

Growth-limiting role of endothelial cells in endoderm development

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ABSTRACT

Endoderm development is dependent on inductive signals from different structures in close vicinity, including the notochord, lateral plate mesoderm and endothelial cells. Recently, we demonstrated that a functional vascular system is necessary for proper pancreas development, and that sphingosine-1-phosphate (S1P) exhibits the traits of a blood vessel-derived molecule involved in early pancreas morphogenesis. To examine whether S1P₁-signaling plays a more general role in endoderm development, S1P₁-deficient mice were analyzed. S1P₁ ablation results in compromised growth of several foregut-derived organs, including the stomach, dorsal and ventral pancreas and liver. Within the developing pancreas the reduction in organ size was due to deficient proliferation of Pdx1⁺ pancreatic progenitors, whereas endocrine cell differentiation was unaffected. Ablation of endothelial cells in vitro did not mimic the S1P₁ phenotype, instead, increased organ size and hyperbranching were observed. Consistent with a negative role for endothelial cells in endoderm organ expansion, excessive vasculature was discovered in S1P₁-deficient embryos. Altogether, our results show that endothelial cell hyperplasia negatively influences organ development in several foregut-derived organs.

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Introduction

Organ and vascular development are an interdependent processes where cross talk between blood vessels and the developing organ anlagen regulates proper organ architecture and function. In the present investigation the effect of a perturbed vascular system on the development of foregut-derived organs was studied in *sphingosine-1-phosphate receptor 1* (S1P₁) deficient embryos.

S1P₁ is a G-coupled receptor and is the founding member of a family of five receptors, S1P_{1–5}, formerly known as EDG receptors. S1P₁'s ligand, sphingosine-1-phosphate (S1P), is a bioactive lipid found at high concentrations in the blood stream where it binds to albumin and high density lipoprotein (HDL). Blood-born S1P is produced and secreted by red blood cells, platelets, monocytes, mast cells, and possibly endothelial cells (Pappu et al., 2007; Venkataraman

et al., 2008; Yatomi et al., 1995). In the surrounding tissues S1P is found at lower levels due to degradation by S1P lyase (Schwab et al., 2005). The conserved and ubiquitously expressed enzymes sphingosine kinase 1 (SphK1) and 2 synthesize S1P by phosphorylation of sphingosine (Spiegel and Milstien, 2002). Upon receptor binding S1P mediates cell proliferation (Zhang et al., 1991), survival (Paris et al., 2002), migration (Sadahira et al., 1992), and morphogenesis (Kupperman et al., 2000). S1P_{1–3} are widely expressed whereas S1P₄ is expressed in lymphoid tissues and S1P₅ in the central nervous system and spleen. S1P_{1–3} play critical roles in vascular maturation. For example, ablating S1P₁ in mice results in massive hemorrhages and destabilization of blood vessels causing lethality before E14.5 (Kono et al., 2004; Liu et al., 2000).

The definitive endoderm gives rise to a number of organs along the primitive gut tube, including the pharynx, thyroid, parathyroid, esophagus, lungs, thymus, stomach, liver, pancreas, small and large intestine. Endoderm development involves axis formation, induction, expansion and differentiation/maturation. Signals from mesodermally-derived tissues in close vicinity to the endoderm, such as the notochord, heart, splanchnic mesenchyme and endothelium, control many aspects of these processes (Deutsch et al., 2001; Golosow and Grobstein, 1962; Hebrok et al., 1998; Kim et al., 1997; Lammert et al., 2001; Wessells and Cohen, 1967; Yoshitomi and Zaret, 2004).

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Reciprocal signaling between the splanchnic mesenchyme and the endoderm mediates the induction of several organ primordia along the anterior–posterior axis of the gut tube. Furthermore, mesenchyme to endoderm signaling, via FGF10, plays a central role in the proliferation of progenitors within the thyroid, thymus, lung, liver, pancreas, stomach and caecum (Berg et al., 2007; Bhushan et al., 2001; Burns et al., 2004; Min et al., 1998; Nyeng et al., 2007; Ohuchi et al., 2000; Revest et al., 2001; Sekine et al., 1999). These observations were recently supported by genetic evidence, demonstrating that failure to form the dorsal pancreatic mesenchyme in *Isl1*-, *N-cadherin*- and *Raldh2*-ablated embryos results in dorsal pancreatic agenesis (Ahlgren et al., 1997; Esni et al., 2001; Martin et al., 2005).

Endothelial cells influence liver development already when the hepatic cells infiltrate the septum transversum before vascular function (Matsumoto et al., 2001). Furthermore, increased liver vasculature results in hyperplasia of the liver due to paracrine factors (LeCouter et al., 2003). In vitro culturing of lung rudiments under hypoxic conditions leads to augmented vessel density and reduced lung epithelial branching. Moreover, over expression of VEGF A in vivo results in increased vascular density and reduced branching of the lung epithelium (Akeson et al., 2003; Zeng et al., 1998). During pancreas development initiation of the dorsal pancreatic bud, maintenance of expression of the crucial pancreatic and duodenal homeobox 1 (Pdx1) transcription factor, induction of the pancreas specific transcription factor 1a (Ptf1a) and endocrine cell specification also depend on endothelial signals (Lammert et al., 2001; Yoshitomi and Zaret, 2004). In contrast, endothelial cells are not necessary for similar events in the ventral bud (Yoshitomi and Zaret, 2004). Endoderm–endothelial interactions continue after the mesenchyme is infiltrated by blood vessels. For example, over expression of VEGF A under the control of the Pdx1 promoter expands the endocrine compartment, whereas the acinar compartment is reduced, suggesting that endocrine and exocrine cell differentiation is controlled by the vasculature. Taken together these data clearly illustrate the importance of well-balanced interactions between the mesenchyme and endothelium with the endoderm for proper induction and expansion of gut-derived organs.

The vasculature signals both via endothelial cells and its content. Recently, we have showed that a functional vascular system plays an important role in dorsal pancreas development by supplying signaling cues from the circulation, and in particular through S1P (Edsbagge et al., 2005).

To directly address the functional role of S1P signaling within endothelial cells during endoderm development, *S1P₁* deficient embryos were analyzed. Here, we demonstrate that *S1P₁*, which is specifically expressed in the endothelium, is required for growth of several foregut derivatives, including stomach, dorsal and ventral pancreas, and liver. We also show that growth of the pancreatic endoderm requires *S1P₁* function, and that reduction in pancreatic volume is caused by a decrease in progenitor proliferation. However, endocrine cell differentiation was unaffected. We were also able to reproduce these results in vitro, thereby excluding indirect effects via changes in the supply of oxygen and nutrients. Surprisingly, endothelial cell ablation failed to mimic the *S1P₁* phenotype. Instead, increased pancreatic organ size and hyper branching were observed. Together with the observation of increased number of endothelial cells in *S1P₁*-deficient embryos, these results point towards a hitherto unknown concept of endothelial cells limiting expansion of foregut-derived organs.

Results

S1P₁ is expressed in endothelial cells during embryogenesis

To determine the *S1P₁* protein expression profile in the developing endoderm, triple immunofluorescence labeling of E-cadherin (Ecad),

S1P₁ and Pecam was conducted. *S1P₁* specifically co-localized with Pecam in endothelial cells at E9.5 and E12.5 (Fig. 1 and S1). During early stages (E9.5) the gut endoderm is primarily exposed to endothelial cells in the form of the dorsal aorta (Fig. 1A), whereas the various endoderm-derived organ anlagen later interact with endothelial cells within the surrounding splanchnic mesenchyme (Figs. 1B–E). *S1P₁* expression was also detected in the central nervous system, but not within the endoderm (Fig. 1 and data not shown).

Growth of foregut-derived organ primordia is compromised in *S1P₁*-deficient embryos

To determine the functional role of *S1P₁* during foregut endoderm development, the epithelial volume of various foregut-derived organ anlagen was measured using Optical Projection Tomography (OPT). Consistent with the original observation, *S1P₁*-deficient embryos died before E14.5 due to cardiovascular defects (Liu et al., 2000). Although the size of the embryos was not severely affected at E12.5, hemorrhages within the central nervous system and the limb buds were observed (Figs. 2A–B). Several of the foregut-derived organs were affected in the *S1P₁* mutants (Figs. 2C–D). Besides, the expression levels of Ecad increased along the anterior–posterior axis of the foregut endoderm. For example, Ecad expression was higher in

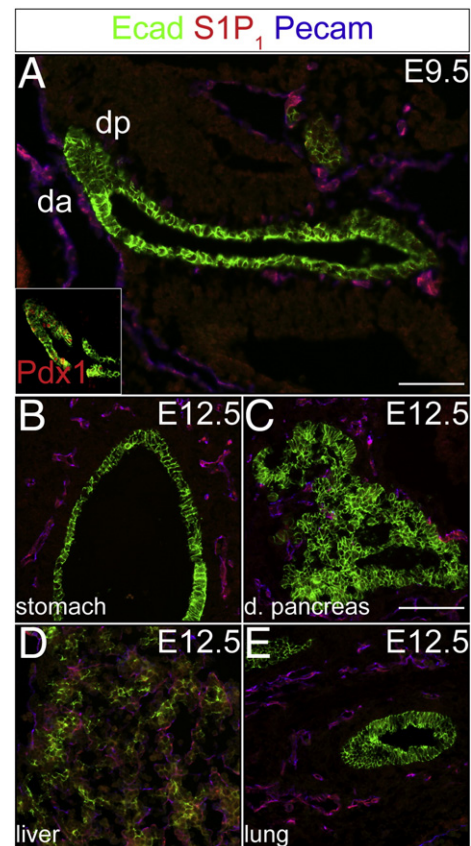


Fig. 1. *S1P₁* is expressed by endothelial cells within the mesenchyme. Immunofluorescence was used to investigate *S1P₁* expression within the embryonic primitive gut tube and its later derived organs. *S1P₁* (red), co-expressed with Pecam positive blood vessels (blue). The endoderm is visualized by Ecad (green). The inset in (A) shows Pdx1 (red) in the part of the endoderm (green), which represents the dorsal pancreas. Throughout the early phase of endodermal development *S1P₁* expression can be detected in blood vessels, neural ectoderm and blood cells. Between E9 and E12 the dorsal pancreas is separated from the *S1P₁*⁺ dorsal aorta by the invading mesenchyme. The mesenchyme is infiltrated by *S1P₁*⁺ blood vessels that reestablish the endoderm–endothelial interaction (C). E12.5 *S1P₁* expressing blood vessels are found in the mesenchyme surrounding both the lung (E) and stomach (B) epithelium as well as in the blood vessels of the liver (D). Dorsal pancreas (dp) and dorsal aorta (da). Scale bars 100 μm.

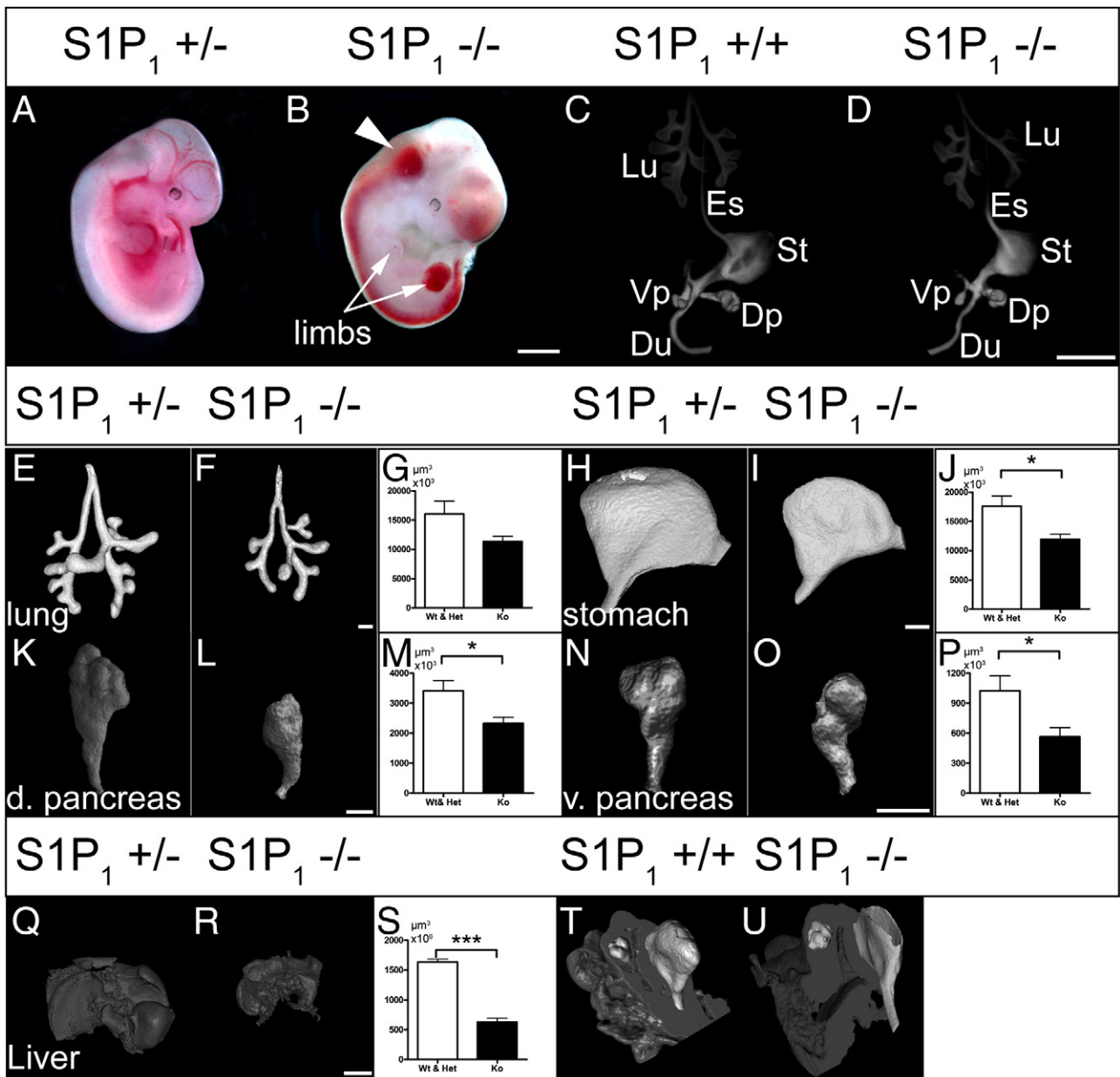


Fig. 2. Quantification of endoderm volumes at E12.5. The *S1P1* deficient embryos suffer from hemorrhages in the central nervous system and in the shortened extremities (A–B). As reported earlier the overall size of the embryos was not severely affected at E12.5. Several foregut-derived organs were affected by *S1P1* ablation (C–D). The dorsal pancreatic bud from *S1P1* deficient embryos protrudes shorter from the duodenum compared to littermate controls (C–D). Note the difference in Ecad intensity between the endoderm organs, with intense Ecad signal in the pancreatic buds, whereas less signal was detected within the lungs. The volume of the endoderm organs; lung, dorsal and ventral pancreas and stomach from *S1P1* deficient embryos was quantified using OPT (E–P). The volume of the *S1P1* deficient dorsal (32%) and ventral (45%) pancreata and stomach (32%), as analyzed by OPT, is significantly reduced compared to the littermate controls (M, P, J), whereas the lung was reduced by 30% but did not reach significance (G). All images are isosurface renderings based on the signal from Ecad stainings. The volume of the liver was quantified using OPT based on endogenous autofluorescence (Q–S). The liver volume was reduced by 61% (S). In order to investigate if *S1P1* ablation resulted in morphological changes in the mesenchyme surrounding the endoderm, a cutting plane was placed in the autofluorescence channel whereas the Ecad channel was kept intact. This analysis revealed no dramatic change in the organization of the mesenchyme surrounding the dorsal pancreas (T–U). Scale bars are 100 μm in all except A–D and Q–R where they are 500 μm. In G, J, M, P and S *S1P1* -/- is compared to *S1P1* +/- and +/- with Student *T*-test. Lung comparison, $p = 0.0989$, dorsal bud comparison, $p = 0.0296$, ventral comparison, $p = 0.0334$, stomach comparison, $p = 0.0239$ and liver comparison, $p = 0.0003$. Error bars represent \pm SEM. *S1P1* -/- $n = 6$ and *S1P1* +/- $n = 8$. Lu = lung, Es = esophagus, St = stomach, Dp = dorsal pancreas, Vp = ventral pancreas and Du = duodenum.

the pancreas compared to in the lung (Figs. 2C–D). The difference in expression levels of Ecad might be due to the fact that lung is a much more branched and tubular organ at this time point, compared to the pancreas. OPT analysis demonstrated that the dorsal (Figs. 2K–M) and ventral (Figs. 2N–P) pancreas and the stomach (Figs. 2H–J) were significantly reduced in size in *S1P1* -/- embryos. The dorsal pancreas was, on average, reduced by 32%, the ventral pancreas by 45% and the stomach by 32%. As a result, the protrusion of the *S1P1* -/- dorsal pancreatic bud was reduced (Figs. 2C–D). The lung (Figs. 2E, and data not shown) showed a similar trend in size reduction (30%)

together with a decline in distal tip number. However, these changes were not statistically significant. For liver volume analyses the high endogenous autofluorescence precluded the use of Ecad stainings. Instead, OPT measurements of the liver volumes are based on endogenous autofluorescence and demonstrated a 61% reduction (Figs. 2Q–S). For an overview of all the organs included in the data set see [Supplementary Figs. S2A–B](#).

To address if *S1P1* ablation resulted in morphological changes in the surrounding endodermal mesenchyme, a cutting plane was placed in the autofluorescence channel with the Ecad channel being kept

intact. This analysis revealed no dramatic changes in the organization of the mesenchyme surrounding the dorsal pancreas (Fig. 2T–U and Supplementary Videos 1–2).

To investigate the onset of perturbed expansion of pancreatic endoderm in $SIP_1^{-/-}$ embryos, quantification of the dorsal pancreatic volume was conducted by whole mount E-cadherin stainings. Whereas no difference in the size of the dorsal pancreas was found at E10.5 (Figs. 3A–C), at E11.5 a 23% reduction was observed in SIP_1 mutants, compared to controls (Figs. 3D–F).

The functional role of SIP_1 in expansion of the pancreatic endoderm is independent of circulatory factors

To further resolve how SIP_1 may control expansion of foregut endoderm derivatives, we focused on one particular foregut-derived organ, i.e. the pancreas. To eliminate the possibility that the endodermal phenotype observed in SIP_1 -deficient embryos was caused by defective circulation, development of the pancreatic endoderm was studied in vitro. At E11.5, no obvious morphological or embryo survival differences were observed between $SIP_1^{-/-}$ embryos and their litter mate controls (insets in Figs. 4A–B) (Liu et al., 2000). However, although control explants consistently developed normally (27 out of 30), the phenotype of the $SIP_1^{-/-}$ explants was rather diverse, spanning from severely compromised growth to merely a reduction in size and degree of branching (Figs. 4A–C and S3A–F). In fact, a high percentage (25%) of the SIP_1 -deficient explants did not grow at all (Fig. 4C). Even when the more naïve pancreatic endoderm was analyzed in vitro similar observations were made. Specifically, analysis of E9.5 SIP_1 mutant explants cultured in vitro for 4 days (corresponding to E11.5–12.5 in vivo), revealed aberrant development of the pancreatic endoderm (Figs. 4D–F).

SIP_1 is required for proliferation of $Pdx1^{+}$ pancreatic progenitors but dispensable for endocrine differentiation

To test if the observed defective growth of the epithelium involved changes in cell proliferation, sections from E12.5 BrdU-pulsed embryos were stained for $Pdx1$ and BrdU. Proliferating cells were observed in both the pancreatic mesenchyme and epithelium, in both genotypes (Figs. 5A–B). However, the proliferative rate of $Pdx1^{+}$ cells

was significantly lower in SIP_1 -deficient embryos compared to their littermate control embryos (Fig. 5C).

Caspase 3 staining of E12.5 dorsal pancreas revealed insignificant apoptotic activities in control and SIP_1 -deficient embryos, thus excluding apoptosis as an explanation for how SIP_1 signaling controls pancreatic endoderm expansion (Fig. S4). To examine whether necrosis was increased in SIP_1 -deficient embryos, expression of the inflammatory marker CD45 was investigated. An increase in CD45⁺ cells within the epithelium would indicate ongoing inflammation and necrosis. However, no increase in the relative number of CD45⁺ cells was found (Fig. S4). Altogether, these data suggests that reduced cell proliferation, but not an increase in apoptosis or necrosis, explains the reduced organ size in SIP_1 -deficient embryos.

At E12.5, glucagon⁺ cells are the most abundant hormone-producing cell type in the dorsal pancreas. To investigate if endocrine differentiation relies on SIP_1 function, whole mount staining of the dorsal pancreatic bud for Ecad and glucagon were conducted (Fig. 5D). At E12.5, SIP_1 deficiency resulted in no significant change in alpha cell differentiation compared to the littermate controls (Fig. 5E). Notably, confocal measurements of the epithelial volume matched our OPT findings (Fig. 5F). To address if beta cell differentiation was affected, E11.5 explants were cultured for two days and analyzed by whole mount staining using insulin and E-cadherin antibodies (Fig. 5G). Consistent with the in vivo analysis, in vitro culture of the dorsal pancreas resulted in reduced volume of the mutant dorsal pancreas (Fig. 5I). However, SIP_1 deficiency resulted in no significant change in the relative number of insulin⁺ cells (5H). QPCR analysis of E12.5 dorsal pancreatic buds was in line with these observations, which demonstrated that *Ngn3*, *Pax4* and 6, *Pdx1* and *insulin* mRNA expression was unchanged in SIP_1 mutants (Figs. 5K–O).

QPCR analysis of the markers for various progenitor populations, including *Cpa1* (tip cells at E12.5 and later acinar cells) (Zhou et al., 2007), *Sox9* (multipotent progenitors at E12.5 and later ducts) (Seymour et al., 2007) as well as the acinar marker *amylase* revealed, no statistical change in the relative mRNA expression (Figs. 5P–R). Furthermore, an analysis of the distribution of the luminal marker, Mucin1, revealed that the overall luminal network in the $SIP_1^{-/-}$ dorsal bud was comparable to the controls (Fig. 5J). However, the proximal part of the $SIP_1^{-/-}$ pancreatic duct was slightly less branched, which probably is secondary to the overall reduction in organ size.

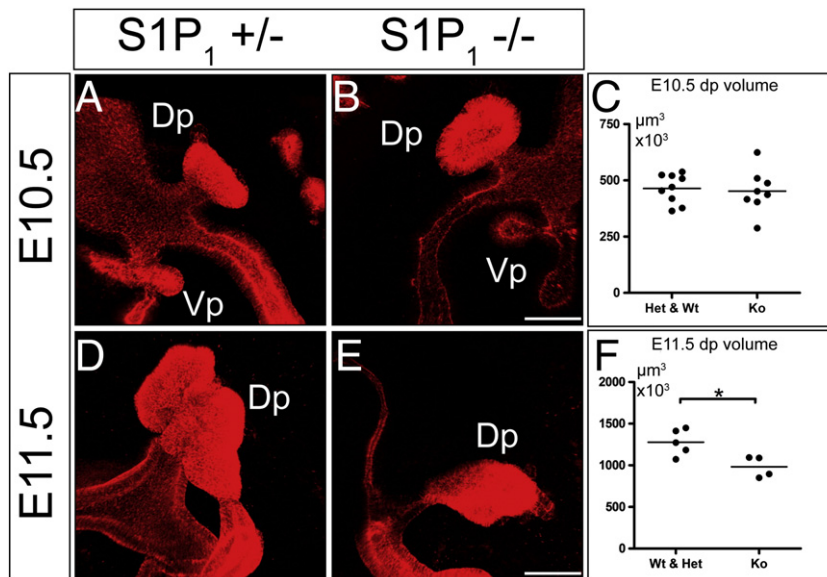


Fig. 3. The SIP_1 endodermal phenotype is detectable at E11.5. To investigate how early the SIP_1 endodermal phenotype could be detected dorsal pancreatic volume was measured after confocal analysis. At E11.5 a reduction of the mutant dorsal pancreatic volume by 23% could be detected (D–F) but not at E10.5 (A–C). Dp = dorsal pancreas and Vp = ventral pancreas. Scale bars 100 μm .

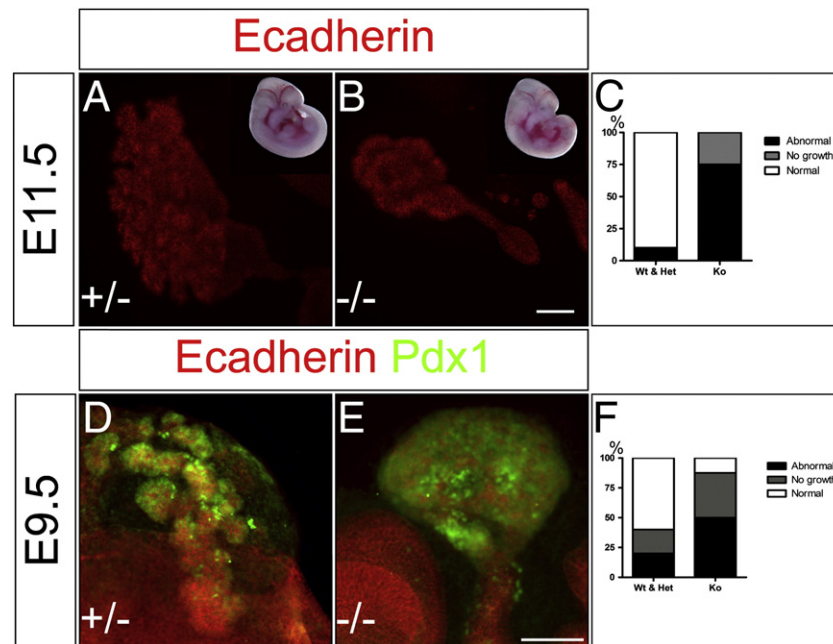


Fig. 4. In vitro culture of E9.5 and E11.5 *S1P1*-deficient pancreatic explants fails to rescue the phenotype. To confirm the reduced growth seen in vivo cultures of E11.5 explanted gut tubes were carried out in vitro. Immunofluorescence analysis of the epithelial marker Ecad (red) concluded that in vitro culture of *S1P1* deficient explants is not sufficient to rescue the pancreatic phenotype (A–C), as insets in A and B the embryos are shown. *S1P1*^{+/+} littermate controls *n* = 8, *+/-* *n* = 22, and *-/-* *n* = 12. To verify that the endoderm had not been permanently damaged already at E11.5, E9.5 cultures were also carried out, Ecad (red) and Pdx1 (green). Even though the success rate in littermate controls was slightly lower, the *S1P1* mutant explants developed notably worse (D–F). *S1P1*^{+/+} littermate controls *n* = 3, *+/-* *n* = 5, and *-/-* *n* = 6. These data support the hypothesis that the phenotype is not just secondary to the cardio-vascular phenotype. Scale bars 100 μ m. The explants shown in A–B are also included in the volume quantification in Fig. 5H.

Thus, *S1P1* is required for proliferation of the *Pdx1*⁺ pancreatic progenitors, but not for early endocrine and exocrine cell specification.

Gene expression analysis of the E10.5 *S1P1*^{-/-} dorsal pancreas

To mechanistically dissect the role of *S1P1* in pancreas development we first examined the mRNA expression levels of genes known to control endoderm development. *Ptf1a* controls dorsal pancreas specification and is expressed in multipotent progenitors but later becomes restricted to acinar cells (Krapp et al., 1996; Yoshitomi and Zaret, 2004). *S1P1* ablation resulted in no change in the mRNA and protein expression of *Ptf1a* (Figs. 6A, H–O), neither was there a change in the mRNA expression of two mesenchymal markers known to play functional roles during pancreatic development, *Isl1* (Ahlgren et al., 1997) and *Wilms tumor 1 homolog* (*Wt1*) (Wagner et al., 2005) (Figs. 6B–C). Consistent with the above, protein levels of *Isl-1*, which is also expressed in endocrine progenitors, were unaffected in *S1P1* mutant pancreatic endoderm and mesenchyme (Figs. 6P–Q). We also analyzed the expression of FGF ligand/receptor family members known to control expansion of the pancreatic epithelium via mesenchyme-to-epithelium signaling (Bhushan et al., 2001; Dichmann et al., 2003; Elghazi et al., 2002; Miralles et al., 1999). However, expression of *FGF10* (*FGF7* could not be detected in all samples and was therefore excluded) and its receptor *FGFR2* were unaffected upon *S1P1* ablation (Figs. 6D–E). Finally, the mRNA expression of two other *S1P* receptors, *S1P2–3*, which are present in the developing pancreatic mesenchyme, showed no compensatory up regulation of these receptors (Figs. 6F–G).

Blood vessel ablation results in increased pancreatic organ size

Our initial hypothesis evolved around the assumption that endothelial cells, via *S1P1*, provide inductive signals necessary for expansion of the pancreatic endoderm. To directly address this hypothesis, we ablated endothelial cells in vitro using 1-[4-(6,7-Dimethoxy-quinolin-4-yloxy)-3-fluoro-phenyl]-3-(2-fluoro-phenyl)-urea (Ilovich et al., 2008), hereafter referred to as quinolin-urea, and

examined the consequences on pancreatic endoderm development. Two days treatment with the quinolin-urea resulted in total ablation of the blood vessels (visualized by Pecan) (Figs. 7A–B). Surprisingly, efficient ablation of endothelial cells at E11.5 did not result in compromised expansion of the epithelium, instead, increased organ size and hyperbranching of the pancreatic endoderm were observed (Figs. 7A–B). Both the ventral and dorsal buds were affected, but the effect was most striking on the ventral bud (Figs. 7C–D).

S1P1 ablation results in increased vascular density in foregut-derived organ anlagen

The endothelial cell ablation results contradicted our initial hypothesis and instead suggested that endothelial cells may limit the growth of foregut-derived organs. Thus, a possible mechanism for how *S1P1* ablation may reduce endoderm organ expansion could involve hypervascularization. Indeed, previous studies demonstrated increased vascular density in the developing *S1P1*^{-/-} limb bud (Chae et al., 2004). Consistent with this observation, we found that *S1P1* ablation results in hypervascularization (2–3-fold increase in blood vessel density) within the mesenchyme surrounding the foregut-derived organs, including the dorsal pancreas (Figs. 8A–C), lung (Figs. 8D–F), stomach (Figs. 8G–I) and liver (Figs. 8J–L).

Discussion

Blood vessels are essential for the developing organism because they provide oxygen and nutrients as well as inductive cues that control cell proliferation and cell fate specification during organogenesis. For example, blood vessels communicate with the surrounding tissue in the developing retina and this interplay controls both differentiation and proliferation of blood vessels and the surrounding astrocytes (West et al., 2005). Furthermore, endothelial cells control the development of endoderm-derived organs, e.g. both liver and dorsal pancreas outgrowth and early pancreatic endocrine cell specification (Lammert et al., 2001; Yoshitomi and Zaret, 2004).

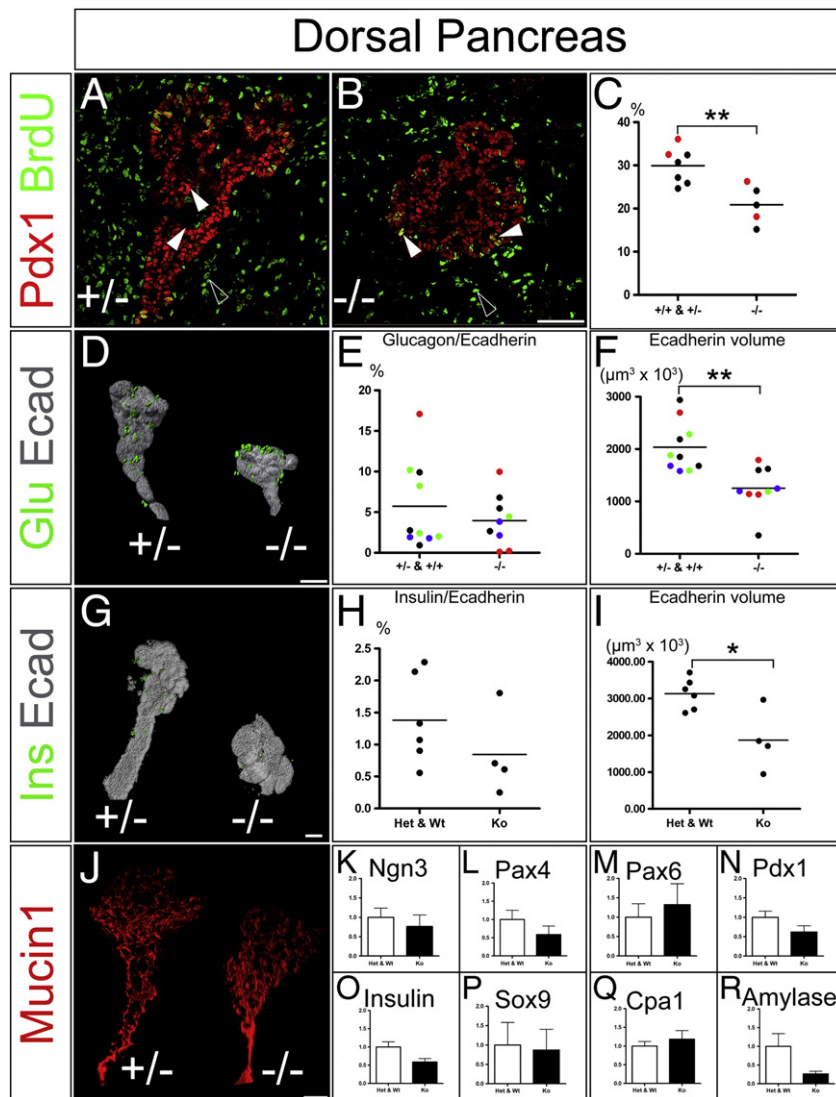


Fig. 5. Proliferation of $Pdx1^{+}$ progenitors is reduced but endocrine differentiation is unaffected in the $S1P1^{-/-}$ dorsal pancreas. Proliferation within the $Pdx1^{+}$ epithelium was analyzed by immunofluorescence of in utero BrdU pulsed embryos. Proliferating cells marked with BrdU (green), were found both inside the $Pdx1^{+}$ epithelium (red), and outside the mesenchyme in both $S1P1$ deficient embryos and littermate controls (A–B). BrdU $^{+}$ cells in the epithelium are indicated with filled arrowheads and in the mesenchyme indicated with open arrowheads. Scale bar 100 μ m. Proliferation of $Pdx1^{+}$ progenitors was significantly reduced (35%) (Student *T*-test) in the $S1P1$ deficient dorsal pancreas (littermate controls $n = 7$, $S1P1$ deficient embryos $n = 5$, $p = 0.0049$). Red and black dots represent different litters (C). To investigate if alpha cell differentiation was affected, glucagon and Ecad volumes of the dorsal bud were estimated by confocal microscopy. No difference between $S1P1$ deficient embryos and littermate controls could be detected in glucagon volume divided by Ecad volume (Student *T*-test, $p = 0.2188$) (E). Note the variation within the littermate control group. Consistent with the OPT analysis a statistically significant decrease in the volume of the $S1P1$ deficient dorsal pancreatic epithelium was detected with confocal microscopy (Student *T*-test, $p = 0.0014$) (F). Each color of the dots represents a different litter (littermate controls $n = 10$, $S1P1$ deficient embryos $n = 9$). Representative samples are shown in D, Ecad (gray) and glucagon (green). Note the unevenly distribution of the endocrine cells which are concentrated to the tip of the bud. Scale bar 100 μ m. To analyze beta cell differentiation, the relative number of insulin $^{+}$ cells (insulin volume divided by Ecad volume) was estimated in E11.5 explants cultured for two days. No difference in the relative number of insulin $^{+}$ cells in $S1P1$ deficient embryos and littermate controls was detected (Student *T*-test, $p = 0.2587$) (H). Consistently, a statistically significant reduction of the dorsal pancreatic epithelium volume was observed (Student *T*-test, $p = 0.0014$) (I). Littermate controls $n = 6$, $S1P1$ deficient embryos $n = 4$. Representative samples are shown in G, Ecad (gray) and insulin (green). In $S1P1$ deficient embryos ductal organization as visualized by Mucin1 staining (red) was unaffected (J). Further analysis of cell differentiation was carried out by QPCR measurements. Expression levels of *Ngn3* (K), *Pax4* (L), *Pax6* (M), *Pdx1* (N), *insulin* (O), *Sox9* (P), *Cpa1* (Q) and *amylase* (R) were measured but no statistical differences were detected. P-values; *ngn3* $p = 0.5503$, *Pax4* $p = 0.3012$, *Pax6* $p = 0.6057$, *Pdx1* $p = 0.1456$, *insulin* $p = 0.0645$, *Sox9* $p = 0.8861$, *Cpa1* $p = 0.4472$ and *amylase* $p = 0.1380$.

However, questions that remain include the identity and nature of these endothelial signals, and whether they exclusively act in a positive manner or play a more dynamic role by also negatively affecting endoderm development. In this study we present evidence in support of endothelial cells playing a hitherto unknown general role in foregut-derived organ development by limiting growth.

Initially, the analysis of $S1P1^{-/-}$ mice, which show destabilized blood vessels and hemorrhages (Liu et al., 2000), revealed a reduction in organ size of the foregut-derived organs, including lung, liver, stomach and pancreas. We specifically investigated the role of this pathway in early pancreas development and now demonstrate that $S1P1$ ablation results in reduced size of the dorsal and ventral

pancreatic buds, but that it has no effect on endocrine or exocrine cell fate specification. The dorsal pancreatic bud size reduction is due to a decrease in proliferation rate of $Pdx1^{+}$ progenitors, indicating that $S1P1$ is required for proliferation, but not differentiation of $Pdx1^{+}$ pancreatic progenitors. The in vivo findings were reproduced in vitro, implying that these findings do not represent indirect effects due to the circulation phenotype of $S1P1$ embryos.

To verify that the requirement of $S1P1$ for pancreatic endoderm expansion is mediated by endothelial cells, which is the only cell type within the developing pancreas where $S1P1$ protein can be detected, blood vessels were ablated at E11.5 in wild type gut/pancreatic explants. Unexpectedly, removing blood vessels did not mimic the

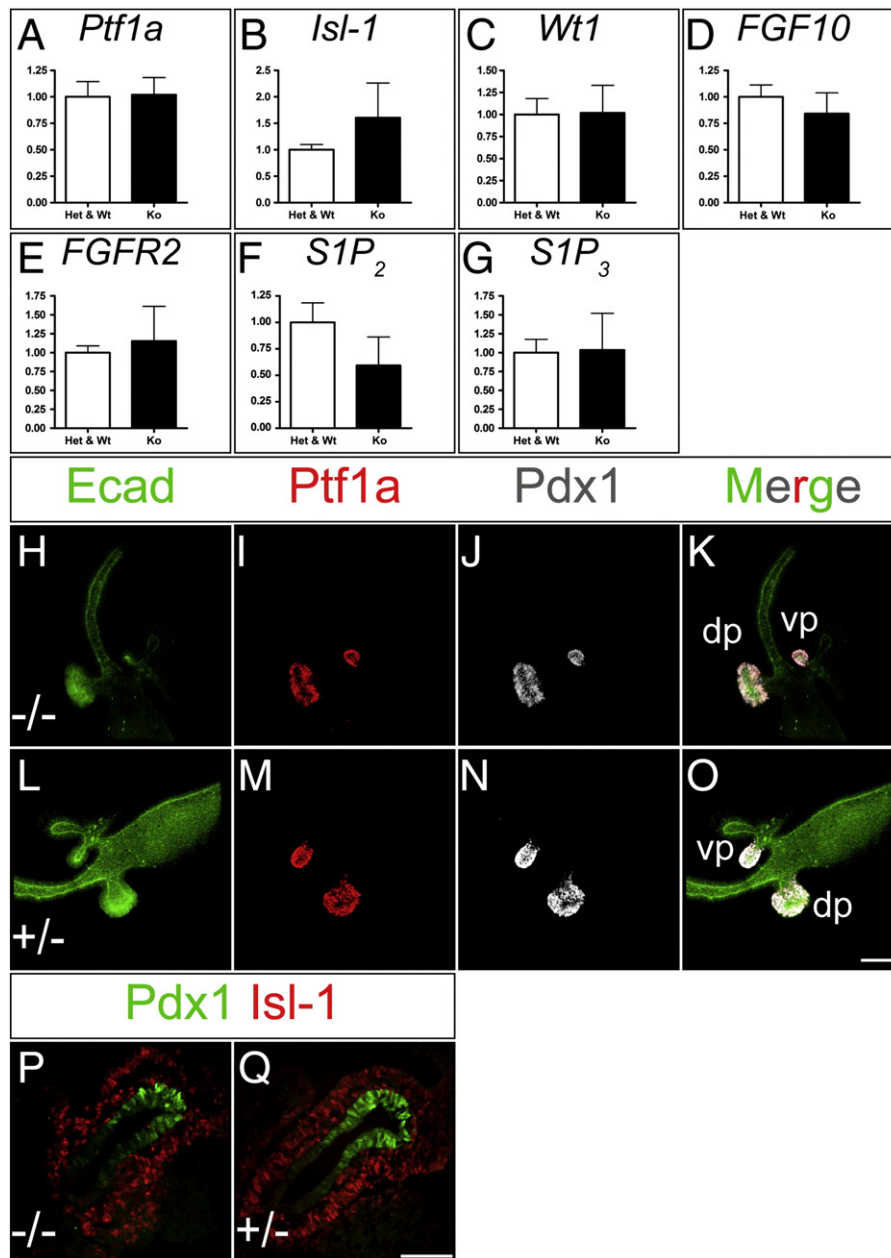


Fig. 6. Marker expression analysis of the *SIP1*-deficient pancreas. QPCR was used to analyze mRNA expression of genes known to be involved in development of the endoderm; *Ptf1a* (A) which is known to be induced by blood vessels, as well as the mesenchymal genes *Isl1* (B) and *Wt1* (C) was quantified. We could not detect any statistical difference in any of these genes. The FGF pathway including *FGF10* (D) and its receptor *FGFR2* (E), known to be important for the expansion of many of the endodermal organs and significantly for the pancreatic progenitors, were measured. In none of these genes we could detect different expression levels between *SIP1* deficient dorsal pancreata and littermate controls. To investigate if *SIP1* deficient dorsal pancreata had an upregulation of the two other pancreatic expressed family members *SIP2* (F) and *SIP3* (G) we used QPCR to analyze the relative expression levels. For *SIP2* and *SIP3* no upregulation was detected. *SIP1*^{+/+} littermate controls n = 5, *SIP1*^{+/-} littermate controls n = 11, and *SIP1*^{-/-} embryos n = 4. Student *T*-test was used; error bars represent standard error of the mean. To verify the mRNA data whole mount immunofluorescence labeling of Ecad (green), Ptf1a (red) and Pdx1 (gray) was used. Whole mount immunofluorescence labeling did not reveal any altered expression levels of Ptf1a in E10.5 embryos (H–O). Neither did protein expression analysis of Isl1 and Pdx1 on E10.5 pancreatic sections (P–Q). Scale bars 100 μm.

SIP1 phenotype. Instead, blood vessel ablation resulted in pancreatic endoderm expansion and hyperbranching, suggesting that endothelial cells limit expansion of the pancreatic epithelium. The blood vessel ablator, quinolin-urea, inhibits VEGFR2 and PDGFRβ (Ilovich et al., 2008) which are expressed by the blood vessels in the pancreas (Lammert et al., 2001). PDGFRβ is additionally also expressed in the pancreatic mesenchyme (Hori et al., 2008). As neither of the receptors is expressed in the pancreatic epithelium, this excludes a direct effect of the quinolin-urea on the epithelium through these receptors. The fact that blood vessel ablation failed to mimic the *SIP1* phenotype suggested that *SIP1* is not directly involved in the reduced growth of

foregut-derived organs. Alternatively, the *SIP1* phenotype may be secondarily caused by an increase in blood vessel density. In fact, this hypothesis is in line with previous work demonstrating that *SIP1*-deficiency results in hypervascularization in the limb bud (Chae et al., 2004). Consistent with this, we show that *SIP1* ablation results in increased number of blood vessels in the mesenchyme that surrounds the foregut-derived organs, including the pancreas, stomach, liver and lung. Altogether, these observations provide evidence (both gain-of-function and loss-of-function) in support of a model where endothelial cells counteract growth-promoting signals – potentially mesenchyme-derived – by providing growth-limiting signals to the pancreatic

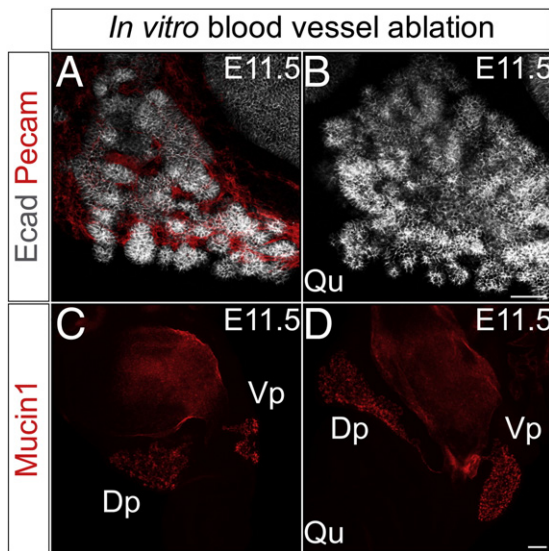


Fig. 7. Blood vessel ablation does not mimic the *SIP₁* phenotype, but results in expansion and hyperbranching of the pancreatic epithelium. Blood vessel ablation did not mimic the phenotype seen in *SIP₁*-deficient explants (A–D). Total ablation of Pecam (red) positive blood vessels was seen after two days of treatment with quinolin-urea (Qu). The epithelium was visualized by Ecad (gray) (A–B). The epithelium was also analyzed using the luminal marker Mucin1 (red). Both dorsal and ventral pancreas are affected by the blood vessel ablation, but the hyperbranching effect is most prominent in the ventral pancreas (C–D). Dp = dorsal pancreas and Vp = ventral pancreas. Scale bars 100 μ m.

endoderm. Endothelial cell ablation experiments will have to confirm whether the same model applies to the other foregut-derived organs as well.

Previously, the consequence of increased vascularization during pancreas development has been studied by expressing VEGF under control of the Pdx1 promoter. The over growth of blood vessels resulted in an increase (three fold) in endocrine mass and a dramatic decrease (seven fold) in acinar mass (Lammert et al., 2001). Although the consequence of *SIP₁* deficiency also involves hypervascularization, no effect on early endocrine or exocrine cell differentiation was observed. However, the early lethality of *SIP₁* mutant animals permitted the analysis of differentiation no later than E12.5. This is at the brink of secondary transition when the variation in endocrine differentiation between embryos is substantial, which might therefore, mask any difference. It is also possible that two opposing mechanisms act in parallel in the *SIP₁* deficient pancreas, i.e. a pro-endocrine effect mediated by the elevated blood vessel density and a negative effect on endocrine differentiation by the hypoxia caused by defective blood vessels (Chae et al., 2004; Heinis et al.). Notably, the decrease in the Pdx1⁺ progenitor pool in *SIP₁*−/− mice is consistent with the observed reduction in acinar cells in adult Pdx1-VEGF mice (Lammert et al., 2001). Endothelial cells also play a functional role during the earlier stages of pancreas development by initiating dorsal pancreas formation via Ptf1a induction (Jacquemin et al., 2006; Yoshitomi and Zaret, 2004).

In the lung, manipulation of blood vessel development has highlighted the need for proper control of density, and spatio-temporal distribution of the blood vessels (Akeson et al., 2003; Schwarz et al., 2000; van Tuyl et al., 2005; Zeng et al., 1998). Taken together it is obvious that the balance between blood vessel growth and organ growth must be tightly controlled. In a previous study it has been shown that induced hypervascularization during adulthood results in liver hyperplasia (LeCouter et al., 2003). Hypervascularization during embryogenesis, in the *SIP₁* mutants, results in hypoplasia of the liver. A possible explanation for these contrary findings could be

that different mechanisms are involved during adulthood and embryogenesis. The influence of the blood vessels on the surrounding tissue might also be different during different phases of development.

It is well established that the development of foregut-derived organs, such as the pancreas, lung, stomach and liver, relies on signals from mesenchymal and endothelial cells (Fukuda and Yasugi, 2005; Gittes, 2009; Shannon and Hyatt, 2004; Warburton et al., 2000; Zaret, 2002, 2008). However, whether endothelial cell signals play a more general role in development of endoderm-derived organs has so far remained unknown. Our results show that endothelial cell hyperplasia negatively influences organ development in several foregut-derived organs, including the pancreas, stomach, lung and liver. This suggest that endothelial cells counterbalance growth stimulatory signals originating from other cells, such as mesenchymal cells (for a schematic illustration see Figs. S3A–C). In the lung and stomach, blood vessels are mainly located within the mesenchyme without direct contact with the epithelium, whereas in the liver and pancreas, the blood vessels are located within both the mesenchyme and the epithelium. It is therefore probable that the molecule, which negatively influences endodermal growth, is a blood vessel-derived diffusible molecule or an extracellular matrix component. Future studies will be necessary to clarify the identity and mechanism of action of the endothelial cell-derived molecule(s) regulating organogenesis of foregut-derived organs.

Materials and methods

Animals

SIP₁-deficient mice were back-crossed for at least 10 generations into C57BL6. Endoderm development was indistinguishable between wild type and *SIP₁*+/− mice. Genotyping of *SIP₁* mice was performed as previously described but with small modifications as (Liu et al., 2000). The mice were housed in the animal facilities at the Lund Biomedical Center, Lund University, Sweden, kept according to animal care guidelines and all experimental procedures were approved by the regional animal ethic committee, Lund and Malmö, Sweden.

Immunofluorescence and immunoreagents

For immunofluorescence, embryos, explants and whole mounts were fixed, sectioned and stained as previously described (Ahnfelt-Ronne et al., 2007; Edsbacke et al., 2005; Esni et al., 2001; Esni et al., 1999). Antibodies used were guinea pig anti Pdx1, 1:1000 (kind gift from Chris Wright); rat anti Ecad, 1:400 (ECCD-2, Takara); mouse anti Ecad, 1:100 (BD Biosciences); rat anti Pecam (CD31), 1:100 (BD Pharmingen); goat anti Pecam (CD31), 1:500 (R&D Systems); rabbit anti *SIP₁*, 1:500 (Santa Cruz Biotechnology); Armenian hamster anti Mucin1, 1:500 (Thermo Fischer Scientific); rabbit anti Ptf1a 1:2500 (BCBC, www.betacell.org/), guinea pig anti glucagon 1:500 (Linco), guinea pig anti insulin 1:800 (Dako), rat anti CD45 1:100 (Abcam) and rabbit anti Isl-1 1:100 (Chemicon). Nuclear staining was performed with 4',6-diamidino-2-phenylindole (DAPI). Proliferating cells were detected with rat anti-BrdU, 1:200 (Nordic BioSite) 50 mg/kg BrdU was injected intraperitoneally 2 hours (h) before the pregnant female was sacrificed. Apoptosis was detected with rabbit anti Caspase 3, 1:1000 (R&D Systems). Secondary antibodies used were from Molecular Probes and Jackson Immuno Research Laboratory Inc. Specimens (sections, whole mounts and cultured pancreatic rudiments) were analyzed using a Zeiss Axioplan microscope and an Apotome unit or a Zeiss LSM 510 confocal microscope. The images were processed using Adobe Photoshop and Imaris. Blood vessel density quantification was performed on sections throughout the entire organ (separated by at least 50 μ m) which were stained with Pecam and E-Cadherin antibodies and DAPI. Axiovision's feather automatic measurement was used. Total blood vessel area for

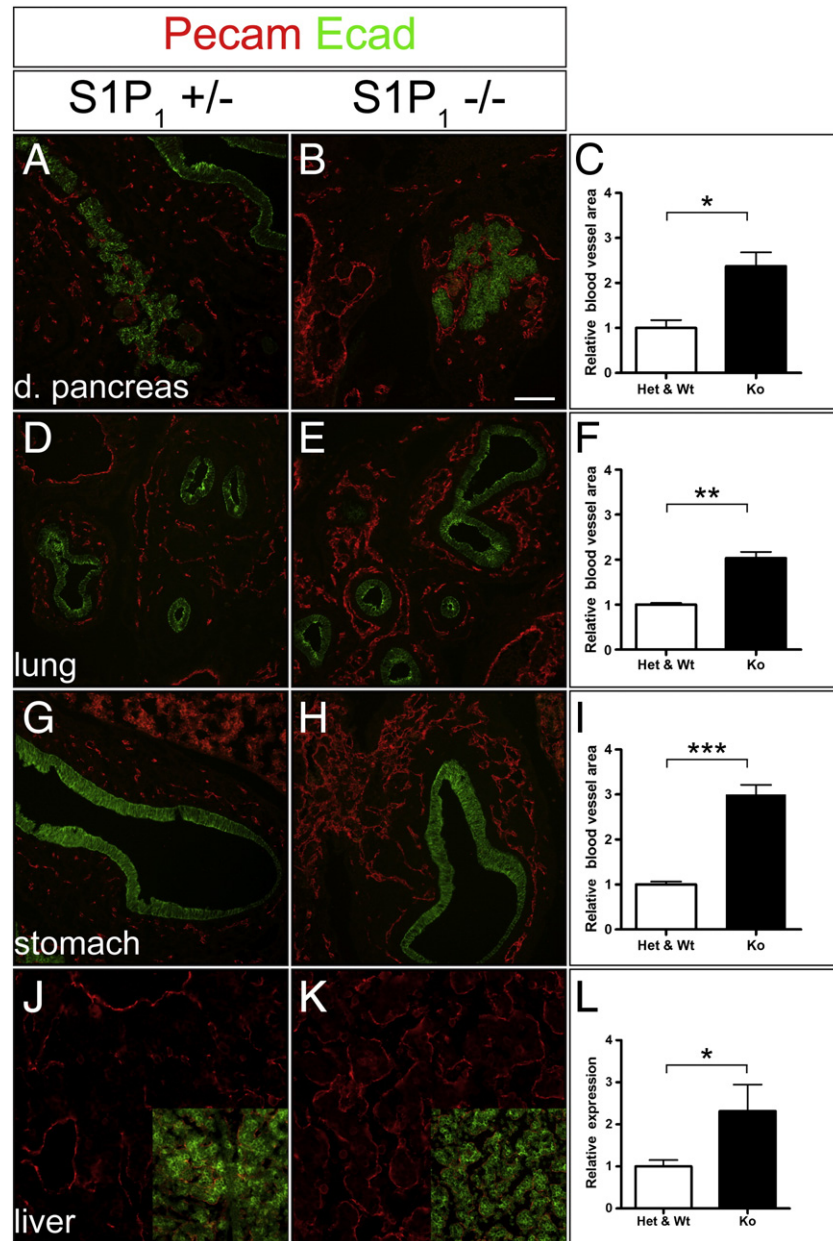


Fig. 8. *S1P₁* ablation results in hypervascularization. In all the investigated endodermal organs *S1P₁* ablation resulted in increased vascular density (A–B, D–E, G–H and I–J). The epithelium is visualized by Ecad (green) and the blood vessels by Pecam (red). For clarity, the liver is shown both without Ecad and in insets with Ecad (I–J). Scale bar 100 μ m. The blood vessel density was quantified by measuring the blood vessel area and dividing it with the total area of the organ — the epithelial area (C, F and I). Due to the endogenous autofluorescence of the liver we used QPCR to quantify the blood vessel marker *Pecam* (K). In all organs we found a 2–3-fold increase in blood vessel density. P values; pancreas $p = 0.0184$, littermate controls $n = 3$, and *S1P₁* -/- embryos $n = 4$, stomach $p = 0.006$, littermate controls $n = 3$, and *S1P₁* -/- embryos $n = 4$, lung $p = 0.0019$, littermate controls $n = 3$, and *S1P₁* -/- embryos $n = 3$ and liver $p = 0.0214$, littermate controls $n = 9$, and *S1P₁* -/- embryos $n = 5$. d. pancreas = dorsal pancreas. Scale bar 100 μ m.

respective organ was divided by total organ area — total epithelial area.

Optical Projection Tomography analysis

E12.5 isolated respiratory- and gastrointestinal tracts were fixed in 4% PFA for 1 h at 4 °C. Fixed tissues were washed in PBS, stepwise dehydrated into Methanol and stored in -20 °C until used. Before staining the tissues were rehydrated into TBST (0.15 M NaCl, 0.1 M Tris pH 7.5, 0.1% Triton X-100) and thoroughly washed. The specimen were blocked in blocking solution (TBST, 10% normal goat serum (Cedar Lane Lab) and 0.01% Sodium azide (Sigma S-8032)) for 24 h at RT, incubated with primary antibody in blocking solution for 24 h at RT and washed extensively for 24 h in TBST. Incubation with

secondary antibody was performed as with primary antibody. The secondary antibody solution was filtered through an Acrodisc syringe filter (Pall life sciences, 0.45 μ m) to avoid precipitate induced artifacts during OPT scanning. Tissues were washed in TBST for 24 h and prepared for OPT scanning as previously described (Alanentalo et al., 2007). Antibodies used were: rat anti Ecad (clone ECCD-2, Zymed) at 1:500 dilution and Alexa 594 goat anti rat (Molecular Probes) at 1:500. For the liver, endogenous autofluorescence was used to define the organ.

The specimen was scanned through 360° using the Bioptonic 3001 OPT scanner (Bioptonic) modified with an exciter D560/40X and emitter E610lpv2 filter (Chroma) for optimal visualization of Alexa 594 fluorescence. All samples were scanned at the same magnification. Tomographic sections (resolution: 1024 × 1024) of the

samples were generated using the NRecon V1.5.0 (SkyScan) software. Orthogonal planes were assessed using DataViewer V1.3.2 (SkyScan). Volume renderings and isosurfaces were created using Volocity v5.3.1 (Improvision, Coventry, United Kingdom). Quantification of organ epithelial volumes, based on the Ecad signal, was performed using the quantification software module for Volocity v5.3.1. The original image stacks of GI tract epithelium were manually cropped into lung, stomach, ventral pancreas and dorsal pancreas. A “find objects by intensity” task was applied to select voxels according to a specified intensity threshold value. The value was chosen to exclude pixels not contributing to the labeled objects (the organ epithelium). A fine filter (3×3 kernel) was also applied to the data sets to avoid selection of background noise voxels with intensities above the threshold level. The volumes of the measured objects were finally exported to the Excel 2007 (Microsoft Corporation, Redmond, Washington) software for statistical analysis.

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). The explants were cultured from 48 h up to six days in a humidified incubator of 37 °C with 5% CO₂. Only *SIP1*-deficient embryos of similar size as their wild type littermates with no apparent external morphological changes, except for shorter limbs and tail and hemorrhages, were chosen for analysis. For the blood vessel ablation experiments, quinolin-urea was added to the culture medium (0.5 μM) at day one of culture.

Quantitative real-time PCR

Dorsal buds from E10.5 or E12.5 embryos were dissected and directly frozen in lysis buffer. Total RNA was extracted with QIAshredder together with RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed in 10 μL reactions with SuperScript III (Invitrogen) according to the manufacturer's instruction using a mixture of 2.5 μM oligo(dT) and random hexamers (Invitrogen). All samples were diluted to 200 μL with water prior to real-time PCR. Real-time PCR measurements were performed on a LightCycler 480 (Roche Diagnostics). 10 μL reactions contained 10 mM Tris (pH 8.3), 50 mM KCl, 3 mM MgCl₂, 0.3 mM dNTP, 1 U JumpStart Taq Polymerase (all Sigma-Aldrich), 0.25 X SYBR Green I (Invitrogen), 400 nM of each primer (Invitrogen) and 2 μL cDNA. Primer sequences are available as supplementary data (Fig. S5). *Actb* and *B2m* were measured using primers contained in the Mouse Endogenous Control Panel supplied by TATAA Biocenter. All samples were run as duplicates. Formation of expected PCR products was confirmed by agarose gel electrophoresis and melting curve analysis for all samples. Gene expression data was normalized against the geometric average of *Actb* and *B2m* and data analysis was performed as described (Nolan et al., 2006; Stahlberg et al., 2008).

For E12.5 samples StepOnePLUS Real Time PCR system from Applied Biosystems was used. The samples were normalized against HPRT and not run in duplicates.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.01.026.

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During the revision process two relevant papers have been published

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