Article

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

SANDER, M, *et al*.

Abstract

Most insulin-producing beta-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 beta-cell precursors and blocks beta-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: beta-cell precursors survive but fail to differentiate into beta-cells throughout development. Together, these experiments reveal two independently controlled pathways for beta-cell differentiation, and place Nkx6.1 downstream of Nkx2.2 in the major pathway of beta-cell differentiation.

Reference

UNIVERSITÉ
DE GENÈVE

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

Maike Sander[1],*, Lori Sussel[1],‡, Jennifer Conners[1], David Scheel[1], Julie Kalamaras[1], Filemon Dela Cruz[1], Valerie Schwitzgebel[1],§, Andrea Hayes-Jordan[1] and Michael German[1,2],¶

[1]Hormone Research Institute, University of California San Francisco, San Francisco, CA 94143-0534, USA
[2]Department of Medicine, University of California San Francisco, San Francisco, CA 94143-0534, USA
*Present address: Center for Molecular Neurobiology, Martinistr. 85, 20251 Hamburg, Germany
‡Present address: Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center, 4200 E. 9th Ave, Denver, CO 80262, USA
§Present address: Pediatric Endocrinology, Children's Hospital, University of Geneva, CH-1211 Geneva, Switzerland
¶Author for correspondence (e-mail: mgerman@biochem.ucsf.edu)

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).

Progressive modifications in gene expression direct the process of development from endoderm precursors to differentiated β-cells. A number of transcription factors have been shown to control pancreas morphogenesis or the differentiation of the endocrine cells (Ahlgren et al., 1997; Edlund, 1998; Gradwohl et al., 2000, Naya, 1997; Harrison et al., 1999; Jonsson et al., 1994; Li et al., 1999; Offield et al., 1996; Sander and German, 1997; Sander et al., 1997; Sosa-Pineda et al., 1997; St-Onge et al., 1997). Although no transcription factor has been identified thus far that selectively controls β-cell formation, the NK-homeodomain factor Nkx2.2 plays an essential role in β-cell development. Nkx2.2 is initially expressed broadly in the pancreatic bud, becomes progressively restricted during the secondary transition, and is eventually limited to the α, β and PP cells of the mature islet. Disruption of the *Nkx2.2* gene leads to the accumulation of incompletely differentiated β-cells that express some β-cell markers, but not insulin (Sussel et al., 1998).

The phenotype of *Nkx2.2* mutant mice may in part result from the loss of other transcriptional regulators, including Nkx6.1 which is absent from the mutant pancreas after the start of the secondary transition (Sussel et al., 1998). Like Nkx2.2, Nkx6.1 is a homeodomain transcription factor, although it falls into a divergent subfamily within the NK homeodomain family (Rudnick et al., 1994). Within the pancreas, Nkx6.1 also follows an expression pattern similar to that of Nkx2.2; the main distinction being that it eventually becomes limited to the β-cells alone (Jensen et al., 1996; Oster et al., 1998; Rudnick 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 *Spe*I/*Eco*RV fragment was cloned into the pBlueScript KS+ cloning vector, and an 800 bp *Not*I 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 give 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 axioscope 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 homeodomain 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

Fig. 1. Initially broad expression of Nkx6.1 becomes restricted after the secondary transition. Immunoperoxidase staining (brown) shows nuclear Nkx6.1 expression in the majority of pancreatic epithelial cells at E10.5 (A) and E12.5 (B). By double immunofluorescence staining at E15.5, Nkx6.1 (green) is co-expressed with insulin (orange) (C), but not with glucagon (orange) (D). Nkx6.1 is also detected in some non-hormone-expressing cells at E15.5. In the adult pancreas, Nkx6.1 (green) is detected exclusively in insulin-expressing cells (orange) (E), and is not co-expressed with glucagon (orange) (F), or somatostatin (red) (G).
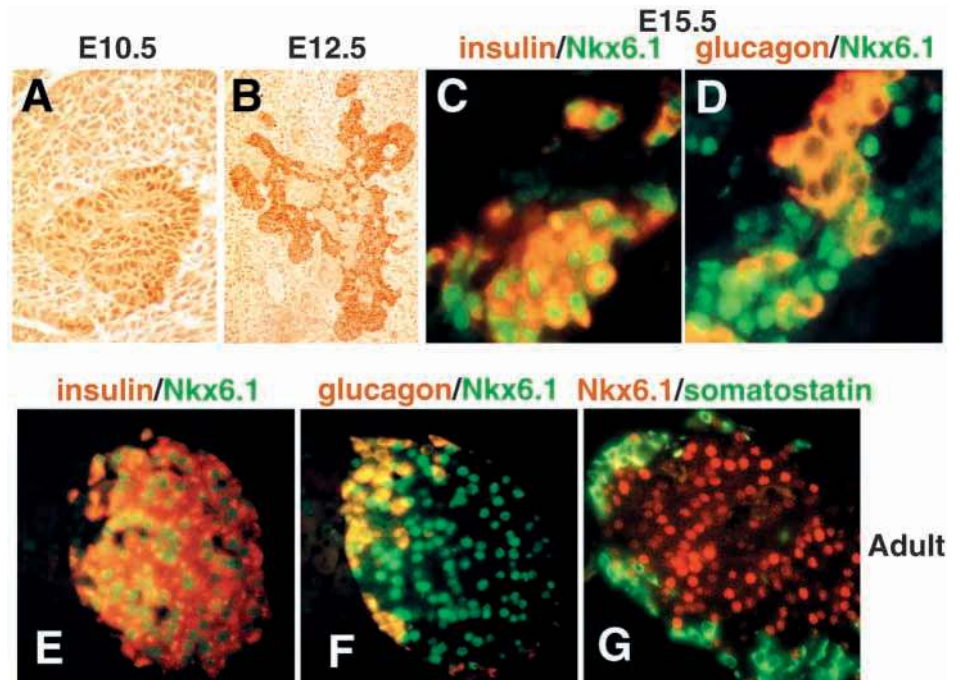
and PP (Fig. 3). When measured by radioimmunoassay in whole pancreas extracts at E18.5, insulin content was similarly decreased: the insulin content of *Nkx6.1* mutant pancreases was 2% of the amount in wild-type pancreases (0.17±0.01 versus 7.6±0.24 µg/mg of protein).

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

	Number of insulin-positive cells/section	
Embryonic day	*Nkx6.1*⁺/⁺	*Nkx6.1*⁻/⁻
E11.5	1.9±1.0	1.7±0.3
E12.5	2.1±0.6	2.9±1.2
E14.5	75±9.0	5.0±0.5
E15.5	83±9.2	5.2±0.4
E16.5	118±11	16±2
E18.5	421±25	29±3

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.
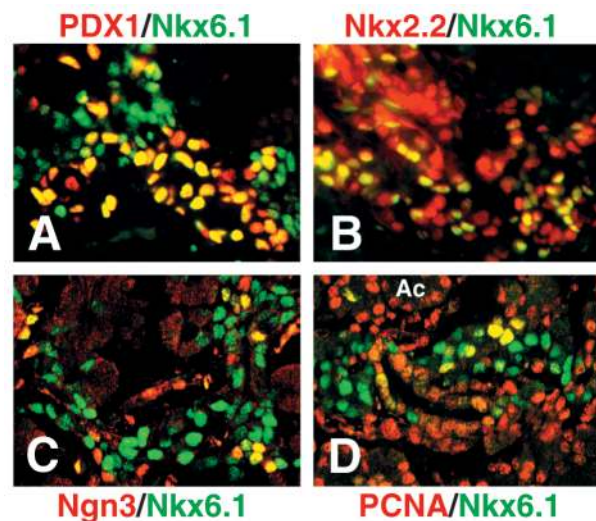

Fig. 2. There are two populations of Nkx6.1-expressing cells at E15.5. The expression of Nkx6.1 (green) and the expression of other islet transcription factors (red) partially overlap (cells with yellow appearing nuclei) at E15.5. Note that a number of Nkx6.1-expressing cells do not express PDX1 (green 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.
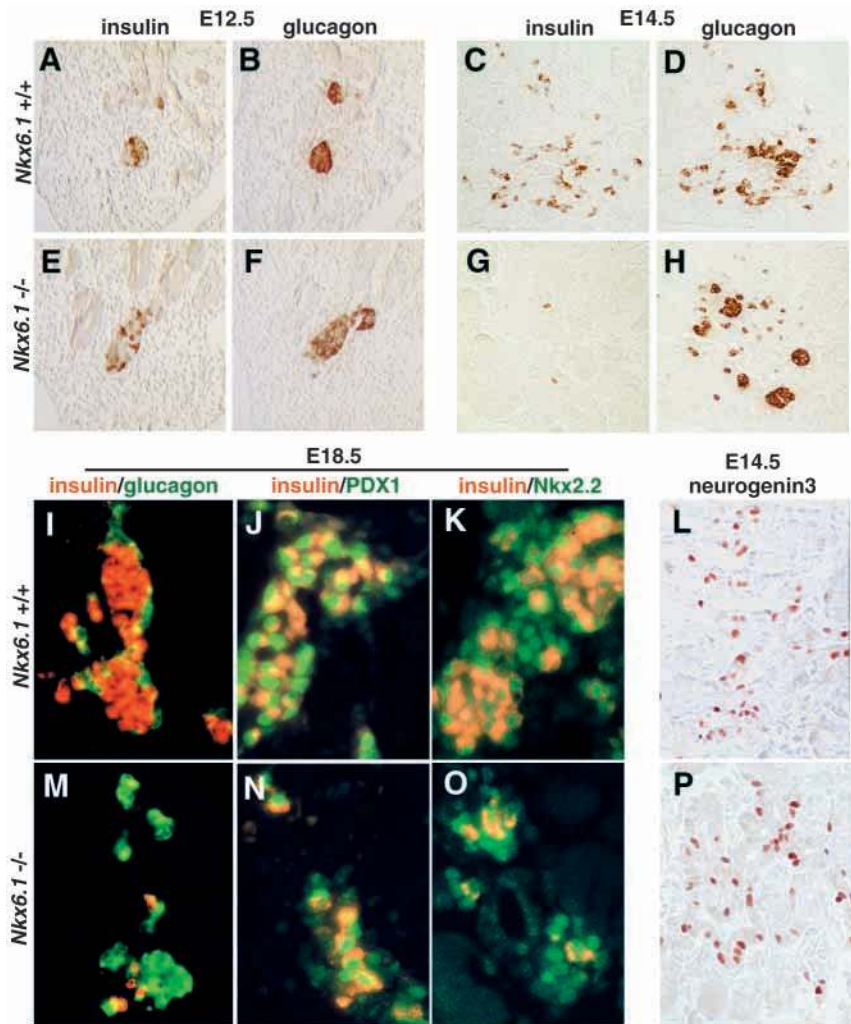
Fig. 3. The number of insulin-expressing cells is markedly reduced in the pancreases of *Nkx6.1* homozygous mutant embryos, starting with the onset of the secondary transition. Until E12.5, immunoperoxidase staining (brown) shows that similar numbers of cells express insulin (A,E), and glucagon (B,F) in the pancreatic buds of wild-type (A,B) and *Nkx6.1* mutant (E,F) pancreases. By E14.5, the number of insulin-expressing cells markedly increases in pancreases of wild type (C), but not of *Nkx6.1* mutant (G) embryos, while the number of glucagon-expressing cells increases in both (D,H). In term embryos, few insulin-expressing cells (red) are detected in pancreases of *Nkx6.1* mutants (M), while glucagon expressing cells (green) appear in normal numbers (I,M). Insulin-expressing cells (orange) in both wild type and *Nkx6.1* mutant pancreases express PDX1 (green) (J,N) and Nkx2.2 (green) (K,O). The number and pattern of cells expressing neurogenin 3 (brown) are the same in both wild-type (L) and *Nkx6.1* mutant (P) embryos, indicating that the initial steps of the β-cell differentiation pathway are unaffected.

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 *Nkx6.1/2.2* 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, *Nkx6.1/2.2* 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

Fig. 4. *Nkx2.2* single mutants and *Nkx2.2* /*Nkx6.1* double mutants accumulate incompletely differentiated islet cells. Fluorescent staining is shown for insulin, glucagon, somatostatin and PP (IGSPP, green) and prohormone convertase 2 (PC2, red, A-D) and IAPP (red, panels E-H) on pancreatic sections of wild-type, *Nkx6.1* mutant, *Nkx2.2* mutant, and *Nkx6.1/Nkx2.2* double mutant homozygous embryos at E18.5. Cells co-expressing one of the four classic islet hormones and PC2 or IAPP appear yellow. Cells expressing PC2 or IAPP but lacking any of the four classic islet hormones appear red and are seen only in the *Nkx2.2* mutant, and *Nkx6.1/Nkx2.2* double mutant homozygous embryos. Islet architecture is also similarly disordered in *Nkx2.2* mutant, and

Nkx6.1/Nkx2.2 double mutant homozygous embryos. Autofluorescent red blood cells appear as small orange cells in some panels.
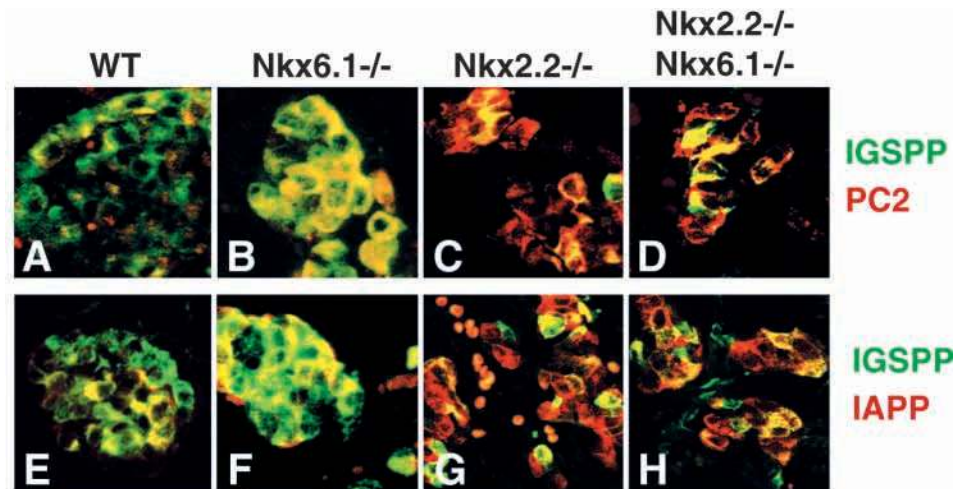
cells at E14.5 and E15.5. Significant β-cell replication was not detected until E18.5, when the replication rate was the same in wild type and mutant embryos. Taken together, the data suggest that the mutant embryos have a decrease in β-cell neogenesis, and that this decrease is due to a defect in the 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. 5. *Nkx2.2* single mutants and *Nkx2.2* /*Nkx6.1* double mutants have the same expression patterns for islet hormones in the pancreas. Immunoperoxidase staining (brown) is shown for the markers indicated on pancreatic sections of wild-type, *Nkx6.1* mutant, *Nkx2.2* mutant, and *Nkx6.1/Nkx2.2* double mutant homozygous embryos at E18.5. Islets structure is intact in the wild-type and *Nkx6.1* mutant embryos, but is disrupted in the *Nkx2.2* mutant and *Nkx6.1/Nkx2.2* double mutant embryos. The number of insulin-expressing cells is reduced in *Nkx6.1* mutants, but no insulin-expressing cells are detected in *Nkx2.2* and *Nkx6.1/Nkx2.2* mutants. *Nkx2.2* and *Nkx6.1/Nkx2.2* mutants show a similar reduction in glucagon- and PP-expressing cells. Cells arrested in differentiation are marked by IAPP expression in the absence of insulin or glucagon in *Nkx2.2* and *Nkx6.1/Nkx2.2* mutant embryos; these cells are not seen in wild-type or *Nkx6.1* mutant embryos. The number of somatostatin-expressing cells is similar in embryos of all four genotypes.
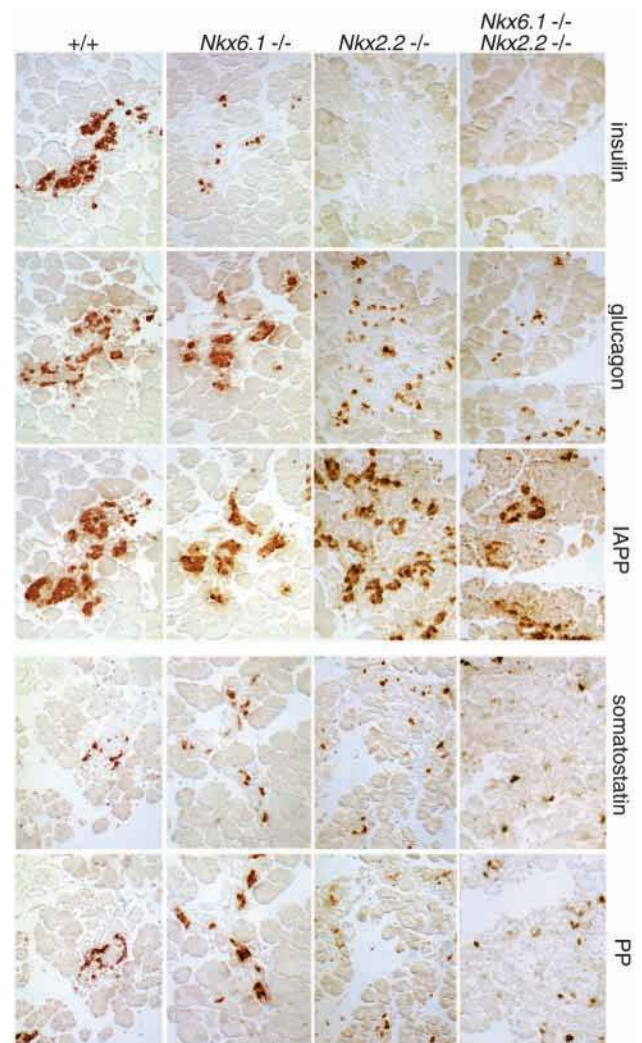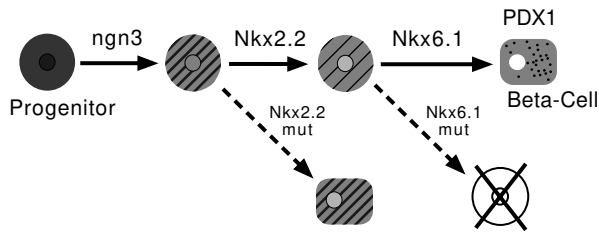
Fig. 6. A proposed model for the major β-cell differentiation pathway. Expression of neurogenin 3 commits endodermal progenitors to an endocrine fate. Nkx2.2 then allows these progenitors to progress to the Nkx6.1 expressing stage. In the absence of Nkx2.2, progenitors committed to a β-cell fate fail 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 two distinct cell populations. The early insulin-expressing cells in the pancreatic bud produce low levels of insulin (Pictet 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 (Oster et al., 1998; Pang et al., 1994). In contrast to these early cells, high insulin-production, as well as Pdx1, Nkx6.1 and

GLUT2 expression, characterize the mature β-cells that form in much larger numbers after E13.

It has been suggested that the early insulin/glucagon co-expressing cells represent a transition state as cells differentiate from the early glucagon expressing cells into mature β-cells (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 2. β-cell proliferation and neogenesis in wild-type and *Nkx6.1* mutant embryos

Embryonic day (BrdU injection)*	Number of BrdU/insulin co-positive cells/section‡		No. of BrdU positive/ insulin positive cells (%)§	
	Nkx6.1$^{+/+}$	*Nkx6.1*$^{-/-}$	*Nkx6.1*$^{+/+}$	*Nkx6.1*$^{-/-}$
E16.5 (E14.5- E16)¶	5.0±0.3	0.52±0.16	4.6±0.3	3.5±0.9
E14.5 (1 hour)**	0.2±0.1	0	0.7±0.5	0
E15.5 (1 hour)**	0.06±0.06	0	0.05±0.05	0
E18.5 (1 hour)**	14±1	0.9±0.16	3.3±0.3	3.0±0.5

 *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

	Number of apoptotic cells/section*	
Embryonic day	*Nkx6.1*[+/+]	*Nkx6.1*[−/−]
E12.5	6.3±0.5	7.5±0.5
E13.5	3±0.3	3.3±0.3
E14.5	2.3±0.6	3.6±0.6
E15.5	2.5±0.5	2.3±0.5

*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. Isl1, 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.

REFERENCES

Ahlgren, U., Jonsson, J., Jonsson, L., Simu, K. and Edlund, H. (1998). β-cell-specific inactivation of the mouse Ipf1/Pdx1 gene results in loss of the beta-cell phenotype and maturity onset diabetes. *Genes Dev.* **12**, 1763-1768.

Ahlgren, U., Pfaff, S. L., Jessell, T. M., Edlund, T. and Edlund, H. (1997). Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells. *Nature* **385**, 257-260.

Alpert, S., Hanahan, D. and Teitelman, G. (1988). Hybrid insulin genes reveal a developmental lineage for pancreatic endocrine cells and imply a relationship with neurons. *Cell* **53**, 295-308.

Apelqvist, A., Li, H., Sommer, L., Beatus, P., Anderson, D. J., Honjo, T., Hrabe de Angelis, M., Lendahl, U. and Edlund, H. (1999). Notch signalling controls pancreatic cell differentiation. *Nature* **400**, 877-881.

Edlund, H. (1998). Transcribing pancreas. *Diabetes* **47**, 1817-1823.

Finegood, D. T., Scaglia, L. and Bonner-Weir, S. (1995). Dynamics of beta-cell mass in the growing rat pancreas. Estimation with a simple mathematical model. *Diabetes* **44**, 249-256.

Gradwohl, G., Dierich, A., LeMeur, M. and Guillemot, F. (2000). Neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. *Proc. Natl. Acad. Sci. USA* **97**, 1607-1611.

Harrison, K. A., Thaler, J., Pfaff, S. L., Gu, H. and Kehrl, J. H. (1999). Pancreas dorsal lobe agenesis and abnormal islets of Langerhans in Hlxb9-deficient mice. *Nat. Genet.* **23**, 71-75.

Herrera, P.-L., Huarte, J., Zufferey, R., Nichols, A., Mermillod, B., Philippe, J., Muniesa, P., Sanvito, F., Orci, L. and Vassalli, J.-D. (1994). Ablation of islet endocrine cells by targeted expression of hormone-promoter-driven toxigenes. *Proc. Natl. Acad. Sci. USA* **91**, 12999-13003.

Herrera, P. L. (2000). Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. *Development* **127**, 2317-2322.

Herrera, P. L., Orci, L. and Vassalli, J. D. (1998). Two transgenic approaches to define the cell lineages in endocrine pancreas development. *Mol. Cell. Endocrinol.* **140**, 45-50.

Jensen, J., Heller, R. S., Funder-Nielsen, T., Pedersen, E. E., Lindsell, C., Weinmaster, G., Madsen, O. D. and Serup, P. (2000). Independent development of pancreatic alpha- and beta-cells from neurogenin3-expressing precursors: a role for the notch pathway in repression of premature differentiation. *Diabetes* **49**, 163-176.

Jensen, J., Serup, P., Karlsen, C., Nielsen, T. F. and Madsen, O. D. (1996). mRNA profiling of rat islet tumors reveals nkx 6.1 as a beta-cell-specific homeodomain transcription factor. *J. Biol. Chem.* **271**, 18749-18758.

Jonsson, J., Carlsson, L., Edlund, T. and Edlund, H. (1994). Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature* **371**, 606-609.

Kash, S. F., Johnson, R. S., Tecott, L. H., Noebels, J. L., Mayfield, R. D., Hanahan, D. and Baekkeskov, S. (1997). Epilepsy in mice deficient in the 65-kDa isoform of glutamic acid decarboxylase. *Proc. Natl. Acad. Sci. USA* **94**, 14060-14065.

Li, H., Arber, S., Jessell, T. M. and Edlund, H. (1999). Selective agenesis of the dorsal pancreas in mice lacking homeobox gene Hlxb9. *Nat. Genet.* **23**, 67-70.

Lin, S. C., Li, S., Drolet, D. W. and Rosenfeld, M. G. (1994). Pituitary ontogeny of the Snell dwarf mouse reveals Pit-1-independent and Pit-1-dependent origins of the thyrotrope. *Development* **120**, 515-522.

Miettinen, P. J., Huotari, M.-A., Koivisto, T., Ustinov, J., Palgi, J., Rasilainen, S., Lehtonen, E., Keski-Oja, J. and Otonkoski, T. (2000). Impaired migration and delayed differentiation of pancreatic islet cells in mice lacking EGF-receptors. *Development* **127**, 2617-2627.

Naya, F. J., Huang, H. P., Qiu, Y., Mutoh, H., DeMayo, F. J., Leiter, A. B. and Tsai, M. J. (1997). Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. *Genes Dev.* **11**, 2323-2334.

Offield, M. F., Jetton, T. L., Labosky, P. A., Ray, M., Stein, R. W., Magnuson, M. A., Hogan, B. L. and Wright, C. V. (1996). PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development* **122**, 983-995.

Oster, A., Jensen, J., Serup, P., Galante, P., Madsen, O. D. and Larsson, L. I. (1998). Rat endocrine pancreatic development in relation to two homeobox gene products (Pdx-1 and Nkx 6.1). *J. Histochem. Cytochem.* **46**, 707-715.

Pang, K., Mukonoweshuro, C. and Wong, G. G. (1994). Beta cells arise from glucose transporter type 2 (Glut2)-expressing epithelial cells of the developing rat pancreas. *Proc. Natl. Acad. Sci. USA* **91**, 9559-9563.

Pictet, R. and Rutter, W. J. (1972). Development of the embryonic endocrine pancreas. In *Handbook of Physiology*. Vol. 1 (eds A. P. Society, D. F. Steiner and N. Frenkel), pp. 25-66. Washington DC: Williams and Wilkins.

Qiu, M., Shimamura, K., Sussel, L., Chen, S. and Rubenstein, J. L. (1998). Control of anteroposterior and dorsoventral domains of Nkx-6.1 gene expression relative to other Nkx genes during vertebrate CNS development. *Mech. Dev.* **72**, 77-88.

Rudnick, A., Ling, T. Y., Odagiri, H., Rutter, W. J. and German, M. S. (1994). Pancreatic beta cells express a diverse set of homeobox genes. *Proc. Natl. Acad. Sci. USA* **91**, 12203-12207.

Sander, M. and German, M. S. (1997). The β-cell transcription factors and development of the pancreas. *J. Mol. Med.* **15**, 327-340.

Sander, M., Neubuser, A., Kalamaras, J., Ee, H. C., Martin, G. R. and German, M. S. (1997). Genetic analysis reveals that PAX6 is required for normal transcription of pancreatic hormone genes and islet development. *Genes Dev.* **11**, 1662-1673.

Schwitzgebel, V. M., Scheel, D. W., Conners, J. R., Kalamaras, J. R., Lee, J. E., Anderson, D. J., Sussel, L., Johnson, J. D. and German, M. S. (2000). Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. *Development* **127**, 3533-3542.

Slack, J. M. (1995). Developmental biology of the pancreas. *Development* **121**, 1569-1580.

Sosa-Pineda, B., Chowdhury, K., Torres, M., Oliver, G. and Gruss, P. (1997). The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas. *Nature* **386**, 399-402.

St-Onge, L., Sosa-Pineda, B., Chowdhury, K., Mansouri, A. and Gruss, P. (1997). Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas. *Nature* **387**, 406-409.

Sussel, L., Kalamaras, J., Hartigan-O'Connor, D. J., Meneses, J. J., Pedersen, R. A., Rubenstein, J. L. and German, M. S. (1998). Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. *Development* **125**, 2213-2221.

Teitelman, G., Alpert, S., Polak, J. M., Martinez, A. and Hanahan, D. (1993). Precursor cells of mouse endocrine pancreas coexpress insulin, glucagon and the neuronal proteins tyrosine hydroxylase and neuropeptide Y, but not pancreatic polypeptide. *Development* **118**, 1031-1039.

Upchurch, B. H., Aponte, G. W. and Leiter, A. B. (1994). Expression of peptide YY in all four islet cell types in the developing mouse pancreas suggests a common peptide YY-producing progenitor. *Development* **120**, 245-252.