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The transcription factors Nkx6.1 and Nkx6.2 possess equivalent activities in promoting beta-cell fate specification in Pdx1$^+$ pancreatic progenitor cells

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Despite much progress in identifying transcriptional regulators that control the specification of the different pancreatic endocrine cell types, the spatiotemporal aspects of endocrine subtype specification have remained largely elusive. Here, we address the mechanism by which the transcription factors Nkx6.1 (Nkx6-1) and Nkx6.2 (Nkx6-2) orchestrate development of the endocrine alpha- and beta-cell lineages. Specifically, we assayed for the rescue of insulin-producing beta-cells in *Nkx6.1* mutant mice upon restoring Nkx6 activity in select progenitor cell populations with different *Nkx6*-expressing transgenes. Beta-cell formation and maturation was restored when Nkx6.1 was expressed in multipotential Pdx1$^+$ pancreatic progenitors, whereas no rescue was observed upon expression in committed Ngn3$^+$ (Neurog3$^+$) endocrine progenitors. Although not excluding additional roles downstream of Ngn3, this finding suggests a first requirement for Nkx6.1 in specifying beta-cell progenitors prior to Ngn3 activation. Surprisingly, although Nkx6.2 only compensates for Nkx6.1 in alpha- but not in beta-cell development in *Nkx6.1*$^{-/-}$ mice, a *Pdx1*-promoter-driven *Nkx6.2* transgene had the same ability to rescue beta-cells as the *Pdx1-Nkx6.1* transgene. This demonstrates that the distinct requirements for Nkx6.1 and Nkx6.2 in endocrine differentiation are a consequence of their divergent spatiotemporal expression domains rather than their biochemical activities and implies that both Nkx6.1 and Nkx6.2 possess alpha- and beta-cell-specifying activities.

KEY WORDS: Nkx6.1, Nkx6.2, Pdx1, Ngn3, Pancreas, Islet, Endocrine, Insulin, Glucagon, Development, Mouse, Transgenic

INTRODUCTION

Recent studies suggest that the most promising approach for the in vitro derivation of insulin-producing pancreatic beta-cells from stem cells is by recapitulating embryonic beta-cell differentiation (D'Amour et al., 2006). The in vitro generation of fully functional beta-cells for transplantation, however, will require further improvement of existing differentiation protocols. Such refinement will require detailed knowledge of the molecular mechanisms that underlie beta-cell differentiation. Lineage-tracing studies in mice have shown that all pancreatic lineages, which include the exocrine and endocrine compartment, arise from a common, proliferative progenitor cell population that is marked by the transcription factor Pdx1 (Gu et al., 2002). Expression of the transcription factor Ngn3 (also known as Neurog3 – Mouse Genome Informatics) further restricts progenitors to five distinct endocrine cell fates: alpha-, beta-, delta-, PP- or epsilon-cells, which produce the hormones glucagon, insulin, somatostatin, pancreatic polypeptide and ghrelin, respectively (Gu et al., 2002; Heller et al., 2005; Prado et al., 2004). Initially, scattered endocrine cells differentiate throughout the organ, but these cells aggregate into so-called islets of Langerhans at the end of gestation (Slack, 1995).

In recent years, major progress has been made in our understanding of the molecular pathways that control endocrine differentiation (Jensen, 2004). Much of this knowledge stems from genetic gain- and loss-of-function experiments in mice. Such studies have shown that Ngn3 activity is essential for the differentiation of all endocrine cells and, conversely, that Ngn3 is sufficient to restrict Pdx1-positive (Pdx1$^+$) progenitors to an endocrine fate (Apelqvist et al., 1999; Gradwohl et al., 2000; Schwitzgebel et al., 2000). Based on the observation that only select endocrine lineages are affected in null mutant mice for *Nkx2.2* (*Nkx2-2*), *NeuroD* (*Neurod1*), *Pax4*, *Arx*, *Hb9* (also known as *Hlxb9* – Mouse Genome Informatics) or *Nkx6.1* (*Nkx6-1*), these transcription factors have been proposed to be downstream effectors of Ngn3 (Collombat et al., 2003; Harrison et al., 1999; Li et al., 1999; Naya et al., 1997; Sander et al., 2000b; Schwitzgebel, 2001; Sosa-Pineda et al., 1997; Sussel et al., 1998; Wilson et al., 2003). Although the loss of *Pax4*, *Arx* and *NeuroD* expression in *Ngn3* mutant mice (Collombat et al., 2003; Gradwohl et al., 2000) indeed suggests a function of these genes downstream of Ngn3 in endocrine differentiation, the evidence for Nkx6.1 being downstream of Ngn3 is less clear.

Deficiency for Nkx6.1 results in a specific abrogation of beta-cell neogenesis during embryogenesis without affecting cell survival or the development of any other cell type in the pancreas (Sander et al., 2000b). In *Nkx6.1* mutant mice, a marked reduction in beta-cell numbers is first apparent at embryonic day (E)14, the time-point at which the first mature beta-cells differentiate. The specific defect in the beta-cell lineage and the maintenance of Ngn3 expression in Nkx6.1-deficient mice have led to the suggestion that Nkx6.1 selectively functions in beta-cell differentiation genetically downstream of Ngn3 (Schwitzgebel, 2001; Wilson et al., 2003). However, recent analysis of compound mutants for *Nkx6.1* and its paralog *Nkx6.2* have suggested that the function of Nkx6.1 is not restricted to beta-cell differentiation (Henseleit et al., 2005). Although the pancreas develops normally in *Nkx6.2* single-mutant mice, *Nkx6.1/Nkx6.2* double-mutant embryos display a severe reduction in alpha-cell number. This demonstrates redundant functions for the two Nkx6 factors and suggests a more general requirement for Nkx6.1 in endocrine differentiation than just in the beta-cell pathway. The expression domains of Nkx6.1 and Nkx6.2

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diverge significantly during development (Henseleit et al., 2005). Co-expression of the two Nkx6 factors is only observed in the early Pdx1$^+$ progenitor cells at E10.5. Thereafter, Nkx6.2 expression becomes downregulated in Pdx1$^+$ progenitor cells, by E15.5 is only detected in a small subset of glucagon$^+$ and exocrine cells, and it is no longer expressed during late gestation or in the adult pancreas. By contrast, Nkx6.1 remains strongly expressed throughout the Pdx1$^+$ epithelium between E11 and E13, and is the only Nkx6 factor expressed in committed endocrine progenitors or in newly differentiated or adult beta-cells (Henseleit et al., 2005; Sander et al., 2000b). Therefore, questions arise as to whether beta-cell differentiation requires sustained Nkx6 activity in Ngn3$^+$ progenitors and whether the inability of Nkx6.2 to compensate for Nkx6.1 in beta-cell differentiation results from differences in their spatiotemporal expression or in their biochemical activities. To determine which progenitor populations require Nkx6 activity for normal beta-cell formation, we used a transgenic approach to restore Nkx6 expression in select progenitor pools in *Nkx6.1* mutants. Surprisingly, we found that expression of Nkx6.1 in the Ngn3$^+$ domain was not sufficient to rescue beta-cell development, whereas the expression of either Nkx6.1 or Nkx6.2 in the Pdx1$^+$ domain resulted in a complete rescue of mature beta-cells. Our findings suggest that beta-cell development requires Nkx6 activity in Pdx1$^+$ progenitors prior to the activation of Ngn3. This study shows that the lack of redundancy between Nkx6.1 and Nkx6.2 in beta-cell development is a result of their divergent expression patterns. Therefore, the Nkx6.1 expression domain rather than its biochemical activity determines the unique requirement for Nkx6.1 in beta-cell specification.

MATERIALS AND METHODS

Transgene construction, mice and BrdU labeling

The *Pdx1-Nkx6.1*-IRES-*lacZ* and *Pdx1-Nkx6.2*-IRES-*lacZ* transgenes were generated by fusion of the mouse *Nkx6.1* or *Nkx6.2* coding region to a 4.3 kb *Xba*I-*Sac*I fragment of the mouse *Pdx1* regulatory sequences and a human beta-globin cassette (–37 bp to +12 bp) (Gannon et al., 2001). An IRES-*lacZ*-polyA cassette was inserted 3′ of the *Nkx6.1* or *Nkx6.2* coding sequence. The *Ngn3-Nkx6.1*-IRES-*eGFP* transgene was generated by fusing the rat *Nkx6.1* coding sequence in frame to the start codon of *Ngn3* preceded by 6.7 kb of mouse *Ngn3* regulatory sequence (Jenny et al., 2002). An IRES-*NLS-GFP*-polyA cassette was placed 3′ of the *Nkx6.1* coding sequence. The transgenic constructs were linearized prior to pronuclear injections into CB6F1 fertilized oocytes (performed by the UC Irvine Transgenic Core Facility). Founders for the *Pdx1-Nkx6.1*, *Pdx1-Nkx6.2* and *Ngn3-Nkx6.1* transgenic lines were identified by PCR with *lacZ* or *GFP* internal primers, respectively.

Nkx6.1 mutant mice have been previously described and were maintained on a CD1 background (Sander et al., 2000b). *Nkx6*-transgene-carrying mice were crossed with *Nkx6.1*$^{+/–}$ mice and *Nkx6.1*$^{+/–}$;transgene$^+$ offspring were used in timed matings with *Nkx6.1*$^{+/–}$ mice. Mid-day of the day on which the vaginal plug was detected was considered as E0.5. In all experiments, embryos of identical genetic background were compared and littermates were used whenever possible. All genotyping was performed by Southern blot analysis as described (Sander et al., 2000b).

Immunohistochemistry and X-gal staining

Pancreata were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 4 hours and immunofluorescence was performed on 10 μm cryopreserved sections. Immunohistochemical detection of proteins was conducted as described previously (Sander et al., 1997). The following primary antibodies were used in these assays: mouse α-glucagon diluted 1:5000 (Sigma); guinea pig α-insulin diluted 1:10,000 (Linco); rabbit α-Nkx6.1 diluted 1:10,000 (Jensen et al., 1996); rabbit α-Pdx1 diluted 1:1000 (Chemicon); guinea pig α-Pdx1 diluted 1:10,000 (C. Wright, Vanderbilt University, Nashville, TN); rabbit α-β-galactosidase diluted 1:500 (ICN, 55976); guinea pig α-Ngn3 diluted 1:1000 (Henseleit et al., 2005); guinea pig α-Nkx6.1 diluted 1:1000 (Henseleit et al., 2005); rabbit α-Glut2 (Slc2a2) diluted 1:200 (Alpha Diagnostics); rabbit α-MafA diluted 1:1500 (R. Stein, Vanderbilt University, Nashville, TN); rabbit α-Myt1 diluted 1:1000 (G. Gu, Vanderbilt University, Nashville, TN); rabbit α-Hb9 diluted 1:8000 (Harrison et al., 1999); rabbit α-Isl1 diluted 1:5000 (Tsuchida et al., 1994); rabbit α-IAPP diluted 1:2000 (Advanced Chemtech); rabbit α-PC1/3 diluted 1:2000 (D. Steiner, University of Chicago, Chicago, IL); and rabbit α-GFP diluted 1:2000 (Molecular Probes, A-6455). Secondary α-IgG antibodies were as follows: Cy3-conjugated α-guinea pig, α-rabbit and α-mouse diluted 1:2000 (Jackson Laboratory); Alexa (488 nm)-conjugated α-guinea pig and α-rabbit diluted 1:2000 (Molecular Probes); biotinylated α-mouse diluted 1:200 (Vector Laboratories). Images were collected on a Zeiss Axioplan2 microscope with a Zeiss AxioCam driven by Zeiss AxioVision v. 3.1 software.

Using 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) as a substrate, whole-mount X-gal staining was performed on either whole embryos or on isolated abdominal organs as described previously (Mombaerts et al., 1996)

Quantitative morphometry and cell counting

Cell counting and/or morphometry was performed on every section through the pancreas at E14.5 or E15.5 and on every tenth section at E18.5 from a minimum of three embryos per genotype. Morphometry was conducted using Image-Pro Plus v. 5.0.1 (Media Cybernetics).

RESULTS

Temporal and spatial relationship of the progenitor markers Pdx1, Ngn3 and Nkx6.1

To better understand the mechanism by which Nkx6.1 controls beta-cell development, we characterized the expression of Nkx6.1 with respect to different progenitor cell markers during pancreatic development. To examine whether endocrine commitment coincides with a loss of Pdx1 expression, we performed co-immunofluorescence staining for Pdx1 and Ngn3 at different stages of pancreatic development. At E10.5, only a few Ngn3$^+$ cells were detected in the Pdx1$^+$ pancreatic epithelium and these early endocrine progenitors co-expressed high levels of Pdx1 (Fig. 1A), therefore suggesting that the earliest endocrine progenitors express high levels of Pdx1. While Pdx1 remained abundantly expressed in the undifferentiated epithelium at E12.5, some Ngn3$^+$ progenitors no longer expressed Pdx1 (Fig. 1B). At E14.5 and at later developmental stages, Ngn3$^+$ progenitors were either Pdx1-negative (Pdx1$^–$) or expressed very low levels of Pdx1 (Fig. 1C and data not shown), thus indicating that commitment to the endocrine lineage is associated with the downregulation of Pdx1. Because Pdx1 is highly expressed in differentiated beta-cells (Guz et al., 1995), our findings suggest that, during beta-cell differentiation, progenitors undergo a progression from Pdx1^{high}/Ngn3$^–$/insulin$^–$ → Pdx1^{high}/Ngn3$^+$/insulin$^–$ → Pdx1^{low}/Ngn3$^+$/insulin$^–$ → Pdx1^{high}/Ngn3$^–$/insulin$^+$.

Next, we characterized the expression of Nkx6.1 with respect to Pdx1 and Ngn3. Nkx6.1 and Pdx1 showed largely overlapping domains of expression during development. Both proteins were co-expressed in the undifferentiated pancreatic epithelium at E10.5 and E12.5 (Fig. 1D and data not shown) and in differentiated beta-cells at E18.5 (Fig. 1F). At E14.5, co-localization of Nkx6.1 and Pdx1 was observed in the majority of cells in the central part of the epithelium, whereas the peripherally located exocrine acinar cells expressed only Pdx1 (Fig. 1E). In contrast to Pdx1, high levels of Nkx6.1 expression were maintained in the majority of Ngn3$^+$ endocrine progenitors until E14.5 (Fig. 1G-I). After E14.5, however, the expression domains of Ngn3 and Nkx6.1 diverged and only 26% of Ngn3$^+$ cells also expressed Nkx6.1 at E16.5 (Fig. 2C). Because

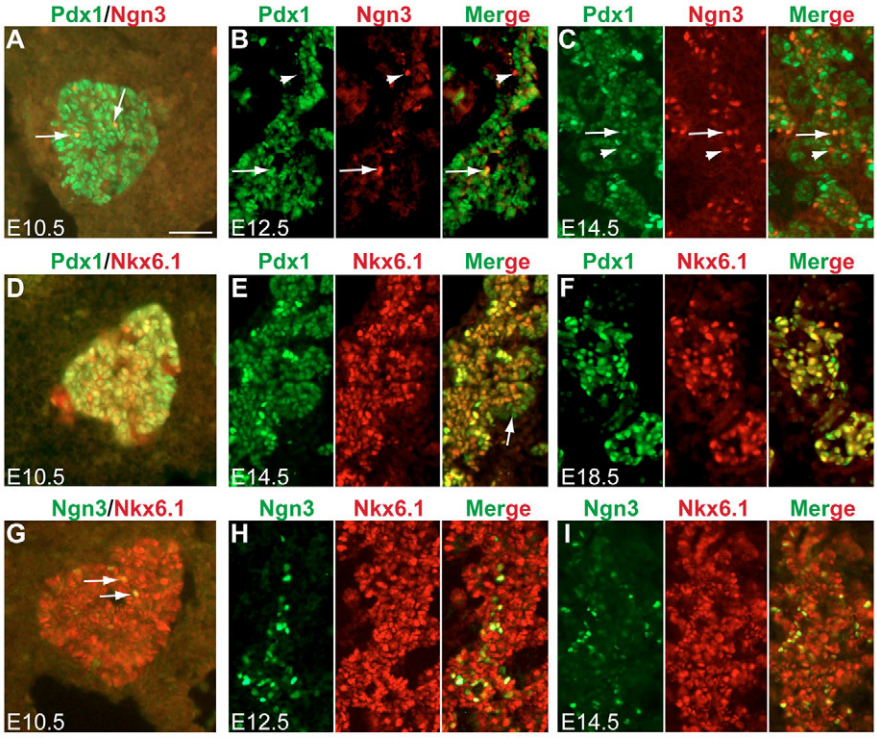

Fig. 1. Nkx6.1 is expressed in similar domains to Ngn3 and Pdx1 during pancreas development. (**A-I**) Immunofluorescence for the indicated proteins in the developing mouse pancreas at E10.5 (dorsal bud; A,D,G), E12.5 (B,H), E14.5 (C,E,I) and E18.5 (F). (A) Ngn3 (red) is co-expressed with Pdx1 (green) at E10.5 (arrows). (B) At E12.5, a subset of Ngn3$^+$ cells does not express Pdx1 (arrowhead), but some Ngn3$^+$ cells maintain Pdx1 expression (arrow). (C) At E14.5, the majority of Ngn3$^+$ cells express low levels of Pdx1. However, some Ngn3$^+$ cells are Pdx1-negative (arrowhead) and some express high levels of Pdx1 (arrow). (D-F) Nkx6.1 (red) is co-expressed with Pdx1 (green) at E10.5 (D), E14.5 (E) and E18.5 (F). Notably, Nkx6.1 is absent from Pdx1$^+$ acini at E14.5 (E, arrow). (G-I) Ngn3 (green) is co-expressed with Nkx6.1 (red) at E10.5 (G, arrows), E12.5 (H) and E14.5 (I). Scale bar: 50 μm.

the restriction of Nkx6.1 to a subset of Ngn3$^+$ cells coincides with the major wave of beta-cell differentiation, we hypothesized that Nkx6.1 activity may commit Ngn3$^+$ endocrine progenitors to a beta-cell fate.

Nkx6.1 expression in endocrine progenitors is not sufficient to specify beta-cell fate

A specific prediction of this hypothesis is that ectopic expression of *Nkx6.1* in all Ngn3$^+$ progenitors will favor their differentiation into beta-cells at the expense of other endocrine lineages. We tested this by directing expression of *Nkx6.1* to the entire Ngn3$^+$ domain with an *Ngn3*-promoter-driven transgene (Fig. 2A). We established five independent transgenic lines and screened each of these lines for expression of the enhanced green fluorescence protein (eGFP) reporter gene in the embryonic pancreas. In two of the mouse lines, we detected high levels of eGFP, which co-localized with Ngn3 in 60% of Ngn3$^+$ cells at E14.5 and E16.5 (Fig. 2B and data not shown). To confirm that not only the eGFP reporter, but also Nkx6.1 protein is expressed from the *Ngn3-Nkx6.1*-IRES-*eGFP* transgene (hereafter abbreviated to *Ngn3-Nkx6.1*), co-staining for Nkx6.1 and Ngn3 was conducted. Because Nkx6.1 was detected in almost all Ngn3$^+$ cells at E14.5 (Fig. 1I), this analysis was carried out at E16.5, when only 26% of Ngn3$^+$ cells co-expressed Nkx6.1 in wild-type embryos (Fig. 2C). In both *Ngn3-Nkx6.1* transgenic lines, the percentage of Ngn3/Nkx6.1 co-positive cells increased to 61% in *Ngn3-Nkx6.1* embryos (Fig. 2D). Our findings confirm previous observations that the 6.7 kb *Ngn3* promoter fragment results in mosaic expression in the Ngn3$^+$ domain (Jenny et al., 2002). Because the *Ngn3-Nkx6.1* transgene did not target all Ngn3$^+$ cells, we selectively studied the fate of transgene-expressing cells by counting their relative contribution to the insulin and glucagon lineages. Although Ngn3 is normally not detected in hormone$^+$ cells (Apelqvist et al., 1999; Gradwohl et al., 2000; Schwitzgebel et al., 2000), *Ngn3*-promoter-directed eGFP can be traced to insulin$^+$ and glucagon$^+$ cells due to the protein stability of eGFP (Li et al., 1998). To study whether expression of Nkx6.1 directs Ngn3$^+$ progenitors to the beta-cell lineage, we compared the ratios of eGFP$^+$;insulin$^+$ and eGFP$^+$;glucagon$^+$ cells to the total number of eGFP$^+$ cells in *Ngn3-Nkx6.1* and in *Ngn3*-IRES-*eGFP* control mice. We did not detect a difference in the percentages of eGFP$^+$;insulin$^+$ and eGFP$^+$;glucagon$^+$ cells or in the total area of insulin$^+$ cells between transgenic and control mice at E18.5 (data not shown), and therefore conclude that Nkx6.1 is not sufficient to confer beta-cell identity to Ngn3$^+$ progenitors.

We next determined whether reconstitution of *Nkx6.1* expression in Ngn3$^+$ progenitors is sufficient to rescue beta-cell development in *Nkx6.1*$^{-/-}$ null mutants. The intercrossing of *Nkx6.1*$^{+/-}$;*Ngn3-Nkx6.1* transgenic mice with *Nkx6.1* heterozygous-mutant mice produced *Nkx6.1*$^{-/-}$;*Ngn3-Nkx6.1* mice, in which all Nkx6.1 protein is derived from the *Ngn3*-promoter-driven transgene. Because Ngn3 expression is not affected in Nkx6.1-deficient embryos (Henseleit et al., 2005; Sander et al., 2000b), the *Ngn3-Nkx6.1* transgene should be similarly expressed in wild-type and in *Nkx6.1*$^{-/-}$ mutant backgrounds. Indeed, 60% of Ngn3$^+$ cells co-expressed Nkx6.1 in *Nkx6.1*$^{-/-}$;*Ngn3-Nkx6.1* embryos (Fig. 3C), which is similar to the fraction of Ngn3$^+$ cells expressing GFP in *Ngn3-Nkx6.1* mice in a wild-type background (Fig. 2B). Consistent with the absence or low abundance of Pdx1 in the Ngn3$^+$ domain in wild-type embryos (Fig. 1C), the cells targeted by the *Ngn3-Nkx6.1* transgene were either Pdx1-negative or expressed low levels of Pdx1 (Fig. 3G). Notably, we observed that the *Ngn3-Nkx6.1* transgene also directed Nkx6.1 expression in a subset of beta-cells (Fig. 3K). This was unexpected, because Ngn3 expression is normally lost before insulin becomes detectable (Apelqvist et al., 1999; Gradwohl et al., 2000; Schwitzgebel et al., 2000). The ectopic expression in insulin$^+$ cells could be explained by differences in protein stability of Nkx6.1 and Ngn3, or alternatively by ectopic expression from the *Ngn3* promoter fragment.

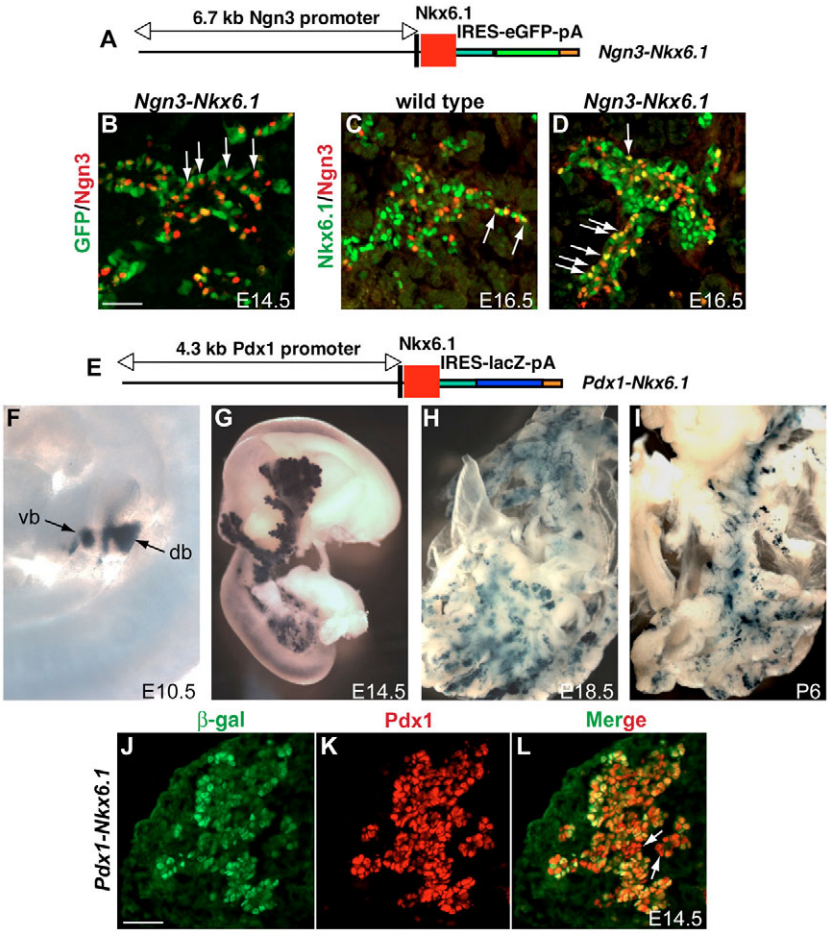

Fig. 2. Characterization of *Ngn3-Nkx6.1* and *Pdx1-Nkx6.1* transgenic mice. (**A**) The *Ngn3-Nkx6.1* transgenic construct comprises the *Ngn3* promoter fused to *Nkx6.1* cDNA followed by an IRES-*eGFP*-polyA cassette. (**B**) In *Ngn3-Nkx6.1* embryos at E14.5, 60% of the transgene-expressing;GFP$^+$ cells express Ngn3 (arrows). (**C**,**D**) Whereas 26% of Ngn3$^+$ cells are co-positive for Nkx6.1 in wild-type embryos at E16.5 (C, arrows), this percentage is increased to 61% in *Ngn3-Nkx6.1* embryos (D, arrows). (**E**) In the *Pdx1-Nkx6.1* transgenic construct, the *Pdx1* promoter drives the expression of *Nkx6.1* cDNA and an IRES-*lacZ*-polyA cassette. (**F-I**) *lacZ* expression in *Pdx1-Nkx6.1* mice is detected in the ventral (vb) and dorsal (db) pancreatic anlagen at E10.5 (F), in the entire pancreatic epithelium at E14.5 (G), and in a subset of pancreatic cells at E18.5 (H) and postnatal day (P)6 (I). (**J-L**) β-galactosidase (β-gal) and Pdx1 are co-expressed in pancreata from *Pdx1-Nkx6.1* mice at E14.5 (J, β-gal in green; K, Pdx1 in red; L, merged). However, some Pdx1$^+$ cells are negative for the β-gal reporter (L, arrows). db/vb, dorsal/ventral pancreatic anlagen. Scale bars: 50 μm in B for B-D and in J for J-L.

Because our analysis demonstrated mainly correct expression of Nkx6.1 from the *Ngn3-Nkx6.1* transgene in Nkx6.1-deficient mice, we next studied whether reconstitution of Nkx6.1 expression in Ngn3$^+$ progenitors is sufficient to rescue beta-cell formation in *Nkx6.1*$^{-/-}$ embryos. Embryos from both *Ngn3-Nkx6.1* lines were analyzed, but each failed to display an obvious rescue of beta-cell number at E14.5 and E18.5 (see Fig. S1A-C in the supplementary material; data not shown). This was corroborated further by quantitative morphometric area measurements for insulin comparing *Nkx6.1*$^{-/-}$;*Ngn3-Nkx6.1* embryos to *Nkx6.1*$^{-/-}$ embryos at E18.5 (see Fig. S1D in the supplementary material). Although it is formally possible that the *Ngn3* promoter specifically fails to target Ngn3$^+$ beta-cell progenitors, the most likely conclusion from these results is that development of the beta-cell lineage requires Nkx6.1 activity outside the Ngn3$^+$ domain.

Beta-cell formation requires Nkx6.1 activity in the Pdx1$^+$ domain

In addition to Ngn3$^+$ progenitors, Nkx6.1 is also widely expressed in Pdx1$^+$ cells (Fig. 1D-F), raising the possibility that the absence of Nkx6.1 in Pdx1$^+$ progenitors causes beta-cell loss in *Nkx6.1*$^{-/-}$ mutants. To examine this possibility, we generated transgenic mice that express *Nkx6.1* under the control of the *Pdx1* promoter. To allow for detection of transgene-expressing cells, an IRES-*lacZ* cassette was introduced downstream of the *Nkx6.1* coding sequence (Fig. 2E). Using X-gal staining on whole embryos, offspring from 5 out of a total of 17 transgenic founders showed specific transgene expression in the dorsal and ventral pancreatic buds (Fig. 2F). X-gal staining of embryos from one of the lines at various developmental stages demonstrated that the transgene recapitulates the pattern of endogenous *Pdx1* expression (Offield et al., 1996) (Fig. 2F-I). This was confirmed by direct co-staining for β-galactosidase (β-gal) and Pdx1 (Fig. 2J-L), which showed β-gal expression in the vast majority of Pdx1$^+$ cells. The pancreas of *Pdx1-Nkx6.1*-IRES-*lacZ* embryos (hereafter abbreviated to *Pdx1-Nkx6.1*) appeared morphologically and histologically normal. Additional morphometric area measurements for insulin and glucagon at E14.5 as well as measurements for pancreatic insulin and glucagon content at birth also failed to show a difference between wild-type and *Pdx1-Nkx6.1* mice (data not shown). We conclude that overexpression of *Nkx6.1* in Pdx1$^+$ cells does not perturb pancreatic morphogenesis and cell differentiation.

To test whether expression of *Nkx6.1* in the Pdx1$^+$ domain is sufficient to restore beta-cell formation in *Nkx6.1*$^{-/-}$-null mice, we crossed four *Pdx1-Nkx6.1* transgenic lines that had confirmed transgene expression at E10.5 into the *Nkx6.1*$^{-/-}$ background. Consistent with the lack of significant Pdx1/Ngn3 co-expression at E14.5 (Fig. 1C), the *Pdx1-Nkx6.1* transgene resulted in a mosaic pattern of very weak or absent Nkx6.1 expression in the Ngn3$^+$ domain (Fig. 3D). Notably, Nkx6.1 protein was absent from the Pdx1$^+$ exocrine acini (Fig. 3H, arrows), although co-staining for β-gal and Pdx1 demonstrated expression of the transgene in the entire Pdx1$^+$ domain (Fig. 2J-L). Because Pdx1 expression is relatively low in acinar cells, this could reflect a difference in the sensitivity of the anti-β-gal and anti-Nkx6.1 antibodies. Consistent with the expression of Pdx1 in beta-cells

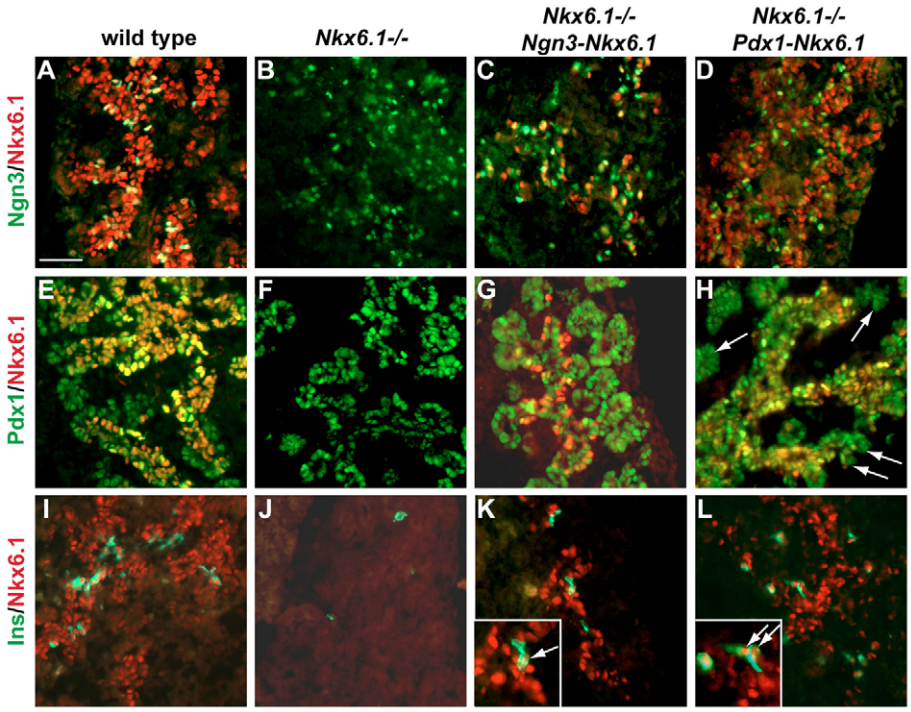

Fig. 3. Characterization of *Ngn3-Nkx6.1* and *Pdx1-Nkx6.1* transgene expression in the *Nkx6.1* mutant background at E14.5. (**A-D**) Immunofluorescence detection of both Nkx6.1 (red; absent in *Nkx6.1*-null mice, B) and Ngn3 (green) shows strong co-expression in wild-type (A) and *Nkx6.1*$^{-/-}$;*Ngn3-Nkx6.1* (C) pancreata, but weak or no co-expression in *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.1* (D) pancreata. A subset of Ngn3^{+} cells is not targeted by the *Ngn3-Nkx6.1* transgene (C). (**E**,**F**,**H**) Nkx6.1 (red; absent in *Nkx6.1*-null mice, F) is co-expressed with Pdx1 (green) in pancreata from wild-type (E) and *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.1* (H) embryos. Notably, Pdx1^{+} acinar cells do not express Nkx6.1 protein in *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.1* embryos (H, arrows). (**G**) *Nkx6.1*$^{-/-}$;*Ngn3-Nkx6.1* embryos display a subset of Nkx6.1^{+} cells (red) with no expression of Pdx1 (green), and another subset with weak Pdx1 expression. (**I**,**J**,**L**) Nkx6.1 (red; absent in *Nkx6.1*-null mice, J) is co-expressed with insulin (Ins, green) in pancreata from wild-type (I) and *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.1* (L, arrows) embryos. (**K**) Occasional co-localization of Nkx6.1 (red) and insulin (green) is also observed in *Nkx6.1*$^{-/-}$;*Ngn3-Nkx6.1* pancreata (arrow). Scale bar: 50 μm.

(Guz et al., 1995), the *Pdx1-Nkx6.1* transgene targeted Nkx6.1 to insulin^{+} cells (Fig. 3L, Fig. 8K). Thus, with the exception of the early acinar cells, the transgene restored Nkx6.1 expression in the entire Pdx1^{+} domain.

Next, we assessed whether *Nkx6.1* expression in the Pdx1^{+} domain can rescue beta-cell formation in *Nkx6.1*$^{-/-}$ mice. In *Nkx6.1*$^{-/-}$ embryos, only one out of three pancreata had detectable insulin^{+} cells at E14.5 (Fig. 4B and data not shown). At this stage, the number of insulin^{+} cells was notably higher in *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.1* embryos from two of the four backcrossed transgenic lines (Fig. 4C), suggesting that the *Pdx1-Nkx6.1* transgene can initiate beta-cell development at the onset of the secondary transition. At E14.5, the average area of insulin^{+} cells was reduced to 3.9% of that of wild type in *Nkx6.1*$^{-/-}$ embryos, but restored to 37% of that of wild type by expression of the *Pdx1-Nkx6.1* transgene (Fig. 4G). Notably, the ability of the transgene to restore beta-cell development at E14.5 varied greatly among individual embryos from one transgenic line, ranging from virtually no rescue to significant rescue. At E18.5, *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.1* embryos displayed a normal-sized pancreas. Embryos from the two

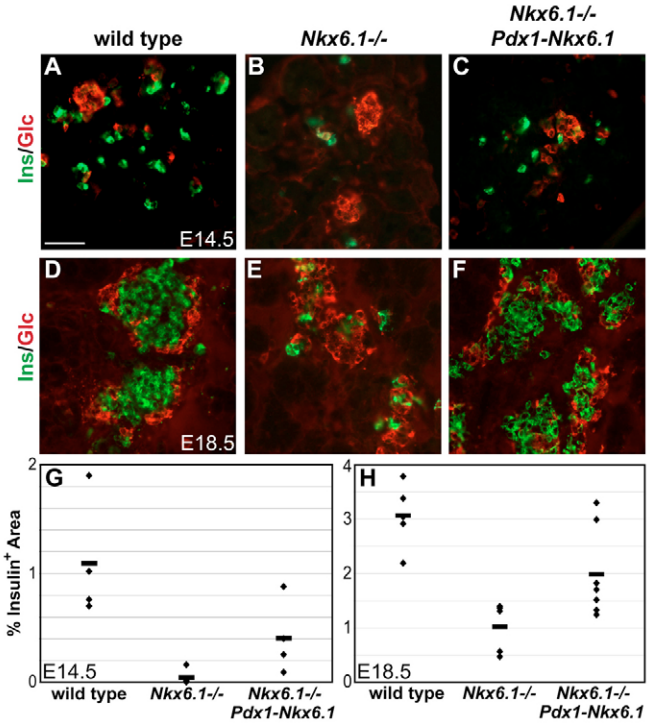

Fig. 4. Expression of Nkx6.1 in the Pdx1^{+} domain can fully restore insulin expression in *Nkx6.1* mutant embryos. (**A-F**) Immunofluorescence detection of insulin (Ins, green) and glucagon (Glc, red). (B,E) *Nkx6.1*$^{-/-}$ embryos have reduced insulin but normal glucagon cell numbers at E14.5 (B) and E18.5 (E) compared to wild type (A,D). (C,F) Expression of the *Pdx1-Nkx6.1* transgene in *Nkx6.1*$^{-/-}$ embryos restores the formation of insulin^{+} cells at E14.5 (C), and restores normal islet morphology and size at E18.5 (F). (**G**) Morphometric quantification of the insulin^{+} area over total pancreatic area at E14.5 reveals an almost complete absence of insulin^{+} cells in *Nkx6.1*$^{-/-}$ embryos and a significant rescue of insulin^{+} cells in a subset of *Nkx6.1*$^{-/-}$ embryos carrying the *Pdx1-Nkx6.1* transgene (n=4). (**H**) At E18.5, the insulin^{+} area is reduced by a threefold average in *Nkx6.1*$^{-/-}$ embryos (n=5) compared to wild-type mice (n=5). The *Pdx1-Nkx6.1* transgene restores the insulin^{+} area in *Nkx6.1*$^{-/-}$ mutants to wild-type values in two embryos, whereas five embryos showed partial or no rescue (n=7). Scale bar: 50 μm.

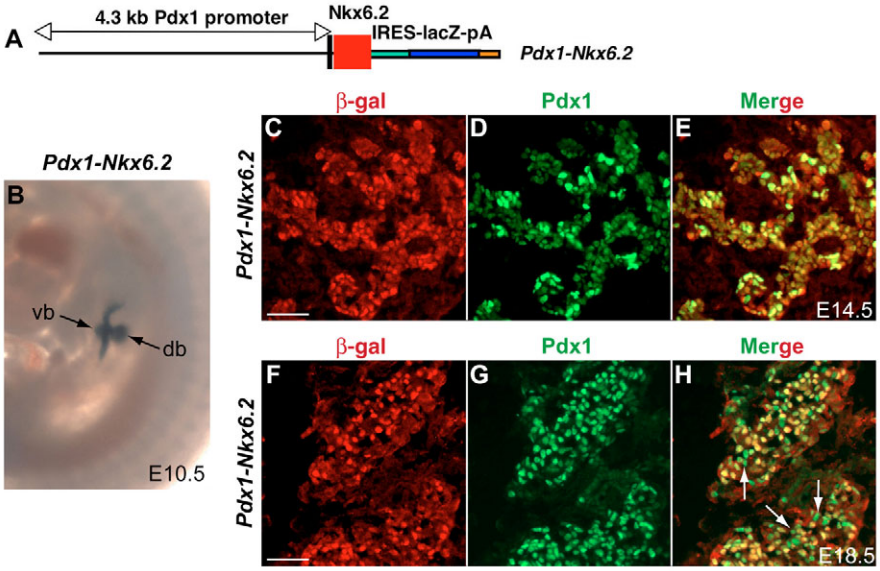

Fig. 5. Characterization of *Pdx1-Nkx6.2* transgenic mice. (**A**) The *Pdx1-Nkx6.2* transgene contains the *Pdx1* promoter fused to *Nkx6.2* cDNA followed by an IRES-*lacZ*-polyA cassette. (**B**) Mirroring the Pdx1$^+$ domain, *lacZ* expression in *Pdx1-Nkx6.2* mice is detected in the ventral (vb) and dorsal (db) pancreas anlagen at E10.5. (**C-H**) In *Pdx1-Nkx6.2* mice, expression of β-galactosidase (β-gal, red) and Pdx1 (green) overlaps completely at E14.5 (C-E), whereas a subset of Pdx1$^+$ cells fails to express β-gal at E18.5 (F-H, arrows in H). db/vb, dorsal/ventral pancreatic anlagen Scale bars: 50 μm.

backcrossed transgenic lines that restored insulin expression at E14.5 showed complete rescue of beta-cells as well as normal islet architecture, with large clusters of insulin$^+$ cells surrounded by glucagon$^+$ cells (Fig. 4F). The number of glucagon-, somatostatin- and PP-producing cells was similar in all genotypes (data not shown). To systematically assess the extent of beta-cell restoration with the two rescuing transgenic lines, we performed quantitative morphometry of insulin cell area in wild-type, *Nkx6.1*$^{-/-}$ and *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.1* embryos. This analysis revealed a cohort with low and a cohort with high insulin cell area within each genotype (Fig. 4H). Some *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.1* embryos showed full beta-cell recovery to wild-type numbers, whereas others were only partially rescued. Comparison of Nkx6.1 expression in different *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.1* embryos from the same transgenic founder revealed variant degrees of mosaic transgene expression (see Fig. S2 in the supplementary material), which could account for the variability in beta-cell rescue. An additional contributing factor could be the outbred genetic background. This idea is supported by our observation that a subset of *Nkx6.1* mutants showed a significantly smaller reduction in beta-cell number at E18.5 than previously observed on the C57BL/6J background (Sander et al., 2000b). Together, our results demonstrate that beta-cell development requires Nkx6.1 activity in the Pdx1$^+$ domain, whereas expression in Ngn3$^+$ progenitors is insufficient to induce beta-cell formation. Notably, because the mice died postnatally due to a defect in motor neuron development (Sander et al., 2000a), we could not conduct studies of beta-cell function in *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.1* mice.

Nkx6.1 and Nkx6.2 have similar biochemical activities in vivo

We have shown previously that the Nkx6.1 paralog Nkx6.2 has fully redundant functions to Nkx6.1 in the development of alpha- but not of beta-cells (Henseleit et al., 2005). The lack of compensation in beta-cell formation could be explained either by differences in the biochemical properties of the two proteins or by their distinct spatial and temporal expression domains in the developing pancreas. To distinguish between these possibilities, we genetically compared the ability of Nkx6.1 and Nkx6.2 to restore beta-cell formation in Nkx6.1-deficient mice. Analogous to the approach described for *Pdx1-Nkx6.1* transgenic mice, we generated *Pdx1-Nkx6.2*-IRES-*lacZ* (*Pdx1-Nkx6.2*) mice (Fig. 5A) and identified four mouse lines that expressed the *lacZ* reporter exclusively in the Pdx1$^+$ domain (Fig. 5B). The transgene was expressed in virtually all Pdx1$^+$ cells at E14.5 (Fig. 5C-E). Similar to the *Pdx1-Nkx6.1* line, mosaic transgene expression in the Pdx1$^+$ domain was observed at E18.5 (Fig. 5F-H).

We crossed the four *Pdx1-Nkx6.2* mouse lines into the *Nkx6.1*$^{-/-}$-null mutant background and compared the ability of the *Pdx1-Nkx6.2* and *Pdx1-Nkx6.1* transgenes to rescue beta-cell formation. Similar to the *Pdx1-Nkx6.1* transgene, expression of the *Pdx1-Nkx6.2* transgene resulted in a significant rescue of insulin$^+$ cells as early as E14.5 (Fig. 6C). At E18.5, *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.2* embryos displayed large islet clusters that were indistinguishable from *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.1* or wild-type embryos (Fig. 6F). As expected, the *Pdx1-Nkx6.2* transgene correctly targeted β-gal expression to the insulin$^+$ cells (Fig. 8L). These results demonstrate that Nkx6.1 and Nkx6.2 have a similar ability to induce beta-cell formation and islet morphogenesis. Again mirroring our observation in *Pdx1-Nkx6.1* rescues, morphometric quantification of five independent embryos from one transgenic founder revealed a cohort with a partially and a cohort with a fully restored insulin cell area in *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.2* embryos (Fig. 6G). Overall, we found that only one out of the four transgenic lines significantly rescued beta-cell development. In lines that failed to rescue, we detected little or no β-gal expression at E18.5 (data not shown).

Nkx6.1 and Nkx6.2 both restore expression of Nkx6.1 targets and beta-cell maturation

To study whether the *Pdx1-Nkx6* transgenes fully restore a normal program of beta-cell differentiation, we analyzed rescued embryos for expression of the endocrine differentiation factor Myt1 (myelin transcription factor 1). Myt1 is a zinc-finger transcription factor that has recently been shown to function as an obligatory cofactor of Ngn3 in endocrine differentiation (Gu et al., 2004). Because pancreatic *Myt1* expression is reduced in *Nkx6.1* mutants (Henseleit et al., 2005), we tested whether the *Pdx1-Nkx6* transgenes restore the expression of *Myt1*. Confirming the findings from our in situ hybridization experiments (Henseleit et al., 2005), immunofluorescence staining with an anti-Myt1 antibody showed a

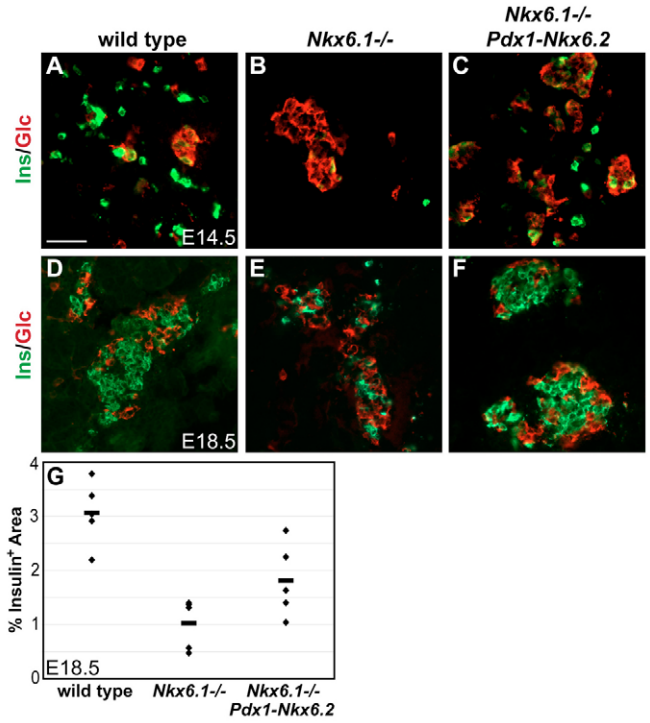

Fig. 6. Expression of Nkx6.2 in the Pdx1$^+$ domain restores insulin expression in *Nkx6.1* mutant embryos. (**A-F**) Immunofluorescence detection of insulin (Ins, green) and glucagon (Glc, red). (B,E) *Nkx6.1*$^{-/-}$ embryos have reduced insulin but normal glucagon cell numbers at E14.5 (B) and E18.5 (E) compared to wild type (A,D). (C,F) Expression of the *Pdx1-Nkx6.2* transgene in *Nkx6.1*$^{-/-}$ embryos restores the formation of insulin$^+$ cells at E14.5 (C) and restores normal islet morphology and size at E18.5 (F). (**G**) Morphometric quantification of the insulin$^+$ area over total pancreatic area in E18.5 embryos reveals an average threefold reduction of insulin$^+$ area in *Nkx6.1*$^{-/-}$ (*n*=5) compared to wild-type mice (*n*=5). The *Pdx1-Nkx6.2* transgene restores the insulin$^+$ area in *Nkx6.1*$^{-/-}$ mutants to wild-type values in two embryos, whereas three embryos showed partial or no rescue (*n*=5). (Compare with Fig. 4.) Scale bar: 50 μm.

marked reduction in the number of Myt1$^+$ cells in *Nkx6.1* mutant embryos (Fig. 7A,B,E). In embryos, in which insulin expression was rescued by the *Pdx1-Nkx6.1* and *Pdx1-Nkx6.2* transgenes, the number of Myt1$^+$ cells was also restored to almost wild-type values (Fig. 7A,C-E). These findings confirm that Myt1 is a target of Nkx6 factors, and demonstrate that Nkx6.1 and Nkx6.2 have similar activity in restoring *Myt1* expression in Nkx6.1-deficient pancreata.

To substantiate further that expression of *Nkx6.1* or *Nkx6.2* in the Pdx1$^+$ domain can fully restore beta-cell development, we studied whether rescued insulin$^+$ cells express common beta-cell markers. At E18.5, beta-cells are marked by the expression of glucose transporter 2 (*Glut2*) and the transcription factor *MafA* (Fig. 8A,E), both of which are absent from the scattered insulin$^+$ cells in *Nkx6.1*$^{-/-}$ mice (Fig. 8B,F). Expression of the *Pdx1-Nkx6.1* or *Pdx1-Nkx6.2* transgene in Nkx6.1-deficient mice restored the expression of both Glut2 and MafA in the majority of beta-cells (Fig. 8C,D,G,H). Likewise, beta-cells in *Pdx1-Nkx6.1*- or *Pdx1-Nkx6.2*-rescued mice expressed the markers Pdx1, Hb9, Isl1, IAPP and PC1/3 (also known as Pcsk1 – Mouse Genome Informatics) the same as in wild-type pancreata (see Fig. S3 in the supplementary material), thus demonstrating that Nkx6.1 and Nkx6.2 both mediate beta-cell maturation, when expressed in the Pdx1$^+$ domain. We noted that a subset of insulin$^+$ cells in *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.1* and *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.2* embryos did not express MafA (Fig. 8G,H). This could be explained by insufficient expression of *Nkx6.1* or *Nkx6.2* from the *Pdx1*-promoter-driven transgene in a subset of beta-cells. Consistent with this idea, Nkx6.1 production from the *Pdx1-Nkx6.1* transgene was not detected in all beta-cells (Fig. 8K), whereas endogenous Nkx6.1 was uniformly expressed in beta-cells of wild-type embryos (Fig. 8I).

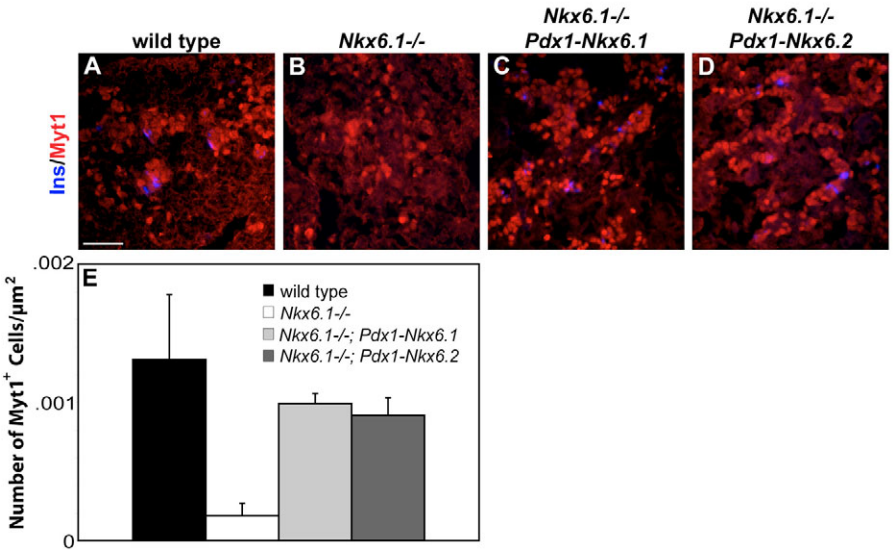

Fig. 7. Expression of Nkx6.1 or Nkx6.2 in the Pdx1 domain restores Myt1 expression in *Nkx6.1* mutant embryos. (**A-D**) Immunofluorescence detection of Myt1 (red) and insulin (Ins, blue) in pancreata from wild-type (A), *Nkx6.1*$^{-/-}$ (B), *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.1* (C) and *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.2* (D) embryos at E14.5. (B-D) *Nkx6.1*$^{-/-}$ embryos have reduced Myt1 expression (B), which is restored by expression of the *Pdx1-Nkx6.1* (C) or *Pdx1-Nkx6.2* (D) transgenes. (**E**) Morphometric quantification of the number of Myt1$^+$ cells over total pancreatic area in E14.5 embryos reveals an average reduction of Myt1$^+$ cells in *Nkx6.1*$^{-/-}$ to 14% of wild-type numbers (*n*=3). The *Pdx1-Nkx6.1* or *Pdx1-Nkx6.2* transgene restores the number of Myt1$^+$ cells in *Nkx6.1*$^{-/-}$ mutants to 75% and 70% of wild-type values, respectively (*n*=3). Values in E represent averages ±s.e.m. Scale bar: 50 μm.

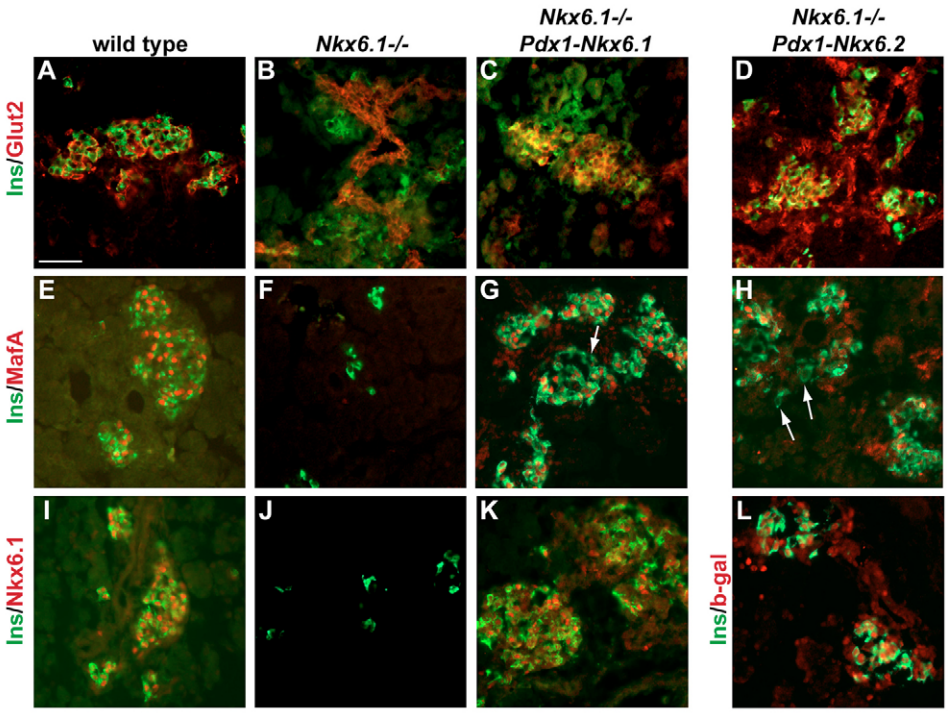

Fig. 8. Expression of Nkx6.1 or Nkx6.2 in the Pdx1$^+$ domain restores beta-cell development and maturation in *Nkx6.1* mutant embryos. Immunofluorescence detection of Glut2 (**A-D**, red), MafA (**E-H**, red), Nkx6.1 (**I-K**, red) or β-galactosidase (β-gal; **L**, red) with insulin (Ins, green) in pancreata from wild-type (A,E,I), *Nkx6.1*$^{-/-}$ (B,F,J), *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.1* (C,G,K) and *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.2* (D,H,L) embryos at E18.5. (A,B) Whereas Glut2 expression is confined to insulin$^+$ cells in wild-type embryos (A), insulin$^+$ cells are Glut2-negative in *Nkx6.1*$^{-/-}$ mutants (B). (B) Notably, *Nkx6.1*$^{-/-}$ embryos display clusters of Glut2$^+$, but insulin-negative, cells adjacent to the insulin$^+$ domain. (C,D) The *Pdx1-Nkx6.1* and *Pdx1-Nkx6.2* transgenes restore the formation of insulin/Glut2-co-expressing cells in the Nkx6.1-deficient background. Some insulin$^+$/Glut2-negative as well as Glut2$^+$/insulin-negative cells remain in transgene-expressing embryos. (F) In *Nkx6.1*$^{-/-}$ embryos, insulin$^+$ cells do not express MafA. (G,H) MafA expression is restored in the majority of, but not in all, insulin$^+$ cells by the *Pdx1-Nkx6.1* and *Pdx1-Nkx6.2* transgenes (arrows point to MafA-negative/insulin$^+$ cells). (K,L) Sustained expression of the *Pdx1-Nkx6.1* and *Pdx1-Nkx6.2* transgenes in rescued insulin$^+$ cells is confirmed by the detection of Nkx6.1 (K) or β-gal (L), respectively. Scale bar: 50 μm.

Interestingly, although absent from beta-cells, 'strings' of Glut2$^+$ cells were found in the vicinity of the developing islets in *Nkx6.1*$^{-/-}$ mutants (Fig. 8B). These cells did not express endocrine hormones, the islet-marker glucagon, chromogranin A, IAPP, PC1/3 or islet transcriptions factors, such as Pdx1, Hb9, Isl1 or Pax6 (data not shown). In wild-type embryos, similar 'strings' of hormone-negative Glut2$^+$ cells were detected in the ductal network of the embryonic pancreas at E14 (Pang et al., 1994) (data not shown), but were no longer present at birth (Fig. 8A). Because beta-cells have been suggested to develop from the Glut2$^+$ ductal network (Pang et al., 1994), it is possible that the Glut2$^+$ cells in *Nkx6.1*$^{-/-}$ mutant pancreata represent arrested precursor cells.

We conclude from our studies that Nkx6.1 and Nkx6.2 have equivalent abilities to induce beta-cell formation and maturation, therefore suggesting that the unique requirement for Nkx6.1 in the development of the beta-cell lineage is a consequence of differences in the spatial and temporal regulation, but not in the biochemical activities, of the two Nkx6 proteins.

DISCUSSION

In this study, we have addressed whether Nkx6 transcription factors control endocrine subtype specification at the level of Ngn3$^+$ progenitors, which have already committed to an endocrine differentiation path. We show that beta-cell development in *Nkx6.1* mutants is rescued upon restoring Nkx6.1 or Nkx6.2 activity in the pancreatic progenitor Pdx1$^+$ domain, but not upon restoring Nkx6.1 in the Ngn3$^+$ domain. We discuss below why our data support the idea that beta-cell fate specification requires Nkx6.1 activity prior to Ngn3-mediated activation of an endocrine program. These findings have important implications for designing appropriate in vitro protocols that aim to differentiate beta-cells from stem cells.

Nkx6.1 and Nkx6.2 possess fully equivalent functions in pancreatic endocrine development

Interestingly, despite the inability of endogenous Nkx6.2 to compensate for Nkx6.1 in beta-cell development, we found that Nkx6.2 restores beta-cell formation and maturation in *Nkx6.1* mutants when expressed under the *Pdx1* promoter. Moreover, Nkx6.2 was able to induce the expression of Nkx6.1 downstream effectors and beta-cell-specific markers. This demonstrates that both Nkx6 factors possess equivalent biochemical activities in endocrine differentiation. Similarly, although only Nkx6.1 is required for motor neuron development in vivo, Nkx6.1 and Nkx6.2 are both able to induce motor neurons when misexpressed in the neural tube (Vallstedt et al., 2001). Hence, the functional differences of Nkx6.1 and Nkx6.2 seem to be largely determined by the divergent expression pattern of the two genes during embryogenesis. Whereas Nkx6.2 is strongly expressed in Pdx1$^+$ pancreatic progenitors between E8.5 and E10.5, its expression rapidly declines thereafter, and Nkx6.1 becomes the predominantly expressed Nkx6 factor between E11.5 and E13 (Henseleit et al., 2005; Pedersen et al., 2005). It is therefore possible that the inability of Nkx6.2 to compensate for Nkx6.1 in beta-cell development is a result of insufficient Nkx6.2 levels in undifferentiated progenitors between E11.5 and E13. This model fits remarkably well with the results of a recent study, which indicates that specification of the different endocrine subtypes is controlled in a temporal manner (Johansson et al., 2007). Via rescue experiments with an inducible *Ngn3* transgene in *Ngn3* mutants, Johansson et al. have shown that induction of the *Ngn3* transgene between E8.5 and E10.5 specifically restores alpha-cell development, whereas beta-cells are restored after E11.5 (Johansson et al., 2007). Thus, the time-window of competence for beta-cell formation correlates with the peak of Nkx6.1 activity and with the downregulation of Nkx6.2 in Pdx1$^+$

pancreatic progenitors. Our observation that Nkx6.1 and Nkx6.2 are functionally equivalent has important implications for understanding the role of Nkx6 factors in endocrine development. Together with our previous finding that alpha-cell development is redundantly controlled by Nkx6.1 and Nkx6.2 (Henseleit et al., 2005), it lends further support to the idea that Nkx6 factors specify the endocrine compartment as a whole, rather than determining the fate choice towards a specific endocrine subtype. This implies that subtype identity is conferred by additional factors that may function in concert with Nkx6.1 or Nkx6.2.

Both with the *Pdx1-Nkx6.1* and the *Pdx1-Nkx6.2* transgenes, we observed incomplete rescue of beta-cell mass in a subset of embryos. This could be explained by the variant degrees of mosaicism in transgene expression that we observed or by differences in endogenous Nkx6.1 protein levels. An alternative possibility is that partial restoration of beta-cell mass in *Nkx6.1* mutants could reflect spatial differences in the expression domains of endogenous *Nkx6.1* and the *Pdx1*-promoter-driven *Nkx6.1* transgene. Indeed, a key difference between the expression of Pdx1 and Nkx6.1 is that Nkx6.1 is expressed at high levels in Ngn3$^+$ endocrine progenitors, whereas Pdx1 expression is markedly downregulated in these cells (see Fig. 1C and Fig. 3D). We consider it unlikely, however, that the lack of Nkx6.1 in Ngn3$^+$ cells accounts for the incomplete rescue with the *Pdx1-Nkx6.1* transgene, because we did not observe a consistent increase in beta-cell mass when an additional *Ngn3-Nkx6.1* transgene was crossed into the *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.1* background (data not shown).

Mechanisms of beta-cell specification

Our finding that the *Pdx1-Nkx6.1*, but not the *Ngn3-Nkx6.1*, transgene restored beta-cell number in *Nkx6.1* mutants implies that beta-cell development requires *Nkx6.1* expression in Pdx1$^+$ progenitors. Because Pdx1 displays a dynamic pattern of expression during beta-cell development, with high levels in undifferentiated, multipotential pancreatic progenitors, absence or low levels in Ngn3$^+$ endocrine progenitors and again high levels in differentiated beta-cells (see Fig. 1) (Jensen et al., 2000; Maestro et al., 2003), this raises the question of which cell population requires Nkx6.1 activity to restore beta-cell formation in *Nkx6.1* mutants? It could reflect a requirement for Nkx6.1 activity either in early multipotential progenitors before *Ngn3* expression is initiated or in late beta-cell progenitors that have reactivated *Pdx1* expression. Several lines of evidence support the notion that the earliest requirement for Nkx6.1 activity is at the level of multipotential Pdx1$^+$ progenitors prior to the activation of *Ngn3*. First, if Nkx6.1 controlled the development of both alpha- and beta-cells exclusively in late progenitors after the downregulation of *Ngn3* expression, one would expect co-expression of Nkx6.1 and Nkx6.2 in early glucagon$^+$ cells. However, whereas Nkx6.1 and Nkx6.2 are co-expressed in early Pdx1$^+$ progenitors, Nkx6.1 expression is absent from the glucagon$^+$ domain (Henseleit et al., 2005; Sander et al., 2000b). Second, if beta-cell specification required Nkx6.1 solely in late beta-cell progenitors, we would expect to have seen some degree of beta-cell rescue with the *Ngn3-Nkx6.1* transgene, because this transgene was ectopically expressed in beta-cells (see Fig. 3K) and therefore targeted at least a subpopulation of late beta-cell progenitors. Therefore, our findings imply that the initiation of beta-cell development requires Nkx6.1 activity in mitotically active, multipotential Pdx1$^+$ pancreatic progenitors. In this respect, the mechanism by which Nkx6.1 controls beta-cell development appears to be distinct from other transcription factors with solely lineage-restricted functions in endocrine differentiation, such as Pax4 and Arx. Unlike Pax4 and Arx, which are both expressed in a *Ngn3*-dependent manner (Collombat et al., 2003; Gradwohl et al., 2000), expression of Nkx6.1 is maintained in Ngn3-deficient embryos (S.B.N., unpublished observation). Moreover, loss of *Pax4* or *Arx* expression is not associated with a reduction in overall endocrine mass, but with an endocrine-fate switch from beta-cells to ghrelin$^+$ cells in *Pax4* mutants and from alpha-cells to beta- and delta-cells in *Arx* mutants (Collombat et al., 2005; Collombat et al., 2003; Prado et al., 2004).

Importantly, a requirement for Nkx6.1 in beta-cell specification before the initiation of *Ngn3* expression does not preclude an additional function for Nkx6.1 in beta-cell maturation. Such an additional role for Nkx6.1 in beta-cells is consistent with our observation that some of the rescued insulin$^+$ clusters in *Nkx6.1*$^{-/-}$;*Pdx1-Nkx6.1* embryos failed to restore *MafA* expression (Fig. 8G). Because expression of the *Pdx1-Nkx6.1* transgene in mature beta-cells is partially mosaic, this could reflect a role for Nkx6.1 in activating *MafA* expression in beta-cells. Moreover, it is possible that the Glut2$^+$/hormone-negative cells that we observed adjacent to the endocrine clusters in *Nkx6.1* mutant embryos represent arrested beta-cell precursors. To substantiate this hypothesis, it needs to be tested whether the Glut2$^+$ cells in *Nkx6.1* mutants arise from Ngn3$^+$ progenitors.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/13/2491/DC1

References

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.

Collombat, P., Mansouri, A., Hecksher-Sorensen, J., Serup, P., Krull, J., Gradwohl, G. and Gruss, P. (2003). Opposing actions of Arx and Pax4 in endocrine pancreas development. *Genes Dev.* **17**, 2591-2603.

Collombat, P., Hecksher-Sorensen, J., Broccoli, V., Krull, J., Ponte, I., Mundiger, T., Smith, J., Gruss, P., Serup, P. and Mansouri, A. (2005). The simultaneous loss of Arx and Pax4 genes promotes a somatostatin-producing cell fate specification at the expense of the alpha- and beta-cell lineages in the mouse endocrine pancreas. *Development* **132**, 2969-2980.

D'Amour, K. A., Bang, A. G., Eliazer, S., Kelly, O. G., Agulnick, A. D., Smart, N. G., Moorman, M. A., Kroon, E., Carpenter, M. K. and Baetge, E. E. (2006). Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat. Biotechnol.* **24**, 1392-1401.

Gannon, M., Gamer, L. W. and Wright, C. V. (2001). Regulatory regions driving developmental and tissue-specific expression of the essential pancreatic gene pdx1. *Dev. Biol.* **238**, 185-201.

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.

Gu, G., Dubauskaite, J. and Melton, D. A. (2002). Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. *Development* **129**, 2447-2457.

Gu, G., Wells, J. M., Dombkowski, D., Preffer, F., Aronow, B. and Melton, D. A. (2004). Global expression analysis of gene regulatory pathways during endocrine pancreatic development. *Development* **131**, 165-179.

Guz, Y., Montminy, M. R., Stein, R., Leonard, J., Gamer, L. W., Wright, C. V. and Teitelman, G. (1995). Expression of murine STF-1, a putative insulin gene transcription factor, in beta cells of pancreas, duodenal epithelium and pancreatic exocrine and endocrine progenitors during ontogeny. *Development* **121**, 11-18.

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.

Heller, R. S., Jenny, M., Collombat, P., Mansouri, A., Tomasetto, C., Madsen, O. D., Mellitzer, G., Gradwohl, G. and Serup, P. (2005). Genetic determinants of pancreatic epsilon-cell development. *Dev. Biol.* **286**, 217-224.

Henseleit, K. D., Nelson, S. B., Kuhlbrodt, K., Hennings, J. C., Ericson, J. and Sander, M. (2005). NKX6 transcription factor activity is required for alpha- and beta-cell development in the pancreas. *Development* **132**, 3139-3149.

Jenny, M., Uhl, C., Roche, C., Duluc, I., Guillermin, V., Guillemot, F., Jensen, J., Kedinger, M. and Gradwohl, G. (2002). Neurogenin3 is differentially required for endocrine cell fate specification in the intestinal and gastric epithelium. *EMBO J.* **21**, 6338-6347.

Jensen, J. (2004). Gene regulatory factors in pancreatic development. *Dev. Dyn.* **229**, 176-200.

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.

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.

Johansson, K. A., Dursun, U., Jordan, N., Gu, G., Beermann, F., Gradwohl, G. and Grapin-Botton, A. (2007). Temporal control of neurogenin3 activity in pancreas progenitors reveals competence windows for the generation of different endocrine cell types. *Dev. Cell* **12**, 457-465.

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.

Li, X., Zhao, X., Fang, Y., Jiang, X., Duong, T., Fan, C., Huang, C. C. and Kain, S. R. (1998). Generation of destabilized green fluorescent protein as a transcription reporter. *J. Biol. Chem.* **273**, 34970-34975.

Maestro, M. A., Boj, S. F., Luco, R. F., Pierreux, C. E., Cabedo, J., Servitja, J. M., German, M. S., Rousseau, G. G., Lemaigre, F. P. and Ferrer, J. (2003). Hnf6 and Tcf2 (MODY5) are linked in a gene network operating in a precursor cell domain of the embryonic pancreas. *Hum. Mol. Genet.* **12**, 3307-3314.

Mombaerts, P., Wang, F., Dulac, C., Chao, S. K., Nemes, A., Mendelsohn, M., Edmondson, J. and Axel, R. (1996). Visualizing an olfactory sensory map. *Cell* **87**, 675-686.

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.

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.

Pedersen, J. K., Nelson, S. B., Jorgensen, M. C., Henseleit, K. D., Fujitani, Y., Wright, C. V., Sander, M. and Serup, P. (2005). Endodermal expression of Nkx6 genes depends differentially on Pdx1. *Dev. Biol.* **288**, 487-501.

Prado, C. L., Pugh-Bernard, A. E., Elghazi, L., Sosa-Pineda, B. and Sussel, L. (2004). Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. *Proc. Natl. Acad. Sci. USA* **101**, 2924-2929.

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.

Sander, M., Paydar, S., Ericson, J., Briscoe, J., Berber, E., German, M., Jessell, T. M. and Rubenstein, J. L. (2000a). Ventral neural patterning by Nkx homeobox genes: Nkx6.1 controls somatic motor neuron and ventral interneuron fates. *Genes Dev.* **14**, 2134-2139.

Sander, M., Sussel, L., Conners, J., Scheel, D., Kalamaras, J., Dela Cruz, F., Schwitzgebel, V., Hayes-Jordan, A. and German, M. (2000b). Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of beta-cell formation in the pancreas. *Development* **127**, 5533-5540.

Schwitzgebel, V. M. (2001). Programming of the pancreas. *Mol. Cell. Endocrinol.* **185**, 99-108.

Schwitzgebel, V. M., Scheel, D. W., Conners, J. R., Kalamaras, J., 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.

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.

Tsuchida, T., Ensini, M., Morton, S. B., Baldassare, M., Edlund, T., Jessell, T. M. and Pfaff, S. L. (1994). Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. *Cell* **79**, 957-970.

Vallstedt, A., Muhr, J., Pattyn, A., Pierani, A., Mendelsohn, M., Sander, M., Jessell, T. M. and Ericson, J. (2001). Different levels of repressor activity assign redundant and specific roles to Nkx6 genes in motor neuron and interneuron specification. *Neuron* **31**, 743-755.

Wilson, M. E., Scheel, D. and German, M. S. (2003). Gene expression cascades in pancreatic development. *Mech. Dev.* **120**, 65-80.