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The concerted activities of Pax4 and Nkx2.2 are essential to initiate pancreatic β-cell differentiation

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Abstract

Pancreatic β cells play a central role in maintaining glucose homeostasis because they secrete insulin in response to increased level of blood glucose; failure of this capacity constitutes a major component of the pathogenesis of diabetes. The identification of key regulators of pancreatic β-cell differentiation is relevant for the overall understanding of this process and for future experiments aimed at regenerating insulin-producing β cells from pancreatic or embryonic stem cells. Several studies using transgenic or knockout mice have established that the development and function of pancreatic β cells are controlled by several genes encoding specific transcription factors. By inactivating the homeobox gene *Pax4*, we previously demonstrated that its function is required for the formation of mature insulin-producing cells. Here, we show that during pancreas ontogeny, *Pax4* is expressed in differentiating endocrine cells, including β cells. Pax4 activity appears essential for appropriate initiation of β-cell differentiation because loss of Pax4 prevents the expression of Pdx1, HB9 and insulin in β-cell precursors. This role of *Pax4* appears to be accomplished via its genetic interaction with another homeobox gene, *Nkx2.2*.
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Introduction

In mammals, the endocrine pancreas is composed of four cell types (β-, α-, δ-, and PP-cells) that collectively regulate glucose homeostasis (Bramblett et al., 2000; Slack, 1995). The role of β cells is fundamental; β cells secrete insulin in response to an increased blood glucose level after food intake, thereby regulating the circulating level of glucose within a very narrow range. Maintenance of the integrity of pancreatic β cells is essential for the prevention of diabetes, a disease prevalent throughout the world. In recent years, significant effort has been made toward identifying the mechanism(s) responsible for the specification and maturation of β cells; this knowledge will provide the molecular basis for generating insulin-producing cells from pancreatic or embryonic stem cells.

The early developing pancreatic epithelium contains multipotent progenitors capable of giving rise to all pancreatic cell lineages (Gu et al., 2002; Herrera, 2002). Therefore, a specific program of gene expression must restrict the developmental potential of pancreatic progenitors and promote their differentiation. The identification and functional characterization of transcription factors expressed in embryonic β cells have yielded important clues about the molecular mechanisms that regulate the development of this cell lineage (Bramblett et al., 2000; Edlund, 1999; Wilson et al., 2003). For example, the basic helix-loop-helix (bHLH) family member Ngn3 specifies an endocrine-cell fate (Apelqvist et al., 1999; Gradwohl et al., 2000); Beta2/NeuroD, another bHLH protein, regulates their appropriate exit from the cell cycle (Naya et al., 1997); and Islet1, a LIM-family member, controls the differentiation of postmi-

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totic endocrine progenitors (Ahlgren et al., 1997). The activity of the NK-family member and homeodomain protein Nkx2.2 is necessary for the maturation of β cells (Sussel et al., 1998), whereas its distant homologue Nkx6.1 controls their expansion (Sander et al., 2000). Finally, the corresponding functions of two members of the Pax family of transcription factors, Pax4 and Pax6, are also important for β-cell development (Dohrmann et al., 2000; Sander et al., 1997; Sosa-Pineda et al., 1997; St-Onge et al., 1997).

Our previous characterization of newborn *Pax4*-deficient (*Pax4*$^{-/-}$) mice showed that their pancreata were almost entirely devoid of mature β cells, a result indicating that Pax4 is a crucial regulator of β-cell development (Sosa-Pineda et al., 1997). Many questions about the function of Pax4 in the pancreas, however, still remain unanswered; for example, what step does Pax4 control during β-cell development, and what are the relative positions of other factors in the Pax4-mediated pathway in endocrine precursors?

To gain further insight into the role of Pax4 in β-cell development, we performed a comparative analysis of gene expression in the pancreata of wild-type and *Pax4*$^{-/-}$ littermates at various developmental stages. We also sought to identify possible genetic interactions between *Pax4* and two other genes (*Pax6* and *Nkx2.2*) that also control β-cell differentiation, by comparing the pancreatic alterations in mice that are deficient in each of these genes and in mice that are double-nullizygous for *Pax4* and *Nkx2.2* (*Pax4*$^{-/-}$ *Nkx2.2*$^{-/-}$). Overall, our results support the proposal that Pax4 and Nkx2.2 are two key components of the molecular machinery responsible for initiating pancreatic β-cell differentiation.

Materials and methods

Animals

Pax4 and *Nkx2.2* heterozygous mice were generated by homologous recombination as previously described (Sosa-Pineda et al., 1997; Sussel et al., 1998); these animals were maintained into an NMRI background. *Sey*$^{NEU/+}$ mice have been previously described (Hill et al., 1991). *Pax4*$^{+/-}$ *Nkx2.2*$^{+/-}$ double heterozygous mice were generated by crossing animals carrying null alleles of *Pax4* (*Pax4*$^{+/-}$) and *Nkx2.2* (*Nkx2.2*$^{+/-}$). Genotyping of mice and embryos was performed as described previously (Hill et al., 1991; Sosa-Pineda et al., 1997; Sussel et al., 1998).

Processing of embryos and pancreatic tissues

Tissues of dissected embryos or pancreata of newborn mice to be analyzed by immunohistochemical methods or in situ hybridization were fixed overnight by immersion in 4% paraformaldehyde at 4°C. After this step, the tissues were subjected to cryoprotection in 30% sucrose in phosphate-buffered saline (PBS) overnight at 4°C and were then embedded in tissue-freezing medium (Tissue-Tek, Triangle Biomedical Sciences). Tissues were cut into sections (section width: 8–12 μm) with a cryostat.

Immunohistochemical analysis

Frozen sections were used for immunohistochemical assays. Primary antibodies were the following: rat anti-uvomorulin/E-cadherin (dilution ratio, 1:1,000; Sigma), guinea pig anti-Islet1 / 2 (dilution ratio, 1:4,000; provided by Jessell), guinea pig anti-Nkx2.2 (raised against a GST-Nkx2.2 fusion protein; dilution ratio, 1:500), rabbit anti-Nkx2.2 (dilution ratio, 1:1,000; provided by Jessell), guinea pig anti-Ngn3 (dilution ratio, 1:2,000; provided by German), rabbit anti-Nkx6.1 (dilution ratio, 1:1,000; provided by Serup), rabbit anti-Pdx1 (dilution ratio, 1:1,000, provided by Wright), rabbit anti-HB9 (dilution ratio, 1:4,000, provided by Kehrl), guinea pig anti-Pax6 (raised against a carboxy-terminus peptide of Pax6, coupled to KLH; dilution ratio, 1:1,000), rabbit anti-Pax6 (dilution ratio, 1:1,000, provided by Mastick), rabbit anti-β-galactosidase (dilution ratio 1:5,000; ICN), guinea pig anti-insulin (dilution ratio, 1:250; DAKO), guinea pig anti-glucagon (dilution ratio, 1:500; LINCO), rabbit anti IAPP (amylin) (dilution ratio, 1:1,000; Advanced ChemTech). For detection of the unlabeled primary antibodies, the following secondary antibodies (dilution ratio 1:200) were used: CY3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.), CY3-conjugated donkey anti-guinea pig IgG (Jackson), Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes), Alexa 488-conjugated goat anti-rat IgG (Molecular Probes), Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes), FITC-conjugated donkey anti-guinea pig IgG (Jackson). Images were obtained either with a Zeiss Axioskop 2 microscope and a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI) or with a Leica TCS confocal laser-scanning microscope. Adobe Photoshop version 5.5 (Adobe Systems, Inc.) was used to process the images.

In situ hybridization

T3 or T7 RNA polymerase was used to produce sense and antisense digoxigenin-labeled mRNA probes in vitro. The templates were DNA subclones containing different coding regions of Pax4 (600 bp fragment containing the paired- and homedomain regions) and Pax6 (Hallonet et al., 1998). Nonradioactive in situ hybridization was performed on 10–12 μm frozen sections as described (Schaeren-Wiemers and Gerfin-Moser, 1993) with the following modifications. Prehybridization was performed in a humidified chamber at 55°C for 1–2 h with 500 μl prehybridization buffer (50% formamide, 5× SSC, 50 μg/ml yeast tRNA, 1%

SDS, 50 μg/ml heparin) per slide for 1 to 2 h. The hybridization solution was prepared by adding 1 ng DIG cRNA per 100 μl prehybridization buffer. The sections were covered with plastic cover slips and not sealed. Post-hybridization, slides were washed as described and then incubated in 1× MAB for 5 min at room temperature before blocking (2% blocking reagent (Roche), 10% heat inactivated sheep serum, 0.1% Tween-20 in 1× MAB) at room temperature for 1 h. Blocking solution was replaced with fresh blocking solution containing anti-DIG antibody (Fab fragments) at a concentration of 1:5000 and incubated overnight at 4°C. The slides were washed three times in 1× MAB with 0.1% Tween-20 for 15 min at room temperature then washed in ddH_2O with 0.1% Tween-20 for 20 min at room temperature. The bound probes were visualized with an alkaline phosphatase-conjugated anti-digoxigenin Fab fragment (Boehringer Mannheim).

Results

Pax4 is transiently expressed in embryonic pancreatic tissue

Using in situ hybridization, we previously showed expression of *Pax4* mRNA in the gut region of E9.5 mouse embryos where the dorsal pancreatic anlage normally emerges (Sosa-Pineda et al., 1997). Using the same approach, we also found that the population of pancreatic *Pax4*-expressing cells increased between E13.5 and E15.5, declined significantly toward the end of gestation, and was absent in the adult organ (Dohrmann et al., 2000; data not shown).

A detailed characterization of pancreatic cells expressing Pax4 has been hampered by the lack of anti-Pax4 antibodies suitable for immunohistochemistry. To overcome this problem, we characterized these cells in pancreatic tissues isolated from $Pax4^{+/-}$ embryos. *Pax4*-heterozygous mice, which otherwise appear normal, have an insertion of the β-galactosidase (*β-gal*) reporter gene in one of two *Pax4* loci (Sosa-Pineda et al., 1997); this strategy allows the identification of Pax4-expressing cells via immunolabeling with anti-β-gal antibodies. We found that Pax4/β-gal proteins were expressed in the same transient manner as *Pax4* transcripts did: at E11.5, Pax4/β-gal^+ cells were scattered throughout the $Pax4^{+/-}$ pancreatic tissue (Fig. 1A); this population reached its maximum between E13.5 and E15.5 (Fig. 1E). Toward the end of gestation, the number of Pax4/β-gal^+ cells decreased (Fig. 1L), and they were undetected in pancreata of adult mice (data not shown).

At E11.5, some of the Pax4/β-gal^+ cells detected in the $Pax4^{+/-}$ pancreata coexpressed Ngn3 (Fig. 1B). Ngn3 is transiently expressed in endocrine precursors and it is required for their specification (Apelqvist et al., 1999; Gradwohl et al., 2000). This result suggests that Pax4 is expressed at around the time of endocrine specification and supports the proposal that Ngn3 activates *Pax4* gene expression (Smith et al., 2003; Wilson et al., 2003). At E11.5, we also found that most Pax4/β-gal^+ cells coexpressed Islet1, a transcription factor expressed in all differentiating pancreatic endocrine cells (Fig. 1C) (Ahlgren et al., 1997; Dohrmann et al., 2000; Grapin-Botton and Melton, 2000; Jensen et al., 2000). This result indicates that in early embryonic pancreata, Pax4-expressing cells largely represent differentiating endocrine precursors. At these stages, low levels of Pax4/β-gal^+ immunoreactivity also seemed to be present in a small number of $glucagon^+$ cells (Fig. 1D); this result suggests that the early expression of Pax4 may not be restricted to the β-cell and δ-cell lineages.

During mouse pancreas ontogeny the peak of Pax4 expression (E13.5-E15.5; Fig. 1E) coincides with a period of massive differentiation of endocrine precursors, the so-called secondary transition (Pictet and Rutter, 1972). Mature β-cells are thought to arise from precursors that initiate differentiation at around this period (Bramblett et al., 2000; Pictet and Rutter, 1972). In E15.5 $Pax4^{+/-}$ pancreata, we observed that the population of Pax4/β-gal^+ cells appeared restricted to regions containing endocrine cells, including differentiating β-cells ($HB9^+$ cells; Fig. 1F); in contrast, Pax4/β-gal^+ cells was excluded from areas containing exocrine cells (compare Figs. 1E and G). This observation suggesting that Pax4 is not expressed in pancreatic exocrine tissue could not be corroborated using double-immunofluorescence due to the lack of exocrine-specific antibodies suitable for these experiments. At E15.5, various endocrine markers such as Ngn3 (Fig. 1H), Islet1 (Fig. 1I), Nkx2.2 (data not shown), and Pax6 (data not shown) colocalized with Pax4/β-gal immunoreactivity; some Pax4/β-gal^+ cells coexpressed insulin (Fig. 1J), and just a few of the $glucagon^+$ cells also appeared to express low levels of Pax4/β-gal (Fig. 1K).

Few days later, at E18.5, numerous Pax 4/β-gal^+ cells coexpressed insulin (Fig. 1M); however, none seemed to express glucagon (Fig. 1N). The fact that Pax4 activity is also required for the formation of pancreatic δ cells (Sosa-Pineda et al., 1997) suggests that in E18.5 $Pax4^{+/-}$ pancreata, the small population of Pax4/β-gal^+ cells devoid of insulin immunoreactivity could represent δ cells.

In summary, these results indicate that in mouse pancreata the expression of Pax4 is largely restricted to embryonic stages. Colocalization of Pax4/β-gal immunoreactivity with various endocrine markers and its exclusion from areas containing exocrine cells indicate that, during pancreas organogenesis, Pax4 expression is largely confined to the endocrine cell population. In mouse embryonic pancreata, the expression of Pax4 peaks during that period when β-cell precursors normally start to differentiate (E13.5-E15.5); moreover, in pancreata of *Pax4*-heterozygous embryos, we detected coexpression of Pax4/β-gal protein and insulin at around those stages. These observations, together with our previous finding indicating that the pancreata of *Pax4*-deficient newborns lack mature β cells (Sosa-Pineda et al., 1997), raise the possibility that Pax4 is an early regulator of β-cell differentiation. This hypothesis is supported by the

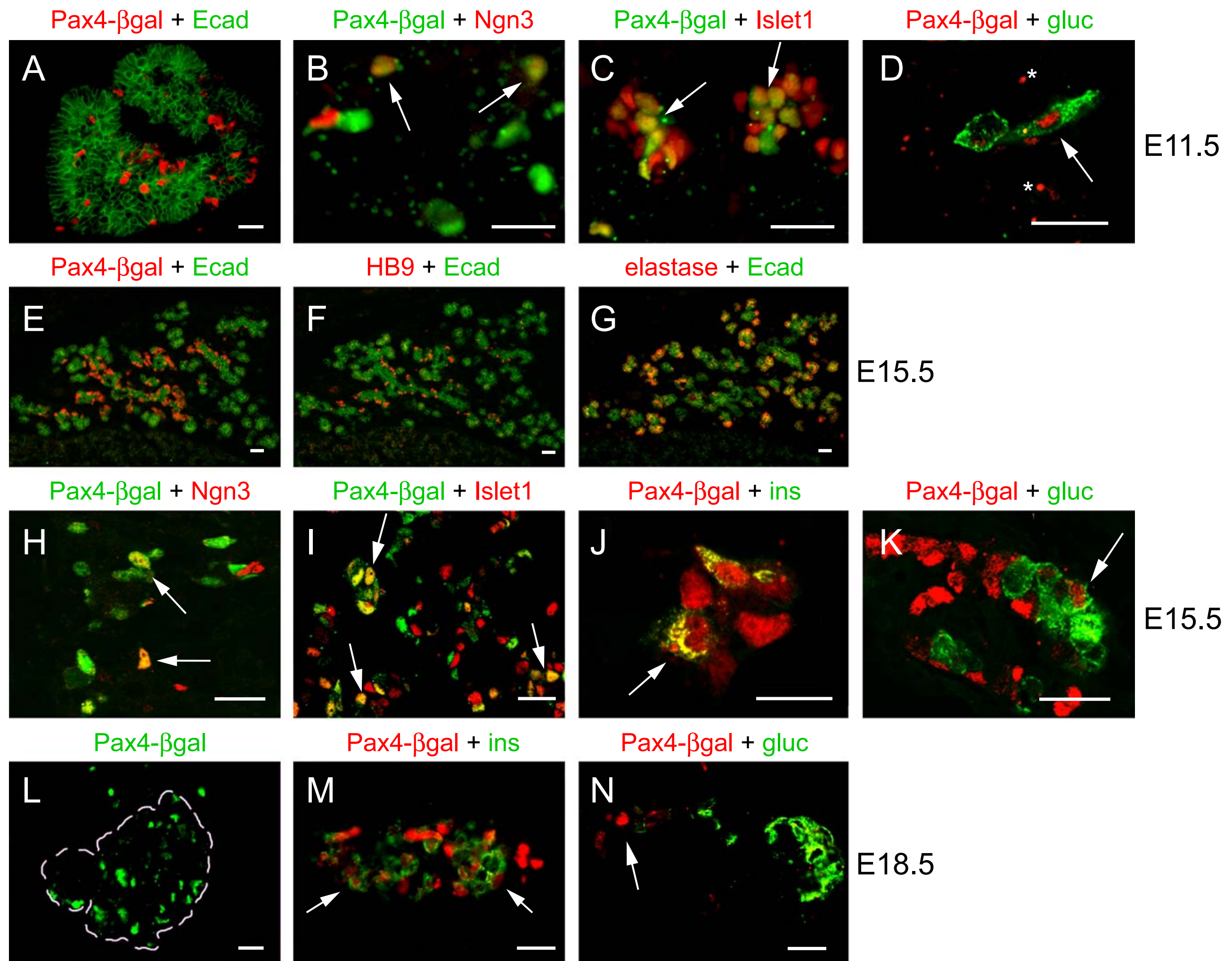

Fig. 1. Pax4 is transiently expressed in endocrine cells during pancreas organogenesis. In mouse developing pancreata, Pax4-expressing cells are numerous at E11.5 (A) and E15.5 (E), but not at E18.5 (L). At E15.5, Pax4 is expressed in areas where endocrine (F), but not exocrine (G) cells differentiate. At E18.5, Pax4-expressing cells locate in areas where islets form (L). Pax4-expressing cells coexpress endocrine markers such as: Ngn3 (B and H, arrows), Islet1 (C and I, arrows) and insulin (J and M, arrows). Coexpression of Pax4 with glucagon was observed in few cells at E11.5 (D, arrow) and E15.5 (K, arrows), but not at E18.5 (N; arrow indicates a Pax4-expressing cell). (A–N) are cryosections of *Pax4*$^{+/-}$ pancreata stained with anti-β-gal antibodies. D, H, I, J, K and N are confocal images (1 μm optical depth). Asterisks in D indicate background staining. Scale bar is 200 μm.

observation that in pancreata of *Pax4*-deficient embryos, the expression of various gene products that are normally detected in early differentiating β cells is missing, as described below.

The lack of functional Pax4 disrupts early β-cell differentiation

Based in our previous results, we sought to determine when during β-cell development is the activity of Pax4 required. To this end, the expression of several β-cell-specific markers was analyzed between E14.5 (early β-cell differentiation) and P1-P2 (islet formation) in pancreata of *Pax4*-nullizygous mice and wild-type littermates.

The β-cell marker Pdx1 is a homeodomain transcription factor originally identified as an activator of the *insulin* and *somatostatin* genes (Bramblett et al., 2000; Leonard et al., 1993; Miller et al., 1994; Ohlsson et al., 1993; Peshavaria et al., 1994). Early in development, Pdx1 is widely expressed in the pancreas and is required for cell proliferation but not for generation of the earliest insulin-synthesizing or glucagon-synthesizing cells (Guz et al., 1995; Jonsson et al., 1994; Offield et al., 1996). At approximately E14.5, high levels of Pdx1 become restricted to differentiating β cells (Ohlsson et al., 1993); high Pdx1 expression remains restricted to β cells in adult pancreata, and its activity is essential for maintaining the functionality of these cells (Ahlgren et al., 1998; Brissova et al., 2002; Dutta et al., 1998; Stoffers et al., 1997). We previously showed that Pdx1 expression was unaltered at E10.5 in *Pax4*$^{-/-}$ pancreata, but was profoundly affected in neonates (Sosa-Pineda et al., 1997). We now extended this study and found that, although numerous cells expressing high levels of Pdx1 were present in the E14.5 wild-type pancreatic epithelia (Fig. 2A), very few were detected in the

Pax4-deficient littermates (Fig. 2E). Similar results were observed at E15.5 when insulin expression was analyzed: few, if any, insulin-expressing cells were detected in *Pax4*-deficient pancreatic tissues (Figs. 2B and F). Thus, the expression levels of Pdx1 are severely affected in early differentiating β cells that are deficient in Pax4 and this alteration could explain, at least partially, the cells' inability to express insulin.

The homeobox gene *Hlxb9*, which encodes the transcription factor HB9, is widely expressed in the pancreatic epithelium during early development; *Hlxb9* function, which acts upstream of *Pdx1*, is required for the formation of the dorsal pancreatic rudiment and cytodifferentiation of the ventral pancreas. HB9 is a β-cell marker, and its persistent expression in mature β cells suggests a role for HB9 in β-cell physiology (Harrison et al., 1999; Li et al., 1999). In *Pax4*-deficient pancreata, the early expression of HB9, like that of Pdx1, was unaffected (data not shown). However, after E14.5, HB9 expression was severely reduced in the $Pax4^{-/-}$ pancreatic epithelium (Fig. 2G), and this defect persisted until birth (Fig. 2H).

The lack of expression of Pdx1, HB9, and insulin did not appear to result from defective proliferation or absence of β-cell precursors, as indicated by the numerous β-gal-expressing cells detected in $Pax4^{-/-}$ pancreata between E13.5 and E15.5 (Figs. 3A and B). Furthermore, no overt apoptosis was detected in this tissue (data not shown). We have also determined that at these stages, *Pax4*-deficient endocrine precursors expressed Ngn3 (Fig. 3C) (Apelqvist et al., 1999; Gradwohl et al., 2000), an indication that these cells are most likely correctly specified. In addition, the *Pax4*-deficient pancreatic cells also exhibited apparently normal expression of the endocrine markers Islet1 (Fig. 3D) (Ahlgren et al., 1997; Dohrmann et al., 2000; Grapin-Botton and Melton, 2000; Jensen et al., 2000), Nkx2.2 (Fig. 3E) (Sussel

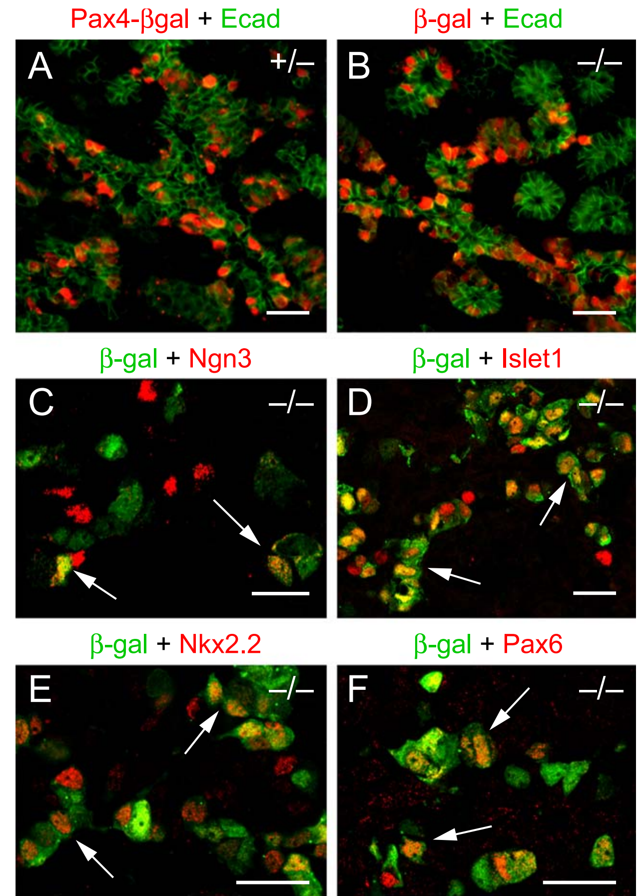

Fig. 3. Loss of functional Pax4 does not prevent the expression of Ngn3, Islet1, Nkx2.2 and Pax6 in pancreatic endocrine cells. At E14.5, a similar population of β-gal^{+} cells is observed between pancreata of $Pax4^{+/-}$ (A) and $Pax4^{-/-}$ (B) embryos. In pancreatic tissue of E14.5-E15.5 $Pax4^{-/-}$ embryos, Pax4-deficient endocrine cells remain expressing Ngn3 (C, arrows), Islet1 (D, arrows), Nkx2.2 (E, arrows) and Pax6 (F, arrows). (C–F) are confocal images (1 μm optical depth). Scale bar is 200 μm.

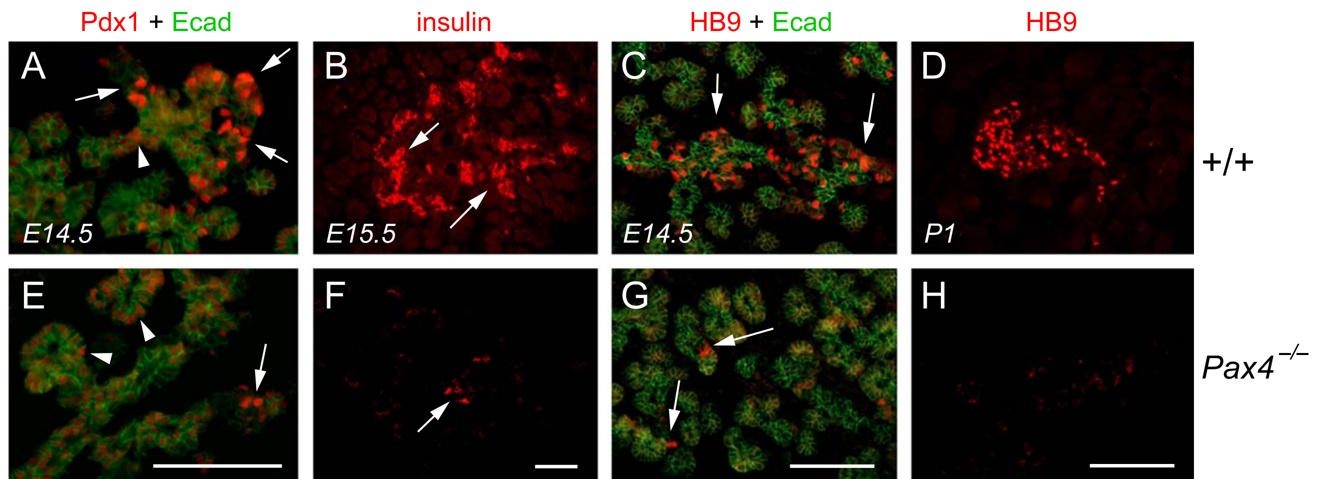

Fig. 2. Lack of functional Pax4 prevents the expression of Pdx1, HB9 and insulin in β-cell precursors. In E14.5 wild-type pancreata, early-differentiating β-cells show high expression of Pdx1 (A, arrows) and HB9 (C, arrows); shortly thereafter, these cells initiate insulin synthesis (B, arrows). In E14.5 to E15.5 $Pax4^{-/-}$ pancreata, only very few cells express high Pdx1 (E, arrow), insulin (F, arrow) or HB9 (G, arrows). The low expression of Pdx1 in non β-cells of these mutants remains unaffected (arrowheads in A and E). P1 wild-type β-cells express HB9 (D); however, no HB9 expression is detected in pancreata of $Pax4^{-/-}$ newborns (H). Scale bar is 100 μm.

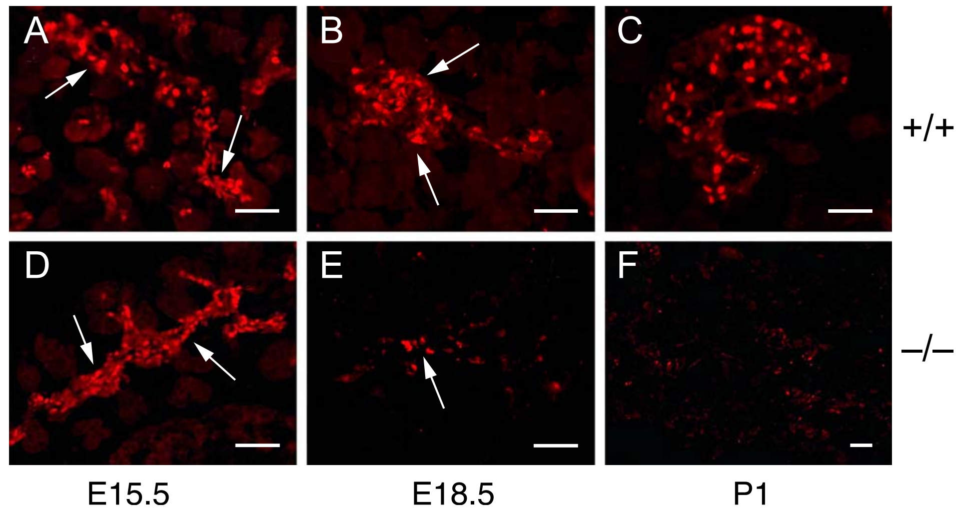

Fig. 4. In *Pax4*$^{-/-}$ pancreatic β-cells, the expression of Nkx6.1 decreases after E15.5. Nkx6.1 becomes restricted to differentiating β-cells at around E14.5 to E15.5, in wild-type (A, arrows) and *Pax4*$^{-/-}$ (D, arrows) pancreata. At E 18.5, the expression of Nkx6.1 is significantly decreased in *Pax4*$^{-/-}$ pancreata (B and E, arrows), and no Nkx6.1 immunoreactivity is detectable in P1 *Pax4*$^{-/-}$ pancreata (C and F). Scale bar is 200 μm.

et al., 1998) and Pax6 (Fig. 3F) (Dohrmann et al., 2000; Sander et al., 1997; St-Onge et al., 1997). Thus, in pancreatic endocrine cells Pax4 does not seem to have a major role in regulating *Islet1*, *Pax6* and *Nkx2.2* gene expression.

In mouse embryos, the transcription factor Nkx6.1 is widely expressed throughout the early pancreatic epithelium; however, at approximately E14.5–E15.5, high levels of Nkx6.1 become restricted to β-cell precursors (Fig.

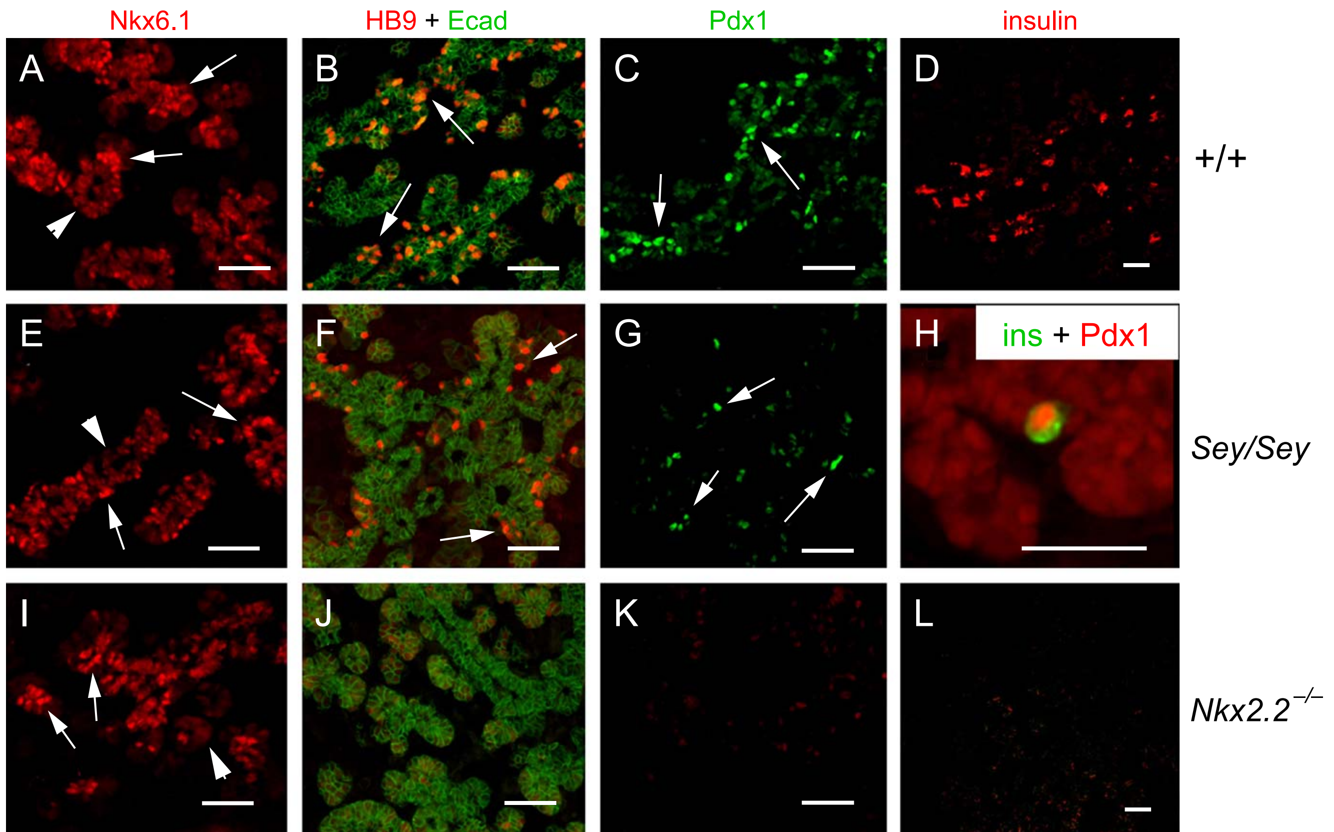

Fig. 5. Early β-cells deficient in Nkx2.2 or Pax6 show altered gene expression. (A) In E14.5 wild-type pancreata, most epithelial cell express low Nkx6.1 (arrowheads); however, differentiating β-cells express high levels of Nkx6.1 (arrows). In E14.5 *Sey*/*Sey* (E) or *Nkx2.2*$^{-/-}$ (I) pancreata, Nkx6.1 expression is unchanged. Differentiating β-cells of *Sey*/*Sey* embryos have normal HB9 expression (B and F); in these embryos, however, fewer β-cells express high Pdx1 (C and G, arrows) and insulin (D and H). β cells lacking Nkx2.2 do not express HB9 (J), Pdx1 (K) or insulin (L). Scale bar is 200 μm.

4A) and are maintained in mature β cells (Fig. 4C) (Edlund, 1998; Jensen et al., 1996, 2000; Oster et al., 1998; Sander et al., 1997, 2000; Wilson et al., 2003). Nkx6.1 activity is necessary for maintaining and expanding the population of β cells (Sander et al., 2000). At E15.5, no obvious changes in the expression of Nkx6.1 were observed in the pancreata of *Pax4*$^{-/-}$ embryos (Fig. 4D). However, starting at around E18.5 the expression of Nkx6.1 appeared drastically decreased (Fig. 4E) and became nearly undetectable at birth (Fig. 4F). These findings indicate that Pax4 contributes to the maintenance of Nkx6.1 expression in differentiating β cells.

Altogether, these results indicate that the activity of Pax4 is essential for initiating the β-cell differentiation program. Pax4 does not appear to control proliferation or the specification of endocrine precursors; however, Pax4 activity in β-cell precursors appears necessary to increase the level of Pdx1 expression, to induce HB9 and insulin expression and to maintain Nkx6.1 expression.

E14.5 Pax6-null pancreata show altered expression of Pdx1 and insulin

Within the Pax family of transcription factors, Pax4 and Pax6, are the closest structural homologues; these proteins are most similar in their corresponding paired and homeodomain DNA-binding domains (Dohrmann et al., 2000; Mansouri et al., 1996). Pax4 and Pax6 are expressed in pancreatic β cells, and their respective activities are necessary for β-cell differentiation (Dohrmann et al., 2000; Sander et al., 1997; Sosa-Pineda et al., 1997; St-Onge et al., 1997). Furthermore, previous evidence suggested that in pancreatic β cells Pax6 participates in controlling *Pdx1*-(Samaras et al., 2002) and *insulin* (Dohrmann et al., 2000; Sander et al., 1997) gene expression. The phenotypic alterations in β cells of newborn mice deficient in Pax4 or Pax6, however, are not identical: in the absence of functional Pax4 insulin-producing cells are nearly absent (Sosa-Pineda et al., 1997), whereas in absence of Pax6 the insulin-expressing cell population is reduced only 3- to 4-fold (Sander et al., 1997; St-Onge et al., 1997). We then decided to determine whether initiation of β-cell differentiation might require the coordinated activities of Pax4 and Pax6. To this end, we performed a detailed expression analysis of E14.5 pancreata from *Sey*^{NEU}/*Sey*^{NEU} (Pax6-deficient) embryos. *Sey*^{NEU} mice have a mutation in Pax6 that disrupts the translation of its encoded protein (Hill et al., 1991); the pancreatic alterations in *Sey*^{NEU} mice are comparable to those found in *Pax6*-nullizygous animals (Sander et al., 1997; St-Onge et al., 1997). In contrast with our previous finding showing that in E14.5 *Pax4*$^{-/-}$ pancreata the expression of HB9 was severely affected, no obvious alterations in HB9 expression were observed in pancreata of *Sey*^{NEU}/*Sey*^{NEU} embryos isolated at this stage (Figs. 5B and F). On the other hand, similar to the loss of Pax4, in pancreata of E14.5 *Pax6*-deficient embryos, the number of cells expressing high levels of Pdx1 was reduced, but only about 70% to 75% (Figs. 5C and G) and these were the only cells that synthesized insulin (Fig. 5H). Also, similar to *Pax4*$^{-/-}$ embryos, the expression of Nkx6.1 was not significantly altered in E14.5 *Sey*^{NEU}/*Sey*^{NEU} pancreata (Figs. 5A and E). Normal expression of *Pax4* transcripts was also observed at this stage in *Pax6*-deficient pancreata (Fig. 6B).

Our finding showing that in β cell precursors the lack of either Pax4- or Pax6 activities affected Pdx1 and insulin expression could suggest that, during β-cell differentiation, these transcription factors have partially redundant functions. However, the fact that early in β-cell differentiation the loss of functional Pax4, but not Pax6, prevented the expression of HB9 also raises the possibility that in β-cell precursors Pax4 and Pax6 do not regulate the same set of genes.

E14.5 Nkx2.2-deficient β cells exhibit similar alterations than those of Pax4

Previous studies indicated that Nkx2.2 is a crucial regulator of β-cell development (Bramblett et al., 2000; Schwitzgebel, 2001; Sussel et al., 1998). Similar to the loss of functional Pax4, the lack of Nkx2.2 activity prevents

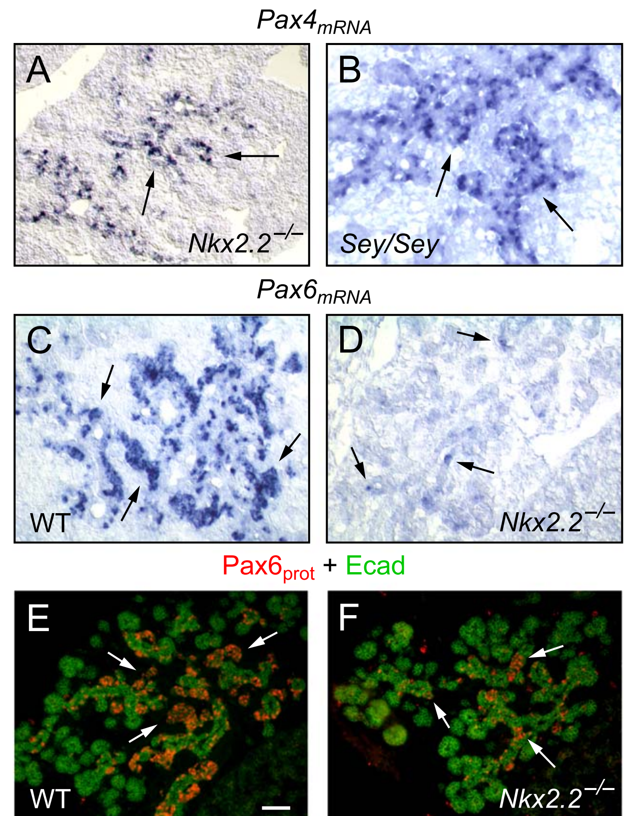

Fig. 6. Pax4 expression is unaltered in E14.5 *Nkx2.2*$^{-/-}$ or *Sey*/*Sey* pancreata; however, Pax6 expression is severely affected in *Nkx2.2*$^{-/-}$ pancreata. At E14.5, normal expression of *Pax4* mRNA is observed in pancreata of *Nkx2.2*$^{-/-}$ (A, arrows) or *Sey*/*Sey* (B, arrows) embryos. In E14.5 *Nkx2.2*$^{-/-}$ pancreata the population of cells expressing *Pax6* mRNA (C and D, arrows) or Pax6 protein (E and F, red and arrows) is reduced. In E and F, the pancreatic epithelium was stained with anti-Ecadherin antibodies (green). Scale bar is 100 μm.

formation of mature pancreatic β cells (Sussel et al., 1998). Because of the similarities between pancreata of *Pax4*-deficient and *Nkx2.2*-deficient newborns and our results showing that Pax4 controls early β-cell differentiation, we sought to analyze whether Nkx2.2 is also required to initiate this differentiation process. With this analysis, we found that in *Nkx2.2*$^{-/--}$ pancreata the expression of HB9 protein was undetectable at E14.5 (Figs. 5B and J). In addition, as previously reported by Sussel et al. (1998) none of the cells in *Nkx2.2*$^{-/-}$ pancreata expressed high levels of Pdx1 (Figs. 5C and K) or insulin (Figs. 5D and L) at around this stage. On the other hand, similar to *Pax4*-deficient pancreata, the expression of Nkx6.1 was not significantly altered in E14.5 *Nkx2.2*$^{-/-}$ pancreata (Figs. 5A and I).

From this analysis, we can conclude that the expression of at least three early markers of β-cell differentiation (HB9, Pdx1 and insulin) is similarly affected in pancreata of E14.5 *Nkx2.2*$^{-/-}$ and *Pax4*$^{-/-}$ embryos. Interestingly, while we found that *Pax4* expression remained unaffected in E14.5 *Nkx2.2*$^{-/-}$ pancreata (Fig. 6A), that of *Pax6* transcripts (Figs. 6C and D) and Pax6 protein was markedly reduced (Figs. 6E and F). These results indicate that in pancreatic endocrine cells, the activity of Nkx2.2 is required to maintain the expression of Pax6 but not that of Pax4, and suggest that the function of Pax4 is essential but not sufficient to promote β-cell differentiation. In addition, this comparison indicates that in the pancreas Pax4 and Nkx2.2 might control similar regulatory pathways.

Pax4$^{-/-}$ *Nkx2.2*$^{-/-}$ *pancreata have identical alterations to those observed in Nkx2.2*$^{-/-}$ *pancreata*

To determine possible genetic interactions between *Pax4* and *Nkx2.2* during β-cell differentiation, we generated *Pax4*$^{-/-}$ *Nkx2.2*$^{-/-}$ double-nullizygous mice. *Pax4*$^{+/-}$*Nkx2.2*$^{+/-}$ double-heterozygous mice appeared normal at birth, survived through adulthood, and did not show any obvious alterations in pancreatic development or glycemia (data not shown). On the contrary and as expected from the corresponding single-gene deficiencies, the *Pax4*$^{-/-}$

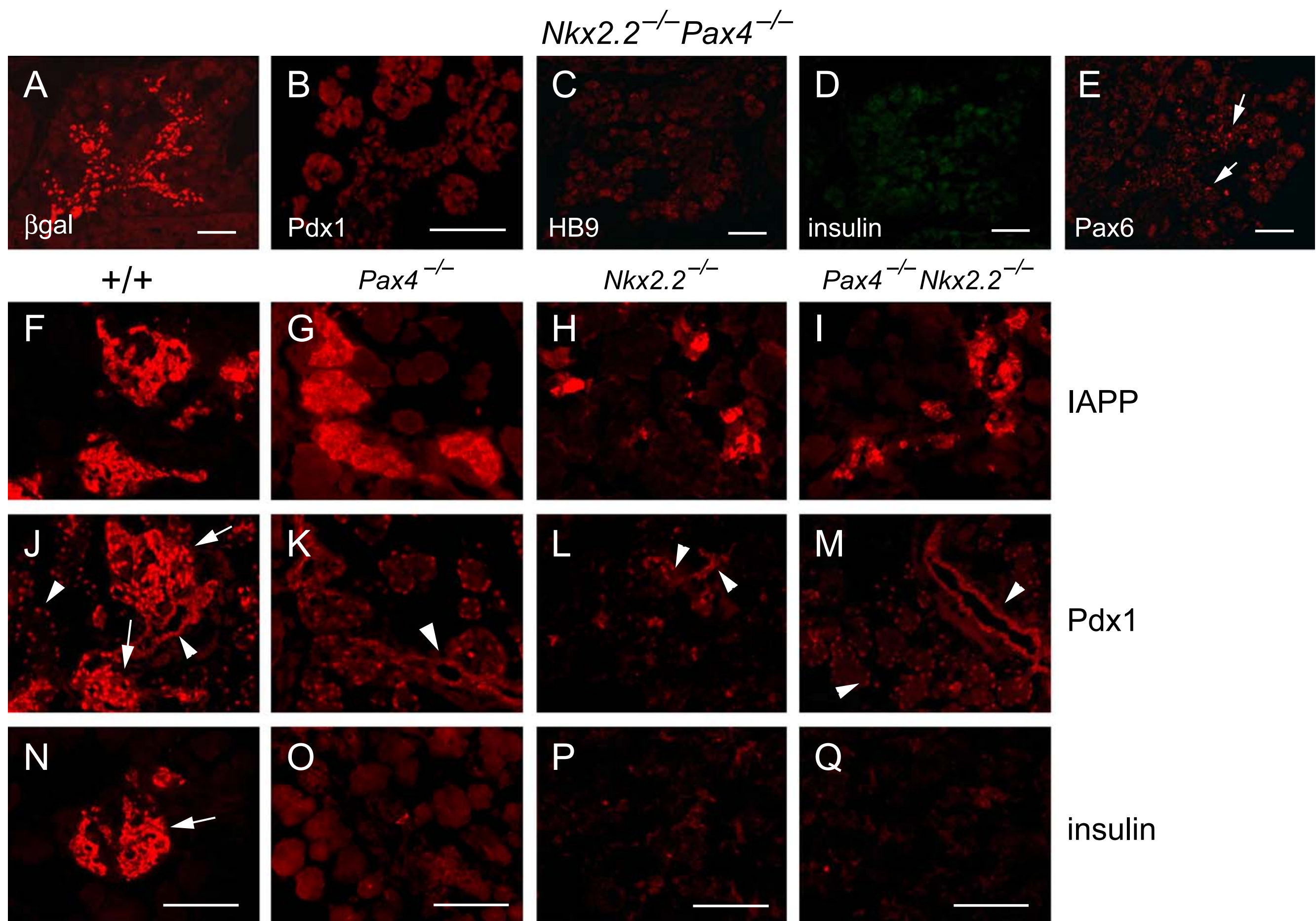

Fig. 7. *Pax4*$^{-/-}$ *Nkx2.2*$^{-/-}$ β-cells recapitulate the alterations of *Nkx2.2*$^{-/-}$ pancreata more closely than those of *Pax4*$^{-/-}$ pancreata. Numerous cells expressing the Pax4-β-gal reporter are observed in pancreata of E14.5 *Pax4*$^{-/-}$*Nkx2.2*$^{-/-}$ embryos (A). These cells do not express Pdx1 (B), HB9 (C), or insulin (D), and show low levels of Pax6 immunoreactivity (E). In pancreata of newborns (P2), mature β-cells express IAPP (F), high levels of Pdx1 (J, arrows) and insulin (N, arrow). Cells expressing IAPP are present in pancreata of *Pax4*$^{-/-}$ (G), *Nkx2.2*$^{-/-}$ (H), and *Pax4*$^{-/-}$ *Nkx2.2*$^{-/-}$ (I) newborns (P2); however, these cells do not express high Pdx1 (K–M) or insulin (O–Q). (F and J), (G, K and O) (H, L and P) and (I and Q) are adjacent sections, respectively. Arrowheads in (J–M) indicate Pdx1 expression in ducts and exocrine cells. Scale bar is 100 μm.

Nkx2.2$^{-/-}$ mice died shortly after birth. Overall, the pancreatic alterations observed in double-nullizygous embryos were identical to those found in *Nkx2.2*$^{-/-}$ littermates. In pancreata of E14.5 *Pax4*$^{-/-}$ *Nkx2.2*$^{-/--}$ embryos, we observed numerous cells expressing the *Pax4/β-gal* reporter gene (the *Nkx2.2* knockout allele does not contain *β-gal*) (Fig. 7A), as well as cells expressing the pan-endocrine marker Islet-1 (data not shown). These results indicate that, similar to the phenotype reported for the functional inactivation of each single gene, the lack of both *Nkx2.2* and *Pax4* does not preclude the formation of pancreatic endocrine cells. However, total lack of cells expressing high levels of Pdx1 (Fig. 7B), HB9 (Fig. 7C), or insulin (Fig. 7D) in pancreata of E14.5 *Pax4*$^{-/-}$ *Nkx2.2*$^{-/-}$ embryos was highly reminiscent of those alterations observed at this stage in *Nkx2.2*$^{-/-}$ pancreata. Furthermore, as in *Nkx2.2*$^{-/--}$ littermates, in the E14.5 double-nullizygous pancreata the expression of Pax6 was also severely reduced (Fig. 7E).

As previously reported by Sussel et al. (1998), in pancreata of *Nkx2.2*-deficient newborns (P2), we also observed cells expressing the β-cell marker islet amyloid polypeptide (IAPP) (Fig. 7H) that were devoid of high levels of Pdx1 (Fig. 7L) or insulin (Fig. 7P) proteins. Those observations led Sussel et al. (1998) to conclude that the lack of functional Nkx2.2 blocks β-cell differentiation. Similarly, we have now also identified clusters of cells expressing IAPP (Figs. 7G and I) that did not express Pdx1 (Figs. 7K and M) or insulin (Figs. 7O and Q) in the pancreata of *Pax4*$^{-/-}$ and *Pax4*$^{-/-}$ *Nkx2.2*$^{-/-}$ neonates (P2). Despite those remarkable similarities between pancreata deficient in *Pax4* and those deficient in *Nkx2.2*, the defects were more profound in the *Nkx2.2*-null background. In *Pax4*-deficient pancreata, just a few cells (at most, 10–15 cells in the whole pancreas; data not shown) coexpressed high levels of Pdx1, HB9, and insulin. Instead, in pancreata of *Nkx2.2*$^{-/-}$ or *Pax4*$^{-/-}$ *Nkx2.2*$^{-/-}$ neonates the expression of those β-cell markers was completely absent. One possibility to explain those differences is that in *Nkx2.2*-deficient pancreata the lack of Pax6 expression enhances the severity of the β-cell phenotype.

Overall, our results from this comparative analysis suggest that Pax4 and Nkx2.2 regulate parallel pathways and that they are both necessary to initiate the β-cell differentiation program; Pax6, on the other hand, appears to be necessary for the correct execution of this program.

Discussion

In this manuscript, we provide evidence to support the proposal that Pax4 regulates an early step in β-cell differentiation: the expression of Pax4 peaks when β cells normally start to differentiate (E13.5-E15.5); the lack of functional Pax4 prevents the expression of three markers of early β-cell differentiation; and Pax4 is expressed in differentiating β cells. However, it is likely that the early expression of Pax4 is not exclusively restricted to β-cell precursors, since we previously showed that Pax4 activity is also required for the formation of somatostatin-producing (δ) cells (Sosa-Pineda et al., 1997). In addition, we now observed that few α-cells (glucagon-expressing cells) also coexpressed low levels of Pax4/β-gal protein in pancreata of E11.5 and E15.5 *Pax4*$^{+/-}$ embryos.

Loss of functional Pax4 does not affect the expression of Ngn3, Islet1, Nkx2.2 or Pax6 in pancreatic endocrine precursors

In mouse embryos, pancreatic endocrine development commences once Ngn3 is expressed in a population of epithelial precursors (Apelqvist et al., 1999; Gradwohl et al., 2000); this results in endocrine-cell specification and in principle, activates *Pax4*-expression (Smith et al., 2003; Wilson et al., 2003). In *Pax4*-deficient pancreatic cells, we detected expression of Ngn3; this result supports the hypothesis that during pancreatic endocrine development Pax4 acts downstream of Ngn3.

The observation that the Nkx family members Nkx2.2 and Nkx6.1 are broadly expressed in the early pancreatic epithelium (where they colocalize with Ngn3) suggested that they are expressed in early pancreatic endocrine precursors (Jensen et al., 2000; Sander et al., 2000; Sussel et al., 1998; Wilson et al., 2003). In those pancreatic endocrine precursors, the expression of the homeobox genes, *Islet1* and *Pax6*, starts to be detected once endocrine differentiation has begun (Ahlgren et al., 1997; Bramblett et al., 2000; Dohrmann et al., 2000; Jensen et al., 2000; Sander et al., 1997; St-Onge et al., 1997; Wilson et al., 2003). Our detailed analysis of the expression of Islet1, Nkx2.2 and Pax6 in E13.5-E15.5 *Pax4*-deficient pancreatic cells did not reveal any obvious alteration in their expression. These results suggest that during pancreatic endocrine development, Pax4 acts downstream of those transcription factors or alternatively, that Pax4 activity is not required to control their expression. In addition, the finding that *Pax4* expression appears normal in pancreatic tissues of E14.5 *Nkx2.2*$^{-/-}$ or *Pax6*-deficient (*Sey*^{NEU}/*Sey*^{NEU}) mouse embryos also suggests that Nkx2.2 and Pax6 do not contribute significantly to activate *Pax4* gene expression.

Loss of functional Pax4 affects the expression of early β-cell markers

In mouse developing pancreata, Nkx6.1 proteins become restricted to differentiating β cells after E14.5 (Jensen et al., 2000; Sander et al., 1997, 2000). Until E14.5–E15.5, no obvious changes in the expression of Nkx6.1 were observed in *Pax4*-nullizygous pancreata. However, starting at approximately E18.5, the level of Nkx6.1 was drastically reduced and it was undetectable in the mutant pancreas at birth. This result indicates that in differentiating β cells the activity of Pax4 contributes to the maintenance of the expression of Nkx6.1.

Table 1
Summary of changes in gene expression in pancreatic β cells of *Nkx2.2*$^{-/-}$, *Pax4*$^{-/-}$, *Sey/Sey* and *Pax4*$^{-/-}$ *Nkx2.2*$^{-/-}$ mice

	Nkx2.2$^{-/-}$		*Pax4*$^{-/-}$		*Sey/Sey*		*Pax4*$^{-/-}$ *Nkx2.2*$^{-/-}$	
	E14.5	E18.5[a]-P1[b]	E14.5	P1	E14.5	E18.5[c]-P1[d]	E14.5	P1
Nkx6.1	+[b]	–[a,b]	+[b]	–[b]	+[b]	Less cells[c]	+[b]	–[b]
Nkx2.2	–	–	+[b]	N.d.	N.d.	N.d.	–	–
Pax4	+[b]	N.d.[e]	–	–	+[b]	N.d.[e]	–	–
Pax6	Low[b]	Low[b]	+[b]	N.d.	–	–	Low[b]	Low[b]
Pdx1	–[b]	–[a,b]	–[b]	–[b,f]	Less cells[b]	Less cells[c]	–[b]	–[b]
HB9	–[b]	–[b]	–[b]	–[b]	+[b]	N.d.	–[b]	–[b]
insulin	–[b]	–[a,b]	–[b]	–[b,f]	Less cells[b]	Less cells[c,d]	–[b]	–[b]
IAPP	N.d.	+[a,b]	N.d.	+[b]	N.d.	N.d.	N.d.	+[b]

[a] Sussel et al., 1998.
[b] This study.
[c] Sander et al., 1997.
[d] St.-Onge et al., 1997.
[e] Expression normally decays at around this stage; N.d., not determined. Yellow boxes indicate gene(s) inactivated in the corresponding mutants.
[f] Sosa-Pineda et al., 1997.

The restriction of high levels of Pdx1 and HB9 proteins to a subpopulation of pancreatic endocrine cells is one of the earliest indications of β-cell differentiation; this event occurs at around E14.5 in mouse development and likely precedes the onset of insulin expression (Gannon et al., 2001; Guz et al., 1995; Harrison et al., 1999; Li et al., 1999; Ohlsson et al., 1993). We have now found that starting at approximately E14.5, the expression of Pdx1, HB9 and insulin was nearly absent in *Pax4*$^{-/-}$ pancreata, and this condition persisted until birth. We have previously shown that insulin^{+} cells were present at E10.5 in *Pax4*-deficient pancreata (Sosa-Pineda et al., 1997). The lack of insulin expression that we now observed in this mutant tissue from E14.5 onwards suggests that Pax4 does not have a direct role in regulating *insulin* gene expression. Altogether, the lack of high levels of Pdx1, HB9 and insulin expression detected after E14.5 in *Pax4*-deficient pancreata supports our hypothesis that Pax4 activity is necessary for initiation of the β-cell differentiation program. Thus, Pax4 seems to occupy a high place in the molecular hierarchy that governs the normal development of pancreatic β cells.

It could be argued that the differences we observed in the expression of some of the analyzed genes in *Pax4*-nullyzygous pancreata, is due to the loss of certain cell types. However, the presence of a large β-gal-expressing cell population in pancreata of E13.5-E15.5 *Pax4*$^{-/-}$ embryos precludes a possible role of Pax4 as a survival factor. Our finding that numerous cells in *Pax4*$^{-/-}$ newborn pancreata continue to express the β-cell marker IAPP raises the possibility that these represent poorly differentiated β cells. Interestingly, Sussel et al. (1998) also reported previously the presence of IAPP-expressing cells in pancreata of *Nkx2.2*$^{-/-}$ newborn mice. This observation and the absence of cells expressing insulin, Pdx1, glucokinase and Glut2 in *Nkx2.2*-deficient pancreata at birth led the authors to propose that the lack of functional Nkx2.2 blocks the maturation of β-cell precursors.

The activities of Pax4 and Nkx2.2 are necessary to initiate β-cell differentiation

Our analysis of gene expression at a stage when β-cell differentiation normally commences showed remarkable similarities between pancreata deficient in *Pax4* and those deficient in *Nkx2.2* (Table 1). These defects, however, were more profound in the *Nkx2.2*-null background. Furthermore, although pancreatic cells deficient in Pax4 remained expressing Pax6, in *Nkx2.2*-deficient pancreata, we only detected a few cells expressing *Pax6* transcripts at E14.5. This result appears to contradict previous data reporting the expression of Pax6 proteins in E18.5 *Nkx2.2*-nullizygous pancreata (Sussel et al., 1998). However, Sussel et al.

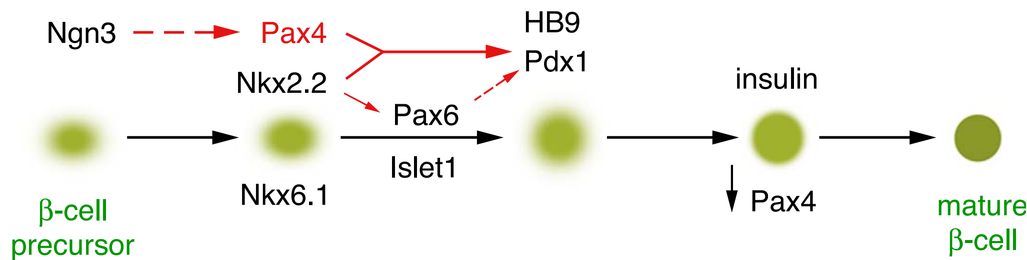

Fig.8. Model of β-cell development. In β-cell precursors, Ngn3 induces the expression of Pax4; these cells also express Nkx2.2 and Nkx6.1, and shortly thereafter, they express Islet1 and Pax6. The parallel activities of Pax4 and Nkx2.2 enable the program of β-cell differentiation, and as a result, the level of Pdx1 increases; the expression of HB9 is induced; and the synthesis of insulin ensues. In fully mature β cells, the activity of Pax4 is no longer required.

(unpublished results) recently corroborated that in E12.5, E15.5, and E18.5 *Nkx2.2*-deficient pancreata the levels of *Pax6* mRNA were drastically reduced.

Taken together, we would like to propose a model of β-cell development (Fig. 8) in which Pax4 is expressed in β-cell precursors after their specification; at around this time, these cells also express Nkx2.2 and Nkx6.1 and shortly thereafter, they express Islet1 and Pax6. The parallel activities of Pax4 and Nkx2.2 enable the program of β-cell differentiation, and as a result, the level of Pdx1 increases; the expression of HB9 is induced; and the synthesis of insulin ensues. In β cells, the expression of Pax4 gradually disappears upon maturation. Although Pax4 and Nkx2.2 appear to act in parallel to increase Pdx1 expression, it is still unclear how this role is accomplished. Pax6, on the other hand, may have a direct effect on Pdx1 expression by transactivating the *Pdx1* gene. In either case, the loss of Pax6 expression in $Nkx2.2^{-/-}$ pancreatic tissues after E14.5 could explain the more severe β-cell phenotype observed in these embryos.

In conclusion, we identified Pax4, Nkx2.2 and Pax6 as crucial regulators of early β-cell development. This knowledge should prove valuable for the development of methods to differentiate pancreatic endocrine precursors into mature β-cells.

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