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An endothelial–mesenchymal relay pathway regulates early phases of pancreas development

Patrick Jacquemin [a,1], Hideyuki Yoshitomi [b,1,2], Yasushige Kashima [b], Guy G. Rousseau [a], Frederic P. Lemaigre [a], Kenneth S. Zaret [b,*]

[a] *Hormone and Metabolic Research Unit, Institute of Cellular Pathology and Université catholique de Louvain, 75 Avenue Hippocrate, B-1200 Brussels, Belgium*
[b] *Cell and Developmental Biology Program, Fox Chase Cancer Center, Room W410, 333 Cottman Avenue, Philadelphia, PA 19111-2497, USA*

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Abstract

Understanding the tissue interactions that induce pancreatic progenitor cells from the embryonic endoderm provides insights into congenital malformations, tissue repair, and differentiating stem cells to a pancreatic fate. The specification of pancreatic progenitors within the dorsal endodermal epithelium has been thought to involve two phases of mesodermal interactions; first with the lateral plate mesoderm and notochord and then with aortic endothelial cells. Afterwards, branching morphogenesis of the pancreatic bud is induced by Isl-1-positive dorsal mesenchyme cells, whose growth is stimulated by factors in the circulation. Using mouse genetic models and embryo tissue explants, we show that the aortic endothelium and dorsal mesenchyme each possess an additional role in pancreatic induction, prior to the branching morphogenesis step. Specifically, we find that aortic endothelial cells promote the survival of nearby, Isl-1-positive dorsal mesenchyme, independently of factors from the circulation. Furthermore, we find that *FGF10* signaling from the mesenchyme cells maintains *Ptf1a* expression in the dorsal pancreatic bud and appears genetically redundant with a role for the transcription factor gene HNF6 in promoting the induction of Pdx-1-positive dorsal endoderm. Together, these studies reveal a relay pathway from aortic endothelium to dorsal mesenchyme and then to the endoderm, along with functions of the dorsal mesenchyme that promote the initial differentiation of the dorsal pancreatic endoderm, prior to organ morphogenesis.
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Keywords: Pancreas; Endothelium; Mesenchyme; Organogenesis; FGF10; Specification

Introduction

In embryonic development, the pancreas, liver, lung, and thyroid emerge from the gut endoderm, the latter being one of the three germ layers formed during gastrulation. Studies of vertebrate organogenesis have revealed that endodermal interactions with different mesodermal cell types pattern the endodermal epithelium into progenitor cell domains and promote local organ outgrowth (Hogan and Zaret, 2002). The purpose of this study was to determine how inductive interactions with different mesoderm cell types are coordinated to initiate pancreatic development. Understanding the processes that cause the endodermal epithelium to stably activate the pancreatic program and undergo morphogenetic development should provide insight into tissue regeneration, oncogenesis, and stem cell differentiation.

In separate dorsal and ventral–lateral domains of the endoderm, the *HNF6* transcription factor gene is required for timely initiation of *Pdx-1* expression (Jacquemin et al., 2003), thereby marking progenitor cells of the pancreas and duodenum (Jonsson et al., 1994; Offield et al., 1996; Ahlgren et al., 1997; Gu et al., 2002). A subset of these cells then expresses other transcription factors, marking both endocrine and exocrine progenitors (Kawaguchi et al., 2002; Chiang and Melton, 2003; Jensen et al., 2004). The cells become columnar, thicken the endodermal epithelium into a tissue bud, and proliferate into the surrounding stromal tissue, thereby initiating pancreas morphogenesis (Slack, 1995).

The dorsal pancreatic endoderm domain is induced by phases of tissue interactions (Wessels and Cohen, 1967; Wells

* Corresponding author. Fax: +1 215 379 4305.
E-mail address: zaret@fccc.edu (K.S. Zaret).
[1] These authors contributed equally to the work.
[2] Current address: Dept. General Surgery, Chiba Univ. Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan.

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and Melton, 2000; Deutsch et al., 2001; Grapin-Botton et al., 2001; Horb and Slack, 2001; Kumar et al., 2003). In mouse embryos around 8.5 days gestation (E8.5), activin-related signaling from the notochord, a mesodermal derivative, represses sonic hedgehog expression in the midgut endoderm and thereby permits the expression of *Pdx-1* and a pancreatic fate in the dorsal endoderm (Kim et al., 1997; Hebrok et al., 1998). Subsequently, around E8.75–9.0 (12–20 somite pairs; 12–20S), aortic endothelial cells, another mesodermal derivative, interact with the dorsal, Pdx-1+ endoderm and induce the essential transcription factor gene *Ptf1a* (Yoshitomi and Zaret, 2004). *flk-1*$^{-/-}$ embryos, which lack endothelial cells and a vasculature (Shalaby et al., 1995), fail to induce *Ptf1a* and develop a dorsal pancreas bud. The ability of isolated, wild-type aortae to induce *Ptf1a* in co-cultured explants of *flk-1*$^{-/-}$ dorsal endoderm shows that the endothelial induction of *Ptf1a* in the dorsal endoderm is specific to the cells' interactions and not to factors in the bloodstream (Yoshitomi and Zaret, 2004).

After specification, morphogenetic induction of the pancreatic buds also involves phases of tissue interactions. Explant recombination studies showed that budding of dorsal pancreatic tissue into the stroma, around E9.5, requires interactions with dorsal mesenchyme cells (Golosow and Grobstein, 1962; Wessels and Cohen, 1967; Spooner et al., 1970; Pictet et al., 1972; Ahlgren et al., 1996), and subsequent proliferation and branching morphogenesis of the pancreatic cells require *FGF10* expressed in the mesenchyme (Bhushan et al., 2001; Ye et al., 2005). Embryos deficient for *Isl-1*, a transcription factor gene normally expressed by dorsal pancreatic mesenchyme and other cell types (Pfaff et al., 1996; Cai et al., 2003), exhibit a marked deficiency in dorsal mesenchyme cells and a failure to develop a dorsal pancreatic bud (Ahlgren et al., 1997). Similarly, embryos deficient for N-cadherin exhibit apoptosis of the dorsal mesenchyme and a failure to develop a dorsal pancreatic bud (Esni et al., 2001). These embryos also have an abnormal aortic endothelium and deficient blood circulation (Edsbagge et al., 2005). Restoration of aortic integrity and circulatory function, in embryos expressing N-cadherin from a cardiac muscle-specific promoter, results in dorsal mesenchyme survival and pancreatic budding, indicating that a systemic factor is involved (Luo et al., 2001; Edsbagge et al., 2005). This factor may be sphingosine-1-phosphate (S1P), a signaling phospholipid found in the blood, for which dorsal mesenchyme and endothelial cells express receptors. Exogenous S1P can stimulate the growth of wild-type dorsal mesenchyme and endothelial cells in explant cultures (Edsbagge et al., 2005). Thus, S1P signaling to mesenchyme, possibly indirectly, via local endothelial cells, supports mesenchyme survival, which in turn promotes pancreatic budding and *FGF10*-induced proliferation.

Although the lack of endothelial cells in *flk-1*$^{-/-}$ embryos is associated with a failure of *Ptf1a* induction and a failure in dorsal pancreas budding (Yoshitomi and Zaret, 2004), the failure in budding is not due to the absence of *Ptf1a* expression, since *Ptf1a*$^{-/-}$ embryos develop an initial dorsal pancreatic bud (Krapp et al., 1998; Kawaguchi et al., 2002). Thus, endothelial cells contribute to a phase of dorsal pancreas development that succeeds *Ptf1a* induction. We therefore investigated a functional connection between aortic endothelial cells and local mesenchyme cells in promoting dorsal pancreatic morphogenesis. This led to the unexpected discovery of a relay pathway whereby endothelial cells directly support mesenchyme cells. Further investigation of the early contributions of the mesenchymal cells to pancreatic development revealed a mechanism whereby the cells promote the initial differentiation of dorsal pancreatic endoderm. We suggest that knowledge of this network can be useful for diverse areas of pancreas biology.

Methods

Embryo isolation and genotyping

Flk-1^{lacZ} mice (gift of J. Rossant) and *Fgf10* mice (gift of N. Itoh and S. Kato) were generated as described (Sekine et al., 1999; Shalaby et al., 1995). *Hnf6*$^{-}$ mice were described (Jacquemin et al., 2000). Noon of the day of the vaginal plug was considered E0.5. Embryos were harvested at E8.5–10.5 and staged according to pairs of somites. Embryos were dissected free of extraembryonic membranes, and the heads were used for genotyping. As the pancreatic phenotype of all heterozygous embryos was normal (Jacquemin et al., 2000; Shalaby et al., 1995), these embryos and wild-type embryos were designated as controls.

In situ hybridization, immunohistochemistry, and TUNEL analysis

Embryos were prepared and analyzed as described (Jacquemin et al., 2003; Yoshitomi and Zaret, 2004). In situ hybridization on sections was performed using a digoxigenin-labeled *fgf10* probe (gift of N. Itoh), and labeling was detected with the TSA Biotin System (NEN). Tissue sections were reacted with mouse monoclonal anti-Isl-1 antibody (Iowa Univ. hybridoma bank) at a 1/2000 dilution or with anti-Pdx-1 antibody (gift from C. Wright) at a 1/5000 dilution in 0.1× blocking buffer at 4°C overnight. Primary antibody was detected with Vectastain Elite ABC kit (Vector, Burlingame, CA) using Vector SG (Vector, Burlingame, CA) or Alexa-Fluor 594 labeled secondary antibodies (Molecular Probes, Eugene, OR). Sections were counterstained with eosin Y or 4′,6-diamino-2 phenylindole (DAPI). Whole mount immunohistochemistry was performed as described (Jacquemin et al., 2003) using affinity-purified rabbit anti-Pdx-1 antibody (gift of H. Edlund). TUNEL analysis with fluorescein was performed with the In situ cell death detection kit (Roche Applied Science).

Tissue dissection and recombinant explants

Embryo tissues were dissected as described (Gittes and Galante, 1993; Yoshitomi and Zaret, 2004). Isolated E9.5 aortae were washed with PBS to remove possibly contaminating blood cells. The dissected tissues were cultured according to Lammert et al. (2001) with the following modifications: The tissues were recombined, embedded in growth-factor reduced Matrigel (Becton Dickinson), and cultured with DMEM media supplemented with 10% calf serum, 1× penicillin–streptomycin (GIBCO BRL) at 37°C with 5% CO_2 for the indicated periods. FGF-10 was from R&D Systems. Media were changed every 2 days of culture.

RT-PCR analysis

RNA was isolated from tissues and explants by using Qiagen RNeasy Micro Kits. PCR analysis and actin primers were as described by Yoshitomi and Zaret (2004). Other primers were *TBP* sense 5′-ACCCTTCACCAATGACTCCTATG-3′, antisense 5′-ATGATGACTGCAGCAAATCGC-3′, 190-bp product; *Pdx-1* sense 5′-AAGAGCCCAACCGCGTCCAGC-3′, antisense 5′-AGTACGGGTCCTCTTGTTTTC-3′ 125-bp product; *Isl-1* sense 5′-GAGCTGGAGACCCTCTCAGTC-3′, antisense 5′-GCGCATCTGGCCGAGGGTTGG-

3′, 200-bp product; *Ptf1a* sense 5′-TGCCATCGAGGCACCCGTTC-3′, antisense 5′-TGAGCTGTTTTTCATCAGTCCAG-3′, 99-bp product. Cycle step titrations were taken for all PCR analyses, and single points in the exponential range, normalized to actin, were chosen for the figures.

Results

Selective loss of dorsal mesenchyme cells in flk-1$^{-/-}$ embryos

When the aorta first interacts with the dorsal endoderm, at the 12–20 somite pair stage (E8.75–9.0), mesenchyme cells begin to accumulate lateral to the endoderm (Wessels and Cohen, 1967). By the 20–25 somite pair stages (E9.5), when the aorta moves dorsally, away from the endoderm, Isl-1-positive mesenchyme cells accumulate between the aorta and the dorsal endoderm (Ahlgren et al., 1997). Although mesenchymal cell survival requires circulating signals via the aorta (Edsbagge et al., 2005), we wished to test the possibility that aortic endothelial cells directly affect dorsal pancreatic mesenchyme cells. We therefore first analyzed the developing pancreatic bud at the 24S stage (E9.5) in wild-type and *flk-1*$^{-/-}$ embryos, which lack endothelial cells and the aorta (Yoshitomi and Zaret, 2004). The dorsal endoderm cells of *flk-1*$^{-/-}$ embryos were weakly Pdx-1-positive and remained in an immature shape (Fig. 1B), in contrast to the columnar wild-type cells at this stage (Fig. 1A). Notably, the mesenchyme that is normally dorsal to the dorsal pancreatic bud at this stage (Fig. 1A) was virtually absent in the *flk-1*$^{-/-}$ embryos (Fig. 1B), with nearly an empty space between the Pdx-1-positive dorsal endoderm and the neural tube (Fig. 1B). By contrast, the ventral and lateral mesenchyme appeared unaffected in *flk-1*$^{-/-}$ embryos, and the ventral midgut endoderm cells expressed high levels of Pdx-1, assumed their normal columnar shape, and initiated the formation of a tissue bud (Figs. 1C, D) (Yoshitomi and Zaret, 2004).

To assess the nature of the dorsal mesenchyme cells in *flk-1*$^{-/-}$ embryos, we performed immunostaining for Isl-1 (Ahlgren et al., 1997). Wild-type embryos at 26S contained Isl-1-positive cells dorsal and lateral to the dorsal pancreatic bud, as well Isl-1-positive pancreatic bud cells (Fig. 1E), as expected (Ahlgren et al., 1997; Pfaff et al., 1996). *flk-1*$^{-/-}$ embryos at 26S had Isl-1-positive cells lateral to the gut tube and within the dorsal endoderm, but the rare mesenchyme cells dorsal to the gut tube were Isl-1-negative (Fig. 1F). We conclude that in the *flk-1*$^{-/-}$ embryos, mesenchymal cells appear lateral to the gut tube, but not dorsally.

Selective apoptosis of local dorsal mesenchyme cells in flk-1$^{-/-}$ embryos

We next investigated the means by which Isl-1-positive mesenchymal cells failed to appear dorsal to the Pdx-1-positive endoderm in *flk-1*$^{-/-}$ embryos. We noted that the dorsal, Isl-1-negative cells were small and round, compared to the Isl-1^{+} cells in control embryos (Figs. 1A, B, E, F), suggesting that they might be apoptotic. At 16–19S (E8.75–9.0), when the aorta normally begins to pull away from the endoderm and Isl-1-positive mesenchyme cells accumulate near the dorsal–lateral endoderm, and begin to appear dorsally (Ahlgren et al., 1997; Pfaff et al., 1996; and see Figs. 4C, F, I below), there were few or no TUNEL-positive cells in dorsal midgut

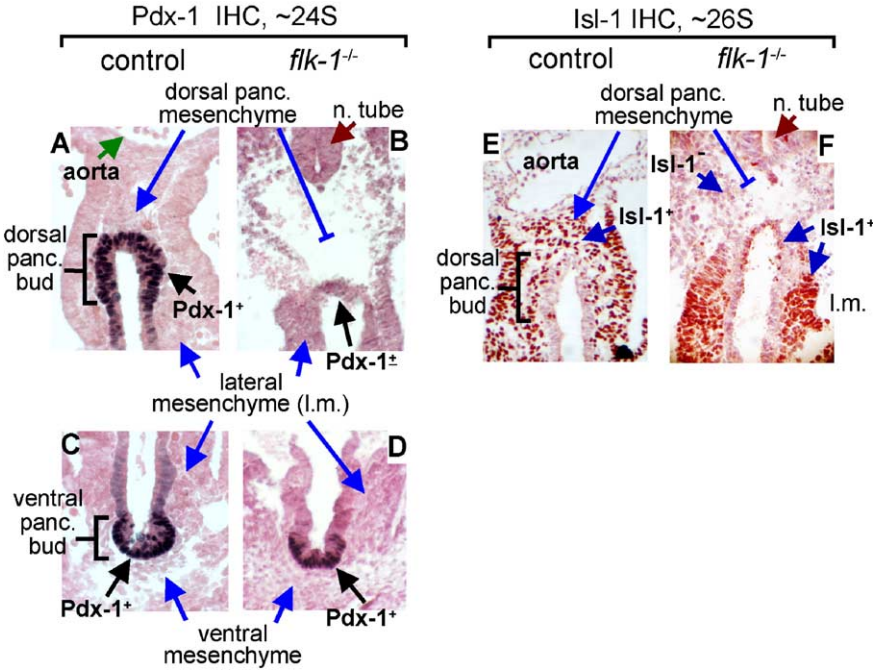

Fig. 1. Dorsal Isl-1^{+} mesenchyme is deficient in *flk-1*$^{-/-}$ embryos. Pdx-1 (A–D) and Isl-1 (E–F) immunostaining of transverse sections of control and *flk-1*$^{-/-}$ embryos at 24 (A–D), 26 (E–H) somite stages. Control embryos have extensive dorsal and lateral Isl-1^{+} mesenchyme cells (brownish-purple staining nuclei, (E)) and a distinct dorsal pancreatic bud (A, E). *flk-1*$^{-/-}$ embryos at 26S have Isl-1^{+} mesenchyme cells lateral to the gut tube and Isl-1^{+} cells within the dorsal endoderm (F), but lack Isl-1^{+} mesenchymal cells dorsally (B, F). In *flk-1*$^{-/-}$ embryos, the ventral pancreatic bud is surrounded by normal amounts of mesenchyme cells and is unaffected (C, D).

mesenchyme of control embryos (Figs. 2A, B; d.m.). However, in *flk-1*$^{-/-}$ embryos at 16S, the mesenchyme cells located dorsal–lateral to the gut tube (Fig. 2C, d.m., right green arrow), as well as dorsal to it (Fig. 2C, d.m., left green arrow), began to appear TUNEL-positive, and by 19S, virtually all such mesenchyme cells were TUNEL-positive, including cells lateral to the dorsal region of the gut tube (Fig. 2D, right green arrow), indicating apoptosis. And as we noted above, by 24S, mesenchymal cells were virtually absent from the dorsal midgut endoderm, whereas normally, the region is filled with Isl-1-positive mesenchyme cells (Figs. 1B, F; Ahlgren et al., 1997; Pfaff et al., 1996). Thus, the timeline for mesenchymal failure is between the 16S and 24S stages, i.e., E9.0–9.5, and it includes both Isl-1-negative cells that are dorsal to the gut tube and a subset of Isl-1-positive cells that are normally dorsal–lateral to the gut tube and that normally appear dorsally after the 16–19S stages (also see Figs. 4C, F, I, below).

To determine the specificity with which apoptotic cells appear dorsally in *flk-1*$^{-/-}$ embryos, we assessed the extent to which apoptotic cells could be detected in the foregut, midgut, and hindgut (Figs. 2G, I, K, M). We used Pdx-1 immunohistochemistry to confirm the midgut position relative to the others (Figs. 2H, J, L). Apoptotic cells could be seen in the dorsal mesenchyme of *flk-1*$^{-/-}$ embryos at the level of the foregut (Fig. 2G, light blue cells denoted by blue arrows) but not in the cardiac tube, despite the fact that cardiac muscle precursor cells are Isl-1-positive (Cai et al., 2003). No apoptotic cells could be found in the dorsal mesenchyme at the level of hindgut (Fig. 2K), where, in wild-type embryos, the aorta is normally narrow and thin. These results indicate that the survival of mesenchyme cells which are normally close to the aorta, rostral to the hindgut, are selectively supported by the presence of the aorta.

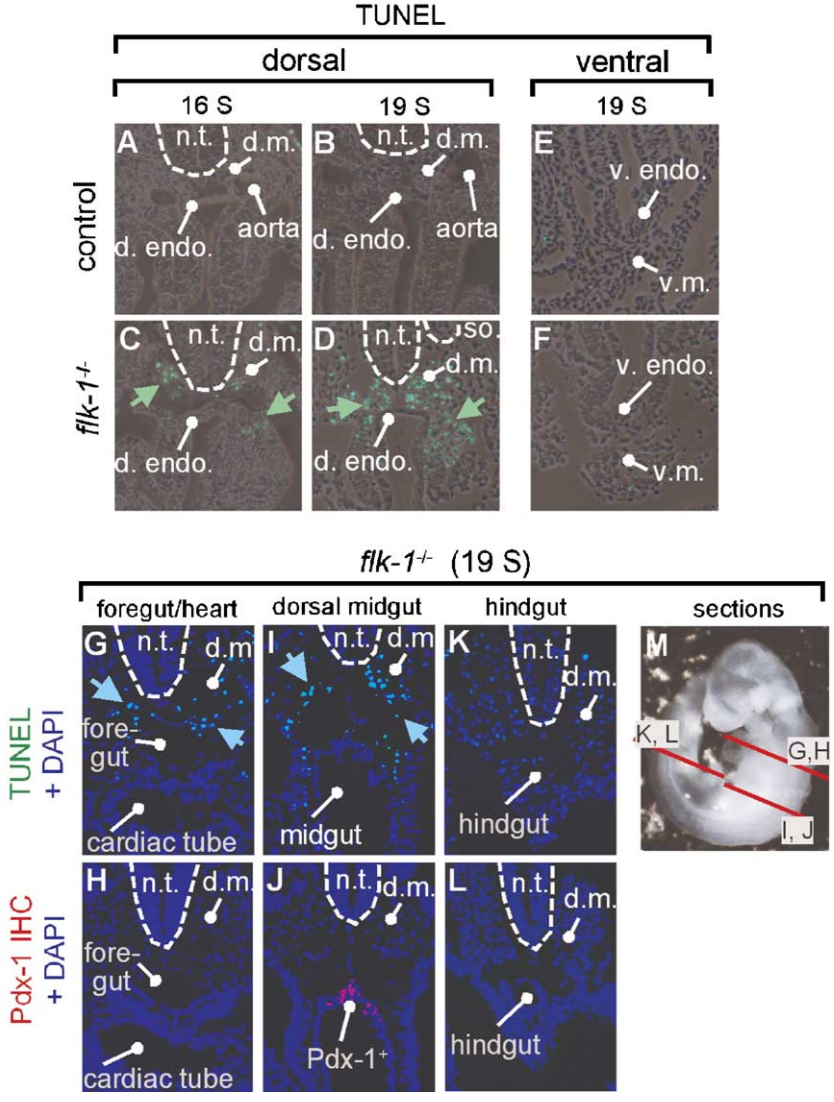

Fig. 2. Presence of aorta supports dorsal mesenchyme cell survival. (A–F) TUNEL analysis on transverse sections of control (A, B, E) and *flk-1*$^{-/-}$ (C, D, F) embryos. At 16S, *flk-1*$^{-/-}$ dorsal mesenchyme cells begin to apoptose (C) and by 19S, most have done so (D). By contrast, no apoptotic cells can be seen ventrally in *flk-1*$^{-/-}$ embryos (F). d.m. and v.m., dorsal and ventral mesenchyme; n.t.; neural tube, d. endo and v. endo, dorsal and ventral endoderm. (G–M) Mesenchymal cells cranial to the hindgut are apoptotic in *flk-1*$^{-/-}$ embryos. TUNEL analysis and DAPI stain (G, I, and K) and Pdx-1 IHC and DAPI stain (H, J, and L) on adjacent transverse sections at different anterior–posterior positions of a 19S *flk-1*$^{-/-}$ embryo, as shown in panel M.

The selectivity of the dorsal mesenchyme's requirement for the vasculature is striking, in that there was not an increased number of TUNEL-positive cells in the dorsal or ventral endoderm (Figs. 2E, F), neural tube (Figs. 2C, D, G, I, K, n.t.), somites (Fig. 2D, so.; and data not shown), or notochord (Figs. 2C, D, G; below n.t.) in *flk-1*$^{-/-}$ embryos, and these tissues are normally as close to the aorta as many of the dorsal mesenchyme cells. We conclude that the presence of the vascular system specifically supports local mesenchyme cells, both Isl-1-positive and -negative, whereas diverse other nearby cell types are unaffected.

Aorta cells support dorsal mesenchyme survival in the absence of circulating factors

Although previous studies indicated that circulating factors support dorsal mesenchyme survival (Edsbagge et al., 2005), the studies left open the possibility that the effect was either secondary to circulating factors required for endothelial maturation or acting in conjunction with direct interactions with endothelial cells (Lammert et al., 2001; Matsumoto et al., 2001; Yoshitomi and Zaret, 2004). To investigate these possibilities, we performed tissue recombination studies. The dorsal mesenchyme and aortae were dissected from midsections of 20–25S wild-type embryos, the aortae were washed to remove blood, and the resultant mesenchyme was shown to be *Pdx-1*-negative and thus free of dorsal pancreatic endoderm cells (Figs. 3A–C). The dorsal mesenchyme and aortae were then cultured individually or together for 8 days and then subjected to Isl-1 immunostaining. Explants of the aorta alone contained a few Isl-1-positive cells (Figs. 3J–L, arrows in L), probably due to a low level of contamination of Isl-1-positive dorsal mesenchyme cells in the dissected aortic tissue (Fig. 3B). Dorsal mesenchyme explants cultured alone exhibited poor survival of Isl-1-positive cells (Figs. 3G–I, arrows in I). By contrast, mesenchyme explants cultured with aorta exhibited abundant Isl-1-positive cells (Figs. 3D–F, arrows in F). Thus, the aortic endothelium can directly promote the survival of Isl-1-positive mesenchymal cells, independent of the endoderm and independent of aortal function as a conduit for factors in the bloodstream.

Expression of Fgf10 by Isl-1-positive dorsal mesenchyme cells well prior to the branching morphogenesis stage of pancreatic differentiation

In light of the classical organogenic view of dorsal mesenchyme cells (Wessels and Cohen, 1967; Pictet et al., 1972), our in vitro explant studies suggested that endothelial cells would promote dorsal pancreas development indirectly, by supporting dorsal mesenchyme cells. The loss of dorsal pancreatic mesenchyme in *flk-1*$^{-/-}$ embryos was associated with a failure of the dorsal, pdx-1-positive endoderm to enter a budding and branching, morphogenetic phase (Fig. 1). This hypothesis led us to investigate growth signals produced by the

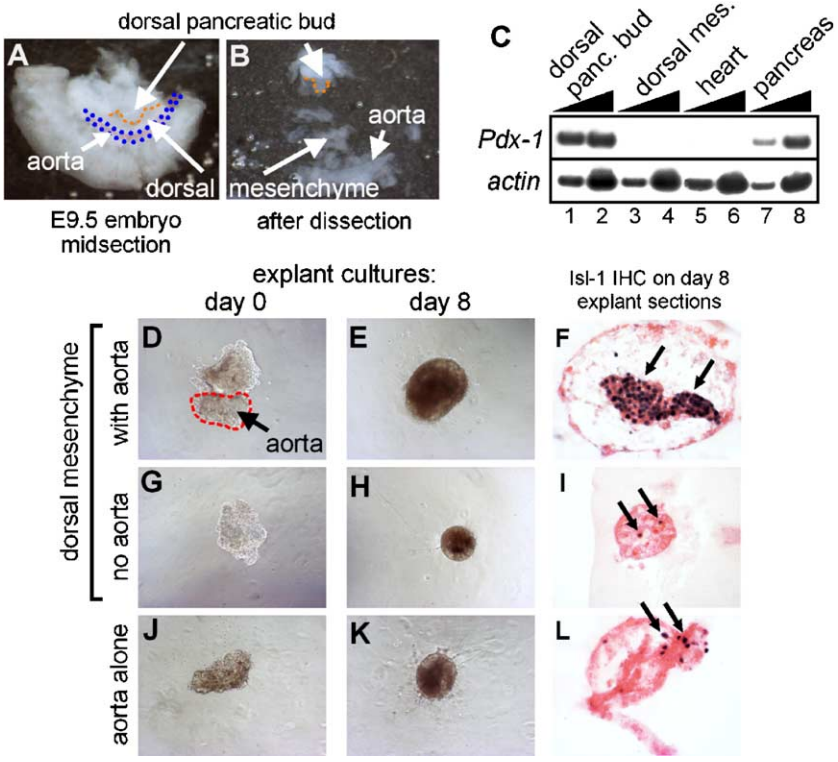

Fig. 3. Isolated aorta maintains the growth of Isl-1-positive dorsal mesenchymal cells in vitro. (A, B) Tissues being dissected from an E9.5 control embryo. (C) RT-PCR analysis shows that *Pdx-1*-positive endodermal cells do not contaminate the dissected dorsal mesenchymal tissue. (D–L) In vitro explant cultures of designated tissues. (D, E, G, H, J, K), whole mount phase contrast views. (F, I, L) Immunohistochemistry for Isl-1 on explant sections. Only the mesenchymal explants cultured with the aorta maintain abundant Isl-1-positive cells (F, shown by arrows).

mesenchyme cells that would be secondary to endothelial and circulating factors. *Fgf10* was previously shown to be expressed at E9.5 in the pancreatic mesenchyme and to be necessary for Pdx-1-positive endoderm proliferation and branching morphogenesis (Bhushan et al., 2001). Also, we note that in those studies, the size of the Pdx-1-positive domain in *Fgf10*$^{-/-}$ embryos appeared smaller than wild type at E10.5, prior to branching morphogenesis (Bhushan et al., 2001; Ye et al., 2005). These observations suggested to us a possible earlier signaling role for *Fgf10*, prior to branching morphogenesis.

Indeed, analysis of sectioned embryos showed that *Fgf10* is expressed in mesenchyme cells when they first appear lateral to the Pdx-1-positive region of the midgut (15S to 17S stage; Figs. 4A, B, D, E; note red hybridization grains to the FGF10 probe). Shortly afterwards, starting at the 21–26S stages, *Fgf10*-positive mesenchyme cells surround the dorsal pancreatic bud, prior to branching morphogenesis (Figs. 4G, H, J, K). *Fgf10* was not expressed around the ventral pancreas bud at E9.5 (26S, Figs. 4M, N). While technical issues prevented colabeling of individual sections, an analysis of sequential serial sections through the dorsal region of wild-type embryos revealed a remarkable concordance between *Fgf10* and *Isl-1* expression in the dorsal mesenchyme cells, at all stages tested (Figs. 4B, C, E, F, H, I). Taken together, these results indicate that the aortic endothelium promotes survival of *Isl-1*- and *fgf10*-positive mesenchymal cells around the dorsal pancreas.

Functional redundancy of Fgf10 and Hnf6 genes in pancreatic specification

To investigate the role of FGF10 in early pancreas development, we analyzed *Fgf10*$^{-/-}$ embryos. At the 19S and 23S stages, pancreas development in *Fgf10*$^{-/-}$ embryos was not affected (compare Figs. 5A, B with D, E), indicating that pancreas specification was normal. At the 27S stage (Figs. 5C, F), the size of the dorsal pancreas was reduced in *Fgf10*$^{-/-}$ embryos, which is consistent with deficient morphogenesis as described earlier (Bhushan et al., 2001). Therefore, analysis of *Fgf10*$^{-/-}$ embryos did not suggest a role for *Fgf10* in expression of pancreatic genes in the endoderm.

However, the timing of dorsal mesenchyme *Fgf10* expression, starting around the 20S stage in normal embryos, correlates with the time of delayed onset of *Pdx-1* expression in *Hnf6*$^{-/-}$ embryos, from the 11S stage to the ~20S stage (Jacquemin et al., 2003). This suggested that *Fgf10* may play a role in inducing Pdx-1 in the endoderm in the absence of *Hnf6*. To test this hypothesis, we generated *Hnf6*$^{-/-}$/*Fgf10*$^{-/-}$ double null embryos. Remarkably, the *Hnf6*$^{-/-}$/*Fgf10*$^{-/-}$ embryos exhibited an apparently complete defect in pancreatic specification. That is, *Hnf6*$^{-/-}$/*Fgf10*$^{-/-}$ embryos failed to activate Pdx-1 expression dorsally or ventrally, even at later stages (Figs. 5G–J for up to 27S; later stages, data not shown), and did not generate a pancreatic bud. Embryo section analysis showed the dorsal pancreatic mesenchyme was intact in the double homozygous embryos (Figs. 5K, L), showing that the failure in pancreatic specification and budding was not due to an absence of mesenchyme. Also, in *Hnf6*$^{-/-}$ embryos, *Fgf10* expression was unaffected (Figs. 5M, N), showing that FGF10 is produced at the time and place when delayed Pdx-1 expression is induced in such embryos. These findings reveal a functional redundancy for the *Hnf6* and *Fgf10* genes, whereby they insure the specification of the pancreas.

FGF10 enhances the expression of Ptf1a

Ptf1a is expressed in progenitors of all pancreas cells and is necessary for the development of all descendant cell types (Krapp et al., 1998; Kawaguchi et al., 2002). We previously found that *Ptf1a* is normally induced in the pancreatic buds at the 15–18 somite stage, which precedes the appearance of *Isl-1*$^{+}$ cells dorsally (Yoshitomi and Zaret, 2004). Interestingly, we discovered that the expression of *Ptf1a* is not properly maintained in *Fgf10*$^{-/-}$ embryos, in that at the 26 somite stage, midgut regions containing the pancreatic buds were markedly deficient in *Ptf1a* mRNA, relative to mRNAs for a control *Tbp* gene or *Pdx-1* (Fig. 6A). This suggested that FGF10 from pancreatic mesenchyme cells might normally help maintain *Ptf1a* expression.

To investigate this possibility more directly, we isolated dorsal pancreatic bud explants and treated them with FGF10. We previously showed that embryonic tissue explants of dorsal, *Pdx-1*-positive endoderm at ~8 somites (E8.5) would induce the de novo expression of the pancreatic specification gene *Ptf1a* (Kawaguchi et al., 2002) if they were cultured in vitro in the presence of aorta tissue (Yoshitomi and Zaret, 2004). This initial induction of *Ptf1a* by the aorta occurs in the apparent absence of dorsal mesenchyme cells. We now find that when dorsal pancreatic bud explants from E9.0–9.5 (20–25S) are cultured for 2 days, they maintain very low levels of *Ptf1a* expression (Fig. 6B, lane 1). The E9.0–9.5 explants contain *Isl-1*$^{+}$ mesenchyme cells (Fig. 6B, lane 1) and express low but detectable levels of *FGF10* mRNA (data not shown). However, when the explants are treated with increasing concentrations of FGF10, they exhibit markedly induced levels of *Ptf1a* mRNA, despite the apparent decrease in *Isl-1* expression or *Isl-1* expressing cells (Fig. 6B, lanes 2–4).

To assess whether FGF10 acts directly upon the endoderm cells or is dependent upon Isl-1^{+} mesenchyme cells or endothelial cells in the explants, we repeated the assay using dorsal pancreatic endoderm isolated from *flk-1*$^{-/-}$ embryos. These explants exhibited low levels of *Ptf1a* expression and little or no *Isl-1* expression (Fig. 6B, lane 5); the latter was due to a lack of mesenchyme and endothelial cells (see Figs. 1–3 and Yoshitomi and Zaret, 2004). However, in separate experiments (Fig. 6B, lanes 6–8 and data not shown), addition of FGF10 to the *flk-1*$^{-/-}$ pancreatic endoderm cultures increased the expression of *Ptf1a*. It is likely that the level of induction was less than seen with wild-type cultures because of the relatively weak survival of dorsal endoderm in the absence of the endothelium and mesenchyme (see Figs. 1B, F). In summary, FGF10 can stimulate

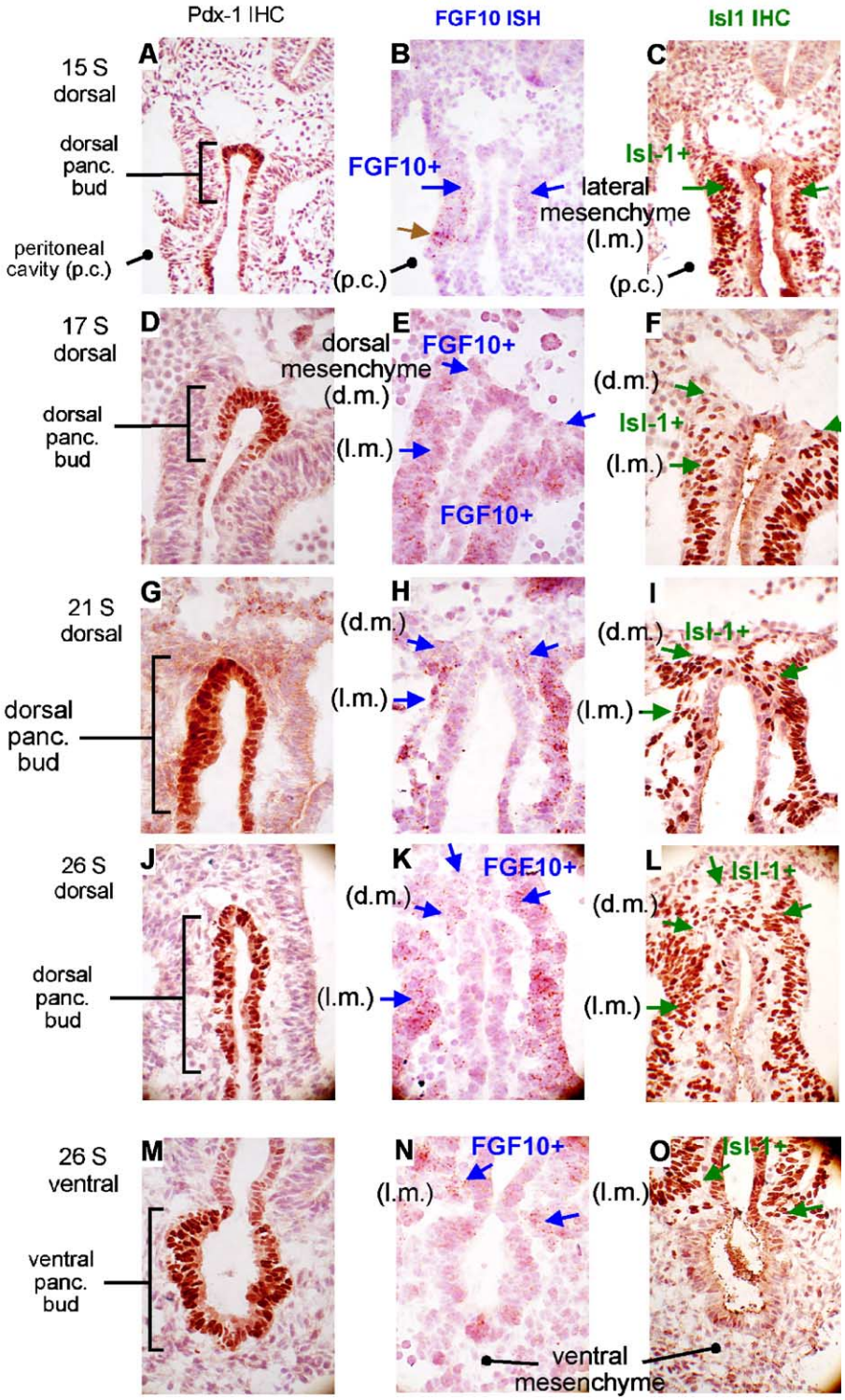

Fig. 4. Fgf10 is expressed in the mesenchyme surrounding the dorsal pancreatic endoderm. Serial sections from wild-type embryos were stained by immunohistochemistry to detect Pdx-1 (A, D, G, J, M) and Isl-1 (C, F, I, L, O), and by in situ hybridization to detect *Fgf10* expression (B, E, H, K, N). *Fgf10* expression is first found in lateral mesenchyme cells (B, E). Starting at the 21S stage, *Fgf10*$^{+}$ mesenchyme cells surround the dorsal pancreatic bud (H, K). The Fgf10^{+} cells are Isl-1^{+} (compare B, E, H, K with C, F, I, L). Some Fgf10 is detected in the mesenchyme around the gut tube, but not around the ventral pancreatic bud (M, N). The ventral pancreas mesenchyme did not express Isl-1 (O). Blue arrows point to *Fgf10* expression, green arrows to Isl-1 expression. l.m. and d.m., lateral and dorsal mesenchyme.

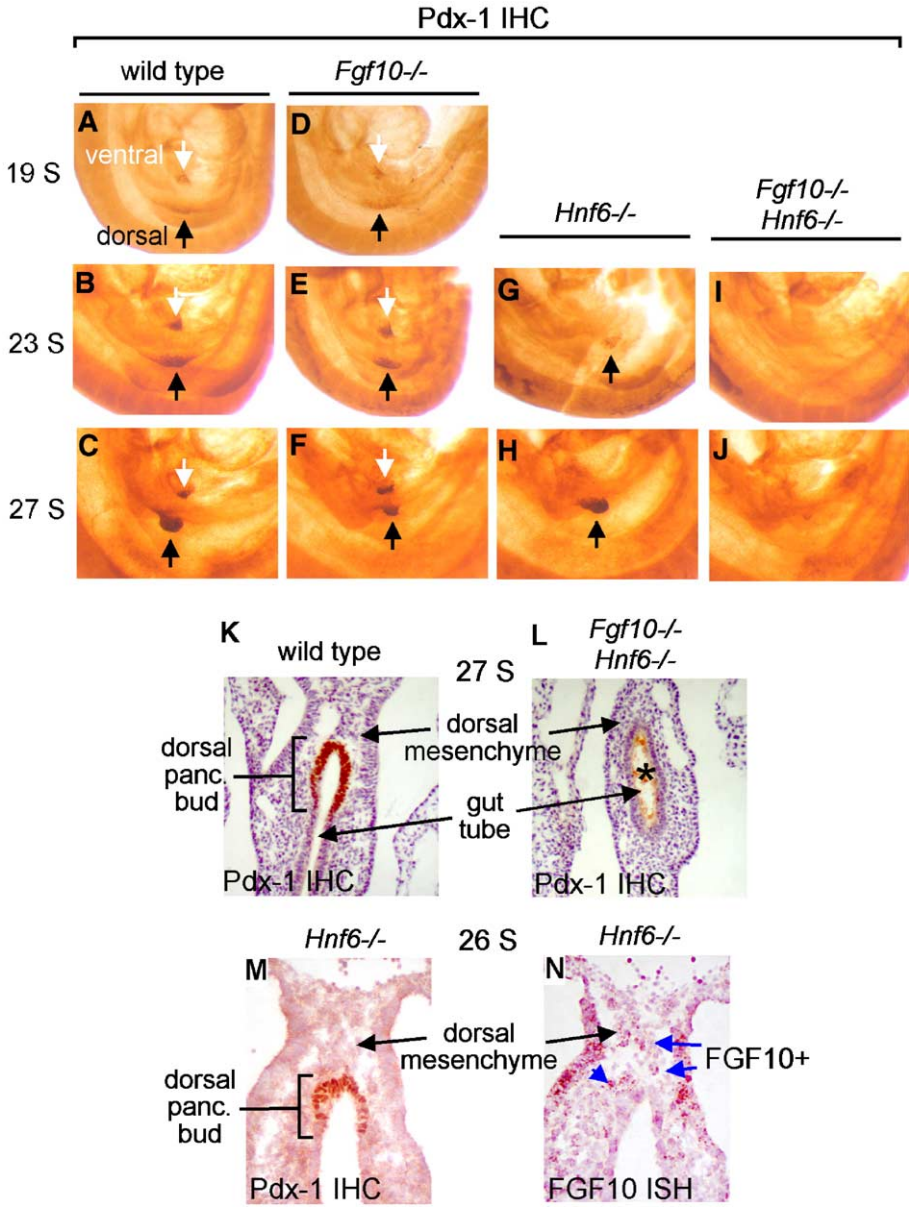

Fig. 5. Failure of pancreatic specification in *Fgf10*$^{-/-}$/*Hnf6*$^{-/-}$ double homozygous embryos. (A–J) Whole mount Pdx-1 immunohistochemistry of embryo midsections. Black arrows, dorsal pancreatic anlagen; white arrows, ventral pancreatic anlagen. (K, L) Section analysis of 27S embryo midguts from the Pdx-1 whole mounts showing an intact dorsal mesenchyme both in control and *Fgf10*$^{-/-}$/*Hnf6*$^{-/-}$ double homozygous embryos. The yellow material marked with an asterisk in panel L is nonspecific debris trapped in the gut tube. No Pdx-1-positive cells or pancreatic bud emerges in *Fgf10*$^{-/-}$/*Hnf6*$^{-/-}$ embryos, even after 36S (data not shown). Serial sections stained for Pdx-1 by immunohistochemistry, and for Fgf10 by in situ hybridization showed that Fgf10 expression is detected in dorsal mesenchyme, even in the absence of HNF-6 (M, N).

the expression of *Ptf1a* by acting upon the dorsal, *Pdx-1*-positive endoderm.

Discussion

Taken together, our data are consistent with endothelial cells having a secondary, indirect inductive influence on pancreatic differentiation that succeeds the endothelial cells' initial influence on pancreatic specification (Lammert et al., 2001; Yoshitomi and Zaret, 2004). We find that endothelial cells support dorsal mesenchyme cells in the midgut that are either Isl-1^{+} or Isl-1^{-} (Figs. 1, 2). The Isl-1-positive population was previously found to be crucial for dorsal pancreatic development (Ahlgren et al., 1997), and here, we show that these cells first appear lateral to the midgut by 15 somites and then proximal to the dorsal Pdx-1-positive cells at around 20 somites (Fig. 4). Our tissue explant assays showed that endothelial cells directly promote the survival of the Isl-1-positive mesenchymal cells, independent of the endoderm and factors in the circulation (Fig. 3). Although circulating factors, including sphingosine-1-phosphate, have recently been shown to also promote dorsal midgut mesenchymal cell survival (Edsbagge et al., 2005), we suggest that such factors work either in parallel with the endothelium, secondarily through it, or both.

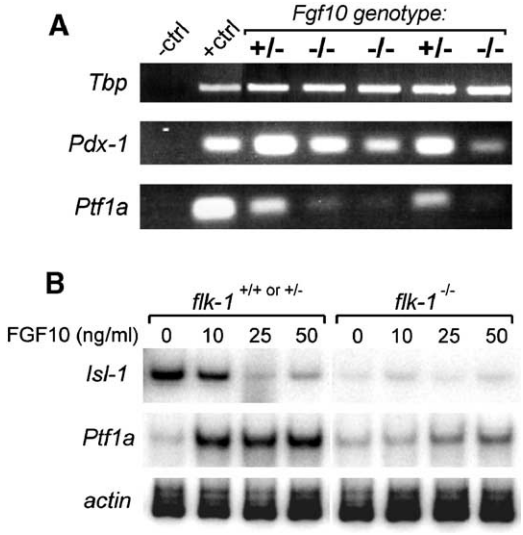

Fig. 6. FGF10 stimulation of *Ptf1a* expression. (A) RT-PCR of midgut mRNAs containing the dorsal and ventral pancreatic buds from E9.5 (26S) embryos of the designated *Fgf10* genotypes. "−ctrl" is with water input; "+ctrl" is with cDNA from E14.5 pancreases. The data show that *Fgf10* is necessary to maintain *Ptf1a* expression. (B) RT-PCR of dorsal pancreatic bud explants of the designated *Flk-1* genotypes cultured for 2 days in the presence of the designated concentrations of FGF10 in the medium. The data show that FGF10 induces *Ptf1a* expression in the endoderm in the absence of endothelium and of significant amounts of Isl-1-positive mesenchyme cells in the explant.

The endothelial support of dorsal mesenchyme survival is remarkably specific, in that diverse other tissues that are normally near the aorta and the endothelium, such as the endoderm, neural tube, notochord, and somites, were not apoptotic in *flk-1*$^{-/-}$ embryos, which lack a vasculature (Figs. 1, 2). The dorsal mesenchyme cells are also different from the ventral, septum transversum mesenchyme cells, which contribute to hepatic endoderm growth (Hentsch et al., 1996) but exhibit no dependence upon endothelial cells (Matsumoto et al., 2001). The lack of dependence of the mammalian ventral pancreatic bud or the zebrafish pancreatic bud on the endothelium (Field et al., 2003; Yoshitomi and Zaret, 2004) further underscores the specific dependence of the dorsal mesenchyme and dorsal pancreas bud on the aorta. Various other examples of direct endothelial cell interactions are emerging (Serluca et al., 2002; LeCouter et al., 2003; Shen et al., 2004), giving rise to the view that different types of endothelium possess distinct inductive capabilities.

We found that the lateral and dorsal Isl-1^{+} mesenchyme cells express FGF10 at around the 15 and 20 somite stages, respectively, which is earlier than had been previously appreciated for this cell domain (Fig. 4). We found that *Fgf10* is necessary at 26 somites to maintain the expression of *Ptf1a*, a transcription factor gene needed to complete pancreatic specification (Kawaguchi et al., 2002). Furthermore, FGF10 treatment of dorsal pancreatic endoderm explants from *flk-1*$^{-/-}$ embryos was sufficient to increase the expression of *Ptf1a*. Based on the timing of normal *Ptf1a* induction, the ability of isolated aorta tissue to induce *Ptf1a* in the absence of mesenchyme (Yoshitomi and Zaret, 2004), and the later appearance of dorsal Isl-1^{+} mesenchyme cells (Fig. 4, this study), we do not believe that the mesenchyme cells promote normal *Ptf1a* induction. Rather, mesenchyme cells may contribute to the maintenance of *Ptf1a* expression. In wild-type explants (Fig. 6), traces of endogenous FGF10 in culture (data not shown) were insufficient to robustly maintain *Ptf1a* expression (Fig. 6), and the ability of exogenous FGF10 to stimulate *Ptf1a* expression may reflect the need for optimal FGF10 concentrations to maintain *Ptf1a* expression.

Previous work had shown that in the absence of HNF-6, dorsal pancreas specification is delayed until the 20S stage (Jacquemin et al., 2003). We now show that the dorsal pancreatic specification in the *Hnf6*$^{-/-}$ background requires *Fgf10* signaling from the mesenchyme (Fig. 5). *Fgf10*, and dorsal pancreatic mesenchyme cells, may possess an unexpected, compensatory activity in terms of insuring dorsal pancreatic specification. Interestingly, ectopic *Fgf10* expression in Pdx-1-positive cells has been found to promote the proliferation of pancreatic progenitor cells and thereby inhibit pancreatic differentiation (Hart et al., 2003; Norgaard et al., 2003). One explanation for these latter results, in light of our findings, is that the FGF10-enhanced progenitor state may include the expression of *Ptf1a*, which is normally down-regulated in endocrine cells after its expression in all pancreatic progenitors (Kawaguchi et al., 2002). Regardless of this speculation, it is interesting to consider how an exogenous factor such as FGF10 can compensate for early pancreas program genes that are normally dependent upon *Hnf6* expression for activity. Presumably, FGF10 activates pathways and transcription factors within undifferentiated endoderm cells that bypass the proper temporal activity of diverse earlier signals that effect the induction of a dorsal Pdx-1-positive endoderm and pancreatic bud formation (Murtaugh and Melton, 2003).

Our discovery of the role of *Fgf10* in *Hnf6* deficient embryos shows that the dorsal endoderm is either continuously subject to pancreatic specification signals that remain present during the 7–20S period, or continuously adaptable to environmental signals that can be re-interpreted for specification in an *Hnf6*$^{-/-}$ background. The plasticity of the endoderm during this period, and the complexity of relay networks, such as from the endothelium to the mesenchyme, underscore the depth to which gut tissue specification has evolved. Far more work is needed to understand these pathways before we can prospectively program cells for biomedical needs with confidence.

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