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The Onecut transcription factor HNF-6 (OC-1) is required for timely specification of the pancreas and acts upstream of Pdx-1 in the specification cascade

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Abstract

The pancreas derives from cells in the ventral and dorsal foregut endoderm that express the transcription factor Pdx-1. These specified cells give rise to the precursors of the endocrine, ductal, and exocrine pancreatic cells. The identification of transcription factors that regulate the onset of Pdx-1 expression is therefore essential to understand pancreas development. No such factor that acts both in the ventral and in the dorsal endoderm is known. We showed previously that the Onecut transcription factor HNF-6 promotes differentiation of the endocrine cell precursors in which it stimulates expression of the proendocrine gene Ngn-3. By analyzing the phenotype of HNF-6 null mice, we now demonstrate that HNF-6 also controls an earlier step in pancreas development. Indeed, the pancreas of *Hnf6*$^{-/-}$ mice was hypoplastic. This did not result from decreased proliferation or from increased apoptosis, but from retarded pancreatic specification of endodermal cells. The onset of Pdx-1 expression was delayed both in the ventral and in the dorsal endoderm, leading to a reduction in the number of endodermal cells expressing Pdx-1 at the time of pancreatic budding. In normal embryos, HNF-6 was detected in the endoderm prior to the expression of Pdx-1. Moreover, HNF-6 could directly stimulate the *Pdx1* promoter. Our data therefore identify HNF-6 as the first factor known to control Pdx-1 expression both in the ventral and in the dorsal endoderm. We conclude that HNF-6 controls the timing of pancreas specification and that HNF-6 acts upstream of Pdx-1 in this developmental process. Together with the known role of HNF-6 in pancreatic endocrine cell differentiation, our data point to HNF-6 as a key regulator of pancreas development.

Keywords: HNF-6; Pdx-1; Endoderm; Mouse; Onecut; Pancreas; Specification

Introduction

In the mouse embryo, the definitive endoderm becomes regionalized by the end of gastrulation. This early patterning results from interactions with the mesoderm and ectoderm. When development proceeds, reciprocal interactions between the endoderm and the mesoderm refine the anteroposterior patterning of the primitive gut. The expression of transcription factors along the anteroposterior axis of the endoderm then specifies the presumptive territories of endoderm-derived organs, such as the pancreas (reviewed by Wells and Melton, 1999; Grapin-Botton and Melton, 2000; Cleaver and Krieg, 2001; Stainier, 2002). Knowing how the expression of these transcription factors is controlled is crucial to understand how organs develop from the endoderm in normal and pathological conditions. In the present paper, we focus on the regulation of pancreas specification.

The pancreas develops from a dorsal region and a ventral region of the posterior foregut endoderm. The endoderm gives rise to a dorsal bud and to a ventral bud, which fuse to form a single organ. Studies of grafted mouse embryonic tissues (Wessells and Cohen, 1967) showed that pancreas specification occurs around the 8-somite stage, at embryonic day (E) 8. The dorsal foregut endoderm is then in contact with the notochord, while the ventral foregut endoderm fated to form the ventral pancreas and the liver is

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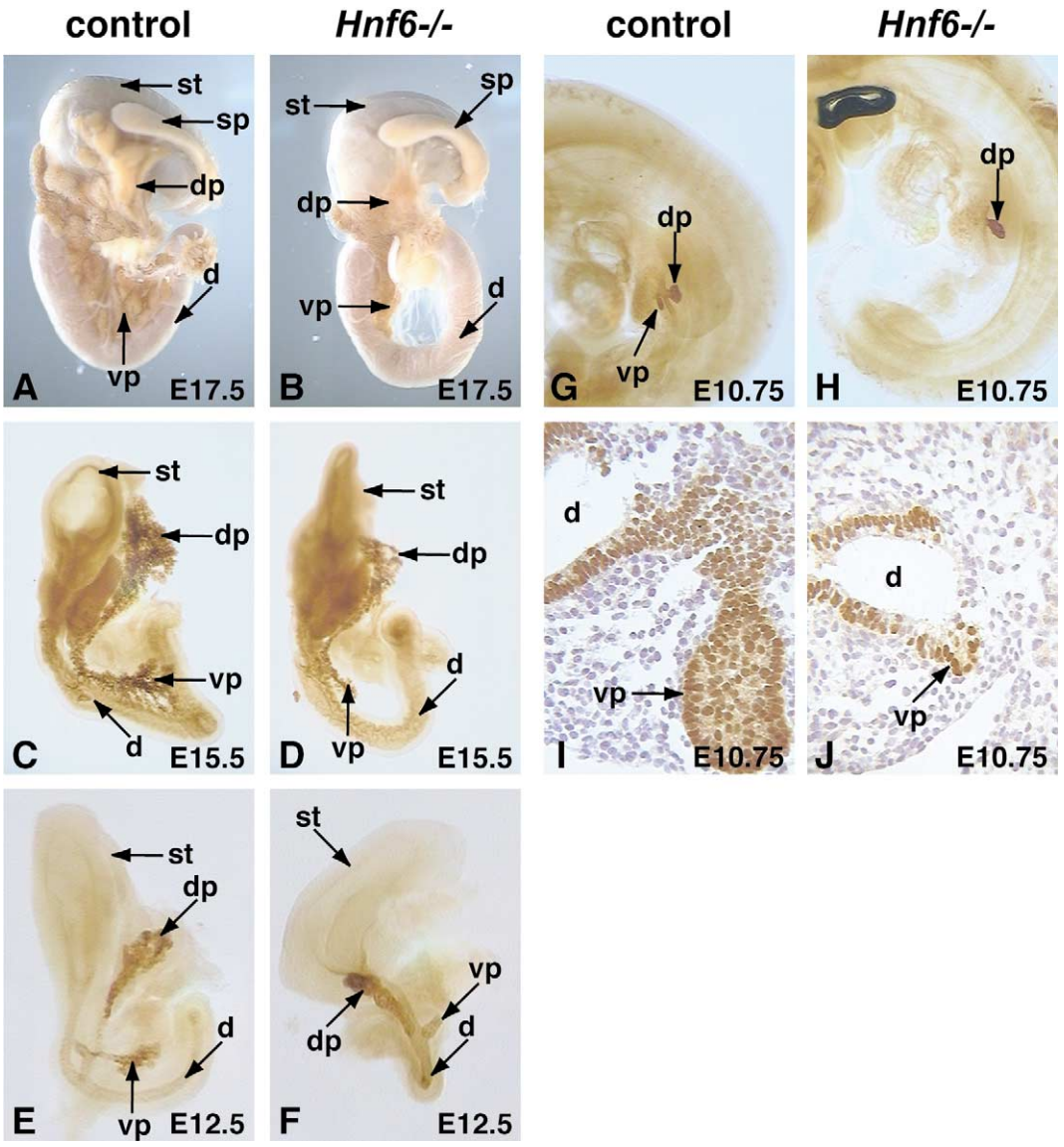

Fig. 1. The pancreas of *Hnf6*$^{-/-}$ embryos is hypoplastic. (A–H) Whole-mount immunohistochemistry experiments were performed on the embryonic days indicated, with an anti-Pdx-1 antibody (brown staining) on digestive tracts dissected from control (A, C, E, and G) or *Hnf6*$^{-/-}$ (B, D, F, and H) embryos. (C–H) were cleared with benzylbenzoate/benzylalcohol, whereas (A) and (B) were not. (I, J) Immunohistochemistry experiments were performed with an anti-Pdx-1 antibody on transverse sections of the ventral pancreatic bud on the day indicated. Pancreatic hypoplasia was striking in *Hnf6*$^{-/-}$ embryos at all the stages examined. At E10.75, the ventral pancreas of *Hnf6*$^{-/-}$ embryos was not visible on whole-mount preparations (H) and was only detected as a small bud on immunostained pancreas sections (J). d, duodenum; dp, dorsal pancreas; st, stomach; sp, spleen; vp, ventral pancreas.

apposed to the cardiac mesoderm. On the dorsal side, signals originating from the notochord, possibly activin-βB and Fibroblast Growth Factor (FGF)-2 (Kim et al., 1997; Hebrok et al., 1998), repress Sonic Hedgehog (Shh) expression in the pancreatic anlage. This repression is essential for pancreas specification (Hebrok et al., 1998; Apelqvist et al., 1997). On the ventral side, the emergence of the pancreas is coordinated with that of the liver (Deutsch et al., 2001). FGF from the cardiac mesoderm induces the local expression of Shh, which represses pancreatic gene expression in the hepatic anlage and induces liver-specific expression. Like for the dorsal pancreas, the ventral pancreas develops only outside the endodermal region that expresses Shh.

The endodermal region committed to a pancreatic fate expresses the transcription factor Pdx-1 (Offield et al., 1996; Ahlgren et al., 1996). In *Pdx1*$^{-/-}$ mouse embryos, the formation of the pancreatic buds occurs, but the subsequent morphogenesis of the pancreas is arrested (Jonsson et al., 1994; Offield et al., 1996; Ahlgren et al., 1996). A role for Pdx-1 in pancreas specification is supported by the observation that ectopic expression of Pdx-1 in chick embryo endoderm causes cells to bud out of the gut epithelium and

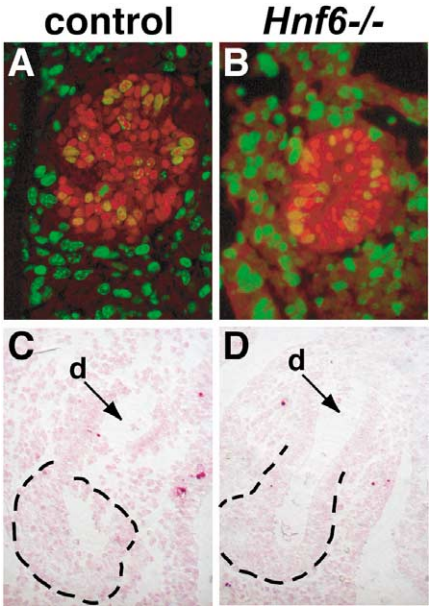

Fig. 2. Pancreatic hypoplasia in *Hnf6*$^{-/-}$ embryos is not due to defective cell proliferation or to excess of apoptosis. (A, B) Immunofluorescence analysis showed cells labeled for Pdx-1 (red), for BrdU (green), or for both (yellow) in the dorsal pancreatic bud of E10.75 control (A) or *Hnf6*$^{-/-}$ (B) embryos. Cell proliferation was the same in control embryos and in *Hnf6*$^{-/-}$ embryos. (C, D) TUNEL assays were performed on E10.75 control (C) or *Hnf6*$^{-/-}$ (D) embryos. Apoptotic cells are labeled in red. The dotted line in (C) and (D) surrounds the dorsal pancreatic bud. Both in control and in *Hnf6*$^{-/-}$ embryos, no apoptotic cells were found in the pancreatic epithelium, whereas but a few were observed in the pancreatic mesenchyme. d, duodenum.

to initiate pancreatic differentiation (Grapin-Botton et al., 2001). In keeping with these observations, lineage-tracing experiments have shown that the three types of pancreatic tissues (exocrine acini, endocrine islets, and ducts) are exclusively derived from Pdx-1-expressing progenitors (Gu et al., 2002).

Another transcription factor that is essential for early pancreas development is Hb9. In the mouse, Hb9 expression is found at the 8-somite stage in the entire dorsal gut endoderm, and in the ventral endoderm at the prospective pancreatic level. In *Hb9*$^{-/-}$ mice, the dorsal region of the gut epithelium fails to express Pdx-1 and to initiate a pancreatic specification program (Li et al., 1999; Harrison et al., 1999). In contrast, the ventral pancreas develops and expresses Pdx-1. This indicates that Hb9 regulates Pdx-1 expression in the dorsal endoderm, so that it is required for specification of the dorsal pancreas, but that transcription factors other than Hb9 stimulate the *Pdx1* gene in the ventral endoderm. The identification of transcription factors that regulate the onset of Pdx-1 expression is therefore essential to understand endoderm patterning and pancreas development.

At later stages of pancreas development, several transcription factors are involved in endocrine and exocrine cell differentiation (Kim and Hebrok, 2001; Kawaguchi et al., 2002; Edlund, 2002; Kim and MacDonald, 2002). The Onecut transcription factor HNF-6 (Onecut 1-Mouse Genome Informatics) (Lemaigre et al., 1996) is one of them. HNF-6 is the prototype of a class of homeoproteins conserved from nematodes to humans (Lannoy et al., 1998). We (Landry et

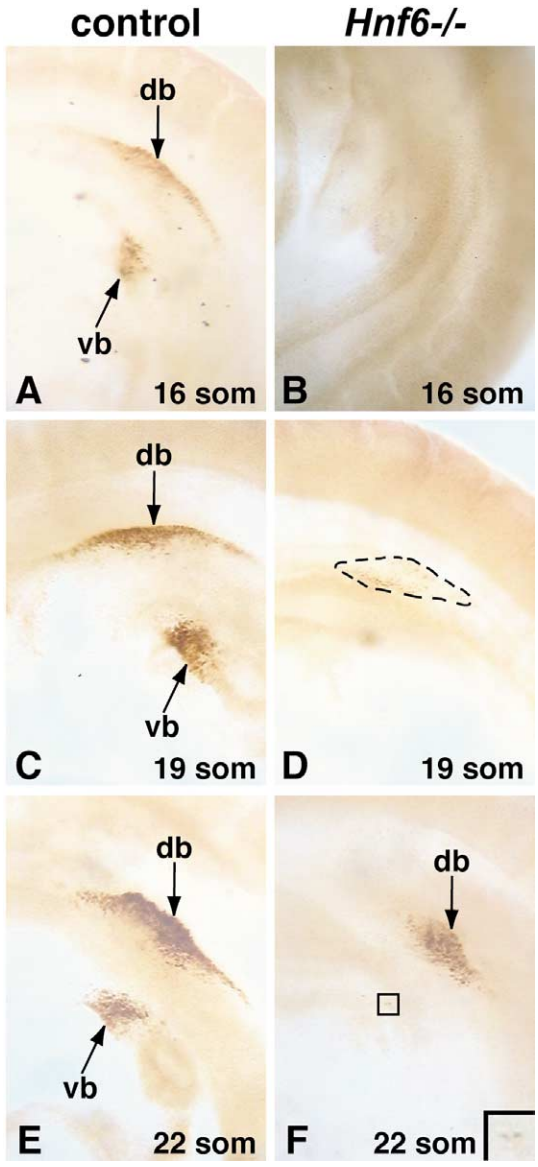

Fig. 3. The onset of Pdx-1 expression is delayed in *Hnf6*$^{-/-}$ mice. (A–F) Whole-mount immunohistochemistry experiments were performed with an anti-Pdx-1 antibody on control (A, C, E) or *Hnf6*$^{-/-}$ (B, D, F) embryos at the stages of development indicated. The anterior part of the embryos is on the left. At 16 somites, *Hnf6*$^{-/-}$ embryos showed no Pdx-1-expressing cell (B), in contrast to the high number of Pdx-1-positive cells present in the ventral and dorsal endoderm of control embryos (A). In *Hnf6*$^{-/-}$ embryos, the first cells that weakly express Pdx-1 (surrounded by the dotted line) were only found at the 19-somite stage in the dorsal endoderm (D), and at the 22-somite stage in the ventral endoderm (F). The inset in (F) is a blow-up of the square that delineates two Pdx-1-positive cells. db, dorsal pancreatic bud; vb, ventral pancreatic bud.

al., 1997) and others (Rausa et al., 1997) have shown that, during early embryonic development, HNF-6 expression is found in the pancreatic buds. At midgestation, HNF-6 is expressed in the protodifferentiated epithelium, while in late gestation, its expression is downregulated in pancreatic endocrine cells. We inactivated the mouse *Hnf6* gene and showed previously that HNF-6 controls pancreatic endocrine cell differentiation at the endocrine precursor stage (Jacquemin et al., 2000). Consistent with this, HNF-6 was shown to stimulate transcription of the proendocrine gene *Ngn3*. Consequently, at birth, the pancreas of *Hnf6*$^{-/-}$ mice contained only a few endocrine cells and the islets of Langerhans were absent. Later on, the number of endocrine cells increased and islets were found in adult *Hnf6*$^{-/-}$ mice. However, the architecture of the islets was perturbed and these mice were diabetic (Jacquemin et al., 2000). In another work using transgenic mice (Gannon et al., 2000). maintenance of HNF-6 expression in endocrine cells after birth led to a similar phenotype, with disruption of islet organization and diabetes. These results showed that HNF-6 plays a role in islet ontogeny and is critical for islet function. In the present paper, we show that HNF-6 is also a critical regulator of the timing of pancreas specification in both the dorsal endoderm and the ventral endoderm, and that HNF-6 acts upstream of Pdx-1 in the specification cascade.

Materials and methods

Generation of Hnf6$^{-/-}$ *mice*

To inactivate the *Hnf6* gene, the proximal promoter region and the first exon were replaced by a neomycine resistance gene (Jacquemin et al., 2000). The phenotype of the *Hnf6*$^{+/-}$ mice was normal. These animals were used as controls together with wild-type littermates.

Immunohistochemistry, in situ hybridization, TUNEL assay, and BrdU detection

Embryos were fixed at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS), incubated in 20% sucrose in PBS at 4°C, embedded in PBS/15% sucrose/7.5% gelatin, and frozen. Sections were cut at 10 μm on a cryostat. Primary antibodies were detected by immunoperoxidase labeling using biotinylated sheep anti-rabbit immunoglobulin G (Roche) and DAB+ (Dako). The following primary antibodies were used: rabbit anti-HNF-6 (gift from R. Costa), affinity-purified rabbit anti-Pdx-1 (gift from H. Edlund, used for immunohistochemistry on whole-mount tissue, see Fig. 1 and 3, and on sections, see Fig. 4), rabbit anti-Pdx-1 (gift from C. Wright, used for experiments described in Fig. 7, and from O. Madsen, used for experiments on sections described in Fig. 1), rabbit anti-Hb9 (gift from J. Kehrl), and rabbit anti-HNF-3β (gift from T. Jessell). Whole-mount immunohistochemistry was performed as described (Ohlsson et al., 1993). Briefly, embryos were fixed in 4% paraformaldehyde in PBS at 4°C. Endogenous peroxidase activity was blocked by immersing the embryos in methanol containing 3% hydrogen peroxide for 1 h. Affinity-purified rabbit anti-Pdx-1 antibody was detected with the Vectastain Elite ABC kit by using DAB (Vector). After each antibody incubation, embryos were washed in TBST (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100) for 5 h with five changes. Nonradioactive in situ hybridization was performed as described (Fode et al., 2000). The *Shh* and *Oc2* antisense RNA probes synthesized, respectively, from the Hh-16.1 plasmid (gift from A. McMahon) and from the pBSmOC2/ISH plasmid (unpublished observations) were labeled with digoxigenin–UTP and detected with the TSA Biotin System (NEN). TUNEL assays on 5-μm paraffin sections were performed by using the in situ cell death detection AP kit from Roche. Apoptotic cells were labeled with Fast Red as substrate of the alkaline phosphatase reaction. BrdU labeling was initiated by intraperitoneal injection (20 μg/g body weight) of pregnant mice 90 min before sacrifice. Embryos were dissected and embedded in paraffin. Double immunofluorescence analysis for Pdx-1/BrdU was performed with rabbit anti-Pdx-1 and mouse anti-BrdU (DSHB) antibodies. The secondary antibodies used were FITC-conjugated anti-mouse (Jackson Laboratory) and Texas Red-conjugated anti-rabbit (Vector) antibodies.

Electrophoretic mobility shift assays (EMSA)

COS-7 cells were transfected with LipofectaminePlus (Gibco-BRL) and 10 μg of the pCMV-HNF-6α expression vector. Twenty-four to 48 h after transfection, the cells were harvested and cell extracts were obtained as described (Jacquemin et al., 2001). Extracts (5 μl) from mock-transfected cells or from cells transfected with the HNF-6 expression vector were added to 15 μl of 10 mM Hepes (pH 7.6), 1 mM dithiothreitol, 1 mM $MgCl_2$, 0.5 mM EDTA, 50 mM KCl, 10% (v/v) glycerol, 4 μg of poly(dI-dC), and ^{32}P-labeled probe (30,000 cpm), and incubated on ice for 20 min. The samples were loaded on a 6% polyacrylamide gel (acrylamide/bisacrylamide ratio of 38:2) in 0.25 × Tris–borate–EDTA buffer and electrophoresed at 200 V for 3 h. For the supershift experiments, the pCMV-MCS vector (Stratagene) containing the rat HNF-6α cDNA cloned downstream and in frame of a Flag-coding sequence was transfected in HEK 293 cells, and cell extracts were obtained as described above. Extracts were incubated with the anti-Flag M2 antibody (0.5 μl) (Sigma) during 15 min prior to probe addition.

Plasmid construction and transfections

A plasmid containing a 9-kb *Xba*I–*Xba*I region of the mouse *Pdx1* gene (gift from C. Wright) was used to obtain

a 4.5-kb *Sma*I–*Sma*I fragment corresponding to the *Pdx1* promoter. This fragment was cloned in pBlueScript (Stratagene). A *Hin*dIII–*Sal*I fragment of pGL3 basic (Promega), containing the luciferase gene and the SV40 polyA signal, was added downstream of the *Pdx1* promoter to generate the pPDX-luc reporter. Rat-1 fibroblasts were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Cells (4×10^4) on 24-well dishes were cotransfected in serum-free DMEM by lipofection using DOTAP (Roche) and 200 ng of pPDX-luc reporter, 150 ng of pECE-HNF6α expression vector, and 30 ng of pRL138 (Lemaigre et al., 1996) coding for *Renilla* luciferase as internal control. After 5 h, the medium was replaced by DMEM plus 10% fetal calf serum, and the cells were incubated for 22 h before measuring luciferase activities using the Dual-Luciferase kit (Promega) and a TD-20/20 luminometer (Promega). Luciferase activities were expressed as the ratio of reporter activity (firefly luciferase) to internal control activity (*Renilla* luciferase).

Results

The pancreas of Hnf6$^{-/-}$ mice is hypoplastic

HNF-6 is expressed in the pancreas during embryogenesis (Landry et al., 1997; Rausa et al., 1997). To evaluate the role of HNF-6 in pancreas development, we inactivated the *Hnf6* gene in the mouse by homologous recombination. Analysis of newborn *Hnf6*$^{-/-}$ mice revealed two distinct pancreatic anomalies. The number of endocrine cells was severely reduced (Jacquemin et al., 2000) and the pancreas was hypoplastic (this paper). This pancreatic hypoplasia persisted in adult *Hnf6*$^{-/-}$ mice. We previously analyzed the endocrine defect and showed that it results from impaired differentiation of the pancreatic endocrine cell precursors (Jacquemin et al., 2000). Since endocrine cells account for less than 10% of the total pancreatic mass (Slack, 1995), the pancreatic hypoplasia in *Hnf6*$^{-/-}$ mice cannot result exclusively from the endocrine defect. In the present paper, we investigate the mechanism of this pancreatic hypoplasia.

We first determined the time at which hypoplasia occurs in the *Hnf6*$^{-/-}$ embryos. We had shown previously that their pancreatic cells express normal levels of Pdx-1 from embryonic day (E) 10.75 to E17.5 (Jacquemin et al., 2000). To visualize the pancreas, we therefore performed whole-mount immunohistochemistry with an anti-Pdx-1 antibody on digestive tracts dissected from control or *Hnf6*$^{-/-}$ embryos. At E17.5, the pancreas was much smaller in *Hnf6*$^{-/-}$ than in control embryos (Fig. 1A and B).Similar observations were made at E15.5 (Fig. 1C and D). At E12.5, before fusion of the pancreatic buds, these buds were smaller and less lobulated in *Hnf6*$^{-/-}$ embryos than in control embryos (Fig. 1E and F). At the 37-somite stage (approximately E10.75), the dorsal and ventral buds could be identified by Pdx-1 labeling of whole-mount tissue (Fig. 1G) in control embryos. In contrast, the ventral pancreas was not visible in *Hnf6*$^{-/-}$ embryos (Fig. 1H). It was only on immunostained pancreas sections that a small ventral bud could be detected in these embryos (Fig. 1J), while the ventral bud of the control embryos was well-developed (Fig. 1I). As to the dorsal bud, it was present in *Hnf6*$^{-/-}$ embryos (Fig. 1H), but it was more elongated and flatter than in the control embryos. To determine whether the dorsal pancreatic bud of *Hnf6*$^{-/-}$ embryos contains less epithelial cells than that of control embryos, we sectioned three control and three *Hnf6*$^{-/-}$ embryos at the 33/34-somite stage (approximately E10.5). Every other section was stained by immunohistochemistry with an anti-Pdx-1-antibody (10–15 sections for the *Hnf6*$^{-/-}$ embryos, and 10–23 sections for the control embryos), and the number of Pdx-1-expressing cells was counted in the dorsal bud. We found that the number of cells in *Hnf6*$^{-/-}$ embryos was about half of that in control embryos (Table 1). We concluded that the absence of HNF-6 leads to pancreatic hypoplasia and that this defect is observed as early as E10.5.

Table 1
Cell count and proliferation index in the pancreas of E10.5 embryos

Control			*Hnf6*$^{-/-}$		
Stage (somites)	Number of cells	Proliferation index	Stage (somites)	Number of cells	Proliferation index
33	1613	18.5	33	749	13.0
33	1223	26.9	33	725	28.3
33	1233	28.6	34	815	20.1

Note. Every other section was used for counting the Pdx-1-expressing cells in the dorsal pancreatic bud. The other sections were used for determining the percentage of BrdU-positive cells in the Pdx-1-positive cell population (proliferation index).

Pdx-1 expression is delayed in Hnf6$^{-/-}$ embryos

We next examined the three possible causes of pancreatic hypoplasia in *Hnf6*$^{-/-}$ embryos, namely defective cell proliferation, excess of apoptosis, or defective pancreatic specification of endodermal cells. The proliferation of the pancreatic epithelium was determined by BrdU incorporation on the remaining sections of the same three control and three *Hnf6*$^{-/-}$ embryos as those used for the cell count experiment (Fig. 2A and B). Every other section was costained by immunofluorescence with anti-Pdx-1 and anti-BrdU antibodies. Results are presented in Table 1. The proliferation index at E10.5 in *Hnf6*$^{-/-}$ embryos was not significantly different from that in control embryos. The same was true at E12.5 (35.6% in *Hnf6*$^{-/-}$ embryos and 35.2% in control embryos). TUNEL assays were performed at E10.75 (Fig. 2C and D) and E12.5 (data not shown) on pancreatic sections from four control embryos and four

Hnf6$^{-/-}$ embryos (two control and two *Hnf6*$^{-/-}$ embryos per stage, two sections per embryo). No apoptotic cells were found in the pancreas of control or *Hnf6*$^{-/-}$ embryos. We concluded that neither defective cell proliferation nor excess of apoptosis accounts for the pancreatic hypoplasia in *Hnf6*$^{-/-}$ embryos.

We were left with the hypothesis that pancreas specification is defective in *Hnf6*$^{-/-}$ embryos. Pdx-1 is the earliest marker of precursor cells of both the ventral and the dorsal pancreas (Ohlsson et al., 1993; Ahlgren et al., 1996). We therefore examined the time course of Pdx-1 expression in control and *Hnf6*$^{-/-}$ embryos, starting from the onset of pancreatic specification of endodermal cells. Control embryos at 16 somites (approximately E9) showed Pdx-1-positive cells in the dorsal and ventral parts of the gut endoderm (Fig. 3A). In contrast, no Pdx-1-expressing cells were observed at that stage in *Hnf6*$^{-/-}$ embryos (Fig. 3B). It was only at the 19-somite stage that a few cells weakly expressing Pdx-1 were found in *Hnf6*$^{-/-}$ embryos, and these cells were confined to the dorsal part of the gut endoderm (Fig. 3D). In control embryos at that stage, the number of Pdx-1-expressing cells had increased as compared with the 16-somite stage (Fig. 3C). At the 22-somite stage (approximately E9.5), control embryos showed numerous Pdx-1-positive cells and typical dorsal and ventral pancreatic buds (Fig. 3E). In *Hnf6*$^{-/-}$ embryos, Pdx-1-expressing cells were present in the dorsal region of the gut endoderm, but their number was much lower than in control embryos (Fig. 3F). Only two Pdx-1-expressing cells were detected in the ventral part of the endoderm (inset in Fig. 3F). These experiments showed that the absence of HNF-6 delays the expression of Pdx-1 from the 11-somite stage to the 19-somite stage in the dorsal endoderm and from the 8-somite stage to the 22-somite stage in the ventral endoderm. This delay reduces the number of Pdx-1-expressing cells at the time when the pancreas buds out of the endoderm.

HNF-6 and Pdx-1 are coexpressed in the endoderm

To approach the mechanism by which HNF-6 controls Pdx-1 expression, we first determined whether these two transcription factors are coexpressed in the endoderm. Adjacent sections of the developing gut were analyzed by immunohistochemistry (Fig. 4). Sections at the 8-somite stage show the dorsal endoderm and the ventral endoderm. The latter includes the prospective hepatic bud, in contact with the cardiac mesoderm, and a lip which extends away from the cardiac mesoderm and which will give rise to the ventral pancreas. HNF-6 expression was detected in the dorsal endoderm, as well as in the prospective hepatic bud and in the endodermal lip (Fig. 4A). At that stage, no other region of the endoderm showed a specific nuclear staining for HNF-6. Pdx-1 expression was detected at the 8-somite stage in a few cells of the endodermal lip (Fig. 4B). These

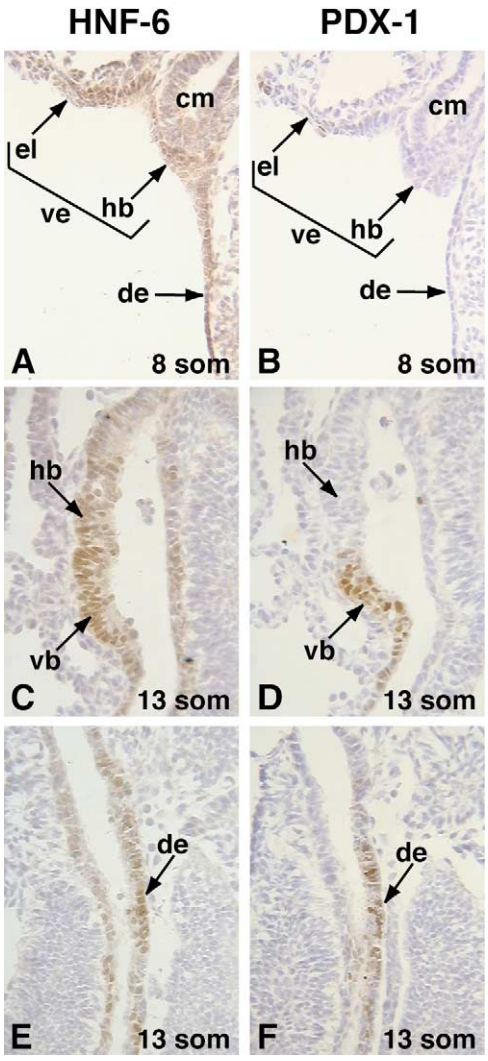

Fig. 4. The expression of HNF-6 in the gut endoderm precedes that of Pdx-1. Immunohistochemistry experiments were performed with anti-HNF-6 (A, C, E) or anti-Pdx-1 (B, D, F) antibodies on sagittal sections of control embryos at the stages of development indicated. HNF-6 and Pdx-1 staining was performed on adjacent sections. The anterior part of the embryos is up. At 8 somites, the endoderm expressed HNF-6, both in the cells of its dorsal part and in the cells of the endodermal lip which will give rise to the ventral bud (A). Some cells of this lip expressed also Pdx-1 (B). At 13 somites, the ventral and dorsal endoderm showed Pdx-1-expressing cells at locations wherefrom the ventral and dorsal pancreas will bud (D, F). HNF-6 was also expressed by these cells (C, E). HNF-6 expression was also detected in adjacent cells, so that its expression domain in the endoderm was larger than that of Pdx-1. cm, cardiac mesoderm; de, dorsal endoderm; el, endodermal lip; hb, prospective hepatic bud; ve, ventral endoderm; vb, ventral pancreatic bud.

cells, which correspond to the first cells that express Pdx-1 in the embryo, will give rise to the ventral pancreatic bud. No Pdx-1-expressing cells were found in the dorsal endoderm (Fig. 4B). This is consistent with the notion that Pdx-1 expression in the ventral endoderm precedes that in the dorsal endoderm, where it appears between the 10- and the 12-somite stages (Li et al., 1999). At the 13-somite

stage, HNF-6 expression was observed in the ventral endoderm at locations wherefrom the liver and the ventral pancreas are budding (Fig. 4C), as well as in the dorsal endoderm (Fig. 4E). At that stage, Pdx-1 was also expressed in the ventral and dorsal endoderm (Fig. 4D and F), but its domain of expression was smaller than that of HNF-6. We concluded that Pdx-1-expressing cells in the prospective pancreas also express HNF-6, and that expression of HNF-6 in the endoderm precedes that of Pdx-1.

The expression of Hb9, HNF-3β, and Shh is normal in Hnf6$^{-/-}$ embryos

The data reported above showed that HNF-6 controls the timing of Pdx-1 expression and that HNF-6 and Pdx-1 are coexpressed in the endoderm. This suggested that HNF-6 could control directly the expression of Pdx-1 in the endoderm but did not rule out that HNF-6 controls Pdx-1 expression via an indirect mechanism. To address the latter possibility, we determined whether Hb9 (Li et al., 1999; Harrison et al., 1999) and Shh (Hebrok et al., 1998; Apelqvist et al., 1997), which are known regulators of Pdx-1 expression in the endoderm, are correctly expressed in *Hnf6*$^{-/-}$ embryos. We also tested the expression of HNF-3β, which controls expression of genes in the primitive gut (reviewed by Zaret, 1999).

Shh has been reported to repress Pdx-1 expression and to exert an inhibitory action on pancreas development (Hebrok et al., 1998; Apelqvist et al., 1997). One possibility was that the lack of Pdx-1 expression before the 19-somite stage in *Hnf6*$^{-/-}$ embryos results from inappropriate expression of Shh in the region of the gut where Pdx-1 is normally present. To test this hypothesis, we performed in situ hybridization experiments with a Shh probe on sections of control and *Hnf6*$^{-/-}$ embryos at 12–13 somites (Fig. 5A and B). Dorsal endoderm at the prospective pancreas level was located by the presence of the notochord, which expresses Shh, and by staining adjacent sections with anti-Pdx-1 or anti-Hb9 antibodies. As expected, Shh was not expressed in the dorsal endoderm of control embryos (Fig. 5A). Likewise, no Shh expression was observed in the dorsal endoderm of *Hnf6*$^{-/-}$ embryos (Fig. 5B). Similar observations were made in the ventral endoderm of these control or *Hnf6*$^{-/-}$ embryos (data not shown).

Expression of the transcription factors Hb9 and HNF-3β was tested by immunohistochemistry. In control embryos at 13–15 somites, cells at the prospective pancreas level in the ventral endoderm and in the dorsal endoderm expressed Hb9 and HNF-3β (Fig. 5C and E), as expected. In *Hnf6*$^{-/-}$ embryos, the expression of Hb9 and of HNF-3β was normal in the ventral endoderm and in the dorsal endoderm (Fig. 5D and F).

These observations indicated that Shh is correctly repressed and that Hb9 and HNF-3β are correctly expressed in *Hnf6*$^{-/-}$ embryos. We therefore excluded that misexpres-

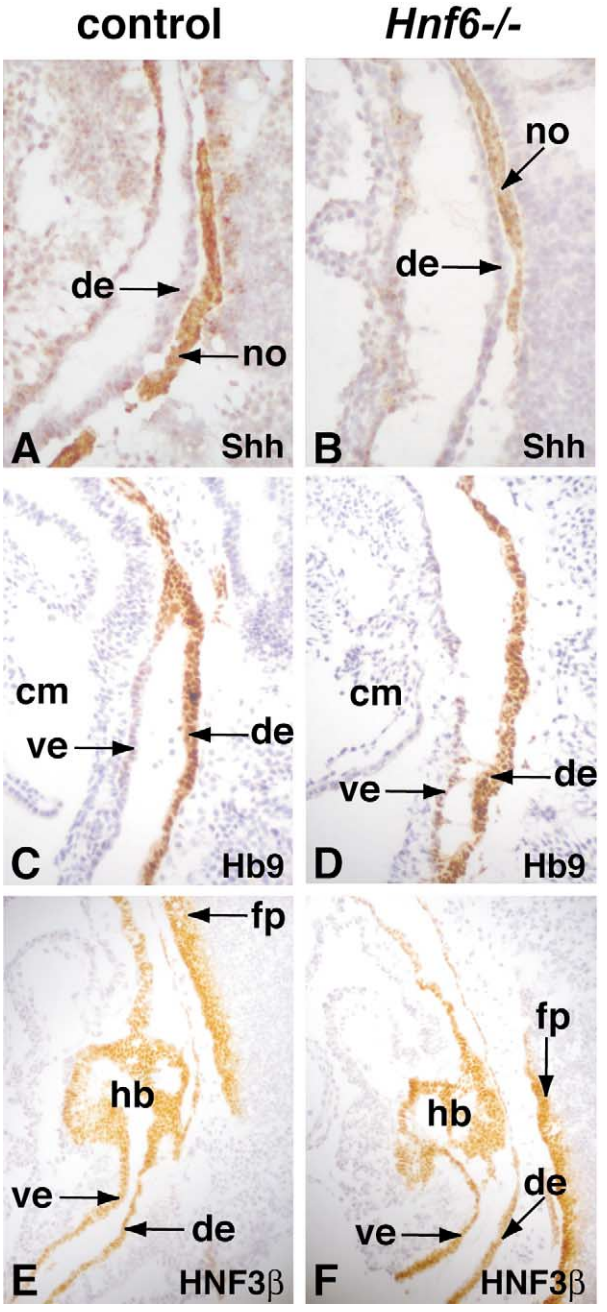

Fig. 5. The expression of Shh, Hb9, and HNF-3β is normal in *Hnf6*$^{-/-}$ embryos. (A, B) In situ hybridization experiments were performed with a digoxigenin-labeled Shh probe on sagittal sections of 13-somite control (A) and 12-somite *Hnf6*$^{-/-}$ (B) embryos. As expected, Shh expression was present in the notochord and no Shh expression was found in the dorsal endoderm of control or *Hnf6*$^{-/-}$ embryos. (C–F) Immunohistochemistry experiments were performed with anti-Hb9 at the 13-somite stage (C, D) or anti-HNF-3β at the 15-somite stage (E, F) antibodies on sagittal sections of control (C, E) or *Hnf6*$^{-/-}$ (D, F) embryos. The pattern of expression of Hb9 and of HNF3β in the endoderm of *Hnf6*$^{-/-}$ embryos was similar to that in the endoderm of control embryos. The region labeled by Hb9 in the ventral endoderm corresponds to the prospective ventral pancreatic bud. The anterior part of the embryos is up. cm, cardiac mesoderm; de, dorsal endoderm; fp, floor plate; hb, hepatic bud; no, notochord; ve, ventral endoderm.

sion of Pdx-1 regulators contributes to the lack of initial expression of Pdx-1 in the endoderm of *Hnf6*$^{-/-}$ embryos.

HNF-6 controls the Pdx1 promoter

The data reported above suggested that HNF-6 controls directly Pdx-1 expression. To test this, we determined whether HNF-6 can bind to the *Pdx1* gene promoter and stimulate its transcriptional activity. Studies on transgenic mice showed that the *cis*-acting elements of the mouse *Pdx1* gene required for its embryonic and adult expression are contained within 4.5 kb of 5′ flanking sequence (Sharma et al., 1996; Wu et al., 1997; Stoffers et al., 1999; Gerrish et al., 2000; Gannon et al., 2001). We therefore searched for potential HNF-6 sites in this region and tested the binding of HNF-6 to the candidate *cis*-acting sequences by EMSA. Three sequences, located from −4205 to −4212 bp (distal site, PDX-D), from −2484 to −2491 bp (middle site, PDX-M), and from −1769 to −1776 bp (proximal site, PDX-P) upstream of the transcription initiation site, matched the HNF-6 consensus (Lannoy et al., 1998). In EMSA, we observed a protein/DNA complex when a probe corresponding to the distal sequence was incubated with extracts of COS-7 cells transfected with an HNF-6 expression vector. This complex corresponded to the binding of HNF-6, as a supershift was observed when an antibody was added in the binding assay (Fig. 6A). Similar HNF-6/DNA complexes were observed with probes corresponding to the middle site or to the proximal site (Fig. 6A). When mutations were introduced in the probes to destroy the HNF-6 binding consensus, these HNF-6/DNA complexes were no longer observed (.Fig. 6A), confirming that the binding of HNF-6 to these *Pdx1* sequences is specific.

We then studied whether HNF-6 transactivates the *Pdx1* gene promoter. The 4.5-kb *Pdx1* promoter sequence was cloned upstream of a luciferase reporter gene to generate pPDX-luc. When pPDX-luc was cotransfected in Rat-1 cells with an HNF-6 expression vector, a 4.5-fold increase in *Pdx1* promoter activity was observed (Fig. 6B), showing that HNF-6 can stimulate the *Pdx1* promoter. We concluded from this set of experiments that HNF-6 can bind to the *Pdx1* promoter and stimulate its activity.

The Onecut transcription factor OC-2 is expressed in the pancreas of Hnf6$^{-/-}$ *embryos*

We have shown above that the control exerted by HNF-6 on the *Pdx1* gene is required only during the initial stages of Pdx-1 expression. This suggested that another factor takes over the role of HNF-6 in wild-type embryos or eventually replaces HNF-6 in *Hnf6*$^{-/-}$ embryos. A candidate was OC-2 (Jacquemin et al., 1999), another member of the Onecut class which displays DNA-binding and transcriptional activation properties similar to those of HNF-6 (Jacquemin et al., 1999).

To test this hypothesis, we performed in situ hybridization experiments with a mouse *Oc2* probe on sections of control or *Hnf6*$^{-/-}$ embryos at the 23-somite stage (Fig. 7A and B). Dorsal pancreatic buds were identified by Pdx-1 immunostaining on adjacent sections (Fig. 7C and D). As expected, the number of Pdx-1-expressing cells in *Hnf6*$^{-/-}$ embryos (Fig. 7D) was smaller than in control embryos (Fig. 7C). *Oc2* was expressed in the dorsal pancreatic bud of control embryos (Fig. 7A) as well as of *Hnf6*$^{-/-}$ embryos (Fig. 7B). These data suggested that the eventual expression of Pdx-1 in *Hnf6*$^{-/-}$ embryos could be explained by a compensatory role of OC-2.

Discussion

Our previous work on *Hnf6*$^{-/-}$ mice has demonstrated that HNF-6 is required in the pancreas for the differentiation of the Ngn3-expressing endocrine cell precursors (Jacquemin et al., 2000). On the other hand, overexpression studies by Gannon et al. (2000) have suggested that downregulation of HNF-6 in the differentiated endocrine cells during islet ontogeny is required for the normal development of islets and the function of β cells. Here, we show that, in addition to its role in pancreatic endocrine cell differentiation, HNF-6 has another function in pancreas development, namely it controls the timing of pancreas specification.

HNF-6 and pancreas specification

Tissue graft experiments have shown that pancreas specification occurs around the 8-somite stage (Wessels and Cohen, 1967). The expression of Pdx-1 in the endoderm coincides with pancreas specification, as it is first detected around this stage. Pdx-1-expressing cells are confined to the prepancreatic regions of the endoderm, the onset of Pdx-1 expression being detected at the 8-somite stage for the ventral endoderm and at the 10- to 12-somite stage for the dorsal endoderm. Here, we show that, in *Hnf6*$^{-/-}$ embryos, the onset of Pdx-1 expression is delayed and that it starts only at the 22-somite stage in the ventral endoderm and at the 19-somite stage in the dorsal endoderm.

As a consequence of the delay in the onset of Pdx-1 expression, the number of Pdx-1-expressing cells available for the formation of the pancreatic buds is reduced in *Hnf6*$^{-/-}$ embryos, and indeed the ventral bud, in which Pdx-1 expression is more delayed, is more affected than the dorsal bud. This leads to hypoplasia of the buds and ultimately to hypoplasia of the entire pancreas at birth. Irrespective of this delay, one could argue that the amount of endoderm present prior to pancreas specification is smaller in *Hnf6*$^{-/-}$ embryos than in control embryos, and that this also contributes to pancreatic hypoplasia. This is unlikely, as development of the stomach and the duodenum, two

organs that also derive from the endoderm, is normal in *Hnf6*$^{-/-}$ mice (Fig. 1A and B).

HNF-6 is only required for the initial expression of Pdx-1, suggesting that another factor compensates for the absence of HNF-6 in the knockout embryos. The data presented here suggest that OC-2, a paralogue of HNF-6, could play this role. As to the signaling mechanisms that trigger pancreas specification in *Hnf6*$^{-/-}$ embryos, one has to consider that in normal embryos Pdx-1 expression is induced at the 8- to 12-somite stage by signaling from the notochord, which is then adjacent to the gut endoderm (Kim et al., 1997). In *Hnf6*$^{-/-}$ embryos, the onset of Pdx-1 expression cannot be ascribed to notochord signaling, since at the 19-somite stage, the notochord and the gut endoderm are separated from each other by the midline fusion of the dorsal aortas. However, mesenchyme-derived signals could play a role. Indeed, mesenchyme development is not affected in *Hnf6*$^{-/-}$ embryos, as suggested by the normal mesenchymal expression of Isl-1 (Jacquemin et al., 2000).

Pdx-1 is the only transcription factor known to functionally delineate the boundaries of the prospective pancreas in the endoderm at the time of pancreas specification. However, pancreas specification also requires the transcription factor Hb9. The lack of Hb9 expression is associated with the absence of Pdx-1 in the dorsal endoderm and with the failure to develop a dorsal pancreatic bud (Li et al., 1999; Harrison et al., 1999), while on the ventral side, Pdx-1 expression and pancreatic budding still occur. The domain of Hb9 expression in the dorsal endoderm is broader than that of HNF-6, which in turn is broader than that of Pdx-1. Therefore, the simple combination of the expression patterns of HNF-6 and Hb9 does not explain the more restricted expression pattern of Pdx-1. Other factors, or the modulation of the activity of HNF-6 and Hb9 by extrinsic signals, could account for the spatially restricted expression of Pdx-1.

To our knowledge, no regulator of Pdx-1 in the ventral endoderm has been identified. The *Hnf6*$^{-/-}$ embryos showed defective expression of Pdx-1 not only in the dorsal endoderm, but also in the ventral endoderm. Our data therefore identify HNF-6 as the first factor known to control both ventral and dorsal pancreas specification.

HNF-6 controls Pdx-1 expression in the endoderm

During pancreas development, the *Pdx1* gene is expressed in the prepancreatic endoderm and in the pluripotent pancreatic epithelium. At later stages, Pdx-1 is predominantly found in β cells. Using transgenic mice, it was shown that a 4.5-kb sequence upstream of the transcription initiation site of the mouse *Pdx1* gene is sufficient to drive expression of a LacZ reporter gene according to the developmental and adult-specific pattern of Pdx-1 (Wu et al., 1997; Stoffers et al., 1999; Gannon et al., 2001). Comparison of the human, chicken, and mouse genes revealed an area of significant sequence identities from −2800 to −1700. Further sequence analysis allowed to divide this area in three subdomains called, from 5′ to 3′, I, II, and III (Gerrish et al., 2000). A gene fragment encompassing subdomains I and II confers expression of a transgene in the pancreatic endocrine cells, while a fragment spanning subdomain III confers expression at E11.5 throughout the pancreatic epithelium (Gannon et al., 2001). The middle (−2484/−2491) and proximal (−1769/−1776) HNF-6 binding sites described here are located within subdomains I and III, respectively. Moreover, these two sites are conserved from mice to humans in these important *cis*-regulatory elements of the *Pdx1* gene, which points to their physiological significance. The distal HNF-6 site (−4205/−4212) is not conserved.

Several tissue-specific transcription factors are known to control the *Pdx1* gene promoter during differentiation of the β cells (Wu et al., 1997; Marshak et al., 2000; Gerrish et al., 2000, 2001; Ben-Shushan et al., 2001; Shih and Stoffel, 2001; Samaras et al., 2002). Among them, only HNF-3β has been shown to control the expression of genes in the endoderm (reviewed by Zaret, 1999). As to Hb9, no direct interaction of this transcription factor with regulatory sequences of the *Pdx1* gene has been described. In fact, it is likely that Hb9 controls the *Pdx1* gene indirectly, as Hb9 reportedly functions as a transcriptional repressor via its interaction with the corepressor Groucho (Muhr et al., 2001). Here, we show that HNF-6 binds to and transactivates the *Pdx1* promoter. These data, and our finding that the onset of Pdx-1 expression in the embryo depends on HNF-6, suggest that HNF-6 is a direct stimulator of *Pdx1* in the endoderm. HNF-6 could trigger chromatin opening over the *Pdx1* gene through its known interaction with the histone acetyltransferases CBP and pCAF (Lannoy et al., 2000), thereby allowing access of other transcription factors to DNA.

Roles of HNF-6 in pancreas development and differentiation

Previous data have shown that HNF-6 regulates pancreatic endocrine cell differentiation and that HNF-6 is also important for the formation of the islets of Langerhans (Jacquemin et al., 2000; Gannon et al., 2000). The present data demonstrate an additional, earlier role for HNF-6 in pancreas development, namely in the timing of pancreas specification. Distinctly timed roles have also been defined for Pdx-1 and for Hb9, as both are required not only for the specification of the pancreas, but also for endocrine cell differentiation or function (reviewed in Edlund, 2002). Like for Pdx-1 and Hb9, HNF-6 exerts its distinct roles by controlling different target genes. It controls the specification process via Pdx-1 expression and endocrine differentiation via Ngn3 expression. In the absence of HNF-6, pancreas specification is delayed, but pancreatic cells express normal

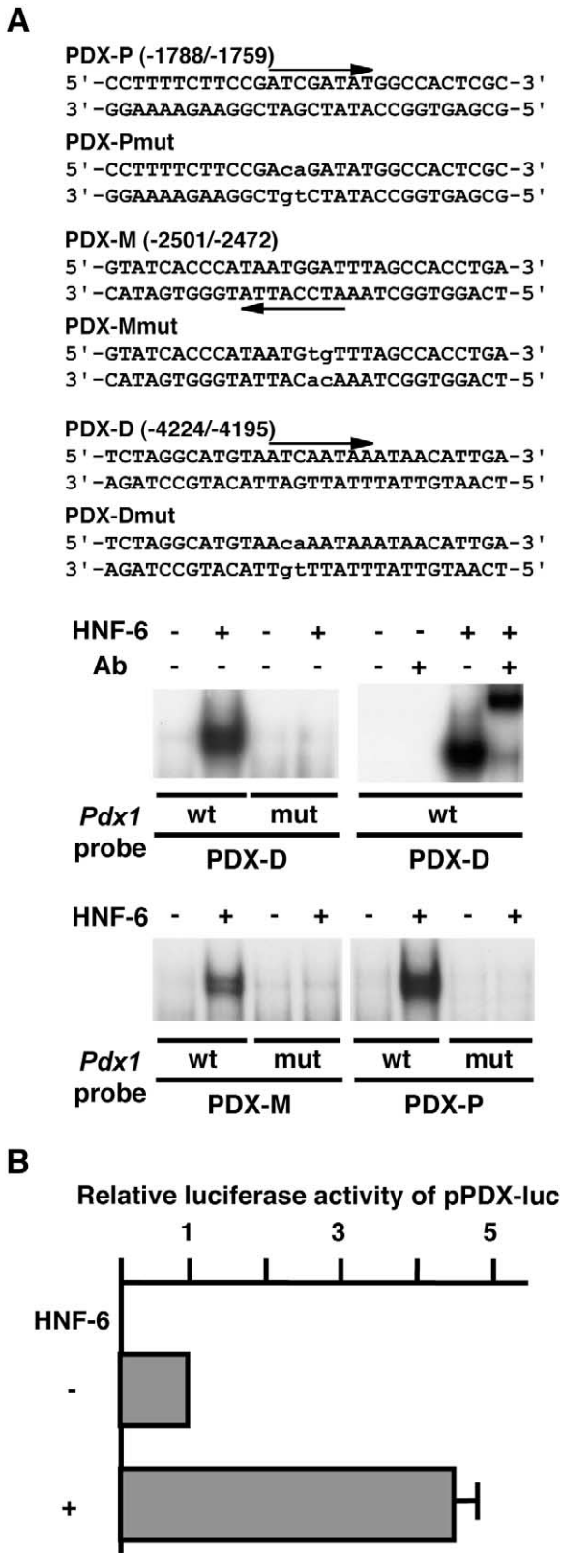

Fig. 6. HNF-6 controls the *Pdx1* gene promoter. (A) HNF-6 binds to three sites in the *Pdxl* gene promoter. The sequence of the proximal (PDX-P), middle (PDX-M), and distal (PDX-D) HNF-6 binding sites in the *Pdxl* promoter is given. Arrows delineate the HNF-6 binding consensus, and lower case letters indicate the nucleotides mutated in the corresponding probes. EMSA were performed with extracts from mock-transfected COS-7 cells (−) or from COS-7 cells transfected with the HNF-6 expression vector (+), as indicated above each lane. The radioactive probes used (wt, wild type; mut, mutated) are indicated below the lanes. An HNF-6–DNA complex was observed with each of the wild-type probes, but not with the mutated probes, in the presence of extracts of cells transfected with the HNF-6 expression vector. A supershift was observed in the presence of an antibody against Flag-tagged HNF-6. (B) HNF-6 stimulates the *Pdxl* promoter. Rat-1 cells were transiently cotransfected with the firefly luciferase reporter containing 4.5 kb of the *Pdxl* promoter sequence (pPDX-luc) and an internal control plasmid coding for *Renilla* luciferase, in the presence of the HNF-6 expression vector (+) or of an empty vector (−) (mean ± s.e.m., $n = 3$). The activity of the *Pdxl* promoter was stimulated 4.5-fold in the presence of the HNF-6 expression vector.

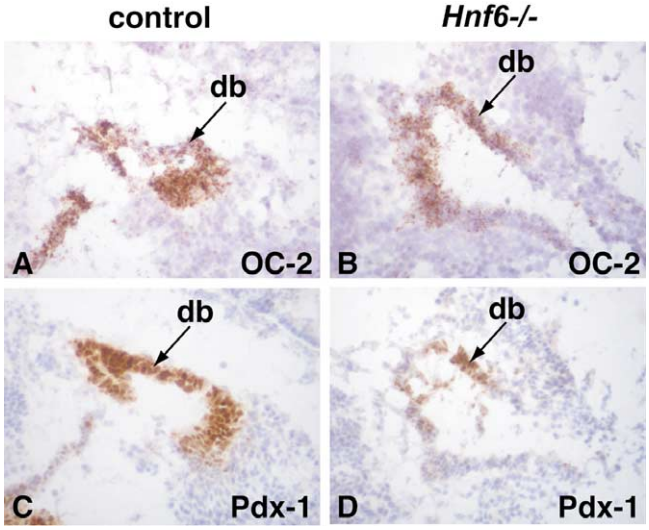

Fig. 7. *Oc2* expression is detected in the pancreas of control embryos and *Hnf6*$^{-/-}$ embryos. In situ hybridization (A, B) and immunohistochemistry (C, D) experiments were performed respectively with a digoxigenin-labeled *Oc2* probe and with anti-Pdx-1 antibodies on sections of 23-somite control (A, C) and *Hnf6*$^{-/-}$ (B, D) embryos. (A, C) and (B, D) are adjacent sections. Pdx-1 labeling was used to localize the dorsal pancreatic bud. The number of Pdx-1 expressing cells was smaller in *Hnf6*$^{-/-}$ embryos than in control embryos, as expected (see Fig. 3). *Oc2* expression was found in the dorsal pancreatic bud and adjacent endoderm of the control (A) and *Hnf6*$^{-/-}$ (B) embryos. db, dorsal pancreatic bud.

levels of Pdx-1 at E10.75 (Jacquemin et al., 2000). It is therefore unlikely that the endocrine defect observed at E12.5 in *Hnf6*$^{-/-}$ embryos results from the delay in pancreas specification. Still, the diabetes observed in adult *Hnf6*$^{-/-}$ mice could result from both defective endocrine differentiation with severe reduction of Glut2 expression (Jacquemin et al., 2000) and pancreas hypoplasia with subsequent reduction in the number of islets of Langerhans. Actually, in these mice, the diabetic syndrome probably also results from the observed decrease in expression of the glucokinase gene in liver (Lannoy et al., 2002).

In conclusion, our present and previous (Jacquemin et al., 2000) results identify HNF-6 as a key regulator of

pancreas specification and endocrine cell differentiation. Perturbations in pancreas function are associated with diabetes. A therapeutic approach aims at engineering cells to produce insulin and transplanting them into patients. It has been suggested that simple gene combinations may be sufficient to generate a pancreatic endocrine cell type from embryonic endodermal cells (Grapin-Botton et al., 2001). The *Hnf6* gene is an obvious candidate in such combinations.

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