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

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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

(Krapp et al., 1998; Kawaguchi et al., 2002). 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; Hebrok et al., 1998; Hebrok et al., 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 (Deutsch 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). Lammert et al. (Lammert 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 *Flk1* (*Kdr* – Mouse Genome Informatics) null mice (Shalaby et al., 1995). *Flk1* 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 *Flk1* 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

Flk1^{lacZ} 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). Embryo 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 extraembryonic 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.

Flk1^{lacZ} expression, immunohistochemistry and in situ hybridization

For assessing expression of the *Flk1*^{lacZ} 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% H_2O_2. 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-AAGAGTGACTTCCAGAC-3′, antisense 5′-GTACCTCGTTACTC-GACAGG-3′; Tal1 sense 5′-AACAACAACCGGGTGAAGAG-3′, antisense 5′-ACTTGGCCAGGAAATTGATG-3′; Pdx1 sense 5′-CA-GGAGGTGCTTACACAGC-3′, antisense 5′-CCCGCTACTACGTT-TCTTATCTTCC-3′ (2 mM $MgCl_2$, 2% DMSO, annealing at 63°C); Ptf1a sense 5′-GGCCCAGAAGGTCATCATCTGC-3′, antisense 5′-AGGAAAGGGAGTGCCCTGCAAG-3′; Prox1 sense 5′-CCCAGC-TGTTGAAAAATAAC-3′, antisense 5′-TCTCAGGTGCTCATCAC-ATA-3′; Ngn3 sense 5′-GGGATACTCTGGTCCCCCGTGC-3′, antisense 5′-GAGCGCATCCAAGGGATGAGGC-3′; Neurod1 sense 5′-CTTGGCCAAGAACTACATCTGG-3′, antisense 5′-GGAGTA-GGGATGCACCGGGAA-3′ (Deutsch et al., 2001); glucagon sense 5′-CATTCACAGGGCACATTCACC-3′, antisense 5′-ACCAGCC-AAGCAATGAATTCCTT-3′ (Herrera et al., 1991); insulin sense 5′-CAGCCCTTAGTGACCAGCT-3′, 5′-TGCTGGTGCAGCACTG-ATC-3′ (Krapp et al., 1998); Hnf6 sense 5′-AGCCCTGGAGC-AAACTCAAGTCG-3′, antisense 5′-TGCATGTAGAGTTCGACG-TTGGAC-3′ (sequences from F. Lemaigre); Wt1 sense 5′-AAC-CACGGTATAGGGTACGA-3′, antisense 5′-CGGCTATGCATCTG-TAAGTG-3′; Hex sense 5′-GCACAAAAGGAAAGGCGGTCAAGT-3′, antisense 5′-ACCTGTTTCAGTCTTCTCCATTTA-3′.

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 cultured with DMEM medium supplemented with 10% calf serum, 1× penicillin-streptomycin (Gibco-BRL) at 37°C with 5% CO_2, for the indicated periods.

Results

Endothelial cells contact nascent dorsal, but not ventral, Pdx1-positive cells extensively

The initial expression of *Pdx1* mRNA can be seen in dorsal and ventral pancreatic progenitor cells at the 10 somite pair stage (10S) and 7S, respectively (E8.5-9.0) (Ohlsson et al., 1993; Ahlgren et al., 1996; Li et al., 1999; Gannon and Wright, 1999; Jacquemin et al., 2003). The dorsal and ventral pancreatic buds become evident as thickenings of the endodermal epithelium by 15-20S, and as marked bulges from the gut tube by 25-30S. As endothelial cells were shown to promote Pdx1 expression in endoderm-aortal explants (Lammert et al., 2001), we wished to assess the extent to which the aortae interact with the dorsal endoderm at the onset of Pdx1 expression in embryos, which is earlier than that assessed by Lammert et al. (Lammert et al., 2001). To assess this sensitively, we double stained $Flk1^{lacZ/-}$ embryos for Pdx1 and β-galactosidase activity (Shalaby et al., 1995). *Flk1* heterozygous embryos express *lacZ* like the native *Flk1* gene, which is highly expressed in embryonic angioblasts and endothelial cells. As previously described, *Flk1* heterozygous embryos grow normally (Shalaby et al., 1995).

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.

Ventral Pdx1-positive endoderm also becomes associated with the vasculature, in this case the vitelline veins, but the association appears different than that of the dorsal endoderm and the aorta. Initially, at 8S, the lateral-most ventral foregut endoderm is near the sinus venosus (Fig. 1F,G), but as the gut closes off and the embryo turns at 10S, the initial few Pdx1-positive cells on the embryo's right side appear distal to the vitelline veins (Fig. 1H,I). By 12S, when a few more Pdx1-positive cells appear, and later, throughout ventral pancreatic bud emergence, we detected a thin line of mesenchymal cells interceding between the ventral Pdx1-positive cells and the vitelline veins (Fig. 1J-M; see green arrow pointing to Pdx1-negative, $Flk1^{lacZ}$-negative cells between the blue stained vitelline veins and the endoderm). At about the 26 somite stage, the ventral pancreatic domain was completely surrounded by mesenchymal cells (Fig. 1N), which, as for the dorsal bud, contained endothelial cells in capillaries (Fig. 1O). These results indicate that during ventral pancreatic development, Pdx1-positive cells emerge from the ventral endodermal cells which, at times, are very close to, but not in apparent contact with, endothelial cells, whereas during dorsal pancreatic development, the aorta is directly adjacent to the Pdx1-positive endoderm. Other markers of dorsal and ventral pancreatic development are described below.

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).

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

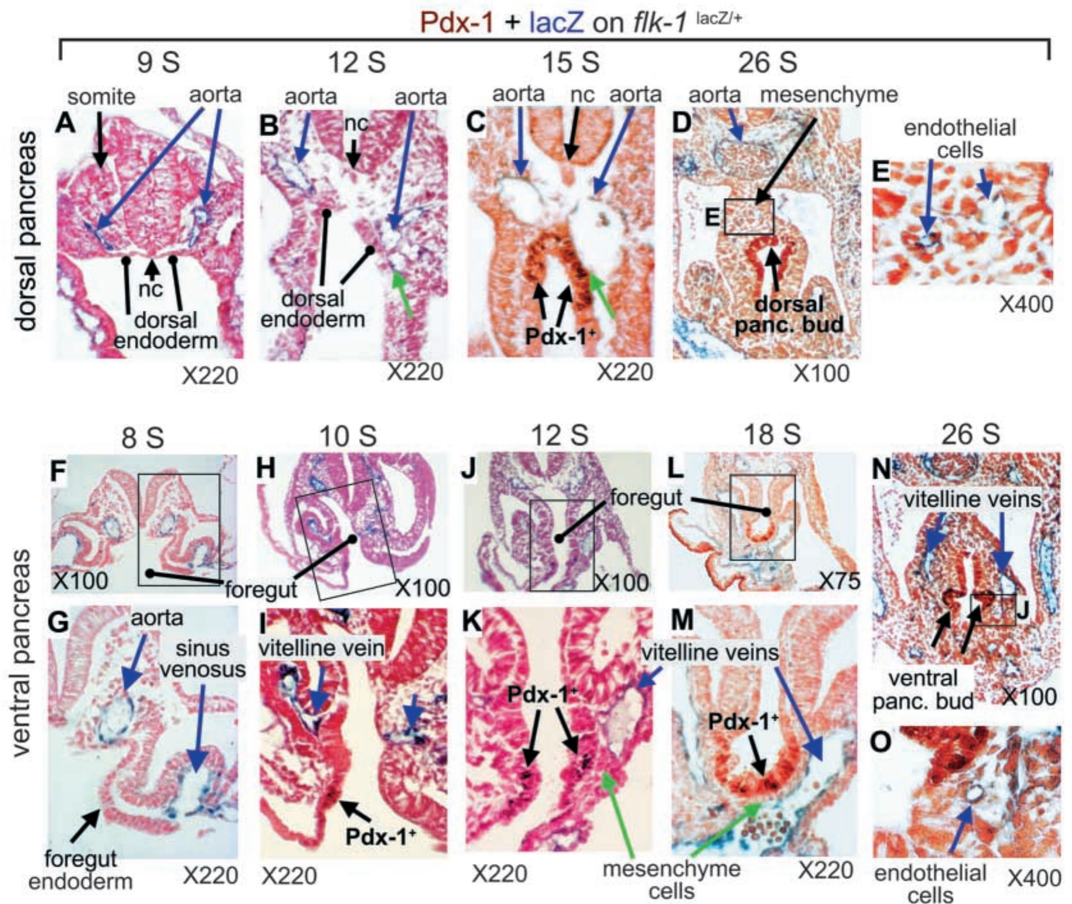

Fig. 1. Dorsal Pdx1-positive endoderm cells interact with endothelial cells more extensively than ventral Pdx1-positive endoderm cells. Double cell labeling for Pdx1 (DAB immunostaining, brown) and β-galactosidase (X-gal, blue) on transverse sections of *Flk1*$^{lacZ/+}$ embryos at E8.5-9.5 (somite stages are indicated on the top of each panel). nc, notochord. The data shown are representative of multiple embryos assayed and sections throughout midgut regions. The boxed regions in D,F,H,J,L,N are magnified in E,G,I,K,M,O, respectively. (A, F) Before the emergence of Pdx1-positive cells, the aorta only has limited contact with the dorsal endoderm, laterally. (B,C) By 12-15S, the aorta moves medially and interacts extensively with the endoderm in a portion of the Pdx1-positive domain, first evident at 15S. No mesenchyme cells were detected between portions of the endoderm and the aorta at this stage (green arrow). (D) As Pdx1-positive cells form the dorsal pancreatic bud, dorsal mesenchyme cells (long black arrow) interpose between them and the fused aorta. At this stage, small capillaries can be found in the dorsal mesenchyme (E, arrows). (F,G) At 8S, the prospective ventral foregut endoderm is in the proximity of the sinus venosus. (H,I) At 10S, during embryo turning, the vitelline vein on the embryo's right side is distal to the initial few Pdx1-positive cells in the ventral endoderm. (J-M) A thin line of mesenchyme cells continuously separates the few, nascent Pdx1-positive endoderm cells from the vitelline veins (K,M; cells at end of green arrow). (N) By 26S, when the ventral pancreatic domain is clearly surrounded by mesenchyme cells, small capillaries can be found in the mesenchyme (O, arrow), as seen in the dorsal region. Original magnifications are indicated at the bottom of each panel.

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 Hnf3β) 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), 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

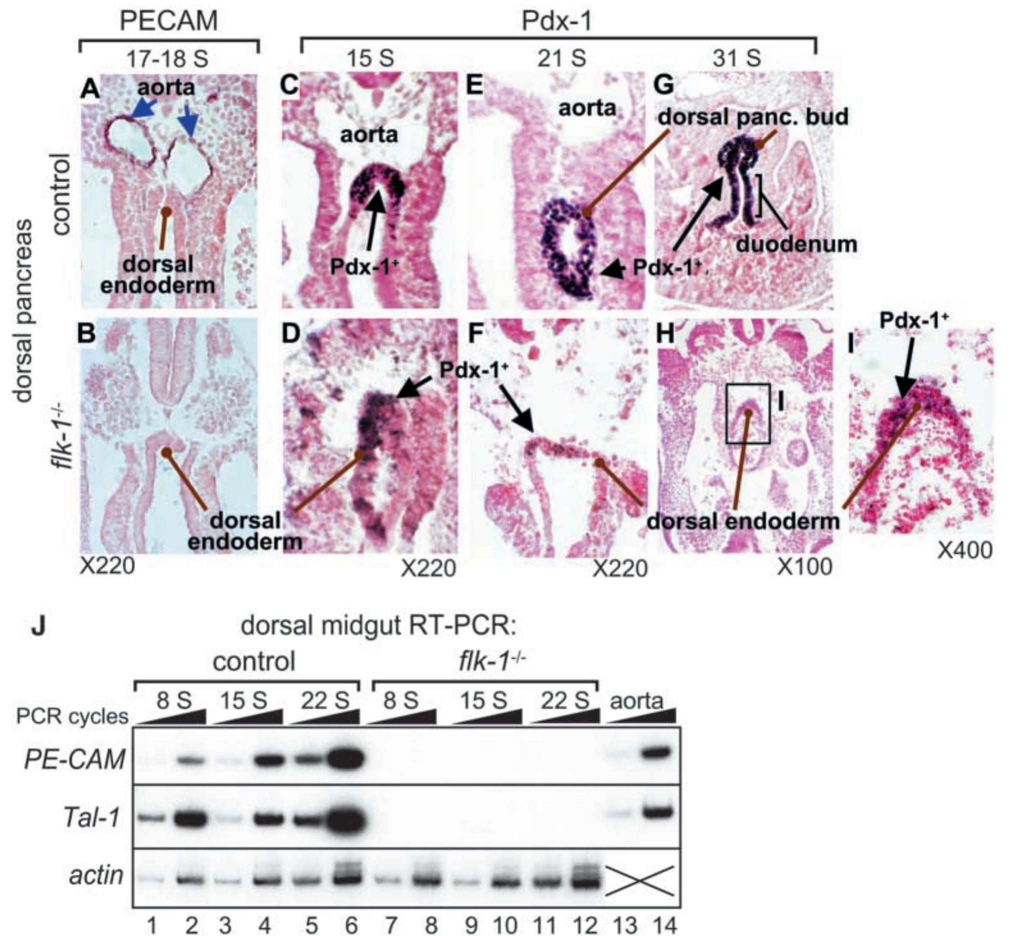

Fig. 2. Dorsal pancreatic development is impaired in *Flk1*$^{-/-}$ embryos after the induction of an initial field of Pdx1-positive cells. Immunostaining of transverse sections of control (upper row) and *Flk1*$^{-/-}$ (lower row) embryos at E9.0-10.0 (somite stages are indicated on the top of each panel). (A,B) Pecam staining to identify blood vessels; no vessels develop in *Flk1*$^{-/-}$ embryos. (C-I) Pdx1 staining. Although at 15S, *Flk1*$^{-/-}$ embryos (D) develop nearly the same size domain of Pdx1-positive cells in the dorsal endoderm as control embryos (C), these cells diminish over time and fail to form a bud (compare G and H). By 31S, very few Pdx1-positive cells can be found in the dorsal endoderm (I). (J) RT-PCR analysis of dorsal midgut RNAs at the designated somite stages (S). The normalizing cycle steps for *Pecam* and *Tal1* were based on prior cycle step analysis of actin. Embryonic aorta served as positive controls. PCR cycle steps: *Pecam*, 30, 33; *Tal1*, 30, 33; actin, 26, 29.

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.

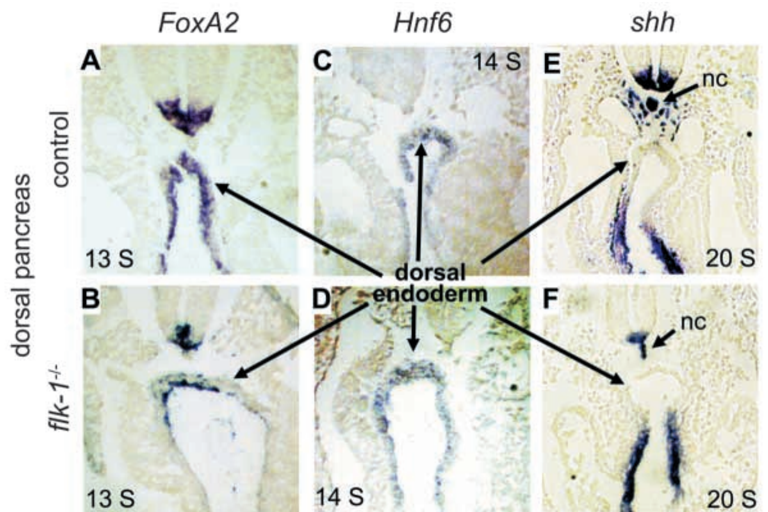

Fig. 3. Dorsal endoderm of *Flk1*$^{-/-}$ embryos expresses *Foxa2* and *Hnf6* and *Shh* expression is repressed, as in control embryos. In situ hybridization of control (A,C,E) and *Flk1*$^{-/-}$ (B,D,F) embryos with probes of *Foxa2* (A,B), *Hnf6* (C,D), and sonic hedgehog (E,F). nc, notochord. Somite stages are indicated in each panel.

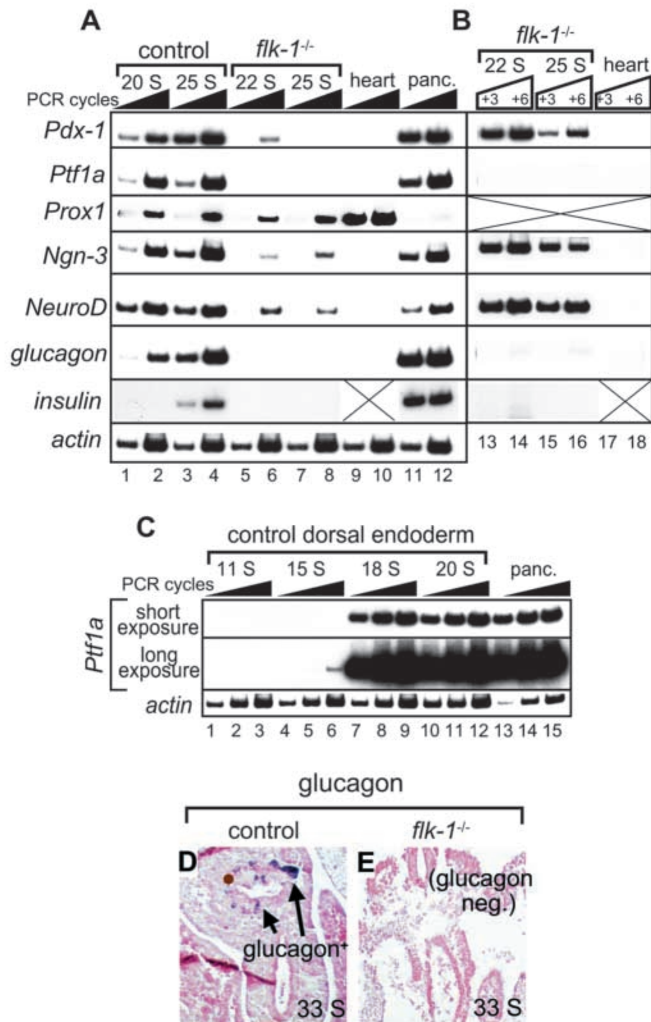

Fig. 4. Dorsal endoderm in *Flk1*$^{-/-}$ embryos does not express *Ptf1a*, insulin and glucagon. (A-C) RT-PCR analysis of the dorsal endoderm in the region of the pancreatic bud. (A) The expression levels of factors involved in early pancreatic development were lower in the dorsal endoderm of *Flk1*$^{-/-}$ embryos than in that of control embryo. (B) The expression of *Ptf1a*, insulin, and glucagon was not observed in the dorsal endoderm of *Flk1*$^{-/-}$ embryos even with three or six additional PCR cycles. PCR cycle ranges of embryo tissues: *Pdx1*, 35-41; *Ptf1a*, 34-40; *Prox1*, 33-39; *Ngn3*, 34-40; *Neurod1*, 34-40; insulin, 33-39; glucagon, 27-33; actin, 23-29. (C) The expression of *Ptf1a* starts by 15S in wild-type embryos, when the aorta has extensive contact with *Pdx1*-positive cells. Short exposure, 12 hours; long, 5 days. In some experiments, *Ptf1a* expression was observed in the 12-15 S range (data not shown). PCR cycles for *Ptf1a*, 39, 42, 45; actin 28, 31, 34. (D,E) Glucagon immunostaining confirms the absence of glucagon expression in the dorsal endoderm of *Flk1*$^{-/-}$ embryos. Somite stages are indicated in the each panel.

Initial ventral pancreas development is not affected in *Flk1*$^{-/-}$ embryos

The absence of endothelial cells and the vitelline vein in the ventral pancreas area of *Flk1*$^{-/-}$ 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*$^{-/-}$ 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*$^{-/-}$ 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*$^{-/-}$ 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*$^{-/-}$ embryos expressed *Ptf1a*, similarly to the wild type (Fig. 5H). These results clearly showed that the initial expression of ventral pancreatic genes, including *Ptf1a*, 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*$^{-/-}$ 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*$^{-/-}$ embryos, but *Ptfla* was only not induced dorsally, endothelial cell interactions appear to control endocrine gene induction at a step later than *Ptfla* expression.

Aorta fragments induce Ptf1a in dorsal endoderm explants of Flk1$^{-/-}$ embryos

To test the hypothesis that the aorta itself, rather than factors in the bloodstream or other secondary effects, regulates the induction of *Ptf1a* in the dorsal endoderm, we performed tissue recombination studies. Dorsal endoderm fragments were dissected from *Flk1*$^{-/-}$ 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*$^{-/-}$ dorsal endoderm expressed *Hnf6*, as expected (Fig. 6D). Owing to the smaller size of the *Flk1*$^{-/-}$ embryos, the endoderm was difficult to dissect. To be sure that the dissected dorsal endoderm, underlying the somites, lacked contaminating

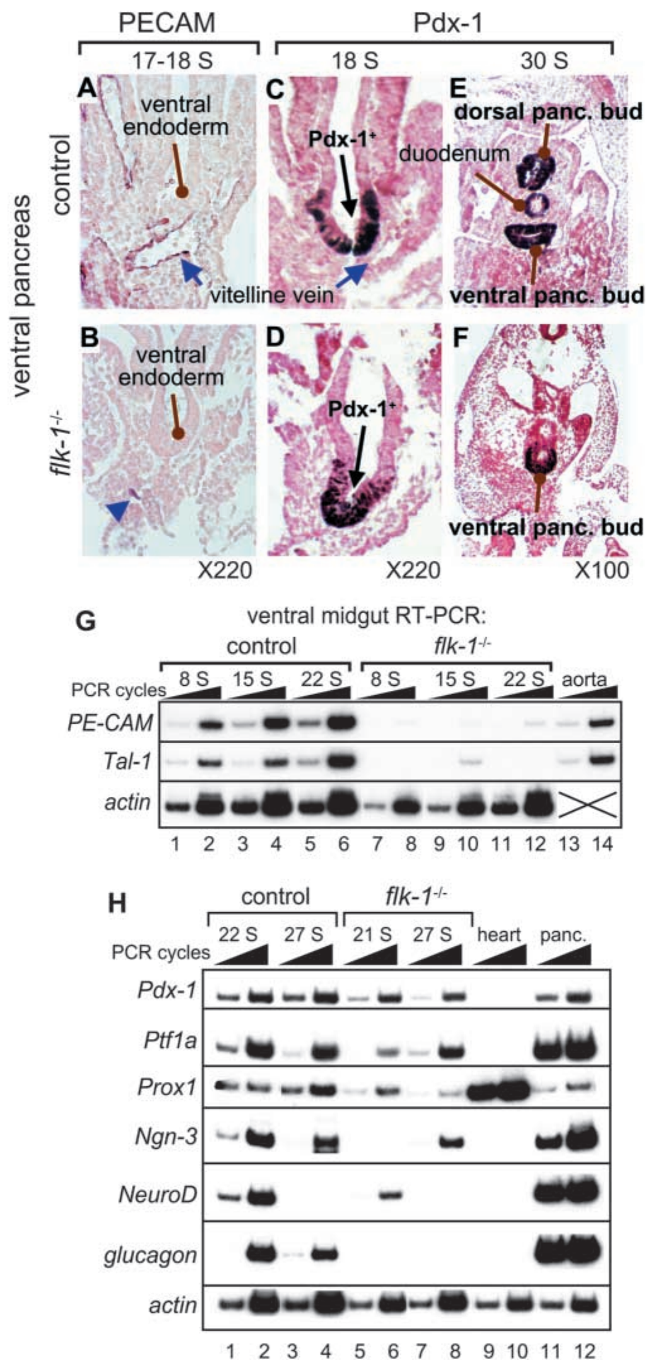

Fig. 5. The initiation of the ventral pancreatic development is not affected in *Flk1*$^{-/-}$ embryos. (A-F) Immunostaining of transverse sections of control (upper row) and *Flk1*$^{-/-}$ (lower row) embryos at E9.0-10.0 (somite stages are indicated on the top of each panel) with Pecam (A,B) and Pdx1 (C-F) antibodies. No endothelial cells or blood vessels were detected in *Flk1*$^{-/-}$ embryos (B). Blue arrowhead denotes a possible extraembryonic cell (see text) staining positive for Pecam; these were occasionally detected and were reported by Shalaby et al. (Shalaby et al., 1995). (G) RT-PCR analysis of *Pecam* and *Tal1* expression in ventral midgut/pancreatic bud tissue. Note faint bands in lanes 10 and 12 (*Flk1* homozygotes). (H) RT-PCR analysis of the ventral region of control and *Flk1*$^{-/-}$ embryos. PCR cycle ranges similar to that in Fig. 4A. Note the induction of *Ptf1a*, but not of glucagon. Insulin is not normally detectable ventrally at these stages (data not shown).

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 cells 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

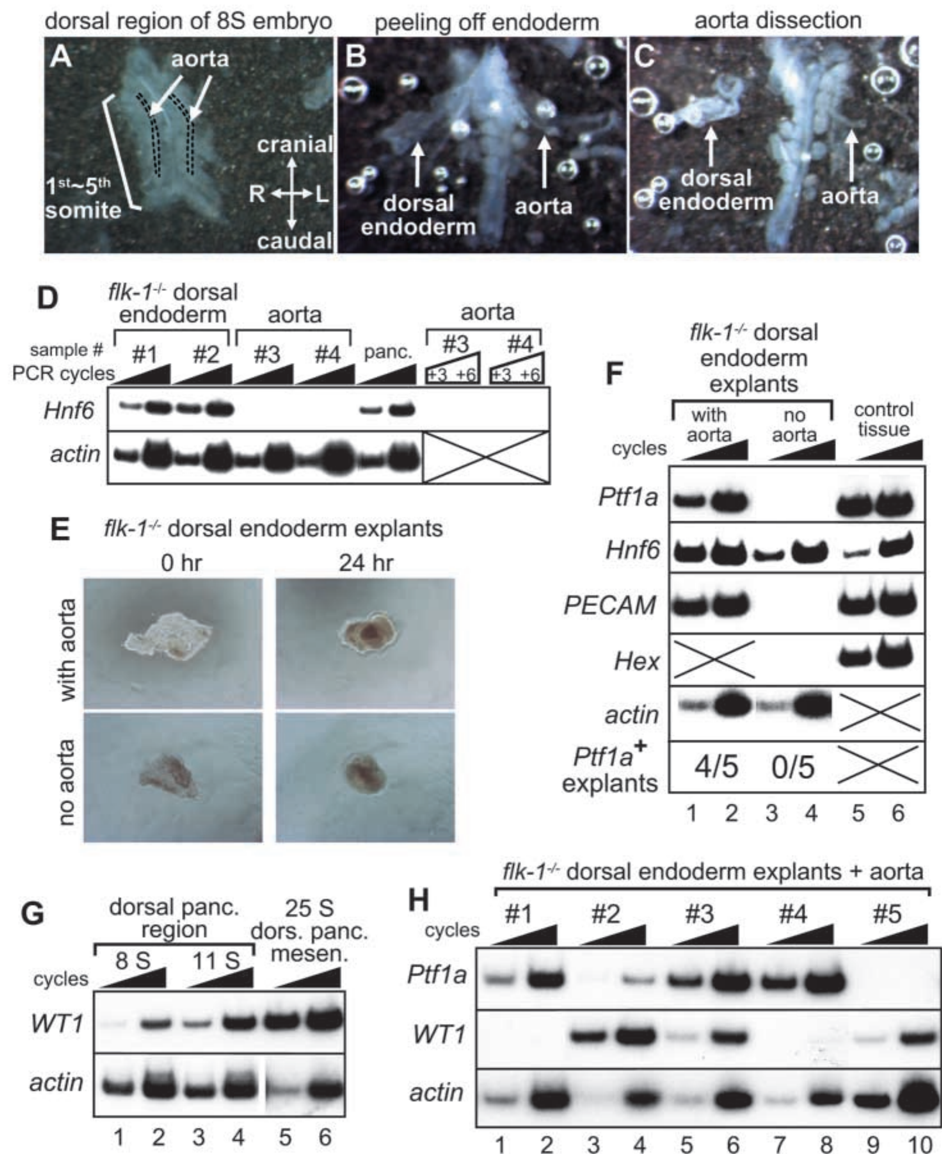

Fig. 6. Dorsal aorta fragments induce *Ptf1a* in dorsal endoderm explants from *Flk1*$^{-/-}$ embryos. (A-C) Dissection of dorsal endoderm and aorta from E8.5 embryos. (D) RT-PCR analysis of *Hnf6* confirms that the gene is expressed in the endoderm fragments and that there is no contamination of endodermal cells in the dissected aorta. PCR cycle ranges for *Hnf6*, 40-45; actin, 26-30. (E) The endoderm explants were cultured with (upper panels) or without (lower panels) the dorsal aorta for 24 hours and photographed. Marked differences in growth were not observed. (F) Analysis of explant cultures. *Hnf6* and *Pecam* RT-PCR confirm the existence of endoderm and aorta, respectively, in explants, and *Hex* analysis of the dorsal endoderm excludes the possibility of contamination by ventral pancreas cells (see text). All explants included in the analysis had the *Hnf6, Pecam, Hex* and actin expression phenotypes shown. For wild-type controls (lanes 5, 6), we used: *Ptf1a*, adult pancreas; *Hnf6*, dorsal endoderm from an 8S embryo; *Pecam*, dorsal aorta from an 8S embryo; *Hex*, liver and ventral pancreatic buds from a 20S embryo. Four out of five *Flk1*$^{-/-}$ dorsal endoderm explants cultured with aorta from control embryos expressed *Ptf1a*, whereas none of the five *Hex*-negative explants cultured without the aorta expressed *Ptf1a*. PCR cycle ranges: *Ptf1a*, 40-43, *Hnf6*, 39-42; *Pecam*, 39-42; *Hex*, 37-40, actin, 28-31. (G) *Wt1*, a mesenchyme marker (Armstrong et al., 1992), is expressed in the dorsal pancreatic region. PCR cycle ranges: *Wt1*, 40, 43; actin, 26, 29. (H) *Wt1*-expressing cells do not persistently contaminate endoderm-aorta explants that induce *Ptf1a* (RT-PCR analysis). PCR cycle ranges: *Ptf1a*, 40-44; *Wt1*, 38-42;

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 the *Flk1*$^{-/-}$ 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 *Flk1*$^{-/-}$ 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 Ptf1a 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 *Flk1*$^{-/-}$ embryos, and both glucagon and insulin were not induce 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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