The role of Prox1 during mouse pancreas organogenesis

De rol van Prox1 in de organogenese van de muis pancreas

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Thesis

to obtain the degree of Doctor from the

Erasmus University Rotterdam by

command of the

rector magnificus

Prof.dr. H.G. Schmidt

and in accordance with the decision of the Doctorate Board

The public defense shall be held on

Wednesday 29 September 2010 at 9:30 o'clock

by

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born in Istanbul, Turkey

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The studies described in this thesis were performed in the Department of Genetics and Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, TN, USA and supported by National Institute of Diabetes and Digestive and Kidney Diseases, NIH (R01DK060542) and the American Lebanese Syrian Associated Charities (ALSAC).

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Scope of this thesis

The pancreas is a mixed (exocrine and endocrine) glandular organ that is important for food digestion and glucose homeostasis. Developmental anomalies or disorders that affect normal pancreas homeostasis may cause various life-threatening diseases such as pancreatitis, diabetes, cystic fibrosis, and pancreatic cancer.

In the past two decades, an increasing number of studies have begun to unravel the molecular and cellular mechanisms regulating mammalian pancreas organogenesis. The information extracted from these studies should be valuable both, to better understand the etiology of some pancreatic diseases, and to design new therapeutic tools to cure those diseases.

A recent study reported expression of the homeodomain transcription factor Prox1 in the presumptive pancreatic region of mouse embryos[1]. This finding raised the possibility that, similar to other tissues, proper pancreas development requires the function of Prox1[2-6]. The studies of my Ph.D thesis sought to uncover the role of Prox1 during mouse pancreas organogenesis.

First, the expression of Prox1 in the developing pancreas was thoroughly characterized and the pancreatic tissues of *Prox1*-nullizygous embryos were analyzed (Chapter 2). Second, since *Prox1*-nullizygous embryos die at around embryonic day (E) 14.5, we generated a novel mouse model (*Prox1*$^{loxP/loxP}$*;Pdx1.Cre*) with conditional inactivation of *Prox1* in pancreatic progenitors, to investigate whether loss of *Prox1* function affects late aspects of pancreas organogenesis (Chapter 3). Third, upon identifying *Opn* as a novel pancreatic gene, we performed an extensive analysis of its expression in the pancreas of mouse embryos and adults and characterized this organ of *Opn*-nullizygous mice (Chapter 4).

Chapter 1

Introduction

1. THE PANCREAS

1.1 Morphology and function of the pancreas

The adult mammalian pancreas (Figure 1) is composed of exocrine and endocrine tissues, which have a completely different morphology and function[7]. The exocrine tissue plays an essential role in food digestion, whereas the endocrine tissue controls glucose homeostasis[8].

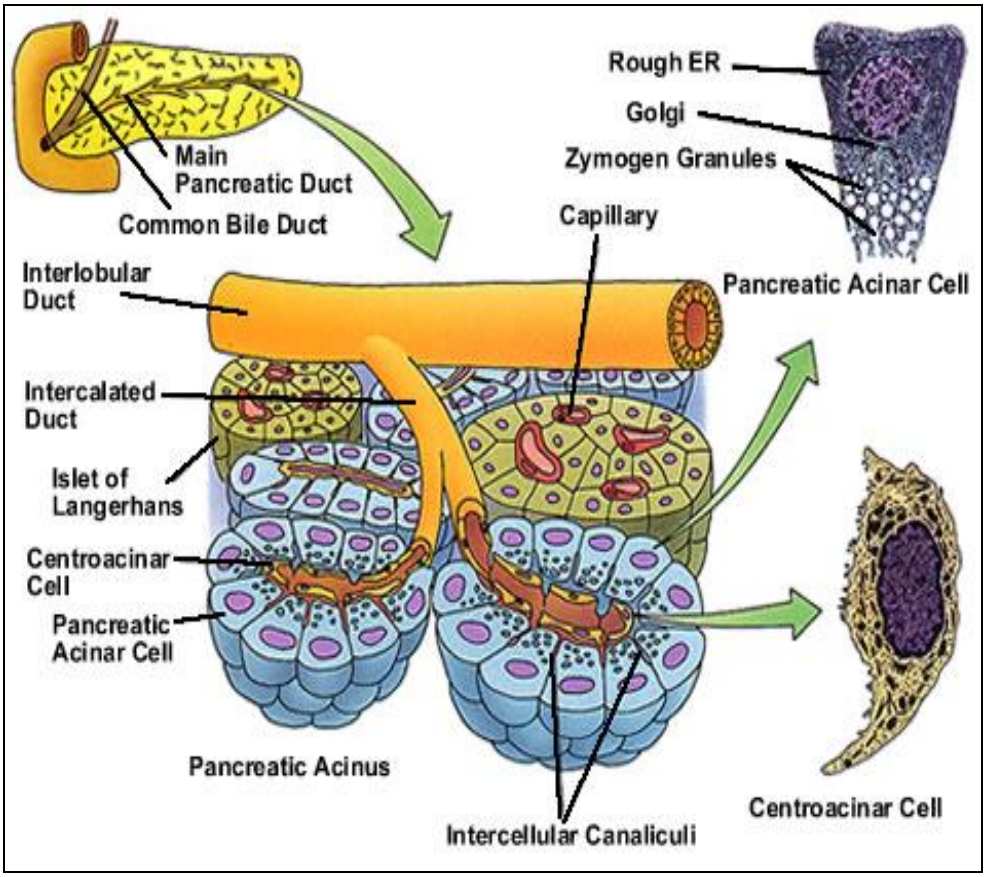

Figure 1. Schematic drawing of pancreas structure (Adapted from reference[9]).

The exocrine pancreas is a lobulated, branched, acinar gland, which makes up almost 95% of the pancreatic mass[10]. The secretory cells of the exocrine pancreas (acini) are pyramidal in shape, have basal nuclei, contain rough endoplasmic reticulum arrays, have extensive Golgi complexes and copious secretory granules containing inactive precursors (zymogens) of digestive enzymes, including proteases, amylases, lipases, and nucleases (Figure 1). Upon hormonal and neuronal stimulation, the enzyme precursors are secreted into the acinar lumen and then drained into small ducts (intercalary ducts) that subsequently merge with the

larger interlobular ducts. The main pancreatic duct collects the secretion from interlobular ducts and carries it into the duodenum, where the inactive enzyme precursors become activated to help the digestion and the absorption of food. Low cuboidal centroacinar (CA) cells are located at the junction of acini and ducts; CA cells are proposed to behave like progenitors in the adult pancreas in response to certain injury conditions[8,10-12].

The endocrine tissue is formed by compact spherical cell clusters called islets of Langerhans, which constitute about 2% of the pancreatic mass[10]. Five different endocrine cell types form these islets: α, β, δ, PP, and ε cells, respectively producing and secreting into the bloodstream the hormones glucagon, insulin, somatostatin, pancreatic polypeptide, and ghrelin. Insulin producing β-cells reside in the center of the islets, and account for the biggest portion (60-80%) of the islet cell mass, while the remaining endocrine cell types are found in the periphery of the islets[13]. The endocrine cells are always found in intimate contact with capillaries. This arrangement provides continuous monitoring of blood glucose levels and efficient hormone secretion[8]. Insulin and glucagon are the key regulators of glucose homeostasis. Upon sensing increased glucose levels in blood, insulin stimulates glucose uptake in muscle and fat, and inhibits hepatic glucose production[14]. Glucagon actions are antagonistic to those of insulin. The secretion of glucagon is stimulated by a fall in the concentration of glucose in the blood. Glucagon increases blood glucose levels by increasing hepatic glycogenolysis[15]. Somatostatin and pancreatic polypeptide exert inhibitory effects on both pancreatic endocrine and exocrine secretion[13]. It is speculated that ghrelin may have a role in regulating insulin secretion[16,17].

1.2 Mouse Pancreas development

The research on pancreas development has dramatically increased in recent years and has contributed to understand the molecular bases of some pancreatic diseases, including diabetes, pancreatitis, and pancreatic carcinoma. These studies also uncovered that some crucial regulators of pancreas organogenesis are also key molecular components of pancreatic diseases[13,18]. Therefore, the identification of molecular and cellular mechanisms regulating mammalian pancreas development should contribute to understand the etiology of pancreatic diseases, and will assist developing new therapies to cure them.

1.2.1 Morphogenesis

1.2.1.1 Pancreatic bud formation

The development of the pancreas is almost identical amongst mammals. In mouse embryos, the early pancreas can be identified at around E9.5 as two outgrowths (dorsal and ventral buds) of the endodermal epithelium located between the stomach and duodenum[10]. The position of the pancreatic buds in the gut endoderm depends both on the inductive signals emanating from neighboring tissues, and the endoderm competence to respond to those signals[19,18].

The differentiation program of the dorsal and ventral pancreatic buds is governed by different inductive signals provided by the surrounding mesodermal tissues[19,18]. Early in mouse development (E8.5), the endoderm that will form the dorsal bud is in direct contact with the notochord[20]. FGF2 and activin βB[21,18] secreted by the notochord inhibit Sonic hedgehog (Shh) expression in the dorsal foregut endoderm; this allows expression of the transcription factor pancreatic–duodenal homeobox 1 (Pdx1) in the region where the dorsal pancreas will form[20,22]. Subsequently, the dorsal pancreatic region becomes in contact with the dorsal aorta, which produces signals necessary for maintenance of Pdx1 expression, dorsal bud formation, and initiation of pancreas transcription factor 1a (Ptf1a*)* expression[23,24].

The ventral pancreatic region is not in contact with either the notochord or the dorsal aorta. The endoderm that will give rise to the ventral pancreas interacts first with the lateral plate mesoderm, which produces activin, bone morphogenetic factor (BMP), and retinoic acid (RA), which assist in establishing the ventral pancreatic domain[25,18]. Both the ventral pancreatic bud and the liver are simultaneously specified in the ventral foregut endoderm (E8-8.5)[26,27,28]. The adjacent cardiac mesoderm produces signals (mainly FGFs) that induce the expression of liver markers and inhibit the expression of pancreatic markers. Ventral endoderm that does not receive signals from the cardiac mesoderm expresses pancreatic markers by default[28].

After formation of the dorsal and ventral pancreatic buds, these tissues start to invade the surrounding mesenchyme. After E10.5, the pancreatic buds expand rapidly and start to form branches. At around E12.0-E13.0, the gut rotates and the dorsal and ventral pancreatic

tissues come into contact and fuse into a single organ. After E14.5, increased production of endocrine cells, branching morphogenesis, exocrine cell differentiation, and primitive duct formation start to occur in the developing pancreas[32,7].

1.2.1.2 Role of the mesenchyme in early pancreas development

Early in development the growing pancreatic epithelium is surrounded by mesenchyme, which produces signals controlling epithelial proliferation, morphogenesis, and cytodifferentiation[29,31,33,10]. For instance, epidermal growth factor (EGF) and fibroblast growth factor 10 (FGF10) stimulate growth and morphogenesis in the developing pancreas[34,35], whereas activin, a member of the TGF-β superfamily, controls proper pancreatic endocrine cell differentiation and also prevents exocrine differentiation[36,37]. In contrast, the TGF-β/activin inhibitor follistatin induces exocrine differentiation and suppresses endocrine differentiation[38].

The transcription factors Islet1 and Pbx1 and the cell adhesion protein N-cadherin, are all expressed in the pancreatic dorsal mesenchyme, and their respective functions are necessary for growth and exocrine differentiation of the dorsal pancreas. In mice lacking Islet1 and N-cadherin, the dorsal mesenchyme does not form[39,40]. *Pbx1*-deficient embryos have disrupted mesenchyme, dorsal and ventral pancreatic hypoplasia due to decreased proliferation, and defects in exocrine differentiation. Similar to the *Islet1*- and *N-cadherin*-deficient pancreas, recombination of mutant dorsal epithelium with wild-type mesenchyme rescued the exocrine differentiation in the *Pbx1*-deficient pancreas[41].

1.2.1.3 Primitive branch formation

Recent studies proposed that primitive branching morphogenesis occurs in the pancreas of zebrafish and mouse embryos through intraepithelial microlumen formation, microlumen fusion into a continuous, larger lumen, and establishment of a ductular network[53,54]. According to this model, scattered microlumens first form in the mouse pancreatic epithelium at around E11.5, which subsequently expand by inducing apical cell polarity in the neighboring epithelial cells. At E12.5, the lumens coalesce into a complex continuous luminal network, which then remodels and matures into a tubular network consisting of a

monolayered, fully polarized epithelium surrounded by a basal lamina (between E13.5 and E15.5) (Figure 2). Notably, Kesavan et al. (2009) showed that the activity of the Rho-GTPase Cdc42 is essential for branch formation in the mouse embryonic pancreas. In addition, other studies uncovered that factors produced by the mesenchyme (including FGF10 and EGF) are also involved in pancreatic branch formation [34,35].

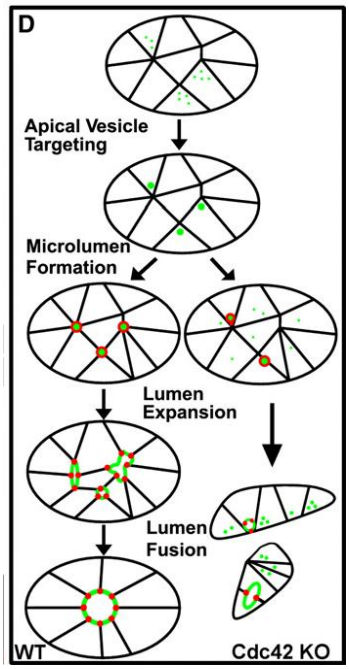

Figure 2. Schematic illustration of microlumen formation. Targeting of vesicles carrying apical proteins (green) to presumptive apical domain (red) establishes apical-basal cell polarity within single cells. Subsequently, neighboring cells undergo cell polarization resulting in the formation of microlumens with a common apical surface. Microlumen expansion and fusion generates a luminal network. In contrast, the Cdc42-deficient pancreatic epithelium fails to generate a multicellular common apical surface, and instead forms intra and intercellular lumens (Adapted from reference[54]).

1.2.2 Cell differentiation

1.2.2.1 Multipotent Progenitors

All epithelial cell types of the pancreas (exocrine, endocrine, and ductal) derive from multipotent progenitors expressing Pdx1[55-58]. Zhou et al. (2007), reported that the tip of the E12.5 pancreatic branches contains progenitors capable of producing all three pancreatic cell types, and showed that those progenitors express Pdx1 and carboxypeptidase 1 (Cpa1), but not the exocrine enzyme amylase (Amy). At around E14.5, those cells located in the tip of the

branches started to express amylase and began to differentiate into exocrine cells. The Zhou study also proposed that proliferation and outgrowth of the multipotent tip cells ($Pdx1^{+}Cpa1^{+}Amy^{-}$) leave behind cells undergoing endocrine and ductal differentiation, and that these cells form the trunk of the branches. The endocrine cells subsequently differentiate and migrate out of the epithelium, leaving the trunk to be made entirely of duct cells[59].

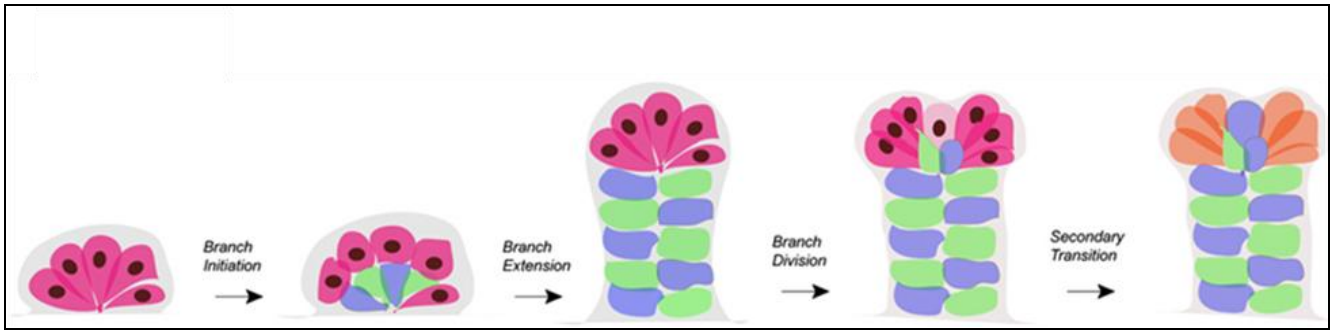

Figure 3. Multipotent progenitors are present in the E12.5 mouse pancreas. Rapid proliferation and differentiation of the progenitors into endocrine and duct cells generate the trunk of the branches. Pink: Cpa1+ multipotent progenitors; blue: duct cells; green: endocrine cells; orange: exocrine cells; light pink: Cpa1 down-regulated cell in the cleft region) (Adapted from reference[59]).

1.2.2.2 Notch signaling and pancreatic cell differentiation

Notch signaling regulates various processes during pancreas organogenesis. The interaction of Notch receptor with its ligand causes cleavage of an intracellular Notch domain (Notch-IC). Upon translocating to the nucleus, Notch-IC binds to the transcription factor Rbp-j to induce the expression of Notch target genes, including those encoding members of the Hes family of transcriptional repressors[42,43].

The Notch1 and Notch2 receptors are expressed in the pancreatic epithelium during pancreatic organogenesis[47]; Notch1 function maintains pancreatic progenitors in an undifferentiated state[48,49,44-46], and its loss-of–function leads to accelerated differentiation of pancreatic progenitors into endocrine cells, and depletion of the pancreatic progenitor pool. Similarly, Rbp-j knockout mice display premature endocrine differentiation, abnormal ductal branching, and a severe decrease in exocrine differentiation in the developing pancreas[44-46].

Conversely, maintaining Notch activity in pancreatic progenitors prevents exocrine and endocrine cell differentiation[50,52].

1.2.2.3 Endocrine cell differentiation

The first wave of endocrine cell genesis occurs between E9.5-11.5 in the mouse pancreas, but the produced cells do not seem to contribute to the mature islets. The second wave of endocrine cell genesis and differentiation begins between E13.5 and E16.5 and gives rise to the vast majority of islet cells[60,61]. At about E18.5, the endocrine cells of the pancreas start to separate from the trunk of the pancreatic branches, migrate into the surrounding acinar tissue, and begin to organize into islets[60,7,61,10].

The basic helix-loop-helix (bHLH) transcription factor neurogenin 3 (Ngn3) is exclusively expressed in endocrine progenitors located within the primitive ductal epithelium. Ngn3 specifies an endocrine cell fate in multipotent pancreatic progenitors, via activating the expression of transcription factors necessary to initiate a core program of endocrine development, and transcription factors that promote the development of specific islet subtypes[62,63] (Figure 4). Loss of Ngn3 function abrogates endocrine genesis in the pancreas, whereas its ectopic expression is sufficient to confer endocrine differentiation in multipotent pancreatic progenitors[48,64,61].

The transcription of *Ngn3* is directly regulated by hepatic nuclear factor 6 (Hnf6/OC-1), a member of the ONECUT family of transcription factors[61,65,66]. Hnf6 is not expressed in mature islet cells and its maintenance in the endocrine lineage leads to disrupted islet architecture and diabetes[61]. OC2, another ONECUT family member, can also bind and stimulate the *Ngn3* promoter, and its function is partially redundant with that of Hnf6 for endocrine development[67].

The development of pancreatic endocrine cells also requires the function of other transcription factors, including: **Islet1**, a LIM homeodomain family member controlling the generation of all endocrine cells[39]; **NeuroD,** a bHLH member that regulates the production of adequate numbers of endocrine cells[68]; **Pax6,** a paired and homeodomain family member

controlling proper islet cell number, islet morphology, and the level of islet hormone synthesis[69,70]**; Pax4,** another paired and homeodomain member that controls the specification of β-cells and δ-cells[71,73]**; Arx**, a homeodomain transcription factor that promotes α-cell specification[74,61]; **Nkx2.2 ,** an **NK** homeodomain protein required for terminal differentiation of β-cells[75]**; Nkx6.1**, another **NK** homeodomain member that controls the formation of appropriate numbers of β-cells[76]**; Hlxb9,** a homeodomain protein required to the formation and maturation of β-cells[77,48]**; MafA,** a basic leucine–zipper transcription factor controlling insulin synthesis and glucose-stimulated insulin secretion; MafA-deficient mice have been shown to display intolerance to glucose and develop diabetes mellitus[78]**;** and **Sox9,** a SOX family transcription factor that maintains the undifferentiated status of pluripotent progenitors and is also required for the specification of endocrine progenitors in the developing pancreas[79,80].

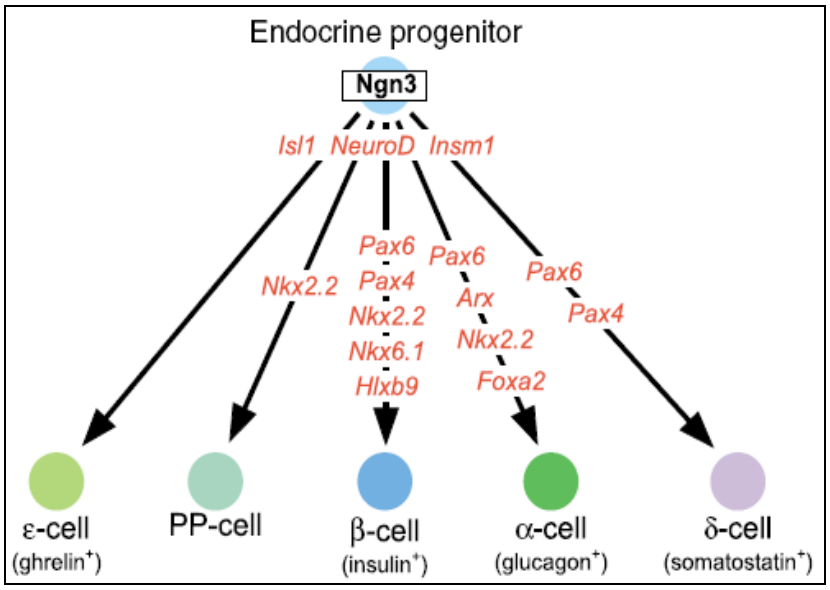

Figure 4. A hypothetical lineage diagram for *Ngn3* positive endocrine precursors. In red are genes required for various steps of endocrine differentiation; some of which (top) appear to function similarly in all subtypes, constituting a core program of endocrine development, whereas others (bottom) are differentially required for specific subtypes (Adapted from reference[62]).

1.2.2.4 Exocrine cell differentiation

Exocrine-specific transcripts can be detected in the mouse embryonic pancreas at around E12.5[81]. At this stage, however, the pancreatic epithelium shows little morphologic evidence of exocrine cell differentiation[33]. After E15.5, pancreatic exocrine cells can be distinguished at the tips of the ductal epithelium by their columnar shape, strongly eosinophilic cytoplasm, appearance of zymogen granules, and formation of amylase$^+$ acinar units[33]. Complete maturation of the pancreatic exocrine tissue includes the expression of digestive enzymes, and the establishment of both cellular polarity and regulated exocytosis[82]. The formation, maintenance, and function of the pancreatic exocrine tissue require the activity of various transcription factors (including Pdx1, Ptf1a/p48, and Mist1), epithelial-mesenchymal interactions, and Notch signaling [83,84,82,38,51,52].

Ptf1 is a hetero-oligomer transcription factor containing three distinct subunits: p75, p64, and p48[85,86]. Ptf1 expression is detected as early as E9.5 in the developing pancreas, several days before the onset of exocrine differentiation. Lineage tracing studies conducted by Kawaguchi et al. (2002) showed that exocrine and most of the endocrine and ductal cells originate from Ptf1 expressing progenitors. This study also showed that the acquisition of a pancreatic fate by the undifferentiated foregut endoderm requires the activity of Ptf1, since *Ptf1*-null cells converted into duodenal cells[88]. Ptf1 binds to transcriptional enhancers of exocrine-specific genes, including elastase and amylase[85]. Its exocrine-specific expression[87] after E15.5 and the absence of exocrine tissue in *Ptf1*-null mutant mice[84] indicates that Ptf1 function is essential for exocrine pancreatic development.

In the pancreas of embryos and adult mice, the expression of **Mist1** (a bHLH transcription factor) is restricted to the exocrine cell lineage[82]. Mist1 function is necessary for complete maturation, maintenance, and proper function of pancreatic exocrine cells[82]. *Mist1* knockout mice exhibit extensive disorganization of the exocrine tissue, defects in regulated exocytosis, intracellular enzyme activation, acinar to ductal metaplasia, and progressive deterioration of the acinar tissue[82].

The function of **Pdx1** is also important for the formation of exocrine tissue, because inactivating its function at the initiation of acinar cell differentiation prevents successful completion of this process[83].

1.2.2.5 Ductal cell differentiation

Duct cells make up a small number of total pancreatic cells (10%), and they have critical roles in the mature pancreas. In addition to forming a network that delivers enzymes into the duodenum, the pancreatic ductal epithelium produces bicarbonate and mucins[89,90]. Goblet cells, which intermingle with ductal cells in the large branches, contribute to mucin production. Pancreatic ductal cells have a single cilium, thought to be important for flow sensing within the ductal lumen[91].

There is currently very limited information on pancreatic duct cell differentiation mostly because of insufficiency of duct specific markers[90]. The lack of definitive ductal precursor markers prevents to determine where and when these cells localize in the developing pancreas. Moreover, the molecular mechanisms directing ductal formation and differentiation are very poorly understood.

Hnf6/OC-1 is expressed in the early embryonic pancreatic epithelium and in developing and mature ducts. Hnf6 is expressed at low levels in pancreatic exocrine cells and is absent in mature islet cells[66]. Hnf6 is an important regulator of pancreatic duct differentiation and its lack of activity promotes dilation and cyst formation in the ducts (most likely due to the lack of primary cilia on ductal epithelial cells)[101]. Zhang et al (2009) showed that mice with pancreas specific inactivation of *Hnf6* had morphological changes in this organ consistent with pancreatitis, including loss of acinar tissue, inflammatory cell infiltration, dilated and very tortuous ducts, cyst formation, and increased expression of connective tissue growth factor (CTGF), metalloproteinase 7 (MMP7), and p8/Nupr1. These authors suggested that defects in primary cilia formation probably led to pancreatitis in those mutant mice[66].

Cano et al. (2006) also showed that preventing cilia formation in pancreatic cells causes severe pancreatic lesions resembling those found in patients with chronic pancreatitis or

cystic fibrosis, including fibrosis, acinar to ductal metaplasia, cyst formation, and abnormal ductal morphology[102].

The function of Cdc42, a Rho-GTPase family member, is required for both, formation of microlumens and primitive ducts and later for maintenance of a polarized tubular phenotype in the developing pancreas[54]. The failure to organize the pancreatic epithelium into tubes in the absence of Cdc42 resulted in a dramatic increase in acinar cell differentiation, and decrease in duct and endocrine cell differentiation. These results indicated that a normal tubular structure of the pancreas is necessary for cell-fate specification by providing the correct microenvironment for multipotent progenitors to differentiate properly[54].

1.3 Diseases of the pancreas

1.3.1 Diabetes

Defects in insulin secretion, insulin action, or both lead to a group of metabolic diseases called diabetes mellitus characterized by hyperglycemia. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels[103]. Type1 diabetes is the result of selective autoimmune destruction of β-cells, whereas Type 2 diabetes occurs when the β-cell population fails to compensate for the increased peripheral insulin resistance[92].

β-cell replacement therapy by pancreatic islet transplantation is a treatment that most closely replicates normal physiological conditions for treatment of Type1 diabetes in a way that is impossible to achieve with exogenous insulin. However, wide clinical application of islet transplantation has been restricted by insufficiency of islet sources and low rates of long-term success. Finding a functional substitute for the missing β-cells or restoring their regeneration capacities is a major goal in the field of diabetes research. Current experiments to achieve these goals are largely focused on ex vivo expansion of β-cells or in vitro differentiation of embryonic and adult stem cells into insulin producing β-cell phenotypes. Despite limited success with these efforts, a much better understanding of the mechanisms that regulate expansion and differentiation of stem/progenitor cells is still needed[104].

1.3.2 Cystic fibrosis

Alterations in the normal physiology of the pancreatic ducts can cause severe diseases, including cystic fibrosis, pancreatitis, and pancreatic adenocarcinoma[90]. Cystic fibrosis (CF) is one of the most common life-shortening genetic diseases. It is an autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*), encoding a membrane-associated chloride ion channel that is also important for bicarbonate secretion[105]. The main targets of CF are exocrine glands, primarily those of the respiratory and gastrointestinal systems. When the CFTR is not functioning, the movement of ions into and out of the cells is blocked, which then disrupts water movement into and out of the cell. This results in thicker mucus production that in turn causes obstruction of the duct lumen, dilation of the duct and acinar lumina, and progressive degradation and atrophy of acini. The damaged acini subsequently are replaced by small cysts, fibrous tissue, and fat[106,90,107].

1.3.3 Pancreatitis

Pancreatitis is an inflammatory disease, which can be acute or chronic. Acute pancreatitis (AP) is usually mild but can be severe or even mortal as a result of multiorgan dysfunction. AP is characterized by edematous fluid in the extracellular space of the pancreas that causes separation of the lobules and acini and by dilation of ductal and acinar lumens. AP may advance to a more severe form with increased acinar cell necrosis and parenchymal inflammatory infiltrates. Chronic pancreatitis (CP) occurs as a result of repeated episodes of acute pancreatitis and is characterized by acinar atrophy and fibrosis[108]. In CP, the dense pancreatic glandular tissue transforms into a mass of almost complete fibrotic tissue. Also, the exocrine cells lose their zymogen granules, are reduced in height, and eventually adopt a duct like morphology. With time, loss of endocrine and exocrine function occurs[109,110].

Gallstones are the major cause of acute pancreatitis, whereas alcohol is associated with acute as well as chronic forms of the disease. In general, pancreatitis appears to initiate when premature activation of digestive enzymes occurs within the acinar cell, which results in autodigestion of the gland. In the case of pancreatic duct obstructions caused by gallstones, exocrine cell exocytosis cannot proceed normally and this results in abnormal colocalization of zymogen and lysosomal granules in those cells. Lysosomal enzymes (particularly

cathepsin B) then activate digestive enzyme zymogens within the acinar cell, which ultimately leads to autodigestion of the gland[111].

Alcohol promotes the formation of protein plugs and stones in the pancreatic ducts, which then cause ulceration, scarring, further obstruction and finally atrophy and fibrosis in the pancreas[111]. Chronic alcohol administration may also cause disturbances in exocytosis of acinar cells[112], decreased pancreatic secretions, accumulation of enzymes within the cell, increased potential of contact between digestive and lysosomal enzymes and, finally, premature intracellular activation of digestive enzymes[113].

Gene mutations increasing the susceptibility to pancreatitis have been found in genes encoding the cationic or anionic trypsinogens[114,115,116], chymotrypsin C[117], trypsin inhibitor[118], and CFTR[119,109].

Chronic pancreatitis is a known risk factor for the development of pancreatic cancer[120,121]. The ensuing inflammation and tissue damage that accompany chronic pancreatitis likely increase genomic instability and proliferation in ductal cells, which in turn raises the risk of neoplastic transformation. Long-standing ductal changes also activate signaling pathways which are normally important during pancreatic development, such as Notch and Hedgehog, that presumably contribute to cellular transformation, formation of acino-ductal metaplasias, pancreatic intraepithelial neoplasias (PanIN) and, finally, pancreatic ductal carcinoma formation[110].

1.3.4 Pancreatic Ductal Adenocarcinoma (PDAC)

Pancreatic Ductal Adenocarcinoma (PDAC) has very poor prognosis because of its diagnosis at late stages of the disease and limited response to chemotherapy and radiotherapy. These tumors are the fifth leading cause of cancer-related death, with less than a 3% 5-year survival rate[122,12].

The cellular origins of PDAC are unknown. Despite the ductal morphology of this tumor[11,123,12], increasing evidence indicates that in the context of chronic inflammation

differentiated pancreatic cell types can become neoplastic and generate tumors[122,124]. Another possible cellular source of tumors are the centroacinar (CA) cells of the pancreas, which are located at the interface of the acini and the ducts and are the only cells retaining Notch activity in the mature pancreas[12,125,126,127] (Figure 1).

2. PROX1

Prox1 is a homeodomain protein. The homeodomain family of transcription factors is characterized by the presence of a 60-amino acid domain, the homeodomain, which is responsible for recognition of and binding to specific DNA sequences[128]. Homeodomain proteins are important transcriptional regulators that control various aspects of morphogenesis and cell differentiation[129,130].

The vertebrate *Prox1* gene encodes a divergent homeodomain transcription factor with homology to the Drosophila *prospero* gene[131,129]. The mouse *Prox1* gene is located on Chromosome 1 and encodes a 737-amino acid protein[131,132]. A recent study identified *Prox2* as a *Prox1* gene homologue expressed in the developing nervous system, adult retina, and adult testes of mice. The function of *Prox2* appears dispensable or redundant for development since *Prox2*-null embryos do not have any obvious phenotype[133].

2.1 Prox1 function in organ development

The function of *Prox1* is critical for the development of multiple organs, and its germline inactivation in mice results in embryonic lethality at around midgestation (E14.5)[2]. The following section describes some of the phenotypes observed in different tissues lacking Prox1 function.

Prox1 in the liver. Mouse Prox1 is expressed very early on in the presumptive hepatic region of the ventral foregut endoderm[1]. Prox1 expression is also detected in hepatic progenitors (a.k.a. hepatoblasts), developing hepatocytes and cholangiocytes, and mature hepatocytes and cholangiocytes (*Seth et al.,* manuscript submitted). Loss of Prox1 function affects the delamination of hepatoblasts from the liver bud and their subsequent migration into the liver lobes. Failure to degrade basal membrane components and persistence of

strong cell-cell interactions might be the cause of this phenotype[6]. Recent studies uncovered that Prox1 also controls the specification of hepatocytes in the fetal liver (*Seth et al.,* manuscript submitted).

Prox1 in the lymphatic system. Prox1 is expressed in a subpopulation of endothelial cells of the mouse embryonic cardinal vein that, upon budding and sprouting, give rise to the lymphatic system. In the absence of Prox1 function, lymphatic vessels and lymphatic capillary plexi do not form[2]. Prox1 activity is essential for the specification of lymphatic endothelial cells, and its absence switches their normal lymphatic differentiation program into a blood vascular program[3].

In vitro studies showed that ectopic Prox1 expression in human blood vascular endothelial cells promotes the expression of lymphatic endothelial cell (LEC) markers and down-regulation of blood endothelial cell (BEC) markers[135]. More recently, Johnson et al. (2008) showed that Prox1 activity is also required to maintain a differentiated LEC phenotype in postnatal and adult mice. These collective data indicate that Prox1 acts as a binary switch to suppress BEC identity, and to promote and maintain LEC identity[136].

Prox1 in the eye. Prox1 is expressed in developing lens cells of mice, and its activity is necessary for progression of terminal lens fiber differentiation and elongation[4]. In *Prox1*-nullizygous mice, the cells in the posterior lens retain proliferative characteristics, down-regulate cell-cycle exit markers (Cdkn1b/p27^{kip1} and Cdkn1c/p57^{kip2}), and fail to polarize and elongate properly. These defects result in formation of a hollowed lens.

Prox1 is expressed in early progenitors and in developing and mature horizontal, amacrine, and bipolar cells of the mouse retina[5]. In this tissue, Prox1 controls cell-cycle exit of progenitor cells and horizontal-cell fate determination. *Prox1*-nullizygous mice do not have horizontal cells in their retina, whereas *Prox1* misexpression in postnatal progenitors promotes horizontal-cell formation[5].

Prox1 in the heart. Prox1 is expressed in the heart of mouse embryos[131,137]. The study of Risebro et al. (2009) suggested that the loss of cardiac *Prox1* function contributes to the early lethality (~E14.5) of *Prox1*-nullizygous mice. By using cardiac specific-deletion approaches, those authors showed disrupted expression and localization of sarcomeric proteins, myofibril disorganization, and growth retardation in hearts lacking Prox1 function {Risebro, 2009 #181}.

Prox1 in the ear. Prox1 is expressed in precursor cells of the developing inner ear sensory epithelium and in differentiated supporting cells, but not in differentiated hair cells[138,139]. Recent studies suggest that Prox1 antagonizes the differentiated hair cell phenotype via repression of Gfi1, a transcription factor critical for hair cell differentiation and survival[140,139].

2.2 Prox1 in tumor formation

A series of recent studies raised the possibility that *Prox1* is a candidate tumor suppressor gene. For instance, various authors reported mutations and aberrant DNA methylation of the human *PROX1* locus[141-146]. In certain human pancreatic cancers, the expression of PROX1 was reduced in comparison to the non-transformed tissue, and this reduction correlated with the differentiation grade of the tumor (i.e., poorly differentiated tumors expressed the lowest levels of Prox1)[141]. Also, mutations in *PROX1* mRNA were found in different human pancreatic cancer cell lines and in human clinical samples taken from pancreatic, esophageal, and colon cancers, and over-expression of wild-type Prox1, but not mutant Prox1, inhibited cell proliferation in pancreatic cell lines and xenografted tumors[141,142]. Shimoda et al. (2006) also found significant correlation between the levels of *Prox1* expression and the differentiation scores of hepatocellular carcinoma (HCC) cell lines. These authors also showed that knockdown of Prox1 protein expression by RNA interference significantly promoted cell proliferation in HCC cell lines, whereas the over-expression of Prox1 greatly suppressed their growth[143]. Finally, *PROX1* was found to be hypermethylated and transcriptionally silenced in carcinomas of the bilary system[145] and also in primary and metastatic breast cancer[146].

In contrast, a study by Petrova et al. (2008) suggested that PROX1 expression increases tumor progression in the colon by disrupting tissue architecture, cell polarity, and adhesion [147]. This study also showed that TCF/β-catenin signaling increases PROX1 expression in intestinal tumors but not in transformed hepatocytes, thereby suggesting a tissue-specific response of *PROX1* to TCF/β-catenin signaling[147].

Overall, the current experimental evidence suggests that the potential role of PROX1 in tumor formation is likely dependent on the cellular context.

2.3 How does Prox1 Function?

2.3.1 Prox1 targets

Currently, only a small number of potential Prox1 target genes have been identified. Co-transfection assays in lens epithelial cell lines showed that Prox1 activates several *γ-crystallin* promoters[148]. In chicken lens fiber cells, Prox1 was shown to bind and activate the *βB1-crystallin* promoter [149]. Prox1 knockdown in human LECs also decreased the expression of integrin α9 and VEGFR3[150]. Shin et al. found four putative Prox1 binding sites in the mouse *FGFR-3* promoter that were simliar to two reported prospero-binding consensus sites: (**C(a/t)(c/t)NN(t/c)** and **(T)AAGACG)**[151,152]. Risebro et al. (2009) identified the genes encoding the structural proteins α-actinin, N-RAP, and Zyxin, as direct targets of Prox1 in the mouse heart[137], and the Sosa-Pineda group recently identified *Follistatin* as a novel Prox1 target gene in mouse hepatoblasts (*Seth et al., submitted*).

2.3.2 Regulators of Prox1 expression

Very little is known about the regulation of Prox1 expression in different tissues. Petrova et al. (2008) showed that, in intestinal tumors, TCF/β catenin signaling increases the expression of PROX1[147]. Francois et al. (2008) also proposed that Sox18 induces development of the lymphatic vasculature via *Prox1* induction. This study showed that *Sox18* directly activates *Prox1* transcription by binding its proximal promoter[153]. Finally, Zhao et al. (2008) proposed that Prox1 might be a downstream target of FGF signaling during mouse lens fiber cell development[154].

2.3.3 Prox1 interactions with Nuclear Receptors

Recent studies uncovered that the interaction of Prox1 protein with certain members of the orphan nuclear receptor family affects the transcriptional activity of those receptors. For example, zebrafish Prox1 was shown to interact with fushi tarazu factor 1b (Ff1b), a zebrafish homolog of steroidogenic factor 1 (SF-1) important for the development of the interrenal organ, and this interaction reduced the transcriptional activity of Ff1b[155]. Steffensen et al. (2004) also demonstrated that Prox1 protein physically interacts with the human LRH1 receptor (hLRH1)[134], and Qin et al.(2004) showed that this interaction impairs binding of LRH1 to the promoter of its target *cholesterol 7-alpha-hydroxylase* (*cyp7a1)*, encoding a critical regulator of bile acid synthesis[156]. Prox1 also appears to antagonize the activity of other nuclear receptors via direct protein-protein interaction, including: hepatocyte nuclear factor 4α (HNF4α), which has a central role in lipoprotein metabolism[157]; peroxisome proliferator activated receptor-γ (PPAR- γ), a regulator of IFN- γ expression, inflammatory responses and immunity[73]; and COUP-TFII, an orphan nuclear receptor required to maintain venous endothelial cell identity[158].

The emergence of Prox1 as a potential regulator of nuclear receptor function indicates that, besides its recognized central role in development, Prox1 probably also has important functions involving metabolism and maintenance of homeostasis in adult stages.

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Chapter 2

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

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Developmental Biology 286 (2005) 182 – 194

Available online at www.sciencedirect.com

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Developmental Biology 286 (2005) 182 – 194

DEVELOPMENTAL BIOLOGY

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

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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

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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

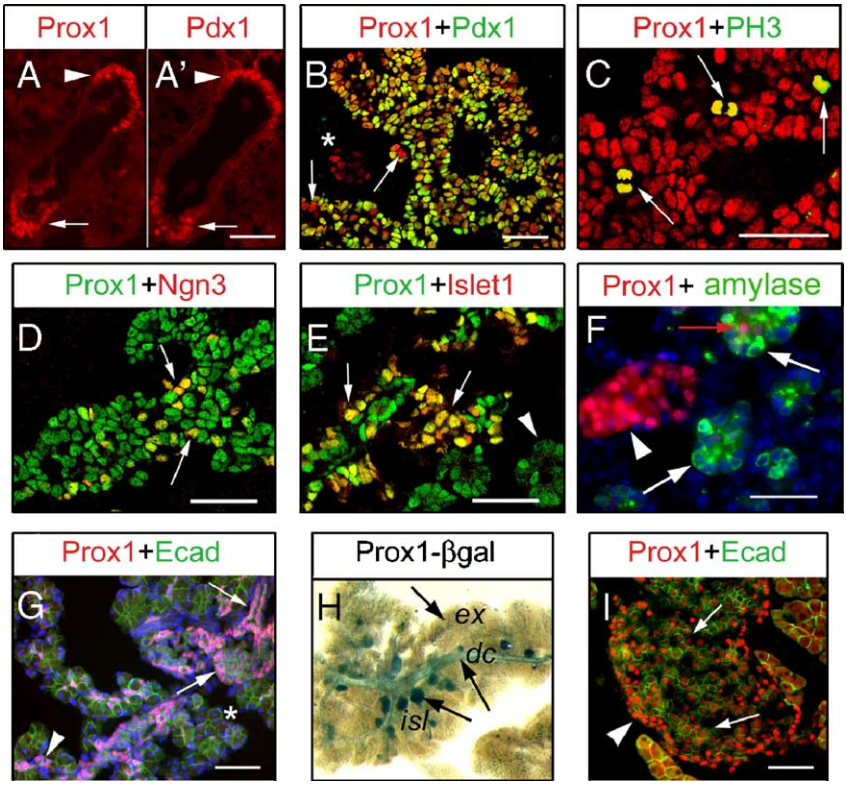

Fig. 1. Temporal expression and distribution of Prox1 during development of the mouse pancreas. (A and A′) At E9.5, Prox1 (red, A) and Pdx1 (red, A′) were similarly expressed in the dorsal (arrowhead) and ventral (arrow) pancreatic regions. (B) At E13.5, Prox1 (red) and Pdx1 (green) were co-expressed 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.

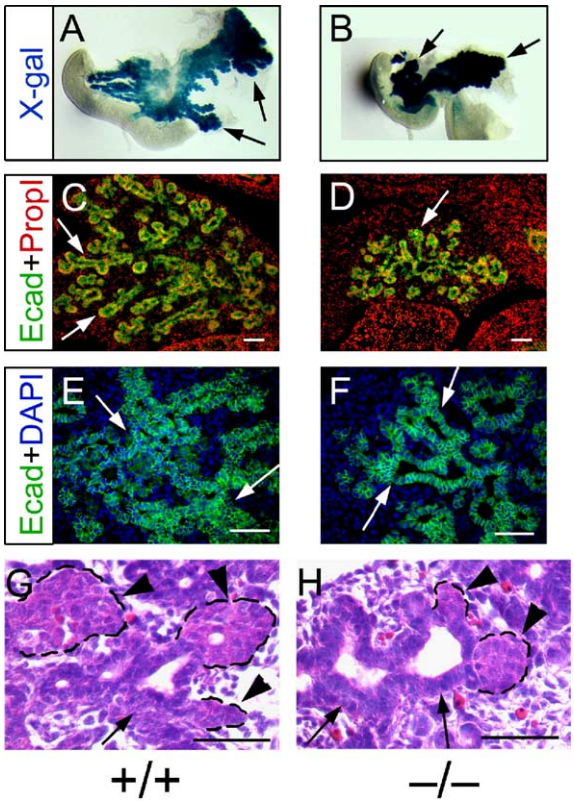

Fig. 2. Abnormalities of E15.0 Prox1-deficient pancreata. (A, C, E, G) The pancreata of E15.0 *Prox1*$^{+/-}$ (A) and wild-type (C, E, G) embryos had numerous branches (arrows in panels A and C). E15.0 wild-type pancreata also have regions of pseudostratified epithelium (arrows in panels E and G). Large 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

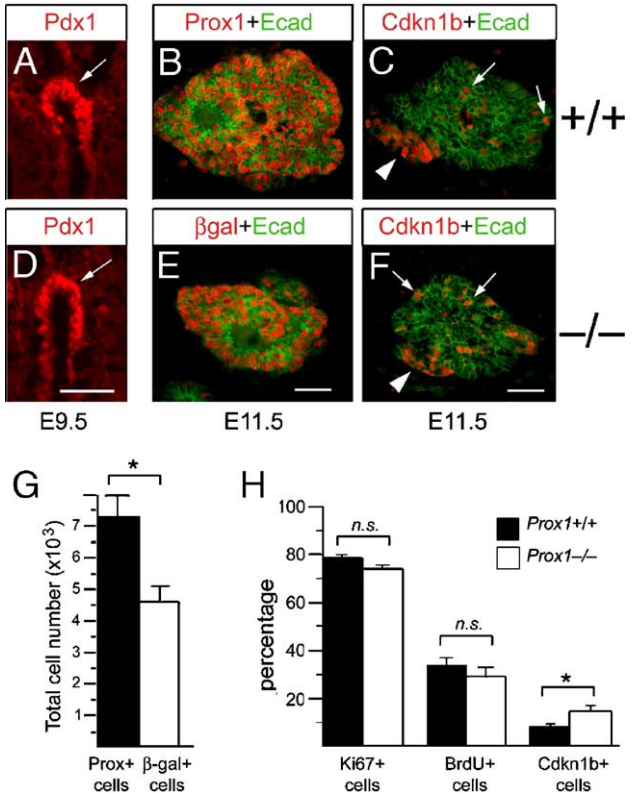

Fig. 3. Lack of Prox1 activity arrests pancreatic growth at around E11.5. (A–F) Frozen sections of wild-type (A–C) and *Prox1*$^{-/-}$ (D–F) pancreata. (A and E) At E9.5, the expression of Pdx1 (red, arrow) was indistinguishable between dorsal pancreata of wild-type (A) and *Prox1*$^{-/-}$ (D) embryos. (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

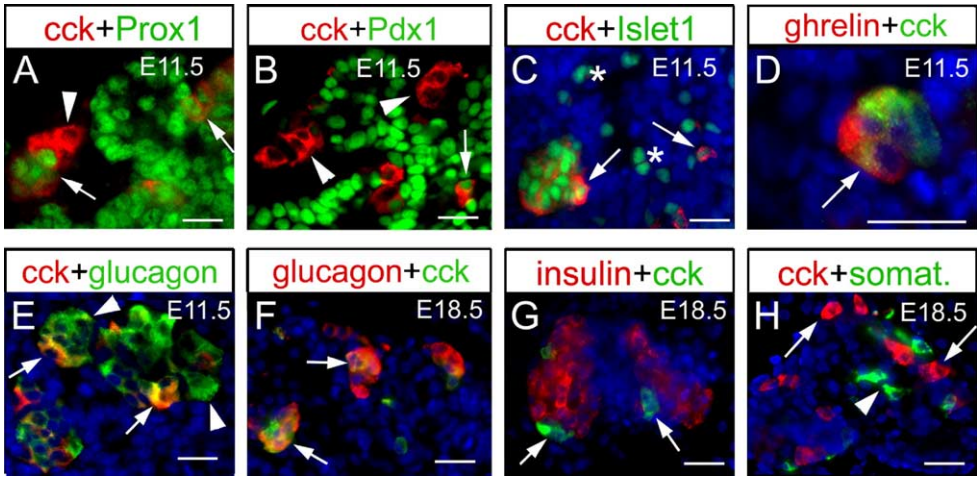

Fig. 4. A population of early pancreatic endocrine cells expressed cholecystokinin (Cck). (A–E) In the pancreata of E11.5 mouse embryos, Cck immunoreactivity (red in panel A) largely colocalized with Prox1 protein (green in panel A; arrows indicate colocalization; arrowhead indicates a Cck^+ cell devoid 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.

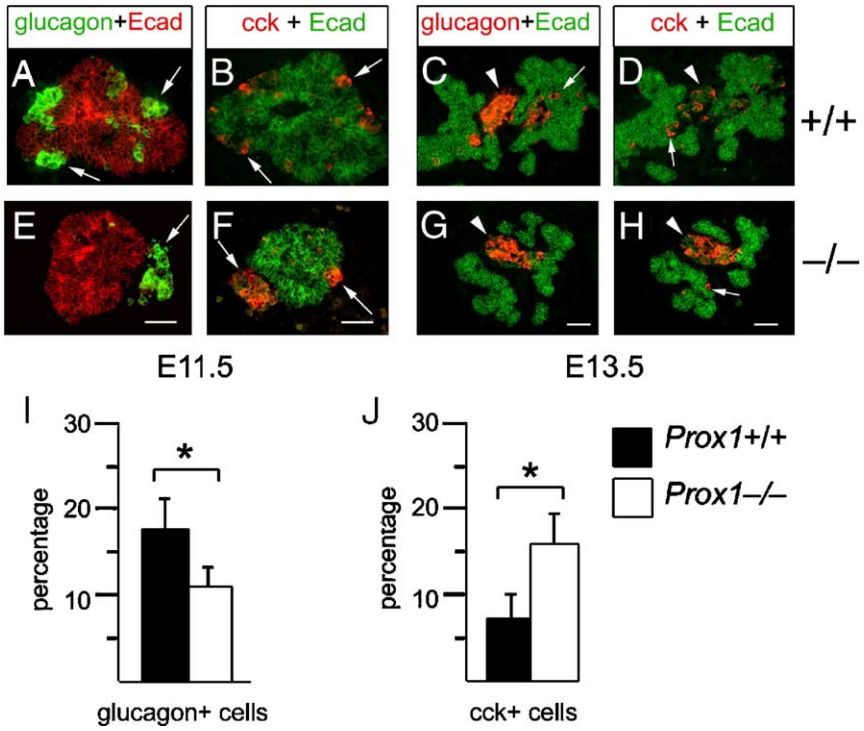

Fig. 5. The absence of Prox1 activity alters the differentiation of early pancreatic endocrine precursors. (A and B) At E11.5, a large fraction of pancreatic endocrine cells expressed glucagon (A, green and arrows), and a small percentage expressed Cck (B, red and arrows). (E–H) The absence of Prox1 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

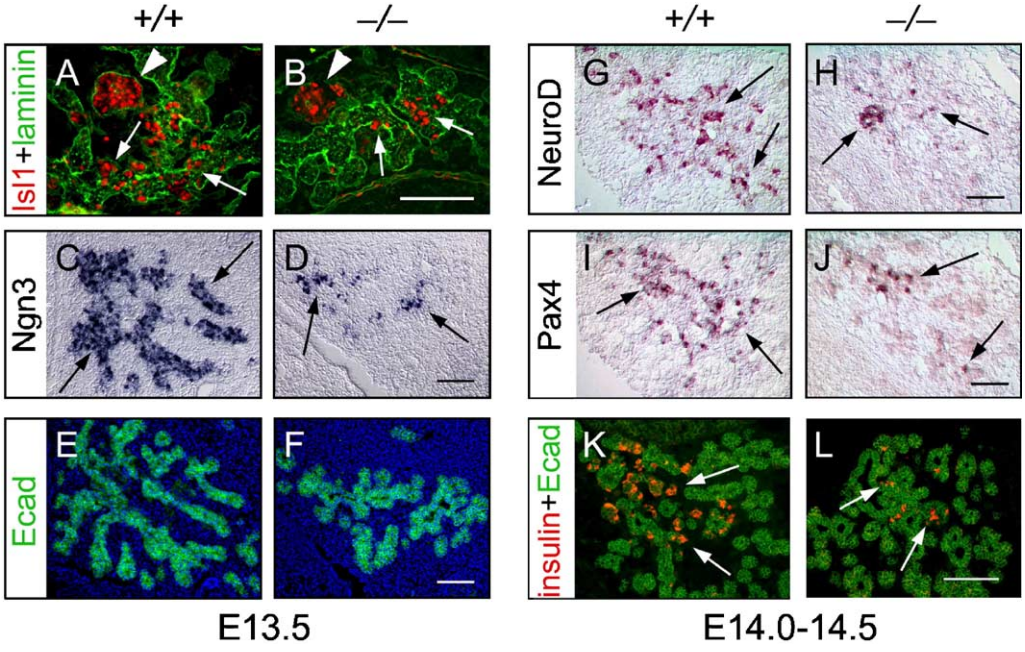

Fig. 6. The formation of endocrine cells in Prox1-deficient pancreatic tissues decreases considerably after E13.5. (A) In the pancreata of E13.5 wild-type embryos, two distinct populations of endocrine cells (Isl1$^+$ cells) are present: those that form large aggregates (arrowhead) and those that are 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.

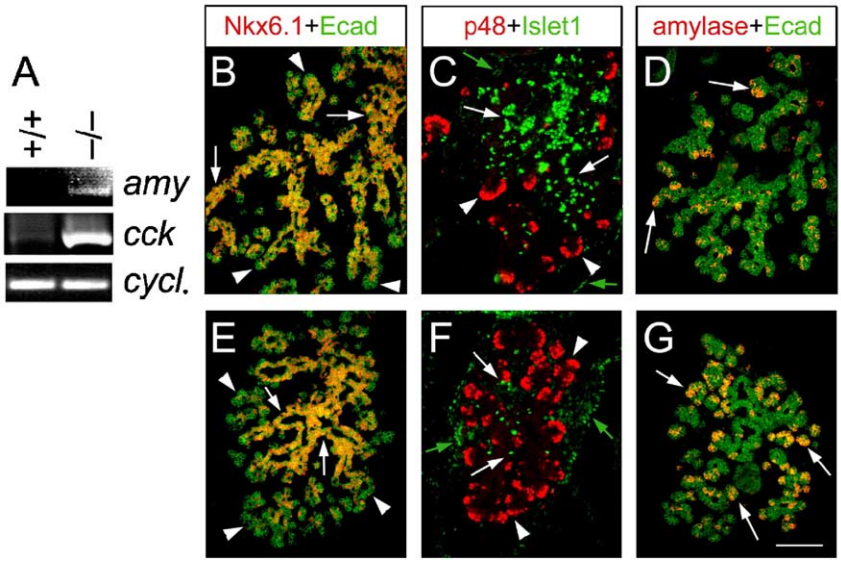

Fig. 7. Exocrine cells differentiate prematurely in Prox1-deficient pancreatic tissues. (A) RT-PCR analysis of E12.5 wild-type and *Prox1*$^{-/-}$ pancreata confirmed the microarray results that indicated increased transcription of amylase (*Amy*) and cholecystokinin (*cck*) in E12.5 Prox1-deficient 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.

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Chapter 3

Loss of Prox1 activity predisposes mice to pancreatitis

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Manuscript in preparation

Loss of Prox1 activity predisposes mice to pancreatitis

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ABSTRACT

The homeodomain transcription factor Prox1 is expressed all through mouse pancreas ontogeny. We deleted *Prox1* in pancreatic progenitors of mice and found that the lack of Prox1 activity affects multiple aspects of pancreas organogenesis including timely exocrine cell differentiation, ductal cell development, islet morphogenesis, and the production of α-cells. Mice lacking Prox1-pancreatic function are viable; however, these mutants gradually loose a large portion of pancreatic exocrine tissue due to apoptosis. Furthermore, we uncovered features indicative of mild, acute pancreatitis in the adult Prox1-deficient pancreas including inflammation, fibrosis, and intrapancreatic activation of protease zymogens. These collective results demonstrate that the function of Prox1 is critical for the formation of a fully functional pancreas.

INTRODUCTION

The mammalian pancreas consists of two types of glandular tissue: exocrine, producer of enzyme precursors required for food digestion, and endocrine, producer of hormones that control blood glucose levels (Slack et al., 1995). The function of the pancreas is critical to support proper nutrition and glucose homeostasis, and congenital conditions that affect its proper development or compromise its function predispose individuals to severe pathologies such as diabetes mellitus, pancreatitis, or pancreatic adenocarcinoma.

Our understanding of early pancreas development and pancreatic endocrine cell differentiation in vertebrates has increased considerably during the last decade (Cano et al., 2007; Murtaugh and Melton, 2003). In contrast, our knowledge of the molecular mechanisms responsible for the formation and maintenance of the exocrine and ductal cells in the pancreas remains poor. Identifying novel regulators of pancreatic exocrine and ductal development is critical, as this information should help deciphering the molecular bases of important human congenital pancreatic diseases.

The function of the divergent homeodomain transcription factor Prox1 is essential for the proper formation of many organs in vertebrates (Dyer et al., 2003; Oliver et al., 1993; Roy et al., 2001; Sosa-Pineda et al., 2000; Wigle and Oliver, 1999; Wigle et al., 1999). We previously reported a dynamic pattern of Prox1 expression throughout most stages of pancreas ontogeny and in the adult organ of mice (Wang et al., 2005). In addition, upon analyzing the pancreata of mouse embryos with a global deletion of *Prox1*, we discovered various important defects, including reduced organ size, poor branching, premature exocrine differentiation, and substantially decreased production of islet cell precursors at the onset of the secondary transition (Wang et al., 2005). These results showed that Prox1 is a novel, key regulator of early pancreas development and predicted that the lack of Prox1 function would have detrimental consequences for overall pancreatic organogenesis. However, this hypothesis could not be explored in *Prox1*-nullizygous embryos, because they die at around E14.5 due to multiple developmental defects (Wigle et al., 1999).

In this study, we inactivated *Prox1* specifically in pancreatic progenitors of mice by using a conditional knock-out approach. We found that the loss of pancreatic Prox1 function affects several aspects of late pancreas organogenesis and predisposes mice to develop a mild form of acute pancreatitis. Our results indicate that Prox1 function is crucial during pancreatic organogenesis for the formation of a fully functional organ, which is capable of maintaining proper homeostasis during postnatal stages.

RESULTS

DEVELOPMENTAL DEFECTS OF *Prox1$^{loxP/loxP}$; Pdx1-Cre* PANCREATA

Transient exocrine defects are observed in the Prox1-deficient embryonic pancreas

Pdx1-Cre transgenic mice were used to delete Prox1 specifically in pancreatic progenitors of *Prox1$^{loxP/loxP}$* mice. We observed broad Prox1 expression in the pancreas of E13.5 wild-type embryos (Fig. 1A), and only a few cells expressing Prox1 in this organ of E13.5 *Prox1$^{loxP/loxP}$;Pdx1-Cre* embryos (Fig. 1B). E13.5 *Prox1$^{loxP/loxP}$;Pdx1-Cre* pancreata also had more cells expressing the exocrine markers amylase or elastase in comparison to E13.5 wild-type pancreata (Fig. 1C,D and data not shown). By E15.5, the exocrine cells of *Prox1$^{loxP/loxP}$;Pdx1-Cre* pancreata expressed numerous exocrine cell products or transcription factors (Fig. 1H and data not shown) (Krapp et al., 1998; Pin et al., 2001). However, these mutant cells also expressed Sox9, a transcription factor normally detected in multipotent progenitors (but not in exocrine cells) of the embryonic pancreas (Fig. 1I, J and data not shown) (Lynn et al., 2007; Seymour et al., 2007). These collective results indicate that the loss of Prox1 function results in precocious expression of exocrine gene products in pancreatic progenitors. Abnormal Sox9 expression in exocrine cells was no longer observed in the *Prox1*-deficient pancreas after E15.5 (data not shown).

E15.5 *Prox1$^{loxP/loxP}$;Pdx1-Cre* pancreata had relatively less exocrine cells mass than E15.5 wild-type pancreata (Fig. 1E-H). However, this reduction in exocrine cell mass was no longer observed in *Prox1$^{loxP/loxP}$;Pdx1-Cre* pancreata at birth (data not shown). We did not detect obvious differences in the expression of the proliferation marker phospho-histone 3 (PH3) between E15.5 and E17.5 *Prox1$^{loxP/loxP}$;Pdx1-Cre* and wild-type pancreata (data not shown). Therefore, we conclude that compensatory mechanisms (including proliferation) enabled the recovery of the exocrine mass of *Prox1$^{loxP/loxP}$;Pdx1-Cre* pancreata after E17.5.

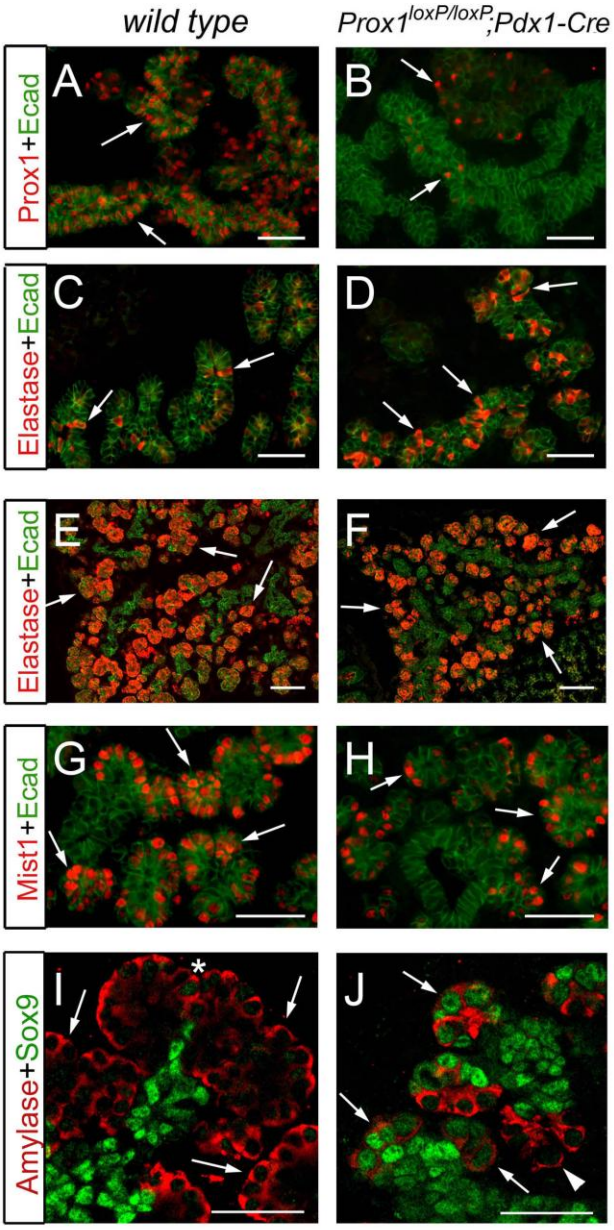

Fig.1. **Exocrine alterations of *Prox1$^{loxP/loxP}$;Pdx1-Cre* embryonic pancreata**. (**A,B**) At E13.5, Prox1 expression is substantially reduced in *Prox1$^{loxP/loxP}$;Pdx1-Cre* pancreata (compare **A** with **B**). (**C,D**) At this stage more cells express elastase in *Prox1$^{loxP/loxP}$;Pdx1-Cre* pancreata (**D**) than in wild-type pancreata (**C**). (**E-H**) At E15.5, exocrine cells expressing elastase (**E,F**) or Mist1 (**G,H**) are more abundant in wild-type pancreata (**E,G**) than in *Prox1$^{loxP/loxP}$;Pdx1-Cre* pancreata (**F,H**). (**I,J**) Numerous exocrine cells of E15.5 *Prox1$^{loxP/loxP}$;Pdx1-Cre* pancreata maintain Sox9 expression (compare **I** with J). Scale bars, 200 μm.

In *Prox1$^{loxP/loxP}$;Pdx1-Cre* newborn pancreata we noticed that some portions of the exocrine tissue appeared underdeveloped. For instance, in these regions we observed acini with smaller exocrine cells or enlarged lumens (Fig. 2A, B), groups of acini surrounding an abnormally dilated intra-acinar

duct (Fig. 2C), or acini showing aberrant coexpression of the exocrine marker elastase and the ductal marker dolichus bifflorus agglutinin (DBA) (data not shown). Microarray analysis results also revealed decreased expression of some exocrine transcripts in the pancreatic tissues of *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* newborns (e.g., *Ptf1a/p48*, *Mist1,* and *GATA4*) (data not shown). However, the previous exocrine defects were no longer observed in *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* pancreata after postnatal (P) day 5 (J. Westmoreland, personal communication). We conclude that the loss of pancreatic Prox1 function transiently delays exocrine cell development.

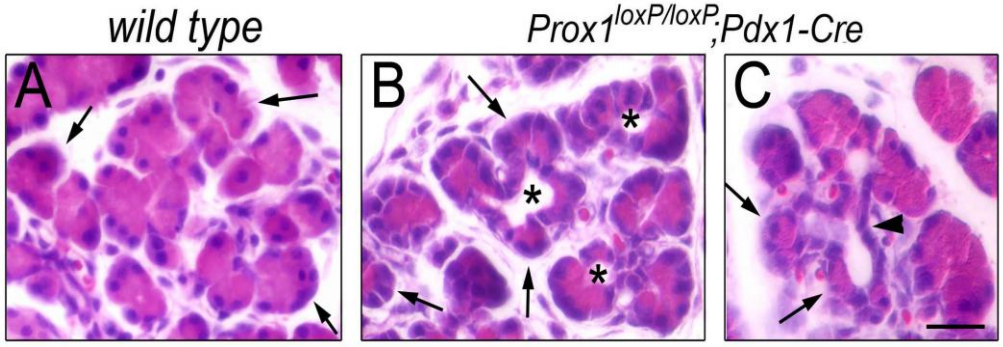

Fig.2. **Some exocrine cells of *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* newborn pancreata seem underdeveloped.** (**A**) Some acini of *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* newborn pancreata have smaller exocrine cells (arrows in **B,C**), or enlarged lumens (asterisks in **B**) in comparison to wild-type newborn pancreata (**A**). The abnormal mutant acini usually appear associated with a dilated intracinar duct (arrowhead in **C**). Scale bar: 400 μm.

Loss of pancreatic Prox1 function affects endocrine cell development

E13.5 *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* pancreata had fewer endocrine precursors (identified by the expression of the proendocrine gene *Ngn3*; Fig. 3A,C) than did their wild-type counterparts. However, Ngn3^{+} cells recovered in the conditional knock-out pancreas after E15.5 (Fig. 3B,D). Since activation of Notch signaling pathway antagonizes the expression of the pro-endocrine *Ngn3* gene in the pancreas, we hypothesized that in E13.5-E14.5 *Prox1*-deficient pancreata Notch activity was probably increased (Apelqvist et al., 1999; Murtaugh and Melton, 2003). However, our results of QRT-PCR analyses showed relatively normal expression of the Notch1 target *Hes-1* or *Notch2* in E13.5 *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* pancreata (data not shown). Furthermore, at birth the *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* pancreata had an islet cell mass that was proportionally similar to that of wild-type littermates (22.2% $\pm$ 3.7% [*wt*] vs 21.5% $\pm$ 3.1% [*mut*]; p=0.9; n=3). Gene profiling and QRT-PCR analyses results showed increased expression of *Ngn3* and *OC-2* (encoding a transcriptional activator of *Ngn3*) in the pancreata of *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* newborn mice (Fig. 3E). We conclude that compensatory mechanisms become activated in the *Prox1*-deficient pancreas after E15.5 to increase endocrine cell genesis.

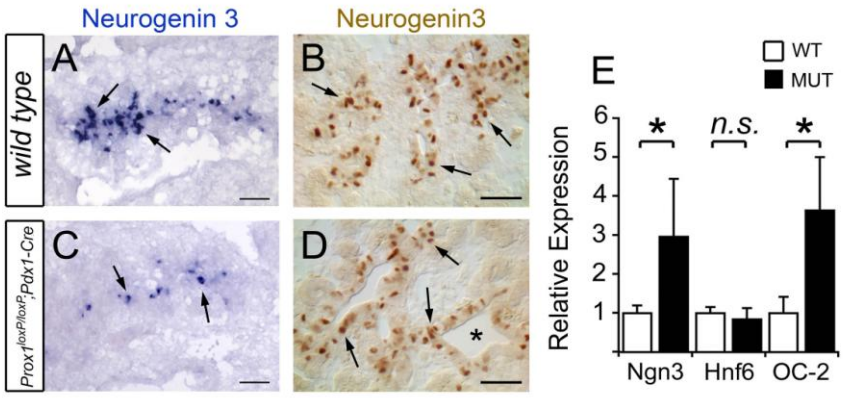

Fig.3. Loss of pancreatic Prox1 function delays the onset of islet cell genesis. (A,C) At E13.5, *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* pancreata (**C**) have fewer endocrine progenitors (Ngn3^{+}) than wild-type pancreata (**A**). (**B,D**) At E15.5, the abundance of Ngn3^{+} cells is similar between *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* (**D**) and wild-type pancreata (**B**). **(E)** QRT-PCR analysis shows increased expression of *Ngn3* and *OC-2* in *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* pancreata at P2. *=*p*<0.05; Scale bars, 200 μm.

In *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* newborn pancreata we observed numerous insulin^{+} cells within the ductal epithelium (Fig. 4D). In similar tissues of wild-type littermates, the insulin^{+} cells were largely incorporated into the islets of Langerhans or occasionally formed small cellular aggregates (Fig. 4A,B). We also found that most islets of *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* newborn pancreata were smaller than those of wild-type pancreata (Fig. 4B,E; the percentage of large islets was 9.57 $\pm$ 1.16 [*wt*] and 4.47 $\pm$ 0.2 [*mut*], p<0.05; and the percentage of small islets was 90.43 $\pm$ 1.16 [*wt*] and 95.43 $\pm$ 0.21 [*mut*], p<0.05; n=3). These results suggest that Prox1 controls the efficient aggregation of pancreatic endocrine cells into large islets.

Prox1 is expressed at high levels in α-cells (glucagon^{+}) and at low levels in β-cells (insulin^{+}) in pancreatic islets of wild-type mice (Wang et al., 2005). We found that the loss of Prox1 function did not affect the expression of various β-cell markers (including insulin; Fig. 4D,E). In contrast, fewer cells expressed glucagon in the islets of P1 *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* pancreata (Fig. 4C,F), and our microarray results revealed a 10-fold reduction in *glucagon* transcripts in that tissue (data not shown). Interestingly, although *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* mice had smaller islets and fewer pancreatic α-cells than wild-type mice, their glucose homeostasis was preserved (data not shown).

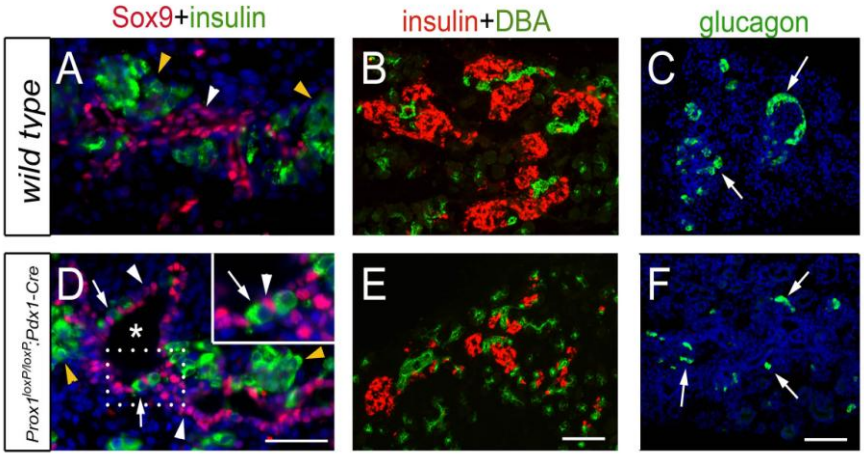

Fig.4. **Islet defects of *Prox1$^{loxP/loxP}$;Pdx1-Cre* newborn pancreata.** (**A,D**) The large ducts (Sox9^{+}) of *Prox1$^{loxP/loxP}$;Pdx1-Cre* pancreata (**D**) (but not those of wild-type pancreata [**A**]) contain insulin^{+} cells. (**B,E**) Most islets of *Prox1$^{loxP/loxP}$;Pdx1-Cre* pancreata (**E**) are smaller than the islets of wild-type pancreata (**B**). (**C,F**) The pancreata of *Prox1$^{loxP/loxP}$;Pdx1-Cre* newborn mice (**F**) have substantially fewer α–cells (glucagon^{+}) in comparison to wild-type pancreata (**C**). Scale bars, 100 μm.

Prox1–deficient pancreatic tissues have numerous ductal defects

Morphogenesis of the pancreatic ductal tree initiates with the formation of "primitive ducts", consisting of polarized epithelial cells expressing the proteins osteopontin (Opn), Mucin1, or dolichos bifflorus agglutinin (DBA) toward the luminal (apical) surface, and the protein laminin on the basal surface (Grapin-Botton, 2005; Kilic et al., 2006; Kesavan et al., 2009). Primitive ducts were visible at E13.5-15.5 in both, wild-type and Prox1-deficient pancreata (Fig. 5B and data not shown). However, although the *Prox1*-deficient primitive ducts appeared correctly polarized, their lumens were abnormally enlarged (Fig. 5A,B). The dilated ductal phenotype of *Prox1*-deficient pancreata was observed all through gestation (Fig. 5B,D). In addition, the large pancreatic ducts of E15.5-P0 *Prox1$^{loxP/loxP}$;Pdx1-Cre* mice contained columnar, not cuboidal epithelium (Fig. 5C,D).

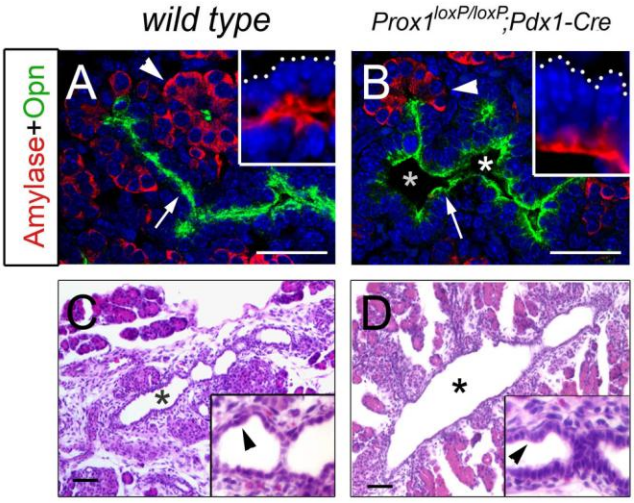

Fig.5. **Morphologic abnormalities of the large ducts of *Prox1$^{loxP/loxP}$;Pdx1-Cre* pancreata. (A,B)** The large developing ducts of E15.5 *Prox1$^{loxP/loxP}$;Pdx1-Cre* pancreata (Opn+) have unusually dilated lumens (asterisks in **B**); columnar, not cuboidal epithelium (insets in **A**,**B**); and normal epithelial cell polarity (compare the apical expression of mucin [red] in insets of [**A,B**]**). (C,D)** The large ducts of *Prox1$^{loxP/loxP}$;Pdx1-Cre* newborn pancreata are slightly hyperplastic and have columnar, not cuboidal epithelium (arrowheads in insets). Scale bars: 200 μm.

In the pancreata of E17.5 *Prox1$^{loxP/loxP}$;Pdx1-Cre* embryos we also noticed alterations in the developing intralobular ducts, including: unusually dilated lumens (Fig. 6A,B), cuboidal epithelial lining instead of low-cuboidal or flattened (Fig. 6C,D), and abnormal cells coexpressing exocrine and ductal markers (Fig. 6D). Similar defects were also observed in the intralobular ducts of *Prox1$^{loxP/loxP}$;Pdx1-Cre* newborn pancreata, as they were dilated and very tortuous, they had abnormal cuboidal epithelial lining (Fig. 6E-H), and their terminal portions contained unusual cells coexpressing ductal and exocrine markers (inset in Fig. 6H). These alterations were not seen in the pancreas of wild-type littermates. Therefore, on the basis of our results we conclude that Prox1 activity is necessary for proper ductal morphogenesis during pancreatic development.

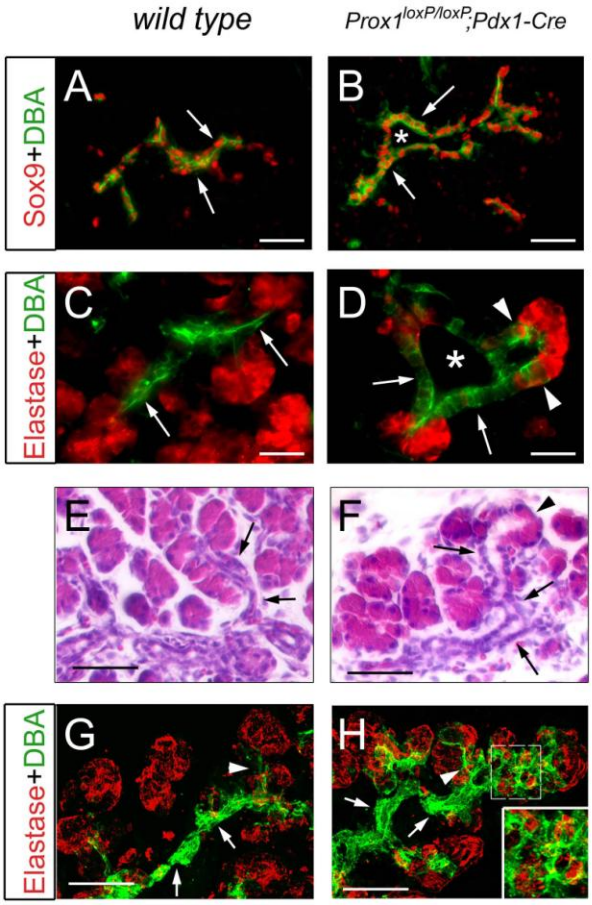

Fig.6. **Morphologic abnormalities of the small ducts of *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* pancreata. (A-D)** The intralobular ducts (Sox9^{+} or DBA^{+}) of E17.5 *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* pancreata have enlarged lumens (asterisks in **B, D**); cuboidal and not flat to low cuboidal epithelium (arrows in **C,D**); and occasional cells co-expressing ductal and exocrine markers (arrowheads in **D**). (**E,F**) The intralobular ducts of *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* newborn pancreata are slightly hyperplastic. (**G,H**) Both, the intralobular ducts (arrows) and the intercalated ducts (arrowheads) of *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* newborn pancreata are very tortuous (compare DBA staining), and have cells co-expressing exocrine and ductal markers in their terminal ends (inset in **H**). Scale bars, 200 μm.

POSTNATAL DEFECTS OF *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* PANCREATA

***Prox1*$^{loxP/loxP}$*;Pdx1-Cre* pancreata undergo extensive loss of exocrine tissue**

Prox1$^{loxP/loxP}$*;Pdx1-Cre* mice were viable, and most reached adulthood. However, at around P21 we detected areas devoid of exocrine tissue (Fig7C-F) or numerous exocrine cells undergoing apoptosis (TUNEL^{+} cells) in every *Prox1*-deficient pancreatic specimen that we analyzed (Fig. 7A,B,G). By 3 months of age, some lobes of *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* pancreata entirely lacked exocrine cells (Fig. 7I,K,M). Staining with Oil Red O revealed that the portions of the mutant pancreas lacking exocrine tissue were extensively infiltrated with adipocytes (inset in Fig. 7I). After 6 months of age, most of the

Prox1$^{loxP/loxP}$*;Pdx1-Cre* pancreatic tissue merely consisted of a few small acini, isolated ducts, and some islets embedded within an extensive mass of adipocytes (Fig. 7K,M). However, all *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* adult mice analyzed retained a variable (but small) portion of relatively intact exocrine tissue (Fig. 7J,L,N).

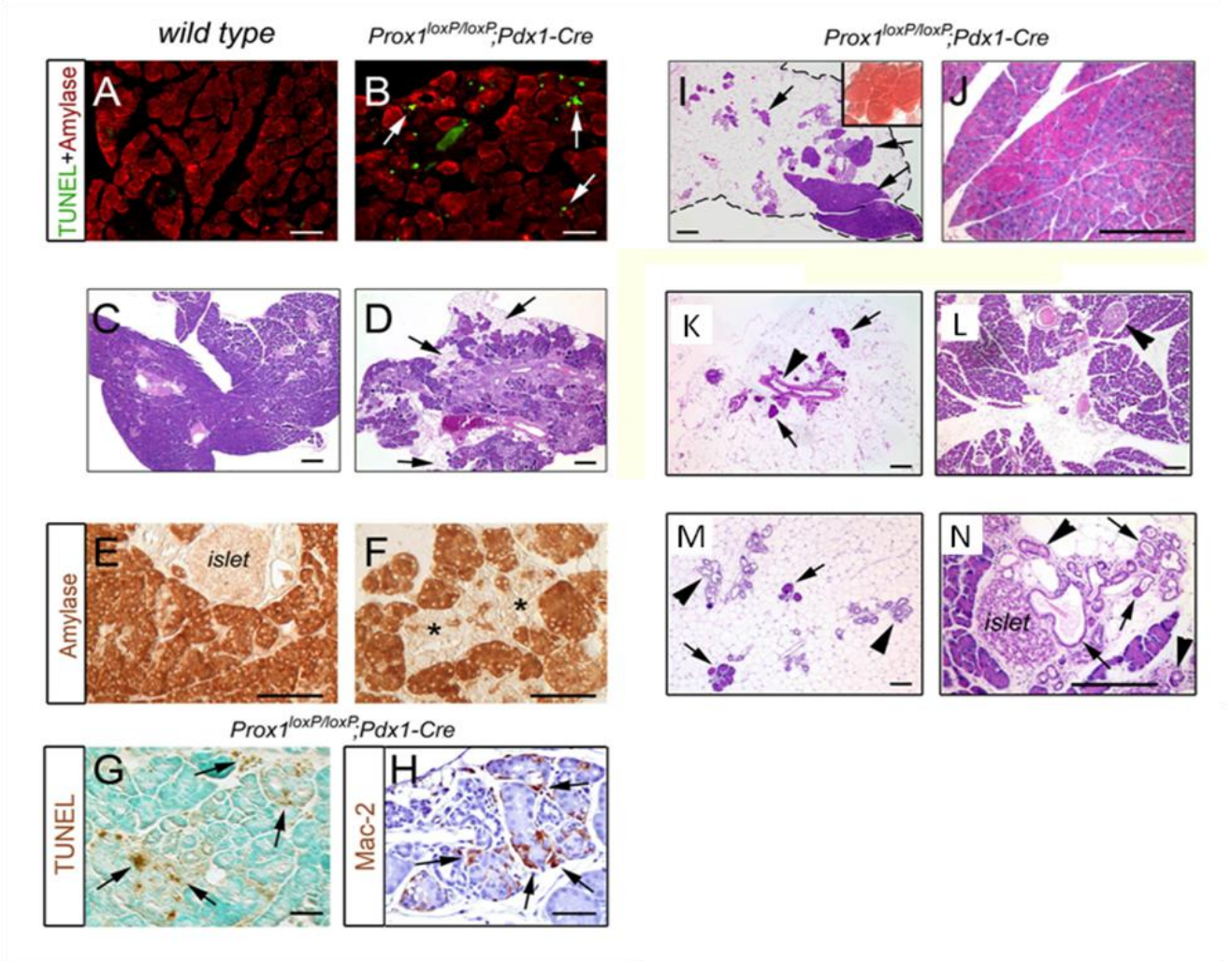

Figure 7. **Prox1-deficient pancreata have massive exocrine tissue loss.** *See text for details*. Scale bars, 50 μm (**C,D,I-N**), 100 μm (**E,F**) or 200 μm (**A,B,G,H**).

Prox1$^{loxP/loxP}$***;Pdx1-Cre* pancreata have features indicative of mild pancreatitis

Most pancreatic ducts in P18 or adult *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* mice appeared hyperplastic, very tortuous, and had abnormal epithelial morphology (Figs. 8A-D and 6E,F, respectively). In the pancreas of *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* adults, some cells within the large ducts expressed insulin, a property that has been reported in the pancreatic ducts of various mouse models of pancreatitis (Fig. 8G) (Chen et al., 2004; Gu and Sarvetnick, 1993; Higuchi et al., 1992). H&E and trichrome staining revealed mild fibrosis, a feature normally associated with pancreatitis, in areas surrounding the large

ducts of *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* adult pancreata (Fig. 8F and data not shown) (Schmid and Whitcomb, 2006). We also observed infiltrates of immune cells (primarily macrophages) in *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* pancreata, and they predominated in areas of noticeable exocrine cell loss (see Fig. 7H) (Vonlaufen et al., 2007). In the lumen of the large ducts of the oldest *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* mouse analyzed (8-14 months old), we noticed extensive cellular debris (Fig. 8H), accumulating cytoplasm in tall columnar cells (Alcian blue^{+}; Fig. 8I), cells resembling intestinal goblet cells (PAS^{+} cells; Fig. 8J), some proliferating cells (Ki67^{+} cells; Fig. 8K), and a few cells expressing phosphorylated Erk1/2 proteins (Fig. 8L). The pancreas of the 14-month-old *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* mouse also contained some cysts (Fig. 8M) and abnormal epithelial ductal structures or acinar-to-ductal metaplasias (Fig. 8N-T). Most of these features have been reported in the pancreas of mice or humans afflicted with certain pathologic conditions and are most likely the result of the activation of specific regenerative processes in combination with low, persistent inflammation (Adsay et al., 2004; Luttges et al., 2000). Collectively, all these histological features suggest that *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* mice are afflicted with a mild form of pancreatitis.

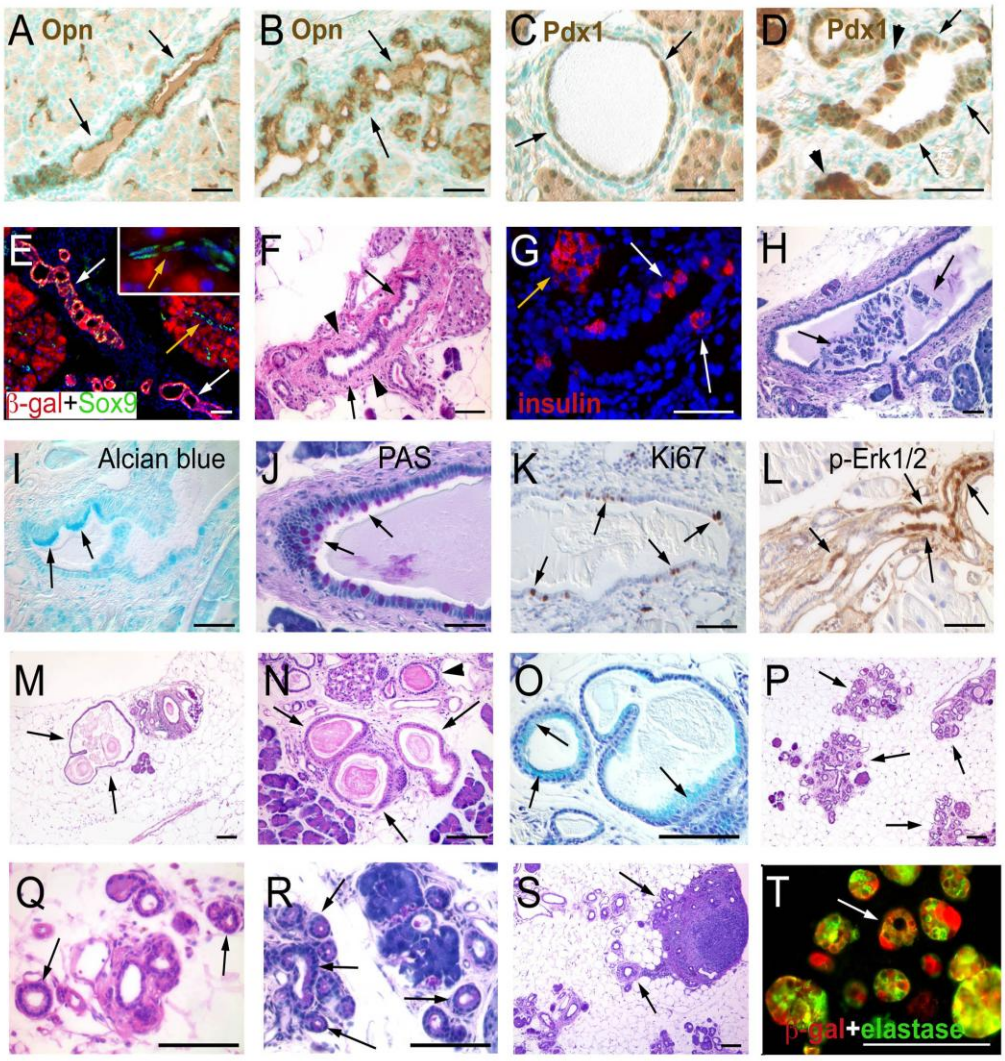

Fig. 8. Duct abnormalities in the pancreas of *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* young and adult mice. *See text for details.* Scale bars, 50 μm (**C,D,I-P**), 100 μm (**E,F**) or 200 μm (**A,B,G,H**).

DISCUSSION

Prox1 controls some aspects of pancreatic endocrine cell development

This study found that the loss of pancreatic Prox1 function delays the onset of the major wave of endocrine cell genesis, and decreases the size of the epithelial branches. It is possible that these two alterations could be interrelated, because a recent study showed that the branches of the developing pancreatic epithelium support endocrine development by providing a microenvironment permissive for endocrine specification (Kesavan et al., 2009). In spite of this alteration, the formation of endocrine cells recovered late in gestation in *Prox1*$^{loxP/loxP}$*,Pdx1-Cre* pancreata. Furthermore, different from wild-

type pancreata, the formation of endocrine precursors extended beyond postnatal stages in the *Prox1*-deficient pancreas. It is noteworthy that the expression of both *Ngn3* and *OC-2* transcripts was increased in *Prox1$^{loxP/loxP}$;Pdx1-Cre* newborn pancreata, because previous studies revealed that OC-2 is a transcriptional activator of *Ngn3* (Vanhorenbeeck et al., 2007). Arguably, the elevated expression of OC-2 probably contributed to enhance the production of endocrine progenitors in *Prox1*-deficient pancreatic tissues.

In the pancreas of *Prox1$^{loxP/loxP}$;Pdx1-Cre* newborn mice we also found that the size of most pancreatic islets was smaller in comparison to wild-type pancreata. It is possible that the loss of Prox1 function affected the efficient aggregation of endocrine cells into islet clusters, because our microarray gene profiling results uncovered decreased expression of integrin alpha 6, a protein implicated in islet cell migration, in the pancreas of *Prox1$^{loxP/loxP}$;Pdx1-Cre* newborn mice (data not shown) (Yebra et al., 2003).

Our results of this study indicate that the lack of Prox1 function is dispensable or redundant for the formation of pancreatic β-cells. In fact, although some *Prox1$^{loxP/loxP}$;Pdx1-Cre* adult mice displayed glucose intolerance, this defect was most likely the consequence of islet damage triggered by exposure to low, ensuing inflammation, as this alteration was not observed in those mutants before 5 months of age. In contrast, Prox1 function appeared to be necessary for the adequate production of pancreatic α-cells, because most *Prox1$^{loxP/loxP}$;Pdx1-Cre* newborn and adult pancreatic tissues had a substantial paucity of glucagon^{+} cells. Unfortunately, this study could not determine how *Prox1* is required during α-cell development because this gene was deleted in just a fraction of α-cells in the pancreas of *Prox1$^{loxP/loxP}$;Pdx1-Cre* mice.

Prox1 controls morphogenesis of the pancreatic ducts

This study uncovered various alterations in the developing ducts of *Prox1$^{loxP/loxP}$;Pdx1-Cre* pancreata including very tortuous shape of the entire ductal tree, abnormal morphology of the epithelial cells (i.e., columnar and not cuboidal in the large ducts) and unusually enlarged lumens. Therefore, we conclude that Prox1 function is necessary for proper pancreatic duct development. It is possible that Prox1 function could also be involved with the specification or proliferation of pancreatic duct epithelial cells, because the ducts of *Prox1$^{loxP/loxP}$;Pdx1-Cre* pancreata are mildly hyperplastic. However, those possibilities could not be explored due to the lack of specific markers identifying duct cell precursors or developing ductal cells of the pancreas.

The loss of pancreatic Prox1 did not affect epithelial polarity or the formation of cilia in pancreatic ductal cells. However, recent results from the Sosa-Pineda laboratory uncovered abnormal, elevated expression of the tight junction protein claudin-2 in the entire *Prox1*-deficient pancreatic ductal tree (J. Westmoreland, personal communication). Increased claudin-2 expression has been linked to some pathologies characterized by defective epithelial permeability, such as Inflammatory Bowel Disease (Weber et al., 2008; Schulzke et al., 2009). Therefore, we hypothesize that the permeability of the *Prox1$^{loxP/loxP}$;Pdx1-Cre* pancreatic ducts was probably affected as a result of excessive claudin-2 expression.

In the pancreatic ducts of older *Prox1$^{loxP/loxP}$;Pdx1-Cre* adult mice we noticed various alterations including cellular debris and abundant secretion in the lumen, mild fibrosis around the ducts, inflammatory infiltrates, acinar-to-ductal metaplasias, and the presence of endocrine cells, goblet cells, and proliferating cells within the ductal epithelium. Although all of these features indicate that the function of the ducts becomes compromised with age in *Prox1$^{loxP/loxP}$;Pdx1-Cre* mice, whether this is the result of the lack of Prox1 function in the duct epithelial cells, inflammation, or both remains to be shown.

Is the pancreatitis of Prox1$^{loxP/loxP}$;Pdx1-Cre mice the result of congenital defects in the pancreatic ducts, the pancreatic exocrine cells, or both?

In pancreatic tissues of *Prox1$^{loxP/loxP}$;Pdx1-Cre* embryos, we observed precocious expression of various exocrine genes. This result indicates that Prox1 function controls the timely activation of some genes in exocrine progenitors. In the pancreas of *Prox1$^{loxP/loxP}$;Pdx1-Cre* newborn mice we also noticed that some exocrine cells had a more immature morphology in comparison to similar cells of wild-type pancreata. However, after P5 these differences were no longer noticed and in subsequent days *Prox1$^{loxP/loxP}$;Pdx1-Cre* mice thrived without showing any symptoms of pancreatic insufficiency or visible exocrine alterations.

Remarkably, starting at around the weaning period *Prox1$^{loxP/loxP}$;Pdx1-Cre* mice underwent ensuing, extensive loss of exocrine tissue due to apoptosis and gradually developed a mild form of acute pancreatitis. It is possible that this alteration was the result of a congenital exocrine defect that prevented these cells to respond properly to the increasing nutritional demands. Although we cannot exclude the previous possibility, thus far we have not detected any obvious defect in the pancreatic exocrine tissue of *Prox1$^{loxP/loxP}$;Pdx1-Cre* juvenile mice. Instead, these mutant mice clearly had defective architecture of the pancreatic ductal tree and they expressed some ductal proteins

abnormally (e.g., claudin-2). Therefore we hypothesize that the pancreatic ductal defects of *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* mice largely contributed to the development of pancreatitis.

Overall, our study revealed a novel requirement of Prox1 function during pancreas organogenesis. Specifically, we uncovered that Prox1 controls the formation of the pancreatic ductal tree, and it participates in some aspects of exocrine cell differentiation, α-cell development and islet morphogenesis. Our future efforts will be directed toward identifying the molecular and cellular bases of the ductal phenotype of *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* mice, and determining whether the congenital defects of these mutant mice increase their susceptibility to certain pancreatic diseases (e.g., pancreatic carcinoma).

MATERIALS AND METHODS

Animals

Prox1$^{loxP/+}$ mice were maintained and genotyped as described in Harvey, N. L. et al (2005). *Pdx1-Cre*$^{+/-}$ mice (Gu, et al., 2002) were obtained from the MMRRC (UC Davis, CA, USA). *Rosa26R*^{LacZ} mice (Soriano, 1999) were purchased from the Jackson Laboratory (Bar Harbor, MA, USA).

Cell counting and Morphometric analysis

Morphometric analysis was conducted using ImageJ 1.37v program. For islet size comparisons, those islets with less than 20% the size of the largest islets were arbitrarily classified as “small” and the rest were classified as “large”.

Glucose Tolerance Testing

Prox1$^{loxP/loxP}$*; Pdx-Cre* mice and age-matched wild-type controls were fasted for 16 hours before intraperitoneal injection of a glucose solution (2 mg per gram body weight). Blood from the tail vein was taken at 15 minutes intervals for 2 hours and glucose levels were measured using a ONE TOUCH Sure Step glucometer (Lifescan).

Processing of embryos and pancreatic tissues

Frozen sections of tissues from dissected embryos or pancreata from newborn mice were prepared for immunohistochemical analysis or in situ hybridization as described in Wang et al., (2005).

Histology

Paraffin sections were stained with hematoxylin and eosin (H&E), with Masson's Trichrome (to examine fibrosis) or with Alcian Blue. Staining for mucin content was performed by using diastase-PAS (Sigma) and for lipid content, by using oil Red O solution (Sigma). For DBA staining we used fluorescein-conjugated Dolichus bifflorus agglutinin.

TUNEL assay

Apoptotic cell death was detected using commercial kits.

Microarray analysis

Gene expression analyses were performed at the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital. Individual pancreata from three P2 wild-type mice and three *Prox1*$^{loxP/loxP}$*; Pdx-Cre* littermates were dissected and processed separately for RNA extraction by using the TRIzol method (Invitrogen). The synthesis of cDNA, generation of biotin-labeled cRNA, hybridization to the GeneChip (Affymetrix MOE-430A 2.0 GeneChip array), incubation with R-phycoerythrin-conjugated streptavidin and with biotin-conjugated anti-streptavidin antibody, scanning of the arrays and comparative analysis of the hybridization results were carried out at the Hartwell Center facility of St. Jude.

Quantitative Real-Time PCR

Total RNA was extracted using TRIzol. The reverse transcriptase reactions were done using TaqMan Reverse Transcription Reagent (Applied Biosystems). Quantitative PCR was performed on an ABI Prism 7900HT Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA). Expression levels were normalized against the expression of the ribosomal coding gene 18S. Quantitative PCR assays were conducted in duplicate for each sample (n=3 samples per genotype) and a mean value was used to calculate mRNA levels. Primers were chosen with the assistance of the computer program Primer Express (PE Applied Biosystems, version 2.0.0).

Primer sequences used for QRT-PCR experiments:

	FORWARD	**REVERSE**
Ngn3	GAACTAGGATGGCGCCTCAT	CGGGAAAAGGTTGTTGTGTCTCT
Hnf6	CGGAGTTCCAGCGCATGT	CCTCTGTCCTTCCCGTGTTC
OC-2	GACCTTCCGCAGGATGTG	GGTTCTTGCTCTTTGCGTTT

Statistical analyses

Statistical significance was determined using the Student's t test (p value < 0.05 indicates statistically significant differences).

ACKNOWLEDGMENTS

We thank L. Bachaboina for excellent technical assistance; A. Kandilci and A. Seth for help with the QRT-PCR analyses and A. McArthur for editing of the manuscript. We also thank the following people for generously providing mice or reagents: D. Melton, G. Gonqiang, C. Wright, M. German, S. Konyeczny and D. Anderson. This project was supported by the National Institute of Diabetes and Digestive and Kidney Diseases (R01DK060542, NIH) and by the American Lebanese Syrian Associated Charities (ALSA

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Chapter 4

Osteopontin is a novel marker of pancreatic ductal tissues and of undifferentiated pancreatic precursors in mice

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Developmental Dynamics 235:1659–1667, 2006

DEVELOPMENTAL DYNAMICS 235:1659–1667, 2006

Osteopontin Is a Novel Marker of Pancreatic Ductal Tissues and of Undifferentiated Pancreatic Precursors in Mice

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Matricellular proteins mediate both tissue morphogenesis and tissue homeostasis in important ways because they modulate cell–matrix and cell–cell interactions. In this study, we found that the matricellular protein osteopontin (Opn) is a novel marker of undifferentiated pancreatic precursors and pancreatic ductal tissues in mice. Our analysis also underscored a specific, dynamic profile of Opn expression in embryonic pancreatic tissues that suggests the participation of this protein's function in processes involving cell migration, cell–cell interactions, or both. Surprisingly, our analysis of Opn-deficient pancreata did not reveal obvious alterations in the morphology or differentiation of these tissues. Therefore, in embryonic pancreatic tissues, it is possible that other proteins act redundantly to Opn or that this protein's function is dispensable for pancreas development. Finally, the maintenance of Opn expression in pancreatic tissues of adults argues for a possible function of this protein in injury and pathologic responses. ***Developmental Dynamics 235:1659–1667, 2006.*** **© 2006 Wiley-Liss, Inc.**

Key words: pancreas; osteopontin; mouse; matricellular proteins; development; pancreatic ducts; pancreatic precursor; embryo

Accepted 27 January 2006

INTRODUCTION

The mature mammalian pancreas is a mixed gland composed of endocrine and exocrine tissues that produce and secrete hormones and enzymes required for nutritional balance. In the mature pancreas, the pancreatic exocrine cells produce the precursors of various digestive enzymes (zymogens), which are collected by a system of intralobular and large ducts and then transported to the duodenum, where they are processed to their active form (Slack, 1995). Pancreatic endocrine cells associate into distinctive spherical structures known as the islets of Langerhans. In these islets, the α cells produce glucagon, the β cells produce insulin, the δ cells produce somatostatin, and the PP cells produce pancreatic polypeptide (Slack, 1995). Because the pancreatic hormones maintain blood glucose homeostasis, their function is crucial to metabolism.

Pancreas organogenesis manifests early in vertebrate development as two protrusions (dorsal and ventral) at the posterior foregut region of the embryo (in mice, this occurs between embryonic days [E] 9.5 and 10.5). After formation of these two primordia, the pancreatic epithelium grows intensely, it undergoes branching morphogenesis and in a nonsynchronous manner it produces the precursors of endocrine, exocrine, and ductal cells (Pictet and Rutter, 1972; Slack, 1995; Murtaugh and Melton, 2003). As demonstrated by the results of numerous in vivo and in vitro studies, the pancreatic epithelium's growth, morphogenesis, and cellular differentiation depend on its interaction with surrounding tissue (Kim and Hebrok, 2001; Edlund, 2002; Murtaugh and Melton, 2003; Wilson et al., 2003). Similarly, evidence gathered from genetic studies supports the notion that some crucial regulators of normal pancreas organogenesis are also key mo-

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Grant sponsor: National Institutes of Health; Grant number: DK60542; Grant sponsor: American Lebanese Syrian Associated Charities.
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DOI 10.1002/dvdy.20729
Published online 3 March 2006 in Wiley InterScience (www.interscience.wiley.com).

lecular components of pancreatic diseases (Habener and Stoffers, 1998; Kim and Hebrok, 2001; Edlund, 2002; Miyamoto et al., 2003). Therefore, identification of the molecular and cellular mechanisms regulating mammalian pancreas development appears necessary, not only to an understanding of the origins of pancreatic dysfunction, but also to the development of tools to improve treatment and prevention of pancreatic diseases (e.g., diabetes mellitus, pancreatitis, or pancreatic cancer).

Cell–cell and cell–matrix interactions play a crucial role in tissue morphogenesis and in homeostasis of adult tissues. The extracellular matrix (ECM) proteins regulate cell function and morphogenesis in important ways, because they contribute directly to the organization or physical properties of structures such as fibrils or basal laminae and because they participate in diverse cellular processes. Indeed, the capacity of cells to adhere to the ECM, crucial to cytoskeletal organization and cellular morphology, is also implicated in a cell's ability to proliferate, migrate, survive, or differentiate (Murphy-Ullrich, 2001). The matricellular proteins integrate a separate subclass of matrix proteins that have no structural role, but they contribute to the regulation of cellular homeostasis. Matricellular proteins bind to many cell-surface receptors, the ECM, growth factors, cytokines, and proteases. They also modulate cell–matrix interactions. These structurally diverse proteins include thrombospondins (TSPs) 1 and 2, the tenascins, SPARC (secreted protein, acidic and rich in cysteine or osteonectin), and osteopontin (Opn; Bornstein and Sage, 2002). In contrast to ECM proteins that generally foster strong cell adhesion, matricellular proteins induce a state of intermediate adhesion that promotes cell motility and that may support cell survival and cell differentiation (Murphy-Ullrich, 2001). The state of intermediate adhesion is characterized by disruption of focal adhesions and reorganization of actin stress fibers. In general, the ability of matricellular proteins to modulate cell adhesion and cytoskeletal organization suggests that they play an important role in developmental and homeostatic processes.

A phosphorylated acidic glycoprotein, Opn can engage several receptors, including members of the integrin receptor family (e.g., $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_4\beta_1$, $\alpha_8\beta_1$ and $\alpha_9\beta_1$) and variants of CD44, the hyaluronic acid receptor. Some Opn interactions involve an RGD (Arg-Gly-Asp) sequence that is also found in various ECM proteins and that binds directly to many integrins (Denhardt et al., 2001). In apparent contradiction to the de-adhesive properties of most matricellular proteins, Opn promotes adhesion of diverse cells to the ECM; however, Opn's interaction with its receptors induces an intermediate state of adhesion that, thus, enables migration, stimulates specific cellular processes, or does both (Bornstein and Sage, 2002). Although osteopontin has been implicated in a broad range of homeostatic (bone remodeling, tissue debridement) and pathologic (cellular immunity, wound healing, cancer metastasis) events, the precise molecular mechanisms underlying its function remain largely unknown (Liaw et al., 1998; Denhardt et al., 2001; Mazzali et al., 2002; Gravallese, 2003; Rittling and Chambers, 2004).

Gu et al. (2004) previously reported that in early mouse embryos (embryonic day [E] 10.5) *Opn* transcripts were expressed in the pancreatic epithelium. The expression of Opn in developing pancreatic tissues of mice suggests a possible involvement of this protein's function in some aspects of pancreas organogenesis. Therefore, in this study, we sought to characterize in detail the expression of *Opn* mRNA and Opn proteins throughout mouse pancreas ontogeny and to determine whether the function of Opn is necessary to pancreas organogenesis by analyzing the pancreatic tissues of Opn-deficient mice.

RESULTS

Dynamic Expression of Osteopontin During Mouse Pancreas Ontogeny

To determine the temporal and spatial pattern of Opn expression during mouse pancreas ontogeny, we used in situ hybridization and immunohistochemical techniques. Our in situ analyses revealed low levels of *Opn* transcripts in pancreatic tissues of embryos as early as E10.5 (data not shown). Two days later, the expression of *Opn* mRNA increased substantially and these transcripts were broadly distributed throughout most of the pancreatic epithelium, with one exception: some large cell clusters located at the periphery of the epithelial tissue were devoid of *Opn* mRNA (Fig. 1A). At E14.5, most pancreatic cells near the epithelial lumen expressed *Opn* mRNA; in contrast, no *Opn* transcripts were detected at the tip of the branches or in cell aggregates on the basolateral side of this tissue (Fig. 1B). After E16.5, *Opn* mRNA's expression became restricted to cells in the ductal portion of the embryonic pancreas (Fig. 1C,D). A similar temporal pattern of expression was also observed for Opn proteins. For example, in the pancreata of embryos isolated at E10.5, we observed Opn immunoreactivity in the cytoplasm of a few cells or in the apical side of cells located toward the lumen of the epithelium (Fig. 1E). From E12.5 until approximately E14.5, the number of pancreatic epithelial cells expressing Opn increased considerably and in the majority of these cells Opn proteins appeared distributed throughout the cytoplasm (Fig. 1F). In contrast, toward the end of gestation (E18.5), all of the pancreatic Opn immunoreactivity appeared deposited on the apical side of ductal epithelial cells (Fig. 1G). In the pancreata of newborn mice we found that the expression of Opn became restricted to ducts and centroacinar cells, and we did not detect these proteins in the endocrine or exocrine compartments (Fig. 1H, and data not shown).

In the pancreas of adult mice, Opn proteins remained expressed in the ductal tissue and they were also detected in the endocrine islets of Langerhans (Fig. 2A). In this organ, low levels of Opn immunoreactivity appeared deposited on the apical side of both intralobular ducts (Fig. 2B) and large ducts (Fig. 2C). In contrast, in pancreatic islet cells Opn proteins were diffusely distributed in the cytoplasm (Fig. 2A,D–F). In most islet cells, Opn proteins colocalized with the β cell markers Pdx1 (Fig. 2D) or insulin (Fig. 2E), although it also coexpressed with the δ cell marker so-

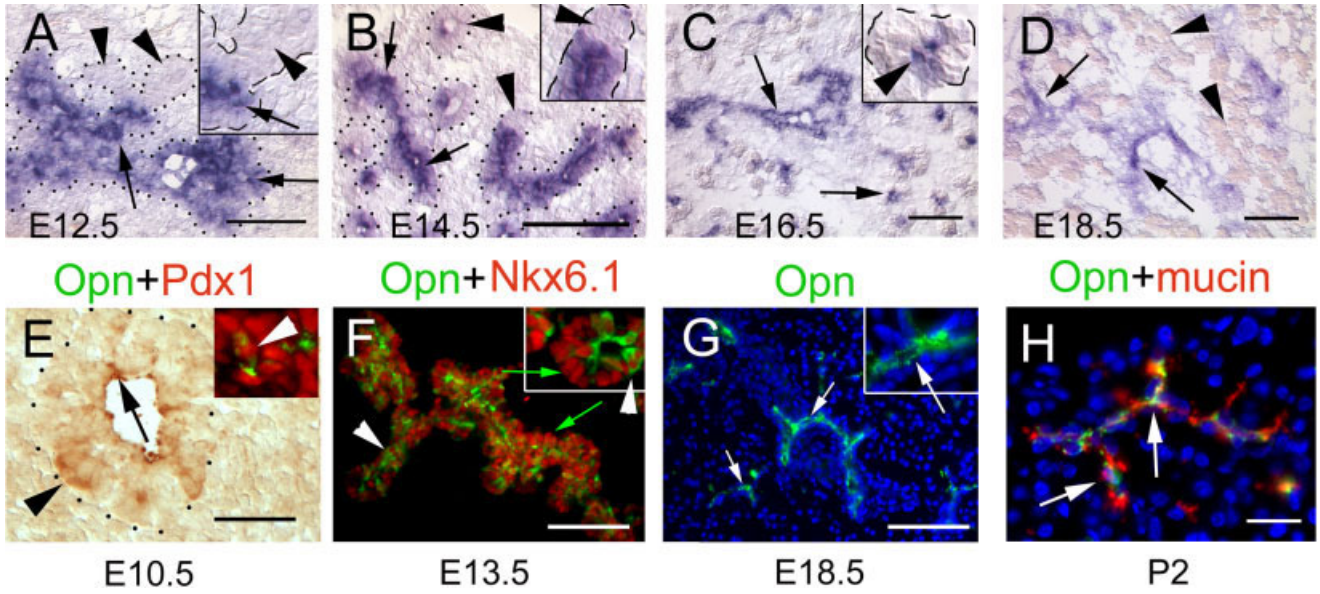

Fig. 1. A–D: Changes in *Opn* mRNA expression between embryonic day (E) 12.5 and E18.5. **A,B:** *Opn* transcripts (arrows in A–D) are broadly distributed in the pancreatic epithelium of embryos dissected between E12.5 (A) and E14.5 (B), with one exception: Clusters of cells on the basolateral side of epithelial tissue of the pancreas or at the tip of the branches (arrowheads and insets in A and B) are devoid of *Opn* mRNAs. **C,D:** After E16.5, *Opn* transcripts become restricted to cells in the lumen of the pancreatic epithelium (arrows in C and D) or to centroacinar cells (arrowhead and inset in C). The developing exocrine acini (arrowheads in D) are devoid of *Opn* expression. E–H: Changes in Opn protein expression between E10.5 and postnatal day (P) 2. In developing pancreata, the expression of osteopontin (Opn) proteins mimics the pattern of *Opn* mRNAs (shown in A–D). **E:** At E10.5, very low levels of Opn proteins are observed in the cytoplasm of some epithelial cells (arrowheads and green fluorescence in inset) or in the lumen (arrow). **F:** At E13.5, a considerably increased population of cells expressing Opn immunoreactivity (green fluorescence and arrowheads) appears dispersed throughout the developing epithelium (labeled with anti-Nkx6.1 antibodies, red fluorescence). In these tissues, most Opn^+ cells express this protein in the cytoplasm (arrowhead in inset), but some epithelial cells are also devoid of Opn immunoreactivity (green arrows and inset). **G:** At E18.5, Opn proteins (green fluorescence) are largely deposited on the apical side of cells of the developing ducts (arrows and inset). **H:** At P2, Opn proteins (green fluorescence) colocalize with the ductal marker mucin (red fluorescence) in small ducts (arrows), in large ducts (not shown) and in centroacinar cells (not shown). Insets in A, B, C, E, F, and G are higher magnification images. In E (inset), the pancreatic epithelium was labeled with anti-Pdx1 antibodies (red fluorescence). In G and H, the cell nuclei were stained with 4′,6-diamidine-2-phenylidole-dihydrochloride (DAPI, blue). Scale bars = 100 μm in A–D,F,G, 200 μm in E, 400 μm in H.

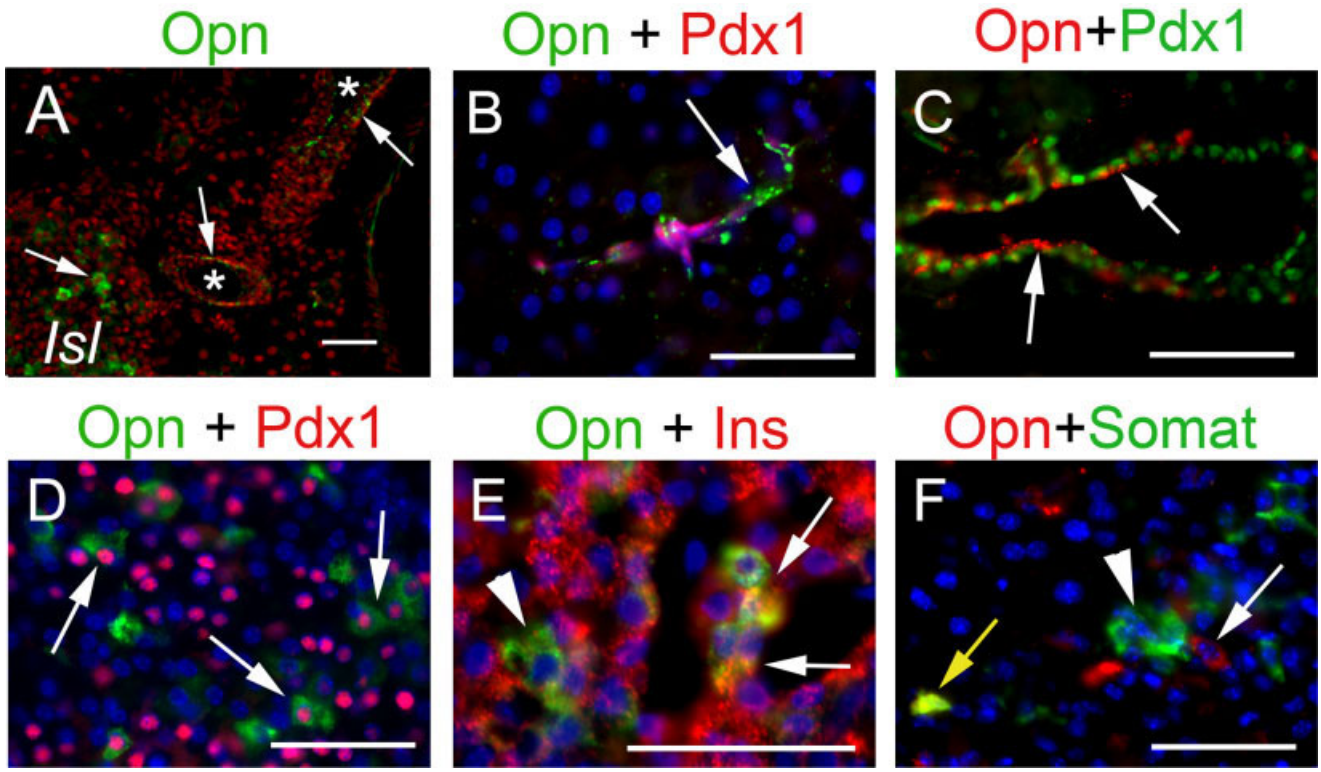

Fig. 2. Osteopontin (Opn) is expressed in adult pancreatic tissues of mice. **A:** Low levels of Opn immunoreactivity (green fluorescence) persist in the pancreatic ductal tissue (asterisk) or in the islets (*Isl*) of Langerhans of adult mice. **B,C:** In small intralobular ducts (B) or in large ducts (C), Opn proteins (arrows; green in B, red in C) colocalize in cells that also express low levels of the transcription factor Pdx1 (red in B or green in C). **D–F:** In adult islets, Opn (green in D and E, red in F) also colocalizes with a subset of $Pdx1^+$ cells (arrows in D). E,F: Some of the Opn^+ islet cells also express insulin (arrows in E) or, occasionally, somatostatin (yellow arrow in F). Arrowheads in E and F indicate Opn^+ cells devoid of insulin (E) or somatostatin (F) expression. In B and C, the signal of Pdx1 expression in the ducts was enhanced to make this protein more visible. Scale bars = 200 μm.

matostatin in a small number of cells (Fig. 2F).

Numerous Pancreatic Cells Express Opn in the Cytoplasm Between E12.5 and E14.5

A detailed analysis of Opn protein distribution in embryonic pancreatic tissue of mice revealed two distinct patterns of expression. During most of pancreas organogenesis (i.e., between E10.5 and postnatal day 2), we noticed deposition of Opn proteins on the apical side of cells located toward the lumen of the epithelium (Figs. 1E–H, 3). However, in the pancreas of embryos dissected between E12.5 and E14.5, we also found that most Opn-immunopositive cells expressed this protein broadly in the cytoplasm (Opn_{cytopl}^{+} cells). Many of these Opn_{cytopl}^{+} cells appeared to be stretching between the luminal and basolateral sides of the epithelium (arrows in Figs. 3A,B,D, 4B,E), often contacting the laminin-rich basal membrane (Fig. 3C), whereas other Opn_{cytopl}^{+} cells seemed to locate only to the basolateral side of this tissue (arrowhead in Fig. 3A).

The Opn_{cytopl}^{+} cell population of the embryonic pancreas is remarkable in another way: most of these cells seemed to represent undifferentiated epithelial precursors. In fact, in the entire pancreas of E13.5 embryos, we found that only a few cells (perhaps no more than four or five epithelial cells) coexpressed Opn_{cytopl} and Ngn3 (a marker of endocrine specification; Fig. 4B), Opn_{cytopl} and p48 (a marker of exocrine specification; Fig. 4D) or Opn_{cytopl} and elastase (an exocrine marker; Fig. 4E), and we never observed colocalization of Opn_{cytopl} with various markers of endocrine differentiation (e.g., Pax6, Islet 1 or glucagon; Fig. 4A,C; data not shown) in the pancreata of embryos isolated between E12.5 and E14.5. In contrast, some Opn_{cytopl}^{+} cells seemed to represent proliferating cells, because they incorporated bromodeoxyuridine (BrdU) after a 1-hr pulse (Fig. 3D). In summary, our analysis of Opn expression throughout mouse pancreas ontogeny revealed a dynamic pattern of this protein's cellular distribution that also suggests a distinctive requirement of Opn function in specific pancreatic cell types of embryos and adults.

Expression of Opn in Other Organs of the Developing Gastrointestinal Tract

Osteopontin exists as an immobilized ECM molecule in mineralized tissues and as a cytokine in body fluids (Denhardt et al., 2001). For instance, in adult humans, OPN proteins can be deposited as a prominent layer at the cell–luminal surface of various epithelia, including those of the gastrointestinal tract, gall bladder, intrahepatic bile ducts, and pancreatic ducts (Brown et al., 1992). In mouse embryos and newborn mice, we found that, in addition to the pancreas, Opn is also expressed in the glandular portion of the stomach and in specific regions of the liver but not in the duodenum (Fig. 5). In the glandular stomach of embryos isolated at E17.5, we observed a prominent expression of *Opn* transcripts in groups of cells at the base of the developing pits (Fig. 5A). In the glandular stomach of newborn mice, we also detected Opn immunoreactivity in mucin-producing cells in the neck/base of the developing gastric units (Fig. 5B) and in groups of cells probably located in the proliferative compartment of the isthmus (Fig. 5C; Falk et al., 1994; Kim et al., 2005).

In the liver of E17.5 embryos or newborns, we detected Opn expression in the developing intrahepatic bile ducts but not in the parenchyma that contains the hepatocytes (Fig. 5D). In the liver of adults the expression of Opn was similar, although the levels of Opn immunoreactivity were substantially lower than those in embryos (data not shown). On the other hand, in the duodenal region of embryos dissected at E18.5, we noticed Opn expression in the terminal region of the pancreatic duct but not in the epithelium of the duodenum (Fig. 5E,F). Overall, the presence of Opn proteins in the epithelium of various digestive organs suggests that this protein's function may be required in the development of these structures, their homeostasis in adult stages, or in both.

Normal Pancreas Development of Opn-deficient Mice

Mice deficient in Opn develop normally, are fertile, and reach adulthood, although there is some evidence of alterations in vascular function, wound healing, and bone resorption in such mice due to the lack of Opn function (Liaw et al., 1998; Yoshitake et al., 1999; Myers et al., 2003). However, to our knowledge, no defects have been reported in the development or the physiology of Opn-deficient pancreatic tissues. Therefore, to determine whether the function of Opn is necessary for pancreas organogenesis, we compared the expression of various morphological or cytodifferentiation markers in pancreatic tissues of wild-type newborn mice to that of Opn-deficient newborn mice.

Our results did not show any obvious morphologic defects in the pancreata dissected from wild-type and Opn-deficient newborns. The architecture of the pancreatic epithelium of wild-type and *Opn*-nullizygous newborn mice were similar. In addition, the lack of Opn function did not seem to affect the generation or spatial organization of exocrine, endocrine, and ductal pancreatic tissues (Fig. 6; data not shown). These results suggest that Opn function is dispensable to pancreas organogenesis or that, in these tissues, other proteins compensate for the lack of Opn activity.

Some candidates that could act redundantly to Opn during organogenesis include other members of the SIBLING (Small Integrin Binding LIgand N-linked Glycoprotein) family of proteins (e.g., bone sialoprotein [Bsp], dentin matrix protein [Dmp] or dentin sialophosphoprotein [Dspp]) (Fisher et al., 2001; Wilson et al., 2005). In developing mice, Opn, Dmp, and Bsp appear to be similarly expressed in specific developing organs (e.g., the testes cords). Thus, it is possible that, during the formation of these structures, the three proteins accomplish analogous functions (Wilson et al., 2005). Another ECM protein (vitronectin [Vn]) could also act redundantly to Opn in developing pancreatic tissues; these two proteins bind to the same integrin receptors, and they are similarly expressed in various em-

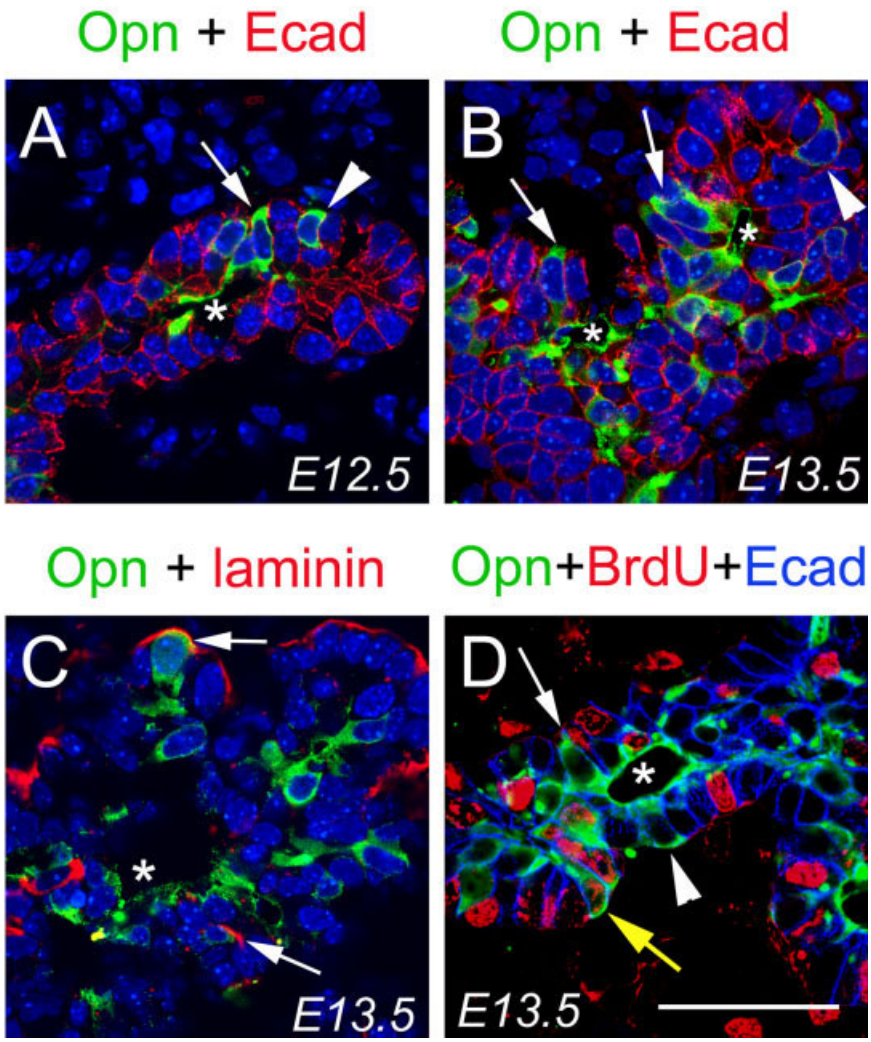

Fig. 3.

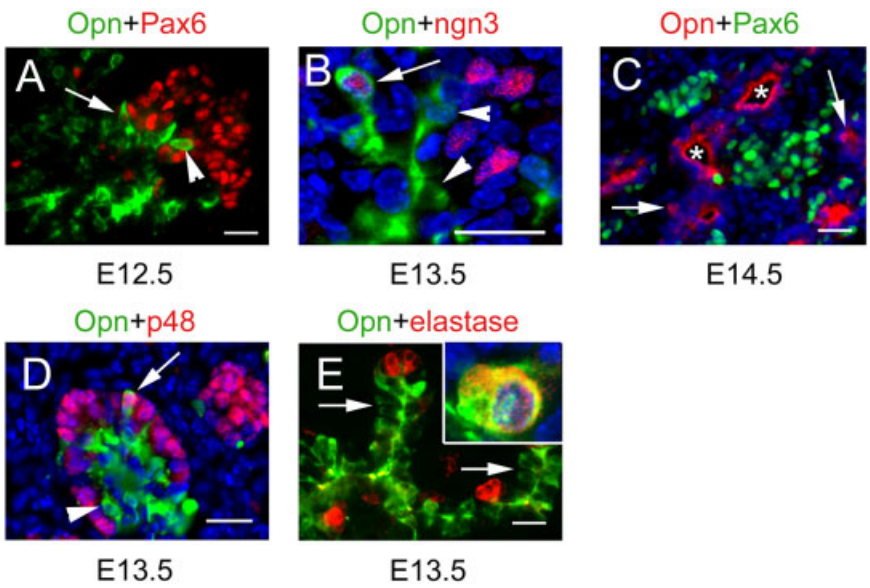

Fig. 4.

bryonic tissues of mice (Liaw et al., 1998). To verify whether Dmp, Bsp, or Vn are also expressed in the developing pancreatic tissue of mice, we performed reverse transcriptase-polymerase chain reaction (RT-PCR) analyses. For these experiments, we used RNA of pancreatic tissue dissected at E14.5, because we previously determined that the number of Opn$^+$ cells in the pancreas is very abundant at this approximate developmental stage (Figs. 1–3). By using this approach, we identified transcription of *Opn* and *Vn* mRNAs, but not of *Dmp* or *Bmp* mRNAs in pancreatic tissues of embryos dissected at E14.5 (Fig. 7A).

Our results showing the presence of *Vn* transcripts in mouse embryonic pancreata are important in light of a study conducted by Cirulli et al.

Fig. 3. In embryonic day (E) 12.5–E13.5 developing pancreata, numerous cells express osteopontin (Opn) proteins in their cytoplasm. **A–D:** Many cells of the E12.5 (A) or E13.5 (B–D) pancreata expressing cytoplasmic Opn (green fluorescence) appear stretching between the luminal side (asterisks) and the basolateral side of the epithelium (arrows in A, B, and D). C: Some of these Opn$^+$ cells (arrows) also appear to be contacting the basal membrane (stained with anti-laminin antibodies, red fluorescence). D: Also, some Opn$^+$ cells (yellow arrow) are proliferating cells that incorporate bromodeoxyuridine (BrdU) after a 1-hr pulse (red fluorescence; arrowhead indicates an Opn$^+$/BrdU-negative cell). In A, B, and D, the pancreatic epithelium was stained with anti-Ecad antibodies (red in A, B; blue in D). In A–C, the cell nuclei were stained with TO-PRO-3. All images were taken with a confocal microscope. Scale bar = 200 μm.

Fig. 4. Most osteopontin-positive (Opn$^+$) cells of embryonic day (E) 12.5–E14.5 pancreatic tissues do not express markers of endocrine or exocrine differentiation. **A,C:** At E12.5 (A) and E14.5 (C), the vast majority of Opn$^+$ cells (arrowheads; green fluorescence in A; red fluorescence in C) do not express the pan-endocrine marker Pax6 (arrow in A points to a unique cell coexpressing Opn and very low levels of Pax6). **B,D,E:** At E13.5, numerous pancreatic epithelial cells express Opn proteins (green fluorescence and arrowheads), but only a few of these Opn$^+$ cells appear to be coexpressing Ngn3 (an endocrine-specification marker; arrow in B), p48 (an exocrine-specification marker; arrow in D), or elastase (inset in E). C: Asterisks denote Opn proteins deposited in the lumen of the pancreatic epithelium. B and inset in E are confocal images. The cell nuclei were stained with TO-PRO-3 (B and inset in E) or with 4′,6-diamidine-2-phenylidole-dihydrochloride (DAPI; C,D). Scale bars = 400 μm.

(2000) in which the authors demonstrated expression of VN in pancreatic epithelial cells of human fetuses. Therefore, by using immunohistochemical analysis, we sought to compare the expression of Opn with that of Vn in pancreatic tissues of E12.5–E14.5 mouse embryos and to determine whether Vn proteins are still expressed in Opn-deficient pancreata. Our results showed that Vn proteins are expressed in the cytoplasm of many pancreatic epithelial cells of E13.5 wild-type mouse embryos (Fig. 7B,E). In addition, in these embryos, we also detected low levels of Vn expression in cells of the mesenchyme surrounding the pancreatic epithelium (arrowheads in Fig. 7B). More important, our results also uncovered that in E13.5 wild-type embryos Vn and Opn proteins colocalize in the cell membrane or in the cytoplasm of pancreatic epithelial cells (Fig. 7D–F). Furthermore, it is also possible that Opn and Vn could act redundantly during pancreas organogenesis to control similar molecular processes, because the lack of Opn did not affect the expression or the distribution of Vn proteins in Opn-deficient embryonic pancreatic tissues (Fig. 7C).

DISCUSSION

Our results showed a distinctive spatial and temporal pattern of Opn expression during mouse pancreas ontogeny. Specifically, in the pancreata of embryos isolated between E12.5 and E14.5, Opn was expressed in numerous undifferentiated cells scattered throughout most of this tissue. Conversely, late in gestation the expression of Opn became restricted to cells of the developing ducts and to centroacinar cells. In the pancreas of adults, Opn was expressed in some islet cells and in the epithelium of small and large ducts. The cellular distribution of Opn proteins also varied during pancreas organogenesis. For instance, in the pancreas of embryos isolated between E12.5 and E14.5, Opn proteins appeared broadly dispersed in the cytoplasm of epithelial cells. Similarly, in islet cells of the adult pancreas, Opn proteins also localized to the cytoplasm. Conversely, in the pancreatic ducts of newborns and adults, the expression of Opn proteins was polarized and it was restricted to the apical side of the epithelium.

The specific distribution of Opn proteins in the cytoplasm of pancreatic epithelial cells is intriguing, because some studies have shown that the interaction of intracellular Opn proteins with specific membrane receptors enables cell migration (e.g., the association of Opn with the CD44 receptor of fibroblasts, macrophages, and osteoclasts facilitates cell motility; Zohar et al., 2000; Denhardt et al., 2001; Suzuki et al., 2002; Fedarko et al., 2004; Zhu et al., 2004). Hence, Opn could favor the motility of pancreatic epithelial cells by means of its interaction with specific intracellular receptors. Opn proteins could also modulate the adhesion/migration properties of pancreatic epithelial cells by means of interacting with integrin receptors. Indeed, integrins are important regulators of pancreas organogenesis, because inhibition of integrin's function alters the migration of endocrine progenitors from the ductal epithelium and it also affects islet morphogenesis (Cirulli et al., 2000). Despite these evidences, in the pancreata of Opn-deficient mice, we did not observe any obvious defects in the architecture or cellular composition of these tissues. Therefore, it is possible that other proteins (e.g., Vn) act redundantly with Opn in developing pancreatic tissues to control certain aspects of this organ's formation or that Opn does not have a major role in the development of pancreatic tissues.

Different studies have shown that most mice lacking the function of a specific matricellular protein have either a grossly normal or a subtle developmental phenotype. However, in many cases these defects became significantly exacerbated when the mutant mice sustained an injury (Bornstein and Sage, 2002). In fact, the study of *Opn*-null mice has revealed important roles for Opn in specific tissues at postnatal stages and in a broad range of homeostatic processes linked by several common themes (e.g., enhanced expression of Opn in response to tissue injury and stimulation of cell motility and cell survival pathways; Liaw et al., 1998; Denhardt et al., 2001; Mazzali et al., 2002; Gravallese, 2003). In addition, in certain human malignancies (including pancreatic adenocarcinoma and gastric carcinoma) OPN proteins were found overexpressed and in some cases the elevated expression of this protein correlated with advanced tumor stages or increased invasiveness of the cancer cells (Coppola et al., 2004; Kolb et al., 2005). OPN was also identified previously as a novel autoantigen of patients with insulin-dependent diabetes mellitus (IDDM), and in this study, we showed expression of Opn proteins in pancreatic islet cells of wild-type adult mice (Fierabracci et al., 2000). Collectively, these results support the proposal that in the pancreas of adults the function of Opn is likely necessary for this organ's repair under pathologic conditions that compromise the integrity or the homeostasis of this tissue (e.g., diabetes, pancreatic cancer, or chronic pancreatitis). In conclusion, our results showed a specific, dynamic profile of Opn expression in pancreatic tissues that suggests the participation of this protein's function in processes involving cell migration, cell–cell interactions, or both, at embryonic stages, and in injury and pathologic responses in the adult.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice were used to analyze the expression of Opn. For timed pregnancies, the day the vaginal plug was discovered was counted as 0.5 days. The *Opn* nullizygous mutant mice were originally obtained from Jackson Laboratory (Bar Harbor, ME). The genotyping of *Opn* mutant mice using tail DNA was performed by PCR using *Neo*-specific forward primer, 5′-GAAGCGGGAAGGGACTGGCT-GCTA-3′ and reverse primer, 5′-CGGGAGCGGCGATACCGTAAAGC-3′, and *Opn*-specific forward primer, 5′-TTCCAAAGAGAGCCAGGAGA-3′ and reverse primer, 5′-TTGGTTACAACGGTGTTTGC-3′. All experimental protocols were approved by the animal care and use committee at St. Jude Children's Research Hospital.

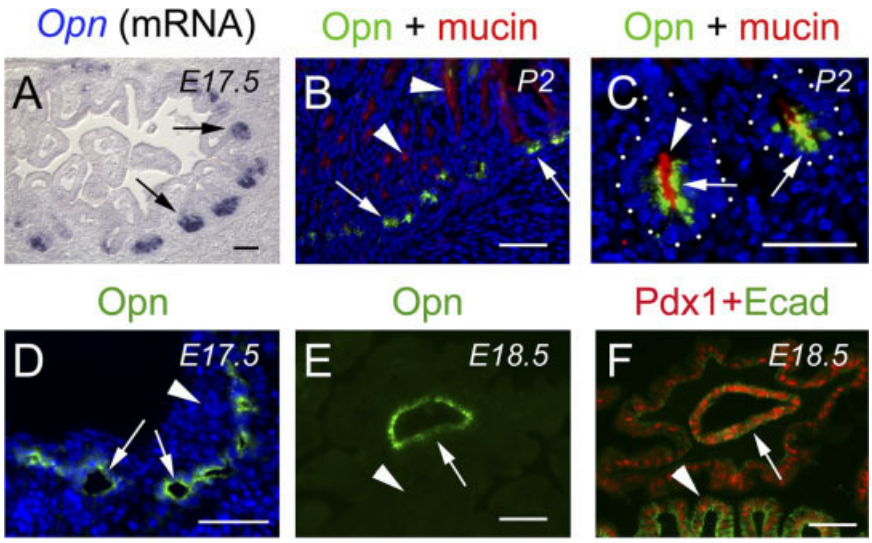

Fig. 5. Osteopontin (Opn) is expressed in the stomach and the hepatobiliary system of mouse embryos or newborn mice. **A–C:** *Opn* transcripts (blue staining in A) or Opn proteins (green fluorescence in B and C) are detected in the glandular portion of the stomach of embryonic day (E) 17.5 embryos (A) or newborns (postnatal day [P] 2; B and C). In this developing organ, the expression of Opn is particularly abundant at the base of the pits (arrows in A and B, dotted outlines in C), and in these areas Opn often colocalizes with mucin (red fluorescence in B and C, arrowhead in C). B: In newborn mice, mucin is also abundantly expressed in the lumen of the stomach (arrowhead). **D:** In the hepatic region of E17.5 embryos, Opn protein expression (green fluorescence) is detected in the developing intrahepatic bile ducts (arrows), but not in the liver parenchyma (arrowhead). **E,F:** In the duodenum of E18.5 embryos, the expression of Opn (green and arrow in E) is detected in the terminal region of the pancreatic duct (visualized in F with anti-Pdx1 antibodies, red and arrow) but not in the epithelia of the duodenum (green folds and arrowhead in F). E and F are adjacent sections. In B–D, the nuclei were stained with 4′,6-diamidine-2-phenylidole-dihydrochloride (DAPI). In F, the epithelium is stained with Ecad antibodies (green fluorescence). Scale bars = 100 μm.

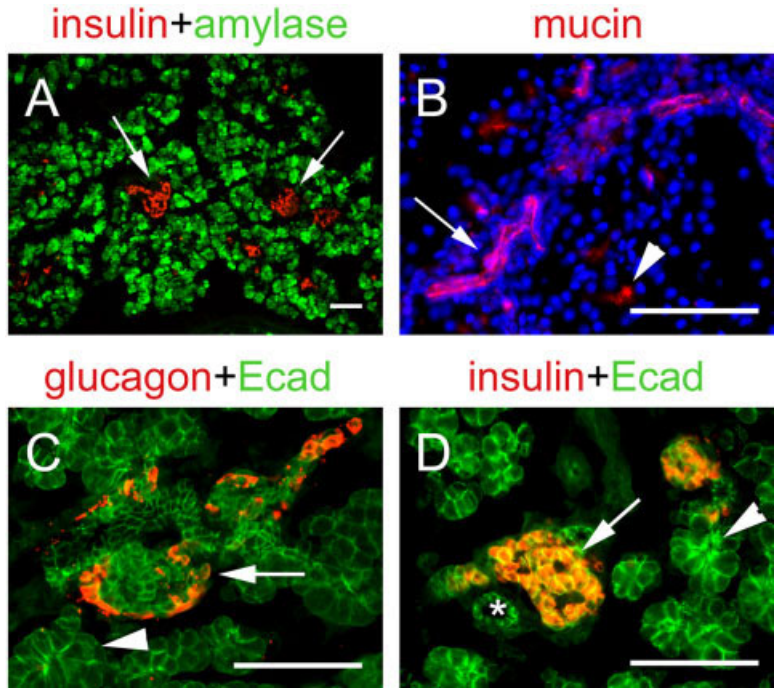

Fig. 6. The loss of osteopontin (Opn) function does not seem to affect pancreas organogenesis. **A:** The pancreata of Opn-deficient newborn mice have a normal distribution of exocrine (green fluorescence) and endocrine (red fluorescence and arrows) tissues. **B:** Such pancreata also have a normal expression of mucin (red fluorescence) in large ducts (arrow) or in centroacinar cells (arrowhead). **C,D:** In addition, in these mutant tissues, the expression of hormones (e.g., glucagon, arrow in C, or insulin, arrow in D) and the architecture of islets (arrows in C and D) or acini (arrowheads in C and D) also seem normal. In B, nuclei were stained with 4′,6-diamidine-2-phenylidole-dihydrochloride (DAPI). In C and D, the epithelia were stained with Ecad antibodies (green fluorescence). Scale bars = 100 μm.

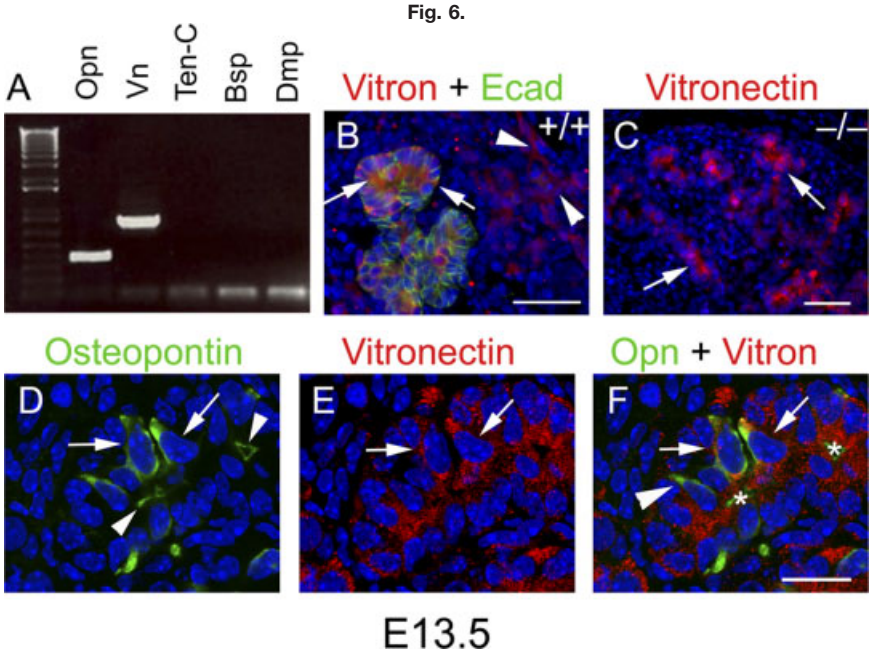

Fig. 7. In developing pancreata, osteopontin (Opn) and vitronectin (Vn) are similarly expressed. **A:** Results of reverse transcriptase-polymerase chain reaction analysis of RNA from embryonic day (E) 14.5 pancreatic tissues show expression of Opn and Vn transcripts but not of those encoding the matricellular protein Tenascin C (*Ten-C*) or the two Small Integrin Binding LIgand N-linked Glycoprotein) (SIBLING) family members *Bsp* or *Dmp*. **B:** In the pancreatic region of E13.5 embryos, expression of Vn proteins (red fluorescence) is detected in the pancreatic epithelium (labeled with green; arrows) and at lower level, in some mesenchymal cells (arrowheads). **C:** Vn (red fluorescence and arrows) is normally expressed in the pancreata of E13.5 Opn-deficient embryos. **D:** At E13.5, expression of Opn proteins (green fluorescence) is detected on the apical side (arrowheads) or in the cytoplasm (arrows) of pancreatic epithelial cells. **E:** In these tissues a large population of epithelial cells also expresses Vn in the cytoplasm (arrows and red fluorescence). **F:** In E13.5 pancreata, many Opn^+ cells coexpress Vn (arrows), although some Opn^+ cells do not (arrowhead). D–F are confocal images of the same section (D and E are separate channels and F shows the overlapped images). Cell nuclei were stained with 4′,6-diamidine-2-phenylidole-dihydrochloride (DAPI; B,C) or with TO-PRO-3 (D–F). Asterisks in F indicate the lumen of the epithelium. Scale bars = 200 μm.

Processing of Embryos and Pancreatic Tissues

Tissues of dissected embryos or 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 overnight at 4°C for cryoprotection, embedded in tissue-freezing medium (Tissue-Tek, Triangle Biomedical Sciences), and cut by a cryostat into sections (8–10 μm for immunohistochemical study and 12 μm for in situ hybridization). Adult mice were perfused manually with cold 4% paraformaldehyde before dissecting their digestive tissues, which were then fixed for 2 more days in 4% paraformaldehyde at 4°C.

Immunohistochemical Analysis

Frozen sections underwent immunohistochemical assays. Primary antibodies were the following: rabbit anti–α-amylase (diluted 1:1,000; Sigma); mouse anti-BrdU (IgG isotype; final concentration, 7 mg/ml; Becton Dickinson); rabbit anti-elastase (1:2,000; AbCam); rabbit anti-glucagon (1:50, Zymed); guinea pig anti-insulin (1:250; DAKO); rabbit anti-mucin (1: 50; Santa Cruz Biotechnology); rabbit anti-laminin (1:2,000; Sigma); guinea pig anti-Ngn3 (1:2,000; provided by M. German); rabbit anti-Nkx6.1 (1:1,000; provided by P. Serup); goat anti-osteopontin (1:25; R&D Systems); rabbit anti-p48 (1:400; provided by H. Edlund); rabbit anti-Pax6 (1:1,000; Covance Research Products); rabbit anti-Pdx1 (1:1,000; provided by C. Wright); rabbit anti-vitronectin (1:100; Chemicon); rabbit anti-somatostatin (1:100; Zymed); and rat anti-uvomorulin/E-cadherin (1:5,000; Sigma). The following secondary antibodies (diluted 1:200) were used for detection: Cy3-conjugated donkey (anti-goat, anti–guinea pig, anti-mouse, anti-rabbit, or anti-rat) IgG from Jackson ImmunoResearch Laboratories, Inc.; Cy5-conjugated donkey anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc); Alexa 488–conjugated goat anti-rabbit IgG (Molecular Probes); Alexa 488–conjugated donkey anti-goat IgG (Molecular Probes). Biotinylated horse anti-goat IgG (Vector Laboratories) was detected by using the VECTASTAIN Elite ABC kit (Vector Laboratories). For nuclear staining, sections were covered with mounting media containing 4′,6-diamidino-2-phenylindole (DAPI) or TO-PRO-3 iodide (carbocyanine monomer), or were counterstained with propidium iodide (Sigma). 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.

In Situ Hybridization

The digoxigenin-labeled *Opn* sense or antisense mRNA probes were transcribed in vitro by using a plasmid containing 300 bp fragment of *Opn* cDNA inserted into the PGEMT-easy vector (Promega). 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 mg/g of body weight) at E11.5 of gestation. Embryos were dissected 1 hr 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 hr.

RT-PCR

Total RNA was isolated from E14.5 pancreata using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The isolated RNA 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: *Bsp*-specific forward primer, 5′-ACACTTACCGAGCTTATGAGG-3′ and reverse primer 5′-TTGCGCAGTTAGCAATAGCAC-3′; *Dmp*-specific forward primer, 5′-TGACAATGACTGTCAGGACGG-3′ and reverse primer 5′-GGCTTTGCTACTGTGGAACCT-3′; *Opn*-specific forward primer, 5′-TTCCAAAGAGAGCCAGGAGA-3′ and reverse primer, 5′-TTGGTTACAACGGTGTTTGC-3′.

ACKNOWLEDGMENTS

We thank L. Bachaboina for assistance with immunostainings and processing of embryos, G. Murti and S. Bhuvanendran for help with confocal microscopy, and M. Carbaugh for editing of the manuscript. We also thank the following people for generously providing reagents: H. Edlund, M. German, P. Serup, and C. V. Wright. This work was supported in part by grant DK60542 (B.S.-P.) from the National Institutes of Health, and by the American Lebanese Syrian Associated Charities (ALSAC).

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Chapter 5

Summary and Discussion

Summary and Discussion

Previously published studies showed that the function of the homeodomain transcription factor Prox1 is crucial for the formation of various organs in mice[1,2]. The studies included in this thesis found that Prox1 is dynamically expressed in the embryonic and adult mouse pancreas[3]. These studies also report that Prox1 is a novel, critical regulator of pancreas development, and that the lack of its activity predisposes mice to pancreatitis.

Prox1 expression in pancreatic tissues

Chapter 2 of my thesis describes the expression of Prox1 in the pancreas of mouse embryos and adults. Prox1 was detected in progenitors of the early developing pancreas, and in pancreatic endocrine, centroacinar, and ductal cells of late gestation embryos, newborns, and adults. In contrast, in developing and mature pancreatic exocrine cells Prox1 expression was not detected. This dynamic pattern of expression suggested that Prox1 controls certain aspects of pancreas development. To explore this possibility, I used two mouse models: *Prox1*$^{-/-}$ mice, carrying *Prox1*-deletion in the germline (Chapter 2), and *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* mice, with *Prox1*-conditional deletion in pancreatic progenitors (Chapter 3).

Role of Prox1 in Early Pancreas Development

The first study of my thesis (Chapter 2), describes the phenotypic alterations in the pancreas of *Prox1*$^{-/-}$ embryos, which survive only until around E14.5 due to malformation of multiple organs. Interestingly, although Prox1 is broadly expressed in the region of the gut endoderm that gives rise to the pancreas and liver, we found that the lack of its activity does not affect pancreas specification. Similarly, our laboratory previously showed that the loss of Prox1 function does not affect liver specification[2] . On the other hand, Prox1 function was found necessary to specify the lymphatic cell fate of mouse venous endothelial precursors, since the lack of its activity switched their differentiation program into that of blood endothelial cells[4]. Moreover, ectopic expression of *Prox1* in primary human blood vascular endothelial cells causes up-regulation of lymphatic endothelial markers and down-regulation of blood vascular endothelial cell markers[4]. Together, these results indicate that Prox1 plays distinct, tissue-specific roles during embryogenesis.

Although pancreas specification occurred normally in the absence of Prox1, we found that the pancreatic buds of *Prox1*$^{-/-}$ embryos were smaller and less branched compared to similar tissues of wild-type littermates (Chapter 2). My studies suggested that those alterations were probably a consequence of insufficient expansion of the pool of pancreatic progenitors due to their premature withdrawal from the cell cycle. Interestingly, our laboratory previously found that Prox1 activity is also necessary to expand the population of liver progenitors (hepatoblasts)[2] . In contrast, the results of other studies suggested that Prox1 normally promotes cell cycle exit in precursors of the retina, lens, or neurons[5, 6, 7] . These opposite roles of Prox1 on cell-cycle regulation provide another example of the tissue-specific functions of this transcription factor.

Role of Prox1 in Endocrine Development

Chapter 2 of my thesis reported that the absence of Prox1 activity altered the differentiation of early pancreatic endocrine precursors by increasing the formation of cholecystokinin (Cck)$^{+}$ cells at the expense of producing glucagon-synthesizing α-cells. Since Cck is a hormone expressed in intestinal but not pancreatic endocrine cells, we hypothesize that Prox1 promotes endocrine differentiation in the early pancreas via preventing the expression of intestinal hormones. This premise is supported by our microarray results showing increased expression of the intestinal peptide Gip in the *Prox1*-deficient E12.5 pancreas.

The loss of Prox1 function also decreased the production of glucagon^{+} cells between E14.5 and P0, during a period recognized as the major wave of pancreatic endocrine cell genesis. In contrast, the formation of insulin-synthesizing β-cells was not affected by the lack of pancreatic Prox1 activity. These results indicated that Prox1 function is required for α-cell development but dispensable or redundant for β-cell development. Unfortunately, *Prox1* was not deleted in all α-cells of *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* pancreatic tissues, and this prevented us from exploring the exact role of Prox1 in those cells. In future studies this caveat might be circumvented via inactivation of Prox1 specifically in α-cells using transgenic mice expressing Cre recombinase under the control of the *glucagon* gene promoter.

We uncovered that although the total islet area was comparable between *Prox1*-deficient and wild-type pancreata, the majority of the *Prox1*-deficient individual islets were smaller than those of wild-type littermates. During pancreas development, islet morphogenesis requires cell migration and cell adhesion events; indeed, the newly formed endocrine cells need to lose their tight association with the ductal epithelium, migrate into the surrounding interstitial matrix and aggregate to form islets[8,9]. Therefore, the change in the islet size without any significant change in the total islet mass suggested that in our *Prox1*-deficient mouse model the adhesion and/or migration of islet cells is defective. Our group previously showed that Prox1 controls the migration of hepatoblasts in the embryonic liver[2]. In the absence of Prox1 activity, the migration of hepatoblasts from the gut epithelium is impaired probably as a result of defective basal membrane turnover and abnormal maintenance of cell-cell contacts mediated by high E-cadherin (Ecad) expression. Our immunohistochemical results did not find any obvious differences in the levels of Ecad expression or in the composition of the basal membrane between wild-type and *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* pancreata. However, our microarray results showed decreased expression of *integrin alpha 6* transcripts in the *Prox1*-deficient newborn pancreas. Integrins are known to participate in both, the migration and organization of endocrine cells into islets[10,11]. It would be interesting to investigate whether integrin alpha 6 is expressed in migrating pancreatic endocrine cells and also, whether *integrin alpha 6*-deficient mice have defective islet cell aggregation. If this will be the case it would suggest direct involvement of integrin alpha 6 downregulation in the observed islet phenotype.

Role of Prox1 in Exocrine Development

The results of Study 1 (Chapter 2) showed that Prox1 is expressed in all progenitors of the early developing pancreas. Based on lineage-tracing results published by others[12], we proposed that the population of Prox1^{+} progenitors include the precursors of the exocrine cell lineage. Study 1 also showed that, once the pancreatic exocrine cells start to differentiate, Prox1 expression rapidly disappears from those cells. Interestingly, we found that the lack of Prox1 activity results in precocious expression of pancreatic exocrine markers. These results suggest that Prox1 expression needs to be downregulated in exocrine progenitors to enable these cells to initiate differentiation.

The premature differentiation of exocrine progenitors reduced the exocrine cell mass of *Prox1*-deficient pancreata transiently (between E14.5-E15.5). However, after E15.5 a recovery of exocrine tissue was noticed and, at birth, we found that the size of the exocrine compartment was comparable between control and *Prox1*-deficient pancreata. Although we did not detect increased proliferation at E15.5 or E17.5 as an explanation for the normalized organ size, it remains possible that the mutant exocrine cell mass recovered via compensatory mechanisms such as increased proliferation, decreased apoptosis, or both after E17.5. These compensatory mechanisms may involve the activity of extrinsic or intrinsic factors that remain to be identified.

We found that some exocrine cells of *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* pancreata appeared slightly underdeveloped and had reduced size at birth. However, these defects recovered shortly thereafter since at later postnatal stages the exocrine cells of *Prox1*-deficient pancreata were indistinguishable from those of wild-type tissues. Presently, we do not know whether this transient developmental delay prevented full maturation or affected the function of the exocrine cells of *Prox1*-deficient pancreata. Moreover, although we could not indentify any obvious alteration in exocrine morphology or exocrine gene expression, all *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* mice showed extensive loss of acinar tissue due to apoptosis after 3 weeks of age. Thus, it is possible that the exocrine cells of *Prox1*-deficient pancreata might not respond adequately to both, increasing nutritional demands and diet changes, as a result of congenital defects that we have not yet identified. It will be important to evaluate additional parameters of exocrine cell maturity and function (including intracellular organization using electron microscopy techniques, or regulated exocytosis) to determine whether the exocrine cells of *Prox1*-deficient pancreata are defective or not.

Role of Prox1 in Ductal Development

We uncovered that the loss of Prox1 function clearly affects pancreatic duct development. Specifically, after E13.5 the lumen of the entire pancreatic ductal tree appeared dilated, and the ductal epithelial cells were taller and more columnar than those of wild-type ducts. Also, E15.5 *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* pancreata had cells at the tips of the branches abnormally co-expressing Sox9 (a progenitor and ductal cell marker) and amylase (an exocrine marker)[13].

At birth, aberrant co-expression of exocrine and ductal markers was observed in cells located at the terminus of small, intercalated ducts in the *Prox1*–deficient pancreas. Also, at both embryonic and postnatal stages the *Prox1*-deficient pancreatic ducts appeared very tortuous and slightly hyperplastic. Finally, our combined results of microarray (Study 2) and immunohistochemical analyses (J.J. Westmoreland and B. Sosa-Pineda, personal communication) uncovered that the expression of the tight junction protein claudin 2 was significantly up-regulated in the *Prox1*-deficient pancreatic ductal epithelium at both, embryonic and postnatal stages. Interestingly, it has been proposed that in liver cells the expression of *claudin2* could be regulated by HNF4α [14], one of those nuclear receptor family members known to physically interact with Prox1. It will be important to investigate whether Prox1 directly or indirectly represses the expression of *claudin2* in pancreatic ductal cells, and whether de-repression of *claudin2* in *Prox1*-deficient pancreata involves the activity of HNF4α or another nuclear receptor family member.

Loss of Prox1 pancreatic function results in mild, acute pancreatitis

Although most *Prox1$^{loxP/loxP}$;Pdx1-Cre* mice survived through adulthood, a few of them became ill and died or had to be euthanized. My analysis of *Prox1$^{loxP/loxP}$;Pdx1-Cre* adult pancreata uncovered various alterations similar to those described in humans or mouse models afflicted with acute pancreatitis (Study 2). More recently, the Sosa-Pineda laboratory determined that abnormal, intra-pancreatic activation of Carboxypeptidase A occurred in the *Prox1*-deficient pancreas (J. Westmoreland and B. Sosa-Pineda, personal communication). The activation of digestive enzymes within pancreatic acinar cells is the major initiating event in the pathogenesis of pancreatitis, as it leads to autodigestion of the gland[15]. Therefore, we conclude that *Prox1$^{loxP/loxP}$;Pdx1-Cre* mice become afflicted with a mild form of acute pancreatitis.

What could be the possible cause of acute pancreatitis in our *Prox1$^{loxP/loxP}$;Pdx1-Cre* mice? Ductal obstruction, caused by gallstones in humans[15] or surgical duct ligation in animal models[16], is a major cause of pancreatitis. In *Prox$^{loxP/loxP}$;Pdx1-Cre* embryos and newborns we discovered important morphologic alterations in the entire pancreatic ductal tree, and it is possible that these defects affected the physiology of the pancreatic ducts. Moreover, the

abnormal, elevated ductal expression of claudin2 could be an important component in the etiology of pancreatitis in *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* mice, because increased expression of this protein was shown to alter epithelial intestinal permeability and was linked to the development of Inflammatory Bowel Disease[17,18]. Therefore, we hypothesize that in *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* mice a defective production and/or transport of pancreatic ductal secretions could lead to intra-pancreatic activation of digestive enzymes and the initiation of events leading to acute pancreatitis. Although we strongly favor this model to explain the cause of pancreatitis in our mutant mice, at present we cannot rule out the contribution of congenital exocrine defects to this pathology.

In summary, the studies included in my thesis uncovered Prox1 as a novel, crucial regulator of pancreas organogenesis. These studies also identified *Opn* as a novel marker of both, pancreatic progenitors and developing pancreatic ductal cells (Chapter 4). I anticipate that the *Prox1*$^{loxP/loxP}$*;Pdx1-Cre* mice generated in this study will be invaluable to identify additional pancreatic ductal markers and to unveil critical cellular and molecular processes involved with pancreas duct development.

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Samenvatting

De pancreas is een ingewikkeld orgaan dat is bestaat uit aparte exocrine en endocrine cellulaire compartimenten. De exocrine pancreas produceert spijsverterings enzymen die via een systeem van vaten naar het duodenum getransporteerd worden waar hun aktivatie helpt voedsel te verteren. De endocrine pancreas bestaat uit de eilandjes van Langerhans die edocrine hormonen aanmaken (insuline, glucagon, somatostatine, alvleesklier eiwitten en ghreline) en uitscheiden in de bloedstroom, wat belangrijk is voor de glucose homeostase.

Ontwikkelings defecten of omstandigheden die de normale orgaan homeostase verstoren kunnen zeer ernstige pancreas aandoeningen veroorzaken zoals pancreatitis, diabetes, cystische fibrose en pancreas kanker. Het onderzoek naar de ontwikkeling van de pancreas staat recentelijk volop in de aandacht omdat het een beter begrip over de moleculaire oorzaak van deze ziekten kan verschaffen en ook om nieuwe behandelings methoden voor hun genezing te ontdekken.

De ontwikkeling van de pancreas wordt gestuurd door verschillende transcriptie- factoren die een cascade van gen-akivatie processen teweegbrengen. De afwijkende homeodomein transcriptiefactor Prox1 wordt geëxpresseerd in verschillende zich ontwikkelende weefsels zoals de lymfevaten, de lens, de retina en de lever, waarbij de funktie van Prox1 cruciaal is voor de normale organogenese. In 2002 rapporteerden Burke en Oliver dat Prox1 in de vermoedelijke pancreas anlage van vroege muize embryos geëxpresseerd wordt. De studies in dit proefschrift stelden zich ten doel om de rol van Prox1 tijdens de ontwikkeling van de muize pancreas te ontrafelen.

Als onderdeel van mijn proefschrift heb ik eerst de expressie van Prox1 tijdens het gehele ontwikkelingsproces van de pancreas gekarakteriseerd (Hoofdstuk 2). Ik vond Prox1 expressie in de voorlopers van de ontwikkelende vroege pancreas, in de pancreas endocrine-, centroacinaire-, en vaat-vormende cellen van late zwangerschaps foetusen, pasgeborenen en volwassenen, maar niet in de exocrine cellen van de volwassen pancreas. Dit dynamische expressie patroon wees erop dat Prox1 bepaalde aspecten van de ontwikkeling van de pancreas reguleert. Door de analyse van Prox1$^{-/-}$ muize pancreata vond

ik dat Prox1 niet de specificatie of de vorming van de pancreas anlage verstoorde, maar wel de groei en morfologie van het ontwikkelende pancreas epitheel en de tijdige differentiatie van de exocrine en endocrine cellen.

Omdat het vrij vroege sterven van Prox1$^{-/-}$ embryos ons ervan weerhield de rol van Prox1 tijdens latere stadia van de pancreas ontwikkeling te kunnen bestuderen, gebruikte ik *Prox1$^{loxP/loxP}$;Pdx1-Cre* muizen om Prox1 conditioneel en specifiek in alle pancreas voorloper cellen te deleteren (Hoofdstuk 3). Door de analyse van de pancreata van deze muizen liet ik zien dat het ontbreken van Prox1 de ontwikkeling van alle drie de weefsel types in de pancreas verstoorde (d.w.z. exocrien, endocrien en vaatweefsel). Om precies te zijn bepaalde ik dat Prox1 nodig is voor de tijdige differentiatie van de exocrine en endocrine voorlopers, de productie van glucagon α-cellen, de morfogenese van de eilandjes van Langerhans en voor de ontwikkeling van het volledige vatenstelsel van de pancreas. Het meest belangrijke was dat deze studies aantoonden dat de *Prox1$^{loxP/loxP}$;Pdx1-Cre* muizen aan een lichte vorm van pancreatitis leden.

Door de studies beschreven in Hoofdstuk 4, identificeerde ik osteopontin (*Opn)* als een nieuwe marker voor zowel pancreas voorloper cellen als vaat-vormende cellen.

Samenvattend lieten de studies in mijn proefschrift zien dat Prox1 een nieuwe en kritieke funktie vervult tijdens de ontwikkeling van de muize pancreas. Ik concludeer ook dat de *Prox1$^{loxP/loxP}$;Pdx1-Cre* muizen, die ontwikkeld werden tijdens deze studie, van nut zullen zijn om de slecht begrepen aspecten van de pancreas organogenese (zoals ontwikkeling van het vaten stelsel) verder te bestuderen of om de cellulaire en moleculaire basis van sommige pancreas ziekten te ontrafelen.

ACKNOWLEDGEMENTS

Working at St Jude Children's Research Hospital has been one of the biggest milestones in my life. In the four and a half years that I have spent in the Department of Genetics and Tumor Cell Biology working on my PhD, I have gained enormous experience both professionally and personally. I am truly happy that I had a chance to meet many wonderful people from all around the world each being an unforgettable piece of my life.

I would like to thank Beatriz, my supervisor, for giving me this great opportunity to work in her lab on my PhD. I am also grateful to her for being supportive during the difficulties I had in my personal life. Many thanks to Gerard for supporting me to start my PhD at Erasmus University, for his guidance during the preparation of my thesis, and most importantly for his encouragements. Thanks also to Guillermo for the suggestions that he made during our lab meetings.

It was a blessing to be surrounded by so many marvelous friends who were always ready to help. I would like to thank all of the Oliver and Sosa-Pineda lab members which I had the opportunity to work with. Thanks to Junfeng for being such a good teacher to me, to Sharla and Jason for helping me go through many difficulties that I had when I first came to the USA, and to Lynda, Qian, Lavanya, Asha, and JJ for their help. I would also like to thank Sathish for answering my endless questions with great patience, Shelly for being such a good friend and for listening and telling me that I will be all right when I was not feeling well, Nikki for the fun we had together and for making me swear that I will never try tequila shots again (!), Miriam for waking up so early to teach me English, and Oleg, Xin, Wei, and Alfonso for helping me whenever I needed. Very special thanks to my dear friends Ayten and Sema. They have been my leaders, the reasons why I am here, and most importantly, they have been my family who was there in my worst days to support me and in my best days to be happy for me. I would like to thank Martijn for being my paranimf and comforting me with his offer to take care of everything on my dissertation day. I would be lost without his help. Many thanks to Chill and Angela for helping me whenever I asked.

Special thanks to Dr. Frank Grosveld for my acceptance to the PhD program at Erasmus University and for being my promoter. I am so grateful to Marike for her endless patience and kindness. I am so appreciative of her answers to my millions of questions and also for being so supportive. Thanks to my committee for reading my thesis and attending my dissertation.

I would like to thank my friends at Northwestern University, Department of Endocrinology for encouraging me.

The success of my PhD would not have been possible without my family. Millions of thanks to my mom, my sisters, my husband, and my parents-in-law for believing in me, for praying for me, and for not letting me give up.

The most difficult times of this period was when I had to sacrifice the moments that I so wanted to be together with my little son, Ege. Thanks my precious baby for bringing such a happiness into my life which overshadowed all the bad and made me stand against difficulties.

I would like to attribute my achievement to my dear dad who sleeps in the deepest of my heart. Dear Dad, no words could be enough to express my appreciation to you. You are my inspiration to be good and to succeed. May heaven be your place where you are in an endless peace. I will love you forever…

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2. **G. Kilic**, C. Sartain, N. Harvey, G. Oliver, B. Sosa-Pineda. “Prox1 is a critical regulator of pancreas organogenesis”. Vanderbilt University Program in Developmental Biology. 10th Annual Scientific Retreat. September 23, 2006, Nashville-TN, USA (ORAL PRESENTATION).

3. **G. Kilic**, C. Sartain, N. Harvey, G. Oliver, B. Sosa-Pineda. “Prox1 is a critical regulator of pancreas organogenesis”. St Jude children`s Research Hospital 2006 Faculty/Postdoctoral Poster Session. November 16, 2006, Memphis-TN, USA (POSTER).

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7. Joby J Westmoreland, **Gamze Kilic**, Jennifer Blain, Beatriz Sosa-Pineda. “Loss of Prox1 activity predisposes mice to pancreatitis”. The first Cold Spring Harbor Meeting on Vertebrate Organogenesis. April 27-May 1, 200. Cold Spring Harbor, NY (POSTER).