A bipotential precursor population for pancreas and liver within the embryonic endoderm

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SUMMARY

The pancreas emerges independently from dorsal and ventral domains of embryonic gut endoderm. Gene inactivation experiments in mice have identified factors required for dorsal pancreas development, but factors that initiate the ventral pancreas have remained elusive. In this study, we investigated the hypothesis that the emergence of the ventral pancreas is related to the emergence of the liver. We find that the liver and ventral pancreas are specified at the same time and in the same general domain of cells. Using embryo tissue explantation experiments, we find that the default fate of the ventral foregut endoderm is to activate the pancreas gene program. FGF signalling from the cardiac mesoderm diverts this endoderm to express genes for liver instead of those for pancreas. No evidence was found to indicate that the cell type choice for pancreas or liver involves a selection for growth or viability. Cardiac mesoderm or FGF induces the local expression of sonic hedgehog, which in turn is inhibitory to pancreas but not to liver. The bipotential precursor cell population for pancreas and liver in embryonic development and its fate selection by FGF has features that appear to be recapitulated in the adult pancreas and are reflected in the evolution of these organs.

Key words: Pancreas, Liver, Organogenesis, Differentiation, Cell signalling, FGF, Shh, Pdx1, Mouse

INTRODUCTION

The signalling mechanisms that control the specification of endoderm-derived organs such as the pancreas, thyroid, liver, lung and intestine have only recently begun to be investigated. While much is known about how these organs undergo differentiation, growth and morphogenesis (Hogan, 1999), we know little about how different domains of gut endoderm give rise to these tissues in the first place (Wells and Melton, 1999; Gannon and Wright, 1999; Zaret, 2000). Understanding how different cell types are specified from the gut endoderm will provide insight into controlling cell differentiation for therapeutic purposes. In this paper we investigate the possibility of a common precursor cell for the pancreas and liver in development and the means by which the cell type choice is made.

The pancreas originates from distinct embryonic outgrowths of the dorsal and ventral regions of the foregut endoderm, with both outgrowths giving rise to endocrine and exocrine cells (Spooner et al., 1970; Slack, 1995). In comparison, the liver develops solely from the ventral foregut endoderm (Zaret, 1996), adjacent to where the ventral pancreas emerges. Various lines of evidence suggest that there are differences in the specification of the dorsal and ventral pancreatic rudiments. In the chick, signals from the overlying notochord promote dorsal

pancreas development by repressing endodermal expression of sonic hedgehog (Kim et al., 1997; Hebrok et al., 1998; Apelqvist et al., 1997; Kim and Melton, 1998); notochord does not influence gene expression in the ventral pancreatic anlage (Kim et al., 1997; Hebrok et al., 1998). Gene inactivation studies in mice have shown that the dorsal pancreatic rudiment is dependent upon the transcription factors Isl1 and Hlxb9; the ventral pancreatic rudiment still develops when these genes are inactivated (Ahlgren et al., 1997; Harrison et al., 1999; Li et al., 1999). The parameters that specify the ventral pancreas are unknown.

Tissue explant experiments show that ventral pancreas specification occurs at the 7- to 9-somite stages (Wessells and Cohen, 1967), whereas morphogenesis of the ventral pancreatic bud is not detected until the 20-somite stage (Spooner et al., 1970). The expression of the earliest known pancreas markers, Hlxb9 and the Pdx1 homeobox protein (Jonsson et al., 1994; Guz et al., 1995; Offield et al., 1996) were reported to be expressed in the ventrolateral foregut at the 8-somite stage (Li et al., 1999). These transcription factors probably respond to the primary signals for pancreatic specification and denote the pancreatic stem cell, prior to morphogenesis. Pdx1 is necessary for the morphogenesis and differentiation of the pancreatic epithelium, after glucagon and insulin expression are initiated at the bud stage (Offield

et al., 1996; Ahlgren et al., 1996). Though the initial Pdx1 expression domain includes part of the future bile duct and duodenum (Offield et al., 1996), Pdx1-positive cells give rise to Isl1- and ngn3-positive cells as progenitors of the endocrine lineage (Ahlgren et al., 1997; Gradwohl et al., 2000) and to p48-positive cells as progenitors of the exocrine lineage (Krapp et al., 1998).

Cell marking studies have definitively shown that both endocrine and exocrine lineages arise from the endoderm (Percival and Slack, 1999). Also, chimeric mouse studies show that islets of endocrine cells are polyclonal in origin (Deltour et al., 1991). These findings predict that a population of uncommitted endoderm cells should behave uniformly as pancreas stem cells. Tissue explant and transplant studies investigating the role of mesenchymal signals in the development of the endocrine and exocrine lineages have employed pancreatic bud tissue isolated well after the time of pancreatic specification (e.g., 15-25 somites or greater; see Wessells and Cohen, 1967; Rutter et al., 1978; Sanvito et al., 1994; Gittes et al., 1996; Miralles et al., 1998; Miralles et al., 1999), leaving open the potential role of mesoderm in controlling the formation of the pancreatic stem cell.

The temporal and physical proximity of budding sites for the liver and ventral pancreas have led us to explore how the specification of these two tissues might be related. In the nascent ventral foregut, the cardiac mesoderm becomes closely apposed to the endoderm, and tissue transplant and explant studies have shown that proximity to the cardiac mesoderm is necessary for the ventral endoderm to develop into the liver (LeDouarin, 1964; LeDouarin, 1975; Gualdi et al., 1996). In mouse embryos, these interactions occur by the 7- to 8-somite stages, at which time liver-specific genes are activated (Cascio and Zaret, 1991; Shiojiri et al., 1991; Gualdi et al., 1996). In an endoderm explant assay, redundant fibroblast growth factor (FGF) signalling from the cardiac mesoderm is necessary and sufficient to induce hepatogenesis within the ventral foregut endoderm (Jung et al., 1999).

In the present study, we investigate how pancreas and liver cells emerge from the ventral foregut. The results indicate that the cell type choice is controlled by signalling from the cardiac mesoderm. Various aspects of this system illustrate that previously documented changes in the differentiation of adult pancreas cells recapitulate a cell type choice in development.

MATERIALS AND METHODS

Isolation and RNA analysis of mouse embryo tissues

After mating C3H mice, noon of the day of the appearance of a vaginal plug was considered as E0.5. Embryo tissues were harvested at E8-8.5 and RNA was isolated and subjected to RT-PCR as described previously (Gualdi et al., 1996; Jung et al., 1999). In some experiments, endoderm was isolated by incubating the anterior portion of the embryo in a solution of 0.125% pancreatin (Sigma), 0.025% polyvinylpyrrolidone-40, and 20 mM Hepes (pH 7.5) in PBS at 4°C for 4-5 minutes. The actin reactions (see Fig. 1B) were the same as those described in Fig. 1B of Gualdi et al. (Gualdi et al., 1996). Pdx1 reactions initially employed primers with the sequences: #436 (bottom strand, for RT) 5′-CCCGCTACTACGT-TTCTTATCTTCC-3′ and #430 (top strand) 5′ GGATGAAA-TCCACCAAAGCTCAC-3′ (285 bp product). In later experiments

we used #556 (top strand) 5′-AATCCACCAAAGCTCACG-CGTGG-3′ (279 bp product).

Embryo tissue cultures and RNA analysis

Embryo tissue explants were cultured in microwells (CoStar) coated with type I collagen (Collaborative Biomedical Products) in DMEM containing 10% calf serum (Hyclone) at 37°C in the presence of 5% CO_2. In some experiments, we added either Matrigel to 10%, recombinant human FGF2 (Boehringer Mannheim) or mouse FGF8b (R & D Systems) to 5 ng/ml, or Shh-N′ peptide as described by Marti et al. (Marti et al., 1995; generously provided by A. McMahon). Endoderm isolated from the midline of the ventral foregut was less frequently positive for pancreatic markers than endoderm fragments that included lateral regions, most likely due to the proximity of the midline ventral endoderm cells to the prominent cardiac bulge. Additional primers for RT-PCR were: Isl1, #547 (bottom strand) 5′-GACTGAGAGGGTCTCCAGCTC-3′ and #546 (top strand) 5′-AGCAAGAACGACTTCGTGATG-3′ (187 bp product) (Karlsson et al., 1990); BETA2/neuroD, #545 (bottom strand) 5′-GGAGTAGGG-ATGCACCGGGAA-3′ and #544 (top strand) 5′-CTTGGCCAAGAA-CTACATCTGG-3′ (228 bp product) (Naya et al., 1997); PTF1-p48, #555 (bottom strand) 5′-AGGAAAGGGAGTGCCCTGCAAG-3′ and #554 (top strand) 5′-GGCCCAGAAGGTCATCATCTGC-3′ (98 bp product) (Krapp et al., 1998); ngn3, #591 (top strand) 5′-CTGCGC-ATAGCGGACCACAGCTTC-3′ and #590 (bottom strand) 5′-CTTC-ACAAGAAGTCTGAGAACACCAG-3′ (233 bp product) (Huang et al., 2000).

Whole-mount staining analysis

Endoderm cultures were fixed overnight at 4°C in 4% paraformaldehyde buffered to pH 7.4 with PBS, then sequentially dehydrated and stored in 100% methanol at −20°C. The rabbit pdx1 antibody was generously provided by C. Wright; the rabbit Shh-N′ antibody was generously provided by A. McMahon; and the guinea pig antibodies to glucagon and insulin were from Linco Research, Inc., with normal guinea pig IgG as a control (the latter giving no signals; data not shown). Antibody reactions were performed as previously reported (Guz et al., 1995) with modifications. Cultures were first incubated with antiserum to pdx1 (1:300) and the bound antibody was visualized with the blue reaction product of a Vector SG substrate. Guinea pig antibodies were visualized with peroxidase-conjugated secondary antibodies (Sigma). Images of the antibody staining were taken and then the same culture was hybridized in situ as described with antisense probes to albumin mRNA (Gualdi et al., 1996) or HNF3β (Foxa2; Ang et al., 1993). The washing and prehybridization procedures eliminated the first antibody signals. Sense and antisense strand RNA probes were labeled with UTP-digoxigenin, used at 0.5-1.0 µg/ml, and detected with alkaline phosphatase-conjugated anti-digoxigenin (Boehringer Mannheim). Color development was for 30 minutes to 2 hour. Tissues were photographed either with a Nikon camera or a Pixera digital device mounted on a Nikon SMZ-U stereomicroscope.

Cell viability and labeling

For Nile Blue Sulfate and BrdU double labeling experiments, at the end of a 24 hour culture period, BrdU was added to a concentration of 10 µM for 30 minutes. After washing the tissues twice in Hank's Balanced Salt Solution (HBSS), they were incubated in 3 µg/ml of a 1 mg/ml Nile Blue Sulfate (Sigma) solution in water diluted either in HBSS or in 10% calf serum for 1 hour at room temperature. After washing the tissues in HBSS twice, they were photographed immediately. Punctate blue staining indicated dead or dying cells that retained Nile Blue. Tissues were then fixed in 70% ethanol/50 mM glycine (pH 2) at −20°C overnight, which removed the Nile Blue. BrdU incorporation was detected using the BrdU labeling and detection kit II (Boehringer Mannheim). Tissues were photographed digitally as described above.

RESULTS

Temporal and spatial relation between pancreas and liver specification

We first sought to define the exact time and place where the earliest markers of the pancreas and liver are activated. RT-PCR was used to investigate *Pdx1* gene expression in endoderm fragments dissected from the ventral foregut of mouse embryos at 8-8.5 days gestation (Fig. 1A). No *Pdx1* product was detected at the 4- to 6-somite stages, relative to the actin control, but abundant *Pdx1* product was evident at the 7- to 8-somite stages (Fig. 1B). Previously we found that serum albumin and other liver-specific genes were activated in the same ventral endoderm population of cells at the 7- to 8-somite stages (Gualdi et al., 1996). Thus, both pancreas- and liver-specific genes are activated in the ventral foregut endoderm at the same time.

We next examined the spatial relationship between the newly specified pancreatic and liver domains. In development, morphogenetic extension of the ventral foregut at the 7- to 8-somite stages results in a 'lip' of endoderm extending away from the cardiac mesoderm and toward the prospective midgut region (Fig. 1C). Considering that the cardiac mesoderm induces the liver, it seemed possible that the hepatic domain would be more closely associated with the nascent heart tissue. We adapted our dissection techniques to isolate the foregut lip of endoderm, distal to the cardiac domain, from the foregut endoderm still in contact with the cardiac mesoderm, using embryos at the 8- to 9-somite stages. (Fig. 1C). RT-PCR analysis of the tissues revealed that only the foregut endoderm near the cardiac mesoderm was albumin positive, whereas only the endoderm at the foregut lip was strongly Pdx1 positive (Fig. 1D). A small amount of Pdx1 expression was detectable in the endoderm sample dissected from the cardiac domain, which could reflect the difficulty in obtaining pure segments of tissue. We conclude that the nascent pancreatic and hepatic domains are, for the most part, non-overlapping.

Previously we described techniques for culturing ventral foregut endoderm and showed that when the cells were isolated after the time of liver gene activation in vivo, they would continue to express liver genes in vitro (Gualdi et al., 1996), demonstrating stable specification. When ventral foregut endoderm fragments were isolated at the 8-somite stage, cultured for 2 days, and doubly labeled for Pdx1 protein and albumin mRNA, a cluster of cells in the explants was frequently positive for Pdx1 protein (Fig. 1E, right panel) and a non-overlapping domain of the explant was positive for albumin mRNA (Fig. 1E, left panel). These data show that after the pancreas and liver are specified in vivo, distinct tissue domains are preserved in endoderm explants cultured in vitro.

Default pancreas fate of the early somite stage, ventral foregut endoderm

Considering that the ventral foregut endoderm from 2- to 6-somite stage embryos fails to activate liver-specific genes when cultured in the absence of cardiac mesoderm or FGF (Gualdi et al., 1996), we wished to determine whether this endoderm would also fail to activate pancreas-specific genes. We expected that it would because of the aforementioned studies of Wessells and Cohen (Wessells and Cohen, 1967) and Spooner et al. (Spooner et al., 1970), which showed that the

ventral foregut endoderm does not become specified to express a pancreatic fate until the 7- to 9-somite stages.

Surprisingly, immunostaining analysis showed that the *Pdx1* gene was activated in 24 of 27 samples of ventral foregut endoderm isolated from 2- to 6-somite stage embryos and cultured for 2 days (Fig. 2A,C,E, blue staining; Table 1). Similar results were obtained with very small explants that consisted almost entirely of endoderm, with just a few
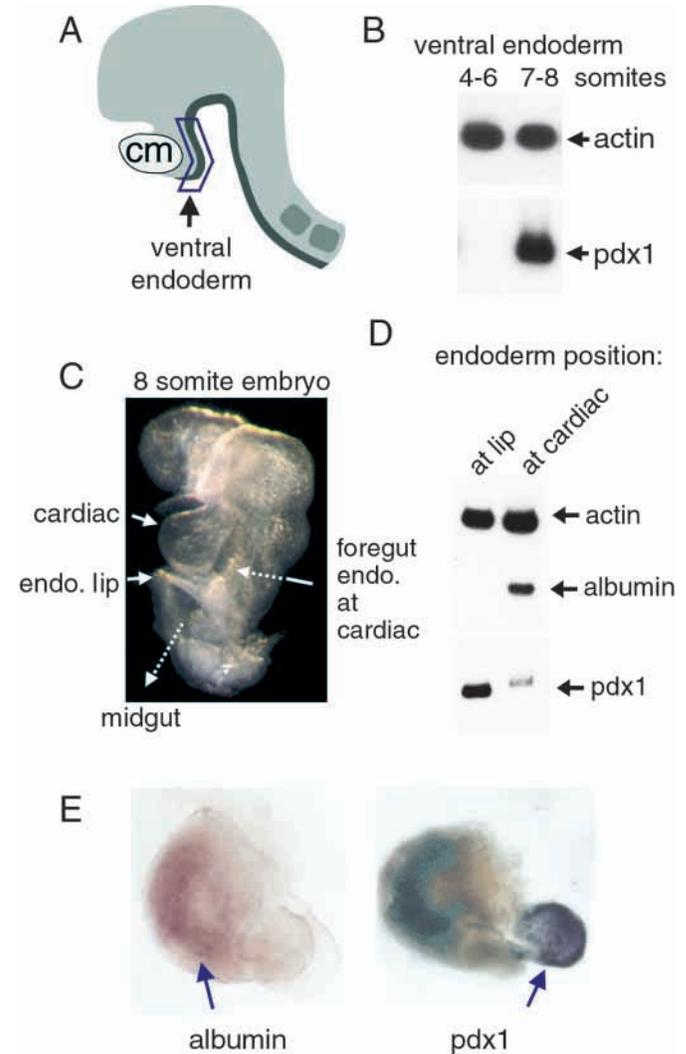

Fig. 1. Activation of Pdx1 at the time of pancreatic specification. (A) Side view of mouse embryo at the 4- to 6-somite stage. Boxed region shows area isolated for analysis; cm, cardiac mesoderm. (B) RT-PCR analysis of RNA from ventral foregut endoderms isolated at the designated somite stages. (C) Anterior half of an 8-somite embryo. Relevant domains are indicated. Dashed arrow for 'foregut endo. at cardiac' indicates that the endoderm is beneath the cells in this external view. (D) RT-PCR analysis of RNA from the foregut lip and the endoderm underlying the cardiac mesoderm. Albumin and actin probes were in the same reaction; Pdx1 was done separately. (E) Double labeling of a single ventral foregut endoderm fragment isolated at the 7- to 8-somite stage and cultured in vitro for 2 days. (Left panel) In situ hybridization for serum albumin mRNA. (Right panel) Immunostaining for Pdx1 protein. Arrows indicate positive domains (pink for albumin, purple for Pdx1). The dark green region in panel E, right, is due to tissue thickness, not Pdx1 staining.
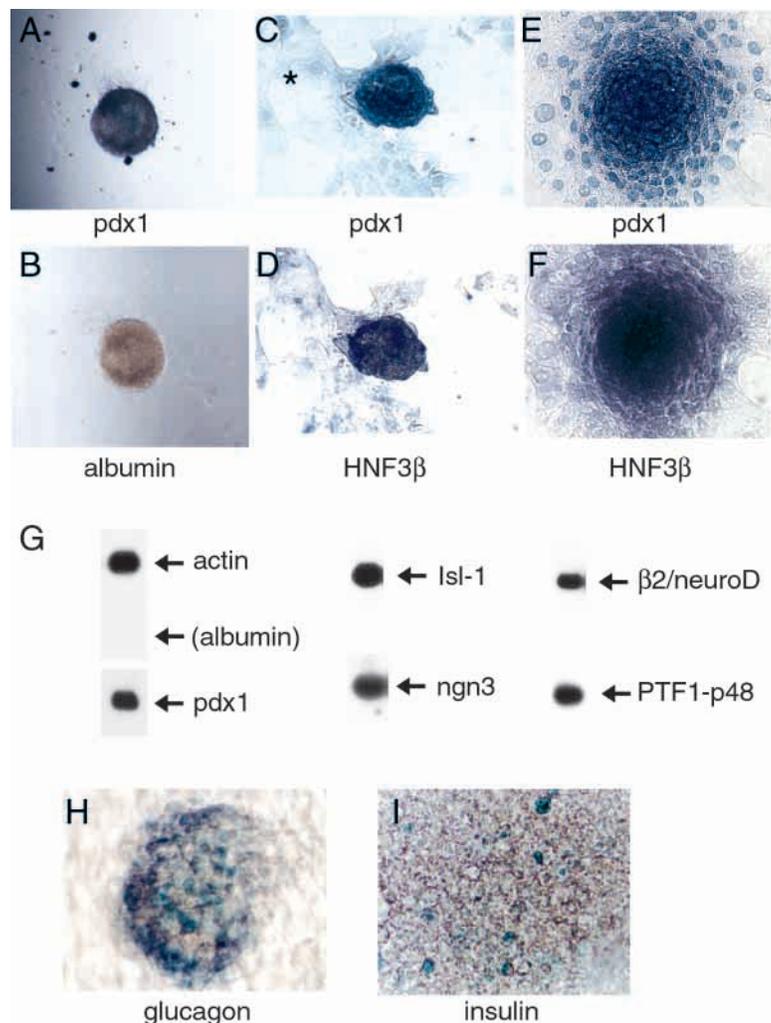
Fig. 2. Default expression of pancreas genes by isolated ventral endoderm. (A-F) Double labeling of ventral foregut endoderm fragments cultured for 2 days. (A,C,E) Immunostaining for Pdx1 protein; most of the cells stain positive (blue). Asterisk in C denotes nonstaining fibroblast cells. (B,D,F) Sequential in situ hybridization for albumin (B) or HNF3β (D,F) mRNA. Purple-brown staining of the epithilium reflects positive hybridization in D and F. (G) RT-PCR of RNA from cultures for designated markers. Note lack of expression of albumin despite addition of relevant primers to the actin control reactions. (H,I) Immunostaining for glucagon and insulin of ventral endoderm explants cultured for 5 days with Matrigel. Blue cells are positively stained. (A-D, original magnification 100×; E,F,H,I, 400×).

fibroblasts that helped the endoderm adhere to the substratum (Fig. 2A), and endoderm explants that existed on a monolayer of fibroblasts (Fig. 2C). Higher magnification views of more spread-out explants showed the expected nuclear staining for Pdx1 (Fig. 2E). Note that in these cultures, which lacked cardiac mesoderm, fibroblasts growing around the epithelium do not stain for Pdx1 (see area denoted by asterisk, Fig. 2C). RT-PCR analysis confirmed the expression of Pdx1 mRNA in the explants and the absence of the liver marker, albumin mRNA (Fig. 2G). Also, all of 13 ventral endoderm samples tested in the absence of cardiac mesoderm were negative for albumin mRNA, as assessed by in situ hybridization (Fig. 2B). In some cultures not all epithelial-like cells stained positive for Pdx1, and in a few cases only 10-20% of the cells were Pdx1 positive. However, double labeling experiments showed that most of the cells that were positive for HNF3β were also positive for Pdx1 (Fig. 2D,F; purple staining). Since HNF3β is expressed by the definitive endoderm (Sasaki and Hogan, 1993; Ang et al., 1993; Monaghan et al., 1993), we conclude that most or all of the endoderm portions of the cultures initiated Pdx1 expression in vitro.

To investigate the degree to which the ventral foregut endoderm would continue pancreatic development in vitro, we assayed other markers. RT-PCR analysis detected transcripts for the endocrine pancreas transcription factors Isl1 (Ahlgren

et al., 1997), ngn3 (Atoh5; Huang et al., 2000), and BETA2/ neuroD (neurod1; Naya et al., 1997) and the exocrine transcription factor PTF1-p48 (Krapp et al., 1998; Fig. 2G). To further characterize their developmental potential, we maintained cultures in 10% Matrigel medium for 6 days as described by Hebrok et al. (Hebrok et al., 1998) and performed immunostaining for glucagon and insulin. As seen in Fig. 2H and I (blue stain), isolated cells and clusters of cells were

Table 1. Summary of in situ immunostaining and hybridization

	Pdx1		Albumin	
Culture conditions	−	+	−	+
Ventral endoderm (VE)	3	24	13	0
Embryo tail sections	10*	0	10*	0
VE+heparan sulfate (h.s.)	0	4	3	0
VE+h.s.+FGF2	15	2‡	0	10
VE+h.s.+FGF8b	6	5	2	4§
VE+cardiac mesoderm	34	9**	3¶	14

*Includes samples cultured in presence of FGF2 and FGF8b.
‡Rare positive cells present.
§See (Jung et al., 1999) for additional samples.
**Positive cells were distant from the cardiac area.
¶While albumin expression in such cultures is sometimes not detectable by in situ hybridization, it is invariably detectable by RT-PCR.

A pdx1   B albumin   C pdx1

◯ = beating cardiac cells

Fig. 3. Proximity to cardiac mesoderm excludes pancreatic fate. Fragments of ventral endoderm and cardiac mesoderm isolated from 2- to 6-somite embryos and cultured for 2 days. (A-C) Dashed circled areas denote beating cardiac cells. Arrows denote areas of positive staining (red-purple, albumin; bluish, Pdx1). The lack of purple staining in A indicates lack of Pdx1 expression, as confirmed by RT-PCR (see Fig. 4G).
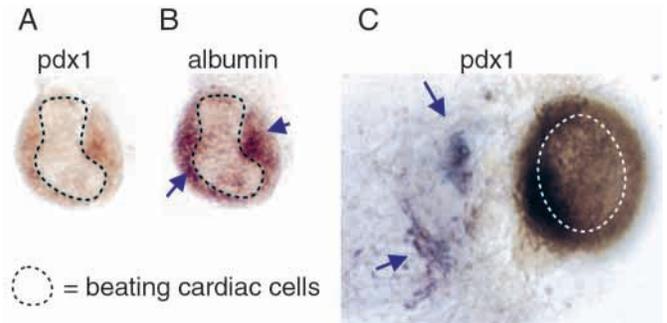
glucagon or insulin positive. These in vitro studies mimic the normal pattern of endocrine development, which is initially evident as individual cells or small clusters that later form islets (Stefan et al., 1983). We conclude that the ventral endoderm explants give rise to various pancreatic endocrine lineages and to the p48-positive stage of the exocrine lineage, as predicted from in vivo studies.

Several lines of evidence indicate that our culture conditions do not induce pancreas development per se. Previously we showed that the prospective dorsoposterior endoderm, which normally develops into the intestine, becomes albumin positive when isolated from inhibitory mesoderm and cultured identically to the conditions described here (Gualdi et al., 1996; Bossard and Zaret, 1998). RT-PCR analysis of RNA from such colonies showed that they are Pdx1 negative (see Fig. 4E, lane 6 below). Also, embryo tail sections failed to stain for Pdx1 antigen after 2 days in culture (Table 1). Thus, our culture conditions do not generally induce pancreatic genes in dorsal endoderm or other tissues.
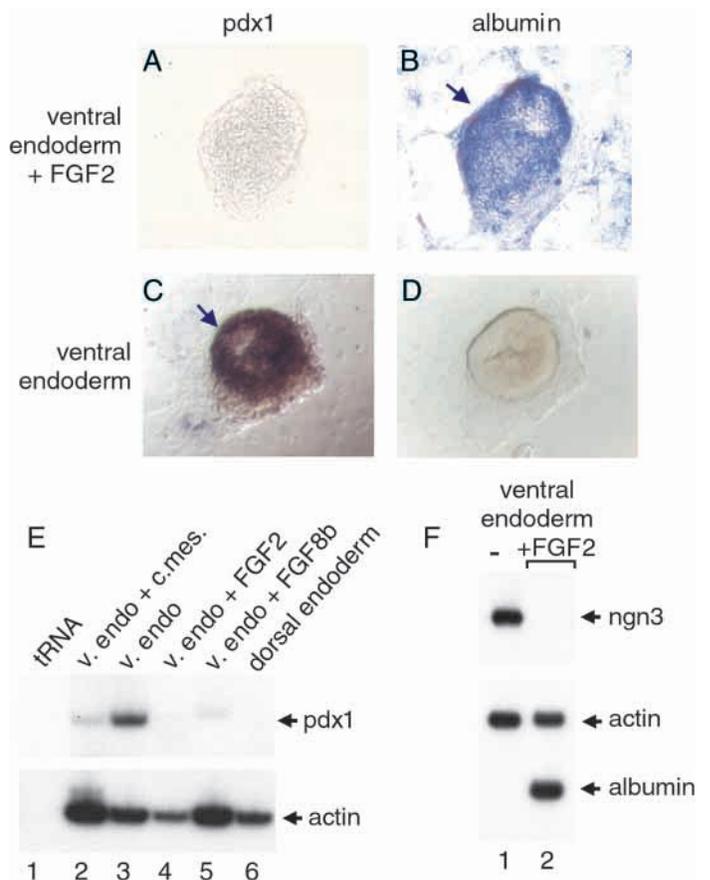
While observing a pancreatic fate prior to the 7- to 9-somite stages might seem at odds with the work of Wessells and Cohen (1967) and Spooner et al. (1970), they stated that their early somite stage ventral endoderm explants consisted of the whole trunk region, which included mesodermal components such as cardiac mesoderm. Signals from another germ layer in their cultures might have restrained the pancreatic fate from being expressed (see below). We conclude that if the ventral foregut endoderm is excised from the embryo after somitogenesis but before the normal time of hepatic specification, it appears predisposed to express pancreas genes, whereas if the endoderm is excised after the time of hepatic specification, it expresses a pancreas fate in a domain that is separate from the nascent hepatic cells.

Close proximity to cardiac mesoderm excludes pancreas

We next asked whether close proximity to the cardiac mesoderm, which normally induces the liver, is inhibitory to the pancreas program within the ventral endoderm. By

establishing ventral endoderm co-cultures with cardiac mesoderm from the 2- to 6-somite stage embryos and allowing hepatic specification to occur in vitro, we could assess the spatial relationship between the cardiac and newly specified pancreatic domains. In such explants, the cardiac mesoderm progresses to the beating stage and therefore the cells are readily distinguishable (Gualdi et al., 1996). As seen in Fig. 3A,B (arrows), where small explants consisted mostly of cardiac cells, the non-beating cells that surrounded the cardiac region were consistently Pdx1 negative (i.e., not blue) and albumin positive (reddish-purple staining). In larger explants, Pdx1 was either not expressed or Pdx1-positive cells occurred in isolated patches at a distance from the cardiac domain (Fig. 3C, purple domains). In a total of 43 cocultures, 34 had no Pdx1 expression and 9 had patchy, distal expression (Table 1). Furthermore, RT-PCR analysis of endoderm co-cultured with cardiac mesoderm showed diminished Pdx1 expression,

Fig. 4. FGF signalling diverts endoderm from pancreas to liver. (A-D) Ventral foregut endoderm fragments from 2- to 6-somite embryos cultured for 2 days with (A,B) or without (C,D) 5 ng/ml FGF2 as indicated, and immunostained for Pdx1 (A,C). The samples were photographed, washed free of stain, then hybridized in situ with an albumin antisense probe (B,D). The sample in B gave more bluish staining (arrow) due to the use of a blue filter. In C, most of the endoderm stained positive (arrow). (E,F) RT-PCR analysis of ventral and dorsal endoderm cultures in the presence or absence (−) of 5 ng/ml FGF2 or cardiac mesoderm (c.mes.).
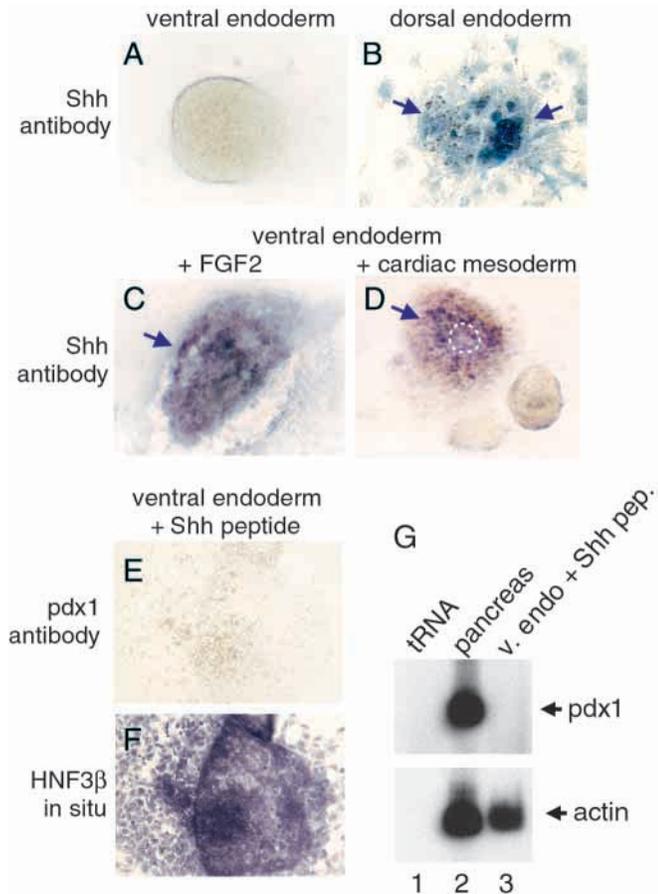
Fig. 5. Induction of Shh in ventral endoderm and suppression of
Pdx1 expression. (A-E) Ventral or dorsal foregut endoderm from 2 to
6-somite embryos cultured alone (A,B), with 5 ng/ml FGF2 (C), with
cardiac mesoderm (D, inside dashed circle) or with 50 ng/ml Shh
peptide (E,F) for 2 days. Explants were stained for Shh or Pdx1
expression with antibodies. Pre-immune Shh antibody gave no signal
(data not shown). Arrows denote areas of positive staining. The
explants in A and E failed to stain with antibody. F shows an in situ
hybridization for HNF3β on the same sample as in E. (G) RT-PCR
analysis showing that Pdx1 expression is suppressed in ventral
foregut endoderm treated with Shh peptide.

relative to endoderm cultured alone (see Fig. 4E, lanes 2, 3
below). Taking the in vivo and in vitro data together, we
conclude that the cardiac mesoderm not only induces the liver
from the endoderm, it also inhibits pancreas development in its
immediate vicinity.

Liver versus pancreas fate selection by FGF signalling

Recently, it was shown that FGF signalling from the cardiac
mesoderm is necessary and sufficient to induce hepatogenesis
within the ventral foregut endoderm, in explant assays (Jung
et al., 1999). Although FGFs are secreted proteins, they are
retained locally by the extracellular matrix and can serve as
short-range signalling molecules (Maciag et al., 1984;
Klagsbrun, 1989; Olwin and Rapraeger, 1992; Bikfalvi et al.,
1997). Since FGF1 or FGF2 can induce multiple liver-specific
genes in foregut endoderm isolated from the 2- to 6-somite
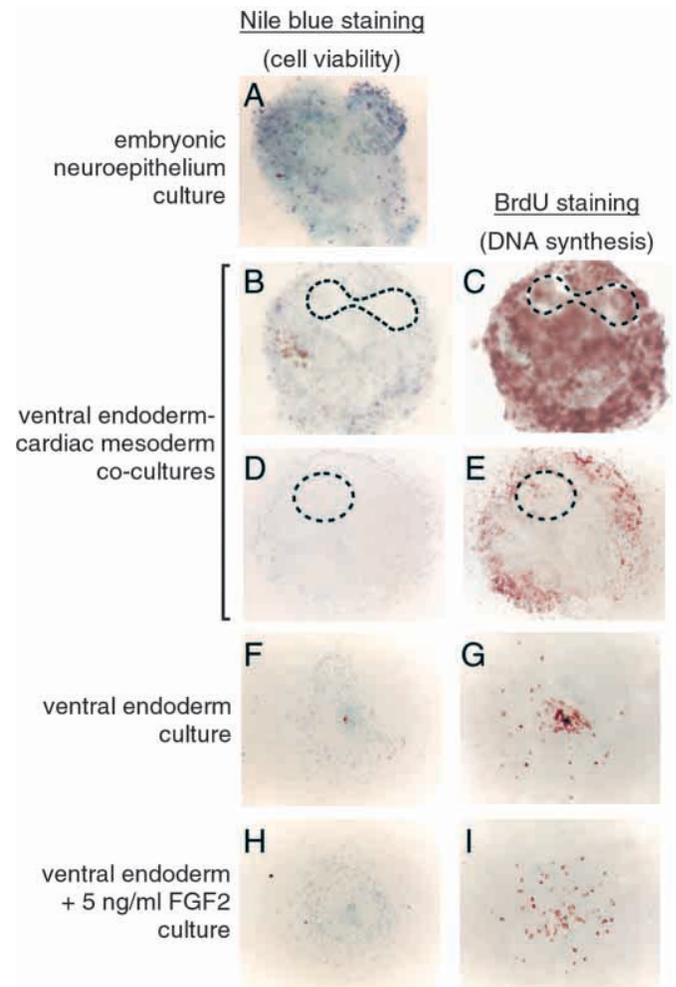stage embryos, we asked whether FGFs affect the expression

Fig. 6. Lack of selection for growth or viability in the differentiating
endoderm. (A,B,D,F,H) Nile Blue staining, which marks dead or
dying cells. Images are of live cultures. (A) Positive control tissue
exhibits punctate Nile Blue staining. (B-I) Endoderm samples that
were doubly stained with Nile Blue and for BrdU incorporation, the
latter marking replicating cells. Brdu staining is of fixed cultures.
(B-E) Areas within the dashed lines are beating cardiac cells. Neither
Nile Blue nor BrdU stain preferentially around the beating areas,
therefore failing to support a cell selection model for the influence of
cardiac mesoderm on ventral endoderm cell fate.

of Pdx1 in the ventral endoderm explants. Importantly, if the
pancreatic and hepatic domains are pre-patterned separately,
FGF signalling should have minimal effect on Pdx1
expression. If, however, a segment of the ventral foregut
endoderm is multipotent with regard to pancreatic and hepatic
fates and if FGF signalling diverts the endoderm cells to a
hepatic fate, uniform exposure of the cells to FGF should
prevent Pdx1 expression throughout the culture.

Ventral foregut endoderm explants from 2- to 6-somite
stage embryos were cultured in vitro and stained with the Pdx1
antibody. In 16 explants cultured in the presence of 5 ng/ml
FGF2, an amount that efficiently activates the liver program
(Jung et al., 1999), 14 explants exhibited no Pdx1 staining and
2 contained only rare Pdx1-positive cells (Table 1; Fig. 4A).
Furthermore, albumin mRNA was typically expressed

Fig. 7. Proposed phases of early ventral pancreas development. (A,B) Diagram of sagittal cross section through ventral foregut region. (A) Early patterning by the mesoderm (arrow) helps create bipotentiality of the ventral foregut endoderm for pancreas and liver. (B) FGF signalling from the cardiac mesoderm diverts proximal endoderm from a pancreatic to a hepatic fate (pink), as evidenced by differences in the expression of the genes shown. Endoderm that is not proximal to the cardiac mesoderm, e.g. at the lip that extends the foregut to the midgut, becomes pancreas (dark green). While the cardiac mesoderm has a net positive effect on hepatic induction (arrow), mechanistically it appears to block an intrinsic program for pancreas (barred lines) and thereby promote liver within the ventral foregut endoderm. (C) Further signalling and morphogenesis results in distinct organs.
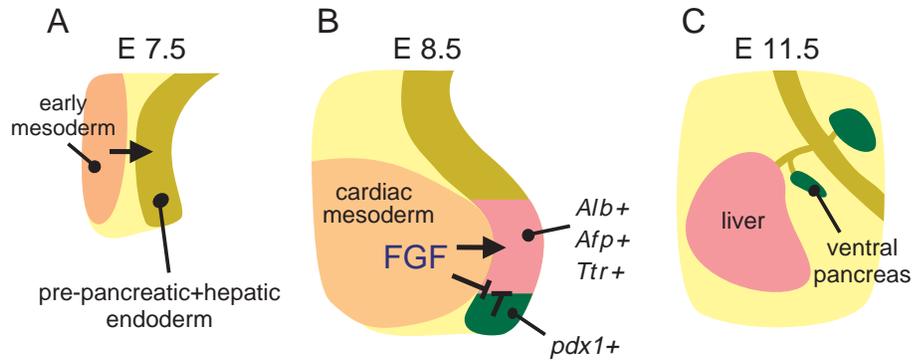
throughout the endoderm explants cultured in the presence of FGF2 (Fig. 4B, arrow; see control in 4D) and other liver markers were positive by RT-PCR (data not shown; see Jung et al., 1999). By contrast, as shown in Fig. 2A,C,E, and in the additional control explant in Fig. 4C (dark staining), Pdx1 was activated in ventral endoderm explants cultured in the absence of FGFs. Previously we found that 5 ng/ml FGF8b did not efficiently induce hepatic gene expression in the ventral foregut endoderm (Jung et al., 1999), and here we also found that FGF8b was less effective in preventing Pdx1 expression, with only 5 out of 11 cultures activating albumin and failing to express Pdx1 (Table 1). RT-PCR analysis confirmed that the FGFs suppressed Pdx1 expression in the ventral endoderm cultures (Fig. 4E, lanes 3-5). In addition, the presence of FGF2 blocked the induction of ngn3 (Atoh5) in most ventral endoderm cultures, while it activated albumin expression (Fig. 4F, lanes 1, 2). Because most of these ventral foregut endoderm cells appear competent to express either liver- or pancreas-specific genes, we suggest that FGF signalling can divert endoderm cells from a default pancreas fate to a hepatic fate.

The default pancreas fate of the ventral foregut endoderm from 2- to 6-somite stage mouse embryos appears distinct from that described in the Introduction for chick dorsal endoderm (Kim et al., 1997; Hebrok et al., 1998). In agreement with these chick studies, we also found that mouse dorsal endoderm, isolated from notochord, fails to elicit Pdx1 expression in culture (Fig. 4E, lane 6). Thus, in the absence of mesodermal signaling, there are distinct default fates for the ventral and dorsal foregut endoderm. These differences seem likely to account for the differences in genetic control of the ventral and dorsal pancreatic lineages (see Introduction).

Sonic hedgehog and ventral pancreas specification

Since the inhibition of sonic hedgehog (Shh) expression within the dorsal foregut endoderm affects dorsal pancreatic specification (Apelqvist et al., 1997; Hebrok et al., 1998; Kim and Melton, 1998), we wished to assess the role of Shh in ventral pancreatic specification. Although inactivation of the *Shh* and/or indian hedgehog genes in mice does not affect pancreas specification, it does enhance the extent of branching of the early pancreatic epithelium, including the ventral domain (Ramalho-Santos et al., 2000; Hebrock et al., 2000). Antibody-based staining of isolated ventral foregut

endoderm after 2 days in culture showed that the cells uniformly did not express Shh antigen (Fig. 5A). By contrast, Hebrok et al. (Hebrok et al., 1998) found that the isolated dorsal endoderm from chick, in culture, did express Shh. Similarly, we observed patchy areas of Shh expression in mouse dorsal endoderm cultured for 2 days (Fig. 5B). The total absence of Shh expression by the isolated ventral endoderm could contribute to the default activation of the pancreas program (Fig. 2). In support of this interpretation, treating isolated ventral endoderm with 5 ng/ml FGF2, which suppresses the pancreas program, activated Shh expression in most of the cells (Fig. 5C, arrow pointing to dark stained area). Also, we reproducibly observed Shh-positive domains of endoderm adjacent to beating cardiac cells in cocultures of ventral foregut endoderm with cardiac mesoderm (Fig. 5D), which is consistent with the absence of Pdx1 expression in that domain (Fig. 3A,C). In Fig. 5D, lower right, note the absence of Shh expression in two endoderm fragments that lacked cardiogenic mesoderm, demonstrating that close proximity to such mesoderm is crucial for activation of Shh expression. Furthermore, we found that in the presence of 50 ng/ml of recombinant Shh peptide, ventral endoderm explants failed to exhibit the default expression of Pdx1, either by in situ antibody analysis (n=4, Fig. 5E), or by RT-PCR of explant RNA (Fig. 5G). Shh did not inhibit the viability of the endoderm explants, as the tissue grew over the 2 day culture period and expressed HNF3β RNA (Fig. 5F). Shh peptide did not activate albumin expression in these explants (data not shown), demonstrating that while the induction of Shh in the ventral endoderm may be downstream of cardiac FGF signalling, it is insufficient to elicit the hepatic program.

We conclude that while Shh is inhibitory to pancreas formation in both the dorsal (Hebrok et al., 1998; Apelqvist et al., 1997; Kim and Melton, 1998) and the ventral endoderm, suppression of default Shh expression in the dorsal foregut allows a domain to develop into pancreas, whereas induction of Shh in the ventral foregut can help exclude a domain from developing into pancreas.

Lack of selection for cell growth or survival in the pancreas versus liver cell choice

We considered the following two models as possible explanations for the ability of cardiac mesoderm/FGF signalling to result in the local exclusion of a pancreatic

domain. (1) The ventral foregut endoderm could consist of two intermingling cell types, one of which is predisposed to make pancreas (pre-pancreatic) and the other predisposed to make liver (pre-hepatic). Cardiac/FGF could cause the selective death of local pre-pancreatic cells and/or the rapid outgrowth of local pre-hepatic cells. (2) Alternatively, the ventral foregut endoderm could consist primarily of a homogenous, bipotential cell population, and cardiac/FGF could cause a diversion of a default pancreatic cell fate to a hepatic cell fate. After culturing thousands of ventral foregut endoderm samples in our laboratory, we have been unable to observe a situation where individual endoderm epithelial cells could survive or give rise to a colony, precluding a direct lineage analysis at the single cell level. We therefore sought to test the first model by asking if marked changes in cell growth or viability accompanied cardiac signalling.

Although the phenotypic assays described in this study so far involved explants cultured for 2 days, preliminary experiments with BrdU, to label replicating cells, indicated that ventral endoderm cells in cocultures with cardiac mesoderm exhibited far more labeling on the first day, compared to the second. Therefore, the following experiments were performed with 1-day cultures, where most of the putative selection would be occurring. Single tissue explants were double labeled with BrdU and Nile Blue, the latter as a whole-mount method to mark dead or dying cells (e.g. Alles and Sulik, 1990; Mori et al., 1995; Vaahtokari et al., 1996). As a positive control for Nile Blue labeling under our conditions, we cultured neuroepithelia from embryonic forebrains and observed punctate blue staining (Fig. 6A), as expected for this cell population in which much growth selection is occurring.

If the cardiac mesoderm promotes the death or inviability of a putative pre-pancreatic subpopulation in its vicinity, Nile Blue staining would be expected to predominate in the cardiac-proximal area. No such cell staining was observed (n=12); occasional Nile Blue-positive cells were scattered throughout the explants and were not reproducibly associated with the beating cardiac domain (Fig. 6B) and in some cultures, hardly any Nile Blue-positive cells were evident (Fig. 6D). If the cardiac mesoderm promotes the enhanced proliferation of a putative pre-hepatic subpopulation in its vicinity, BrdU labeling would be expected to predominate adjacent to the beating cardiac cells. As seen in Fig. 6C, no selective staining was observed (n=8); rather, in many samples, most of the endodermal cells in the culture were replicating their DNA. In some samples, BrdU staining was enriched near the periphery (Fig. 6E). We conclude that there is an apparent lack of selection for growth or survival in the endodermal domain near the cardiac mesoderm, which fails to support a cell selection model for the exclusion of a pancreatic fate.

To investigate whether FGF signalling elicited a growth selection within the ventral foregut endoderm, we cultured the endoderm as isolated explants in the presence or absence of FGF2. The endoderm alone exhibited little Nile Blue staining and the presence of FGF2 had no effect (Fig. 6F,H). Double-labeling with BrdU revealed similar sporadic staining in both the endoderm alone and in FGF2 treated cultures (Fig. 6G,I). Taken together, the present data are consistent with the hypothesis that cardiac/FGF signalling elicits a cell fate choice within the ventral endoderm, rather than promoting selective outgrowth of distinct, pre-determined cell subpopulations.

DISCUSSION

Little is known about how the ventral foregut endoderm is patterned to develop into the pancreas and liver. Of all of the gene inactivation studies in mice, so far none have revealed mutations that block the initial development of these tissues. We provide evidence for a domain of ventral endoderm possessing bipotentiality for pancreas and liver and provide an explanation for how the cell type choice is made (Fig. 7). Explant culture experiments lead us to propose that by the early somite stages, the default program for the ventral domain of foregut endoderm is to become pancreas. Endoderm from outside the foregut, cultured similarly, failed to initiate pancreas gene expression, showing that the culture conditions alone do not generally induce pancreas. The default pancreas program of the somite-stage ventral endoderm is diverted by proximity to the cardiac mesoderm, causing the adjacent endoderm to express the liver program instead (Fig. 7B). During this period in embryogenesis, the cardiac mesoderm moves from lateral domains to the midline, resulting in cardiac tube formation. We suggest that the endoderm that is on either side of the nascent cardiac tube and at the lip that extends the foregut to the midgut (Figs 1C, 7B, dark green segment) will be sufficiently distal to cardiac mesoderm to permit the expression of the pancreas program. It remains to be determined whether there is a discrete threshhold of FGF concentration that determines the boundary between one cell fate and another (see Fig. 3C). However, further morphogenetic movement and budding of the nascent tissues clearly results in a distinct liver and ventral pancreas (Fig. 7C).

As described in the Introduction, there is much evidence to show that both the endocrine and exocrine pancreas lineages are derived from a single pancreatic stem cell, and that hepatocytes and bile duct cells are derived from a single liver stem cell type, or hepatoblast (Shiojiri, 1984; Germain et al., 1988). Here we have focused on the initial specification of the pancreas by showing that the isolated endoderm could give rise to cells expressing markers for endocrine lineages (Pdx1, ngn3, Isl1, BETA2/neuroD, glucagon, and insulin) and the initial p48 marker of the exocrine lineage. We have not focused on the further development of the exocrine lineage. Because in our hands, the Pdx1 expression domain nearly encompassed that of HNF3β, the latter being a marker of definitive endoderm, most of the ventral foregut endoderm in our isolations was competent to express the pancreatic marker. Although even a few fibroblast cells present in our cultures may help promote the pancreatic program, the same cultures of endoderm are largely competent to initiate liver development, demonstrating bipotentiality. In preliminary studies, we have found that patches of this endoderm in the presence of cardiac mesoderm also begin to express Ttf1, an early thyroid and lung transcription factor (Mizuno et al., 1991; Lazzaro et al., 1991; Kimura et al., 1996), but we have yet to discern the relationship between the Ttf1, Pdx1, liver and cardiac domains (G. Deutsch, unpublished).

We note that the default pancreatic program of the early somite stage, ventral endoderm at E8.5 probably reflects previous patterning from the presomitic stages (Fig. 7A). Shortly after gastrulation, the ventrolateral endoderm is surrounded by splanchnic mesoderm. Wells and Melton (2000) recently showed that presomitic endoderm from E7.5 would

progress to a Pdx1-positive stage in vitro only when cultured in the presence of fragments of mesoderm and ectoderm. None of the FGF, BMP, or other factors tested could induce Pdx1 expression in isolated E7.5 endoderm. Taken together, the data indicate that the default pancreas fate at the E8.5 stage requires earlier, as yet undefined signals from other germ layers.

The ability of cardiac FGF signalling to promote hepatogenesis (Jung et al., 1999) could have been interpreted as permitting the expression of a default hepatic program or activating liver genes in a cell population already committed to the liver. The data in this paper contradict those possibilities, because we find that the default path for ventral foregut endoderm is the pancreatic program and that cardiac FGF signalling diverts the cell population from this fate. This, and the lack of effect of cardiac mesoderm or FGF on the viability and growth of the proximal endoderm, support the concept that FGF promotes liver differentiation within the ventral endoderm population. Indeed, the data explains a curious observation from our previous studies. Gualdi et al. (1996) and Jung et al. (1999) found that prior to hepatogenesis, the ventral foregut endoderm expresses, at a very low level, the liver genes for alpha-fetoprotein (Afp) and transthyretin (Ttr). Yet in culture, AFP and TTR expression became rapidly extinguished in the absence of cardiac mesoderm or FGF signalling. The data in this paper explain the loss of AFP and TTR in the isolated endoderm as part of the cell type choice toward pancreas, which apparently excludes the expression of genes for the liver.

The default pancreatic fate of the ventral foregut endoderm cells in mouse and the apparent fate diversion by FGF2 agrees with previous studies of endoderm development in *Xenopus*. Mesoderm-free vegetal explants from early blastula stage embryos, cultured in isolation, initiate the expression of the Pdx1 homolog, XlHbox8 (Gamer and Wright, 1995). Although additional pancreas markers were not analyzed, FGF2 potently inhibited XlHbox8 expression, similar to the effects we observed with FGF2 on the ventral foregut endoderm in the mouse. In another study, isolated vegetal pole explants also autonomously activated Xlhbox8 as well as insulin (Henry et al., 1996). Thus, a default pancreatic fate by the ventral endoderm and its suppression by FGF may be evolutionary conserved. This is in contrast to the situation of the dorsal endoderm, which fails to express a default pancreatic fate in isolation (Kim et al., 1997) and where very low levels of FGF2 are stimulatory to Pdx1 expression (Hebrok et al., 1998). At later developmental stages, FGF signalling is critical for the development of the exocrine lineage (Miralles et al., 1999).

A further difference between the dorsal and ventral endoderm foregut domains is our finding that by default, the ventral endoderm does not express Shh; by contrast, Hebrok et al. (1998) and we found that the isolated dorsal endoderm does express Shh. While notochord represses dorsal Shh expression, permitting pancreas development in vitro (Hebrok et al., 1998; Kim and Melton, 1998), we find that cardiac mesoderm induces local Shh expression (Fig. 5D), which may contribute to the local exclusion of pancreas development. Indeed, we found that Shh treament of isolated foregut endoderm was sufficient to repress Pdx1 expression (Fig. 5E,G). We suggest that the well-documented observation of Shh expression in the ventral foregut endoderm in mouse embryos (Echelard et al., 1993; Goodrich et al., 1996) is due to the extensive interacting surface with the cardiac mesoderm, leading to Shh induction

in much of the ventral endoderm. The *Shh* gene is essential for the morphogenesis of the trachea, lung and esophagus (Chiang et al., 1996; Litingtung et al., 1998), while the combination of a *Shh* homozygous null allele with a *Ihh* heterozygous null allele causes enhanced early ventral pancreatic branching (Ramalho-Santos et al., 2000; Hebrock et al., 2000), consistent with hedgehog signaling being inhibitory to early pancreas development. However, ectopic Shh within the Pdx1 expression domain inhibited but did not delete pancreas development in mouse embryos, apparently by perturbing adjacent mesenchyme differentiation (Apelqvist et al., 1997). Analogously, we found that in our endoderm-cardiac mesoderm co-cultures, the expression of Ptc-2 (Ptch2), a Shh receptor and responsive gene (Motoyama et al., 1998; Carpenter et al., 1998), occurred in the mesenchyme cells surrounding the Shh-positive endodermal epithelium (J. Jung, unpublished). Also, homozygous mutants for *Ptc-1* (*Ptch1*) are deficient in early pancreas gene expression (Hebrok et al., 2000). Further studies will be required to assess the complex relationship between Shh, responsive mesenchyme, and pancreatic development.

Although both FGF1 and FGF2 are induced in the cardiac mesoderm at the time of hepatic and pancreatic initiation in the mouse embryo (Jung et al., 1999), inactivation of either or both of the genes for these proteins is not detrimental to gut organ development (Ortega et al., 1998; Dono et al., 1998; Miller et al., 2000). However, the cardiac mesoderm also expresses FGF4 (Zhu et al., 1996), FGF17 (Maruoka et al., 1998), and a low level of FGF8 (Crossley and Martin, 1995) and a general FGF signalling antagonist is inhibitory to hepatogenesis in vitro (Jung et al., 1999); thus, cardiac FGF signalling appears highly redundant.

The common embryological origin of the ventral pancreas and the liver may be reflected in certain evolutionary and pathological states. Primitive invertebrates such as molluscs have a single organ, the hepatopancreas, that serves functions of both organs (Hoar, 1975). The caudal pancreas in the sea lamprey apparently develops by transdifferentiation of cells in the hepatic duct (Elliot and Youson, 1993). The partitioning of liver and ventral pancreas into separate organs may have evolved from a common cell population, like that described here, and the dorsal pancreatic rudiment may be a late evolutionary adaptation.

When rats are subjected to a certain diet that induces pancreatic damage, they develop clusters of hepatocytes within their pancreas (Rao et al., 1989; Dabeva et al., 1995; Dabeva et al., 1997). Also, transgenic overexpression of an FGF in the adult mouse pancreas leads to the appearance of hepatocytes in the islets (Krakowski et al., 1999). The latter finding underscores the role of FGF signalling in promoting hepatic versus pancreatic differentiation. In these cases, it is unknown whether the pancreatic hepatocytes emerge due to trans-differentiation of pancreatic duct cells or to the alternative specification of an as yet undefined stem cell population that may associate with the duct compartment (e.g. see Slack, 1985; Gu and Sarvetnick, 1993; Kritzik et al., 1999). Nonetheless, the examples of liver cells appearing in the adult pancreas illustrate how understanding the related specification of these tissues in development, as revealed here, should provide new insights into how cell differentiation might be controlled at will for cell-based therapies for disease.

Note added in proof

The following paper underscores the developmental relationship between the pancreas and the liver.

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