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The morphogenesis of the pancreatic mesenchyme is uncoupled from that of the pancreatic epithelium in IPF1/PDX1-deficient mice

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SUMMARY

We have previously shown that mice carrying a null mutation in the homeobox gene *ipf1*, now renamed to *pdx1*, selectively lack a pancreas. To elucidate the level at which PDX1 is required during the development of the pancreas, we have in this study analyzed the early stages of pancreas ontogeny in PDX$^{-/-}$ mice. These analyses have revealed that the early inductive events leading to the formation of the pancreatic buds and the appearance of the early insulin and glucagon cells occur in the PDX1-deficient embryos. However, the subsequent morphogenesis of the pancreatic epithelium and the progression of differentiation of the endocrine cells are arrested in the *pdx1*$^{-/-}$ embryos. In contrast, the pancreatic mesenchyme grows and develops, both morphologically and functionally, independently of the epithelium. We also show that the pancreatic epithelium in the *pdx1* mutants is unable to respond to the mesenchymal-derived signal(s) which normally promote pancreatic morphogenesis. Together these data provide evidence that PDX-1 acts cell autonomously and that the lack of a pancreas in the *pdx1*$^{-/-}$ mice is due to a defect in the pancreatic epithelium.

Key words: homeodomain, *ipf1/pdx1*, apancreatic, epithelium, mesenchyme, morphogenesis, epitheliomesenchymal interactions, mouse, pancreas

INTRODUCTION

In the mouse, the first histological indication of morphogenesis of the dorsal pancreas is evident at the 22- to 25-somite stage, embryonic day 9.5 (e9.5), as a dorsal evagination of the duodenal epithelium at the level of the liver (Wessells and Cohen, 1967). Shortly thereafter a similar evagination occurs on the ventral side, as a derivative of the liver primordium. During the following 2 days, the pancreatic epithelium proliferates and invades the surrounding mesenchyme. Several independent studies have shown that the mesenchyme signals to the pancreatic epithelium and hence promotes cytodifferentiation and morphogenesis of the pancreas, i.e. the growth of epithelial cells and the organization of exocrine cells into acini (Golosow and Grobstein, 1962; Wessells and Cohen, 1967; Levine et al., 1973). This epitheliomesenchymal interaction is essential for both cytodifferentiation and morphogenesis, since, in the absence of mesenchyme, the epithelial cells fail to grow and differentiate (Golosow and Grobstein, 1962; Wessells and Cohen, 1967). The ability of the primitive gut epithelium with its surrounding mesenchyme to give rise to the pancreas has been studied in vitro both in the rat and the mouse (Wessells and Cohen 1967; Spooner et al., 1970). These studies show that the primitive gut epithelium acquires the ability to give rise to the pancreas by the 10- to 12-somite stage, provided it is co-cultured with the surrounding mesenchyme. It has also been shown that the appearance of endocrine cells such as glucagon and early insulin cells precede the morphogenesis of the pancreas (Alpert et al., 1988; Herrera et al., 1991; Gittes and Rutter, 1992; Teitelman et al., 1993).

We have previously shown that the homeodomain protein PDX1, in mouse originally isolated as IPF1 (Ohlsson et. 1993) and in rat as STF-1/IDX-1 (Leonard et al., 1993; Miller et al., 1994), is required for pancreatic development (Jonsson et al., 1994). In the adult mouse pancreas, PDX1 is preferentially expressed in the β-cells where, as has been shown in vitro using cell lines, it binds to and transactivates the insulin promoter (Ohlsson et al., 1993). We now show that, in mouse embryos, the onset of both PDX1 expression and the evagination of the pancreatic epithelium is initiated before the 15 somites stage. Between e10 and e11 PDX1 expression is down-regulated, but later reappears in the differentiated β-cells (Ohlsson et al., 1993). Mice homozygous for a targeted mutation in the *pdx1* gene selectively lack a pancreas (Jonsson et al., 1994).

The lack of a pancreas in the *pdx1*$^{-/-}$ mutants indicates that PDX1 is fundamental to pancreas development. In this study, we wanted to elucidate (i) at what stage of pancreas development PDX1 is required, (ii) if PDX1 acts cell autonomously and (iii) the requirement for PDX1 in differentiation of the glucagon- and insulin-producing cells. We now show that, in *pdx1*$^{-/-}$ mutants, the initial stage of pancreatic bud formation occurs, but that the subsequent morphogenesis of these buds is inhibited. Despite the arrested development of the pancreatic epithelium, the corresponding mesenchyme develops normally, both morphologically and functionally. This suggests that the lack of a pancreas in the PDX1-deficient mice results from a defect in the pancreatic epithelium. Finally, we show that the glucagon cells and a large portion the early insulin cells do not express PDX1 and that they appear in the

pdx1$^{-/-}$ embryos but that the progression of differentiation of these cells is arrested in the mutant embryos.

MATERIALS AND METHODS

Animals and isolation of embryos

The generation of *pdx1*$^{-/-}$ mice has been described elsewhere (Jonsson et al., 1994). Wild-type, +/– and –/– mice were obtained from our local breeding colony of *pdx1*$^{+/-}$ mice. For the isolation of embryos at different stages, *pdx1*$^{+/-}$ mice were mated and the morning of the vaginal plug was designated as embryonic day 0 (e0). Pregnant females were killed and the uteri were transferred to sterile, ice-cold Lebowitz L15 medium (GibcoBRL). The embryos were subsequently isolated and the alimentary tract or the pancreatic rudiments were dissected out and used in immunohistochemical analysis or for recombination experiments (see below).

Genotyping

DNA was isolated from the yolk sac of embryos or the tail tip of 2- to 4-week-old mice by proteinase K (Boehringer) digestion followed by phenol extraction and ethanol precipitation. The genomic DNA was analysed by PCR using two internal *pdx1* primers, both located within exon #2, 5′-GCGGGATCCCGAGTGGGGGCGGT-3′and 5′-CGGGATCCCAGACCCGCTCACCC-3′, and two internal neo primers, 5′-TGAATGAACTGCAGGACGAGG-3′and 5′-AAGGTGAGATGACAGGAGATC-3′.

Immunohistochemistry and in situ hybridization

Intact e9 embryos or dorsal pancreatic buds from embryos at stages e11 and e15 were dissected under ice-cold Lebowitz L15 medium (Gibco) and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at +4°C for 1-2 hours. Immunohistochemistry was carried out essentially as described previously (Ohlsson et al., 1993; Jonsson et al., 1994). The antisera used were diluted as follows; GP anti-rat insulin C-peptide serum (Linco) 1:200, GP anti-glucagon antibodies (Linco) 1:10000, affinity-purified rabbit anti-PDX1 antibodies (Ohlsson et al., 1993) 1:400 and rabbit anti-human α-amylase antibodies (Sigma) 1:4000. Whenever double labellings were performed a blocking step using swine anti-rabbit IgG (DAKO) diluted 1:20 was included. As secondary antibodies, biotinylated anti-GP (Vector) 1:200 and biotinylated anti-rabbit antibodies (Vector) 1:200 were used. The fluorophores used were: fluorescein anti-GP antibodies (Vector) 1:100, strepavidin-FITC (Southern Biotech.) 1:200 and Cy3 anti-rabbit antibodies (Jackson) 1:200-400. The slides were subsequently mounted with 'ready-to-use' fluorescent mounting medium (DAKO).

In situ hybridization using a full-length rat *shh*-probe, kindly provided by T. Jessell, was performed essentially as described (Schaeren-Wiemers and Gerfin-Moser, 1993). Double-label immunohistochemistry and in situ hybridization was performed as described by Tsuchida et al. (1994).

Whole-mount immunohistochemistry

Whole-mount immunohistochemistry was performed as described previously (Ohlsson et al., 1993). Intact e8-e9 wild-type embryos or the gastrointestinal tract, including the lower part of the esophageal ventricle, pancreas and duodenum, from mutant and wild-type e10 to e13.5 mouse embryos, were dissected out under Lebowitz L15 medium (Gibco). Primary antibodies against rat insulin C-peptide were diluted 1:400 and primary antibodies against glucagon were diluted 1:20000. Affinity-purified rat anti-PDX1 (Ohlsson et al., 1993) and affinity-purified rat anti-HNF3-β antibodies, kindly provided by T. M. Jessell, were both diluted 1:800. The primary antibodies were detected with the ABC immunoperoxidase system (Vector Lab. Inc., USA) as described earlier (Ohlsson et al., 1993).

Confocal microscopy analysis

Longitudinal sections of dorsal pancreatic buds and recombinants were immunostained as described above. Specimens were analyzed using an epifluorescence microscope (Zeiss axioplan) or a confocal laser scanning microscope equipped with dual detectors and an argon-krypton (Ar/Kr) laser for simultaneous scanning of two different fluorochromes (Multiprobe 2001, Molecular Dynamics Sunnyvale, CA). Confocal microscopy was used to analyse co-localisation between PDX-1 and glucagon or insulin in centrally located endocrine cells. Sets of fluorescent images were acquired simultaneously for Cy3 and FITC-tagged markers. Companion images (Image size 1025×1024) were scanned with pixel size 0.11 and 1 μm step size. The images were digitally optimised and assigned red and green pseudocolors for Cy3 and fluorescein respectively. Approximately 500 (e15) and 100 (e11) different cells were analysed by counting cells immunopositive for the endocrine markers alone ($Glu^+/PDX1^-$, $INS^+/PDX1^-$) or together with PDX1 ($Glu^+/PDX1^+$, $INS^+/PDX1^+$).

Isolation, recombination and culture of pancreatic rudiments

The dorsal pancreatic buds of e10 embryos were dissected out essentially as described by Gittes and Galantes (1993). The mesoderm was removed from the epithelium by soaking in 1% trypsin in Dulbecco's PBS (GibcoBRL) for 20 minutes at +4°C. The trypsinisation was inhibited by transferring the buds to pancreas culture media (PCM); Medium 199 (GibcoBRL), supplemented with 10% fetal calf serum

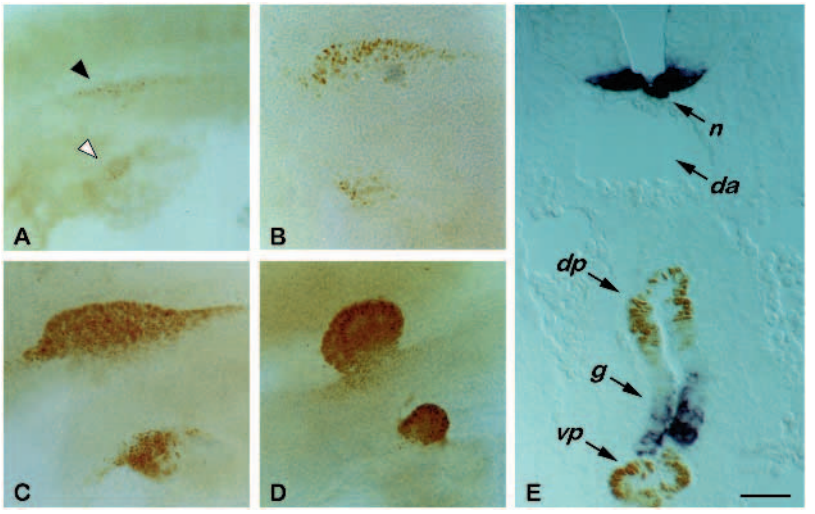

Fig. 1. Early embryonic expression of PDX1 is restricted to the developing pancreatic buds. Whole-mount immunohistochemistry on early wild-type embryos using anti-PDX1 antibodies. (A-D) PDX1 is selectively expressed in the developing pancreatic buds. (A) ~10 somites. The black arrowhead indicates the dorsal pancreatic epithelium and the white arrowhead indicates the ventral pancreatic epithelium. (B) ~15 somites; (C) ~20 somites and (D) ~25 somites. The embryos shown in B-D are oriented as in A. Note that in all these stages (A-D) no or very little PDX1 expression is observed outside of the developing pancreatic buds. (E) Double-label in situ hybridization and immunohistochemistry using anti-PDX1 (brown) antibodies and a *shh* (dark-blue) probe on a transverse section of a ~25 somites embryo showing the expressing of PDX1 in the dorsal and ventral epithelium. *shh* is shown as a known marker for the gut epithelium (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994). Abbreviations: da, dorsal aorta, n, notochord, dp, dorsal pancreas, vp, ventral pancreas, g, gut. Scale bar; E, 10 μm.

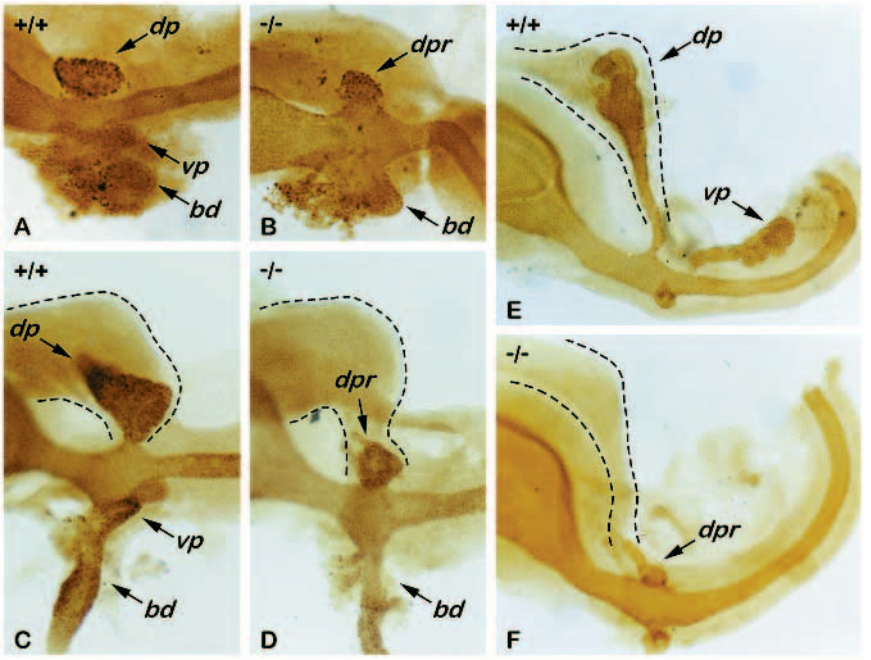

Fig. 2. The morphogenesis of the pancreatic epithelium is arrested in the *pdx1*$^{-/-}$ embryos. Whole-mount immunohistochemistry on e10 (A,B), e11 (C,D) and e12 (E,F), wild-type (A,C,E) and *pdx1*$^{-/-}$ (B,D,F) embryos using HNF 3β-antibodies. In the wild-type embryos, the dorsal pancreatic epithelium grows and becomes lobulated (A,C,E). The dorsal pancreatic epithelium in the *pdx1*$^{-/-}$ embryos forms a bud but then becomes growth arrested and does not lobulate (B,D,F). Note that the ventral pancreatic bud can not be detected in the *pdx1*$^{-/-}$ embryos at the e10-e12 stage. Abbreviations: dp, dorsal pancreas; vp, ventral pancreas; dpr, dorsal pancreatic rudiment; bd, bile duct. Broken line indicates the dorsal pancreatic mesenchyme.

(GibcoBRL), penicillin G-streptomycin 50 U/ml-50 μg/ml (GibcoBRL), fungizone 1.25 μg/ml (GibcoBRL) and Redu-Ser II 1:250 (Upstate Biotech. Inc.). The tissues were allowed to recover in PCM for 30-60 minutes at room temperature before the mesenchyme was carefully dissected away from the epithelium using tungsten needles. Dorsal pancreatic mesenchyme from PDX1-deficient e13.5 embryos was obtained by isolating the hollow mesenchymal bud. Isolated pancreatic epithelia that were completely free of mesenchyme were directly recombined with mesenchyme on Millicell CM culture inserts (Millipore) which had been individually placed in 24-well plates with 300 μl of PCM as described by Gittes and Galante (1993). The cultures were then maintained for 6 days in a humidified incubator at 37°C with 5% CO_2 with a daily change of PCM. On day 6, the cultures were photographed and the cell inserts were then removed from the wells and the filters of the inserts were cut so that the cultured recombinants could be isolated. The different recombinants were then fixed in 4% paraformaldehyde in 0.1 M PB at +4°C for 30-60 minutes, cryoprotected in 30% sucrose in 0.1 M PB for 24 hours at +4°C, embedded in mounting media, frozen and sectioned. Immunohistochemistry on sectioned recombinants was performed as described above.

RESULTS

The early onset of PDX1 expression is correlated to pancreatic commitment of the foregut epithelium

The apancreatic phenotype observed in *pdx1*$^{-/-}$ mice (Jonsson et al., 1994) indicated that PDX1 has an early function in the initial stages of pancreas development. To correlate the temporal pattern of PDX1 expression in the foregut epithelium to the stage at which the gut is assumed to commit to a pancreatic fate, we used the anti-PDX1 antibodies in whole-mount immunohistochemistry on mouse embryos at different stages of development. Using whole-mount immunohistochemistry, a few PDX1-positive cells can first be detected at around the 10-somite stage in the dorsal and ventral gut epithelium from which the pancreatic buds evaginates. (Fig. 1A). The number of PDX1-expressing cells increases as embryogenesis proceeds. At the 15-somite stage (Fig. 1B), PDX1 expression

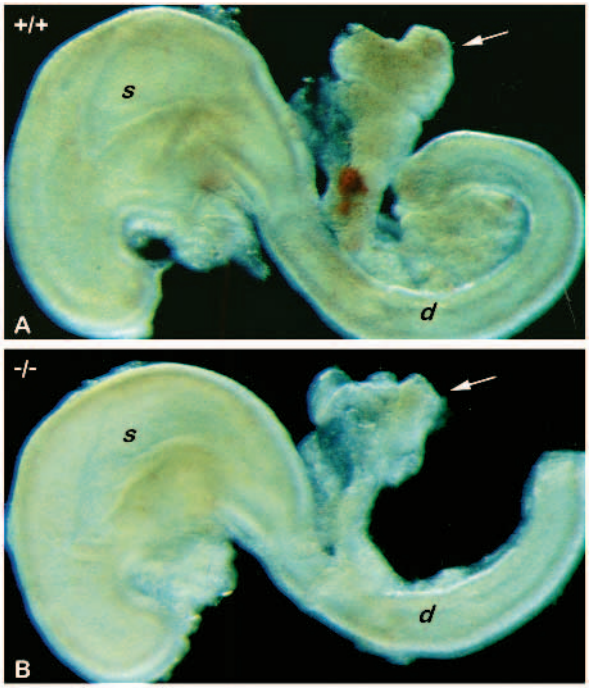

Fig. 3. The pancreatic mesenchyme develops normally in the *pdx1*$^{-/-}$ embryos. (A) Photograph showing the pancreatic bud of an e13.5 *pdx1*$^{+/+}$ embryo. Note the lobulation and vascularisation of the pancreatic epithelium. (B) In e13.5 *pdx1*$^{-/-}$ embryos, the pancreatic mesenchyme forms a hollow bud independently of the pancreatic epithelium The arrow indicates the dorsal pancreatic bud. Abbreviations: d, duodenum; s, stomach.

is clearly evident in both dorsal and ventral foregut endoderm and at 18-20 somites (Fig. 1C) PDX1 is strongly expressed in both pancreas primordia, which now appear as protrusions of the dorsal and ventral foregut. High level PDX1 expression is maintained in the majority of the cells in the pancreatic buds

at the 25-somite stage (Fig. 1D). Thus, PDX1 is one of the earliest markers of pancreas development and the onset of PDX1 expression correlates very well in time with the commitment of this part of the gut to a pancreatic fate. Throughout the development of the pancreas, PDX1 is selectively expressed in the epithelium and at no stage is PDX1 expression observed in the pancreatic mesenchyme (Fig. 1E, Ohlsson et al., 1993 and data not shown). Also at the early stages (e8-e9) of pancreas development, no PDX1 expression is observed outside the protruding pancreatic buds (Fig. 1A-D).

Both the onset of PDX1 expression and the evagination of the dorsal epithelium is initiated before the 15-somite stage (Fig. 1A,B), which means that this induction occurs prior to the existence of any condensed dorsal mesenchyme (Wessells and Cohen, 1967; Slack, 1995). Before the 15-somite stage, the dorsal pancreatic epithelium is instead in direct contact with the notochord (Wessells and Cohen, 1967; Slack, 1995). The notochord then becomes separated from the dorsal pancreatic epithelium and is subsequently kept separated from the pancreatic epithelium, which now express high levels of PDX1, first by the dorsal aorta and later also by the accumulated mesenchyme as is shown in Fig. 1E (see also Wessells and Cohen, 1967; Slack, 1995).

Pancreas morphogenesis is inhibited in the *pdx1*$^{-/-}$ embryos

To elucidate why the *pdx1*$^{-/-}$ mice fail to develop a pancreas, we set out to examine at what stage the effects of the mutated *pdx1* gene becomes manifest. A number of PDX1-negative embryos from stage e10 to e12 were analysed by whole-mount immunohistochemistry using anti-HNF-3β antibodies. HNF-3β is a member of the winged helix family of transcription factors and is expressed in both embryonic and adult endoderm (Ruiz i Altaba et al., 1993; Ang et al., 1993; Monaghan et al., 1993; Sasaki and Hogan, 1993). Expression of HNF-3β was analyzed in e10 (Fig. 2A,B) e11 (Fig. 2C,D) and e12 (Fig. 2E,F) wild-type (Fig. 2A,C,E) and mutant embryos (Fig. 2B,D,F) in order to monitor the morphogenesis of the pancreatic buds. Fig. 2A,B clearly shows that the initial evaginations that leads to the formation of the dorsal pancreatic buds occur in the *pdx1*$^{-/-}$ embryos (Fig. 2B). However, the subsequent growth and morphogenesis of the buds in *pdx1*$^{-/-}$ embryos are strongly inhibited over the following 2 days, since at both e11 (Fig. 2D) and e12 (Fig. 2F) the dorsal bud is very much reduced in size compared to the wild type (Fig. 2C,E). The remaining, growth-arrested bud in the mutant embryos is unbranched (see Fig. 2D,F) indicating that, in the absence of PDX1, the pancreatic epithelium is unable to grow and differentiate.

The ventral pancreatic bud is more difficult to detect and does not seem to be present at e10 (Fig. 2B). Although the dorsal pancreatic bud is present in the *pdx1*$^{-/-}$ embryos at e10, it is smaller than the corresponding dorsal bud in the *pdx1*$^{+/+}$ embryos (Fig. 2A,B). This indicates that, in the *pdx1*$^{-/-}$, the pancreatic buds are growth-arrested already at e10 and, since the ventral bud in wild-type embryos is very small at this stage, we cannot conclusively distinguish the ventral bud from the growing foregut and bile duct in the *pdx1*$^{-/-}$ mutants.

The development of the pancreatic mesenchyme is independent of that of the epithelium

Although the pancreatic epithelium is growth-arrested in the PDX1-negative embryos, the dorsal pancreatic mesenchyme apparently grows and develops normally (Fig. 2B,D,F). Analysis of e13.5 mutant embryos showed that the dorsal pancreatic mesenchyme is present as a 'hollow pocket' that can be dissected away from the stomach and duodenum (Fig. 3B). In wild-type e13-e14 embryos, this mesenchymal pocket is filled by the pancreatic epithelium (compare Fig. 3A and B). Interestingly, the dorsal pancreatic mesenchyme of the *pdx1*$^{-/-}$ mutants does not only grow independently of the pancreatic epithelium, it also exhibits a morphologically normal, bud-like shape and occupies its normal position (Fig. 3B and data not shown).

The pancreatic epithelium is defective in *pdx1*$^{-/-}$ embryos

The fact that the mesenchyme seems to develop morphologically normally in the PDX1-negative embryos may indicate that the lack of pancreatic development in these mutants results solely from a defect in the epithelium. To test this hypothesis, we performed a series of recombination experiments. Dorsal pancreatic mesenchyme from e10 wild-type embryos was recombined with dorsal pancreatic epithelium from e10 *pdx1*$^{-/-}$ embryos and vice versa. Wild-type epithelium cultured in the absence of mesenchyme failed to grow and differentiate (data not shown) as has been described previously (Golosow and Grobstein, 1962). Recombination of mesenchyme and epithelium from e10 wild-type embryos resulted in both growth and branching of the epithelium (Fig. 4A,B), as well as the appearance of insulin- and amylase-expressing cells (Fig. 4C). Identical results were observed when mutant mesenchyme and wild-type epithelium from e10 embryos were recombined (Fig. 4D-F). The hollow mesenchymal pocket from e13.5 mutant mouse embryos was also able to promote pancreatic growth and differentiation giving rise to both insulin- and amylase-expressing cells when recombined with dorsal pancreatic epithelium from wild-type e10 embryos (Fig. 4G-I). In contrast, wild-type mesenchyme (e10) was unable to rescue mutant epithelium (e10) and no growth or differentiation occurred (Fig. 4J-L). Note that, when recombining mutant epithelium with wild-type mesenchyme, the mesenchyme does not grow (Fig. 4J,K), which is in contrast with the unaffected growth of the pancreatic mesenchyme observed in the *pdx1*$^{-/-}$ mice (see Figs 2, 3). This discrepancy indicates that the dorsal pancreatic mesenchyme requires signals from neighbouring tissues in order to be able to grow. In all recombination events described above, except when using mutant epithelium, other pancreatic cell types expressing glucagon, somatostatin and carboxypeptidase A also appeared (data not shown).

Hence the dorsal pancreatic mesenchyme in PDX1-negative embryos not only develops morphologically normally in the absence of the concomitant morphogenesis of the pancreatic epithelium, but it is also functionally active and able to promote morphogenesis and differentiation of wild-type epithelium. In contrast, the pancreatic epithelium in these mutants is defective and unable to respond to both wild-type and mutant mesenchyme.

The glucagon cells and the early insulin cells are generated in the *pdx1*$^{-/-}$ embryos

Our previous analysis of e15 and neonatal *pdx1*$^{-/-}$ mice failed to reveal any insulin- or amylase-expressing cells (Jonsson et al., 1994). However, since the early pancreatic buds are indeed

formed in the *pdx1*$^{-/-}$ embryos and pancreatic glucagon and insulin cells are known to appear prior to the morphogenesis of the pancreas (Alpert et al., 1988; Herrera et al., 1991; Gittes and Rutter, 1992; Teitelman et al., 1993), we screened young *pdx1*$^{-/-}$ embryos for the appearance of these early pancreatic endocrine cells. Whole-mount immunohistochemistry was performed on e11 and e13 wild-type and PDX1-negative mouse embryos in order to detect the presence of insulin- and glucagon-positive cells. Using anti-glucagon antibodies on wild-type e11 embryos, glucagon-positive cells were shown to be present at a position corresponding to the dorsal pancreatic bud (Fig. 5A). Similarly, in e11 *pdx1*$^{-/-}$ embryos, glucagon-positive cells are clearly present at a similar position as in the wild type (Fig. 5B). At e11, only a few cells expressing insulin are present in the wild-type embryos (Fig. 5C) and in the *pdx1*$^{-/-}$ embryos also few but clearly detectable insulin-expressing cells are observed (Fig. 5D). This indicates that these early glucagon and insulin cells develop independently of PDX1. In e13 wild-type embryos (Fig. 5E,G), the number of glucagon- (Fig. 5E) and insulin- (Fig. 5G) positive cells had increased and now colonized both the dorsal and the ventral pancreatic buds. In contrast, very few glucagon-positive cells (Fig. 5F) could be detected in the e13 *pdx1*$^{-/-}$ embryos (Fig. 5F,H) at the level of the initial dorsal evagination and no insulin-positive cells were detected (Fig. 5H).

These data confirm that the initial stages of pancreatic development occur in the *pdx1*$^{-/-}$ mutants and that the appearance of the early glucagon and insulin cells are independent of PDX1 but that these cells require PDX1 for their survival and/or propagation. We have analysed *pdx1*$^{-/-}$ embryos at stage e11 to e13 with the TUNEL assay to elucidate whether the early pancreatic cells become apoptotic in the absence of PDX1. This does not seem to occur since, at least with this technique, we can not confirm any increased apoptosis in the *pdx1*$^{-/-}$ mice (data not shown).

The early developing pancreatic endocrine cells are PDX1-negative

During normal embryonic development, the first glucagon-producing cells can be detected around e9.5 in the mouse and the insulin-producing cells appear 1-2 days later (for review see Slack, 1995 and references therein). It has been proposed, but not conclusively shown, that the pancreatic endocrine cells arise from common progenitor cells which differentiate into the different hormone-producing cell types (Alpert et al., 1988; Herrera et al., 1991; Gittes and Rutter, 1992; Teitelman et al., 1993). The results of a set of ablation experiments using the promoters of the different pancreatic hormone genes to drive the expression of the diphtheria toxin in transgenic mice contest this theory or at least indicate that the different pancreatic endocrine cells diverge from each other early in development (Herrera et al., 1994). We have previously shown that PDX1 is preferentially expressed in the β-cells of the adult mouse pancreas and that there is no co-expression of glucagon and PDX1 in the embryonic or adult mouse (Ohlsson et al., 1993; Serup et al., 1995). Others have reported some degree of co-expression of PDX1 and glucagon as well as co-expression of PDX1 and other pancreatic hormone markers during the development of the pancreas (Guz et al., 1995). Based on these observations, it has been proposed that the early PDX1-positive cells represent pancreatic stem cells (Guz et al., 1995).

To test this, we investigated in detail the relationship between PDX1 and the early glucagon- and insulin-positive cells using confocal microscopy. Double-immunohistochemistry on e11 mouse pancreas using anti-PDX1 and anti-glucagon antibodies (Fig. 6A) or anti-insulin C-peptide antibodies (Fig. 6B) showed that, at this embryonic stage, the glucagon-positive cells do not express PDX1. The majority of the insulin-positive cells were also PDX1-negative although a low level of PDX1 expression was observed in some insulin cells (data not shown). This result is consistent with the observation that glucagon and insulin cells appear in the *pdx1*$^{-/-}$ mutant. At e15, roughly 90% of the insulin cells expressed high levels of PDX1 (Fig. 6D) whereas no co-expression (less than 1%) of glucagon and PDX1 was observed (Fig. 6C). In summary, these data support the findings that the early glucagon and insulin cells are independent of PDX1.

DISCUSSION

The region of the embryonic foregut from which the pancreas develops is specified prior to any morphological manifestation since, by the 10- to 12-somite stage (e8.5), this part of the gut will, when cultured in vitro, give rise to a normally developed pancreas (Wessells and Cohen, 1967). In this paper, we show that, in the mouse, the expression of PDX1 is initiated around 10-12 somites in the gut wall epithelium where the dorsal and ventral pancreatic evaginations will later appear. Although PDX1 have been reported to be expressed in other duodenal epithelial cells than the pancreas at later stages (Guz et al., 1995), no or very little PDX1 expression is found in other parts of the duodenum than the pancreatic buds in early embryos, e8-e9. We also show that both the onset of PDX1 expression and the evagination of the epithelium on the dorsal side occur prior to the accumulation of any dorsal mesenchyme. This suggests that the instructive signal(s) that potentially induce both the onset of PDX1 expression and the evagination of the epithelium might not emanate from the mesenchyme (see Fig. 7). Instead, such an instructive signal is, as we have suggested earlier (Ohlsson et al., 1993), more likely to originate from the notochord since at this stage, 10-13 somites, the notochord is in direct contact with the dorsal pancreatic epithelium (Wessells and Cohen, 1967; Slack, 1995).

The results presented in this paper clearly show that the dorsal pancreatic bud is formed in the PDX1-deficient embryos but that the subsequent morphogenesis of the bud is inhibited. The fact that the initial steps in the formation of the pancreatic buds still occurs in the PDX1-deficient mice argues against a function for PDX1 in the specification of the foregut to a pancreatic fate. We propose that the gut epithelium is patterned prior to the stage at which it has been shown to be competent to form a pancreas (e.g. 7-8 somites) and that, in response to appropriate instructive signals from neighbouring tissues, it will initiate the pancreatic program. These inductive events result in the onset of PDX1 expression and the evagination of the pancreatic epithelium. We suggest that, during the initial stages of pancreas development (e8-e10), a functional *pdx1* gene is required for the expression of gene(s) which makes the epithelial cells able to grow, branch and differentiate (see Fig. 7).

The pancreatic mesenchyme in the *pdx1*$^{-/-}$ mice grows and forms a hollow bud-like structure in the absence of a growing and differentiating epithelium. The mesenchyme from *pdx1*$^{-/-}$

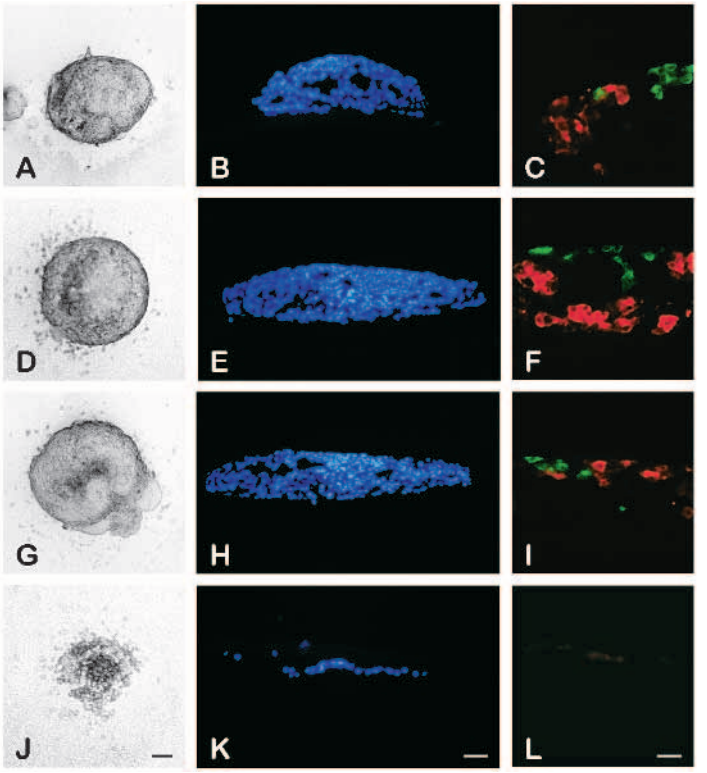

Fig. 4. The pancreatic epithelium in *pdx1*$^{-/-}$ mice is defective. Photograph showing cultured recombinants (A,D,G,J) on day 6. Sectioned recombinants stained with DAPI (B,E,H,K), and the same sections double stained for insulin (green) and amylase (red) (C,F,I,L) Wild-type e10 pancreatic epithelium (A-I) recombined with wild-type e10 mesenchyme (*n*=18) (A-C), mutant e10 mesenchyme (*n*=8) (D-F) or mutant e13 mesenchyme (*n*=5) (G-I) results in both morphogenesis (A,B,D,E,G,H) and differentiation (C,F,I). In contrast, mutant e10 pancreatic epithelium recombined with wild-type e10 mesenchyme (*n*= 6) (J-L) is unable to grow (J,K) and differentiate (L). Scale bars; A,D,G,J, 100 μm; B,E,H,K, 40 μm; C,F,I,L, 20 μm.

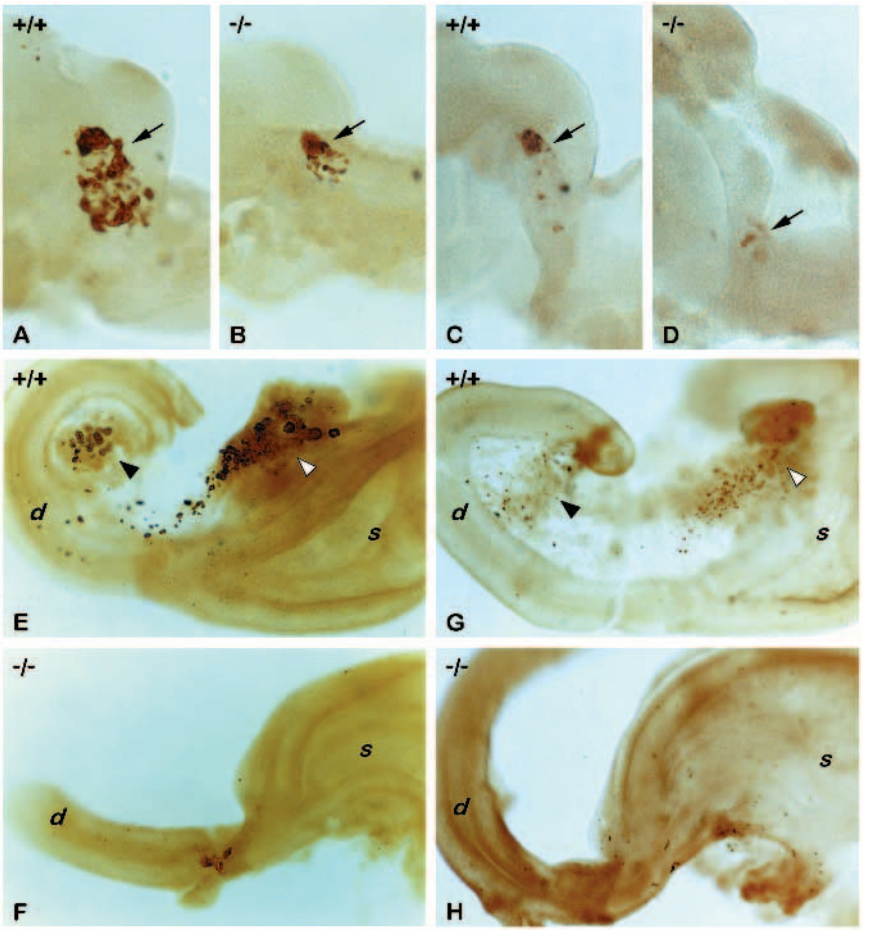

Fig. 5. The early glucagon and insulin cells are generated in the *pdx1*$^{-/-}$ embryos. Whole-mount immunohistochemistry of e10 (A-D) and e13 (E-H), gastrointestinal tract derived from wild-type (A,C,E,G) and *pdx1* mutant (B,D,F,H) embryos using anti-glucagon (A,B,E,F) and anti-insulin C-peptide (C,D,G,H) antibodies. The early glucagon cells appear in the *pdx1*$^{-/-}$ embryos at e10 (B) but are unable to proliferate and/or differentiate (F). A few protodifferentiated insulin cells appear in the PDX1-deficient embryos at e 10 (D) but are no longer detectable at e13 (H). Note! The dark-brown staining seen in H does not correspond to insulin-expressing cells but is the result of contaminating, insoluble particles that became associated with the tissue during the whole-mount procedure. (A-D) The arrow indicates glucagon- or insulin-positive cells in the dorsal pancreatic bud. (E,G) The white arrowhead indicates the dorsal pancreatic bud and the black arrowhead indicates the ventral pancreatic bud. Abbreviations: d, duodenum; s, stomach.

mice is also functionally active in promoting morphogenesis and cytodifferentiation of pancreatic epithelium when recombined in vitro. In contrast, the mutant pancreatic epithelium can not be rescued by the addition of wild-type mesenchymal in a similar experiment. Together, these data suggest (i) that the *pdx1* gene acts cell autonomously by exerting its effects within the epithelium where it is expressed, and (ii) that the growth and development of the pancreatic mesenchyme is independent both of PDX1 expression and the concomitant morphogenesis of the pancreatic epithelium.

Glucagon and insulin cells are generated in the *pdx1*$^{-/-}$ mice again raising the issue of a common origin of the pancreatic endocrine cells. It has been proposed that the early PDX1-positive cells represent a common pancreatic stem cell population from which both the pancreatic endocrine and exocrine cells are generated (Guz et al., 1995). The apparent lack of PDX1 expression in glucagon and early insulin cells and the presence of both of these cell types in the *pdx1*$^{-/-}$ mutants indicate that, if such a pancreatic stem cell does exist, it is not dependent on PDX1. The results of cell ablation experiments using diphtheria toxin genes (Herrera et al., 1994) also argue against a common stem cell origin for the pancreatic endocrine cells. The profiles

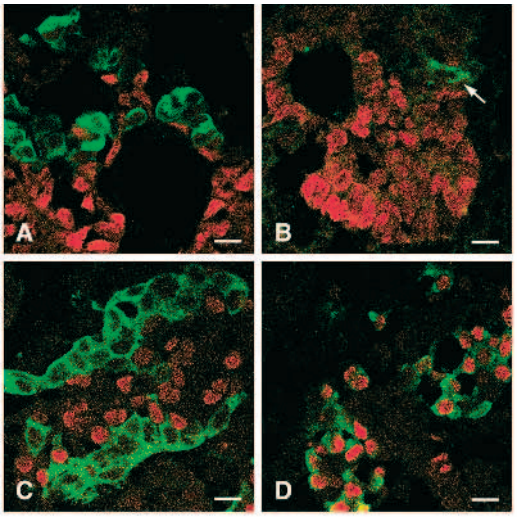

Fig. 6. The early glucagon and insulin cells do not co-express PDX1. Confocal imaged immuno colocalization of e11 (A,B) and e15 (C,D) sectioned dorsal pancreas using anti-PDX1 (A-D) and anti-glucagon (A,C) or anti-insulin C-peptide (B,D) antibodies. (A,C) Note that the glucagon-expressing cells at e11 and e15 do not express PDX1 (less than 1%). (B,D) At e11, very few insulin-expressing cells have appeared (indicated by the arrow in B) and the majority of these do not express PDX1 whereas, at e15, the majority of the insulin-expressing cells are PDX1 positive (D). Scale bar, 10 μm.

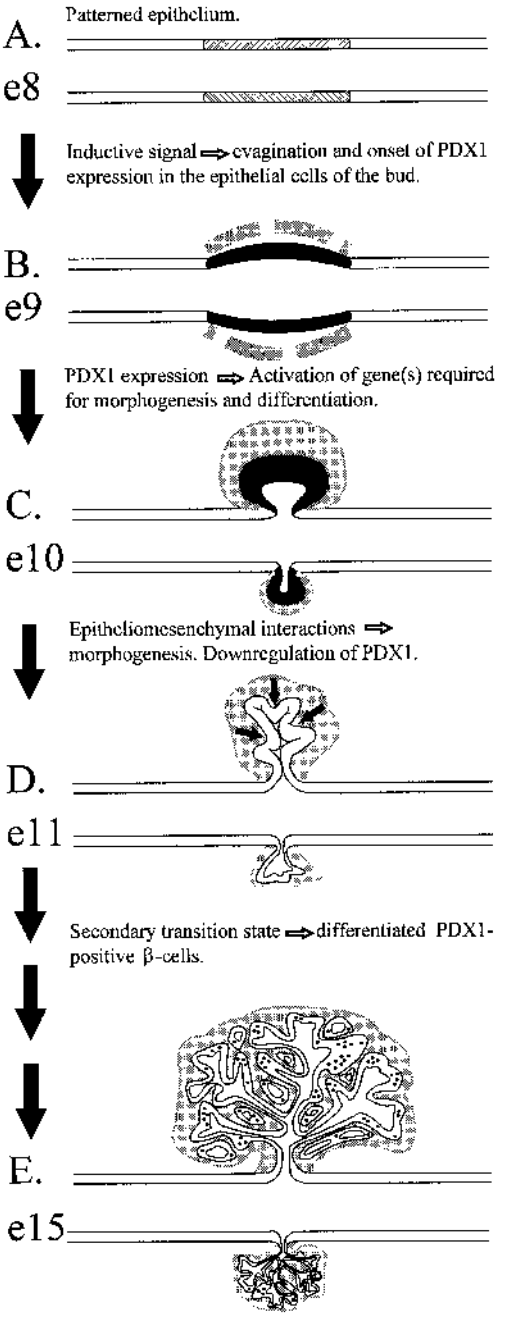

Fig. 7. Model for the role of PDX1 during the development of the pancreas. Summary of the expression profile of PDX1 and different inductive events involved in the development of the pancreas from e8 to e15. This model proposes a dual role for the homeobox gene *pdx1* during pancreas development. (A) e8: Patterned embryonic gut epithelium (striped) receives an inductive signal(s) prior to 15 somites, which leads to the onset of PDX1 in the dorsal and ventral gut wall and the initiation of evagination (Compare with Fig. 1A,B). At this stage, no pancreatic mesenchyme is associated with the dorsal gut tissue, which instead is in direct contact with the notochord (Wessells and Cohen, 1967; Slack, 1995). (B) e9: High level of PDX1 expression (dark grey) in the protruding dorsal and ventral pancreas diverticulum (see also Fig. 1B-D). Mesenchymal cells (light grey) gradually accumulates adjacent to the dorsal gut epithelium (Wesells and Cohen, 1967). Glucagon-expressing cells appear. (C) e10: PDX1 has activated gene(s) that makes the pancreatic epithelium able to grow, branch and differentiate. The early insulin cells appear. (D) e11: Epitheliomesenchymal interactions leads to morphogenesis and differentiation of the pancreas. PDX1 is down-regulated (Ohlsson et al., 1993). During the following days the secondary transition state occurs. This leads to a marked increase in the relative numbers of insulin-positive cells that are now PDX1 positive. (E) e15: The pancreatic buds are heavily lobulated and differentiated exocrine cells have appeared. The majority of the insulin-expressing cells (dark-grey dots) are now PDX1-positive and produce high amounts of the hormone.

of glucagon and insulin gene expression during development are also strikingly different. The glucagon cells reach the adult levels of hormone expression in a single transition early in development, whereas the insulin cells go through a secondary transition later in development in order to reach the adult levels of insulin production (Pictet and Rutter 1972).

We have previously shown that high level PDX1 expression persists until around e10.5, after which it becomes downregulated (Ohlsson et al., 1993). The secondary transition of the insulin cells (Pictet and Rutter, 1972) correlates well in time with the onset of high level PDX1 expression which now is particular to the differentiating, insulin-producing β-cells (Ohlsson et al., 1993) suggesting a role for PDX1 in the terminal differentiation of the β-cells and the regulation of insulin gene expression. We have earlier also shown that, at least in vitro, PDX1 binds to and regulates the expression of the insulin gene (Ohlsson et al., 1993). However, the relationship between the early PDX1-expressing cells, the early insulin cells and the later, more differentiated insulin cells remains unresolved.

Together, the data presented in this paper suggest that the role of PDX1 during the first days of pancreatic development is to impart competence to the early pancreatic epithelial cells to grow, branch and differentiate. At this early stage of development, PDX1 could transcriptionally regulate a gene(s) encoding a receptor(s) and/or other components involved in a signal transduction machinery which transmits mesenchymal signal(s) and hence promotes morphogenesis of the pancreatic epithelium. Alternatively, PDX1 controls the expression of adhesion molecules, growth factors, morphogens, all of which could be key for the morphogenesis and differentiation of the pancreas. The nature of this gene (or genes) remains unknown, but we are presently isolating genes regulated by PDX1. The second proposed function of PDX1, which is restricted to the fully differentiated β-cells, remains to be defined using more specific mutants.

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