Hex homeobox gene-dependent tissue positioning is required for organogenesis of the ventral pancreas

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Summary

In animal development, digestive tissues emerge from different positions of the endoderm as a result of patterning signals from overlying mesoderm. Although embryonic tissue movement during gastrulation generates an initial positional relationship between the endoderm and mesoderm, the role of subsequent endoderm movement against the mesoderm in patterning is unknown. At embryonic day 8.5 in the mouse, proliferation of cells at the leading edge of ventral-lateral endoderm, where the liver and ventral pancreas emerge, helps close off the foregut. During this time, the endoderm grows adjacent to and beyond the cardiogenic mesoderm, an inducer of the liver program and an inhibitor of the pancreas program. The homeobox gene *Hex* is expressed in this endoderm cell domain and in the liver and ventral pancreas buds, after organogenesis. We have found that in *Hex*$^{-/-}$ embryos, there is a complete failure in ventral pancreatic specification, while the liver program is still induced. However, when *Hex*-null ventral endoderm is isolated prior to its interaction with cardiogenic mesoderm and is cultured in vitro, it activates early pancreas genes. We found that *Hex* controls the proliferation rate, and thus the positioning, of the leading edge of endoderm cells that grow beyond the cardiogenic mesoderm, during gut tube closure. Thus, *Hex*-controlled positioning of endoderm cells beyond cardiogenic mesoderm dictates ventral pancreas specification. Other endodermal transcription factors may also function morphogenetically rather than by directly regulating tissue-specific programs.

Key words: Homeobox, Differentiation, Endoderm, Pancreas, Morphogenesis

Introduction

Understanding the basis for gut tissue induction by the endoderm germ layer provides insight into development, disease, and possible strategies for cell-based therapies. Embryo tissue explant studies have revealed the multipotency of endoderm and the importance of patterning signals emanating from different portions of the overlying mesoderm (Wells and Melton, 2000; Hogan and Zaret, 2002; Kumar et al., 2003). Presently we know virtually nothing about how the differential growth of the endoderm and mesoderm may control the juxtaposition of signaling and receptive domains, thereby determining where gut tissues will form. It is well established that signaling and cell adhesion molecules can govern the positioning of cells for proper inductive events (Amthor et al., 1998; Batlle et al., 2002; Lele et al., 2002), but there are few examples of regulatory transcription factors that govern cell differentiation by such a morphogenetic mechanism (e.g. McLarren et al., 2003). In genetic studies, transcription factors found to be required for specification of gut tissues are usually assumed to directly induce tissue-specific target genes (Edlund, 2002; Wilson et al., 2003; Zaret, 2002). In the study described here, we employed both genetic and embryo tissue explant approaches to investigate the role of a transcription factor in the specification of a pancreatic cell fate.

In vertebrates, the pancreas emerges from ventral and dorsal domains of the gut endoderm epithelium (Slack, 1995). The early pancreatic transcription factor Pdx1 (also known as Ipf1) (Ohlsson et al., 1993; Offield et al., 1996) is first detected in ventral-lateral domains of endoderm in mouse embryos at the 7 somite pair stage (7S), approximately day 8.5 of gestation (E8.5) (Gannon and Wright, 1999), and marks the ventral pancreatic progenitor cells (Gu et al., 2002). This is coincident with the first appearance of hepatic gene expression in an adjacent domain of ventral foregut endoderm cells (Gualdi et al., 1996; Deutsch et al., 2001). During this period, the ventral foregut endoderm cells constitute a leading edge of epithelium that grows ventrally, causing the curved foregut sheet to form a tube. At the 6-7S stage, the cardiogenic mesoderm elaborates fibroblast growth factors that can induce the liver program in explants of adjacent endoderm and simultaneously inhibit the pancreas program (Jung et al., 1999; Deutsch et al., 2001). Ventral endoderm cells that become positioned past the cardiogenic mesoderm, prior to this point, can execute the pancreas program. Presently there is no understanding of whether a specific morphogenetic control mechanism allows

the prospective ventral pancreatic domain to be positioned appropriately during specification.

Mouse genetic studies have shown that ventral and dorsal pancreatic patterning requires different sets of transcription factors. The bHLH transcription factor gene *Ptf1a*^{p48} is necessary for the specification of all ventral but not all dorsal pancreatic cells (Kawaguchi et al., 2002; Krapp et al., 1998); the homeobox genes *Isl1* and *Hlxb*9 are necessary for the specification of all dorsal but not ventral pancreatic cells (Ahlgren et al., 1997; Harrison et al., 1999; Li et al., 1999); and the cut-homeodomain factor gene *Hnf6* (*Onecut1*) controls the timing of appearance of both ventral and dorsal pancreatic cells (Jacquemin et al., 2003), as well as aspects of early liver development (Clotman et al., 2002). Ectopic expression of the homeobox gene *Pdx1* expands the domain of dorsal pancreatic progenitors (Ferber et al., 2000; Grapin-Botton et al., 2001; Horb et al., 2003). The particular cell functions that are regulated by these pancreatic factors are unknown, and it is unclear whether they directly or indirectly activate the pancreatic gene program.

While homeobox transcription factors can directly control the expression of genes that confer cell-specific differentiation (Garcia-Bellido, 1975), they also control genes that affect the growth, movement, or death of aggregates of cells (Graba et al., 1997), and thus are hypothesized to control differentiation at the level of tissue morphology (Weatherbee et al., 1998). *Hex* is a homeobox-containing gene in the *Antennapedia/Ftz* class (Crompton et al., 1992) that is expressed in anterior endoderm cells at embryonic day 7.0 of mouse gestation (E7.0) and subsequently in the ventral-lateral foregut (Thomas et al., 1998) that gives rise to the ventral pancreas and the liver. *Hex* is expressed in the liver bud (Thomas et al., 1998; Bogue et al., 2000), but whether *Hex* is expressed in the nascent ventral pancreas has been unknown. In *Hex*-null mouse embryos at E7.5, the definitive endoderm appears compromised in its anterior displacement of the visceral endoderm (yolk sac), as seen by the expression pattern of *Foxa2* (Martinez-Barbera et al., 2000). Chimeric embryo experiments have demonstrated that these morphological defects are intrinsic to the definitive endoderm and were not due to defective signaling from the visceral endoderm (Martinez-Barbera et al., 2000). *Hex*-null embryos grow to the E11.5 stage and undergo normal turning and gut tube closure, but they lack a liver, a thyroid, and parts of the forebrain (Keng et al., 2000; Martinez-Barbera et al., 2000). Thus, while *Hex* may be transiently required for morphogenesis of the anterior definitive endoderm, the basis for this control is not understood and it appears not to grossly affect the morphogenesis of the embryo; nor is it known how *Hex* may control gut tissue development.

While studying the basis for the liver defect in *Hex*-null embryos, we discovered that *Hex* controls the growth, rather than the specification, of the liver bud. We further discovered that *Hex* controls the proliferation of ventral foregut endoderm cells prior to liver induction and that, unexpectedly, *Hex* controls the specification of the ventral pancreas. However, embryo tissue explant assays show that *Hex* operates as a pancreatic specification factor by a heretofore-unappreciated mechanism; by allowing a subset of endoderm cells to grow past a mesodermal signaling center in the embryo. The approaches taken here may be used to determine whether other developmental transcription factors act at a morphogenetic level during endoderm patterning, rather than as direct regulators of tissue-specific genes.

Materials and methods

Mouse phenotypes and genotyping

Martinez-Barbera et al. (Martinez-Barbera et al., 2000) placed *Hex* homozygous null embryos into three classes: class I lacked a rostral forebrain; class II had a reduced forebrain and no abnormalities in the first branchial arch; class III had a small telencephalic vesicle and olfactory placode, but a normal-appearing forebrain. We backcrossed *Hex*^{lacZ} C57BL/6 mice to C57BL/6 mice to obtain a more consistent phenotype (new/previous percentage): class I (82/30), class II (10/30), class III (7/30) and no phenotype (0/10). Embryos were dissected and genotyped as described by Rossi et al. (Rossi et al., 2001) and Martinez-Barbera et al. (Martinez-Barbera et al., 2000).

Hex^{lacZ} expression and histology

Embryos were fixed in 4% paraformaldehyde in PBS at 4°C for 30-60 minutes, washed with PBS on ice twice for 10 minutes, then stained in X-gal solution in PBS: 0.2% X-gal, 2 mM $MgCl_2$, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 0.02% NP-40, overnight at 37°C. Embryos were rinsed twice with PBS for 10 minutes, post-fixed in 4% paraformaldehyde in PBS, and photographed digitally. For immunostaining, embryos were dehydrated through a PBT (PBS +0.1% Tween 20)-methanol series and stored in methanol at –20°C. Embryos were embedded in paraffin wax and sectioned at 4-7 μm. Slides were immunostained with peroxidase or alkaline phosphatase using the avidin-biotin-HRP method. Boiling in 10 mM sodium citrate buffer pH 6.0 for 10 minutes was used for unmasking; secondary antibody was used at 1:2000 dilution. The bound peroxidase was visualized by reaction with a Vector-SG substrate, while the alkaline phosphatase was visualized using BM-Purple (Roche); sections were counterstained with Eosin and mounted with Permount. Primary antibodies to the following antigens (made in rabbit unless otherwise indicated) were used at the indicated dilutions: Pdx1 (a generous gift from C. Wright, Vanderbilt University, TN), 1:5000; Hlxb9 (a generous gift from J. Kehrl), 1:8000; glucagon (Maine Biotechnologies) 1:180; Isl1 (40.2D6 from the Hybridoma Bank at the University of Iowa) 1:2000; Foxa2 (Santa Cruz; made in goat) 1:150 and Gata4 (Santa Cruz; made in goat) 1:150.

In situ hybridization

Whole-mount in situ hybridization on embryos (Wilkinson, 1992) and explants (Rossi et al., 2001) has been described previously. Probes included *Mrg1* (Dunwoodie et al., 1998), *Hex* (Thomas et al., 1998), *Foxa2* (Ang et al., 1993; Dufort et al., 1998), albumin (*Alb*) (Cascio and Zaret, 1991) and α-fetoprotein (Cascio and Zaret, 1991). A *Pdx1* cDNA probe from nt 132-863 of the coding region was cloned from embryonic RNA by RT-PCR into the pCR-Script plasmid. After whole-mount in situ hybridization, embryos were post-fixed in 4% PFA, photographed with a Pixera Pro150ES camera mounted on a Nikon SMZ-U stereomicroscope, dehydrated in ethanol, cleared in xylenes, embedded in paraffin wax, and sectioned at 6 μm.

RNA isolation and RT-PCR cycle step analysis

RNA was isolated as described (Gualdi et al., 1996) and resuspended in 10 μl of nuclease free water (Ambion). One μl of the total RNA was quantified using RiboGreen® RNA Quantitation Kit (Molecular Probes). RNA (5-10 ng for cultured explants and 50-60 ng for primary embryonic tissue) was reverse transcribed using Superscript II (Invitrogen) and oligo(dT_{15}). Reverse transcribed cDNA (1 μl) was amplified in 40 μl of 20 mM Tris-HCl (pH 8.4) containing 50 mM KCl, 1.5 mM $MgCl_2$, 50 μM of each deoxynucleotide triphosphate,

1 U Amplitaq DNA polymerase (Roche), 8 pmol of each specific oligonucleotide, and 0.3 pmol of oligonucleotide phosphorylated with [γ-^{32}P]ATP. Multiple PCR cycle steps were analyzed by gel electrophoresis, to be sure that the reactions were in the exponential range of PCR (Gualdi et al., 1996). Cycle number for each sample was normalized with actin levels as follows. A first round of PCR reactions for actin was done and the cycle range with an exponential increase of PCR product was assigned to each sample (approx. 28-32 cycles). Next, the other genes were assayed adjusting the cycle number in each sample to the inter-sample variability found with actin (total range used was 30-42 cycles). When actin and albumin were assayed in the same PCR reaction, actin primers were added after 6-8 cycles with albumin primers (Jung et al., 1999). Other primers were described by Deutsch et al. (Deutsch et al., 2001) except: *lacZ* #825 (bottom strand, for RT) 5′-ACTCCAACGCAGCACCATCAC-3′ and #824 (top strand) 5′-TACTGTCGTCGTCCCCTCAAA-3′ (331 bp product) and *Prox1* #829 (bottom strand, for RT) 5′-CAGAGATGAGCAGGAACCAACAG-3′ and #824 (top strand) 5′-GCACTACAACAAAGCAAATGACT-3′ (361 bp product).

Embryo tissue isolation and culture

After mating *Hex^{lacZ}* mice, noon of the day of the appearance of a vaginal plug was considered as E0.5. For endoderm explant culture experiments, embryo tissues were harvested at E8.5 and staged by somite number; only tissue from embryos younger than 7S were used. Embryo tissues were dissected at 37°C in PBS under a dissecting microscope (60× magnification) with electrolytically etched tungsten needles, and cultured as described previously (Gualdi et al., 1996). In some experiments, endoderm alone was isolated by incubating the anterior portion of the embryo in 0.125% pancreatin (Sigma), 0.025% polyvinylpyrrolidone-40, and 20 mM Hepes (pH 7.5) in PBS at 4°C for 2 minutes, prior to dissection. Explants were cultured in 8-well glass microwell slides (LabTek) coated with type I rat tail collagen (Collaborative Biomedical Products) in DMEM (Invitrogen) containing 10% calf serum (Hyclone) (Gualdi et al., 1996). Beating cardiac mesoderm cells in live explants, previously shown to be α-actin positive (Gualdi et al., 1996), were evident by microscopy and annotated on photographs of explants. Cultures were maintained at 37°C and 5% CO_2 in air.

Cell proliferation and apoptosis analyses

BrdU incorporation was detected using the BrdU Labeling and Detection Kit II (Roche). Pregnant animals were injected with BrdU reagent and sacrificed after 2 or 3.5 hours, and embryos were processed following standard histological procedures (see above). BrdU immunostaining was done using the manufacturer's protocol. Immunostaining of phospho-histone H3 (Cell Signaling) was performed as described above at a 1:50 dilution. BrdU and Phospho-H3 were quantified by alternately taking digital images of a section with bright-field (to determine the positive-stained cells) and phase contrast (to determine the total number of cells) illumination. The limits of the ventral endoderm domain selected for quantification at 5-8S were established caudally by the visceral/definitive endoderm border (Foxa2-negative/Foxa2-positive transition) in neighboring sections and rostrally by mapping the *Hex*-positive domain observed in the wild-type embryos. At least three *Hex$^{-/-}$* embryos were analyzed for each stage and assay. Probability value was determined by the homoscedastic one tailed t-test. Apoptotic cells were detected using the In Situ Cell Death Detection Kit (Roche).

Results

Hepatic differentiation begins, but liver growth fails, in *Hex$^{-/-}$* embryos

Previous studies indicated that *Hex$^{-/-}$* mouse embryos at E9.5 did not express liver genes, suggesting a defect in differentiation, as there was only a thickening of the endodermal epithelium as evidence of possible hepatic induction (Martinez-Barbera et al., 2000; Keng et al., 2000). A similar phenotype was observed in zebrafish embryos treated with an antisense morpholino to *Hex* (Wallace et al., 2001). The expression of liver and pancreas genes in the ventral endoderm is normally first detectable by RT-PCR at the 7-8S stage (E8.5) (Gualdi et al., 1996; Deutsch et al., 2001). We found that albumin, transthyretin (*Ttr*), and *Prox1*, three of the earliest expressed liver genes (Jung et al., 1999; Sosa-Pineda et al., 2000), are induced in the ventral foregut endoderm of *Hex$^{-/-}$* embryos by the 10S stage (late E8.5); dorsal endoderm served as negative controls (Fig. 1A, lanes 4-9 for *Alb* and *Ttr*; data not shown for *Prox1*). Also, ventral foregut endoderm explanted from *Hex*-null embryos at the 2-6S stages, prior to hepatic induction, and co-cultured for 48 hours with its adjacent cardiogenic mesoderm, an inducer of the liver (Le Douarin, 1975; Gualdi et al., 1996), was competent to activate albumin and α-fetoprotein genes (Fig. 1B; arrows denote positive signals; see also Fig. 4D, lanes 16-19 below). However, in vivo, the thickening hepatic endoderm region of *Hex*-null embryos at 18S (E9.0) was dramatically smaller than the emerging liver bud of heterozygous embryos at the same stage (Fig. 1D,E; red boxes), and similar in size to the hepatic endoderm region of earlier, 14S heterozygous embryos (Fig. 1C,D; red boxes), suggesting a defect in growth.

We found that *Hex$^{-/-}$* hepatic endoderm cells at the 14S and 18S stages have a markedly lower rate of incorporation of bromodeoxyuridine (BrdU), compared to wild-type or heterozygous counterparts (Fig. 1F,G; red bars; $P<0.01$). Furthermore, there was a lower rate of BrdU incorporation in the *Hex$^{-/-}$* hepatic endoderm compared with the non-hepatic lateral gut endoderm (Fig. 1C,E; blue boxes and Fig. 1F,G; blue bars), where *Hex* is not expressed [data not shown (Thomas et al., 1998)]. No TUNEL staining, indicative of apoptosis, was observed in the hepatic endoderm region of either heterozygous or *Hex$^{-/-}$* embryos, but it was readily detected in the neural tube and amnion, which served as controls (Fig. 1H-J). Thus, the liver becomes specified and the hepatic endoderm begins to differentiate in *Hex$^{-/-}$* embryos, but a localized deficiency in the proliferation of the hepatic endoderm leads to a defect in liver bud development.

Defect in ventral-lateral endoderm proliferation and positioning in *Hex$^{-/-}$* embryos

Considering that *Hex* is expressed in the ventral-lateral endoderm (Fig. 2A) (Thomas et al., 1998), we wished to determine whether there is a growth defect in *Hex$^{-/-}$* endoderm prior to and during organogenic patterning. We first used *Foxa2/Hnf3b* expression as a marker of ventral definitive endoderm cells (Ang et al., 1993), as *Foxa2* is intrinsically necessary for ventral foregut endoderm development (Dufort et al., 1998). At the 8S stage (E8.5), the domain of ventral-lateral foregut cells expressing *Foxa2* was clearly reduced in *Hex$^{-/-}$* embryos, compared to wild type, but other *Foxa2*-positive domains, such as the notochordal plate, were normal (Fig. 2B,D; ventral foregut, red arrows; notochordal plate, arrowheads). By contrast, there was no change in *Mrg1* mRNA expression, either spatially or quantitatively, which is a marker of the adjacent septum transversum mesenchyme in foregut mesoderm cells (Fig. 2C,E; red arrows) (Dunwoodie et al.,

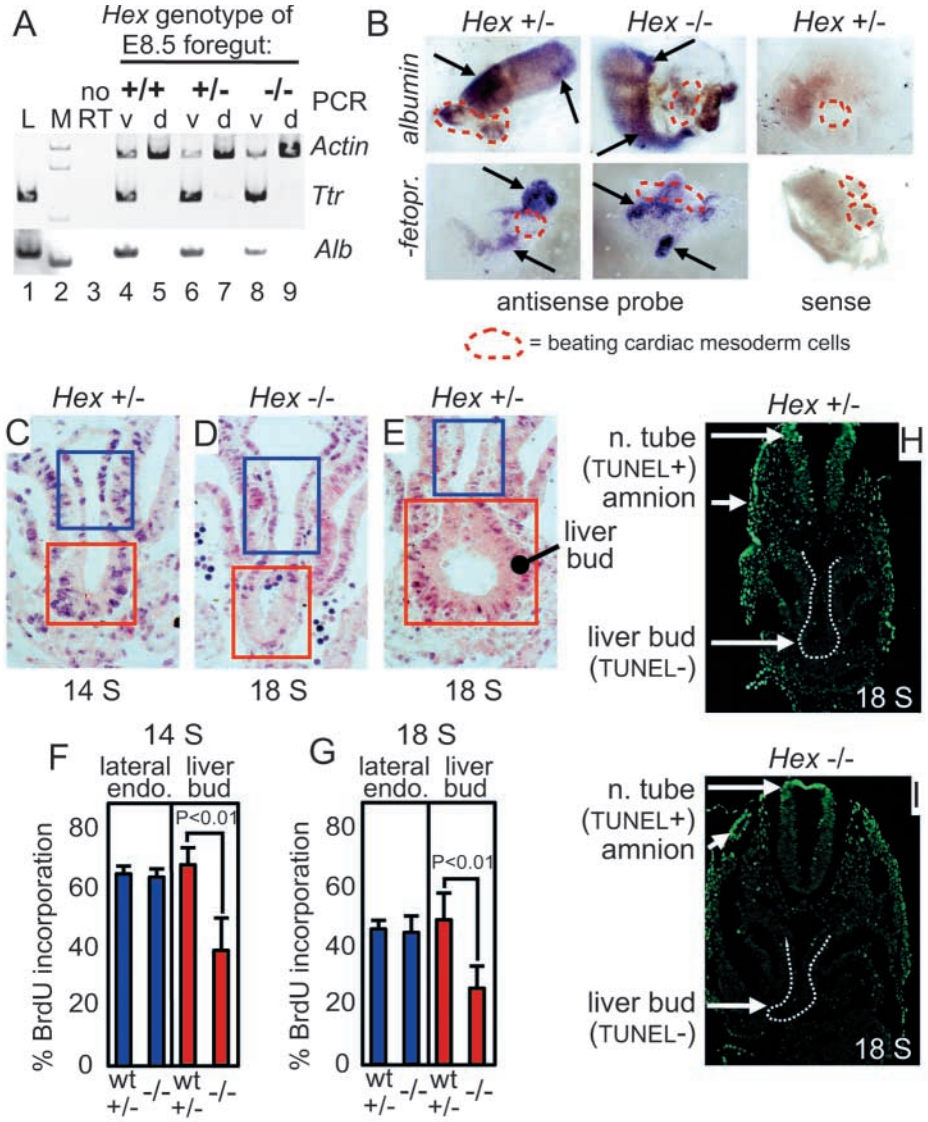

Fig. 1. Liver is specified in *Hex*$^{-/-}$ embryos but fails to grow because of a cell proliferation defect. (A) RT-PCR analysis of RNA from ventral and dorsal foregut domains of 10-somite stage (S) embryos (lanes 3-8). *Albumin* and *transthyretin* were expressed in the hepatic primordium of *Hex*$^{-/-}$ embryos at 10S (*n*=8, two representative samples depicted). L, embryonic liver mRNA; no RT, not reverse transcribed RNA; v, ventral; d, dorsal. (B) In situ hybridization for *Albumin* and *α-fetoprotein* on isolated ventral endoderm from 2-6S embryos cultured for 48 hours with cardiac mesoderm. Beating cardiac domains are outlined in red and sometimes lie above or below endoderm domains; all purple staining is positive, with a subset denoted by arrows. (C-E) Embryos were exposed in vivo to BrdU for 2 hours, harvested, fixed, sectioned and stained for BrdU incorporation (purple nuclei). Blue boxes denote lateral gut endoderm; red boxes denote hepatic endoderm. (F,G) Quantitation of BrdU-positive endoderm cells relative to total cells in the hepatic (red bars) and lateral (blue bars) endodermal domains. A total of 24 sections from wild-type (*n*=3) and *Hex*$^{-/-}$(*n*=3) embryos were evaluated; P value was determined by the homoscedastic one tailed t-test. A lower proliferation rate was detected in the hepatic bud of *Hex*$^{-/-}$ embryos. (H,I) TUNEL assay in the hepatic bud of 18S embryos. The regions with a white dotted outline correspond to the blue and red boxed regions in panels D,E. No evidence for enhanced apoptosis was detected in *Hex*$^{-/-}$ embryos; note the positive staining in the neural tube and amnion in both embryos.

1998), nor in the position of the cardiogenic mesoderm (Martinez-Barbera et al., 2000) (also see below).

These whole-mount data were confirmed by a detailed immunohistochemical analysis of sagittal sections. At 5-6S, just prior to liver and pancreatic patterning (Deutsch et al., 2001; Gualdi et al., 1996), the Foxa2-positive endodermal epithelium in wild-type embryos progresses ventrally around the cardiogenic mesoderm (Fig. 2G; ventral to left, see 'v.e.' cells with Foxa2-positive black nuclei adjacent to red arrow) and terminates where the large, rounder, Foxa2-negative visceral endoderm cells began (Fig. 2G; 'visc.' cells adjacent to black arrow). This leading edge of ventral definitive endoderm cells also expresses *Hex* (Fig. 2F; 'v.e.' cells with blue stain adjacent to red arrow). In *Hex*$^{-/-}$ embryos, the ventral definitive endoderm domain was reduced and did not completely surround the cardiogenic mesoderm (Fig. 2H; cells with Foxa2-positive black nuclei adjacent to red arrow). By the 8-9S stage, when patterning would have normally occurred, wild-type definitive endoderm grows caudal to and beyond the cardiogenic mesoderm, extending the anterior intestinal portal (Fig. 2P; near head of red arrow). Again, these ventral definitive endoderm cells are Hex-positive (Fig. 2O, adjacent to red arrow). By contrast, in *Hex*$^{-/-}$ embryos the definitive endoderm cells exhibited a marked failure to grow caudal to the cardiac domain (Fig. 2Q, cells adjacent to red arrow) and the anterior intestinal portal now contained visceral endoderm cells (Fig. 2Q, cells adjacent to black arrow); the latter being detected by strong immunohistochemical staining for Gata4 (Fig. 2R; cells adjacent to black arrow). Other embryonic endoderm domains in *Hex*$^{-/-}$ embryos, where *Hex* is not normally expressed, appeared morphologically normal (Martinez-Barbera et al., 2000) (data not shown). The persistent expression of *Hex* in wild-type cells constituting the leading edge of the ventral-lateral definitive endoderm and the diminished numbers of such cells in *Hex*$^{-/-}$ embryos suggests that *Hex* specifically controls the advancement of the endodermal epithelium towards gut closure and, consequently, the positioning of this endodermal domain beyond the cardiac mesoderm.

Hex control of ventral endoderm morphogenesis could be at the level of changes in cell shape, size, migration or proliferation. We found that the leading edge of ventral

endoderm cells in *Hex*$^{-/-}$ embryos appeared identical in shape and size to those in wild type (Fig. 2G,H,P,Q). We therefore investigated the proliferative rate of the endoderm cells. A total of 80 sections of wild-type and *Hex*-null embryos were scored for phosphorylated histone H3 (P-H3), a mitotic marker (Schmiesing et al., 2000), in the ventral-lateral endoderm cells that normally express *Hex*. As seen in Fig. 2I,J (red boxed regions) and quantitated in Fig. 2M, the leading ventral-lateral edge of definitive endoderm cells exhibited a statistically significant decrease in P-H3 labeling in *Hex*$^{-/-}$ embryos, compared with wild type, at the 5-6S stage (P<0.05). The same domain of cells also exhibited a statistically significant decrease in BrdU incorporation in *Hex*$^{-/-}$ embryos (Fig. 2K,L, red boxed regions; N, 'ventral-lateral', red bars;

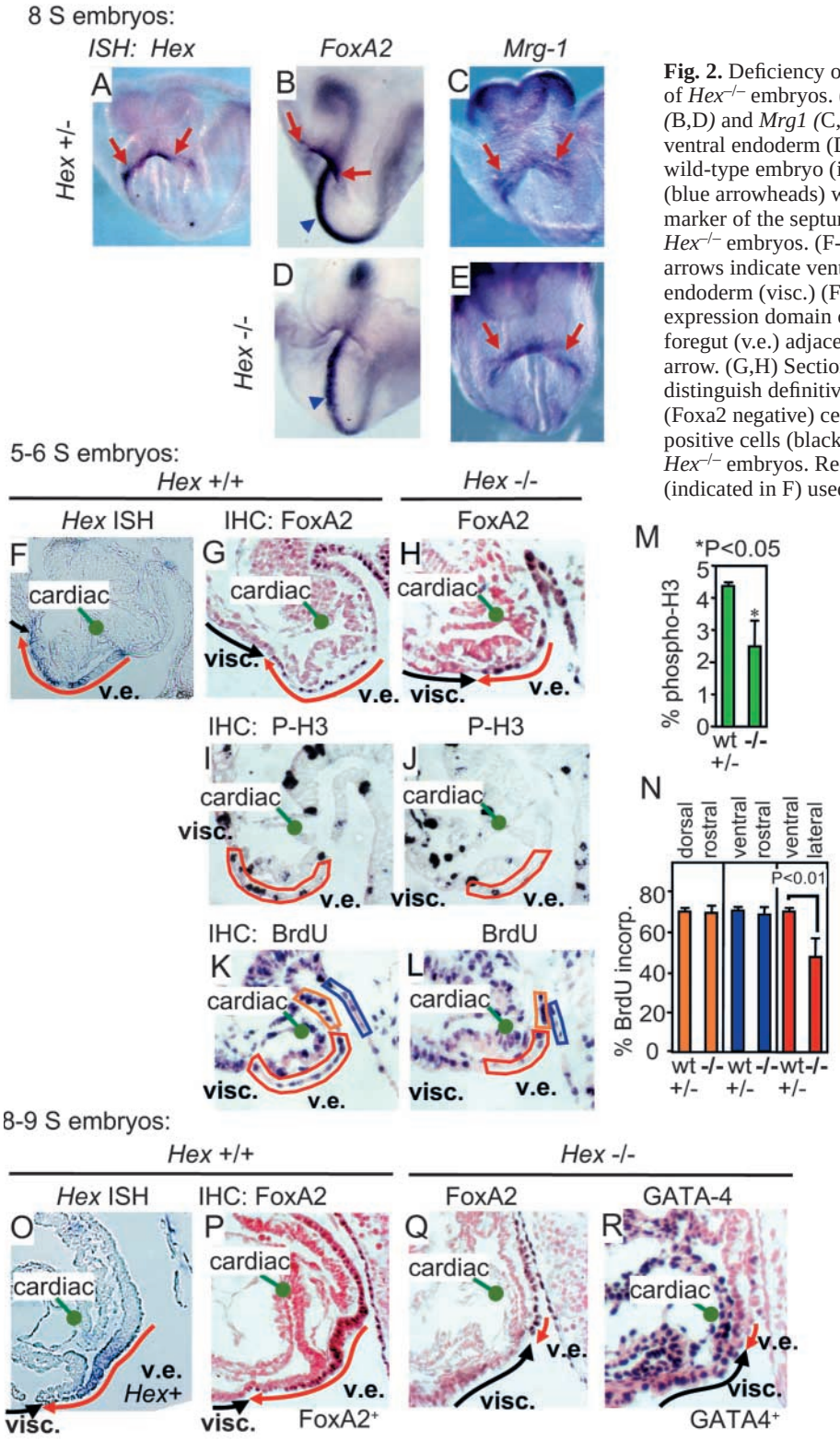

Fig. 2. Deficiency of definitive endoderm cells in the ventral foregut of *Hex*$^{-/-}$ embryos. (A-E) in situ hybridization for *Hex* (A), *Foxa2* (B,D) and *Mrg1* (C,E). Reduced Foxa2-positive domain in the ventral endoderm (D) corresponds to the *Hex*-positive domain of the wild-type embryo (in A); Foxa2 expression in the notochordal plate (blue arrowheads) was not affected. The *Mrg1* expression domain, a marker of the septum transversum mesenchyme, was not affected in *Hex*$^{-/-}$ embryos. (F-H) Sagittal sections of a 5-6S embryos; red arrows indicate ventral endoderm (v.e.) and black arrows, visceral endoderm (visc.) (F) In situ hybridization for *Hex* showing the expression domain of *Hex* in definitive endoderm cells of the ventral foregut (v.e.) adjacent to the visceral endoderm, contiguous to red arrow. (G,H) Sections immunostained for *Foxa2* (blue nuclei) to distinguish definitive (Foxa2 positive) and visceral endoderm (Foxa2 negative) cell domains. (I,J) Diminished phospho-H3 positive cells (black nuclei) are found in the ventral endoderm of *Hex*$^{-/-}$ embryos. Red boxes denote the *Hex*-positive domain (indicated in F) used in the quantitation analysis in M. (K,L) Embryos were exposed in vivo to BrdU for 3.5 hours, harvested, fixed, sectioned and stained for BrdU incorporation (purple nuclei). The colored boxes in K indicate the endoderm domains used for in the quantitation analysis in N. (M) Percentage of phospho-H3-positive cells in the ventral lateral endoderm (red boxes in I and J). A total of 80 sections from wild-type (n=3) and *Hex*$^{-/-}$ (n=4) embryos were counted. A statistically significant decrease of phospho-H3-positive cells is found in the ventral endoderm of *Hex*$^{-/-}$ embryos. (N) Percentage of BrdU-positive cells in different endodermal domains: dorsal-rostral endoderm (blue box), ventral-rostral endoderm (orange box) and ventral-lateral endoderm (red box). A total of 70 sections from wild-type (n=3) and *Hex*$^{-/-}$ (n=4) embryos were counted. P value was determined by the homoscedastic t-test (one tail distribution). A statistically significant decreased BrdU incorporation is found in the ventral-lateral endoderm of *Hex*$^{-/-}$ embryos; the lower proliferation rate is not found in other domains of *Hex*$^{-/-}$ embryos. (O) In situ hybridization for *Hex*. Sagittal section showing the expression domain of *Hex* in the ventral endoderm at 8-9S. (P-R) Immunostaining of sagittal sections from 8-9S embryos comparing the anterior visceral/definitive endoderm border in the wild-type and *Hex*$^{-/-}$ embryos. In the *Hex*$^{-/-}$ embryo, visceral endoderm cells (strongly Gata4 positive) occupy the position where the definitive endoderm (Foxa2 positive) normally occurs in the ventral foregut. In all sections, ventral is to the left, dorsal is to the right.

P<0.01). BrdU incorporation in cells located in dorsal-rostral or ventral-rostral endodermal domains, where *Hex* is not normally expressed (Fig. 2F), did not have a decreased proliferation rate (Fig. 2K,L; orange and blue boxed regions; Fig. 2N, orange and blue bars). Thus, the reduced ventral-lateral endoderm domain in *Hex*-null embryos and the excess of visceral endoderm cells in the gut is due to a localized defect in cell proliferation of the definitive endoderm. These results emphasize how *Hex* controls the growth of a discrete domain of endoderm.

Failure of ventral pancreatic specification and morphogenesis in *Hex*$^{-/-}$ embryos

The *Hex*$^{-/-}$ deficiency in ventral foregut endoderm that is normally caudal to the cardiogenic mesoderm led us to investigate the consequences for pancreas development. As expected (Ohlsson et al., 1993; Offield et al., 1996), we found that in wild-type E8.5 embryos (9S), *Pdx1* mRNA is expressed in the ventral-most cells in the anterior intestinal portal (Fig. 3A; 'ventral'); the same cells that normally express *Hex* (Fig. 3B) and fail to become positioned there in *Hex*-null embryos

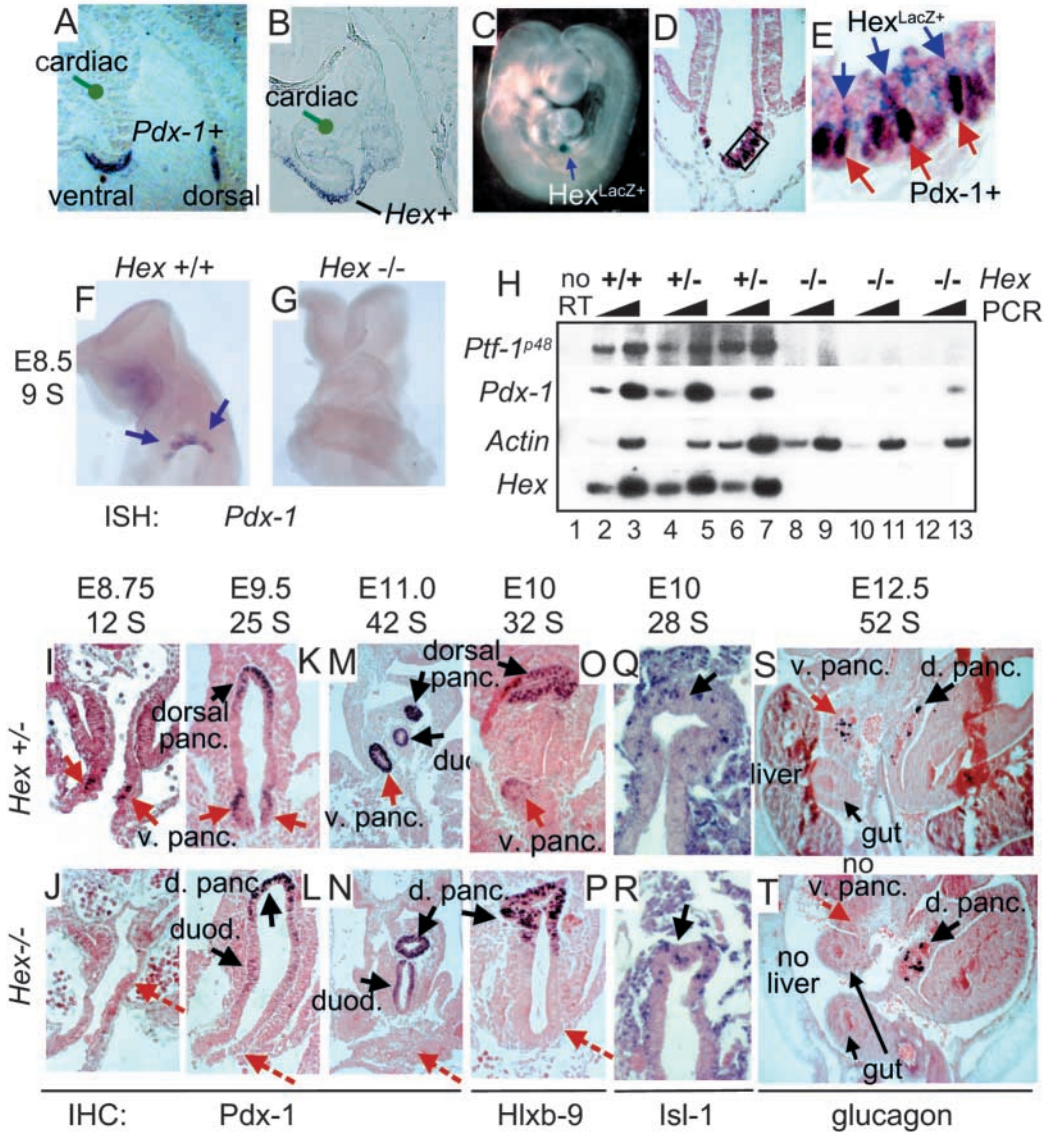

Fig. 3. Ventral pancreas is not specified in *Hex*$^{-/-}$ embryos. (A,B) Sagittal sections comparing the *Pdx1* and *Hex* expression domains in wild-type embryos. The *Pdx1*-positive domain in the ventral endoderm (A) corresponds to the most anterior-ventral positive domain of *Hex*-positive cells (purple staining in B). Pdx1-positive cells also occur in the dorsal pancreatic endoderm. (C,E) *lacZ* expression in a *Hex*$^{+/-}$ embryo. (C) Note the caudal extension of the blue *lacZ*-positive cells. Arrow indicates level of section in D. (D) Transverse section of the embryo in C immunostained for Pdx1; the boxed region is magnified in E. (E) Cells doubly positive for Pdx1 (nuclear, dark) and *Hex*^{lacZ} (cytoplasmic, blue) are present in the ventral endoderm of the gut. (F,G) In situ hybridization for *Pdx1*. Arrows point to *Pdx*-positive cells at the exit of the foregut (as in A). (H) RT-PCR analysis of *Ptf1*^{p48} in ventral foregut endoderm from 12-16S embryos of the designated genotypes. The lowest panel depicts endogenous *Hex* expression. (I-T) Immunohistochemical staining for designated markers. Complete section series of three to four embryos were analyzed for each stage and marker, all with the same results as those shown. Dashed arrow indicates regions where pancreatic genes or cells would normally occur, but where they are absent in *Hex*-null embryos. Original magnifications: I-L, O-R, 200×; M, N, 100×; S, T, 40×. No evidence for pancreatic gene expression or morphogenesis was detected ventrally in *Hex*$^{-/-}$ embryos.

(Fig. 2Q). E9.5 embryos, heterozygous for the *Hex*-null allele with *lacZ* under the control of the *Hex* promoter, exhibited cytoplasmic staining for β-galactosidase in a caudal extension of the hepatic bud (Fig. 3C; blue labeled cells). Nuclear Pdx1 protein was also present in the cells of the caudal extension (Fig. 3D,E), but not the liver bud (data not shown). By contrast, the dorsal pancreatic endoderm was Pdx1 positive, but Hex^{lacZ} negative (Fig. 3C; data not shown). These results were confirmed by in situ hybridization for endogenous *Hex* mRNA and immunostaining for Pdx1 (data not shown). Thus, *Hex* is expressed in ventral, but not dorsal, pancreatic progenitor cells.

Pdx1 mRNA was undetectable in 9S $Hex^{-/-}$ embryos (E8.5) by in situ hybridization (Fig. 3G, compare region denoted by blue arrows in wild type in 3F). Pdx1 protein was detected in wild-type ventral and dorsal pancreatic regions from 12S onwards, coinciding with the thickening of those endodermal domains into pancreatic buds (Fig. 3I,K,M; red arrows). However, Pdx1 was not detected in the ventral gut of $Hex^{-/-}$ embryos at any stage, nor was there any evidence for thickening of the endoderm where the pancreas bud would normally form (Fig. 3J,L,N; dashed arrows). Pdx1 expression in the dorsal pancreatic endoderm and in the prospective duodenal cells (Offield et al., 1996) was normal at the 25S stage and later in $Hex^{-/-}$ embryos (Fig. 3K-N). Expression of *Ptf1a*, a pancreatic specification factor (Kawaguchi et al., 2002), was detected by RT-PCR in the *Pdx1*-positive, ventral endoderm of wild-type embryos in the 12-16S range, but not in ventral gut tissue of *Hex* nulls (Fig. 3H, lanes 8-13), and it remained undetectable in $Hex^{-/-}$ at E9.5 (data not shown). Occasionally, we saw low amounts of *Pdx1* RT-PCR products in 12-16S dissected *Hex*-null ventral gut tissues (Fig. 3H, lane 13). In light of never having seen Pdx1 protein expression in *Hex*-null ventral foreguts, or any other pancreas marker (see below), we attribute the weak *Pdx1*-positive signal in our dissection to duodenal progenitors (Offield et al., 1996), because hours later, these laterally distinct cells are Pdx1-positive in *Hex*-null embryos (see Fig. 3L,N, black arrows). *Hlxb9*, which is expressed in wild-type pancreatic buds (Harrison et al., 1999; Li et al., 1999), was also absent from the ventral but not dorsal region of $Hex^{-/-}$ embryos (Fig. 3O,P). In summary, the absence of ventral expression of these earliest regulatory genes specific to the pancreas suggests that, in contrast to the situation with the liver, *Hex* controls the initial specification and differentiation of ventral pancreatic endoderm.

Glucagon-positive cells first appear in the ventral pancreas at E10.5-11.0 of wild-type embryos (data not shown), but they were not found ventrally in $Hex^{-/-}$ embryos, even in a rare embryo surviving to E12.5 (Fig. 3S,T, dashed arrow points to absence of glucagon cells and of any pancreatic mass). Significantly, after analyzing all fore- and midgut sections from multiple embryos stained for the above markers, we conclude that there was no budding or morphogenesis of the prospective ventral pancreas domain in any $Hex^{-/-}$ embryo (n=40; Fig. 3J,L,N,P,T; data not shown). By contrast, the budding and branching of the dorsal pancreas

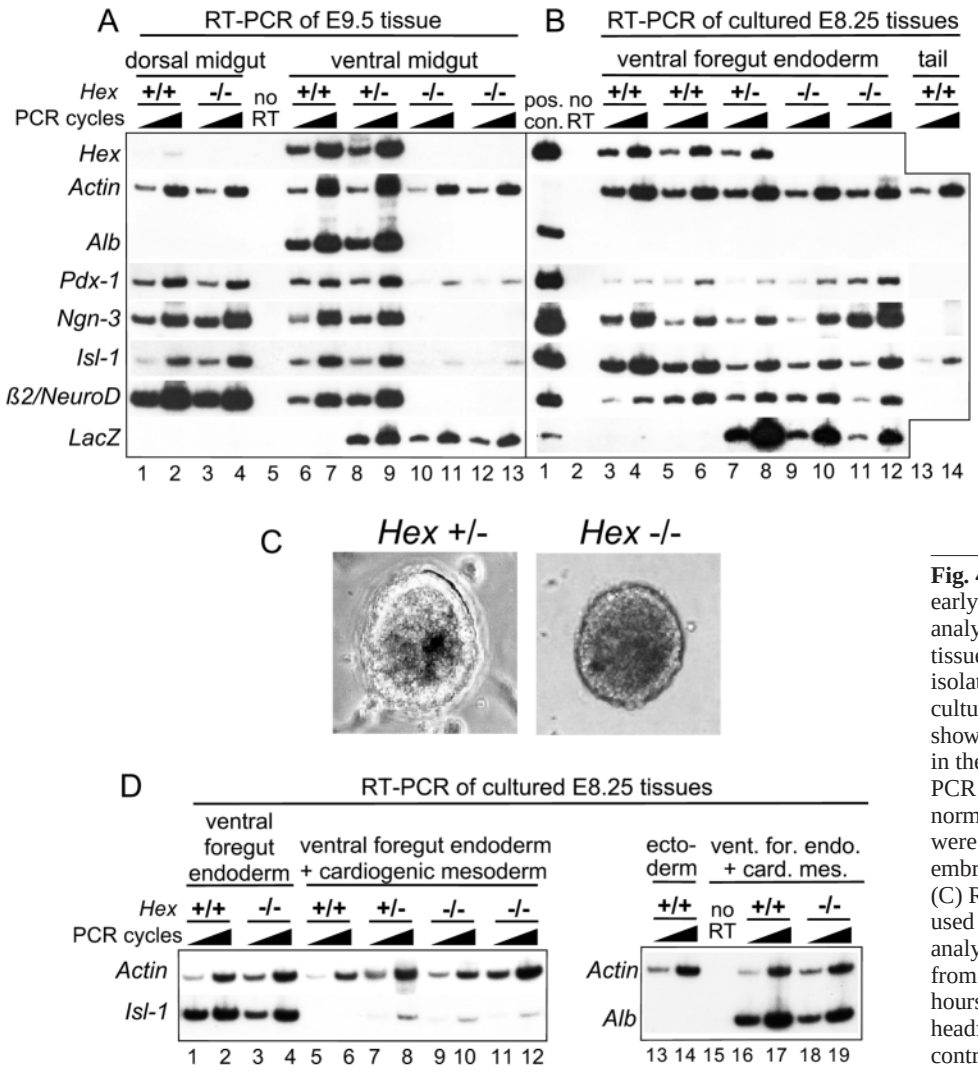

Fig. 4. *Hex* is not required for expression of early pancreatic genes in vitro. (A,B) RT-PCR analysis of E9.5 dorsal and ventral midgut tissues and of ventral endoderm explants isolated from E8.25 (2-6S) embryos and cultured for 48 hours. Different cycle steps are shown; albumin and actin PCR was performed in the same PCR reaction (Gualdi et al., 1996). PCR cycle numbers for other markers were normalized by actin levels. Positive controls were embryonic liver for albumin and E13.5 embryonic RNA for the other genes. (C) Representative ventral endoderm cultures used for the RT-PCR shown in B. (D) RT-PCR analysis of ventral endoderm explants isolated from E8.25 (2-6S) embryos and cultured for 48 hours with or without cardiogenic mesoderm; a headfold ectoderm explant served as a negative control.

and the expression of dorsal pancreatic factors, such as Isl1 at these stages, was normal (Fig. 3Q,R), indicating that the midgestation lethality of the *Hex* mutation does not block pancreatic development per se. We conclude that *Hex* is required for the initial specification of the ventral pancreatic endoderm, and in the absence of such, there is a complete failure in pancreatic morphogenesis and differentiation.

Hex is not necessary to activate the ventral pancreatic program

Although the pancreatic specification defect in *Hex*-null embryos superficially might resemble that seen with other transcription factor mutants that block dorsal or ventral pancreatic development (Edlund, 2002; Wilson et al., 2003), we wished to rigorously assess whether *Hex* is directly required to activate early pancreas genes, or if it controls pancreatic fate by an indirect, morphogenetic mechanism. We therefore used an embryonic tissue explant system (Gualdi et al., 1996; Deutsch et al., 2001). Ventral-lateral, definitive endoderm was isolated from 4-6S embryos, prior to the time of pancreatic and hepatic induction, and was cultured for 48 hours in the absence of cardiogenic mesoderm. We previously showed that such *Foxa2*-positive explants contain trace amounts of septum transversum mesenchyme cells (Rossi et al., 2001) and default to a pancreatic fate (Deutsch et al., 2001). The $Hex^{-/-}$ endoderm explants showed normal viability and, like wild-type endoderm explants, exhibited little if any growth (Fig. 4C) (Jung et al., 1999; Deutsch et al., 2001). The ability of the dissected ventral-lateral endoderm to initiate hepatic gene expression, if cultured with cardiogenic mesoderm (Fig. 1B; Fig. 4D, lanes 16-19), proves that we were isolating prospective ventral foregut endoderm from the *Hex*-null embryos. We performed RT-PCR analysis on ventral endoderm explants without cardiogenic mesoderm and compared the gene expression patterns with those of ventral and dorsal midgut domains of intact E9.5 embryos, which represent a comparable developmental stage (Deutsch et al., 2001). We note that the *Hex*-null endoderm explants without cardiogenic mesoderm remained *Alb* negative, like wild-type endoderm explants (Fig. 4B, lanes 3-12) (Jung et al., 1999). The wild-type, E9.5 ventral midguts (uncultured) expressed *Alb*, as expected, whereas the comparable tissue from *Hex*-null embryos at E9.5 did not, as previously reported (Fig. 4A, lanes 6-13) (Keng et al., 2000). Also, the *Hex*-null explants expressed the Hex^{lacZ} marker (Fig. 4B, lanes 9-12), further emphasizing that we isolated the correct cell population (see Fig. 2A).

Strikingly, RT-PCR analysis of explant RNAs showed that the *Hex*-null ventral endoderm appeared fully competent to activate early pancreas genes, including *Pdx1* and the pro-endocrine genes *Isl1*, *Ngn3*, and *β2/NeuroD* (Ahlgren et al., 1997; Gradwohl et al., 2000; Naya et al., 1995; Schwitzgebel et al., 2000) (Fig. 4B, lanes 9-12). This was in sharp contrast to the in vivo situation, where, by E9.5, the $Hex^{-/-}$ ventral endoderm failed to activate these genes (Fig. 4A, lanes 10-13). The low levels of *Pdx1* and *Isl1* expression seen in the ventral midgut RNAs of E9.5 $Hex^{-/-}$ embryos in Fig. 4A can be accounted for by the normal expression of these genes in prospective duodenal (Fig. 3K) and lateral mesenchymal cells (Fig. 3Q), respectively (Ahlgren et al., 1997; Offield et al., 1996). In addition, the induction of high levels of *Isl1* expression in the $Hex^{-/-}$ ventral endoderm explants was suppressed when cardiogenic mesoderm was included (Fig. 4D, compare lanes 3, 4 with 9-12), as in control embryos (Fig. 4D, lanes 1, 2, 5-8) (Deutsch et al., 2001).

These data show that despite the complete absence of ventral pancreas development in *Hex*-null embryos, *Hex* is not necessary for the ventral foregut endoderm to activate early pancreatic genes. We conclude that in *Hex*-null embryos, the failure of an endodermal domain to be positioned beyond the cardiogenic mesoderm, and escape its hepatic-inducing signaling, results in the absence of the pancreatic program. Thus, *Hex* normally controls the growth and positioning of a specific endodermal domain to allow ventral pancreas organogenesis.

Discussion

Many *Hox* and other homeobox-containing genes are expressed in the endoderm and are important for gut organ induction (Wells and Melton, 1999), yet little is known about how these genes control organogenesis. We have shown that the homeobox gene *Hex* controls the growth and positioning of a limited domain of the endodermal epithelium, to allow tissue specification. As summarized in Fig. 5, ventral endoderm that grows sufficiently past the cardiogenic mesoderm at the 6-7S stage, when the mesoderm elaborates a hepatogenic signal (Jung et al., 1999), will execute the pancreas program (Fig. 5A,B). In *Hex*-null embryos (Fig. 5C,D), a defect in definitive endoderm growth beyond cardiogenic mesoderm allows for hepatic but not pancreatic induction, despite the intrinsic competence of the mutant

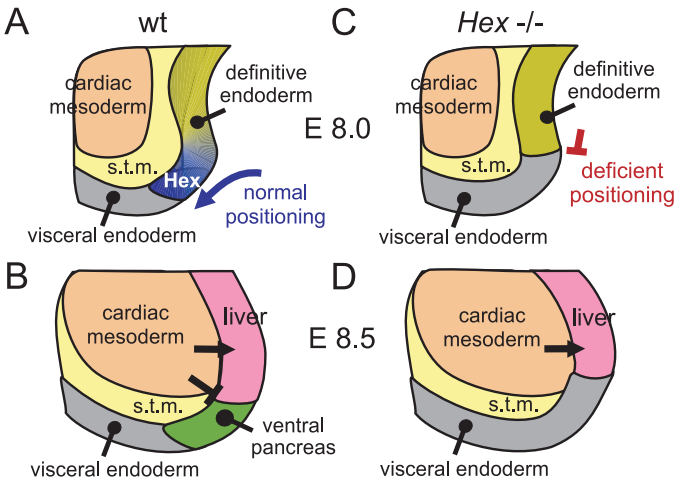

Fig. 5. Hex control of ventral foregut morphogenesis and patterning. (A,B) In wild-type embryos, *Hex* (expressed in the blue domain) maintains the proliferation rate of the leading edge of ventral definitive endoderm cells during gut closure, resulting in the normal positioning of cells beyond the cardiogenic mesoderm and allowing the execution of the ventral pancreatic fate. (C,D) Owing to a defect in the proliferative rate of ventral definitive endoderm cells in $Hex^{-/-}$ embryos, the cells do not become positioned beyond the hepatogenic influence of the cardiac mesoderm and into the normal position of pancreatic induction, and the pancreas is not specified. Liver cells are still induced, but they continue to be less proliferative. s.t.m., septum transversum mesenchyme.

endoderm to initiate the pancreatic program. These findings reveal how tissue positioning and the timing of cell interactions are precisely controlled in the embryo to pattern the gut, and support a role for *Hex* in controlling endodermal morphogenesis, rather than tissue-specific gene programs.

Although both the pancreas and liver phenotypes in *Hex*-null embryos appear to be caused by the common defect in localized endoderm cell proliferation, the phenotypes manifest themselves differently. None of the earliest genes tested that are specific to pancreas are activated in the ventral endoderm in $Hex^{-/-}$ embryos, at E8.5 or later, neither is there ventral pancreatic bud morphogenesis, indicating a defect that prevents all ventral pancreatic organogenesis (Fig. 3, Fig. 4A). By contrast, all of the earliest genes tested that are specific to liver are activated in the ventral endoderm of $Hex^{-/-}$ embryos (Fig. 1A,B), and the endoderm exhibits clear thickening as the hepatic endoderm cells become columnar (Fig. 1D). Further analysis will determine whether once the liver domain is specified, poor proliferation or migration of the hepatic endoderm cells causes them to be diluted out, or if the cells de-differentiate, by E9.5.

Although Hex, like other homeobox factors, was originally considered to regulate differentiation genes (Brickman et al., 2000; Denson et al., 2000), there are examples of Hex controlling cell proliferation in other developmental contexts. Newman et al. (Newman et al., 1997) showed that in *Xenopus* embyros, *Hex* is expressed in vascular endothelial cells and that overexpression increases endothelial cell numbers. *Hex* is expressed in basal cells of the developing chick dermis (Obinata et al., 2002) and overexpression in those cells in culture increases both their proliferative rate and the epidermal area. We found that when *Hex* is deleted, the proliferation of endodermal epithelium cells decreases and there is a decrease in the area spanned by those cells. Thus, *Hex*-controlled proliferation, at the cellular level, dictates the rate at which the leading edge of the endodermal epithelium is positioned relative to the cardiogenic mesoderm. This apparently becomes a timing mechanism so that a segment of the endoderm can avoid the onset of hepatogenic signaling by the cardiac mesoderm at the 6-7 S stage (Jung et al., 1999) and thereby initiate pancreatic development.

The ability of *Hex*-null ventral endoderm to activate liver but not pancreas genes in vivo, and pancreas but not liver genes in vitro (in the absence of cardiac mesoderm), provides further evidence that this endoderm cell population is bipotent for hepatic and pancreatic fates (Deutsch et al., 2001). Lineage tracing studies of Kawaguchi et al. (Kawaguchi et al., 2002) show that in *Ptf1a*-null embryos, which fail to specify a ventral pancreas but have normal amounts of ventral endoderm, and thus are unlike *Hex*-null embryos, the ventral endoderm cells adopt a duodenal-intestinal fate. Taking all of these studies together, the data suggest that prospective ventral-lateral endoderm cells may be competent to execute at least three different fates. Hepatogenic signaling induces the liver fate, an as yet unidentified signal induces or permits the pancreatic fate (Lammert et al., 2001; Kumar et al., 2003), and in the absence of both cardiac signaling and the expression of *Ptf1a*, the cells execute an intestinal-duodenal fate.

In summary, the *Hex* homeobox gene controls the ventral pancreatic program indirectly, by maintaining the proliferation rate and consequently the positioning of ventral foregut endoderm cells relative to the mesoderm. It remains to be seen whether other endodermal transcription factors besides *Hex* control the position of the endoderm relative to other mesodermal and ectodermal signaling centers. An HMG box-containing gene that controls endoderm development, *Sox17* (Kanai-Azuma et al., 2002), also affects the amount of definitive endoderm cells and the activation of *Pdx1*. *Sox17* also affects endodermal apoptosis, though how this may be connected to patterning is unknown. We suggest that *Sox17* and perhaps other early endodermal transcription factors (Wells and Melton, 1999) could be more critical for the morphogenetic control of tissue interactions than for the direct regulation of cell type-specific genes during patterning. Continuing with this theme, at later stages in development or in adults, such transcription factors could be more important for the growth of different cells within an emerging or regenerating tissue, thereby affecting the timing and extent of cell interactions that are required for further differentiation events.

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