Endothelial cell interactions initiate dorsal pancreas development by selectively inducing the transcription factor Ptf1a

Hideyuki Yoshitomi and Kenneth S. Zaret*

Cell and Developmental Biology Program, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111, USA

*Author for correspondence (e-mail: zaret@fccc.edu)

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Summary

Dorsal and ventral pancreatic bud development from the endoderm requires inductive interactions with diverse mesodermal cell types and the action of transcription factors expressed within the endoderm. Presently it is unclear which mesodermal interactions activate which pancreatic transcription factors, and whether such inductions are common for initiating dorsal and ventral pancreas development. Previous studies of Lammert et al. (Lammert, E., Cleaver, O. and Melton, D. (2001) Science 294, 564-567) showed that signaling from embryonic blood vessel cells, derived from the mesoderm, promotes pancreatic bud development. Using a combination of mouse Flk1–/– embryos lacking endothelial cells and tissue recombination experiments, we discovered that the initial induction of dorsal endoderm cells positive for the pancreatic and duodenal transcription factor Pdx1 does not require aorta or endothelial cell interactions, but dorsal pancreatic bud emergence and the maintenance of Pdx1 expression does. Aortal endothelial cells induce the crucial pancreatic transcription factor Ptf1a in the dorsal pancreatic endoderm; whereas the vitelline veins, which are normally adjacent to the emerging ventral pancreatic bud, are unnecessary for ventral Ptf1a induction or for ventral pancreatic bud initiation. We find that the aorta cells themselves, apart from the blood supply, cause the induction of Ptf1a in dorsal endoderm explants. Thus, endothelial cell interactions specifically promote early dorsal pancreatic development, at least in part, by inducing Ptf1a+ pancreatic progenitors. Additionally, we find that endothelial cells are necessary for the induction of both the insulin and glucagon genes.

Key words: Pancreas, Endothelial, Endoderm, Transcription factor, Ptf1a

Introduction

Patterning of the endoderm germ layer into different tissues is dependent upon inductive interactions with mesodermal cells that induce different transcriptional programs in cells along the gut (Wells and Melton, 1999; Hogan and Zaret, 2002). Understanding the diverse inductive steps and how they promote tissue development can provide insight into ontogeny, regenerative responses to tissue damage, and directed control of cell differentiation. Classic embryo tissue recombination studies identified mesenchyme cells that induce the pancreas from the endoderm (Golosow and Grobstein, 1962; Wessels and Cohen, 1967; Spooner et al., 1970; Gittes et al., 1996; Kumar et al., 2003) and more recent studies showed that endodermal interactions with notochord (Kim et al., 1997) and endothelial cells (Lammert et al., 2001) are also necessary. Gene inactivation experiments have identified transcription factors required for pancreatic induction (Edlund, 2002; Kim and MacDonald, 2002; Wilson et al., 2003), but presently there is a gap in understanding which of the inductive interactions with the mesoderm induce which of the pancreatic transcription factors in the endoderm. Furthermore, tissue buds for the vertebrate pancreas arise independently from dorsal and ventral-lateral domains of the gut epithelium, and it is unknown whether factors necessary for development of both domains are induced by similar mesodermal interactions, dorsally and ventrally. Understanding these issues is crucial for a synthetic view of pancreatic organogenesis.

The dorsal and ventral pancreatic buds arise between embryonic days 8.5 and 9.5 of gestation (E8.5-E9.5) of the mouse and eventually combine to generate the mature organ (Wessels and Cohen, 1967; Rugh, 1968; Pictet et al., 1972). The initial fields of prospective pancreatic endoderm consist of progenitors of endocrine and exocrine cells (Deltour et al., 1991; Percival and Slack, 1999; Herrera, 2000; Gu et al., 2002). These cells express the homeobox containing transcription factor Pdx1, which is also expressed in duodenal progenitors and is necessary for pancreatic and duodenal differentiation after the formation of the dorsal and ventral pancreatic buds (Wright et al., 1989; Ohlsson et al., 1993; Jonsson et al., 1994; Ahlgren et al., 1996; Offield et al., 1996). The dorsal and ventral endodermal expression of the homeodomain factor Hnf6 (Rausa et al., 1997; Landry et al., 1997) is necessary for the timely initiation of Pdx1 expression (Ohlsson et al., 1993; Ahlgren et al., 1996; Li et al., 1999; Gannon and Wright, 1999; Jacquemin et al., 2003). The dorsal and ventral endodermal expression of the basic helix-loop-helix transcription factor Ptf1a, originally discovered by its function in the exocrine pancreas (Krapp et al., 1996; Rose et al., 2001), was recently shown to be necessary for the development of endocrine, exocrine and duct cell lineages both dorsally and ventrally.
Although Pdx1, Hnf6 and Ptf1a function in both dorsal and ventral pancreatic endoderm, the mesodermal inducers of these factors in the embryo remain unknown.

Despite the commonalities, the dorsal and ventral pancreatic buds have some different mesodermal inducers and transcriptional effectors. The homeodomain protein Hb9 is necessary for dorsal, but not ventral, pancreatic bud emergence (Li et al., 1999; Harrison et al., 1999). The notochord, a mesodermal derivative, is required to inhibit the expression of sonic hedgehog in the dorsal endoderm, thereby allowing pancreatic development (Kim and Melton, 1998; Hebros et al., 1998; Hebros and Melton, 2000). Deleting notochord has no effect on ventral pancreatic development (Kim et al., 1997). Genetic mutations that deplete dorsal mesenchyme cells cause deficiencies in dorsal but not ventral pancreatic bud development (Ahlgren et al., 1997; Esni et al., 2001). The ventral pancreas emerges near the liver, and tissue explant studies showed that pancreatic induction occurs in ventral foregut endoderm distal to cardiogenic mesoderm (Deuch et al., 2001; Kumar et al., 2003), the normal inducer of the liver (Le Douarin, 1975; Fukuda, 1979; Houssaint, 1980; Gualdi et al., 1966). These and other studies show that dorsal pancreatic endoderm is induced by notochord and mesenchyme, whereas ventral pancreatic endoderm patterning is permitted by the absence of cardiogenic mesoderm and perhaps the presence of local mesenchyme cells (Rossi et al., 2001; Kumar et al., 2003).

In addition, endothelial cells, another mesodermal derivative, provide organogenic stimuli for the pancreas (Cleaver and Melton, 2003). Lannert et al. (Lannert et al., 2001) showed that at E9.0-E10 in the mouse, the aorta and the vitelline veins are near the emerging dorsal and ventral pancreatic buds, respectively. Using tissue recombination experiments, they showed that fragments of aorta induce the expression of Pdx1 and insulin in dorsal endoderm cultures from mouse embryos. Removal of the aorta from Xenopus embryos causes a failure to induce certain pro-endocrine transcription factors and insulin, in vivo. They also showed that transgenic mice over-expressing vascular endothelial growth factor (Vegfa) in pancreatic endocrine cells causes hypertrophy of the pancreatic islets. These studies showed that endothelial cells provide inductive signals for pancreatic development, apart from the ability of blood vessels to serve as conduits for nutrients, oxygen and soluble signaling molecules.

The pioneering studies of endothelial cells in pancreas development did not address the following questions. (1) Although blood vessels are adjacent to where both dorsal and ventral pancreatic buds develop, do endothelial cells similarly promote organogenesis in both contexts? (2) What are the transcription factor gene targets required for early pancreatic development that are induced by endothelial cell interactions?

To address these issues, we used Fkl1 (Kdr – Mouse Genome Informatics) null mice (Shalaby et al., 1995). Fkl1 encodes a receptor for Vegfs and is expressed in endothelial cells (Millauer et al., 1993; Quinn et al., 1993; Oelrichs et al., 1993; Yamaguchi et al., 1993). A homozygous null Fkl1 mutation blocks endothelial cell development at the angioblast stage, preventing the formation of mature endothelial cells and blood vessels (Shalaby et al., 1995). The genetic ablation approach is important because we previously found that during tissue bud development, pockets of endothelial cells and nascent capillaries can be detected within the nascent hepatic mesenchyme, making it difficult to completely dissect away endothelial cells for tissue explant studies (Matsumoto et al., 2001).

For the present study, we performed a detailed temporal and functional analysis of blood vessel interactions with prospective pancreatic endoderm in the mouse embryo. We find that endothelial cell interactions are more critical for dorsal than ventral pancreatic development and that endothelial cells induce different pancreatic transcription factors in the endoderm than those induced by other mesodermal cell types. The studies described here provide a link in understanding how mesodermal interactions with the endoderm lead to specific transcription factor inductions within the network required for pancreatic organogenesis.

**Materials and methods**

**Embryo isolation and genotyping**

Fkl1lacZ mice were generated as described previously (Shalaby et al., 1995) and kindly provided by J. Rossant. Noon of the day of the vaginal plug was considered as day 0.5 of gestation (E0.5). Embryos were harvested at E8.5-10.0 and staged according to the number of pairs of somites, which provides a far more precise measure of relevant developmental stages than the day of gestation. Embryos were dissected free of extrabryonic membranes, the head portion was removed from each embryo and subjected to genotyping by PCR as described previously (Shalaby et al., 1995). As the phenotype of the heterozygous embryos were normal (Shalaby et al., 1995), these embryos and wild-type embryos were designated as controls.

**Fkl1lacZ expression, immunohistochemistry and in situ hybridization**

For assessing expression of the Fkl1lacZ allele, embryos were fixed, stained in X-gal solution, and embedded in paraffin as described previously (Rossi et al., 2001). For immunostaining, embryos were fixed in 4% paraformaldehyde buffered at pH 7.4 with phosphate-buffered saline (PBS) at 4°C overnight, washed twice with 1× PBS at 4°C for 10 minutes, dehydrated through a PBT (1× PBS + 0.1% Tween-20)-methanol gradient, embedded in paraffin, and sectioned at 7 μm by standard procedures. The following primary antibodies and dilution were used: rabbit polyclonal anti-Pdx1, 1:5000 (gift from C. Wright), rabbit polyclonal anti-glucagon, 1:180 (Maine Biotechnology Services, Portland, ME). Briefly, after deparaffinization and rehydration, sections were boiled in citrate buffer (10 mM sodium citrate, pH 6.0) for 10 minutes and endogenous peroxidase activity was blocked by immersing sections in 0.3% H2O2. Following treatment with blocking buffer (10% heat inactivated goat serum, 1% ovalbumin in PBT), sections were reacted with primary antibodies in 0.1× blocking buffer at 4°C overnight. Primary antibodies were detected with Vectastain Elite ABC kit (Vector, Burlingame, CA) using either DAB (Sigma, St. Louis, MO) or Vector SG (Vector, Burlingame, CA). Sections were counterstained with Eosin Y. Whole embryos were immunostained for Pecam as described previously (Sato and Bartunkova, 2000) using 30 μg/ml of antibody (BD Pharmingen, San Diego, CA), embedded in paraffin, sectioned and counterstained. In situ hybridization was performed as described previously (Ang et al., 1993; Jung et al., 1999) with Foxa2, Hnf6 and Shh riboprobes reported previously (Ang et al., 1993; Echelard et al., 1993; Rausa et al., 1997); then embryos were sectioned.

**RT-PCR analysis**

RNA was isolated by cesium chloride gradient centrifugation, reverse transcribed with oligo(dT) primers, and subjected to PCR as described previously (Gualdi et al., 1996). PCR was performed with 1.5 mM...
Mg\(^{2+}\) at 60°C for annealing, unless indicated otherwise below. PCR cycle ranges for each gene and tissue fragment were determined by first analyzing actin mRNA in each sample at three cycle steps over a nine cycle range, quantitating electrophoretic products with a phosphorimager, and determining cycle ranges to use for other primer sets; the latter, in turn, were analyzed at multiple cycle steps, usually differing by three cycles, to ensure that the reactions were in the exponential range of PCR. The sequences of the primers (and certain cases of specific conditions for PCR) were: Pecam sense 5'-GCA-AAGATGACTTCCAGAC-3', antisense 5'-GTACCTGCTTACTGCAGCGG-3', Tal1 sense 5'-AAACAACCCGGTTGAAGGC-3', antisense 5'-ACTTGGCAGAAGATTGATG-3', Pdx1 sense 5'-CA-GGAGGTGTCAATACACG-3', antisense 5'-CCCCCTACTACGTTCT-3' (2 mM MgCl\(_2\), 2% DMSO, annealing at 63°C); Ptfla sense 5'-GGCCCAAGAAGGTCTCATCTGC-3', antisense 5'-AGGAAAGGATGACCTGCAAG-3'; Prox1 sense 5'-CCAGCTGTGTGAAAAAT-3', antisense 5'-TCTCAAGTGCTCATCATCATA-3'; Ngn3 sense 5'-GGGATATCTCGTCCCGTGTC-3', antisense 5'-GAGGCCATCAAAGGAGGC-3'; Neurod1 sense 5'-TGCTGGTGCAGCACTG-3', antisense 5'-ACCTGTTTCAGTCTTCTCCA-3'; Pecam sense 5'-AACACAGGCGCTACATTACC-3', antisense 5'-ACCCAGCAGCATTGGGAAA-3'; Pecam antisense 5'-GCA-3'; Ngn3 antisense 5'-GCGCCCTTAGTGACCAGCT-3'; glucagon sense 5'-GACAGG-3', antisense 5'-ACTTGGCCAGGAAA TTGA TG-3' (Deutsch et al., 2001); Ptf1a sense 5'-GGCCCAGAAGGTCA TCTGC-3', antisense 5'-ACCTGTTTCAGTCTTCTCCA-3' (Lammert et al., 2001). To assess this Pdx1 expression in embryos, which is earlier than that assessed by Lammert et al. (Lammert et al., 2001), we wished to assess the extent to which Pdx1 expression in endoderm-aortal explants promote Pdx1 expression in endodermal epithelium by 15-20S, and as marked bulges from 1993; Ahlgren et al., 1996; Li et al., 1999; Gannon and Wright, 1999; Jacquemin et al., 2003). The dorsal and ventral endodermal markers of dorsal and ventral pancreatic development are directly adjacent to the Pdx1-positive endoderm. Other markers of dorsal and ventral pancreatic development are described below.

Significantly, at 15S, when Pdx1-positive cells normally appear in the dorsal endoderm, Flk1 null embryos exhibited

At 8-9S, just prior to induction of Pdx1 dorsally, the left and right aortae were lateral to the notochord, somites and dorsal-medial endoderm (Fig. 1A), and thus only in partial contact with the dorsal pancreatic progenitor domain, which has been mapped to the endoderm adjacent to the notochord and underlying the somites (Matsushita, 1996; Kumar et al., 2003). By 12-15S, the aorta contacted the dorsal endoderm extensively, without intervening cells (Fig. 1B,C; green arrows), and Pdx1-positive cells appeared by 15S in the dorsal endoderm near and beyond the area of contact with the aorta. At 26S, the fused aorta was clearly separated from the dorsal bud (Fig. 1D). We note that by 26S, capillaries with Flk1-positive endothelial cells were detected in the dorsal mesenchyme near the pancreatic bud (Fig. 1E), emanating from the aorta (data not shown). These observations show that endothelial cells of the aorta gain a tight association with dorsal endoderm during the period when Pdx1 is induced.

Tissue dissection and recombinant explants

Embryo tissues were dissected according to the method of Gittes and Galante (Gittes and Galante, 1993). The dissected tissues were cultured as described previously (Lammert et al., 2001) with the following modifications. The tissues were recombined, embedded in growth-factor reduced matrigel matrix (Becton Dickinson) and following modifications. The tissues were recombined, embedded in Galante (Gittes and Galante, 1993). The dissected tissues were dissection and recombinant explants.

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The presence of the aorta induces Ptf1a dorsally and the outgrowth of the pancreatic bud, but not the initial field of Pdx1-positive cells

To examine the roles of endothelial cells in pancreas development in vivo, we studied Flk1 homozygous null mutant embryos, which lack endothelial cells. Fig. 2A,B shows the absence of the aorta in the dorsal region of Flk1 mutant embryos, compared to wild type, which was confirmed by a lack of cells expressing platelet endothelial cell adhesion molecule (Pecam; CD31), a marker for embryonic endothelial cells (Vecchi et al., 1994). RT-PCR products for Pecam and Tal1, the latter encoding an early endothelial transcription factor (Visvader et al., 1998), were detectable in the dorsal midgut region of control embryos, but not in that of Flk1 homozygous nulls (Fig. 2J).
nearly the same size domain of Pdx1-positive cells as control embryos (Fig. 2C,D; also compare with wild type in Fig. 1C). Thus the initial field of dorsal Pdx1-positive cells appears to be induced in the absence of the dorsal aorta. However, by 20-25S, when a pancreatic bud normally begins to develop, fewer Pdx1-positive cells were detected in the dorsal endoderm of Flk1–/– embryos, compared to wild type, and no bud was evident (compare Fig. 2E,F). By 31S (E10), there was still no morphological evidence for pancreatic bud formation in the dorsal endoderm of null mutant embryos, and few Pdx1-positive cells remained (Fig. 2H, magnified in I).

The expression of Pdx1 is regulated by transcription factors expressed in the dorsal endoderm, including Foxa2 (formerly Hnf3\beta) and Hnf6 (Wu et al., 1997; Gerrish et al., 2000; Jacquemin et al., 2003). In situ hybridization showed that at 13-14S, the expression of Foxa2 was a bit weaker than in wild type, but still positive in Flk1–/– embryos (Fig. 3A,B). Also, Hnf6 was expressed in the Flk1 null embryos in nearly the same domain of cells as in the controls (Fig. 3C,D). These expression patterns are consistent with the observation that endothelial cells are not necessary for the initial field of dorsal endoderm-expressing Pdx1.

The notochord was not affected morphologically in Flk1–/– embryos, and Shh was repressed in the dorsal endoderm as in wild type (Fig. 3E,F; arrows to dorsal endoderm) (Kim et al., 1997; Apelqvist et al., 1997; Hebrok et al., 2000). Thus, notochord interactions with the dorsal endoderm physically and functionally precede those of the dorsal aorta, as originally suggested by Lammert et al. (Lammert et al., 2001).

The transcription factor genes Prox1 (Burke and Oliver, 2002) (Fig. 4A, lanes 6, 8) and Hb9 (data not shown), a regulator of pancreatic development (Li et al., 1999; Harrison et al., 1999; Li et al., 1999), were also induced in the dorsal endoderm of Flk1–/– embryos. As expected from the fewer Pdx1-positive cells seen by 20S, the expression level of Pdx1 mRNA in the dorsal endoderm was lower in Flk1–/– embryos than in controls (Fig. 4A), requiring additional PCR cycles for the RT-PCR products from Flk1–/– dorsal endoderm to equal that from control endoderm (Fig. 4B). The expression of the early endocrine transcription factor genes Ngn3 (Apelqvist et al., 1997; Gradwohl et al., 2000; Schwitzgebel et al., 2000; Gu et
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al., 2002) and Neurod1 (Naya et al., 1997) was detectable but also lower in Flk1+/− embryos than in control embryos (Fig. 4A,B), consistent with the fewer numbers of Pdx1-positive cells dorsally.

Interestingly, of the eight early pancreatic transcription factor genes we assessed, Ptf1a was the only one that appeared to be completely inactive in Flk1−/− embryos (Fig. 4A), even when assayed at very high cycles of PCR (Fig. 4B). Thus, there was a complete failure to induce Ptf1a in the dorsal endoderm in the absence of the aorta. These data show that endothelial cell interactions, or a functional aorta, are specifically required for Ptf1a induction dorsally. Supporting this, we found that in wild-type embryos, the expression of Ptf1a in the dorsal endoderm normally first becomes detectable at 12-15S (Fig. 4C; and data not shown), when the dorsal aorta is tightly juxtaposed to the dorsal endoderm (Fig. 1B).

RT-PCR (Fig. 4A,B) and immunohistochemistry studies (Fig. 4D,E) showed that insulin and glucagon are also not detectably induced in Flk1+/− embryos. This contrasts with the phenotype of mouse embryos containing a null mutation of the Ptf1a gene, in which some insulin and glucagon-positive cells develop dorsally, although pancreatic development is disrupted there (Krapp et al., 1998; Kawaguchi et al., 2002). Thus, endothelial cells and/or the aorta induce or permit the expression of dorsal Ptf1a, pancreatic bud outgrowth, and critical endocrine genes.
Initial ventral pancreas development is not affected in Flk1<sup>−/−</sup> embryos

The absence of endothelial cells and the vitelline vein in the ventral pancreas area of Flk1<sup>−/−</sup> embryos was confirmed by staining for Pecam (Fig. 5A,B). Occasionally we saw a Pecam-positive cell that was distal to the ventral pancreatic bud (Fig. 5B, blue arrowhead). Also, while RT-PCR showed a strong decrease in the expression of Pecam and Tal1 in ventral regions of dissected embryos (Fig. 5G), at higher PCR cycles and long exposures we could detect some products (Fig. 5G, lanes 10, 12), consistent with the previously reported appearance of some angioblasts in the umbilicus near the gut in Flk1 embryos (Shalaby et al., 1995). Thus while the vitelline veins and other local vasculatures are completely absent, a few distal angioblasts persist ventrally in Flk1 homozygotes.

Despite the lack of local vasculatures and in contrast to the situation dorsally, at 18-20S the ventral domain of Pdx1-positive cells in Flk1<sup>−/−</sup> embryos remained the same size as in control embryos (Fig. 5C,D and data not shown). In the Flk1 null mutant, these Pdx1-positive cells began to proliferate and develop into a bud (Fig. 5E,F). The ventral pancreatic bud of Flk1<sup>−/−</sup> embryos was smaller than in control embryos, probably linked to the generally impaired growth of the null mutants (see comparable magnifications of entire embryo sections in Fig. 5E,F). RT-PCR analysis showed that the Flk1<sup>−/−</sup> ventral endoderm cells expressed early pancreatic transcription factors at nearly the same level as in control embryos (Fig. 5H), with Ngn3 and Neurod1 expression at this stage being inconsistent ventrally even in wild-type embryos (Fig. 5H) (Naya et al., 1997; Apelqvist et al., 1997). Most significantly, the ventral pancreatic progenitors in Flk1<sup>−/−</sup> embryos expressed Ptf1α, similarly to the wild type (Fig. 5H). These results clearly showed that the initial expression of ventral pancreatic genes, including Ptf1α, was not markedly affected by the absence of local endothelial cells. Also, the normal, albeit smaller, progression of ventral pancreatic bud development in the Flk1<sup>−/−</sup> embryos, despite the absence of a vasculature through this stage, shows that the ensuing embryonic lethality of the genetic model is not generally inhibitory to pancreatic development per se.

We note that the absence of endothelial cells did prevent the induction of glucagon ventrally (Fig. 5G). Insulin was not yet expressed ventrally in wild-type embryos during the period (data not shown). Since glucagon was not induced either dorsally or ventrally in Flk1<sup>−/−</sup> embryos, but Ptf1α was only not induced dorsally, endothelial cell interactions appear to control endocrine gene induction at a step later than Ptf1α expression.

**Aorta fragments induce Ptf1α in dorsal endoderm explants of Flk1<sup>−/−</sup> embryos**

To test the hypothesis that the aorta itself, rather than factors in the bloodstream or other secondary effects, regulates the induction of Ptf1α in the dorsal endoderm, we performed tissue recombination studies. Dorsal endoderm fragments were dissected from Flk1<sup>−/−</sup> embryos at 7-10S (Fig. 6A-C) and cultured for 24 hours with or without aortae dissected from control embryos (Fig. 6E), which is comparable to the E8.5-9.5 transition in vivo. By this strategy, the isolated dorsal endoderm is genetically depleted of endothelial cells, which was confirmed by RT-PCR analysis showing the absence of Pecam expression (Fig. 6F, lanes 3, 4; ‘no aorta’ explants). RT-PCR analysis also showed that the dissected aorta from wild-type embryos was Hnf6 negative, and thus was not contaminated with endoderm, and that the isolated Flk1<sup>−/−</sup> dorsal endoderm expressed Hnf6, as expected (Fig. 6D). Owing to the smaller size of the Flk1<sup>−/−</sup> embryos, the endoderm was difficult to dissect. To be sure that the dissected dorsal endoderm, underlying the somites, lacked contaminating...
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ventral-lateral endoderm, which does not require endothelial cells for Ptf1a induction (Fig. 5H), we selected only those Hnf6-positive, endoderm-only explants which, upon culture, were negative for the expression of Hex (Fig. 6F), as Hex marks both hepatic and ventral pancreatic progenitor cells and not the dorsal pancreatic progenitors (Martinez-Barbera et al., 2000; Bort et al., 2004). We also assessed the presence of mesenchyme cells by RT-PCR for Wt1, which in situ hybridization has shown to be expressed specifically in prospective dorsal and lateral mesenchyme cells (Armstrong et al., 1992). We found that Wt1 levels in the dorsal pancreatic region normally increase during E8.5-9.5 (Fig. 6G), consistent with the appearance of mesenchymal cells around the dorsal pancreatic endoderm (Fig. 1B,C).

All five dorsal endoderm explants from Flk1–/– embryos that were cultured without aorta lacked Ptf1a expression (Fig. 5F, lanes 3, 4). By contrast, of five explants of Flk1–/– dorsal endoderm recombined with wild-type Pecam-positive aortae, four expressed Ptf1a (Fig. 5F,H). Of the four Ptf1a-expressing explants, two contained Wt1-expressing cells and two contained none or undetectable levels of such cells (Fig. 5H, lanes 1-8). A recombinant explant that did not express Ptf1a contained Wt1-expressing cells (Fig. 6H, lanes 9, 10). Taking together the in vivo and in vitro data, the results indicate that the aorta induces Ptf1a expression in the dorsal endoderm independently of the presence of mesenchyme cells. The ability of the aorta to induce Ptf1a in explant experiments demonstrates that cellular interactions with the endothelium are critical, and not aorta function, in terms of carrying blood and oxygen.

**Discussion**

As described in the Introduction, pancreatic development from the endoderm, like that of other gut-derived tissues, requires inductions from diverse mesodermal cell types. Lammert et al. (Lammert et al., 2001) originally demonstrated that aortal endothelial cells contribute to pancreatic development. We have used different approaches that confirm their general findings and make the new discovery that the local vascular endothelium provides inductive interactions that are critical for the initial differentiation of dorsal pancreatic progenitors, but not for ventral pancreatic progenitors. We find that the endothelium promotes dorsal pancreatic development by specifically inducing the transcription factor Ptf1a, and not various other early factors, revealing how particular mesodermal interactions activate subsets of the dorsal pancreatic transcriptional program. Our ability to reconstitute the inductive step in vitro indicates that the inductive interactions are from the endothelial cells themselves, and not a consequence of vascular function.

We find that the aortal endothelium induces Ptf1a in a field of dorsal endoderm cells that, by the 13-15S stage, already have Shh expression repressed and Hnf6 and Pdx1 expression induced (Fig. 3B,D,F). Genetic lineage tracing studies have shown that early endoderm cells expressing Ptf1a contribute to all pancreatic cell lineages and thereby constitute the definitive pancreatic progenitor population (Krapp et al., 1998; Kawaguchi et al., 2002). These and other studies (Chiang and Melton, 2003) also showed that Ptf1a expression normally initiates in the Pdx1-positive cell population. Taken together,
these points show that endothelial cell interactions in vivo (Fig. 4A,B) and in vitro (Fig. 6E,F), contribute to the initial differentiation of dorsal pancreatic progenitor cells from the endoderm by inducing the transcription factor gene Ptf1a.

From our embryonic tissue recombination studies, it appears that the aortal endothelium induces Ptf1a directly in the dorsal endoderm, rather than through an intermediary cell such as lateral mesenchyme cells, which are necessary for ventral pancreatic development (Kumar et al., 2003). Also, the aorta exhibits an extensive and apparently direct interaction with the Pdx1-positive dorsal endoderm during the 12-15S stage (Fig. 1B), when Ptf1a is first induced (Fig. 4C, lane 6), apparently prior to mesenchyme cells interposing extensively between the tissues (Fig. 1C). By contrast, although the vitelline veins are near the ventral Pdx1-positive endoderm cells, we can discern mesenchyme cells interposing between the tissues at all stages (Fig. 1E-I) and our data show that the vitelline veins and other local vasculatures are not required for ventral Ptf1a induction (Fig. 5G). It remains possible that rare, distal angioblasts that persist in Flk1−/− embryos could promote ventral pancreatic development, perhaps in an analogous manner to that described recently in normal early pancreatic bud development in cloche mutant zebrafish embryos that have a greatly diminished vasculature (Field et al., 2003). Alternatively, initial ventral pancreatic bud development in the mouse, and pancreatic bud development in general in zebrafish, may be independent of vascular cell signaling. Regardless, it thus appears that, as for other pancreatic transcription factors described in the Introduction, the induction of Ptf1a, which is necessary for pancreatic differentiation in general (Krapp et al., 1998; Kawaguchi et al., 2002), is differentially dependent upon vascular cells for the mouse dorsal and ventral pancreatic progenitor cell populations.

We found that the aorta, endothelial cells, and/or Ptf1a expression were necessary to promote the outgrowth of dorsal endoderm explants into a pancreatic bud (Fig. 2F,H). This effect was only marginal in the ventral endoderm, where the Pdx1 cell population appeared to be about half the normal size by
30S, but the cells were within a morphologically distinct pancreatic bud (Fig. 5F). The ability of Pdx1-positive cells to be maintained ventrally and generate a pancreatic bud-like structure shows that the embryonic lethality of Fkll−/− mutation, and the lack of endothelial cells or a blood supply, is not generally deleterious to the initiation of pancreatic morphogenesis. Ventral Ptf1a homozygous null cells in the previous lineage studies (Kawaguchi et al., 2002) exhibited a fate change to an intestinal cell type, and therefore formed part of the gut tube instead of budding into a pancreas. While most of the dorsal Ptf1a homozygous null cells that expressed a Ptf1a-cre lineage marker also formed gut cells, a subpopulation eventually grew out and exclusively expressed endocrine genes, as previously observed (Krapp et al., 1998; Kawaguchi et al., 2002). However, the endocrine gene-expressing cells in the Ptf1a null embryos did not form a normal dorsal pancreas bud or tissue; instead, by E14.5-16.5 the cells grew into an extended rudiment and eventually the cells became incorporated into the spleen. Consistent with these observations, we found that the residual dorsal Ptf1a-negative cells in Fkll−/− embryos expressed the endocrine progenitor genes Pdx1, Ngn3 and Neurod1 (Fig. 2D; Fig. 4A,B). The failure to generate a bud in the dorsal Pdx1-positive, Ptf1a-negative cell population in such embryos could explain the mouse tissue recombination and Xenopus aorta excision results of Lammert et al. (Lammert et al., 2001), which resulted in diminished Pdx1-positive endocrine cells. We suggest that the aorta did not induce a field of cells capable of initiating Pdx1 expression, but rather specifically induced Ptf1α resulting in the subsequent ability of those cells to maintain normal amounts of dorsal pancreatic tissue.

Although the initial development of the ventral pancreas is not strongly affected by the absence of a vasculature, glucagon, an early endocrine cell marker, was not induced in the ventral pancreatic bud of Fkll−/− embryos, and both glucagon and insulin were not induced dorsally. The data indicate that endothelial cells serve a distinct function in promoting endocrine gene activation, in agreement with the studies of Lammert et al. (Lammert et al., 2001), particularly as we observed a capillary network beginning to form in the early, emerging pancreatic buds at 26S (Fig. 1D,J).

We note that various earlier studies of mesenchymal cell induction of pancreatic development employed mesenchyme tissue explants isolated from embryos at E9.5 and onwards, which we have shown to contain endothelial cells (Fig. 1D,J). Thus it is possible that at least some of the inductive effects previously attributed to mesenchyme cells could be due to the presence of endothelial cells within the explants.

In summary, we provide evidence that during dorsal pancreatic development, the vascular endothelium plays a central role in activating Ptf1a, one of the initial genes required to specify the pancreatic lineage, yet it does so only for the dorsal endoderm. Specific questions for the future include: What leads to the recruitment of endothelial cells and vessels by endodermal domains that give rise to vascularized tissues? What explains our observations of differences in a requirement for the aorta, but not the vitelline veins, for the initial expression of Ptf1a? What are the signaling molecules involved? In addition, it will be interesting to identify mesodermal inducers of other transcription factors required for the initiation of pancreatic differentiation from the endoderm, and thus understand how distinct mesodermal interactions lead to the activation of the transcription factor network that promotes pancreatic development.

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