryonic development and early postnatal stages.

Keywords: Pax6; Cre/loxP; Lineage tracing; Endocrine cells; Pancreas

Introduction

The pancreas is composed of the endocrine acinar and ductal components. There are four types of endocrine cells: β, α, δ, and PP that secrete, respectively, insulin, glucagon, somatostatin, and pancreatic polypeptide into the bloodstream. These hormone-secreting cells are organized in the islets of Langerhans and are surrounded by exocrine cells that secrete digestive enzymes through the ducts into the intestine (Slack, 1995). The pancreas originates during embryogenesis as dorsal and ventral budding from the duodenum, posterior to the stomach. The pancreatic cells evolve gradually from the multipotent pool of progenitors that reside in this region. The endocrine cells originate from different populations of progenitor cells, because α and β cells seem to originate from different lineages, and the expression of transcription factors that mark the progenitor cells do not always overlap (Herrera, 2000; Jensen et al., 2000).

Endocrine cell markers are the first to be detected in the mouse around embryonic day 9.5 (E9.5). These cells appear to coexpress insulin and glucagon, but not other differentiation markers. Only at mid gestation (around E13.5), fully differentiated β and α cells, as well as exocrine cells, are apparent (Bort and Zaret, 2002; Herrera, 2002). This phase of differentiation is termed “secondary transition”; however, the lineage relationship between the early appearing cells and this later pancreatic cell population is not yet known. Endocrine cells continue to accumulate throughout embryonic development; however, the typical islets of Langerhans with the centrally located insulin producing cells surrounded by other endocrine cell types are apparent only close to birth (from E18.5). During the first weeks of life, the pancreas...
undergoes a rapid growth spurt. Initially, the total mass of islets continues to grow relatively more than the rest of the pancreas (Rosselin and Emami, 1997). This phase is followed by a new growing period, during which the weight of the pancreas and the different cell type populations increase rapidly. This growth phase continues until weaning (Rosselin and Emami, 1997).

Mutant and transgenic studies have identified several transcription factors that play roles in pancreas development, and the genetic hierarchy between them has been partially clarified (Edlund, 2002; Sander and German, 1997). The homeodomain transcription factor Pdx1 is required for the formation of all progenitor cells, as null mutants of this gene have no pancreas (Jonsson et al., 1994; Offield et al., 1996). Hlx9, like Pdx1, is required for the formation of the dorsal lobe and for endocrine cell differentiation in the ventral bud (Harrison et al., 1999; Li et al., 1999). Other factors required for endocrine cell number and final differentiation include members of the Nkx and the Pax homeobox gene families, Nkx2.2, Nkx6.1, Pax6, and Pax4.

The Nkx gene family members are involved in the development of the ventral CNS and the pancreas. Nkx2.2 is expressed during embryogenesis in the pancreas and is involved in the formation of three islet cell types, excluding the somatostatin-producing cells. Nkx2.2 seems to regulate the expression of Nkx6.1, and both appear to be essential for the final differentiation of the $\beta$ cells (Sander et al., 2000; Susel et al., 1998). Pax6 is essential for CNS and pancreas development as well. Its expression in the pancreas is initiated in the pancreatic progenitors, concomitant with the onset of hormone expression, after which the expression persists in all endocrine cells throughout development and in the adult pancreas. The phenotype of Pax6 mutants suggested a specific role for this gene in the differentiation of $\alpha$ cells, for the normal numbers and hormone expression of other endocrine cell types, and for organization of the endocrine cells in the islets (Sander et al., 1997; St-Onge et al., 1997). Pax4 is expressed transiently during embryogenesis only. Mice with a disrupted Pax4 gene have no $\beta$ cells and $\delta$ cells, and the number of $\alpha$ cells is increased (Sosa-Pineda et al., 1997). Mice lacking both Pax6 and Pax4 are devoid of all endocrine cell types, suggesting some overlap in the roles of the two genes in endocrine cell genesis (St-Onge et al., 1997). The persistent expression of Pax6 only in the adult endocrine cells suggests a unique role for Pax6 in endocrine cell function at postnatal stages.

The perinatal death of homozygotes for Pax6 null mutations restricted the study of Pax6 function in the postnatal pancreas. To investigate the Pax6 requirement during postnatal stages, with respect to endocrine cell function and islet morphology, as well as to follow the fate of the Pax6-deficient cells, we employed the Cre/loxP approach to inactivate Pax6 in the endocrine pancreas only. The regulatory regions of the Pax6 and Pdx1 genes were employed to activate Cre and to inactivate Pax6, specifically from the developing pancreas of $\text{Pax6}^{\text{floox/lox}}$ mice (Ashery-Padan et al., 2000), thus avoiding the perinatal death. The inactivation of Pax6 exclusively in the endocrine cell types prolonged the life of the mutants by only a few days, as they died suffering from an overt diabetic phenotype. This suggests that postnatal neogenesis does not seem to compensate for the early developmental defects in the endocrine pancreas of the Pax6-deficient mice. Lineage tracing of the Pax6-deficient cells using the Z/AP reporter line revealed that Pax6 is not required for the specification, formation, or survival of $\beta$ cells. However, it is essential for the normal expression of final differentiation markers such as insulin and glucose-transporter type-2 in these cells. Furthermore, our findings suggest that Pax6 function is in parallel to Nkx2.2, Nkx6.1, and Pdx1 in some of the $\beta$ cells during the late stages of pancreas development.

Materials and methods

The $\text{Pax6}^{\text{floox/lox}}$ mice and the Le-Cre-GFP transgene were established as described (Ashery-Padan et al., 2000). The Pdx1-Cre construct was described (Gannon et al., 2000), although the line presented here was derived from a different founder in which the recombination detected is not mosaic but exists in almost all pancreatic cells (Herrera, 2002). Detection of GFP was conducted as described (Ashery-Padan, 2002). $\text{hAP}$ detection was described (Lobe et al., 1999). The proportion of hAP-stained cells was calculated by monitoring hAP activity in every fifth cryostat section (14 $\mu$m) thus representing whole E18.5 or P1 pancreases (26 sections for control Le-Cre-GFP/Z/AP and 16 sections for $\text{Pax6}^{\text{floox/lox}}, \text{Le-Cre-GFP,Z/AP}$ litter mates at E18.5, and 52,36 control and 59,74 mutant sections from two P1 pancreases for each genotype). The images were analyzed using the image analysis system “AnalySIS” (SIS, Muenster, Germany). For this purpose, shading correction was achieved by subtraction of a background image without specimen from the image to be analyzed. Next, the threshold between hAP-labeled pancreatic regions and nonlabeled tissue was defined. The proportion of hAP-positive regions in the total pancreatic tissue was calculated for all sections of each pancreas and then average, and standard deviation was calculated for each genotype. Antibody staining of paraffin sections was as described (Ashery-Padan et al., 2000); the antibodies used were mouse anti-insulin (Sigma 1:200), rabbit anti-insulin (DAKO 1:100), rabbit antiguacagon (DAKO 1:100), mouse anti-NKX2.2 (74.5A5) (developmental studies hybridoma bank 1:100), rabbit anti Nkx6.1 (kind gift from Palle Serup 1:100), rabbit anti-Pdx1 (kind gift from Chris Wright 1:500), mouse anti-hAP (Sigma 1:300), rabbit anti-Glut2 (Chemicon 1:500), rabbit anti-Pax6 (BAbCO 1:200). Secondary antibodies conjugated to rhodamine red-X or Cy2 were from Jackson Immuno Research Laboratories. The slides were viewed with an Olympus BX61 fluorescent microscope. Glucose levels
were measured with the glucometer (Bayer). Protein from the pancreas was extracted, and concentrations of insulin were determined by radioimmunoassay (RIA), using the commercial kit from Linco. Urine ketones (acetone) were measured with strips (Bayer).

Results

**Le-Cre-GFP recombination pattern identifies endocrine precursors at E9.5**

The early expression of Pax6 in the pancreatic progenitors seems to correspond with endocrine cell development and function (Sander et al., 1997; St-Onge et al., 1997). However, the detection of Pax6 as early as E9.5 in cells residing in the pancreatic bud may reflect a function for this factor in multipotent pancreatic progenitors. To define the developmental potential of the Pax6-expressing cells, we employed a Cre/loxP-based approach to trace cells that express Cre under the control of the Pax6 regulatory domain.

We employed the Le-Cre-GFP transgenic mouse line (Fig. 1A and Ashery-Padan et al., 2000), in which the genomic fragment used to activate Cre and GFP includes all the domains identified so far as mediating the expression of Pax6 in the pancreas (Kammandel et al., 1999; Xu et al., 1999; Zhang et al., 2003). The GFP was colocalized with Pax6-positive cells at E9.5, suggesting that the regulatory region indeed recapitulates the Pax6 expression pattern from the early onset of its expression (Fig. 1D). GFP was also detected in adult islets (Fig. 4), corresponding to Pax6 expression in the adult. This line allowed us to follow the fate of cells that express Pax6 during pancreas genesis by monitoring Cre-mediated recombination in double transgenic animals carrying both the Le-Cre-GFP transgene and the Z/AP reporter strain (Fig. 1B and Lobe et al., 1999), in which human alkaline phosphatase (hAP) is expressed following Cre activity.

The hAP activity detected in the Le-Cre-GFP;Z/AP embryos was evident at E9.5 in few cells within the dorsal bud endoderm (Figs. 1C–E). The expression of hAP then persisted in the endocrine cell population, and this reporter protein is detected in all adult endocrine cell types, as shown in double immunolabeling with antibodies against hAP (red) and somatostatin, glucagon, and pancreatic polypeptide (the three cell types are labeled green, thus, the co-labeling is imaged as orange shown in the insets).
gene expression was restricted to the forming islets at P3 (Fig. 1F). In the adult (P20), hAP activity was detected only in islets (Fig. 1G) in all endocrine cells, including the PP, somatostatin, and α cells (Figs. 1H–J). This suggests that Pax6, from the early stages of development, is expressed in cells already specified to an endocrine cell fate but not in cells destined to become acinar or ductal. The phenotype observed in the Pax6 pancreas mutants, therefore, appears to be due to its autonomous function in endocrine cells.

Pax6 is essential for the function of β cells after birth

Pax6 has been demonstrated both to play an essential role in cell genesis and endocrine cell organization and to influence β cell numbers. In these studies, the pancreatic phenotype of Sey
\textsuperscript{pop} and Pax6
\textsuperscript{lacZ} was analyzed, and the insulin concentration in the pancreas was found to be 13% and 30%, respectively, compared to the normal (Sander et al., 1997; St-Onge et al., 1997). This reduction in insulin content may result in an overt diabetic phenotype, although mice seem to maintain normal glucose levels even with low levels of insulin (Sreenan et al., 1999). Due to several developmental defects, the Pax6 mutant mice die immediately after birth. This precluded analysis of Pax6’s role in the final stages of pancreas development, especially the last phase of endocrine cell growth and cellular organization in the islets of Langerhans.

To study the effects of Pax6 inactivation during postnatal development, we examined the pancreatic phenotype of Pax6
\textsuperscript{fllox/lacZ};Le-Cre-GFP mice (Fig. 2A). The LacZ allele is considered to be a null allele, which means that only one allele must be deleted by Cre in the Pax6
\textsuperscript{fllox/lacZ};Le-Cre-GFP genotype for complete inactivation of Pax6 in the cells. This genotype is expected to allow for higher efficiency of Pax6 inactivation and low variability of the phenotype. Subsequent analysis, however, revealed the same phenotype in the Pax6
\textsuperscript{fllox/lox};Le-Cre-GFP new born mice.

The Pax6
\textsuperscript{fllox/lox};Le-Cre-GFP mutants were moving and feeding, as evidenced by milk in their stomachs (Fig. 2B). However, 2–6 days after birth, severe growth retardation was evident, and the pups were found to suffer from hyperglycemia and hypoinsulinemia (Table 1). This condition deteriorated over the next 2 days, as levels of insulin in the pancreas further decreased from 18% of normal at P1 to 6% of normal at P3 (Table 1). At P3, ketones (acetone) were detected in all animals with glucose levels above 20 mM (Table 1). The animals usually died 3–6 days after birth, suffering from an overt diabetic phenotype. To verify that death was due to pancreatic deficiency, we investigated the phenotype of the Pax6
\textsuperscript{flox/flox};Pdx-Cre mice in which Cre was activated in all pancreatic progenitors from as early as E9.5, when the pancreatic buds begin to form (Herrera, 2002). The Pax6
\textsuperscript{flox/flox};Pdx-Cre mice also died after birth, and the pancreatic phenotype was similar to the phenotype of Pax6
\textsuperscript{fllox/lacZ};Le-Cre-GFP mice (Fig. 3). The pancreatic phenotype of the newborn Pax6
\textsuperscript{fllox/lacZ};Le-

### Table 1

<table>
<thead>
<tr>
<th>Age</th>
<th>Genotype</th>
<th>Blood glucose mM</th>
<th>Pancreatic insulin content µg/mg</th>
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<tr>
<td>P0–P1</td>
<td>control</td>
<td>4.5</td>
<td>17.3 (-4.2; n = 5)</td>
</tr>
</tbody>
</table>
|      | Pax6
\textsuperscript{fllox/lacZ};LeCre-GFP | (±1.4; n = 7) | (±1.7; n = 5) |
| P3   | control | 4.1 | 27 (±1.14; n = 3) |
|      | Pax6
\textsuperscript{fllox/lacZ};LeCre-GFP | 23 | 1.47 (±0.9; n = 4) |

The levels of glucose in the blood are expressed as mmol/liter from nonfasted neonates. The levels of glucose increased rapidly in the Pax6
\textsuperscript{fllox/lacZ};Le-Cre-GFP (Le-mutant) neonates from an average of 11.12 mM at P1 to 23 mM and more at P3 (*2 of 11 P3 mice had blood glucose levels higher than could be measured), compared with average glucose levels in the control litter mates of 4.1–4.5 mM. Insulin is expressed as µg/mg pancreas protein from nonfasted animals. Corresponding with the high glucose, insulin levels were reduced in the pancreas of the Pax6
\textsuperscript{fllox/lacZ};Le-Cre-GFP neonates. The concentration of insulin in the pancreas reduced rapidly from 3.2 µg/mg protein at P0–P1 to 1.47 µg/mg protein at P3. The number shown represents the mean number ± standard deviation from the mean values for blood glucose or insulin content obtained from the indicated (n) number of mice. At P3, ketones were detected in the urine of all animals with glucose levels above 20 mM (n = 8). Death of the neonates occurred between postnatal days 3 and 6.
Cre-GFP and the Pax6<sup>flox/flox</sup>,Pdx-Cre pups was compared, with respect to expression of glucagon and insulin. In both mutants, very few α cells were identified and the number of insulin-expressing cells was reduced (Fig. 3). These findings support the notion that Pax6 function in the pancreas is essential for normal β cell function and, thus, for the survival of the postnatal mice.

**Pax6 is required for β cell function, but not for neogenesis of endocrine cells**

The change in number and endocrine cell types in the Pax6-deficient mice was based in previous studies on the identification of hormones, insulin somatostatin, glucagons, and PP. This precluded the identification of mutant cells that had lost the expression of these final differentiation markers.

In order to trace and identify all of the Pax6-deficient cells, we followed hAP distribution and activity in the Pax6<sup>flox/flox</sup>,Le-Cre-GFP;Z/AP, as compared with the Pax6<sup>+/+</sup>;Le-Cre-GFP;Z/AP (Fig. 3). These genotypes allowed tracing of the Cre-mediated recombinant cells and their progeny, as all of these cells express the hAP reporter. In the Pax6<sup>flox/flox</sup>,Le-Cre-GFP;Z/AP, the hAP<sup>+</sup> (Pax6<sup>−</sup>) cells were organized in small islets which had string-like morphology; however, the cells seem to adhere to each other and were not completely dispersed, as suggested from the scattered insulin-positive cells identified in the mutant pancreas (Fig. 3). The proportion of hAP-expressing cells from the total pancreatic tissue was determined (see Materials and methods). The difference in the ratio of hAP/total between the mutant as compared to control was not found to be significant. This suggests that islet cell neogenesis is not significantly hampered at this late stage of development despite the loss of Pax6 activity. Scale bar indicates 20 μm in A–C and 80 μm in D–E.

**Fig. 3. Reduction in number of insulin-expressing cells and nearly no α cells in islets of the Pax6<sup>flox/LacZ</sup>,Le-Cre-GFP, Pax6<sup>flox/LacZ</sup>,Pdx-Cre. (A–C) Glucagon (red) and insulin (green) are detected, using double immunolabeling on 6 μm paraffin sections of pancreas from (A) wt P1, (B) Pax6<sup>flox/LacZ</sup>,Le-Cre-GFP, P1, (C) Pax6<sup>flox/flox</sup>,Pdx-Cre, P1. α Cells are only rarely detected, and β cells are reduced in number in the mutant pancreas. Lineage tracing of Cre activity on frozen sections of E18.5 pancreas of the (D) control Pax6<sup>+/+</sup>;Le-Cre-GFP;Z/AP and the (E) mutant Pax6<sup>flox/flox</sup>,Le-Cre-GFP;Z/AP reveal that the mutant cells are localized in small clumps and string like islets. (F) The proportion of hAP<sup>+</sup> regions from the total pancreatic tissue was determined (see Materials and methods). The difference in the ratio of hAP/total between the mutant as compared to control was not found to be significant. This suggests that islet cell neogenesis is not significantly hampered at this late stage of development despite the loss of Pax6 activity. Scale bar indicates 20 μm in A–C and 80 μm in D–E.**
Change in progenitor cell fate was reported in the Pax4 mutant pancreas where more \(\alpha\) cells were identified, possibly at the expense of \(\alpha\) cells (Sosa-Pineda et al., 1997). To investigate this possibility, the expression of PP and somatostatin were documented in the mutant, as compared with the control (Fig. 4). Normal numbers of PP\(^{+}\) and somatostatin\(^{+}\) cells were detected, in Pax6\(^{\text{flox/flox}}\);Le-Cre-GFP mice, thus not supporting a change in endocrine cell fate in the Pax6-deficient cells. Furthermore, some GFP-positive cells coexpressed insulin, which excluded the possibility that insulin-expressing cells escaped the Pax6 inactivation. Interestingly, expression of GFP from the Le-Cre-GFP transgene was maintained in the Pax6-deficient pancreas. Thus, it seems that Pax6 is not required for the activity of the Pax6 regulatory region in the pancreas.

**Pax6 functions along with Pdx1, Nkx2.2, Nkx6.1, and Islet1 in postnatal development**

Several transcription factors are known to be required for the generation of the normal number of endocrine cell types, their differentiation, and the proper physiology of adult islet cells. Pdx1 is required for pancreas formation, for maintaining hormone cell function, and for Glut2 expression (Ahlgren et al., 1998). Furthermore, Isl1 is detected in postmitotic pancreatic progenitors and is required for endocrine cell formation (Ahlgren et al., 1997). Another example is Nkx2.2, which is required for the number of endocrine cell types, for the final differentiation of \(\beta\) cells, and for normal expression of Glut2 (Sander et al., 2000). Nkx2.2 regulates Nkx6.1, which is required for \(\beta\) cell neogenesis, during the secondary transition (Sander et al., 2000). The similarity in the pancreatic phenotype among these factors suggests possible genetic interactions among them. Indeed, it has been suggested that Pax6 regulates Pdx1 expression (Samaras et al., 2002), as Pax6 binding sites have been detected in the Pdx promoter, and Nkx2.2 and Nkx6.1 expression domains seem to be regulated by Pax6 activity in the spinal cord (Briscoc et al., 1999; Takahashi and Osumi, 2002).

We characterized the expression of Pdx1, Nkx2.2, Nkx6.1, and Isl1 in Pax6-deficient islets (Fig. 5). Interestingly, the expression of Nkx2.2, which is expressed in pancreatic epithelial progenitors and is required for generating normal cell numbers of \(\beta\), \(\alpha\), and PP cells (Sussel et al., 1998), did not completely overlap with Pax6 distribution in the normal pancreas at E15.5 (Fig. 5A). This staining pattern could be explained by the fact that at E15.5, the pancreas still contains a certain number of Nkx2.2 expressing epithelial progenitors concomitantly with the presence of developed endocrine cells, expressing Pax6. We could...
Fig. 5. Nkx2.2 and Pax6 expressions partially overlap in the endocrine progenitors during development, and the expression of NKX2.2 and other endocrine transcription factors is maintained in Pax6<sup>-</sup> endocrine cells. (A) Nkx2.2 and Pax6 are expressed in partially overlapping populations of endocrine cells in the pancreas of E15.5 wild-type embryos. Some cells express only Nkx2.2 (green arrow) or Pax6 (red arrow), and other cells express both (yellow arrow), suggesting heterogeneity of the endocrine progenitors. (B–E) In both the normal P1 pancreas and in the (F–I) Pax6 mutant pancreas, the expression of Nkx2.2 (B, F), Isl1 (C, G), Pdx1 (D, H), and Nkx6.1 (E, I) is detected. The markers colocalize with the Pax6-deficient cells that express GFP from the Le-Cre-GFP transgene, as shown here for Nkx6.1 (GFP green, Nkx6.1 red in E, I). Thus, the Nkx6.1-expressing cells are mutants for Pax6, rather than cells that escaped inactivation. Scale bar indicates 20 μm in A and 40 μm in B–I.
not identify complete loss of the expression of Nkx2.2, Nkx6.1, Islet1, and Pdx1 in the Pax6-deficient pancreas (Fig. 5 and Sander and German, 1997) as some cells continue to express these markers. This suggests that at least during early postnatal development, these factors function along with Pax6 in the endocrine cells.

Glucose-transporter type-2 expression is abnormal in the Pax6-deficient pancreas

Glut2 is a low-affinity transporter suggested to mediate glucose-stimulated insulin secretion. This transporter is essential for normal glucose homeostasis and pancreas endocrine function (Guillam et al., 1997). Its expression was found to be reduced in glucose-unresponsive islets from different animal models of diabetes (Nkx2.2, Pdx1, FoxA2; Ahlgren et al., 1998; Shih et al., 2001; Sussel et al., 1998), suggesting that several transcription factors function together to regulate the expression of this transporter. We investigated the expression of Glut2 in the Pax6-deficient pancreas (Fig. 6). The islet morphology in the mutant was identified using antibodies against Nkx2.2, as the expression of Nkx2.2 is not disrupted in the Pax6 islets. Glut2 protein was not detected in the Pax6-deficient pancreas (Fig. 6), suggesting that Pax6 plays a role in regulating the expression of this transporter. The Pax6-deficient islets did, however, maintain the expression of convertase PC1/3, a proinsulin processing enzyme (not shown). Thus, the reduction in insulin in this mutant is not connected to the processing of the hormone, but rather, may be due to direct changes in insulin expression and/or the inability of these cells to respond to high blood glucose levels because of reduced Glut2 expression.

Discussion

Previous studies established essential roles for Pax6 and Pax4 in normal endocrine development. Pax4 has been related mostly to β and δ cell differentiation, and Pax6 has been associated with the formation of α cells, organization of the endocrine cell types in the islets, and with the normal numbers of β cells. The last phase of islet of Langerhans formation and function could not be addressed in both mutants because of their early death. It has been unclear whether the postnatal neogenesis would have taken place in Pax6-deficient mice and whether the postnatal β cells would have been able to function normally in the absence of Pax6. In addition, the perturbed islet morphology observed in the Pax6 mutants could have been attributed either to the reduced cell number or to developmental delay, rather than to the intrinsic requirement of Pax6 for islet morphology. In this study, we demonstrated that Pax6 activity is in cells already destined to an endocrine cell fate. In these cells, it is not required for endocrine cell genesis nor for cell type specification, but is mainly essential for maintaining expression of molecules necessary for the function of the β and α cells, thus, ultimately, assuring nutrient homeostasis and preventing a diabetic phenotype.

We employed the Pax6 regulatory regions to activate Cre in Pax6 expressing cells. The activity of the Pax6 regulatory element was restricted to cells of an already established endocrine fate, as the hAP expression was exclusively observed in endocrine cell population. It is interesting that the Pax6-expressing cells that are apparent in the E9.5 pancreatic bud do not seem to contribute to populations of non-endocrine cell types. This is either because these early cells exist only transiently or because the dissociation of endocrine and non-endocrine lineages takes place in the early progenitors of the E9.5 pancreatic bud.

While the data presented speaks against a role for Pax6 in any non-endocrine progenitor cells, it is possible that this factor does play a role in endocrine cell type specification. We could not however find support for this option, as the expression of endocrine cell type markers somatostatin and pancreatic polypeptide seem to be unperturbed in the Pax6-deficient endocrine cells. Nevertheless, identification of markers that are expressed during the development of the endocrine cells subtypes is required to exclude subtype changes that do not allow final differentiation and to determine the identity of the Pax6-deficient cells that do not express any of the final differentiation markers.

It has been suggested previously that Pax6 is required for the formation of most α cells and part of β cell populations.
The reduction in these cell numbers seems also to result in abnormal organization of the cells within the islets. In the same line, the reduction we have observed in insulin content in the Pax6+ pancreas between P1 and P3 might reflect an arrest in the rapid growth of the endocrine cell population that normally takes place after birth (Kaung, 1994). Nevertheless, as long as the identification of the cell types was dependent on identification of the final differentiation markers, the actual fate of the mutant cells was not known. The use of the Z/AP reporter to trace cell fate allowed us to directly investigate the fate of Pax6-deficient cells. The results were intriguing, as the number of mutant cells was not perturbed in the mutants as compared to controls during late embryogenesis and early postnatal life. The findings presented here do not exclude an early role for Pax6 in the formation of specific endocrine cells, as it is possible that an early developmental delay is compensated for by a later growth phase. It is also possible that in the conditional mutant, not all cells undergo recombination. Thus, those that escape the mutation reach final stages of development. Although, at this time, we cannot exclude these possibilities, the findings presented strongly suggest that Pax6 is required at least during the last phase of pancreas growth to maintain functional β cell rather than their genesis.

Pax6 also seems to function in parallel with the transcription factors Nkx2.2, Isl1, and Pdx1, which are known to be essential for endocrine cell development. Thus, in postnatal stages, Pax6 seems to be required directly for normal β cell function, rather than indirectly by controlling these transcriptional regulators. These findings are in agreement with the suggestion that Pax6 plays a role in regulating the expression of functional genes (insulin, Glut2, glucagon), rather than in the specification of endocrine cell types.

Other studies suggest a role for Pax6 in endocrine cell maintenance and renewal after birth. Overexpression of Pax6 using the Pdx1 promoter was shown to result in diabetes and pancreatic tumors in mice (Yamaoka et al., 2000). Expression of Pax6 was detected in interferon-gamma-expressing transgenic mice that exhibit new islet growth and expansion in adult animals (Kritzik et al., 2000). The study of Pax6 mutants implicated a role for Pax6 in the synthesis of glucagon and insulin, based on the identification of Pax6 binding sites in the promoters of the two genes (Sander and German, 1997). This direct role in β cell function is supported and extended in our study, as the marked decrease in expression of Glut2 might contribute to the reduced expression of insulin as well.

Several developmental transcription factors, such as Pdx1, NeuroD/beta2, and hepatocyte nuclear factor 1a and 4a, have been found to be mutated in patients who suffer from heritable diabetes with dominant inheritance (MODY). Mutations in Pax4 were recently shown to be associated with type 2 diabetes (Kanatsuka et al., 2002; Shimajiri et al., 2001), and heterozygous mutations in the Pax6 gene were found to be associated with glucose intolerance in human carriers of these mutations (Yasuda et al., 2002). The findings described here further suggest that Pax6 may also be affected in patients suffering from diabetic conditions, which, however, may be obscured by pleiotropic requirements in other organ systems. The similar cellular phenotype of the pancreas in Pax6loxZ/LoxZ;Le-Cre-GFP, Pax6loxP/loxP;Pdx-Cre, and Pax6laclacZ/lacZ suggested that the regulatory domains employed in the Le-Cre-GFP transgene probably include all of the regions essential for Pax6 expression in the developing islets. Two regions have been identified so far that mediate the transcriptional activity in the pancreas (Kammandel et al., 1999; Xu et al., 1999; Zhang et al., 2003), and recently, NeuroD/beta2 has been shown to bind directly to one of these elements (Marsich et al., 2003). These domains should be considered in future attempts to identify mutations in human patients suffering from familial neonatal diabetes.

Further delineation of the cell autonomous mechanisms dependent on Pax6 for the failure of β cell function and investigation of the relationship between α and β cells is in progress, using somatic inactivation of Pax6 in each of these cell types and in the adult. Understanding the requirement for transcriptional regulators, such as Pax6 for the maintenance of endocrine cell function, may ultimately lead to novel therapies aimed at delaying or treating some aspects of diabetic phenotypes.

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