Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Biology 286 (2005) 182 – 194

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Prox1 activity controls pancreas morphogenesis and participates in the production of "secondary transition" pancreatic endocrine cells

Junfeng Wang[a], Gamze Kilic[a], Muge Aydin[b], Zoe Burke[c], Guillermo Oliver[a], Beatriz Sosa-Pineda[a,*]

[a]*Department of Genetics and Tumor Cell Biology, St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, TN 38105-2794, USA*
[b]*Department of Genetics, Institute for Experimental Medical Research, Istanbul University, Istanbul, Turkey*
[c]*Centre for Regenerative Medicine, Department of Biology and Biochemistry, University of Bath, Bath BA27AY, UK*

Received for publication 22 March 2005, revised 15 July 2005, accepted 18 July 2005
Available online 24 August 2005

Abstract

The development of the mammalian pancreas is governed by various signaling processes and by a cascade of gene activation events controlled by different transcription factors. Here we show that the divergent homeodomain transcription factor Prox1 is a novel, crucial regulator of mouse pancreas organogenesis. Loss of Prox1 function severely disrupted epithelial pancreas morphology and hindered pancreatic growth without affecting significantly the genesis of endocrine cells before E11.5. Conversely, the lack of Prox1 activity substantially decreased the formation of islet cell precursors after E13.5, during a period known as the "secondary transition". Notably, this defect occurred concurrently with an abnormal increment of exocrine cells. Hence, it is possible that Prox1 contributes to the allocation of an adequate supply of islet cells throughout pancreas ontogeny by preventing exocrine cell differentiation of multipotent pancreatic progenitors. Prox1 thus appears to be an essential component of a genetic program destined to produce the cellular complexity of the mammalian pancreas.

Keywords: Pancreas; Prox1; Mouse; Endocrine; Exocrine; Development; Ontogeny; Embryo; Cholecystokinin

Introduction

The murine pancreas arises from two evaginations (dorsal and ventral) that begin to form at the foregut/midgut region of the embryo at approximately embryonic day 9 (E9.0). This process is followed by intense growth and branching and by subsequent rotation and fusion of the primordia into a single organ (Murtaugh and Melton, 2003; Slack, 1995). The growth of the pancreatic epithelium is accompanied by the asynchronous production of distinct pancreatic cell types: endocrine (alpha, beta, delta, and PP), exocrine, and ductal cells (Murtaugh and Melton, 2003; Pictet and Rutter, 1972). Whereas a handful of glucagon-producing (alpha) cells can be detected as early as E8.5, insulin-producing (beta) cells appear in increasing numbers beginning at approximately E13.5. In contrast, pancreatic exocrine cells, somatostatin-producing (delta) cells, and pancreatic polypeptide-producing (PP) cells start to appear only shortly thereafter (Pictet and Rutter, 1972; Slack, 1995). This changing character of pancreatic differentiation probably reflects changes over time in both the inductive milieu and the potential of pancreatic progenitors. Similarly, the proliferation of pancreatic progenitor cells also appears to be governed by complex interactions involving extrinsic (mesenchymal) cues and intrinsic factors (Edlund, 2002; Kim and Hebrok, 2001; Murtaugh and Melton, 2003). Crucial for the characterization of the intricate molecular mechanisms governing the fate and expansion of pancreatic progenitors is the identification of key components participating in these processes. In recent years and largely in part

* Corresponding author. Fax: +1 901 495 2907.
E-mail address: Beatriz.Sosa-Pineda@stjude.org (B. Sosa-Pineda).

0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2005.07.021

through the use of mouse genetics, an increasing number of gene functions have been identified that appear to be necessary for the correct execution of a specific aspect(s) of pancreas organogenesis (Edlund, 2002; Habener et al., 2005; Murtaugh and Melton, 2003).

The divergent homeodomain transcription factor Prox1 is expressed in the mouse pancreatic region even before the pancreatic bud arises (Burke and Oliver, 2002). Prox1 is also expressed in various developing tissues where its function appears to be essential for normal organogenesis (Oliver et al., 1993). Hence, Prox1 activity is required for specification of the lymphatic vasculature (Wigle and Oliver, 1999; Wigle et al., 2002), lens fiber cell differentiation (Wigle et al., 1999), retinal cell-type specification (Dyer et al., 2003), and hepatic morphogenesis (Sosa-Pineda et al., 2000). In the study described here, we characterized the expression of Prox1 throughout the development of the mouse pancreas and thoroughly analyzed the pancreata of *Prox1*-nullizygous embryos. Our results showed that the lack of pancreatic Prox1 function hindered pancreatic epithelial growth and disrupted overall pancreas morphology. In addition, Prox1-deficient pancreata had severe disturbances in endocrine cell genesis and increased production of exocrine precursors. Our studies thus unveiled a novel function of Prox1 that not only seems to impinge on various aspects of pancreas morphogenesis, but also appears to be necessary to preserve the cellular complexity of this organ.

Materials and methods

Generation and genotyping of Prox1 mutant mice

Functional inactivation of *Prox1* and genotyping of offspring by PCR or Southern blot analysis of genomic DNA were previously described (Wigle et al., 1999). The experimental protocols were approved by the animal care and use committee at St. Jude Children's Research Hospital.

Processing of embryos and pancreatic tissues

Tissues of dissected embryos or pancreata of newborn mice were prepared for immunohistochemical analysis or in situ hybridization by fixation overnight in 4% paraformaldehyde at 4°C. Tissues were then immersed in 30% sucrose in phosphate-buffered saline (PBS) overnight at 4°C for cryoprotection, embedded in tissue-freezing medium (Tissue-Tek, Triangle Biomedical Sciences), and cut by a cryostat into sections (8 μm for immunohistochemical study and 12 μm for in situ hybridization).

Histologic analysis

E15.0 pancreata were dissected, fixed overnight in 4% paraformaldehyde, and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin, examined by using a Zeiss Axioskop 2 microscope, and photographed with a SPOT digital camera (Diagnostic Instruments).

Detection of β-galactosidase activity

Whole E15.0 embryos (*Prox1*$^{+/-}$ or *Prox1*$^{-/-}$) or dissected pancreata of adults (*Prox1*$^{+/-}$) were incubated for 60 min at 4°C with fixative solution (1% formaldehyde, 0.2% glutaraldehyde, 0.2% NP-40, and 0.1% SDS in PBS). Tissues or embryos were then washed twice at room temperature for 20 min with PBS and incubated overnight at 30°C with staining solution (1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.2% NP-40, 0.1% SDS in PBS). The digestive tracts of E15.0 embryos were dissected and photographed. Adult pancreata were washed with 20% and 50% glycerol for 2 h and with 80% glycerol overnight and were then photographed with a Leica MZFLIII stereomicroscope equipped with a Hamamatsu C5810 color digital camera (Hamamatsu Photonics K.K.).

Immunohistochemical analysis

Frozen sections underwent immunohistochemical assays. Primary antibodies were the following: rabbit anti-α-amylase (diluted 1:250; Sigma); rabbit anti-β-galactosidase (1:5000; ICN); mouse anti-bromodeoxyuridine (IgG isotype; final concentration, 7 μg/ml; Becton Dickinson); rabbit anti-Cdkn1b (1:200; Santa Cruz Biotechnology); rabbit anti-cholecystokinin (1:50; Lab Vision Corporation); guinea pig anti-glucagon (1:500; LINCO Research, Inc); guinea pig anti-insulin (1:250; DAKO); guinea pig anti-Isl1 (1:4000; provided by T. Jessell); mouse anti-Ki67 (1:50; Pharmingen); rat anti-laminin (1:200; BIODESIGN); guinea pig anti-Ngn3 (1:2000; provided by M. German); rabbit anti-Nkx2.2 (1:1000; provided by T. Jessell); rabbit anti-Nkx6.1 (1:1000; provided by P. Serup); rabbit anti-p48 (1:400; provided by H. Edlund); rabbit anti-Pax6 (1:1000; Covance Research Products); rabbit anti-phospho-histone3 (1:2000; Upstate Biotechnology); rabbit anti-Pdx1 (1:1000; provided by C. Wright); goat anti-Pdx1 (1:10,000; provided by C. Wright); rabbit anti-Prox1 (1:5000; Covance Research Products); guinea-pig anti-Prox1 (1:500); goat anti-somatostatin (1:250; Santa Cruz Biotechnology); and rat anti-uvomorulin/E-cadherin (1:1000; Sigma). The following secondary antibodies (diluted 1:200) were used for detection: Cy3-conjugated donkey anti-guinea pig IgG (Jackson ImmunoResearch Laboratories, Inc.); Cy3-conjugated donkey anti-mouse IgG (Jackson); Cy3-conjugated donkey anti-rabbit IgG (Jackson); Cy3-conjugated donkey anti-rat 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-guinea pig IgG (Molecular Probes); and Alexa 488-conjugated donkey anti-goat IgG (Molecular Probes). Biotinylated donkey anti-rabbit IgG (Jackson) was detected

by using the VECTASTAIN Elite ABC kit (Vector Laboratories). For nuclear staining, sections were covered with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (VECTASHIELD; Vector Laboratories). Images were obtained either with the Zeiss Axioskop 2 microscope or with a Leica TCS confocal laser-scanning microscope. Adobe Photoshop version 7.0 (Adobe Systems, Inc.) was used to process the images.

Cell counting

Whole pancreata of wild-type embryos and *Prox1*$^{-/-}$ littermates were sectioned (8 μm), and each third (E9.5) or fifth (E10.5 and E11.5) consecutive section was incubated with antibodies recognizing specific molecular markers (Prox1 in wild-type tissues, β-galactosidase in *Prox1*$^{-/-}$ tissues, Ki67, bromodeoxyuridine [BrdU], Cdkn1b, Islet1, Ngn3, glucagon, or cholecystokinin [Cck]) to estimate the percentage of cells that expressed each marker in the entire pancreas. The number of Prox1^{+} and β-gal^{+} cells was an estimate of the total number of pancreatic cells in wild-type and *Prox1*$^{-/-}$ pancreata, respectively.

In situ hybridization

Digoxigenin-labeled antisense mRNA probes were transcribed in vitro by using plasmids kindly provided by D. Anderson (neurogenin 3 [Ngn3]), M.J. Tsai (NeuroD/Beta2), J. Jensen (Hes-1), J. Hald (Notch1), and U. Lendahl (Notch2). The production of Pax4 antisense riboprobes was previously described (Wang et al., 2004). The probes were used for nonradioactive in situ hybridization on 12-μm frozen sections as previously described (Wang et al., 2004).

BrdU staining

Pregnant females were injected with BrdU (100 μg/g of body weight) at E11.5 of gestation. Embryos were dissected 2 h later and processed for cryosectioning as previously described. Frozen sections were incubated in blocking solution (20% fetal bovine serum and 2% Boehringer Blocking Powder) for 30 min, washed with Tris-buffered saline with 0.1% Tween-20, incubated in 2 N HCl for 15 min and rinsed four times with 0.1 M sodium borate solution (pH 8.5). After this rinsing, sections were incubated with anti-BrdU antibody overnight and then with secondary Cy3-labeled anti-mouse IgG antibody for 3 h.

Microarray analysis

Gene expression analyses were performed at the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital. The Affymetrix MOE-430A GeneChip array, which contains probes for 14,484 well-characterized mouse genes, was used in the analysis. Dorsal pancreata from four E12.5 wild-type embryos and four *Prox1*$^{-/-}$ littermates were dissected. After genotyping, pancreata of the same genotype were pooled, and RNA was extracted. Total RNA was prepared by using the TRIzol method (Invitrogen) and quantified by spectrophotometry. An Agilent 2100 Bioanalyzer evaluated RNA quality (i.e., the integrity and relative abundance of the 28S and 18S ribosomal RNAs). Two hundred nanograms of total RNA were subjected to two rounds of linear amplification as described in the Affymetrix small-sample version 2.0 protocol. Briefly, RNA was annealed to a T7-oligo(dT) primer, and double-stranded cDNA was generated by using the SuperScript II cDNA synthesis kit according to the manufacturer's instructions (Invitrogen). After ethanol precipitation, the cDNA, which served as a template, and the MEGAscript T7 kit (Ambion) were used to synthesize cRNA. After purification, 400 ng of cRNA served as the starting template for a second round of cDNA synthesis in which random hexamers initiated reverse transcription and the T7-oligo(dT) primer completed second-strand synthesis. The resulting cDNA was ethanol precipitated and used as a template to generate biotin-labeled cRNA (T7 RNA polymerase-based Bioarray HighYield RNA Transcript Labeling Kit; ENZO Diagnostics, Inc). Ten micrograms of biotin-labeled cRNA was fragmented by heating and metal-induced hydrolysis, added to a hybridization cocktail that contained probe array controls and blocking agents, and incubated overnight at 45°C on a GeneChip array. After hybridization, a GeneChip Fluidics Station 400 washed the arrays automatically under high-stringency conditions to remove nonhybridized labeled cRNA. Arrays were incubated with R-phycoerythrin conjugates of streptavidin (SAPE, Molecular Probes), washed, and incubated with biotin-conjugated anti-streptavidin antibody. After removal of the antibody solution, the arrays were restained with SAPE, washed again, and scanned by the Affymetrix GeneChip Scanner 3000. Expression signals for each gene were calculated by the Affymetrix GCOS software (version 1.1) that uses the global scaling method with the 2% trimmed average signal set to 500. By establishing values of the wild-type sample as the baseline and by using the GCOS software, we evaluated changes in gene expression. Detection and change calls for each probe set were determined by using the default parameters of the software and as recommended by the Affymetrix GeneChip protocol. To compare gene expression at E12.5, we compared the hybridization results of the wild-type sample with those of the mutant sample.

RT-PCR

The total RNA isolated from four E12.5 wild-type or *Prox1*$^{-/-}$ pancreata was treated with RNAse-free DNAse and then used with the Advantage RT-for-PCR kit (Clontech) and random hexamer primers to synthesize cDNA. Two microliters of this reaction product served as a template for PCR using the following primers: amylase-specific forward

primer, 5′-TGTCCTATTTAAAGAACTGG-3′, and reverse primer, 5′-CTTCTTTTGTTACTCCATTG-3′; *Cck*-specific forward primer, 5′-GCACTGCTAGCGCGATACAT-3′, and reverse primer, 5′-GGCTGAGATGTGGCTGCATT-3′; and cyclophilin-specific forward primer, 5′-CAGGTCCTGG-CATCTTGTCC-3′, and reverse primer, 5′-TTGCTGGTC-TTGCCATTCCT-3′. During each cycle of PCR, the samples were incubated at 94°C for 45 s, at 60°C for 45 s, and at 72°C for 45 s. Twenty-eight cycles were conducted to amplify cyclophilin cDNA; 40 cycles were performed to amplify *Cck* cDNA; and 50 cycles were done to amplify amylase cDNA. Amplification was followed by a 10-min period of extension at 72°C.

Statistical analyses

The Student's *t* test was used to compare the proportions of cells that expressed markers of interest in *Prox1*$^{+/+}$ and *Prox1*$^{-/-}$ pancreata. A P value < 0.05 indicated a statistically significant difference.

URLs

Complete details about the Affymetrix MOE-430A GeneChip array are available at http://www.affymetrix.com.

Results

Prox1 is widely expressed throughout the ontogeny of the mouse pancreas

As previously reported (Burke and Oliver, 2002), Prox1 protein was expressed in the dorsal pancreatic anlagen of E9.5 embryos (Fig. 1A); this expression was similar to that of Pdx1 (Fig. 1A′), one of the earliest pancreatic markers

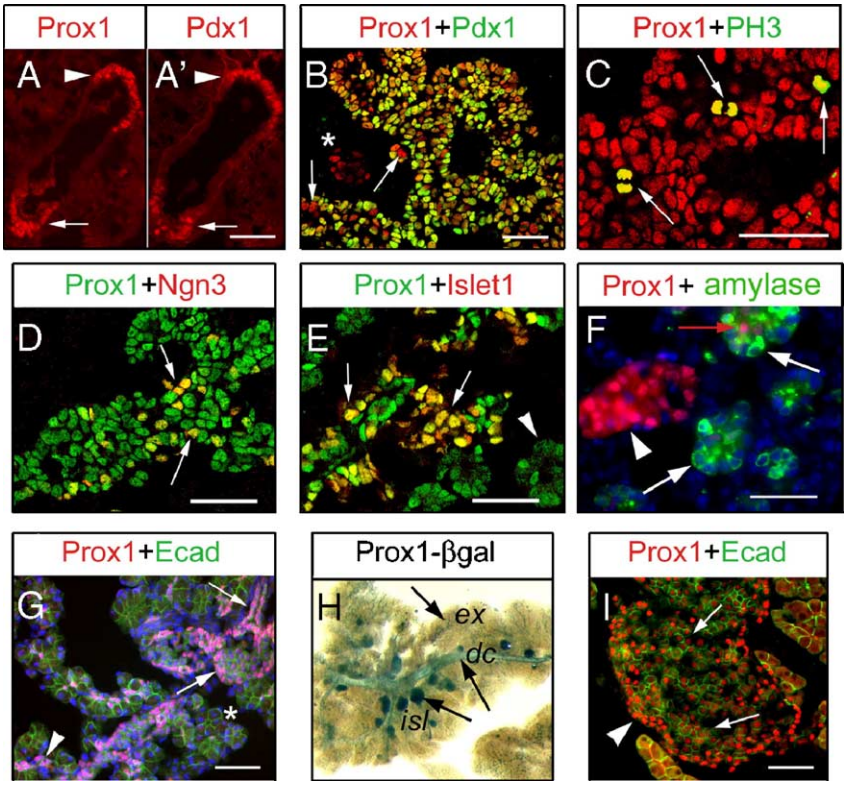

Fig. 1. Temporal expression and distribution of Prox1 during development of the mouse pancreas. (A and A′) At E9.5, Prox1 (red, A) and Pdx1 (red, A′) were similarly expressed in the dorsal (arrowhead) and ventral (arrow) pancreatic regions. (B) At E13.5, Prox1 (red) and Pdx1 (green) were co-expressed in most pancreatic epithelial cells, but a small number of cells located in large clusters (asterisk) and within the epithelium (arrows) expressed only Prox1. (C and D) At E13.5, Prox1 proteins (red in panel C, green in panel D) were visible in cells undergoing mitosis (phospho-histone3^{+} [PH3^{+}] cells, arrows and yellow in panel C) and in newly specified endocrine precursors (Ngn3^{+} cells, arrows and yellow in panel D). (E) In E15.5 pancreata, Prox1 protein (green) was abundant in cells expressing the pan-endocrine marker Isl1 (yellow, arrows), but Prox1 expression in other regions of this tissue (arrowhead) was low. (F) At E15.5, Prox1 protein (red) was expressed at high levels in clusters of cells (arrowhead) and at medium or low levels (red arrow) in cells located at the center of exocrine acini. Conversely, in cells expressing the exocrine marker amylase (arrows and green), the levels of Prox1 proteins were very low or absent. (G) In newborn pancreata, Prox1 was expressed at high levels in developing islets and ducts (arrows and pink) and in centroacinar cells (arrowhead), and at very low levels in exocrine acini (asterisk). (H) In adult pancreata, the expression of Prox1 was observed in islets (*isl*), in large ducts (*dc*), and in small ducts distributed within the exocrine tissue (*ex*). (I) In adult pancreatic islets, Prox1 (red) was expressed at high levels in cells located at the periphery (arrowhead) and at lower levels in the core of insulin-producing cells (arrows). (A–G, I) Frozen sections of the pancreata of wild-type embryos and of adults stained with specific antibodies. (F and G) Cell nuclei were stained with DAPI. (H) Whole *Prox1*$^{+/-}$ pancreas stained with X-gal. Confocal images are shown in B-E. Ecad, E-cadherin; Ngn3, neurogenin3; βgal, β-galactosidase. Scale bars, 200 μm.

(Edlund, 2002; Murtaugh and Melton, 2003; Offield et al., 1996). Later in development, co-expression of Pdx1 and Prox1 proteins was detected in most of the E13.5 pancreatic epithelium (Fig. 1B), with the exception of a few isolated cells and of cells arranged in clusters that expressed Prox1 but were devoid of Pdx1 immunoreactivity (Fig. 1B). In E13.5 pancreata, we detected Prox1 immunoreactivity in proliferating cells (identified by expression of the mitotic marker phosphohistone 3; Fig. 1C) and in newly specified endocrine precursors that expressed the basic helix–loop–helix protein neurogenin3 (Ngn3) (Fig. 1D) (Wilson et al., 2003). Starting at around E15.5, endocrine cells (identified by expression of the pan-endocrine marker islet1 [Isl1]; Ahlgren et al., 1997) expressed high levels of Prox1 protein (Fig. 1E), but in differentiating exocrine cells the expression of Prox1 was nearly absent (Fig. 1F). In newborn pancreata, high levels of Prox1 were detected in ducts from which endocrine islets seemed to emerge, in cells located at the periphery of these islets, and in centroacinar cells (Fig. 1G). In contrast, cells that were located at the core of the islets and corresponded to insulin-producing beta cells expressed only moderate levels of Prox1; pancreatic exocrine acini were almost devoid of this protein (Fig. 1G). The same expression profile remained in adult pancreata (Figs. 1H and I). Previous studies demonstrated that the activity of Prox1 is essential for the appropriate formation of various organs (Dyer et al., 2003; Sosa-Pineda et al., 2000; Wigle and Oliver, 1999; Wigle et al., 1999). Therefore, we hypothesized that Prox1 also controls certain aspects of pancreatic ontogeny. To investigate this premise, we undertook an extensive molecular analysis of Prox1-deficient pancreata.

Loss of functional Prox1 disrupts pancreas morphology

The death of *Prox1*$^{-/-}$ embryos at approximately E15.0 is probably the result of multiple developmental alterations (Wigle et al., 1999). In wild-type embryos at this stage, the pancreatic epithelium has developed numerous branches that normally harbor a mixed population of undifferentiated and differentiating precursors (Figs. 2A and C). The histological and immunohistochemical analyses of E15.0 wild-type pancreata revealed regions of stratified epithelium (Figs. 2E and G) and large, eosinophilic cell aggregates emerging from the basolateral side of this tissue (Fig. 2G). In contrast, E15.0 Prox1-deficient pancreata were smaller than those of wild-type or heterozygous embryos and almost entirely lacked branches (Figs. 2B and D). In addition, the pancreata of E15.0 *Prox1*$^{-/-}$ littermates not only appeared underdeveloped but also possessed large areas of simple columnar epithelium with a ductal appearance (Figs. 2D and F). Likewise, in these mutant tissues the majority of eosinophilic clusters were smaller than those of wild-type littermates (compare Fig. 2G with H). Together, these alterations indicate that Prox1 activity is required to control the size and appropriate morphogenesis of the normal pancreas.

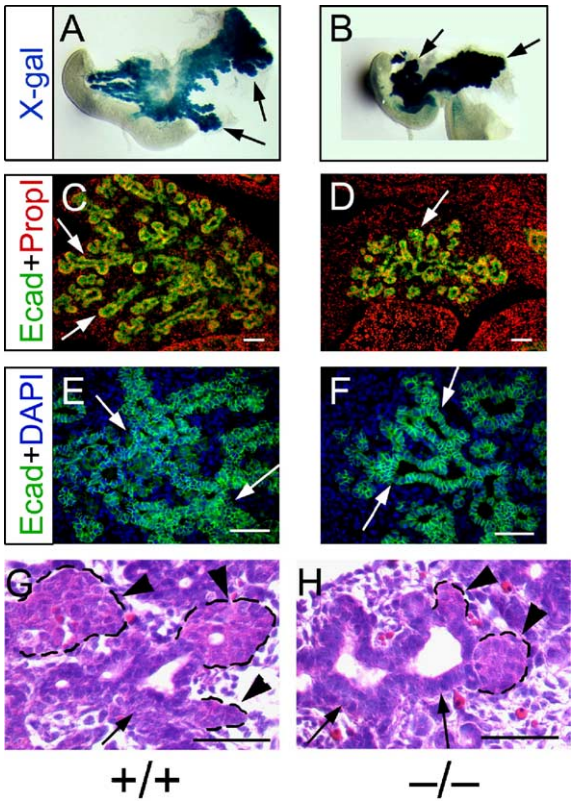

Fig. 2. Abnormalities of E15.0 Prox1-deficient pancreata. (A, C, E, G) The pancreata of E15.0 *Prox1*$^{+/-}$ (A) and wild-type (C, E, G) embryos had numerous branches (arrows in panels A and C). E15.0 wild-type pancreata also have regions of pseudostratified epithelium (arrows in panels E and G). Large clusters of cells containing abundant cytoplasm are also visible in the vicinity of the pancreatic epithelium or emerging from it (arrowheads in panel G). (B, D, F, H) In contrast, E15.0 *Prox1*$^{-/-}$ pancreata were smaller (compare panel A with B) and almost devoid of branches (arrows in panels B and D). E15.0 *Prox1*$^{-/-}$ pancreata appeared to be less developed than those of wild-type littermates, because it contained large portions of poorly stratified epithelium with a ductal appearance (arrows in panel F) and smaller endocrine cell aggregates (arrowheads in panel H). (A and B) Whole pancreata of *Prox1*$^{+/-}$ (A) and *Prox1*$^{-/-}$ (B) embryos stained with X-gal. (G and H) Paraffin sections of E15.0 wild-type (G) or *Prox1*$^{-/-}$ (H) embryos stained with hematoxylin–eosin. (C–F) Frozen sections stained with antibody to E-cadherin (green) and with a nuclear marker (propidium iodide, red in panels C and D; DAPI, blue in panels E and F). Scale bars, 200 μm.

Loss of functional Prox1 hinders pancreatic growth

Our analysis of E9.5 *Prox1*-nullizygous mice did not show obvious alterations in the morphology or size of the pancreatic anlagen (Figs. 3A and D). However, after E11.5 the pancreatic tissues of *Prox1*-nullizygous mice were consistently smaller than those of wild-type littermates (Figs. 3B and E). Moreover, quantitative analysis of pancreatic epithelial cells (i.e., Prox1-positive cells of wild-type pancreata [Fig. 3B] or β-galactosidase [β-gal]-positive cells of *Prox1*$^{-/-}$ pancreata [Fig. 3E]) revealed that the size of the E11.5 *Prox1*-nullizygous pancreas was approximately 63% of that of wild-type littermates (7162 ± 899 cells [wild type] vs. 4560 ± 560 cells

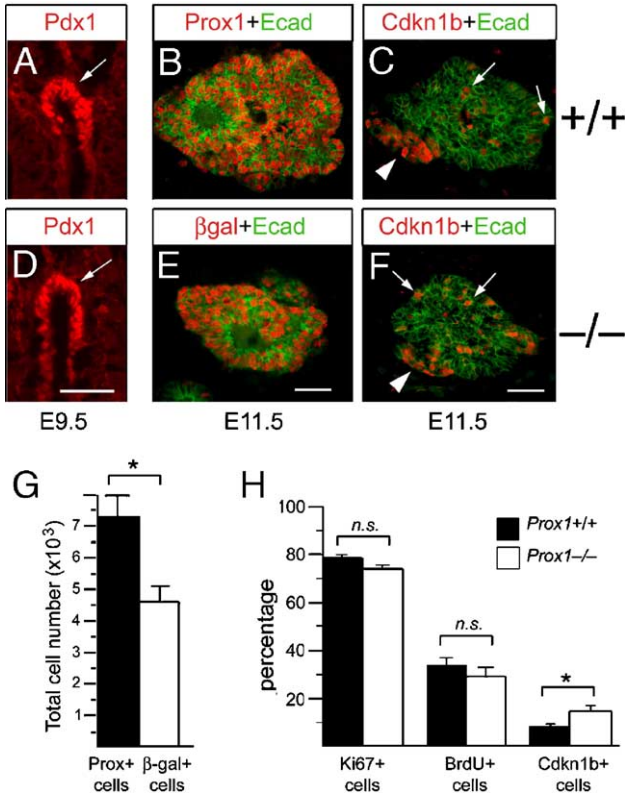

Fig. 3. Lack of Prox1 activity arrests pancreatic growth at around E11.5. (A–F) Frozen sections of wild-type (A–C) and *Prox1*$^{-/-}$ (D–F) pancreata. (A and E) At E9.5, the expression of Pdx1 (red, arrow) was indistinguishable between dorsal pancreata of wild-type (A) and *Prox1*$^{-/-}$ (D) embryos. (B and E) At E11.5, Prox1 (red in panel B) was detected in all pancreatic cells of wild-type embryos, and β-gal was detected in all pancreatic cells of *Prox1*$^{-/-}$ embryos (red in panel E). (G) By estimating the total number of cells that expressed Prox1 (wild type) or β-gal (*Prox1*$^{-/-}$), we determined that the size of E11.5 *Prox1*$^{-/-}$ pancreata was approximately 63% of that of wild-type pancreata. (H) The proliferation index (i.e., the percentage of Ki67^{+} cells or of cells that incorporated BrdU) in E11.5 *Prox1*$^{-/-}$ pancreata was not significantly different than that in pancreata of wild-type littermates. (H) However, in E11.5 *Prox1*$^{-/-}$ pancreata, the proportion of cells that expressed Cdkn1b, an indicator of exit from the cell cycle (red in panels C and F), was almost twice that in wild-type pancreata. (B, C, E, F) Pancreatic epithelia were stained with antibody to E-cadherin (green). Asterisks indicate statistically significant differences (i.e., $P < 0.05$ in comparisons of mutant and wild-type tissues); n.s. indicates differences that were not statistically significant (as determined by the Student's *t* test). Scale bars, 200 μm.

[*Prox1*$^{-/-}$]; $P < 0.01$, 7 embryos per group) (Fig. 3G). To determine whether the reduced size of Prox1-deficient pancreatic tissues was the result of defective proliferation, we compared the proliferation index (i.e., the proportion of cells that expressed Ki67 or that incorporated BrdU) between E11.5 *Prox1*$^{-/-}$ pancreata and wild-type pancreata. Our results showed no significant difference in the proliferation index of E11.5 *Prox1*$^{-/-}$ pancreata (Ki67: 78.5% ± 1.5% [wild type] vs. 75.6% ± 0.4% [*Prox1*$^{-/-}$]; $P = 0.36$. BrdU: 33.5% ± 2.8% [wild type] vs. 29.1% ± 3.2% [*Prox1*$^{-/-}$]; $P = 0.37$; 3 embryos per group) (Fig. 3H). Similarly, the reduced size of *Prox1*$^{-/-}$ pancreata also did not seem to result from increased cell death, as no overt apoptosis was detected in these mutant tissues between E11.5 and E15.0 (data not shown). In contrast, our quantitative analysis showed a significant increase (by a factor of approximately 1.8) in the proportion of cells that expressed the cell cycle-exit indicator Cdkn1b in E11.5 *Prox1*-deficient pancreatic tissues (9.1% ± 1.3% [wild type] vs. 15.2% ± 1.9% [*Prox1*$^{-/-}$]; $P < 0.01$; 3 embryos per group) (Figs. 3C, F, H). Cdkn1b (also known as p27^{Kip1}) is a cyclin kinase inhibitor of the Cip/Kip family that causes cell cycle arrest by blocking phosphorylation of the retinoblastoma protein (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996; Sherr and Roberts, 1995). This result raises the possibility that the inability of some epithelial progenitors to undergo additional rounds of cell division contributed to the decrease in size of the *Prox1*-deficient pancreata.

In early pancreatic tissues, the activity of Notch maintains the undifferentiated state of progenitors and, conversely, the absence of Notch activity prevents pancreatic growth by promoting en masse endocrine differentiation of most pancreatic progenitors (Apelqvist et al., 1999; Esni et al., 2004; Hald et al., 2003; Jensen et al., 2000; Murtaugh et al., 2003). Therefore, one possible explanation for the reduced size of *Prox1*-deficient pancreatic epithelia is that the absence of Prox1 activity disrupted *Notch* signaling. However, this possibility seems unlikely because in *Prox1*-deficient pancreata we did not detect a substantial increase in the number of Isl1^{+} cells between E9.5 and E11.5 (Supplementary Figs. 1A, B, D, E, and Table 1), in the proportion of Pax6^{+} cells at E11.5 (18.3% ± 3.6% of wild-type vs. 19.3% ± 3.4% of *Prox1*$^{-/-}$ embryos; $P = 0.4$; 3 embryos per group; Table 1), or in the proportion of Ngn3^{+} endocrine progenitors at E11.5 (Supplementary Figs. 1C, F, and Table 1). Moreover, by using in situ hybridization, we found that the expression of three components of the *Notch* signaling pathway (*Notch1*, *Notch2*, *and Hes-1*; Lammert et al., 2000) was comparable between pancreata of E13.5–E14.5 wild-type embryos and *Prox1*-nullizygous littermates (Supplementary Fig. 2).

Table 1
Endocrine cell counting

Stage	Marker	No. of positive cells		
		Wild type	*Prox1*$^{-/-}$	*n*
E9.5[a]	Islet1	26	30	1
E10.5[b]	Islet1	104	107	1
E11.5[c]	Islet1	191.3 ± 47.4	161.0 ± 45.4	3
E11.5[b]	Ngn3	28 (7.5)[d]	22 (7.4)[d]	1
E11.5[a]	Pax6	263.0 ± 93.6 (18.3 ± 3.6)[e]	174.7 ± 29.0 (19.3 ± 3.4)[e]	3

n, pairs of littermates.
[a] 6 sections.
[b] 3 sections.
[c] 5 sections.
[d] Percentage of Ngn3^{+} cells in the entire pancreatic cell population (as determined by the total number of Prox1^{+} cells of wild-type tissue or β-gal + cells of *Prox1*$^{-/-}$ tissue).
[e] Percentage of Pax6^{+} cells in the entire pancreatic cell population.

Taken together, our results indicate that the reduced size of *Prox1*-deficient pancreatic tissues did not result from defective cell proliferation or from the absence of Notch activity. However, it is possible that the arrest in this organ's growth resulted in part from premature withdrawal of epithelial progenitors from the cell cycle, as indicated by the increase in the proportion of cells that expressed Cdkn1b/p27 in *Prox1*-deficient pancreata at E11.5.

Comparative microarray analysis revealed increased expression of Cck (cholecystokinin) in Prox1-deficient pancreata

To identify additional alterations in gene expression that resulted from the lack of pancreatic Prox1 activity, we conducted a comparative gene-profiling analysis by using dorsal pancreata dissected from E12.5 wild-type and *Prox1*-nullizygous embryos. After hybridizing wild-type and mutant cRNAs to an Affymetrix MOE-430A GeneChip and comparing the results of the hybridization as described in the Materials and methods section, we created a list of genes whose expression was consistently increased or decreased by more than a factor of 2.0 in the *Prox1*-deficient pancreata (Table 2, Supplementary data). Overall, these results did not show significant alterations in the expression of genes encoding products known to be required for cell proliferation. In contrast, our microarray results indicated a significant enrichment (by a factor of approximately 5.7) of *Cck* transcripts in RNA isolated from *Prox1*-nullizygous mice (Table 2, Supplementary data). In wild-type mice, the hormone Cck is largely produced by mature enteroendocrine duodenal cells (Rindi et al., 2004). In addition, expression of Cck has also been observed in a few cells of the mouse pancreata toward the end of gestation and at postnatal stages (Liu et al., 2001). To our knowledge, no studies have shown that Cck is expressed early in pancreas development. Thus, by using immunohistochemistry, we sought to characterize the expression of Cck in pancreatic tissues of wild-type mouse embryos between E11.5 and E18.5 (Fig. 4).

The differentiation of various types of pancreatic endocrine cells occurs in a time-restricted manner (Pictet and Rutter, 1972; Slack, 1995). Between E9.5 and E12.5 in mouse development, an early wave of differentiation produces endocrine cells that synthesize mainly glucagon, although a reduced number of early pancreatic endocrine cells also express ghrelin, insulin, or islet amyloid polypeptide, either alone or in combination with glucagon (Pictet and Rutter, 1972; Wilson et al., 2002; Prado et al., 2004). We have now determined that a small population of Cck-expressing cells is also present at around E11.5 in the mouse pancreata (Figs. 4A–E). In these tissues, a few Cck-positive cells were found scattered throughout the pancreatic epithelium; however, the majority of Cck^+ cells appeared associated with clusters of cells (Figs. 4A–E). The association of pancreatic Cck^+ cells with large endocrine cell clusters was also observed at E13.5 (Fig. 5D). Previous studies of cell lineage showed that all pancreatic epithelial cells originate from $Pdx1^+$ precursors (Gu et al., 2002). In E11.5 wild-type pancreatic tissues, we found that most Cck^+ cells co-expressed Prox1 proteins (Fig. 4A), and conversely the majority of Cck-expressing pancreatic cells did not

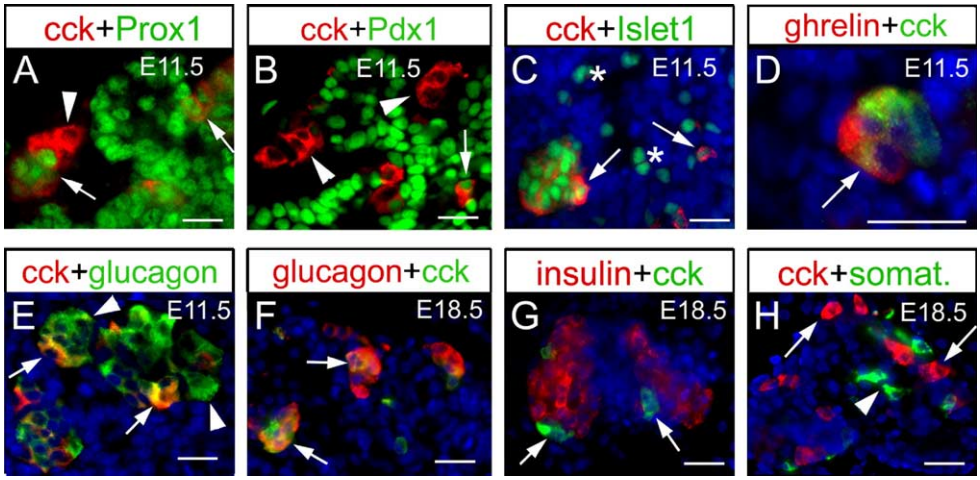

Fig. 4. A population of early pancreatic endocrine cells expressed cholecystokinin (Cck). (A–E) In the pancreata of E11.5 mouse embryos, Cck immunoreactivity (red in panel A) largely colocalized with Prox1 protein (green in panel A; arrows indicate colocalization; arrowhead indicates a Cck^+ cell devoid of Prox1 immunoreactivity). (B) In contrast, most Cck^+ cells (red) did not express Pdx1 (green; arrowheads indicate $Cck^+/Pdx1^-$ cells; the arrow points to a Cck^+ cell that co-expressed a low level of Pdx1 protein). (C) In pancreatic tissues, Cck-expressing cells (red) were endocrine because they all expressed the pan-endocrine marker Isl1 (green; arrows indicate colocalization of Cck and Isl1 proteins; the asterisks point to $Isl1^+/Cck^-$ cells). (D and E) Additionally, Cck^+ cells (green in panel D and red in panel E) largely co-expressed glucagon (arrows in panel E; arrowheads point to $glucagon^+/Cck^-$ cells) or, in rare occasions, ghrelin (arrow in panel D). (F–H) In pancreatic tissues of E18.5 embryos, Cck immunoreactivity (red in panel H and green in panels F and G) largely colocalized with glucagon (red in panel F, arrows). In contrast, at this stage Cck did not co-localize with insulin (red in panel G; arrows point to $Cck^+/insulin^-$ cells) or somatostatin (green in panel H; arrows point to $Cck^+/somatostatin^-$ cells and arrowhead points to $somatostatin^+/Cck^-$ cells). (C–H) Cell nuclei were stained with DAPI. Scale bars, 400 μm.

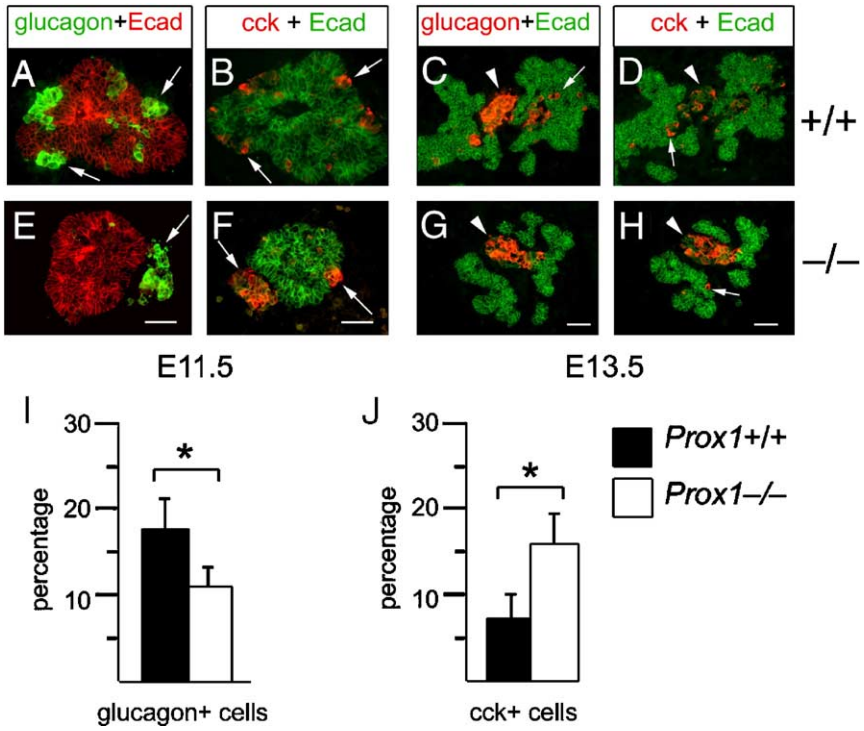

Fig. 5. The absence of Prox1 activity alters the differentiation of early pancreatic endocrine precursors. (A and B) At E11.5, a large fraction of pancreatic endocrine cells expressed glucagon (A, green and arrows), and a small percentage expressed Cck (B, red and arrows). (E–H) The absence of Prox1 activity did not preclude the formation of early pancreatic endocrine cells or their aggregation into clusters (arrows in panels E and F and arrowheads in panels G and H). However, early endocrine differentiation was affected by the absence of Prox1 function: in E11.5 *Prox1*$^{-/-}$ pancreata, more endocrine cells expressed Cck (F and J) than glucagon (E and I). This alteration was also observed at E13.5 in *Prox1*$^{-/-}$ pancreata (compare the expression of Cck [red] between wild-type [arrow and arrowhead in panel D] and *Prox1*$^{-/-}$ pancreata [arrow and arrowhead in panel H], and that of glucagon [red] between wild-type [arrow and arrowhead in panel C] and *Prox1*$^{-/-}$ [arrow and arrowhead in panel G] pancreata). (A–H) Pancreatic epithelia were stained with antibody to E-cadherin (green in panels B–D, F–H; red in panels A and E). Asterisks indicate statistically significant differences (i.e., $P < 0.05$ in comparisons of mutant and wild-type tissues); n.s. indicates differences that were not statistically significant (as determined by the Student's *t* test). Scale bars, 200 μm.

express Pdx1 (Fig. 4B). Thus, it appears that the expression of Cck follows a rapid decline in the level of Pdx1 proteins in pancreatic tissues. In addition, pancreatic Cck-expressing cells are largely post-mitotic; that is, they do not co-express the mitotic marker phospho-histone3 (data not shown). Not surprisingly, Cck-expressing pancreatic cells appeared to be endocrine cells-all co-expressed islet1 (Fig. 4C). Moreover, most Cck^{+} cells in embryonic pancreatic tissues co-expressed glucagon (Fig. 4E) or, occasionally, ghrelin (Fig. 4D). This observation is consistent with previous reports that indicated that cells expressing multiple hormones are present in early pancreatic tissues of mouse embryos (Herrera, 2002; Prado et al., 2004; Wilson et al., 2002). Although numerous pancreatic Cck^{+} cells also co-expressed glucagon at E18.5 (Fig. 4F), colocalization of Cck and ghrelin proteins was rare in these tissues (data not shown), and we did not detect co-expression of either of Cck and insulin or Cck and somatostatin (Figs. 4G and H). Hence, these results suggest that Cck^{+} pancreatic cells represent a subpopulation of alpha (i.e., glucagon-expressing) cells.

Remarkably, the proportion of Cck-expressing cells was larger in the pancreata of E11.5 *Prox1*$^{-/-}$ embryos than that in the pancreata of wild-type littermates (7.1% ± 3.9% [wild type] vs. 17.1% ± 2.7% [*Prox1*$^{-/-}$]; $P = 0.01$; 3 embryos per group) (Figs. 5B, F, J). This increase did not seem to result from an abnormal expansion of the population of endocrine cells because E11.5 Prox1-deficient pancreata also had proportionately fewer glucagon-synthesizing cells than their wild-type counterparts (18.1% ± 5.2% [wild type] vs. 11.1% ± 2.1% [*Prox1*$^{-/-}$]; P=0.049; 3 embryos per group) (Figs. 5A, E, I). This altered proportion of Cck^{+} and glucagon^{+} cells was also observed in E13.5 Prox1-deficient pancreata (compare Figs. 5C and D with Figs. 5G and H). Together, these results suggest that although Prox1 activity is dispensable for the formation of early pancreatic endocrine cells, it probably influences cell fate decisions by antagonizing the expression of hormones normally enriched in more posterior regions of the gastrointestinal tract.

Loss of functional Prox1 affects islet cell genesis

In mouse embryos, a second wave of pancreatic endocrine differentiation (the "secondary transition") commences at around E13.5 and persists throughout gestation (Pictet and Rutter, 1972). During this period, the pancreatic precursors gradually differentiate into one of the four main types of

islet cells (Pictet and Rutter, 1972; Murtaugh and Melton, 2003). In wild-type embryos at this stage, the pancreatic endocrine cell population is composed of large clusters of hormone-producing/Isl1$^+$ cells located towards the basolateral side of the pancreatic epithelium (arrowheads in Figs. 5C, D, and 6A), scattered endocrine cells (Isl1$^+$) (arrows in Fig. 6A), and numerous, newly specified endocrine precursors (Ngn3$^+$) that appear dispersed throughout most of the epithelium (Fig. 6C). In the pancreata of E13.5 *Prox1*-deficient embryos, some Isl1$^+$ cell clusters were still present (arrowhead in Fig. 6B); however, only a small number of individual endocrine (Isl1$^+$) cells or very few endocrine precursors (Ngn3$^+$) were detected in these mutant tissues (Figs. 6B and D). This observation suggests that after E13.5, the loss of pancreatic Prox1 reduces the production of islet cell precursors. To further investigate this possibility, we compared the expression of two other early endocrine markers (NeuroD and Pax4; Wilson et al., 2003) or that of insulin between E13.5 and E14.5 wild-type and *Prox1*$^{-/-}$ pancreata. Accordingly, while numerous cells of wild-type pancreata expressed any of those three markers (Figs. 6G, I, K), a substantial reduction in the number of cells expressing NeuroD (Fig. 6H), Pax4 (Fig. 6J), or insulin (Fig. 6L) was observed in E14.0–E14.5 Prox1-deficient pancreata. Together, these results indicate that after E13.5 the activity of Prox1 is necessary for the genesis of pancreatic islet cell precursors, for their maintenance, or for both processes.

Loss of functional Prox1 increases exocrine cell genesis

The results of our microarray analysis revealed a substantial, abnormal increase in exocrine-specific transcripts in the pancreata of E12.5 Prox1-deficient embryos (e.g., amylase [*Amy2*] transcripts were increased by a factor of 2.5; serine protease 2 [*Prss2*], by a factor of 3.0–48.5; and trypsin 4 [*Try4*], by a factor of 3.0 [Table 2, Supplementary data]). By using semi-quantitative RT-PCR, we further confirmed that the loss of pancreatic Prox1 activity noticeably increased the expression of amylase RNA at E12.5 (Fig. 7A). These results raised the possibility that, contrary to its effects on islet cell genesis, the loss of Prox1 activity in pancreatic tissues increases the production of exocrine cells. To test this hypothesis, we analyzed the expression of various exocrine markers in the pancreata of *Prox1*-nullizygous embryos and wild-type littermates at E13.5 or E14.5.

In E13.5–14.5 pancreata, the population of endocrine, islet cell precursors (Isl1$^+$) normally disperses within a broad “endocrine territory” distinguished by the expression of the homeodomain transcription factor Nkx6.1 (Figs. 7B

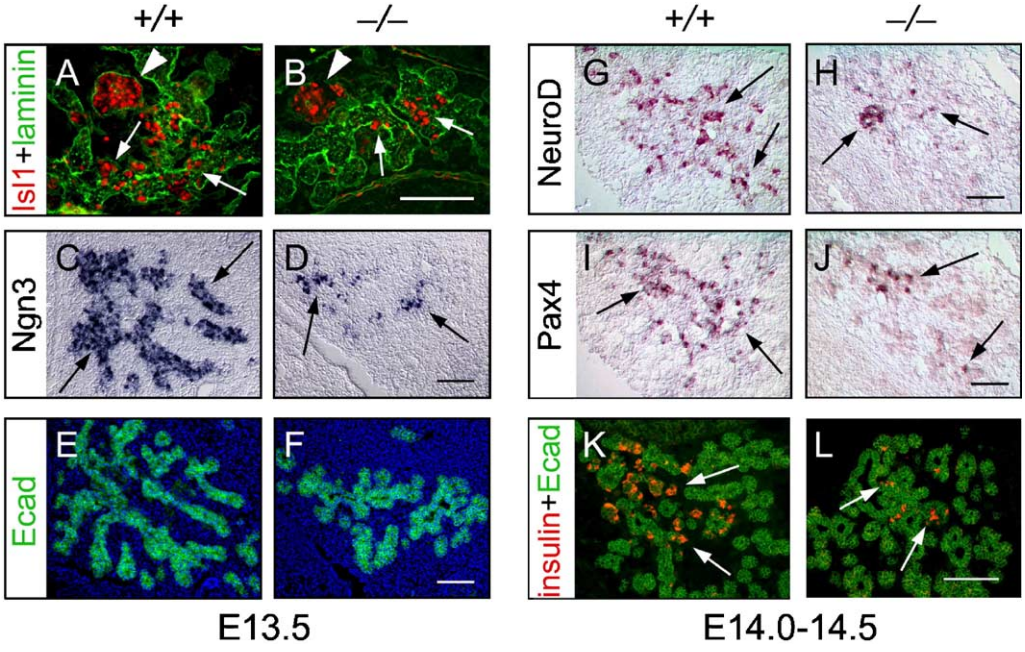

Fig. 6. The formation of endocrine cells in Prox1-deficient pancreatic tissues decreases considerably after E13.5. (A) In the pancreata of E13.5 wild-type embryos, two distinct populations of endocrine cells (Isl1$^+$ cells) are present: those that form large aggregates (arrowhead) and those that are scattered within the epithelium (arrows). (B) While E13.5 *Prox1*$^{-/-}$ pancreata still had some aggregates of endocrine cells (arrowhead) only a few Isl1$^+$ cells were dispersed within the epithelium (arrows). (C) In wild-type pancreata, the production of endocrine progenitors (Ngn3$^+$ cells, arrows) increased significantly at around E13.5. At E14.0, numerous endocrine precursors expressed NeuroD (arrows in panel G) or Pax4 (I). (K) At E14.5, some endocrine precursors started to differentiate into insulin-producing beta cells (red and arrows). Conversely, the pancreata of E13.5–E14.5 Prox1-deficient embryos had significantly fewer endocrine progenitors (arrows in panel D) and only a very small number of cells expressed NeuroD (arrows in panel H), Pax4 (arrows in panel J), or insulin (arrows in panel L). The pancreatic epithelia were visualized by staining with anti-laminin antibodies (A and B) or anti-E-cadherin (Ecad) antibodies (E, F, K, L). (K and L) Anti-insulin antibodies were used to stain differentiating beta cells. Transcripts for Ngn3 (C and D), NeuroD (G and H) or Pax4 (I and J) were detected by digoxigenin-labeled antisense probes. Sequential, adjacent sections are shown in panels C and E and in panels D and F. Scale bars, 100 μm.

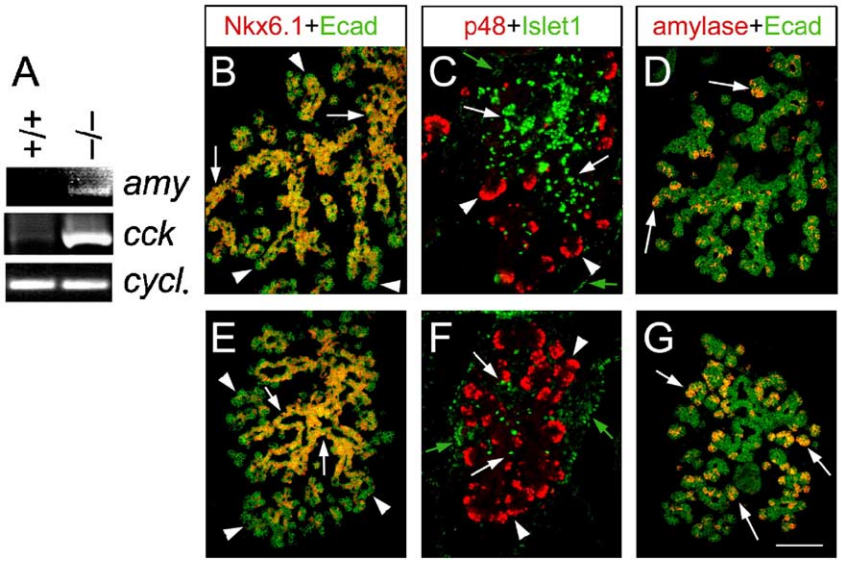

Fig. 7. Exocrine cells differentiate prematurely in Prox1-deficient pancreatic tissues. (A) RT-PCR analysis of E12.5 wild-type and *Prox1*$^{-/-}$ pancreata confirmed the microarray results that indicated increased transcription of amylase (*Amy*) and cholecystokinin (*cck*) in E12.5 Prox1-deficient tissues. (B and C) In the pancreatic epithelia of E14.5 wild-type embryos, Isl1^{+} cells (arrows in panel C) appeared to be included within an "endocrine territory" that expressed Nkx6.1 protein (orange and arrows in panel B). In contrast, the tips of the branches that were largely devoid of Nkx6.1 immunoreactivity (arrowheads in panel B) had numerous cells that expressed the exocrine marker p48 (arrowheads in panel C). In these tissues, endocrine cells (Isl1^{+} cells, arrows and green in panel C) clearly outnumbered exocrine cell precursors (p48^{+}, red and arrowheads in panel C). Conversely, the domain of Nkx6.1 expression in the pancreata of E14.5 Prox1-deficient embryos (orange and arrows in panel E) was reduced and was virtually absent at the periphery of these epithelia (arrowheads in panel E). Also, the ratio of Isl1^{+} cells to p48^{+} cells in these mutant tissues was inverted in comparison with the ratio in pancreata of wild-type littermates (compare panel C with F). (D and G) Few exocrine cells (amylase^{+}) were visible in the pancreatic epithelia of E14.5 (arrows in panel D) wild-type embryos. In contrast, the population of exocrine cells (amylase^{+}; orange and arrows in panel G) was abnormally increased in the pancreata of E14.5 Prox1-deficient embryos. In (B, D, E, G) pancreatic epithelia were visualized by staining with anti-E-cadherin antibodies (green). (C and F) Green arrows indicate Isl1 expression in mesenchymal cells. In (B and E) the image of Nkx6.1 staining was enhanced to show areas of low-level and high-level immunoreactivity. Scale bars, 100 μm.

and C) (Sander et al., 2000). Conversely, the increasing population of exocrine precursors (expressing the basic helix-loop-helix transcription factor p48) localizes to the tip of the elongating branches (arrowheads in Figs. 7B and C) and is largely excluded from areas expressing Nkx6.1 (arrows in Fig. 7B) or Isl1 (arrows in Fig. 7C) (Krapp et al., 1998; our own observations). Unexpectedly, in E14.5 *Prox1*$^{-/-}$ pancreata the domain of Nkx6.1 expression was more restricted (arrows in Fig. 7E), there were significantly fewer Isl1^{+} cells (arrows in Fig. 7F), and those areas devoid of Nkx6.1 immunoreactivity (arrowheads in Fig. 7E) contained numerous cells that expressed p48 (arrowhead in Fig. 7F). Accordingly, in pancreatic tissues of *Prox1*-nullizygous embryos isolated between E13.5 and E14.5, we observed a substantial increase in the number of pancreatic cells that expressed the exocrine differentiation markers carboxypeptidase A (data not shown) or amylase (compare Fig. 7D with G). Altogether, these results raise the intriguing possibility that the loss of Prox1 activity favors exocrine cell genesis at the expense of the production of new islet cell precursors.

Of note, our microarray data also indicated reduced expression of *neurogenin3* (−1.87) and *Nkx6.1* transcripts (−9.8-fold, "Absent" call) and increased expression of *Ptf1/p48* (+1.7) in *Prox1*-deficient pancreata already at E12.5. However, these specific alterations were not indicated in Table 2 in the Supplementary data section because this table includes only 2-fold changes or "Present" calls.

Discussion

Prox1 activity controls pancreatic growth and pancreas morphogenesis

In this study, we showed that Prox1 is expressed in nearly all pancreatic progenitor cells early in development whereas in pancreatic tissues of late-gestation embryos or adults, Prox1 expression becomes restricted to endocrine islets and to cells located within ducts. The extensive expression of Prox1 in early pancreatic tissues suggests an involvement of Prox1 activity in the specification of pancreatic progenitors, in their proliferation, or in both processes. However, our analysis of *Prox1*-deficient mouse embryos revealed that Prox1 activity is dispensable for pancreas specification because in the absence of Prox1 the initial stages of pancreas organogenesis (i.e., the formation of the dorsal and ventral pancreatic primordia) proceeded normally. Conversely, a prominent delay in pancreatic growth occurred in *Prox1*-deficient embryos after E11.5. As a result, *Prox1*-nullizygous mice had considerably smaller pancreata at the time of their death (at around E15.0) than did their wild-type littermates. In addition, these

mutant tissues had severely impaired morphology: they almost entirely lacked branches and the epithelia appeared to be poorly developed. Thus, our studies determined that Prox1 activity supports pancreatic growth and is also required for appropriate pancreas morphogenesis.

Does Prox1 prevent premature withdrawal of progenitors from the cell cycle?

The lack of pancreatic Prox1 function hindered epithelial growth and prevented the elongation of branches. These two alterations could indicate defective cell proliferation, because previous studies showed that the expansion of the pancreatic primordia and the formation of branches require cell division (Edlund, 2002; Horb and Slack, 2000). Despite this evidence, the results of our comparative microarray analysis did not show any major alterations in the expression of genes encoding cell cycle regulators or components of growth-promoting signaling pathways in the pancreata of E12.5 *Prox1*-deficient embryos (Table 2, Supplementary data). Additionally, our quantitative analyses did not show significant differences in the proliferation index between pancreatic tissues of E11.5 *Prox1*-deficient embryos and their wild-type littermates (Fig. 3H). Overall, the results of these studies indicate that Prox1 does not participate in promoting cell proliferation or cell survival of early pancreatic cells.

Conversely, in E11.5 *Prox1*-deficient pancreata there was an increase in the fraction of cells that expressed Cdkn1b/p27 protein. Interestingly, a number of studies of cultured cells have shown that the expression of Cdkn1b increases in response to extracellular anti-proliferative signals (Fero et al., 1996; Nourse et al., 1994). Therefore, we could hypothesize that in pancreatic progenitors Prox1 normally enables additional rounds of cell division by antagonizing anti-proliferative signals. Alternatively, Prox1 activity could directly increase or stabilize the expression of Cdkn1b/p27 or could contribute to the increase in the pool of pancreatic cells by stimulating the formation of progenitors with self-renewal capacity. Although it is not clear how the activity of Prox1 in pancreatic tissue would prevent the accumulation of Cdkn1b/p27 protein (thereby allowing further rounds of cell division), it is intriguing that in other types of progenitor cells (e.g., neural cells and lens cells) Prox1 or its *Drosophila* homolog prospero does not prevent but rather promotes cell cycle exit (Torii et al., 1999; Wigle et al., 1999; Li and Vaessin, 2000; Cremisi et al., 2003). Conversely, in *Drosophila* glial cell precursors, prospero prevents cell cycle withdrawal by antagonizing the expression of Dacapo, a Cdkn1b homolog (Griffiths and Hidalgo, 2004). Thus, it appears that the influence of Prox1 function (or that of prospero) on cell cycle progression during development is largely dependent on the cell-type context.

Although the lack of antibodies suitable for double-labeling experiments precluded the identification of those Cdkn1b/p27^{+} cells present in developing pancreatic tissue, we speculate that at least some of these cells represent post-mitotic endocrine progenitors. In support of this proposal, Cdkn1b/p27 immunoreactivity was detected within large cell aggregates that also expressed Isl1 in the pancreata of E11.5 *Prox1*-deficient or wild-type mice. Notwithstanding this evidence, it is also possible that some pancreatic Cdkn1b/p27^{+} cells may also represent exocrine progenitors withdrawing prematurely from the cell cycle. Indeed, our microarray data indicated increased differentiation of exocrine progenitors as early as E12.5 in the absence of Prox1 activity (Table 2, Supplementary data).

Previous studies in *Xenopus* and mice have shown that Cip/Kip kinase inhibitors have dual roles in certain tissues: the inhibitors not only promote cell cycle exit, but also enable differentiation of cells into specific subtypes. For example, in *Xenopus* retinal progenitors, the maintenance of high levels of p27^{Xic1} proteins (the homolog of Cdkn1b) promotes a “late”, amacrine cell fate (Ohnuma et al., 1999). In retinal progenitors of mice, Cdkn1c/p57 plays two roles: first, it acts as a cyclin kinase inhibitor in dividing cells, and later it promotes the amacrine interneuronal cell fate of progenitors (Dyer and Cepko, 2000). Thus, it is possible that the abnormal, premature accumulation of Cdkn1b/p27 proteins in *Prox1*-deficient pancreata not only promotes premature cell cycle exit of progenitors, but also enables the “Cck” type of differentiation of these cells. The possibility that Prox1 activity influences endocrine cell fate decisions in early pancreatic tissues is similar to the previously reported function of Prox1 in retinal tissues: Prox1 not only induces the exit of retinal progenitor cells from the cell cycle, but also specifies the fate of horizontal cells (Dyer et al., 2003).

Our microarray results revealed an anomalous increase in transcripts encoding gastric inhibitory peptide (Gip, another hormone normally enriched in enteroendocrine duodenal cells; Rindi et al., 2004) and reduced expression of glucagon (−1.74; data not shown) and islet amyloid polypeptide (−2.64 and −4.0) in E12.5 *Prox1*-deficient pancreata (Table 2, Supplementary data). Unfortunately, we could not verify whether an abnormal increase in the number of Gip-producing cells also occurred in these mutant tissues, because our immunohistochemical analyses failed to detect low levels of this protein (data not shown). Likewise, we could not perform a rigorous, quantitative comparative analysis of IAPP expression between pancreata of *Prox1*-deficient and wild-type littermates, because the number of IAPP^{+} cells is small and varies in early (E10.5–E13.5) pancreatic tissues. However, these specific alterations in gene expression provide further support to our proposal that the loss of Prox1 activity altered the differentiation of early pancreatic endocrine precursors. Overall, the identification of Prox1 as a transcription factor required to determine the fates of various subtypes of pancreatic cells provides a valuable tool to help in characterizing the molecular and cellular events influencing cell fate decisions in the developing pancreas.

Prox1 activity supports endocrine cell genesis during the "secondary transition"

The lack of Prox1 activity did not seem to prevent "early" pancreatic endocrine cell genesis (i.e., the production of those hormone-expressing cells formed between E8.5 and E11.5 in mouse pancreatic tissues) (Murtaugh and Melton, 2003; Wilson et al., 2002) or their aggregation into clusters, as indicated by the normal expression of Isl1 in the pancreata of *Prox1*$^{-/-}$ embryos between E9.5 and E11.5 (Supplementary Fig. 1 and Table 1) (Ahlgren et al., 1997). However, the increase in the fraction of Cck-expressing endocrine cells and the concomitant decrease in glucagon-expressing cells observed in E11.5–E13.5 *Prox1*$^{-/-}$ pancreata suggest a specific requirement of Prox1 activity in the establishment of "early" endocrine cell fates. In contrast, the finding that after E13.5 *Prox1*-deficient pancreata had a substantial decrease in the number of Ngn3-, Pax4-, NeuroD-, or insulin-expressing cells and a more restricted expression of Nkx6.1 also indicated that in these mutant tissues endocrine cell genesis was significantly affected during the period corresponding to the "secondary transition".

Results of our combined microarray and immunohistochemical analyses also identified a substantial increase in the number of pancreatic exocrine cells in *Prox1*-nullizygous embryos and a dramatic increase in exocrine-specific transcripts as early as E12.5. Therefore, how did the removal of Prox1 activity selectively reduce the number of "late" endocrine precursors and simultaneously increase exocrine cell genesis? One possible explanation for the aforementioned results is that Prox1 activity normally enables endocrine cell differentiation of pancreatic bipotent progenitors (i.e., maintains or stabilizes Ngn3 expression) by repressing exocrine cell differentiation (e.g., by antagonizing PITF1/p48 expression) or by counteracting signals that normally promote exocrine cell differentiation in early pancreatic tissues (Kim and Hebrok, 2001). This premise is supported by our finding that the levels of Prox1 protein during normal pancreas development remained high in newly specified endocrine precursors but were markedly reduced in cells that expressed early markers of exocrine differentiation (e.g., amylase or elastase). In addition, previous cell lineage studies using retroviral labeling of pancreatic cells have also suggested the existence of bipotent progenitor cells capable of producing both endocrine and exocrine cell progeny in pancreatic epithelia (Fishman and Melton, 2002; Murtaugh and Melton, 2003). Nonetheless, our results do not rule out the possibility that Prox1 has entirely different functions in endocrine and exocrine pancreatic precursors. For instance, while Prox1 could contribute to maintenance of Ngn3 expression in newly specified endocrine precursors produced after E13.5, in exocrine precursor cells Prox1 could stimulate additional rounds of division before they began to differentiate. In this case, the increment of exocrine cells observed in E13.5–E14.5 *Prox1*$^{-/-}$ pancreata would be merely an indication of premature differentiation of exocrine precursors as a result of premature withdrawal from the cell cycle.

In summary, our studies identified Prox1 as a crucial, novel regulator of multiple processes during early pancreas organogenesis. First and foremost, we propose that the activity of Prox1 is necessary to maintain an expanding pool of undifferentiated pancreatic progenitor cells. In other tissues, particularly the developing mammalian cortex, maintaining the progenitor state of epithelial cells appears necessary not only to ensure appropriate growth, but also to generate cellular diversity (Cremisi et al., 2003; Bajjalieh, 2004). We conclude that the lack of Prox1 activity prevents pancreatic growth and affects the cellular composition of this tissue; therefore, Prox1 is a crucial component of a genetic program that is destined to produce the cellular complexity of the mammalian pancreas.

Acknowledgments

We thank A. Demirkan and K. Ure for assistance with immunostaining and cloning; S. Holloway, S. Kalloway, and J. Shelton for technical assistance; Geoffrey Neale for assistance with microarray screening and microarray data analyses; G. Murti and K. Barnes for help with confocal microscopy; and J.C. Jones for editing of the manuscript. We also thank the following people for generously providing reagents: D. Anderson, H. Edlund, M. German, J. Jensen, T. Jessell, J. Hald, U. Lendahl, D. Mastick, P. Serup, M-J. Tsai, and C. V. Wright. This work was supported in part by grants DK60542 (B.S.-P.) and HL073402 (G.O.) from the National Institutes of Health and by the American Lebanese Syrian Associated Charities (ALSAC).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.07.021.

References

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

Apelqvist, A., Li, H., Sommer, L., Beatus, P., Anderson, D.J., Honjo, T., de Angelis, M.H., Lendahl, U., Edlund, H., 1999. Notch signaling controls pancreatic cell differentiation. Nature 400, 877–881.

Bajjalieh, S., 2004. Trafficking in cell fate. Nat. Genet. 36, 216–217.

Burke, Z., Oliver, G., 2002. Prox1 is an early specific marker for the developing liver and pancreas in the mammalian foregut endoderm. Mech. Dev. 118, 147–155.

Cremisi, F., Philpott, A., Ohnuma, S., 2003. Cell cycle and cell fate interactions in neural development. Curr. Opin. Neurobiol. 13, 26–33.

Dyer, M.A., Cepko, C.L., 2000. $p27^{Kip2}$ regulates progenitor cell proliferation and amacrine interneuron development in the mouse retina. Development 127, 3593–3605.

Dyer, M.A., Livesey, F.J., Cepko, C.L., Oliver, G., 2003. Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina. Nat. Genet. 34, 53–58.

Edlund, H., 2002. Pancreatic organogenesis—Developmental mechanisms and implications for therapy. Nat. Rev. 3, 524–532.

Esni, F., Ghosh, B., Biankin, A.V., Lin, J.W., Albert, M.A., Yu, X., MacDonald, R.J., Civin, C.I., Real, F.X., Pack, M.A., Ball, D.W., Leach, S.D., 2004. Notch inhibits Ptf1 function and acinar cell differentiation in developing mouse and zebrafish pancreas. Development 131, 4213–4224.

Fero, M.L., Rivkin, M., Tasch, M., Porter, P., Carow, C.E., Firpo, E., Polyak, K., Tsai, L.-H., Broudy, V., Perlmutter, R.M., Kaushansky, K., Roberts, J.M., 1996. A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in $p27^{Kip1}$-deficient mice. Cell 85, 733–744.

Fishman, M.P., Melton, D.A., 2002. Pancreatic lineage analysis using a retroviral vector in embryonic mice demonstrates a common progenitor for endocrine and exocrine cells. Int. J. Dev. Biol. 46, 201–207.

Griffiths, R.L., Hidalgo, A., 2004. Prospero maintains the mitotic potential of glial precursors enabling them to respond to neurons. EMBO J. 23, 2440–2450.

Gu, G., Dubauskaite, J., 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.

Habener, J.F., Kemp, D.M., Thomas, M.K., 2005. Minireview: transcriptional regulation in pancreatic development. Endocrinology 146 (3), 1025–1034.

Hald, J., Hjorth, J.P., German, M.S., Madsen, O.D., Serup, P., Jensen, J., 2003. Activated Notch1 prevents differentiation of pancreatic acinar cells and attenuate endocrine development. Dev. Biol. 260, 426–437.

Herrera, P., 2002. Defining the cell lineages of the islets of Langerhans using transgenic mice. Int. J. Dev. Biol. 46, 97–103.

Horb, L.D., Slack, J.M., 2000. Role of cell division in branching morphogenesis and differentiation of the embryonic pancreas. Int. J. Dev. Biol. 44, 791–796.

Jensen, J., Pederson, E.E., Galante, P., Hald, J., Heller, R.S., Ishibashi, M., Kageyama, R., Guillemot, F., Serup, P., Madsen, O., 2000. Control of endodermal endocrine development by Hes-1. Nat. Genet. 24, 36–44.

Kim, S.K., Hebrok, M., 2001. Intercellular signals regulating pancreas development and function. Genes Dev. 15, 111–127.

Kiyokawa, H., Kineman, R.D., Manova-Todorova, K.O., Soares, V.C., Hoffman, E.S., Ono, M., Khanam, D., Hayday, A.C., Frohman, L.A., Koff, A., 1996. Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of $p27^{Kip1}$. Cell 85, 721–732.

Krapp, A., Knofler, M., Ledermann, B., Burki, K., Berney, C., Zoerkler, N., Hagenbuchle, O., Wellauer, P.K., 1998. The bHLH protein PTF1-p48 is essential for the ormation of the exocrine and the correct spatial organization of the endocrine pancreas. Genes Dev. 12, 3752–3763.

Lammert, E., Brown, J., Melton, D.A., 2000. Notch gene expression during pancreas organogenesis. Mech. Dev. 94, 199–203.

Li, L., Vaessin, H., 2000. Pan-neural Prospero terminates cell proliferation during *Drosophila* neurogenesis. Genes Dev. 14, 147–151.

Liu, G., Pakala, S.V., Gu, D., Krahl, T., Mocnik, L., Sarvetnick, N., 2001. Cholecystokinin expression in the developing and regenerating pancreas and intestine. J. Endocrinol. 169, 233–240.

Murtaugh, L.C., Melton, D.A., 2003. Genes, signals, and lineages in pancreas development. Annu. Rev. Cell Dev. Biol. 19, 71–89.

Murtaugh, L.C., Stanger, B.Z., Kwan, K.M., Melton, D.A., 2003. Notch signaling controls multiple steps of pancreatic differentiation. Proc. Nat. Acad. Sci. U. S. A. 100, 14920–14925.

Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Shishido, N., Horii, I., Loh, D.Y., Nakayama, K., 1996. Mice lacking $p27^{Kip1}$ display increased body size, multiple organ hyperplasia, retinal dysplasia and pituitary tumors. Cell 85, 707–720.

Nourse, J., Firpo, E., Flanagan, M., Meyerson, M., Polyak, K., Lee, M.-H., Massague, J., Crabtree, G., Roberts, J., 1994. IL-2 mediated elimination of the $p27^{Kip1}$ cyclin-Cdk kinase inhibitor prevented by rapamycin. Nature 372, 570–573.

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

Ohnuma, S., Philpott, A., Wang, K., Holt, C.E., Harris, W.A., 1999. $p27^{Xic1}$, a Cdk inhibitor, promotes the determination of glial cells in *Xenopus* retina. Cell 99, 499–510.

Oliver, G., Sosa-Pineda, B., Geisendorf, S., Spana, E.P., Doe, C.Q., Gruss, P., 1993. Prox1, a prospero-related homeobox gene expressed during mouse development. Mech. Dev. 44, 3–16.

Pictet, R., Rutter, W.J., 1972. Development of the embryonic endocrine pancreas. In: Steiner, D.F., Frenkel, N. (Eds.), Handbook of Physiology. Williams and Wilkiins, Washington, DC, pp. 25–66.

Prado, C.L., Pugh-Bernard, A.E., Elghazi, L., Sosa-Pineda, B., Sussel, L., 2004. Ghrelin cells replace insulin-producing β cells in two mouse models of pancreas development. Proc. Natl. Acad. Sci. U. S. A. 101, 4679–4684.

Rindi, G., Leiter, A.B., Kopin, A.S., Bordi, C., Solcia, E., 2004. The "normal" endocrine cell of the gut. Changing concepts and new evidences. Ann. N. Y. Acad. Sci. 1014, 1–12.

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

Sherr, C.J., Roberts, J.M., 1995. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev. 9, 1149–1163.

Slack, J.M., 1995. Developmental biology of the pancreas. Development 121, 1569–1580.

Sosa-Pineda, B., Wigle, J.T., Oliver, G., 2000. Hepatocyte migration during liver development requires Prox1. Nat. Genet. 25, 254–255.

Torii, M., Matsuzaki, F., Osumi, N., Kaibuchi, K., Nakamura, S., Casarosa, S., Guillemot, F., Nakafuku, M., 1999. Transcription factors Mash-1 and Prox-1 delineate early steps in differentiation of neural stem cells in the developing central nervous system. Development 126, 443–456.

Wang, J., Elghazi, L., Parker, S.E., Kizilocak, H., Asano, M., Sussel, L., Sosa-Pineda, B., 2004. The concerted activities of Pax4 and Nkx2.2 are essential to initiate pancreatic beta-cell differentiation. Dev. Biol. 266, 178–189.

Wigle, J.T., Oliver, G., 1999. Prox1 function is required for the development of the murine lymphatic system. Cell 98, 769–778.

Wigle, J.T., Chowdhury, K., Gruss, P., Oliver, G., 1999. Prox1 function is crucial for mouse lens-fibre elongation. Nat. Genet. 21, 318–322.

Wigle, J.T., Harvey, N., Detmar, M., Lagutina, I., Grosveld, G., Gunn, M.D., Jackson, D.G., Oliver, G., 2002. An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotpe. EMBO J. 21, 1505–1513.

Wilson, M.E., Kalamaras, J.A., German, M.S., 2002. Expression pattern of IAPP and prohormone convertase 1/3 reveals a distinctive set of endocrine cells in the embryonic pancreas. Mech. Dev. 115, 171–176.

Wilson, M.E., Scheel, D., German, M.S., 2003. Gene expression cascades in pancreatic development. Mech. Dev. 120, 65–80.