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Abstract

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Reference


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Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of β-cell formation in the pancreas

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SUMMARY

Most insulin-producing β-cells in the fetal mouse pancreas arise during the secondary transition, a wave of differentiation starting at embryonic day 13. Here, we show that disruption of homeobox gene Nkx6.1 in mice leads to loss of β-cell precursors and blocks β-cell neogenesis specifically during the secondary transition. In contrast, islet development in Nkx6.1/Nkx2.2 double mutant embryos is identical to Nkx2.2 single mutant islet development: β-cell precursors survive but fail to differentiate into β-cells throughout development. Together, these experiments reveal two independently controlled pathways for β-cell differentiation, and place Nkx6.1 downstream of Nkx2.2 in the major pathway of β-cell differentiation.

Key words: Islet, β-cell, Insulin, Transcription factor, Mouse

INTRODUCTION

The islets of Langerhans in the pancreas are specialized endocrine micro-organs composed of four distinct cell types: insulin-producing β-cells, glucagon-producing α-cells, somatostatin-producing δ-cells and pancreatic polypeptide-producing (PP) cells. The β-cells are key metabolic regulators, and their loss or dysfunction leads to diabetes mellitus.

During fetal development, all four endocrine cell types, as well as the more abundant exocrine cells of the pancreas derive from a common set of epithelial cells that originate in the early gut endoderm (for a review of pancreatic development see Slack, 1995). The first morphological evidence of the future pancreas appears as a small dorsal bud at the foregut-midgut junction at embryonic day E9.5 in the fetal mouse. A few insulin-expressing cells appear within a day after bud formation, and these early insulin-expressing cells often co-express glucagon (Teitelman et al., 1993) but lack the expression of some other β-cell markers (Oster et al., 1998; Pang et al., 1994). Fully differentiated β-cells first appear around E13 at the start of a massive wave of β-cell differentiation termed the ‘secondary transition’ (Pictet and Rutter, 1972). Although it has been proposed that the insulin-expressing cells generated in the early pancreatic bud function as progenitors for the β-cells that appear later during the secondary transition (Pictet and Rutter, 1972; Teitelman et al., 1993; Upchurch et al., 1994), this relationship has never been proven by direct lineage tracing, leaving the possibility that the two populations form via distinct pathways (Pang et al., 1994).
In this study, we have examined the role of Nkx6.1 in the hierarchy of transcriptional events leading to β-cell differentiation. Consistent with its specific expression in β-cells, homozygous mutation of the Nkx6.1 gene in mice profoundly inhibits β-cell formation; but this defect only becomes evident after the start of the secondary transition. The pancreatic phenotype of mice with homozygous mutations in both the Nkx6.1 and Nkx2.2 genes is identical to the phenotype of Nkx2.2 homozygous single mutant mice. These studies provide conclusive genetic evidence that Nkx6.1 lies downstream of Nkx2.2 in the major pathway of β-cell formation.

**MATERIALS AND METHODS**

**Nkx6.1 gene targeting**

To generate Nkx6.1 mutant mice, a genomic clone containing exon1 and 2 of the Nkx6.1 gene was isolated from a 129J mouse genomic library in lambda Dash (Stratagene) by Southern hybridization screening. An 8.5 kb SpeI/EcoRV fragment was cloned into the pBlueScript KS+ cloning vector, and an 800 bp NotI fragment containing part of exon1 was replaced by a PGK-neo cassette. Recombinant ES cell clones and Nkx6.1 mutant mice were generated as described previously (Kash et al., 1997), and maintained on a C57BL/6J mouse background. All ES cell clones and mice were genotyped by Southern blotting with a probe external to the targeting construct. No Nkx6.1 protein can be detected in the homozygous mutant embryos using antisera directed at the C-terminal end of the molecule (data not shown), showing that the mutation encodes for a null allele.

**Immunohistochemistry and TUNEL assays**

Immunohistochemical and immunofluorescence analyses were performed on paraffin sections as described previously (Sander et al., 1997). The primary antibodies used in these assays were the following: rabbit anti-NKX6.1 diluted 1:4000; guinea pig anti-insulin diluted 1:10000 (Linco); guinea pig anti-glucagon diluted 1:10000 (Linco); mouse anti-somatostatin diluted 1:100 (Fitzgerald); rabbit anti-PP diluted 1:2000 (Dako); rabbit anti-IAPP diluted 1:4000 (Advanced Chemtech), rabbit anti-PC1/3 diluted 1:2000 (kindly provided by Donald Steiner); mouse anti-BrDU diluted 1:200 (Chemicon), rabbit anti-PAX6 diluted 1:4000 (kindly provided by Simon Saule); monoclonal anti-NKX2.2 diluted 1:50 (kindly provided by Thomas Jessell); and monoclonal anti-ISL1 diluted 1:100 (Developmental Studies Hybridoma Bank).

Neurogenin 3 and PDX1 antigens were produced by inserting the coding sequence for the N-terminal 95 amino acids (neurogenin3) and the C-terminal 80 amino acids (PDX1) from the mouse genes downstream of the glutathione-S-transferase coding sequence in the pGEX-2T vector (Pharmacia). The resulting fusion proteins were purified from E. coli and injected into rabbits (neurogenin 3) and guinea pigs (PDX1). Rabbit anti-neurogenin 3 was used at a 1:5000 dilution, and guinea pig anti-PDX1 was used at a 1:4000 dilution. Pre-immune sera gave no staining at the same concentrations.

For immunohistochemistry biotinylated anti-rabbit, anti-guinea pig or anti-mouse antibodies (Vector) were used at a 1:200 dilution, and detected with the ABC Elite immunoperoxidase system (Vector). The secondary antibodies used for immunofluorescence were as follows: FITC-conjugated anti-rabbit, anti-mouse or anti-guinea pig diluted 1:100 (Jackson Laboratory); Cy3-conjugated anti-rabbit diluted 1:200 (Jackson Laboratory); and rhodamine-conjugated anti-guinea pig diluted 1:300 (Cappel). Fluorescence was visualized with a Zeiss axioskope and a Leica confocal microscope.

TUNEL assays on tissue sections were performed using a commercially available kit (Oncor). For cell counting, representative sections throughout the entire organ were chosen for immunohistochemistry with an anti-insulin antibody. The average number of insulin-positive cells per section was determined by counting stained cells in eight sections from an individual pancreas.

**Quantification of insulin concentrations**

Protein was extracted as described before (Sander et al., 1997). The concentration of insulin was determined by radioimmunoassay using a commercially available kit (Linco).

**RESULTS**

Nkx6.1 is expressed in the developing and mature pancreas and the central nervous system (Oster et al., 1998; Qiu et al., 1998; Rudnick et al., 1994). In the developing mouse pancreas, Nkx6.1 protein could be detected as early as embryonic day 10.5 (E10.5) in the majority of epithelial cells (Fig. 1A). This pattern of broad expression within the pancreatic epithelium persisted through E12.5 (Fig. 1B). With the start of the secondary transition, around E13, Nkx6.1 expression became restricted: by E15.5 it was exclusively detected in insulin-expressing cells and scattered ductal and periductal cells (Fig. 1C,D).

At E15.5, near the peak of new β-cell formation in the fetal pancreas, all Nkx6.1-expressing cells co-expressed Nkx2.2, although many Nkx2.2-positive cells did not express Nkx6.1 (Fig. 2). At the same embryonic age, Nkx6.1 expression partially overlapped with the expression of two other pancreatic transcription factors: the pancreatic-duodenal homeomain factor PDX1 and the bHLH factor neurogenin 3, a marker of islet cell progenitors (Schwitzgebel et al., 2000; Fig. 2).

Since PDX1 and neurogenin 3 are not found in the same cells at this stage in pancreatic development (Schwitzgebel et al., 2000), these two factors define two populations of Nkx6.1-expressing cells: immature progenitor cells co-expressing neurogenin 3, and more mature cells co-expressing insulin and PDX1. Many of the insulin-negative/Nkx6.1-positive cells also expressed a marker of replicating cells, proliferating cell nuclear antigen (PCNA) (Fig. 2). PCNA was not detected in the insulin-expressing cells at this stage (data not shown).

At later developmental stages, and in the adult pancreas, Nkx6.1 became completely restricted to insulin-expressing cells (Fig. 1 and data not shown). In this regard Nkx6.1 is unique: no other transcriptional regulators are known to be restricted exclusively to the β-cells within the pancreas.

To test the role of Nkx6.1 in pancreatic development, we generated mice with a null allele for Nkx6.1 by homologous recombination in ES cells. A portion of exon 1 including the translation initiation site was deleted and replaced by a PGK-neomycin resistance cassette.

At E18.5, one day prior to birth, the pancreases of homozygous Nkx6.1 mutant embryos were normal in size and gross appearance. Histologically, the pancreatic islets were smaller than normal, but the endocrine cells were organized into islet-like clusters. Immunohistochemistry, however, revealed a profound deficiency of insulin-expressing cells in Nkx6.1 mutant embryos at E18.5 (Fig. 3 and Table 1). No differences between wild type and Nkx6.1 mutant embryos could be detected in the expression of glucagon, somatostatin
and PP (Fig. 3). When measured by radioimmunoassay in whole pancreas extracts at E18.5, insulin content was similarly decreased: the insulin content of Nkx6.1 mutant pancreases was 2% of the amount in wild-type pancreases (0.17±0.01 versus 7.6±0.24 µg/mg of protein).

The deficiency in β-cells first appeared at the start of the secondary transition. In both wild-type and mutant embryos, the first glucagon-expressing cells appeared at E9.5 in the dorsal pancreatic bud, and insulin-producing cells appeared a day later (data not shown). Until E12.5, the pancreatic buds of wild type and Nkx6.1 mutant embryos contained similar numbers of insulin-producing cells (Fig. 3 and Table 1). While the number of insulin-expressing cells increases exponentially after E13 in wild-type embryos, Nkx6.1 mutant embryos failed to show this expansion of the β-cell population (Fig. 3 and Table 1).

The normal initial development of insulin-expressing cells suggests that Nkx6.1 may be exclusively required by a second embryonic phase of β-cell neogenesis. Unlike the β-cells that form after E13, early-generated insulin-producing cells lack PDX1 and Nkx6.1 expression (Oster et al., 1998) (data not shown), and may co-express glucagon (Teitelman et al., 1993). These early insulin-expressing cells were unaffected in Nkx6.1 mutant embryos, demonstrating the presence of two distinct pathways for generating insulin-expressing cells in the embryonic pancreas.

Table 1. Number of insulin-expressing cells in wild type and Nkx6.1 mutant embryos

<table>
<thead>
<tr>
<th>Embryonic day</th>
<th>Number of insulin-positive cells/section</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nkx6.1+/+</td>
</tr>
<tr>
<td>E11.5</td>
<td>1.9±1.0</td>
</tr>
<tr>
<td>E12.5</td>
<td>2.1±0.6</td>
</tr>
<tr>
<td>E14.5</td>
<td>7.5±0.9</td>
</tr>
<tr>
<td>E15.5</td>
<td>5.2±0.4</td>
</tr>
<tr>
<td>E16.5</td>
<td>83±9.2</td>
</tr>
<tr>
<td>E18.5</td>
<td>42±2</td>
</tr>
</tbody>
</table>

The mean number of insulin-positive cells per section (detected by immunostaining) was determined by counting stained cells in 12 representative sections through an individual pancreas from wild-type (+/+) or mutant (−/−) embryos. The numbers shown represent the mean number±standard error of the mean of insulin-positive cells per section from four independent pancreases.

Fig. 2. There are two populations of Nkx6.1-expressing cells at E15.5. The expression of Nkx6.1 (green) and the expression of other islet transcription factors (red) partially overlap (cells with yellow appearing nuclei) at E15.5. Note that a number of Nkx6.1-expressing cells do not express PDX1 (green appearing cells in A), whereas all Nkx6.1 expressing cells co-express Nkx2.2 (B). Many of the Nkx6.1-expressing cells that do not express PDX1 express neurogenin3 (C) and the proliferating cell nuclear antigen PCNA (D). At this same time, the acinar cells are replicating rapidly, as demonstrated by the PNA labeling of an acinus (Ac) in D.
The few β-cells present in the Nkx6.1 mutant embryos at E18.5 appeared to be fully mature: in addition to insulin, they expressed islet amyloid polypeptide, prohormone convertases 1/3 and 2, and the transcription factors PDX1 and Nkx2.2 (Fig. 3 and data not shown). Two transcription factors associated with mature islet cells, Pax6 and Isl1, were also expressed normally in the Nkx6.1 mutant pancreas (data not shown), demonstrating that Nkx6.1 is not required for the expression of any of these transcription factors.

The presence of Nkx2.2 in the Nkx6.1 mutant pancreas suggests that Nkx6.1 functions downstream of Nkx2.2 in β-cell development. To establish the genetic hierarchy between Nkx6.1 and Nkx2.2, we generated embryos homozygous for disruptions of both genes. Nkx2.2 single mutant embryos do not form any insulin-expressing cells in the pancreas throughout development, and instead accumulate incompletely differentiated β-cells. Despite the absence of any of the four classic islet hormones, insulin, glucagon, somatostatin and PP, these incompletely differentiated cells can be identified by their expression of the pan-islet prohormone convertase PC2, as well as their abundant expression of PC1/3 and IAPP (Sussel et al., 1998; Fig. 4).

The pancreatic phenotype of Nkx6.1/2 double mutant embryos was indistinguishable from the Nkx2.2 single mutant phenotype (Figs 4, 5). In Nkx6.1 single mutant pancreases, cells expressing the four classic islet hormones accounted for all IAPP-positive and PC2-positive cells. By contrast, identical to Nkx2.2 single mutants, Nkx6.1/2 double mutants accumulated hormone-negative islet cells that expressed IAPP and PC2 in their pancreases. The failure of the Nkx6.1 gene mutation to affect the development of the pancreas in Nkx2.2 mutant embryos demonstrates that Nkx6.1 functions genetically downstream of Nkx2.2 in pancreatic development.

Unlike the Nkx2.2 mutant embryos, the Nkx6.1 single mutant embryos did not accumulate incompletely differentiated β-cell precursors. Nor was there an increase in any of the other endocrine cell types to suggest that β-cell precursors deviate to an alternate cellular fate (Figs 3, 5), as seen in mice lacking the paired-homeodomain transcription factor Pax4 (Sosa-Pineda et al., 1997). At the same time, β-cell precursors formed at a normal rate, since the mutant embryos had normal pancreatic expression of neurogenin 3 (Fig. 3), a marker for early stage precursors, cells at the earliest step of the islet cell differentiation pathway (Apelqvist et al., 1999; Gradwohl et al., 2000; Schwitzgebel et al., 2000; Fig. 3B). Therefore, the cells are lost at a later stage in the pathway, either as late-stage β-cell precursors (cells that no longer express neurogenin 3 but do not yet express insulin) or as completely differentiated, insulin-expressing β-cells.

To distinguish these two possibilities, we compared the rate of new β-cell formation after the secondary transition in wild-type and Nkx6.1 mutant embryos. To label newly formed β-cells, pregnant mice were injected with BrdU five times per day between E14.5 and E16. At E16.5, the number of insulin-expressing cells labeled with BrdU was greatly reduced in the Nkx6.1 mutant embryos compared with their wild-type littermates (Table 2), indicating that β-cell formation is greatly reduced in the Nkx6.1 mutant embryos. During this period, the replication rate of pre-existing β-cells is extremely low, as evidenced by the 1 hour BrdU labeling of insulin-expressing
cells at E14.5 and E15.5. Significant β-cell replication was not detected until E18.5, when the replication rate was the same in wild type and mutant embryos. Taken together, the data suggest that the mutant embryos have a decrease in β-cell neogenesis, and that this decrease is due to a defect in the late-stage precursors that have progressed beyond the neurogenin 3 stage (Fig. 6).

Finally, to exclude increased cell death as a cause for β-cell loss, we tested directly the rate of apoptosis in the fetal pancreas by the TUNEL assay. Embryonic Nkx6.1 mutant pancreases had no gross increase in apoptotic cells relative to wild-type littermates (Table 3). This low rate of apoptosis cannot account for a substantial loss of mature β-cells, adding further support to a role for Nkx6.1 in β-cell neogenesis.

**DISCUSSION**

In this study we have determined the function of Nkx6.1 in pancreatic development. Nkx6.1 was expressed in three populations of cells during pancreatic development: first broadly in the undifferentiated epithelial cells of the early pancreatic buds; then, after the secondary transition, in a subset of proliferating islet cell progenitors; and finally in the differentiated β-cells. Deletion of the Nkx6.1 gene in mice caused a marked reduction of β-cells after the secondary transition. We excluded a role for Nkx6.1 in the formation of islet cell progenitors or in the proliferation or survival of the differentiated β-cells, and propose instead that the loss of
Fig. 6. A proposed model for the major β-cell differentiation pathway. Expression of neurogenin 3 commits endodermal progenitors to an endocrine fate. Nkx2.2 then allows these progenitors to progress to the Nkx6.1 expressing stage. In the absence of Nkx2.2, progenitors committed to a β-cell fate fail to differentiate into β-cells, and instead develop into incompletely differentiated cells, but stable cells characterized by IAPP expression. Downstream of Nkx2.2, Nkx6.1 ensures the expansion and progression of Nkx2.2-expressing β-cell precursors to mature β-cells. In the absence of Nkx6.1, most of the late stage precursors are lost, and in contrast to precursors in Nkx2.2 mutants, do not adopt an arrested or alternate fate.

Nkx6.1 affects cells in transition from early neurogenin 3-expressing precursors to differentiated β-cells. Because Nkx2.2/Nkx6.1 double mutants display the same phenotype as Nkx2.2 single mutants, we conclude that Nkx6.1 functions downstream of Nkx2.2 in pancreatic development. Our analysis defines a function for Nkx6.1 downstream of Nkx2.2 in the expansion and final differentiation of β-cell progenitors.

Distinct pathways for β-cell formation

In the developing pancreas, the first insulin-expressing cells appear as early as the bud stage, but significant β-cell numbers cannot be detected until E13. These early and late cells expressing insulin appear to represent distinct cell populations. The early insulin-expressing cells in the pancreatic bud produce low levels of insulin (Picet and Rutter, 1972), and often co-express glucagon (Alpert et al., 1988; Teitelman et al., 1993), but do not express the transcription factors PDX1 and Nkx6.1, or the glucose transporter GLUT2 (Alpert et al., 1988; Teitelman et al., 1993). Our data do not support this model. Because we do not detect significant replication of insulin-expressing cells prior to E18, maturation of the small early population of insulin/glucagon co-expressing cells cannot explain the much larger number of mature β-cells that appear after E13. Instead we propose that most of the β-cells in the late fetal pancreas must develop from non-hormone-expressing progenitor cells. In support of this conclusion, Herrera and colleagues have used lineage tracing in transgenic mouse models to show that the glucagon promoter is not active in the progenitor cells for mature β-cells (Herrera et al., 1994, 1998; Herrera, 2000).

In Nkx6.1 mutant embryos, the development of the early insulin-expressing cells remains unaffected, but β-cell numbers fail to increase with the secondary transition. Similarly, formation of the early population requires neither Pax4 (Sosa-Pineda et al., 1997) nor PDX1 (Offield et al., 1996), although Pax4 is required for formation of the late population of β-cells (Sosa-Pineda et al., 1997), and PDX1 is required for their maintenance (Ahlgren et al., 1998). Together, these findings suggest that distinct genetic programs control the formation of the early and late populations of insulin-producing cells during embryogenesis. Analogous dual pathways have been described in the development of thyrotrope cells of the pituitary gland. In the pituitary, an early population develops independently of the Pou-homeodomain transcription factor Pit1, whereas thyrotropes arising later in embryogenesis require Pit1 for their development (Lin et al., 1994).

In late embryogenesis and early postnatal life, as the wave of β-cell neogenesis that initiated with the secondary transition starts to wane, the proliferation of pre-existing β-cells produces a further increase in β-cell numbers (Finegood et al., 1995). Supporting this notion, we observed BrdU uptake in 3% of all β-cells at E18.5. β-cell proliferation, the third wave of β-cell formation, does not require Nkx6.1. Those few β-cells that do arise during the secondary transition in Nkx6.1 mutant embryos enter the pool of replicating cells at the same rate as in wild-

<table>
<thead>
<tr>
<th>Embryonic day (BrdU injection)*</th>
<th>Number of BrdU/insulin co-positive cells/section‡</th>
<th>No. of BrdU positive/insulin positive cells (%)§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nkx6.1+/+</td>
<td>Nkx6.1+/−</td>
</tr>
<tr>
<td></td>
<td>Nkx6.1+/−</td>
<td>Nkx6.1+/-</td>
</tr>
<tr>
<td>E16.5 (E14.5- E16)‡‡</td>
<td>5.0±0.3</td>
<td>5.2±0.16</td>
</tr>
<tr>
<td>E14.5 (1 hour)**</td>
<td>0.2±0.1</td>
<td>0</td>
</tr>
<tr>
<td>E15.5 (1 hour)**</td>
<td>0.06±0.06</td>
<td>0.05±0.05</td>
</tr>
<tr>
<td>E18.5 (1 hour)**</td>
<td>1.4±1</td>
<td>0.9±0.16</td>
</tr>
</tbody>
</table>

*Pregnant mice were injected intraperitoneally with 0.5 μg of BrdU per gram of body weight every 5 hours between E14.5 and E16, and then embryos were harvested 12 hours after the last injection, or were injected one hour prior to harvesting the embryos**. The first experiment labels precursor cells, the second experiment labels preexisting β-cells.

†Pancreatic sections were co-immuno-stained with an anti-insulin and an anti-BrdU antibody. The mean number of insulin-positive and BrdU/insulin co-positive cells per section was determined by counting stained cells in 10 representative sections through four pancreases for each data point. The mean±standard error of the mean is shown. When precursors are labeled (labeled E14.5-E16 and harvested E16.5), the total number of BrdU/insulin co-positive cells per section in the Nkx6.1 mutant homozygotes (−/−) is markedly reduced relative to wild type(+/+), indicating that a reduced number of precursors are progressing to mature β-cells.

§The mean percentage of insulin-positive cells that are BrdU co-positive±standard error of the mean is shown. A significant decrease in the half-life of insulin-expressing cells would cause a decrease in the average age of the cells and therefore an increase in the percentage of newly formed cells, as indicated by BrdU labeling. The similarity in percentage of BrdU-labeled cells in wild-type and Nkx6.1 mutant homozygous embryos at E16.5 indicates that the half-life of the β-cells is unaffected by the absence of Nkx6.1.
Table 3. Apoptosis in wild type and Nkx6.1 mutant pancreases

<table>
<thead>
<tr>
<th>Embryonic day</th>
<th>Number of apoptotic cells/section*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nkx6.1+/+</td>
</tr>
<tr>
<td>E12.5</td>
<td>6.3±0.5</td>
</tr>
<tr>
<td>E13.5</td>
<td>3.4±0.3</td>
</tr>
<tr>
<td>E14.5</td>
<td>2.3±0.6</td>
</tr>
<tr>
<td>E15.5</td>
<td>2.5±0.5</td>
</tr>
</tbody>
</table>

*Pancreatic sections were assayed by TUNEL assay for apoptotic cells. The mean number of apoptotic cells per section was determined by counting stained cells in 10 representative sections through four pancreases for each data point. The mean±standard error of the mean is shown.

type embryos. To date, little is known about the factors involved in perinatal β-cell proliferation, although a recent study by Miettinen et al. (2000) has implicated EGF signaling.

**Function of Nkx6.1 in β-cell neogenesis**

Defects at several points in the progression of cells from undifferentiated epithelial precursors to mature β-cells could account for the reduction in β-cell numbers seen in Nkx6.1 mutant embryos. Progenitor cells that have initiated the program of endocrine differentiation can be identified by their transient expression of the pro-endocrine bHLH transcription factor neurogenin 3, which is expressed in a subset of epithelial cells prior to expression of any of the hormone genes (Apelqvist et al., 1999; Gradwohl et al., 2000; Jensen et al., 2000; Schwitzgebel et al., 2000). The pancreases of Nkx6.1 mutant embryos contain normal numbers of neurogenin 3-expressing cells during the secondary transition, suggesting that the loss of Nkx6.1 does not impact the initiation of endocrine cell differentiation and the generation of neurogenin 3-expressing progenitor cells (Fig. 6).

It is conceivable that increased cell death of differentiated β-cells could account for the reduction in β-cell numbers in Nkx6.1 mutant embryos. However, our data provide no evidence that Nkx6.1 functions to maintain β-cells once they have differentiated. Both the BrdU labeling data in Table 2 and the apoptosis studies detailed in Table 3 demonstrate that excess loss of differentiated β-cells cannot account for the reduction in β-cell numbers seen in Nkx6.1 mutant embryos. Therefore, Nkx6.1 functions at some point in the differentiation pathway after the generation of neurogenin 3-expressing progenitors but prior to the appearance of differentiated β-cells.

But what happens in the Nkx6.1 mutants to the neurogenin 3-expressing cells that are normally destined to become β-cells? We have no evidence that the absence of Nkx6.1 causes β-cell precursors to deviate to an alternate endocrine cell fate, as occurs in embryos lacking Pax4 (Sosa-Pineda et al., 1997). Nor do incompletely differentiated β-cell precursors accumulate in the pancreas, as occurs in embryos lacking Nkx2.2 (Sussel et al., 1998). It appears instead that Nkx6.1 is required for maintaining and expanding the population of β-cell precursors as they progress from neurogenin 3-expressing progenitors to differentiated β-cells. In the absence of Nkx6.1, most of these β-cell precursors are lost. This loss could result from early apoptosis, prior to the expansion of the β-cell precursor population, or from a block in the replication of β-cell precursors, or from a combination of both defects, thereby preventing the normal expansion of the β-cell population starting at E13. In the Nkx2.2 mutants, β-cell precursors apparently escape this fate, possibly because the absence of Nkx2.2 prevents them from reaching the Nkx6.1-dependent stage.

**Hierarchy of transcription factors**

During embryonic development, a cascade of transcriptional events controls β-cell formation in the pancreas. Different transcription factors control distinct checkpoints along the pathway to the differentiated β-cells. The first step of pancreatic epithelial cells towards an endocrine fate is controlled by neurogenin 3. Loss of neurogenin 3 function in mice results in a complete absence of endocrine cell differentiation (Gradwohl et al., 2000). We have shown that neurogenin 3 expression in the pancreas does not depend on Nkx6.1.

Several transcription factors control the subsequent differentiation of precursor cells into the four islet cell types. Loss of function studies have shown that most transcription factors control differentiation of more than one islet cell type. Is11, Pax6 and NeuroD (Neurod1 – Mouse Genome Informatics), for example, affect the development of all pancreatic endocrine cells (Ahlgren et al., 1997; Naya et al., 1997; Sander et al., 1997; St-Onge et al., 1997). Other factors, such as Pax4 and Nkx2.2 predominantly control the differentiation of β-cells (Sosa-Pineda et al., 1997; Sussel et al., 1998).

In contrast to Pax4 and Nkx6.1, Nkx2.2 is required for the development of both the early insulin-expressing cells and the later mature β-cells (Sussel et al., 1998). In the absence of Nkx2.2, β-cell differentiation is arrested, leading to the accumulation of IAPP-positive, but insulin-negative islet cells. The presence of these incompletely differentiated islet cells has led to the hypothesis that Nkx2.2 functions in the later steps of β-cell differentiation. In Nkx6.1 single mutant pancreases, β-cells can escape the complete block in differentiation that occurs in Nkx2.2 mutant embryos, apparently because expression of Nkx2.2 is maintained.

While Nkx2.2 expression is maintained in Nkx6.1 mutant pancreases, Nkx6.1 expression is lost after the secondary transition in Nkx2.2 mutant pancreases, suggesting that Nkx6.1 may function downstream of Nkx2.2 in β-cell development. In addition, the pancreatic phenotype of Nkx2.2/Nkx6.1 double mutant embryos is indistinguishable from Nkx2.2 single mutant embryos, demonstrating an epistatic relationship of Nkx6.1 downstream of Nkx2.2 in β-cell differentiation (Fig. 6).

In summary, our analysis establishes a requirement for Nkx6.1 downstream of Nkx2.2 in pancreatic β-cell development. After E13, the rate of β-cell differentiation increases exponentially. In this wave of β-cell neogenesis, Nkx6.1 mediates the expansion and final differentiation of β-cell progenitors. Our experiments outline three pathways for the formation of insulin-expressing cells under the control of distinct molecular programs: an early minor pathway involving Nkx2.2 but not Pax4, PDX1 or Nkx6.1; the major pathway for neogenesis during the secondary transition that requires Nkx6.1; and the late proliferation of pre-existing β-cells.

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