Nkx6 transcription factor activity is required for α- and β-cell development in the pancreas

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Summary

In diabetic individuals, the imbalance in glucose homeostasis is caused by loss or dysfunction of insulin-secreting β-cells of the pancreatic islets. As successful generation of insulin-producing cells in vitro could constitute a cure for diabetes, recent studies have explored the molecular program that underlies β-cell formation. From these studies, the homeodomain transcription factor NKX6.1 has proven to be a key player. In Nkx6.1 mutants, β-cell numbers are selectively reduced, while other islet cell types develop normally. However, the molecular events downstream of NKX6.1, as well as the molecular pathways that ensure residual β-cell formation in the absence of NKX6.1 are largely unknown. Here, we show that the Nkx6.1 paralog, Nkx6.2, is expressed during pancreas development and partially compensates for NKX6.1 function. Surprisingly, our analysis of Nkx6 compound mutant mice revealed a previously unrecognized requirement for NKX6 activity in α-cell formation. This finding suggests a more general role for NKX6 factors in endocrine cell differentiation than formerly suggested. Similar to NKX6 factors, the transcription factor MYT1 has recently been shown to regulate α- as well as β-cell development. We demonstrate that expression of Myt1 depends on overall Nkx6 gene dose, and therefore identify Myt1 as a possible downstream target of Nkx6 genes in the endocrine differentiation pathway.

Key words: Nkx6.1, Nkx6.2, Myt1, Pancreas, Islet, Endocrine, Insulin, Glucagon, Development, Mouse

Introduction

The possibility of developing a cell-based therapy for treatment of diabetes mellitus has recently generated interest in identifying the molecular pathways that control development of endocrine cells in the pancreas. In mammalian pancreas, endocrine cells are clustered in the islets of Langerhans, in which a large core of insulin-producing β-cells is surrounded by α-, δ- and PP-cells that produce glucagon, somatostatin and pancreatic polypeptide (PP), respectively. Because of their role in the pathogenesis of diabetes mellitus, the mechanisms that underlie formation of β-cells have been studied in most detail. Although β-cell proliferation appears to be the predominant mechanism of β-cell regeneration during adulthood (Dor et al., 2004), β-cell formation from multipotent precursors accounts for the vast majority of β-cells formed during embryogenesis (Jensen, 2004; Kim and MacDonald, 2002). Therefore, most factors that control β-cell differentiation have been identified through studies in the embryo.

Formation of the mouse pancreas begins at embryonic day (E) 9.5 as separate dorsal and ventral evaginations from the foregut endoderm (Slack, 1995). At this stage, the epithelium contains multipotent progenitors that express the transcription factor PDX1 and have the potential to give rise to all pancreatic lineages, comprising endocrine and exocrine cells, as well as cells of the pancreatic ducts (Gu et al., 2002; Herrera, 2002). The first endocrine cells appear as early as E10.5 and produce glucagon- and/or insulin, but lack specific products characteristic of mature hormone-producing cells (Oster et al., 1998; Wilson et al., 2002). Mature insulin- and glucagon-producing cells, as well as cells expressing the exocrine-specific markers amylase and carboxypeptidase A are first detected around E13.5. The first δ- and PP-cells are found at E15.5 and E18.5, respectively (Pictet and Rutter, 1972; Slack, 1995).

It has been shown that specific transcription factors restrict the developmental potential of the initially multipotent pancreatic progenitors and promote their differentiation into specific cell types (Jensen, 2004). This is exemplified by the basic helix-loop-helix (bHLH) transcription factor NGN3, which limits the potential of a specific subset of pancreatic precursors to undergo endocrine differentiation while prohibiting an exocrine or ductal cell fate (Gu et al., 2002). The complete absence of hormone-producing cells from the pancreas of Ngn3−/− mice further supports a role for Ngn3 in endocrine specification (Gradwohl et al., 2000). Interestingly, ectopic expression of Ngn3 in all pancreatic precursors
largely results in excess differentiation of α-cells, but not of other endocrine cell types. This indicates that although able to confer endocrine identity to early pancreatic progenitors, NGN3 requires additional factors for the differentiation of β-, δ- and PP-cells (Apelqvist et al., 1999; Schwitzgebel et al., 2000).

Among the transcription factors that are required for β-cell development are NKX2.2, PAX4, HB9 and NKX6.1. In Nkx2.2 and Pax4 mutants, loss of β-cells results from a switch to an alternative endocrine fate (Prado et al., 2004; Sosa-Pineda et al., 1997), whereas HB9 has been implicated in the control of β-cell maturation (Harrison et al., 1999; Li et al., 1999). Mice deficient for the NK-homeodomain factor Nkx6.1 have a specific defect in β-cell neogenesis, while all other endocrine cell types develop normally (Sander et al., 2000). In Nkx6.1 mutants, β-cell development is disrupted only after E13.5, when the first mature β-cells appear. This suggests that the differentiation of early-appearing insulin-positive cells and mature β-cells is controlled by independent mechanisms and implies a selective role for NKX6.1 in just the major β-cell differentiation pathway.

The observation that a significant number of β-cells still form in the absence of NKX6.1 even after E13.5 points to the existence of an NKX6.1-independent pathway of β-cell development. In this study, we examined the Nkx6.1 paralog Nkx6.2 for its function and possible synergy with NKX6.1 in pancreatic development. In contrast to NKX6.1, we found NKX6.2 function to be dispensable for normal endocrine development. However, our analysis of Nkx6.1;Nkx6.2 double nullizygous mice not only revealed a partially compensatory role for NkX6.2 in β-cell development, but an as of yet unappreciated requirement for NKX6 activity in α-cell formation. We further discovered that NKX6 activity controls expression of the NGN3 co-factor MYT1 in a dose-dependent manner, thus providing a possible mechanism by which NKX6 activity may control endocrine development.

Materials and methods

Mutant mice and BrdU injections

Nnkx6.1 and Nnkx6.2 mutant mice were generated by gene targeting as previously described (Sander et al., 2000; Vallstedt et al., 2001). Compound Nkx6 mutant embryos were obtained by crossing Nkx6.2+/–;Nnkx6.1+– mice in timed matings. Mid-day of the day on which the vaginal plug was detected was considered as day E0.5. All genotyping was performed by Southern blot analysis as described (Sander et al., 1997). The following primary cDNA probes were used: Ngn3 (Susens et al., 1997). For BrdU labeling experiments, pregnant females were injected with 50 µg BrdU per gram of body weight i.p., and embryos harvested 45 minutes after injection.

Immunohistochemistry, in situ hybridization, TUNEL assay and X-gal staining

Pancreata were removed from adult mice and embryos at E15.5 and later stages; pancreatic tissue in embryos at earlier stages was studied in whole embryos. Samples were fixed in 4% paraformaldehyde in PBS and either paraffin-embedded or frozen in OCT.

Immunohistochemical detection of proteins was performed as described previously (Sander et al., 1997). The following primary antibodies were used in these assays: rabbit α-amylin (IAPP) diluted 1:2000 (Peninsula); rabbit α-Hb9 diluted 1:8000 (Harrison et al., 1999); rabbit α-amylose (Sigma) diluted 1:500; goat α-grehlin diluted 1:1000 (Santa Cruz); guinea pig α-glucagon diluted 1:3000 (Linco); mouse α-glucagon diluted 1:8000 (Sigma); mouse α-insulin diluted 1:8000 (Linco); mouse α-insulin diluted 1:8000 (Sigma); rabbit α-ISL1 diluted 1:5000 (Tsuchida et al., 1994); rabbit α-NKX6.1 diluted 1:3000 (Jensen et al., 1996); guinea pig α-NKX6.2 diluted 1:4000 (Vallstedt et al., 2001); rabbit α-pancreatic polypeptide diluted 1:2000 (Dako); rabbit α-PDX1 diluted 1:3000 (Ohlsson et al., 1993); rabbit α-somatostatin diluted 1:3000 (Dako); rabbit α-NGN3 diluted 1:3000 (Sander et al., 2000); rabbit α-PAX6 diluted 1:3000 (S. Saule); mouse α-BrdU diluted 1:200 (Chemicon); mouse α-β-galactosidase diluted 1:200 (ICN); NGN3 and NKX6.1 antigens were produced by inserting the coding sequence for the N-terminal 95 amino acids (NGN3) and the C-terminal 66 amino acids (NKX6.1) 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 guinea pigs; guinea pig α-NGN3 and guinea pig α-NKX6.1 were diluted 1:1000.

Secondary antibodies used for immunofluorescence were as follows: Cy3-conjugated α-guinea pig, α-rabbit and α-mouse diluted 1:2000 (Jackson Laboratory); Cy5 conjugated α-rabbit diluted 1:200 (Jackson Laboratory); Alexa (488 nm)-conjugated α-mouse, α-guinea pig and α-rabbit diluted 1:2000 (Molecular Probes). Images were collected on a Zeiss Axioplan2 microscope with a Zeiss Axiocam or a Leica confocal microscope (Leica TCS NT).

TUNEL assays on tissue sections were performed using a commercially available kit (Oncor).

Whole-mount X-gal staining was performed on Nkx6.2+/– mice, in which the TaelacZ gene was inserted into the Nkx6.2 locus. Using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as a substrate, staining was performed on either whole embryos or isolated abdominal organs as described previously (Mombaerts et al., 1996). In situ hybridizations with either digoxigenin- (Gradwohl et al., 1996; Wilkinson, 1992) or [α-35S]UTP-labeled antisense riboprobes (Susens et al., 1997) were performed on 10 µm cryosections as described. The following cDNA probes were used: Mty1 (Gu et al., 2004), Ngn3 (Gradwohl et al., 2000) and Pxx4 (Wang et al., 2004).

RNA preparation and real-time quantitative PCR

Total RNA from dissected pancreatic anlagen was extracted with the RNeasy kit (Qiagen) and treated with DNase. cDNA was prepared by in vitro transcription using SuperscriptII reverse transcriptase (Invitrogen). PCR reactions were performed in triplicate in a total reaction volume of 50 µl, and amplifications performed in an ABI Prism 7700 sequence detection system (Applied Biosystems). With the exception of Mty1, which was detected with Taqman Universal PCR Mastermix (Applied Biosystems), all transcripts were amplified with 1× SYBR Green PCR master mix (Applied Biosystems) and 300 nM of each primer. To exclude contamination with non-specific PCR products, melting curves were analyzed for all PCR products. The following cycle was used for the amplification: 50°C for 2 minutes; 95°C for 10 minutes; followed by 40 cycles of denaturation at 95°C for 15 seconds; and primer extension at 60°C for 1 minute. For each reaction, a parallel reaction that lacked template was performed as a negative control. Relative changes in gene expression were calculated by the comparative ΔCt method in which γ-actin was used for normalization with the SYBR Green method and HPRT with the Taqman method (Livak and Schmittgen, 2001). Listed 5′ to 3′, primer sequences were as follows: γ-actin forward, GCACCCCGGTGTCC-TGAC; γ-actin reverse, CAGGATGCATAACAGGAC; glucagon forward, TTCCGACAGAAGTCCGACTT; glucagon reverse, TC-CTGTTGGCCAGATTACCT; insulin forward, CCACCCAGGC-TTTGCTCAA; insulin reverse, CCCAGTCCTGGTTGTCCCA. The Taqman gene expression IDs were Mm00456190_m1 for Mty1 and Mm00446968_m1 for HPRT.

Hormone quantification and cell counting

Protein was extracted from individual pancreata of E18.5 embryos using acid extraction and protein concentration was then determined.
by the Bradford dye-binding assay. The concentrations of insulin and glucagon were determined by radioimmunoassay (RIA) using commercially available kits (Linco).

To obtain a representative average of the number of hormone-positive cells, an entire pancreas was used for quantification. Immunofluorescence staining was performed on 10 µm sections and positive cells counted on every ninth section throughout the pancreas at E16.5 and E18.5, every fifth section at E12.5 and E14.5, and all sections at E10.5. The average cell number per section was determined for all sections counted from each individual pancreas. Mean differences were tested for statistical significance using the Student’s t-test.

Results
Nkx6.2 is transiently expressed in the developing mouse pancreas
To determine the pattern of Nkx6.2 expression, we first performed enzymatic X-gal staining on mouse embryos, in which the Nkx6.2-coding sequence was replaced by a Tau-lacZ cassette (Nkx6.2tlz mice) (Vallstedt et al., 2001). At E9.5, β-galactosidase (β-gal) activity was selectively detected in both the dorsal and the ventral pancreatic rudiments (Fig. IA). By E15.5, β-gal was expressed at high levels in the dorsal and at lower levels in the ventral aspect of the pancreas (Fig. IF). Additional β-gal activity was found in the distal part of the stomach and the duodenum (Fig. IF). In contrast to Nkx6.1, which is maintained in adult β-cells (Sander et al., 2000), Nkx6.2 expression rapidly declined in late embryogenesis. Only few scattered β-gal-positive (+) cells were detected at E18.5 and no expression was seen in the adult pancreas (data not shown).

To examine which pancreatic cell types express NKX6.2, we performed co-immunofluorescence analyses with an anti-NKX6.2 antibody, together with antibodies against various pancreatic markers. Demonstrating specificity of the anti-NKX6.2 antibody, NKX6.2 and β-gal colocalized in cells of
the distal stomach epithelium and dorsal pancreas in Nkx6.2+/+ embryos (Fig. 1B), whereas no NKX6.2 staining was detected in Nkx6.2−/− embryos (see Fig. S1B in the supplementary material). At E10.5, PDX1 marks the entire pancreatic epithelium as well as the prospective distal stomach and duodenum (Fig. 1C; see Fig. S1C in the supplementary material) (Offield et al., 1996), NKX6.2 colocalized with PDX1 in the pancreatic and stomach epithelium (Fig. 1C). Notably, NKX6.2 was found in a large percentage (~70%) of, but not in all, PDX1+ cells, and was absent from the early glucagon-expressing cells at E10.5 (Fig. 1D). By E12.5, NKX6.2 expression became restricted to a few epithelial cells, of which a significant proportion expressed glucagon (Fig. 1E). At E15.5, the pancreatic epithelium contains undifferentiated pancreatic progenitors, which include the NGN3+ endocrine progenitors, as well as already differentiated α-, β-, and exocrine cells. Nkx6.2 is not part of the endocrine progenitor pool at E15.5 or prior, as NKX6.2 does not colocalize with NGN3 (Fig. 1G, data not shown). Likewise, Nkx6.2 was not detected in insulin-producing β-cells (Fig. 1H). Instead, we found that all NKX6.2+ cells co-expressed either glucagon or amylase (Fig. 1I). As both endocrine and exocrine cells express PDX1 at E15.5, this finding is consistent with the observation that the vast majority of NKX6.2+ cells were PDX1+ (Fig. 1J). Interestingly, only a subset of, but not all α- and exocrine cells expressed NKX6.2 (Fig. 1I). Together with the finding that NKX6.2 expression disappears from the pancreas around birth, these observations indicate that maturation of α- and exocrine cells coincides with the downregulation of NKX6.2.

As NKX6.2 is not expressed in NGN3+ endocrine progenitors or β-cells (Fig. 1G,H), we asked whether PDX1/NKX6.2 co-expressing progenitors could give rise to NGN3+ or insulin+ cells. To test this issue, we used the highly stable β-gal protein as a tracer for the fate of Nkx6.2-expressing cells. Similar to NKX6.2 protein (Fig. 1I), we found β-gal to colocalize with glucagon and amylase (Fig. 1M,N). However, in contrast to NKX6.2 protein, a number of β-gal+ cells co-expressed either NGN3 or insulin (Fig. 1K,L). Given the stability of β-gal protein, these data are consistent with the idea that Nkx6.2-expressing cells differentiate into NGN3+ endocrine progenitors and ultimately into β-cells.

**NKX6.1 and NKX6.2 have distinct domains in the pancreas**

The finding that NKX6.2 colocalizes with glucagon and amylase, but is absent from insulin-producing cells and NGN3+ endocrine progenitors suggests that it is expressed in a separate domain from NKX6.1, which is found in β-cells and NGN3+ cells (Fig. 3A,B). We directly tested this hypothesis by co-immunofluorescence staining for NKX6.1 and NKX6.2. At E10.5, we observed five different populations of cells in the pancreatic epithelium (Fig. 2A,B): first, cells that exclusively expressed either NKX6.1 (~33% of all NKX6+ cells) or NKX6.2 (~16%); second, a small population of cells that expressed both NKX6 proteins at similar levels; and finally, cells that produced low levels of one NKX6 factor, but high levels of the other (all three populations of co-expressing cells account for ~51% of NKX6+ cells). At E12.5, the vast majority of pancreatic epithelial cells exclusively expressed NKX6.1 (~86% of all NKX6+ cells), and only a few, scattered cells co-expressed NKX6.1 and NKX6.2 (~3%) (Fig. 2C). Consistent with the absence of NKX6.2 from insulin+ and NGN3+ cells (Fig. 1G,H), NKX6.1 and NKX6.2 were found in entirely separate domains at E15.5 (Fig. 2D). Thus, with the exception of the early, undifferentiated epithelium, NKX6.1 and NKX6.2 mark different cell populations in the pancreas.

**Nkx6.2 is regulated by NKX6.1**

Similar to our observations in the pancreas, a progressive segregation of the NKX6.1 and NKX6.2 expression domains was also noted during spinal cord development (Vallstedt et al., 2001). Moreover, it was observed that NKX6.1 represses the expression of NKX6.2 in the spinal cord, providing a possible mechanism for how their exclusive expression domains are established and maintained.

To determine if a similar mechanism operates in the pancreas, we examined whether absence of NKX6.1 affects expression of NKX6.2. Although we did not detect any difference in the number of NKX6.2+ cells between wild-type and Nkx6.1−/− mutants at E10.5 (Fig. 3Q), their number was increased in Nkx6.1−/− mutants at E12.5 (Fig. 3E,F,Q). Likewise, microarray experiments from whole pancreatic epithelium showed a significant upregulation of Nkx6.2 mRNA in Nkx6.1−/− mutants at E13.5 (2.5-fold) and E15.5 (2.8-fold) (data not shown). To test if there is mutual cross-repression between the two NKX6 factors, we also analyzed the expression of NKX6.1 in Nkx6.2−/− mutants, but did not detect an increase in the number of NKX6.1+ cells (data not shown). Therefore, similar to spinal cord (Vallstedt et al., 2001), NKX6.1 represses Nkx6.2, but NKX6.2 does not repress Nkx6.1 in the pancreas.

Next, we examined whether the normal expression domain of NKX6.1 is fully reconstituted by NKX6.2 in Nkx6.1−/− mutants. Between E10.5 and E15.5, NKX6.1 is normally found in a large percentage of undifferentiated epithelial cells, which
Fig. 3. Upregulation of NKX6.2 expression in Nkx6.1 mutant embryos.

Immunofluorescence detection of NKX6.1 with NGN3 (A), insulin (Ins) (B), glucagon (Glc) (C) or amylase (Amy) (D) in pancreas from E14.5 (A) and E15.5 (B-D) embryos. NKX6.1 is expressed in a subset of NGN3+ cells (A), in all insulin+ cells (B), and in very few glucagon+ cells (arrowhead in C). No co-expression of NKX6.1 and amylase is observed (D). (E-P) Confocal images showing immunofluorescence staining for NKX6.2 together with various pancreatic markers in wild-type and Nkx6.1−/− embryos. At E12.5, the number of NKX6.2+ cells is increased in the pancreatic epithelium of Nkx6.1 mutants (E,F). Upregulation of NKX6.2 in Nkx6.1 mutants is not seen in insulin+ (Ins) (arrowheads in G,H indicate insulin+/NKX6.2− cells) or glucagon+ (Glc) cells (arrowheads in K,L indicate glucagon+/NKX6.2− cells). NGN3+ cells are NKX6.2− in wild type and Nkx6.1 mutants (I,J). The ectopic Nkx6.2+ cells in Nkx6.1 mutants do not produce amylase (Amy) (M,N). At E15.5, the majority of ectopic NKX6.2-expressing cells co-express PDX1 (O,P).

Quantification of NKX6.2+ cells in wild-type and Nkx6.1−/− embryos. The average number of NKX6.2+ cells per section was determined by counting immunofluorescence-labeled cells at E10.5, E12.5 and E15.5. Four independent pancreata were evaluated for each genotype. The average number of cells are shown as a % of wild type (arbitrarily set to 100%) ±s.e.m. Scale bar: 50 μm.

includes the majority of NGN3+ endocrine progenitors (Fig. 3A; data not shown). In addition, NKX6.1 was expressed in all insulin+ cells, occasionally in glucagon+ cells, but was absent from the exocrine lineage (Fig. 3B-D). In Nkx6.1 mutants, NKX6.2 was not detected in insulin+ or NGN3+ cells at E15.5 or prior (Fig. 3HJ; data not shown), indicating that the absence of NKX6.1 does not result in a full reconstitution of the genuine NKX6.1 expression domain by NKX6.2.

To reveal the identity of the NKX6.2+ cells in Nkx6.1 mutants, we next examined whether ectopic NKX6.2 expression occurs in α- or exocrine cells. This did not appear to be the case, as a similar fraction of glucagon- and amylase-producing cells stained positive for NKX6.2 in wild type and Nkx6.1 mutants (Fig. 3K-N). Together, these findings suggest that NKX6.2 is not ectopically activated in differentiated cells. Therefore, we tested if NKX6.2+ cells co-expressed PDX1, which in addition to β- and exocrine cells, marks pancreatic progenitors at E15.5. Indeed, more than 90% of NKX6.2+ cells in Nkx6.1 mutants were also PDX1+ (Fig. 3P). As we did not observe ectopic expression of NKX6.2 in β- or exocrine cells, we can infer that upregulation of NKX6.2 in the absence of NKX6.1 most probably occurs in pancreatic progenitors.
Notably, at E18.5, NKX6.2 is no longer detected in either wild-type or Nkx6.1 mutant pancreas, suggesting that NKX6.2 is only transiently upregulated during development.

**NKX6.1 and NKX6.2 have partially redundant functions in endocrine differentiation**

As Nkx6.2 has been previously shown to compensate for Nkx6.1 during motoneuron development (Vallstedt et al., 2001), we next examined whether Nkx6.1 and Nkx6.2 also cooperate during development of the pancreas. First, we tested if deletion of Nkx6.2 alone affects pancreatic morphogenesis or cell differentiation. The pancreas of Nkx6.2 mutants was of normal size, morphology and histology both at E18.5 and in adult mice (data not shown). Furthermore, immunofluorescence staining for islet cell hormones at E14.5 and E18.5 revealed normal numbers and distribution of the endocrine cells (Fig. 4A,B,E,G,H; Fig. 5A,B,E,F). Finally, there was no difference in pancreatic insulin and glucagon content between wild type (insulin, 7.8±2.8 µg/mg protein; glucagon, 329±55 ng/mg protein; n=4) and Nkx6.2 mutant pancreata (insulin, 6.8±1.2 µg/mg protein; glucagon, 300±49 ng/mg protein; n=4) at E18.5.

These findings demonstrate that NKX6.2 is dispensable for normal pancreas development, but do not exclude a compensatory function of NKX6.2 in the absence of NKX6.1 activity. To test if Nkx6.1 and Nkx6.2 have partially redundant functions, we compared pancreatic endocrine development Nkx6.1 single and Nkx6.1;Nkx6.2 double nullizygous embryos. As in Nkx6.1 and Nkx6.2 single mutants, the pancreas was of normal size in Nkx6.1–/–;Nkx6.2–/– embryos at E18.5. In the absence of NKX6.1 alone, the number of insulin+ cells was reduced by ~85%, while the number of glucagon+, somatostatin + and PP + cells was normal (Fig. 4A,C,E; Fig. 5A,C,E,G). Additional deletion of Nkx6.2 in an Nkx6.1 mutant background resulted in a significant further reduction of insulin+ cells to only ~8% of wild-type embryos (Fig. 4C,D,E). Notably, the insulin+ cells in both Nkx6.1–/– and Nkx6.1–/–;Nkx6.2–/– embryos lacked expression of the mature β-cell marker MAFA and Glut2, but were PDX1-, PAX6- and HB9-positive (data not shown). Surprisingly, we also observed a drastic (~65%) reduction in glucagon cell numbers in Nkx6.1–/–;Nkx6.2–/– embryos (Fig. 4D,E), a phenotype that was not observed in either of the two Nkx6 single mutants (Fig. 4B,C,E). Development of somatostatin- and PP-producing cells was also reduced, albeit less severely (Fig. 4D,E).
cells was not affected in Nkx6.1\(^{-/-}\);Nkx6.2\(^{-/-}\) embryos (Fig. 5D,H). Similar to cell numbers, pancreatic insulin and glucagon mRNA levels were also significantly lower in Nkx6.1\(^{-/-}\);Nkx6.2\(^{-/-}\) than in Nkx6.1\(^{-/-}\) embryos (Fig. 4F). Thus, our present findings reveal a requirement for NKX6 activity in the development of both the insulin and the glucagon lineages. The role of NKX6 proteins in \(\alpha\)-cell development could not be revealed from the analysis of either Nkx6 single mutant, as the other NKX6 factor fully compensates.

Marker gene analysis and lineage tracing experiments suggest that the scattered insulin- and glucagon-producing cells that are detected prior to E13.5 are different from mature \(\alpha\)- and \(\beta\)-cells, which only form later during embryogenesis (Herrera, 2000; Oster et al., 1998; Wilson et al., 2002). To determine the time point at which NKX6.2 compensates for NKX6.1 in endocrine differentiation, we analyzed Nkx6 compound mutants at different stages of development. In both Nkx6.1\(^{-/-}\) and Nkx6.1\(^{-/-}\);Nkx6.2\(^{-/-}\) embryos, the number of early endocrine cells at E12.5 were similar to wild-type littermates (data not shown), suggesting an NKX6-independent mechanism for their development. Absence of NKX6.1 alone resulted in a drastic reduction in \(\beta\)-cell numbers by E14.5, while glucagon\(^+\) cells were not affected (Fig. 4G,I) (Sander et al., 2000). In contrast to our observations at E18.5, Nkx6.1\(^{-/-}\) and Nkx6.1\(^{-/-}\);Nkx6.2\(^{-/-}\) embryos did not differ with respect to their insulin and glucagon cell numbers or hormone mRNA levels at E14.5 or E15.5 (Fig. 4J,K), therefore demonstrating that the additional loss of endocrine cells in Nkx6.1\(^{-/-}\);Nkx6.2\(^{-/-}\) embryos occurs between E14.5 and birth.

No evidence for endocrine cell conversion in the absence of Nkx6 activity

A possible mechanism that could account for the reduction in insulin- and glucagon-producing cells is that progenitors differentiate along an alternate path. Such fate conversion has recently been shown to be the cause of \(\beta\)-cell loss in Nkx2.2 and Pax4 mutant mice (Prado et al., 2004), where cells producing the hormone ghrelin are formed at the expense of \(\beta\)-cells. To determine if a similar fate conversion underlies the \(\beta\)- and \(\alpha\)-cell loss in the absence of NKX6 activity, we analyzed the expression of ghrelin in Nkx6 compound mutants, but did not detect an increase in ghrelin\(^+\) cells (Fig. 5I-L). Together with our finding that the number of somatostatin- and PP-producing cells were normal in Nkx6.1\(^{-/-}\);Nkx6.2\(^{-/-}\) embryos (Fig. 5D,H), these results indicate that endocrine fate conversion does not account for the reduction in insulin- and glucagon-producing cells in NKX6-deficient mice.

An alternative explanation for the reduction in insulin and glucagon cell numbers is that endocrine cells are arrested in their final steps of differentiation and therefore fail to produce hormones. If endocrine precursors were arrested before their final differentiation, one would expect to detect cells that have initiated expression of some endocrine markers, such as IAPP, ISL1 or PAX6. However, we found that the number of IAPP-, ISL1- and PAX6-producing cells mirrored the number of hormone-positive cells in all Nkx6 compound mutants. Although Nkx6.2 mutants resembled wild-type embryos, a gradual reduction in the number of IAPP-, ISL1- and PAX6-producing cells mirrored the number of hormone-positive cells in all Nkx6 compound mutants. Although Nkx6.2 mutants resembled wild-type embryos, a gradual reduction in the number of IAPP-, ISL1- and PAX6-producing cells mirrored the number of hormone-positive cells in all Nkx6 compound mutants. Although Nkx6.2 mutants resembled wild-type embryos, a gradual reduction in the number of IAPP-, ISL1- and PAX6-producing cells mirrored the number of hormone-positive cells in all Nkx6 compound mutants.
affects the formation of NGN3+ endocrine progenitors. As determined by in situ hybridization and immunofluorescence staining, all Nkx6 compound mutants had similar numbers of NGN3-expressing cells as did wild-type embryos at E14.5 (Fig. 7A-D; NGN3+ cell numbers as determined by immunofluorescence: Nkx6.2–/– ~80% of wild type; Nkx6.1–/– ~80% of wild type; Nkx6.1–/–;Nkx6.2–/– ~92% of wild type). Likewise, in our microarray experiments we found no reduction in Ngn3 mRNA levels in Nkx6.1 mutant pancreas at E13.5 or E15.5 (data not shown). At E16.5, we consistently observed a slight (~20%), but not statistically significant reduction in the number of NGN3+ cells in Nkx6.1–/– and Nkx6.1–/–;Nkx6.2–/– embryos (Table 1). Notably, in all genotypes, more than 95% of all NGN3+ or PAX6+ cells were BrdU negative (data not shown), indicating that the majority of endocrine progenitors are not in S-phase of the cell cycle. It is therefore unlikely that differences in the proliferative rate of NGN3+ progenitors account for the loss of endocrine cells in Nkx6 mutants. Likewise, as there is no increase in the number of TUNEL+ cells (Table 1), cell death does not appear to be the underlying mechanism.

Next, we analyzed whether NKX6 activity could be required for the expression of important endocrine differentiation factors. First, we tested whether the β-cell differentiation factors Pax4 and HB9 are expressed in Nkx6-deficient mice. Both factors were normally expressed at E14.5 (see Fig. S2 in the supplementary material), therefore suggesting that Pax4 and HB9 are regulated independently of Nkx6 factors.

Recent findings indicate that NGN3 alone is insufficient to induce endocrine differentiation, but requires the zinc-finger transcription factor MYT1 as a co-factor. Expression of a dominant-negative form of Myt1 (DnMYT1) reduces the ability of NGN3 to induce ectopic glucagon expression from chicken endoderm (Gu et al., 2004). Moreover, inhibition of MYT1 activity in endocrine progenitors by DnMYT1 in transgenic mice results in a severe reduction in the number of insulin- and glucagon-producing cells (Gu et al., 2004). As the phenotype caused by expression of DnMYT1 resembles our observations in Nkx6.1–/–;Nkx6.2–/– embryos, we considered the possibility that Nkx6 genes function in a common genetic pathway with Myt1. A first hint that NKX6 factors regulate Myt1 came from our microarray experiments, which showed a significant reduction of Myt1 expression in Nkx6.1 mutants at E13.5 and E15.5 (1.7- and 1.8-fold, respectively; data not shown). To test whether Myt1 expression depends on overall Nkx6 gene dose, we analyzed Myt1 in pancreas of Nkx6 compound mutants by in situ hybridization. Corresponding to the microarray data, we found that the number of Myt1-expressing cells was mildly reduced in Nkx6.1–/– and Nkx6.1–/–;Nkx6.2–/– embryos (Fig. 7E,G), while severely diminished in Nkx6.1–/–;Nkx6.2–/– embryos (Fig. 7E,H). Such dose-dependent regulation of Myt1 expression by Nkx6 compound mutants by in situ hybridization. Corresponding to the microarray data, we found that the number of Myt1-expressing cells was mildly reduced in Nkx6.1 mutants at E13.5 and E15.5 (1.7- and 1.8-fold, respectively; data not shown). To test whether Myt1 expression depends on overall Nkx6 gene dose, we analyzed Myt1 in pancreas of Nkx6 compound mutants by in situ hybridization. Corresponding to the microarray data, we found that the number of Myt1-expressing cells was mildly reduced in Nkx6.1–/– and Nkx6.1–/–;Nkx6.2–/– embryos (Fig. 7E,G), while severely diminished in Nkx6.1–/–;Nkx6.2–/– embryos (Fig. 7E,H). Such dose-dependent regulation of Myt1 expression by Nkx6 activity was confirmed by quantitative RT-PCR, which showed a 1.5-fold and 3.3-fold reduction in Myt1 mRNA levels in Nkx6.1–/– and Nkx6.1–/–;Nkx6.2–/– embryos, respectively (Fig. 7I). These findings suggest that Nkx6 factors control α- and β-cell differentiation by either directly or indirectly regulating the expression of the NGN3 co-factor MYT1.

Discussion

In this study, we demonstrate that the endocrine differentiation program in the mouse pancreas requires activity of Nkx6 class proteins. Although a requirement for Nkx6.1 in β-cell differentiation has been previously shown (Sander et al., 2000), our present study provides evidence that redundant activity of the Nkx6.1 paralog Nkx6.2 masks a more general
requirement for NKX6 activity in pancreatic endocrine differentiation. This is supported by the finding that α-cell numbers are normal in either Nkx6.1 or Nkx6.2 single null mutants, but markedly reduced in the absence of both NKX6 factors. Based on the observation that NKX6 factors control pancreatic expression of the NGN3 co-factor Myt1 in a dose-dependent fashion, we propose that NKX6 factors function in endocrine differentiation through regulation of Myt1.

**Distinct and redundant activities of NKX6.1 and NKX6.2 proteins in pancreatic development**

In this study, we show that proper α- and β-cell development requires the combined activities of both NKX6.1 and NKX6.2. Although NKX6.2 fully rescues α-cell formation in the absence of NKX6.1, it only partially compensates for NKX6.1 in β-cell development. One possible explanation for the only partial rescue of β-cells is that both NKX6 factors have distinct biological functions. Although we cannot exclude this possibility, there is currently little biochemical or biological evidence to suggest that NKX6.1 and NKX6.2 have disparate activities. First, both NKX6 factors share almost identical DNA-binding homeodomains, bind to similar target sequences (Awatramani et al., 2000; Jorgensen et al., 1999; Mirmira et al., 2000), and function both as transcriptional repressors through interaction with Gro/TLE co-repressor proteins (Muhr et al., 2001). Second, when transfected into the developing neural tube, NKX6.1 and NKX6.2 have qualitatively similar activities in inducing motoneurons (Vallstedt et al., 2001). A second potential mechanism that could account for the inability of NKX6.2 to compensate for NKX6.1 in β-cell differentiation is the difference in their spatial expression domains. In support of this idea, we found only NKX6.1, but not NKX6.2, to be expressed in NGN3+ endocrine progenitors and β-cells. It is therefore possible that normal development of the β-cell lineage requires sustained expression of NKX6 factors in endocrine progenitors and/or β-cells, while α-cell development requires only NKX6 activity in PDX1+ progenitors. Consistent with this view, ectopic expression of Ngn3 under control of the Pdx1 promoter leads to premature formation of α- but not β-cells (Apelqvist et al., 1999; Schwitzgebel et al., 2000).

### Table 1. Number of NGN3* and apoptotic cells in pancreata from E16.5 Nkx6 compound mutant embryos and their wild-type littermates

| Cell number/section* | +/+ (±/±) | Nkx6.2−/− (% of wild type) | Nkx6.1−/− (% of wild type) | Nkx6.1−/− Nkx6.2−/− (% of wild type) |
|---------------------|--------|-----------------|-----------------|-------------------------------|
| NGN3 positive cells | 49±5.69 | 49±5.69 (100%) | 38.8±8.13 (79%) | 37.4±5.26 (76%) |
| Apoptotic cells     | 6.9±0.59 | 9.0±1.63 (130%) | 5.4±0.88 (78%) | 5.0±1.68 (102%) |

*Pancreatic sections were immunostained with an anti-NGN3, or assayed by TUNEL staining for apoptotic cells. The mean number of NGN3-positive, or TUNEL-positive cells per section was determined by counting stained cells from three pancreata for each data point. The mean±s.e.m. is shown.

Fig. 7. In situ hybridization for Ngn3 (A-D) and Myt1 (E-H) in pancreas from wild-type, Nkx6.2−/−, Nkx6.1−/− and Nkx6.1−/−;Nkx6.2−/− embryos at E14.5. Ngn3 expression is similar in wild type and all Nkx6 compound mutants (A-D). By contrast, expression of Myt1 is reduced in Nkx6.1−/− and almost absent in Nkx6.1−/−;Nkx6.2−/− embryos (E-H). I Quantitative RT-PCR analysis for Myt1 mRNA in pancreata from Nkx6 compound mutants at E13.5. Myt1 levels are shown relative to the levels for HPRT mRNA from three independent measurements of three pancreata for each genotype. Values are shown as a % of wild type (arbitrarily set to 100%)±s.e.m. *P<0.01, as determined by Student’s t-test. Scale bar: 50 µm.
Development may be mediated through as yet unknown NKX6.1 may not involve direct transcriptional repression, but Together, these findings suggest that repression of expressed in branchiomotor neurons and therefore do not have function by NKX6.2 occurs in PDX1 + pancreatic progenitors, while NKX6.2 is expressed only in PDX1+ progenitors, NKX6 factors may regulate Myt1 expression through a non-cell-autonomous mechanism. A factor (X) that mediates the signal between PDX1+ and Ngn3+ progenitors remains to be identified. Myt1 in Ngn3+ progenitors is required for their differentiation into α- and β-cells.

Several observations indicate that compensation for NKX6.1 function by NKX6.2 occurs in PDX1+ pancreatic progenitors (Fig. 8). First, co-expression of NKX6.1 and NKX6.2 is only found in PDX1+ cells of the early pancreatic epithelium. Second, we show that derepression of NKX6.2 in Nkx6.1 mutants is limited to PDX1+ progenitors. Finally, sustained expression of β-gal in Ngn3+ precursors of Nkx6.2+ embryos suggests that NKX6.2/PDX1 co-positive cells are precursors for a subset of Ngn3+ endocrine progenitors.

**Regulation of Nkx6.2 by NKX6.1**

The transcriptional repression of Nkx6.2 by NKX6.1 could either occur directly by binding of NKX6.1 to Nkx6.2 regulatory sequences or indirectly through regulation of additional genes. To test whether NKX6.1 could directly regulate Nkx6.2 transcription, we examined the mouse and human 5′ upstream sequences for conserved NKX6 consensus binding sites, but were unable to identify such elements within 10 kb 5′ of the Nkx6.2 transcription start site (K.D.H. and M.S., unpublished). Recent studies on Nkx6 gene function in the mouse hindbrain show that NKX6.1 and NKX6.2 are co-expressed in branchiomotor neurons and therefore do not have mutually exclusive expression domains (Mueller et al., 2003). Together, these findings suggest that repression of Nkx6.2 by NKX6.1 may not involve direct transcriptional repression, but may be mediated through as yet unknown Nkx6 target genes.

**NKX6 and MYT1 function in pancreatic endocrine development**

Based upon the finding that Nkx6.1 mutants display a selective reduction in β-cells, it was suggested that NKX6.1 functions exclusively in the β-cell differentiation pathway (Sander et al., 2000). Our present results demonstrate a previously unrecognized requirement for NKX6 activity in α-cell formation, therefore suggesting a more general role for NKX6 factors in pancreatic endocrine development. A possible mechanism for how NKX6 activity may control α- and β-cell differentiation is provided by our finding that NKX6 proteins regulate expression of the neurogenin (NGN) co-factor MYT1 in a dose-dependent manner. Both neurogenesis and pancreatic endocrine differentiation require the combined activities of MYT1 and NGN (Bellefroid et al., 1996; Gu et al., 2004). In the nervous system, NGN can induce the expression of Myt1, which suggests that they may function in a linear genetic pathway (Bellefroid et al., 1996). If regulation of these factors in the pancreas is similar to the nervous system, and Ngn3 would be able to induce the expression of Myt1, it would explain why ectopic expression of Ngn3 alone is sufficient to induce pancreatic endocrine differentiation. However, from our results we can conclude that regulation of Myt1 by NKX6 factors is independent of Ngn3, as Ngn3 is normally expressed in Nkx6 single and in Nkx6.1;Nkx6.2 double mutant mice. Thus, we propose that Ngn3 is not sufficient to induce and/or maintain Myt1 in the pancreas, but that Myt1 expression requires the activity of NKX6 factors. Although we cannot exclude a cell-autonomous mechanism, our findings that NKX6.2 is expressed only in PDX1+, but absent from Ngn3+ progenitors, while Myt1 is enriched in Ngn3-expressing cells (Gu et al., 2004), suggests that NKX6 factors regulate Myt1 through a non-cell-autonomous mechanism (Fig. 8).

If NKX6 activity is necessary for Myt1 expression, how can we reconcile the fact that Myt1 is still present in Nkx6.1;Nkx6.2 double mutant mice? One possible explanation is that NKX6.1 and NKX6.2 do not account for all NKX6 activity in the pancreas. The mouse genome indeed contains a third Nkx6 class gene that we found to be expressed in E10.5 and E14.5 pancreas by RT-PCR (K.D.H. and M.S., unpublished). This residual NKX6 activity could account for the low levels of Myt1 as well as for the small numbers of α- and β-cells that still differentiate in the pancreas of Nkx6.1–/–;Nkx6.2+ embryos. It remains to be shown if deletion of all three Nkx6 genes in mice will result in a complete absence of Myt1 expression and a subsequent block of all endocrine cell differentiation, or whether, alternatively, NKX6 factors have a specific role in the development of just the α- and β-cell lineages, while δ- and PP-cell differentiation are controlled by a NKX6-independent mechanism.

In contrast to Myt1, Ngn3 expression appears to be independent of NKX6 activity. This is supported by our finding that Ngn3 expression is normal in Nkx6 mutants until E15.5 and only slightly (~20%) reduced at E16.5. As the reduction in Ngn3 cell numbers at E16.5 is small and observed to the same extent in Nkx6.1–/– and Nkx6.1–/–;Nkx6.2+ embryos, it is unlikely to account for the loss of glucagon+ cells, which is only seen in Nkx6.1–/–;Nkx6.2+ embryos. This raises the issue of how absence of NKX6 activity affects the fate of Ngn3+ cells. In our analyses, we did not find any evidence for persistence of Ngn3+ cells at later developmental time points (data not shown) or a subsequent arrest in their endocrine differentiation. Moreover, our observation that the number of apoptotic, somatostatin-, PP- or ghrelin-producing cells was normal in Nkx6 mutants, argues against cell death or endocrine fate conversion as an underlying cause of α- and β-cell loss. This leaves the possibility that absence of NKX6 activity leads to the differentiation of Ngn3+ progenitors into non-endocrine cells; a hypothesis that we are currently testing.
Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/13/3139/DC1

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Development and disease

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