Vascular function and sphingosine-1-phosphate regulate development of the dorsal pancreatic mesenchyme

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Summary

Early growth and differentiation of the pancreatic endoderm is regulated by soluble factors from the pancreatic mesenchyme. Previously, we demonstrated that N-cadherin-deficient mice lack a dorsal pancreas, due to a critical role of N-cadherin in dorsal pancreatic mesenchymal cell survival. Here, we show that restoring cardiac and circulatory function in N-cadherin null mice by cardiac-specific expression of N-cadherin, rescues formation of the dorsal pancreas, indicating that the phenotype is secondary to defects related to cardiac/vascular function. Based on this observation, we demonstrate that soluble factors present in plasma, such as sphingosine-1-phosphate, rescue formation of the dorsal pancreas in N-cadherin-deficient mice. We also show that sphingosine-1-phosphate indirectly promotes budding of the pancreatic endoderm by stimulating pancreatic mesenchymal cell proliferation. Finally, we identify sphingosine-1-phosphate receptors within the mesenchyme and show that pertussis toxin blocks the sphingosine-1-phosphate-induced actions, suggesting the involvement of G-protein-coupled sphingosine-1-phosphate receptors. Thus, we propose a new model where blood vessel-derived sphingosine-1-phosphate stimulates growth and budding of the dorsal pancreatic endoderm by induction of mesenchymal cell proliferation.

Key words: Pancreas, Morphogenesis, Blood vessel, Mesenchyme, Sphingosine-1-phosphate, Mouse

Introduction

Differentiation and growth of the pancreatic endoderm is regulated by different sources of mesenchymal signals. Initially, the notochord and lateral plate mesenchyme regulate commitment of the dorsal and ventral pancreatic endoderm, respectively. For example, activin-βB and FGF2 secreted from the notochord inhibit expression of sonic hedgehog in the presumptive dorsal pancreatic endoderm, which results in induction of the pancreatic and duodenal transcription factor Pdx1 in this region (Hebrok et al., 1998; Kumar and Melton, 2003). Subsequently, both the ventral and dorsal pancreatic endoderm come in close proximity to blood vessel endothelial cells. Using a combination of in vivo and in vitro experiments, it was recently demonstrated that endothelial cells regulate subsequent steps in differentiation of the pancreatic endoderm. Coculture of prepatterned pancreatic endoderm and aorta endothelium resulted in endocrine cell differentiation. Moreover, removal of endothelial cell precursors for the dorsal aorta in *Xenopus* embryos resulted in inhibition of endocrine cell differentiation in the endoderm. Finally, ectopic attraction of endothelial cells to the stomach and duodenum, by the transgenic expression of Vegfa under the control of the Pdx1-promoter, resulted in ectopic insulin-expressing cells (Lammert et al., 2001). Analysis of *Flk*$^{-/-}$ embryos, which lack endothelial cells, further revealed that maintenance, but not induction, of Pdx1, Ptf1a initiation, and bud emergence, require aorta or endothelial cell interactions. Notably, in contrast to the dorsal pancreatic bud, induction of Ptf1a and emergence of the ventral bud do not require endothelial cell interactions (Yoshitomi and Zaret, 2004). The identity of the endothelium-derived molecules has yet to be identified.

Soon after cell fate determination of the pancreatic endoderm, morphogenesis begins with folding of both the dorsal and ventral pancreatic endoderm, processes regulated by soluble factors (members of the FGF and activin families) secreted from the mesenchyme (Le Bras et al., 1998; Miralles et al., 1999; Scharfmann, 2000). Whereas the lateral and ventral gut mesenchyme is splanchnic-derived and present before gut closure and pancreas commitment, the dorsal pancreatic mesenchyme is recruited from a hitherto unknown subpopulation of lateral plate mesenchymal cells.

Mice deficient for islet 1 (Isl1) and the cell adhesion molecule N-cadherin lack a dorsal pancreas due to failure to form the dorsal pancreatic mesenchyme (Ahlgren et al., 1997; Esni et al., 2001). Recently, we showed that N-cadherin is required for formation of the dorsal pancreas, by mediating

dorsal pancreatic mesenchymal cell survival (Esni et al., 2001). Notably, whereas Pdx1 expression is initiated within the N-cadherin null (*Cdh2*$^{-/-}$) dorsal pancreatic endoderm, it later disappears (at 9.5 days postcoitum, dpc) (Esni et al., 2001). *Cdh2*$^{-/-}$ mice die of cell adhesion defects in the heart around 9.5 dpc (Radice et al., 1997). However, these mice exhibit additional defects in development of the nervous system, somites and yolk sac. Importantly, when cadherin function was restored within the heart of N-cadherin-deficient mice by expressing either N-cadherin or E-cadherin under the regulation of a cardiac-specific promoter (α myosin heavy chain) (cardiac-rescued *Cdh2*$^{-/-}$ mice), the embryos survived until 10.5-11 dpc due to rescued heart and vascular function (Luo et al., 2001).

To address whether the pancreatic phenotype in N-cadherin-deficient mice reflects a cell-autonomous function of N-cadherin within the mesenchyme or if it is secondary to other defects, e.g. cardiac and/or vascular function, we analysed pancreas development in cardiac-rescued *Cdh2*$^{-/-}$ mice. Unexpectedly, morphogenesis of the dorsal pancreatic bud was rescued in 9.5 dpc cardiac-rescued *Cdh2*$^{-/-}$ mice, suggesting that the pancreatic phenotype of N-cadherin-deficient mice was secondary to the cardiac/vascular defects. Using an in vitro pancreatic explant model, we show that sphingosine-1-phosphate (S1P)-mediated G-protein-coupled signalling rescues formation of the dorsal pancreas in N-cadherin-deficient mice in vitro, by specifically triggering proliferation of the dorsal pancreatic mesenchyme.

Materials and methods

Animals

Pancreatic development was indistinguishable between wild-type and *Cdh2*$^{+/-}$ mice. Genotyping of N-cadherin knockout and cardiac-rescued *Cdh2*$^{-/-}$ mice was as previously described (Luo et al., 2001; Radice et al., 1997).

Immunohistochemistry and immunoreagents

For immunohistochemistry, embryos were fixed and sectioned as previously described (Esni et al., 1999). Antibodies directed against N-cadherin, Pdx1 and Isl1 were used as previously described (Esni et al., 1999; Esni et al., 2001). Endothelial cells, F-actin, and proliferating cells were detected with anti-CD31 (PECAM, PharMingen, 1:100), endomucin, V.5C7 (Morgan et al., 1999), Phalloidin-Alexa 568 (Molecular probes, 1:40) and anti-Ki67 (Novocastra Laboratories Ltd, 1:200), respectively. Biotin-conjugated anti-rat (1:1000) and anti-rabbit (1:1000) antibodies were purchased from Jacksons Immuno Research Laboratory Inc. Alkaline-phosphatase conjugated anti-rat (1:1000) was purchased from Southern Biotechnology Associates, Inc. The Vectastain ABC and Dab substrate kit for peroxidase were from Vector Laboratories Inc. and used according to the manufacturer's instructions. Endomucin was detected with a TSA kit from Molecular probes and used according to the manufacturer's instructions with slight modifications. Sections were permeabilized and blocked in 5% skim milk dissolved in PBST (PBS pH 7.4, 1% Triton X-100) and subsequently incubated with PBSLEC (PBS pH 6.8, 1% Triton X-100, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.1 $MnCl_2$) for 60 minutes at room temperature. The endomucin antibody was diluted 1:20 and incubated overnight at 4°C. Hematoxylin and eosin staining was performed by standard procedure.

Whole-mount Immunohistochemistry

Whole-mount immunohistochemistry was performed as previously described (Ohlsson et al., 1993).

Preparation of agarose-beads

Agarose-beads from Bio-Rad were rinsed three times in PBS and added onto a bacterial plate, where they were dried prior to being soaked in different factors. Beads were incubated in plasma (prepared from 15.5 dpc wild-type embryos), S1P (Sigma, final concentration 0.1 μM) dissolved in PBS supplemented with 100 μg/ml BSA and in pertussis toxin (Sigma, final concentration 0.2 μg/ml) dissolved in PBS supplemented with 100 μg/ml bovine serum albumin (BSA) at 37°C for one hour and stored at 4°C. Beads were prepared weekly.

Culture of pancreatic rudiments

Isolation, recombination and culture of pancreatic rudiments were carried out essentially as previously described (Ahlgren et al., 1996; Esni et al., 2001; Gittes and Galante, 1993). Pre-soaked agarose-beads (see above) were added to the rudiments. The explants were cultured for 48 hours in a humidified incubator at 37°C with 5% CO_2 and subsequently whole-mount stained with Pdx1 and CD31 antibodies.

For cultures of dorsal pancreatic mesenchyme, the same mesenchyme was removed from 10.5 dpc wild-type endoderm in ice-cold PBS without using trypsin. Mesenchyme pooled from several embryos was plated onto 0.1%-gelatine-coated coverslips positioned in a 24-well tissue-culture plate and cultured in '199' medium (Gibco), supplemented with 10% inactivated fetal calf serum (FCS), 50 U/ml penicillin/streptomycin (Gibco), and 1.25 μg/ml fungizone (Gibco), for 24 hours in a humidified incubator at 37°C prior to the addition of different factors. The factors were added for another 24 hours before the samples were processed for further analysis.

Proliferation assay

BrdU-labelling was used to detect proliferating cells in two-day-old cultures of pancreatic mesenchyme. Cells were incubated with BrdU (Roche) for 1 hour, fixed and stained for proliferating cells according to the manufacturer's recommendations. To stain the nuclei, DAPI (1:1000) was added in the last wash. The cells were photographed, counted, and the proliferation index was determined as the relative number of BrdU-labelled cells in the cultures. The assay was repeated three times for each factor, n=3.

RNA preparation and RT-PCR

RNA was prepared from dorsal pancreatic mesenchyme of 10.5 dpc wild-type embryos by using the Qiagene RNA isolation kit (Qiagene). cDNA synthesis was performed by using Superscript II from Gibco. PCR was run with primers specific for SIP_{1-5}, Pdx1, Isl1, and β-actin.

5′-TAGCAGCTATGGTGTCCACTAG-3′ (sense) and
5′-GATCCTGCAGTAGAGGATGGC-3′ (anti-sense) for SIP_1,
5′-ATGGCAACCACGCATGCGCAG-3′ (sense) and 5′-GGCGGTGAAGATACTGATGAG-3′ (anti-sense) for SIP_3,
5′-ATGGGCGGCTTATACTCAGAG-3′ (sense) and 5′-GACGGAGAAGATGGTGACCAC-3′ (anti-sense) for SIP_2, 5′-CTCCTCATTGTCCTGCACTA-3′ (sense) and 5′-GATCATCAGCACGGTGTTGA-3′ (anti-sense) for SIP_4, 5′-TGTTTCTGCTCCTTGGCAGC-3′ (sense) and 5′-ATCTCGGTTGGTGAAGGTGT-3′ (anti-sense) for SIP_5, 5′-CCACCCCAGTTTACAAGCTC-3′ (sense) and 5′-TGTAGGCAGTACGGGTCCTC-3′ (anti-sense) for Pdx1,
5′-ATGGTGGTTTACAGGCGAACC-3′ (sense) and
5′-GGGCAGAAACAACATCAGAACCTG-3′ (anti-sense) for Isl1,
5′-GTGGGCCGCTCTAGGCACCAA-3′ (sense) and
5′-CTCTTTGATGTCACGCACGATTTC-3′ (anti-sense) for β-actin.

In situ hybridization

In situ RNA hybridization was performed with slight modifications of previously described protocols (Bostrom et al., 1996). Hybridization solution consisted of 55% formamide, 20% dextran sulphate, 1 mg/ml yeast tRNA, 1× Denhardt's solution, 5 mM EDTA, 0.2 M NaCl, 0.013 M Tris-HCl, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, and probes were denaturized at 80°C for 5 minutes. Digoxigenin-labelled probes of

SIP_1 and SIP_3 were generated from cDNA fragments in the pBluescript KS+ vector. The anti-sense probes were generated by T3 RNA polymerase-mediated transcription after *Not*I digestion, whereas the sense probes were generated by *T7* RNA polymerase-mediated transcription after *Eco*RI digestion. SIP_2 probes were generated from *S1P2* cDNA in the pCMV SPORT 6 vector. The anti-sense probe was generated by *T7* RNA polymerase-mediated transcription after *Sal*I digestion and the sense probe was generated by *Sp6* RNA polymerase-mediated transcription after *Not*I digestion. *SIP1* and *SIP3* cDNAs were gifts from M. Takemoto, and *SIP2* cDNA was purchased from Invitrogen life technologies. Hybridization temperatures were: SIP_1, 57°C; SIP_2 and SIP_3, 61°C.

Results

Dorsal pancreatic bud emergence is rescued in cardiac-rescued *Cdh2*$^{-/-}$ mice

To investigate whether the lack of the dorsal pancreas in N-cadherin-deficient mice reflects a cell-autonomous function within the mesenchyme or if it is secondary to other defects, e.g. cardiac and/or vascular function, we analysed pancreas development in cardiac-rescued *Cdh2*$^{-/-}$ mice. Surprisingly, formation of the dorsal pancreatic bud was rescued in cardiac-rescued *Cdh2*$^{-/-}$ mice (Fig. 1A-L). The fact that the dorsal pancreatic mesenchymal cell death observed in *Cdh2*$^{-/-}$ embryos was prevented in the cardiac-rescued *Cdh2*$^{-/-}$ embryos indicates that N-cadherin does not play a cell-autonomous role in mesenchymal cell survival (Fig. 1P,Q). The dorsal aorta endothelium was present in all three genotypes, however, in cardiac-rescued *Cdh2*$^{-/-}$ embryos, the endothelium appeared healthier than in *Cdh2*$^{-/-}$ embryos (Fig. 1M-O). In conclusion, the finding that the dorsal N-cadherin-deficient pancreatic mesenchyme survived and was recruited to the dorsal pancreatic endoderm in cardiac-rescued *Cdh2*$^{-/-}$ embryos indicates that the pancreatic phenotype of N-cadherin-deficient mice was secondary to the cardiac/vascular defects.

Plasma rescues formation of the dorsal pancreatic bud in *Cdh2*$^{-/-}$ pancreatic explants

To identify the mechanism for how cardiac/vascular function may affect pancreatic development, the following findings were considered. First, the dorsal aorta lies in close vicinity to the endoderm (Fig. 1M) and it was recently reported that signals from endothelial cells induce pancreatic (Lammert et al., 2001; Yoshitomi and Zaret, 2004) and liver (Matsumoto et al., 2001) cell differentiation. Second, in contrast to *Cdh2*$^{-/-}$ embryos, the cardiac-rescued *Cdh2*$^{-/-}$ embryos developed an intact circulatory system (Luo et al., 2001). Third, mesenchymal cells that differentiate into vascular smooth muscle cells surrounding the dorsal aorta endothelium are recruited through lysophospholipid-mediated signalling from ligands present in the blood (Liu et al., 2000). Altogether these facts suggest that either the vascular endothelium or blood-borne factors could be responsible for proper recruitment/survival of the dorsal pancreatic mesenchyme. Since the most dramatic consequence of expressing N-cadherin within the heart of *Cdh2*$^{-/-}$ embryos was the restoration of an intact circulatory system, we investigated whether factors present in the blood were responsible for formation of the early dorsal pancreas. Indeed, when *Cdh2*$^{-/-}$ pancreatic and gut explants were incubated with beads soaked in plasma from wild-type embryos, dorsal pancreas morphogenesis was rescued (% ventral and dorsal pancreas: *Cdh2*$^{-/-}$ (17%) vs.

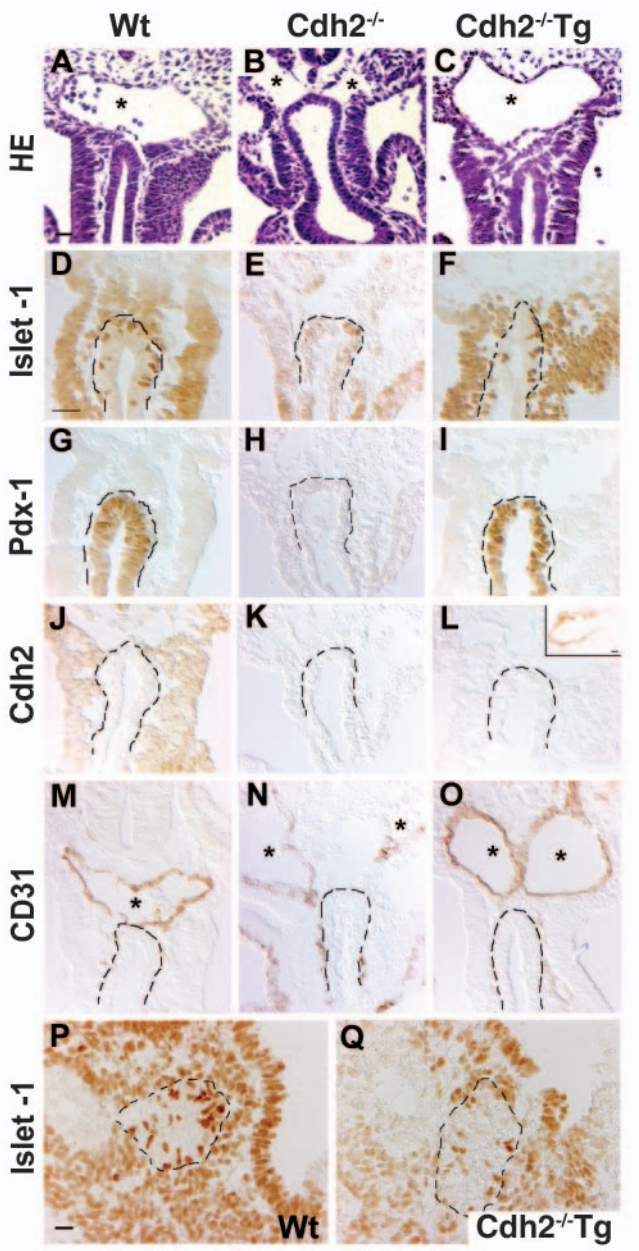

Fig. 1. Restored cardiac/vascular function in N-cadherin-deficient embryos rescues formation of the dorsal pancreas. Histochemically and immunohistochemically stained sections of the dorsal pancreatic region from 9.5 dpc wild-type (A,D,G,J,M), *Cdh2*$^{-/-}$ (B,E,H,K,N), cardiac-rescued *Cdh2*$^{-/-}$ (Cdh2$^{-/-}$Tg) (C,F,I,L,O) embryos, and from 10.5 dpc wild type (P) and cardiac-rescued *Cdh2*$^{-/-}$ (Q) embryos. (A-C) Sections were stained with hematoxylin and eosin (HE). (D-Q) Immunohistochemical staining of sections with antibodies against Isl1 (D-F,P,Q), Pdx1 (G-I), N-cadherin (Cdh2; J-L; inset shows expression of the *Cdh2* transgene in the heart) and CD31 (M-O). Despite the lack of N-cadherin expression within the dorsal pancreatic mesenchyme, dorsal pancreas formation is rescued in cardiac-rescued *Cdh2*$^{-/-}$ embryos (G-L). The dorsal pancreatic endoderm and dorsal aorta are indicated by broken line and asterisk, respectively. Scale bars: in A, 30 μm for A-C; in D, 20 μm for D-O; in P, 20 μm for P,Q; inset in L, 40 μm.

Table 1. In vitro analysis of early dorsal bud formation

	Wild type (*n*)	*Cdh2*$^{-/-}$ (*n*)	*Cdh2*$^{-/-}$ + plasma (*n*)	*Cdh2*$^{-/-}$ + S1P (*n*)	*Cdh2*$^{-/-}$ + PTX + S1P (*n*)	WT + PTX (*n*)
db	20 (21)	3 (18)	5 (10)	9 (14)	2 (6)	7 (10)
% db*	95%	17%	50%	64%	33%	70%

db, early dorsal bud formation.
*Percentage of explants that develop a dorsal bud in addition to a ventral bud.

Cdh2$^{-/-}$+plasma (50%); Table 1, Fig. 2A-D). Components present in the plasma appeared to act primarily on the mesenchyme, since growth of mesenchyme-free pancreatic endoderm was unaffected in the presence of plasma (Fig. 3A-E).

S1P rescues formation of the dorsal pancreatic bud in *Cdh2*$^{-/-}$ pancreatic explants

In analogy with the recruitment of dorsal pancreatic mesenchyme, vascular smooth muscle cells of the dorsal aorta are derived from mesenchymal cells in close proximity. Recently, it was demonstrated that vascular maturation is deficient in mice lacking the S1P receptor $S1P_1$ due to deficient vascular smooth muscle cell recruitment (Liu et al., 2000). S1P is an extracellular lipid mediator present in the circulation that signals through distinct members of the S1P subfamily of G-protein-coupled receptors (GPCRs) (Pyne and Pyne, 2000; Spiegel and Milstien, 2003). Activation of the S1P receptors triggers diverse cellular effects including proliferation, survival, migration, adhesion molecule expression, and cytoskeletal changes (Paris et al., 2002; Sadahira et al., 1992; Spiegel and Milstien, 2003; Zhang et al., 1991). To test whether the plasma-induced rescue of the dorsal pancreas could be mediated by S1P, we incubated *Cdh2*$^{-/-}$ explants with beads soaked in S1P. The data show that S1P rescued dorsal pancreas morphogenesis with somewhat higher efficiency than plasma (% ventral and dorsal pancreas: *Cdh2*$^{-/-}$ (17%) vs *Cdh2*$^{-/-}$+S1P (64%); Table 1, Fig. 2E). As with plasma, S1P appeared to specifically affect the mesenchyme since no effect was seen on mesenchyme-free endoderm (Fig. 3F).

Members of the S1P receptor subfamily that bind S1P with high affinity include $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$ (Pyne and Pyne, 2000; Spiegel and Milstien, 2003). RT-PCR and in situ hybridization analyses of the 10.5 dpc wild-type dorsal pancreatic bud revealed that *S1P*$_1$, *S1P*$_2$, and *S1P*$_3$ mRNAs were all expressed within the mesenchyme (Fig. 4). Whereas *S1P*$_1$ mRNA was specifically expressed in endothelial cells, both *S1P*$_2$, and *S1P*$_3$ mRNAs were preferentially expressed in the mesenchyme (Fig. 4B-J). The fact that pre-incubation with pertussis toxin (PTX), which ADP-ribosylates and inactivates G_i, blocked the S1P-mediated rescue of the dorsal pancreas in *Cdh2*$^{-/-}$ explants (% ventral and dorsal pancreas: *Cdh2*$^{-/-}$+S1P (64%) vs *Cdh2*$^{-/-}$+PTX and S1P (33%); Table 1, Fig. 2F), and that PTX blocked early dorsal bud formation in wild-type explants (30%, Table 1, Fig. 2G), suggest that G-protein-coupled S1P receptors play a role in early pancreas development.

S1P regulates dorsal pancreatic mesenchymal cell proliferation

The observations that: (1) growth of the dorsal pancreatic endoderm requires signals from the dorsal pancreatic mesenchyme (Scharfmann, 2000), (2) dorsal pancreatic

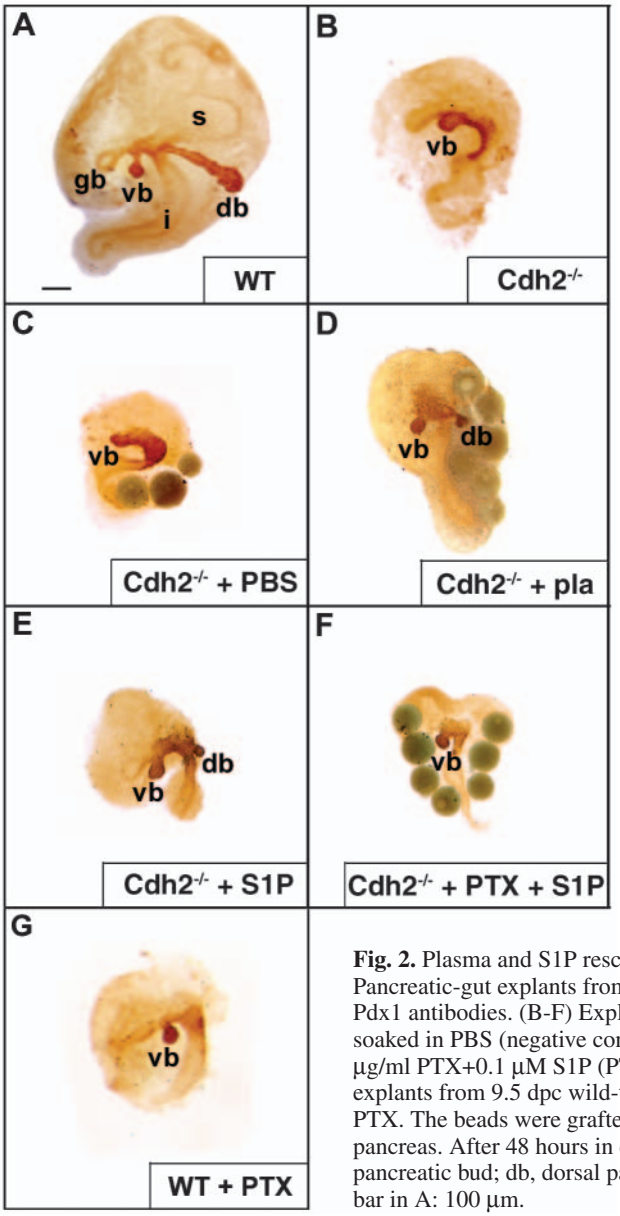

Fig. 2. Plasma and S1P rescue early morphogenesis of the dorsal pancreas in *Cdh2*$^{-/-}$ explants. Pancreatic-gut explants from 9.5 dpc wild-type (WT; A,G) and *Cdh2*$^{-/-}$ (B-F) embryos stained with Pdx1 antibodies. (B-F) Explants from 9.5 dpc *Cdh2*$^{-/-}$ embryos incubated in the presence of beads soaked in PBS (negative control; C), plasma from 15.5 dpc wild-type embryos (D), 0.1 μM S1P (E), 0.2 μg/ml PTX+0.1 μM S1P (PTX beads were pre-grafted 2 hours before S1P beads were added) (F), and explants from 9.5 dpc wild-type embryos incubated with (G) or without (A) beads soaked in 0.2 μg/ml PTX. The beads were grafted on the dorsal side of the gut, at the site of the presumptive dorsal pancreas. After 48 hours in culture, the explants were stained with Pdx1 and photographed. Vb, ventral pancreatic bud; db, dorsal pancreatic bud; s, stomach; i, intestine; gb, gall bladder; pla, plasma. Scale bar in A: 100 μm.

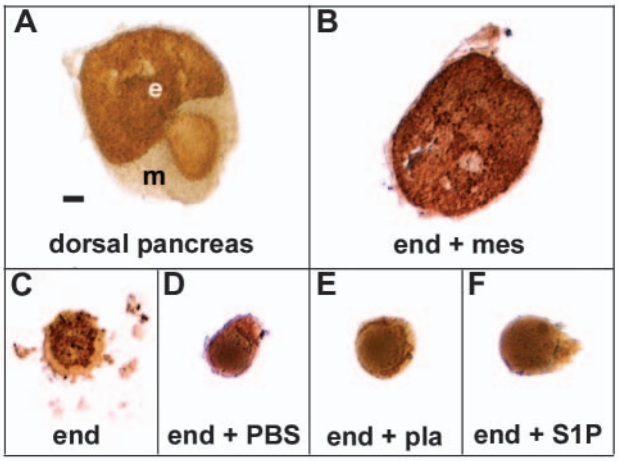

Fig. 3. Plasma and S1P cannot induce growth/differentiation of mesenchyme-free dorsal pancreatic endoderm. Isolated intact (A) or mesenchyme-stripped wild-type 10.5 dpc dorsal pancreatic bud were cultured for 48 hours with isolated 10.5 dorsal pancreatic mesenchyme (B), no addition (C), with beads soaked in PBS (D), plasma (E), or 0.1 μM S1P (F) and stained with Pdx1 antibodies. Whereas the intact dorsal pancreatic bud (A) as well as mesenchyme-free dorsal pancreatic bud endoderm recombined with dorsal pancreatic mesenchyme (B) grew, mesenchyme-free dorsal pancreatic endoderm failed to grow alone (C) or in the presence of PBS (D), plasma (E) and S1P (F), respectively. Of note, in B the mesenchyme was lost during the staining procedure. e and end, dorsal pancreatic endoderm; m and mes, dorsal pancreatic mesenchyme; pla, plasma from 15.5 wild-type embryos. Scale bar: 50 μm.

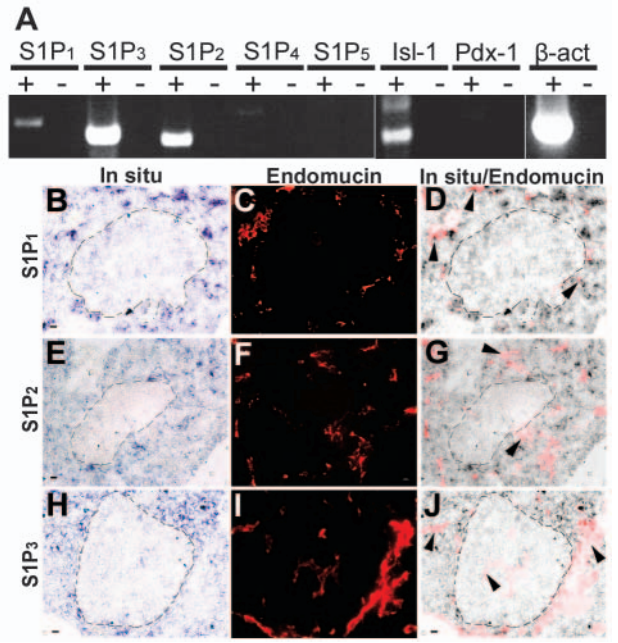

Fig. 4. $S1P_{1\text{-}3}$ are expressed in the dorsal pancreatic mesenchyme. (A) RT-PCR detection of mRNAs for *S1P*$_{1\text{-}5}$ in 10.5 dpc wild-type dorsal pancreatic mesenchyme. Mesenchyme of the dorsal pancreas was separated from the endoderm and total RNA was isolated. Analyses of *Isl1* and *β-actin* mRNAs were used as positive controls, whereas detection of *Pdx1* mRNA was used for excluding endoderm contamination. The presence or absence of reverse transcriptase is indicated by (+) and (–), respectively. (B-J) Double in situ hybridization and immunofluorescence analysis of *S1P*$_1$ (B), *S1P*$_2$ (E) and *S1P*$_3$ (H) mRNA and endomucin (marker for endothelial and hematopoietic progenitor cells; C,F,I) (Brachtendorf et al., 2001), respectively, in 10.5 dpc wild-type embryos. (D,G,J) Overlay of in situ hybridization and endomucin staining images to visualize possible colocalization of $S1P_1$ (D), $S1P_2$ (G) and $S1P_3$ (J) with endothelial cells. Whereas *S1P*$_1$ mRNA was localized in the endothelium of major and minor blood vessels in the embryo, such as the aorta (not shown) and vessels in the mesenchyme surrounding the dorsal pancreas (B-D), mRNAs for *S1P*$_2$ and *S1P*$_3$ were expressed in the mesenchyme surrounding the dorsal pancreas (E-J). Arrowheads indicate colocalization of *S1P*$_1$ mRNA with endothelial cells in D, and absence of $S1P_2$ and $S1P_3$ in endothelial cells in G and J, respectively. The dorsal pancreatic endoderm is indicated by striped lines. Scale bar in H: 10 μm for B-J.

mesenchyme was rescued in cardiac-rescued *Cdh2*$^{-/-}$ embryos (Fig. 1), (3) S1P-mediated G-protein-coupled receptor-signalling rescued growth of the dorsal pancreas in *Cdh2*$^{-/-}$ embryos (Fig. 2), (4) the S1P-mediated G-protein-coupled receptor-mediated rescue of the dorsal pancreas was not mediated by a direct effect on the endoderm (Fig. 3) and (5) the dorsal pancreatic mesenchyme expressed receptors for S1P ($S1P_1$, $S1P_2$, $S1P_3$) (Fig. 4), suggested that the mechanism by which S1P rescued dorsal pancreatic growth may involve a direct effect on the mesenchyme. Development of the dorsal pancreatic mesenchyme involves both cell migration and proliferation, and to study these processes in vitro, we developed a system for culturing primary dorsal pancreatic mesenchymal cells. Since no dorsal pancreatic mesenchyme could be recovered from *Cdh2*$^{-/-}$ embryos, mesenchyme from wild-type embryos was used. BrdU-labelling experiments demonstrated that the number of proliferating cells within the dorsal pancreatic mesenchyme significantly increased in the presence of S1P (Fig. 5A). Importantly, this effect was inhibited by pertussis toxin (Fig. 5A), suggesting that cell proliferation was regulated by S1P-ligand-induced S1P GPCR signalling. The mesenchyme consists of both mesenchymal and endothelial cells that express distinct S1P receptors. The fact that both mesenchymal and endothelial cells underwent mitosis, as revealed by co-expression of Ki67 with Isl1 and CD31, respectively, (Fig. 5B,C) suggested that both cell types could contribute to the increased number of proliferating cells in the presence of S1P. However, considering the few proliferating endothelial cells in comparison to proliferating mesenchymal cells and an estimated ratio between mesenchymal and endothelial cells of 9:1, it seems reasonable to assume that the increase in BrdU-positive cells in the presence of S1P was due to a predominant expansion of mesenchymal cells.

To study whether S1P regulates mesenchymal cell migration, we investigated the consequence of incubating the dorsal pancreatic mesenchyme in different concentrations of S1P, on cellular morphology and actin organization. However, no change in cellular morphology, migratory behaviour or actin organization was observed, indicating that at least under current experimental conditions cell migration was unaffected (Fig. 5D,E).

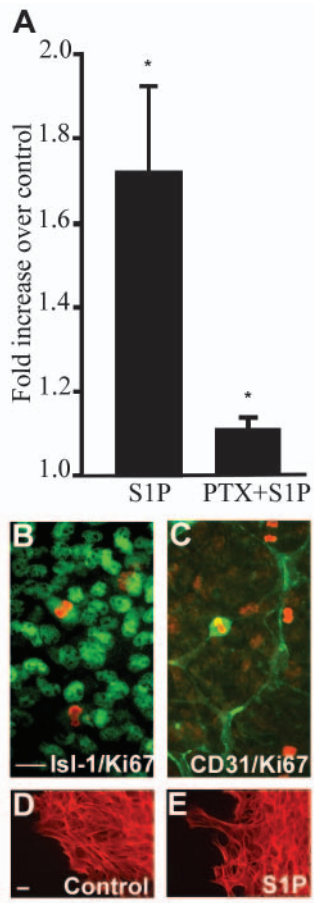

Fig. 5. S1P stimulates proliferation of dorsal pancreatic mesenchymal cells in a pertussis toxin-sensitive manner. (A-C) Primary cultures of 10.5 dpc wild-type dorsal pancreatic mesenchymal cells were established and incubated with or without 0.5 μM S1P or 0.2 μg/ml PTX (including a 2 hour pre-incubation)+0.5 μM S1P for 24 hours. Quantification of the total number of cells and the number of proliferating cells were estimated by counting DAPI- and BrdU-positive cells, respectively. Data are presented as fold increase over control. Asterisks indicate that S1P significantly increases cell proliferation and that PTX inhibits this effect, as determined by two-tailed *t*-test ($P<0.05$). Error bars represent s.e.m. To detect proliferating mesenchymal and endothelial cells, double immunofluorescence was performed with Isl1 (mesenchymal marker) and Ki67 (B), and CD31 (endothelial marker) and Ki67 (C). The ratio of mesenchymal and endothelial cells was estimated to be 9:1. Although both mesenchymal and endothelial cells underwent mitosis, the majority of the proliferating cells were of mesenchymal cell origin. (D,E) To detect changes in the migratory behaviour of the mesenchymal cells, F-actin was visualised by Phalloidin staining after a 24-hour incubation with (E) or without (D) 0.5 μM S1P. No change in F-actin rearrangement could be distinguished. Scale bars: in B, 20 μm for B,C; in D, 20 μm for D,E.

S1P-induced rescue of the *Cdh2*$^{-/-}$ dorsal pancreatic bud outgrowth does not correlate with apparent changes in vascular density

To begin to explore whether S1P may affect endoderm-endothelial cell interactions, we examined the distribution of blood vessels in our in vitro system. To visualize blood vessels around the pancreatic buds, we colabelled explants with antibodies against Pdx1 and CD31. In addition to rescuing formation of the *Cdh2*$^{-/-}$ dorsal pancreatic endoderm, S1P rescued growth of *Cdh2*$^{-/-}$ mesenchyme and endothelium (Fig. 6). Importantly, the distribution of endothelial cells and blood vessels around the presumptive dorsal and ventral pancreas was apparently unaffected by N-cadherin ablation and addition of S1P (Figs 1, 6), suggesting that N-cadherin and S1P are not primarily involved in the localization of endothelial cells/blood vessels near the pancreatic endoderm.

Discussion

Several experimental strategies were employed to follow up our recent finding that N-cadherin is required for dorsal pancreatic mesenchymal cell survival and dorsal pancreas formation, and to identify S1P-mediated signalling as an important regulator of early pancreas development. Initially, we showed that restoring cardiac and circulatory function in *Cdh2*$^{-/-}$ mice by cardiac-specific expression of N-cadherin, rescues formation of the dorsal pancreas, indicating that the phenotype is secondary to defects related to cardiac/vascular function. Based on this observation, we demonstrate that plasma and S1P-mediated G-protein-coupled signalling rescues formation of the dorsal pancreatic bud in N-cadherin-deficient mice in vitro, indicating a functional role of S1P in dorsal pancreatic morphogenesis. Importantly, the in vitro results indicate that dorsal pancreas agenesis in *Cdh2*$^{-/-}$ embryos is independent of the supply of oxygen and nutrients.

Soon after specification of the dorsal pancreatic endoderm, the dorsal pancreatic mesenchyme is recruited around the endoderm. Intimate reciprocal interactions between the epithelium (endoderm) and the mesenchyme mediate further

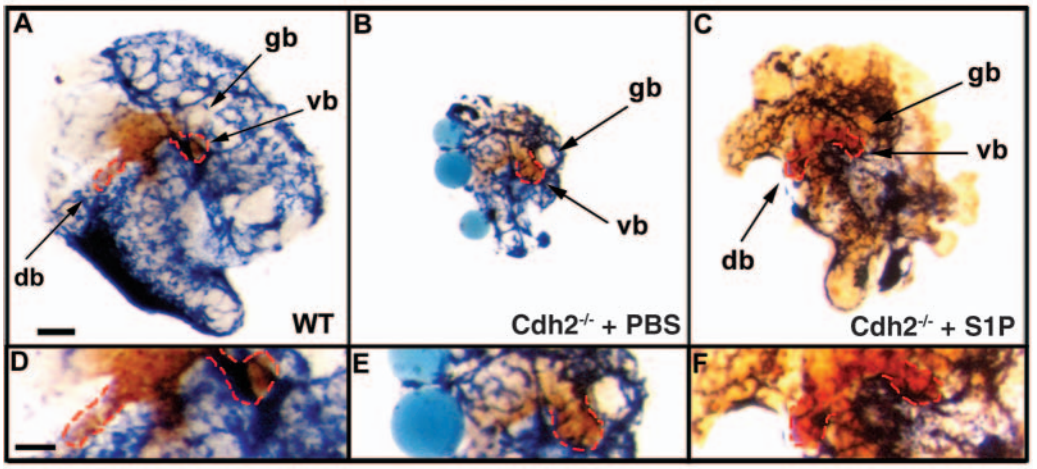

Fig. 6. Blood vessels are present but disorganized around *Cdh2*$^{-/-}$ pancreatic buds independent of the presence or absence of S1P. Pancreatic-gut explants from wild type (WT; A) and *Cdh2*$^{-/-}$ embryos incubated with beads soaked in PBS (B) or S1P (C) were analysed for the presence and distribution of blood vessels by double immunostainings with CD31 (blue) and Pdx1 (brown) antibodies. Higher magnification of the pancreatic region in A, B, and C is shown in D, E, and F, respectively. Whereas blood vessels were present nearby the pancreatic endoderm independent of the genotype and presence of S1P, N-cadherin-deficiency resulted in a disorganised network of blood vessels. S1P stimulated an overall growth of the pancreatic-gut explants, including endoderm, mesenchyme and blood vessels. db, dorsal pancreatic bud; vb, ventral pancreatic bud; gb, gall bladder. Both the dorsal (left) and ventral (right) pancreas is delineated with red broken lines. Scale bars: in A, 100 μm for A-C; in D, 30 μm for D-F.

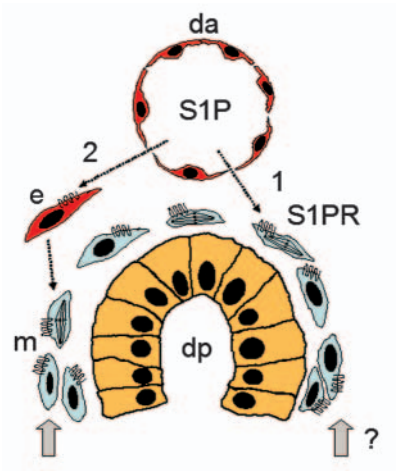

Fig. 7. Proposed model for how blood vessel function contributes to pancreas development subsequent to endoderm specification. Blood vessel (aorta)-derived S1P binds S1P receptors on dorsal pancreatic mesenchymal cells (1) or endothelial cells (2), resulting in heterotrimeric G-protein-mediated intracellular signalling and enhanced mesenchymal cell proliferation/survival in a cell-autonomous manner (1) or indirectly via S1PR-triggered signalling from endothelial cells (2). Mesenchymal cells accumulate around the committed dorsal pancreatic endoderm and secrete soluble factors necessary for further growth/differentiation of the endoderm. Da, dorsal aorta; m, mesenchymal cells; e, endothelial cells; dp, dorsal pancreas; arrows, recruitment of dorsal pancreatic mesenchymal cells from lateral plate mesenchyme.

growth and differentiation of the dorsal pancreas. Previous studies have demonstrated that in the absence of the mesenchyme, the dorsal pancreas does not emerge (Ahlgren et al., 1997; Esni et al., 2001; Scharfmann, 2000). Consequently, it was necessary to address whether the S1P-mediated rescue of the dorsal pancreatic bud formation in *Cdh2*$^{-/-}$ explants was specifically mediated by an effect on the endoderm or mesenchyme. Indeed, using whole pancreatic and mesenchyme explants, we show that S1P exerted its effect specifically on the mesenchyme by stimulating mesenchymal cell proliferation.

Although we have not identified the cellular source of the S1P involved in pancreas development, the following observations indicate that it is blood vessel-derived. First, S1P is produced and secreted by cells of haematopoietic origin, such as activated platelets, monocytic and mast cells (Yatomi et al., 1995). Second, at the time of dorsal pancreatic mesenchyme recruitment (20-25 somites), immature endothelial cell-cell contacts and holes in endothelial cells (P. Wastesson, personal communication) result in leakage of smaller molecules, such as S1P, from the circulation (dorsal aorta) (Fig. 7). Third, rescue of the dorsal pancreatic bud in cardiac-rescued *Cdh2*$^{-/-}$ embryos correlates with rescue of an intact circulatory system (Luo et al., 2001). Finally, the dorsal pancreatic bud in *Cdh2*$^{-/-}$ embryos was rescued with similar efficiencies in vitro by plasma from wild-type embryos and S1P.

S1P signals in a paracrine and autocrine manner through several GPCRs, including $S1P_{1\text{-}5}$ (Pyne and Pyne, 2000). The observations that PTX, an inhibitor of G_i, blocked S1P-mediated rescue of dorsal pancreatic bud formation in *Cdh2*$^{-/-}$ embryos and that S1P GPCRs, $S1P_{1\text{-}3}$, were specifically expressed in the dorsal pancreatic mesenchyme, suggest that S1P's effect on dorsal pancreas development is mediated through S1P GPCR-mediated signalling in the mesenchyme. In support of this model, we show that budding of the wild-type dorsal pancreatic endoderm was inhibited by PTX. Whereas *S1P*$_2$ and *S1P*$_3$ mRNAs were expressed in mesenchymal cells, *S1P*$_1$ mRNA was confined to endothelial cells, suggesting that both a cell-autonomous role of S1P in mesenchymal cells and an indirect effect via endothelial cells must be considered. To address the latter, we attempted to ablate endothelial cells in vitro by using a potent Vegf antagonist, the recombinant soluble Vegfr1 (Flt-1)/Fc chimera, which due to high affinity to Vegf can block Vegf-mediated endothelial cell proliferation and survival (Ferrara and Davis-Smyth, 1997; He et al., 1999). However, the present experimental conditions do not allow for efficient ablation of endothelial cells within the dorsal pancreatic mesenchyme by the Vegf antagonist (J. Edsbagge, unpublished).

A functional role of blood vessels in early pancreatic development has previously been reported to involve cell interactions between the endoderm and endothelial cells, and to be independent of the supply of oxygen and nutrients (Lammert et al., 2001; Yoshitomi and Zaret, 2004). These studies showed that endothelial cell interactions are required for induction and maintenance of transcription factors known to regulate pancreatic endoderm specification, such as Pdx1 and Ptf1a, and endocrine cell differentiation. Moreover, it was demonstrated that specification of the dorsal, but not the ventral, pancreatic endoderm requires signals from the endothelial cells (Yoshitomi and Zaret, 2004). Thus, these studies demonstrate that independent of vascular function, endothelial cells directly regulate the specification and differentiation of the early pancreatic endoderm.

These studies did not, however, address the functional role of blood vessels on subsequent steps in pancreas development, such as formation of the dorsal pancreatic mesenchyme and emergence of the dorsal pancreas. Concomitant with the recruitment of the dorsal pancreatic mesenchyme, the aorta becomes displaced by the invading mesenchymal cells, which through reciprocal interactions with the endoderm stimulates dorsal pancreatic bud emergence (Scharfmann, 2000). Based on our findings the following model for how blood vessel function contributes to pancreas development subsequent to endoderm specification is proposed (Fig. 7). Blood vessel (aorta)-derived S1P escapes from the circulation through immature endothelial cell-cell contacts and holes in endothelial cells, and binds S1P receptors (S1PRs) on dorsal pancreatic mesenchymal cells or endothelial cells. Activation of heterotrimeric G-protein-coupled S1PR-mediated intracellular signalling in mesenchymal and/or endothelial cells results in enhanced mesenchymal cell proliferation/survival in a cell-autonomous or indirect manner, respectively. In analogy with many epithelial organs, such as the lung, kidney, hair and gut, the accumulation/condensation of mesenchymal cells is necessary for proper signalling, i.e. the release of soluble growth factors, to stimulate growth and differentiation of the epithelium, in this particular case the dorsal pancreatic endoderm. Whether S1P, in addition to its role in mesenchymal cell proliferation/survival, participates in the recruitment of the lateral plate mesenchyme-derived dorsal pancreatic

mesenchyme by stimulating cell migration remains to be shown.

In conclusion, we provide evidence that adds new dimensions to how blood vessels control pancreatic development. Our findings suggest that subsequent to dorsal pancreatic endoderm specification by endothelial cell interactions, blood vessel-derived S1P regulates dorsal pancreatic mesenchyme formation and thereby emergence of the dorsal pancreatic bud. Taken together, all available data on the functional role of blood vessels in pancreatic development suggest that the patterning and function of blood vessels concomitant with organogenesis may be of general importance for guiding differentiation and morphogenesis.

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