Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas

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The generation of the pancreas and small intestine from the embryonic gut depends on intercellular signalling between the endodermal and mesodermal cells of the gut [1–5]. In particular, the differentiation of intestinal mesoderm into smooth muscle has been suggested to depend on signals from adjacent endodermal cells [1–3]. One candidate mediator of endodermally derived signals in the embryonic hindgut is the secreted protein Sonic hedgehog (Shh) [6]. The *Shh* gene is expressed throughout the embryonic gut endoderm [7,8] with the exception of the pancreatic bud endoderm, which instead expresses high levels of the homeodomain protein Ipf1/Pdx1 (insulin promoter factor 1/pancreatic and duodenal homeobox 1), an essential regulator of early pancreatic development [9–12]. Here, we have examined whether the differential expression of *Shh* in the embryonic gut tube controls the differentiation of the surrounding mesoderm into specialised mesoderm derivatives of the small intestine and pancreas. To test this, we used the promoter of the *Ipf1/Pdx1* gene to selectively express *Shh* in the developing pancreatic epithelium. In *Ipf1/Pdx1–Shh* transgenic mice, the pancreatic mesoderm developed into smooth muscle and interstitial cells of Cajal, characteristic of the intestine, rather than into pancreatic mesenchyme and spleen. Also, pancreatic explants exposed to Shh underwent a similar program of intestinal differentiation. These results provide evidence that the differential expression of endodermally derived *Shh* controls the fate of adjacent mesoderm at different regions of the gut tube.

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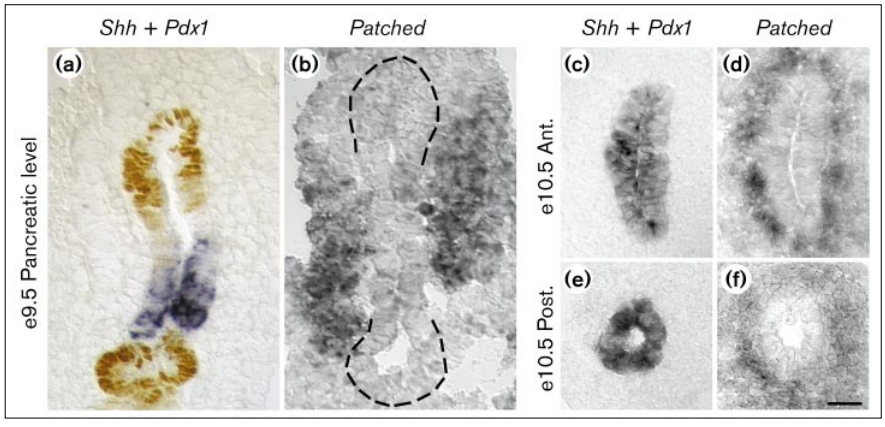
Results and discussion

Dorsal and ventral pancreatic buds do not express *Shh* or *Patched*

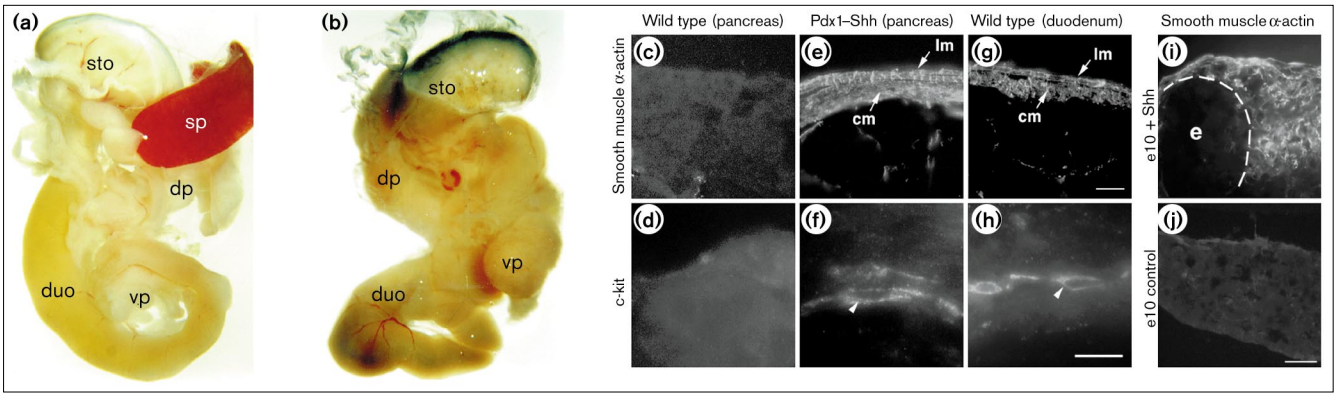
In early mouse embryos, *Shh* is initially expressed in the ventral part of the foregut endoderm [7,8], but from embryonic day 10.5 (e10.5), the expression is uniform at anterior and posterior levels to the pancreatic buds (Figure 1c,e). In contrast, both the dorsal and ventral pancreatic endoderm lack *Shh* expression throughout development, and instead express high levels of *Ipf1/Pdx1* (Figure 1a) [9–12]. Expression of *Indian hedgehog* (*Ihh*) partially overlaps with that of *Shh* in the early foregut endoderm [8], and *Ihh* is also excluded from the developing pancreatic endoderm (unpublished observations). The Hedgehog (Hh) proteins bind to the transmembrane protein Patched (Ptc); in addition, the expression of the *Ptc* gene is induced in cells exposed to all three mouse Hh proteins [13]. The elevation of *Ptc* transcription therefore provides an indication of active Hh-mediated signalling. Consistent

Figure 1



Shh expression is excluded from the developing pancreatic anlagen. **(a)** Double-label immunohistochemistry and *in situ* hybridization on a transverse section of an e9.5 wild-type embryo showing that Shh expression at the pancreatic anterior–posterior (A–P) level is restricted to the lateral gut endoderm (dark blue). Note that the dorsal and ventral pancreatic epithelium express high levels of Ipf1/Pdx1 (dark brown) at this stage. **(b)** *In situ* hybridization of a transverse section of an e9.5 wild-type embryo showing that *Patched* expression is restricted to the mesoderm flanking the Shh^+ lateral endoderm whereas the mesoderm surrounding the pancreatic buds (broken lines) is Ptc^-. At e10.5 anterior and posterior of the pancreatic A–P level, Shh and Ptc are expressed throughout the gut tube. **(c–f)** Transverse sections of an e10.5 embryo showing (c,e) Shh and (d,f) Ptc expression (c,d) anterior and (e,f) posterior of the pancreatic A–P level. Abbreviations: ant, anterior; post, posterior. The scale bar in (f) corresponds with 80 μm. In all figures, dorsal is uppermost.

Figure 2



Pancreatic mesoderm differentiates into smooth muscle in *Ipf1/Pdx1–Shh* transgenic mice. The stomach–duodenal region of **(a)** a 3 week old control mouse and **(b)** a *Ipf1/Pdx1–Shh* transgenic mouse as it appears after removal from the abdomen. Note the intestinal like appearance of the pancreatic buds and the absence of the spleen in (b). Immunohistochemistry on sections of 3 week old (c–f) pancreas and (g,h) duodenum from (c,d,g,h) wild-type or (e,f) transgenic mice using antibodies against (c,e,g) α-smooth muscle actin and (d,f,h) c-kit. The transgenic epithelium is surrounded by a smooth muscle layer, including ICCs, similar to that of the normal duodenum – compare (e,f) with (g,h). **(i)** In pancreatic explants exposed to Shh, the mesodermal compartment expresses smooth muscle α-actin. **(j)** Pancreatic explants grown in the absence of Shh do not express smooth muscle α-actin. See supplementary material for a movie showing the response of a pancreatic explant to soluble Shh. Abbreviations: sto, stomach; sp, spleen; duo, duodenum; dp, dorsal pancreas; vp, ventral pancreas; lm, longitudinal muscle layer; cm, circular muscle layer; e, endoderm. The scale bar in (c,e,g) corresponds to 40 μm; in (d,f,h), 10 μm; and in (i,j), 20 μm.

with the distribution of *Shh*, *Ptc* was expressed in the gut mesoderm (Figure 1b,d,f and [13]), but not in the pancreatic mesenchyme or the splenic anlage (Figure 1b). Thus, the duodenum and pancreas differ in the expression pattern of *Shh* and *Ihh* in the endoderm, and also in the developmental fates of their derivative epithelia and mesenchyme.

Ectopic expression of *Shh* in the pancreatic endoderm converts pancreatic mesoderm into intestinal mesenchyme

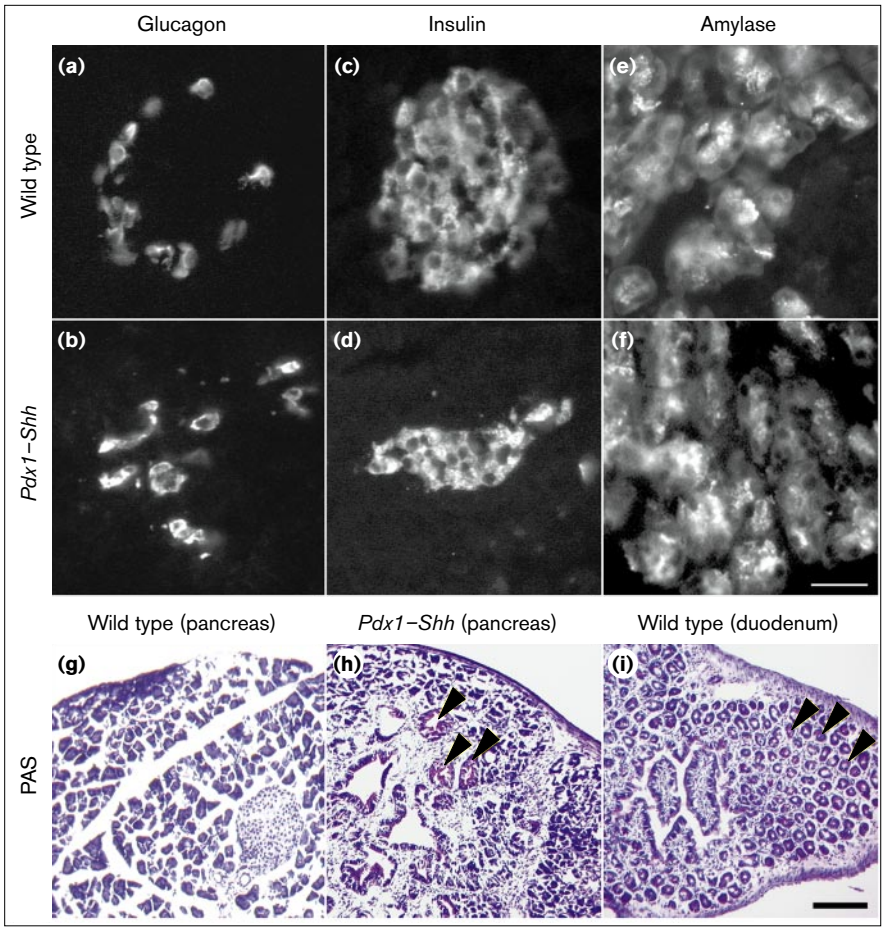
To examine whether Shh directs the region-specific differentiation of the gut endoderm and/or mesoderm, we selectively expressed *Shh* in the pancreatic endoderm under the control of the specific promoter of the *Ipf1/Pdx1* gene. Transgenic mice were viable and gut-tube differentiation was examined in mice that were 3 weeks old. Transgenic *Ipf1/Pdx1–Shh* pups lacked any sign of a spleen and two intestinal-like appendages were associated with the duodenum in the region normally occupied by the pancreas (Figure 2a,b). Histological analysis revealed that the transgenic pancreatic epithelium was surrounded by a distinct mantle of smooth-muscle-like cells with a cellular organisation that closely resembled the smooth-muscle layer of the intestine (Figure 2c–h and data not shown). These cells expressed the smooth-muscle-specific markers α-actin (Figure 2e,g) and myosin (data not shown), confirming that this mantle layer was indeed composed of smooth muscle cells, apparently organised in a longitudinal and a circular muscle layer. Smooth muscle cells also formed an interstitial network that spanned the epithelial part of the bud (data not shown). Moreover, analysis of c-kit expression in the transgenic buds revealed the presence of cells that morphologically resembled the interstitial cells of Cajal (ICCs) [14–18]; these cells, like those in the normal intestine, were located between the longitudinal and circular muscle layers (Figure 2f,h). No c-kit$^+$ ICC-like cells were found in the wild-type pancreas (Figure 2d). Thus, ectopic expression of *Shh* in the endoderm inhibits spleen development and converts pancreatic mesoderm into intestinal mesenchyme.

ICCs are thought to control the pacemaker activity of the gut [14–18]. To test whether Shh induces the conversion of pancreatic mesoderm to intestinal mesoderm in terms of function as well as structure, we cultured explants of e10 dorsal pancreatic buds *in vitro* for 7 days in the absence or presence of Shh (10^{-8} M). As in the *Ipf1/Pdx1–Shh* transgenic mice (Figure 2e), the mesodermal part of the pancreatic bud explant was converted into smooth muscle cells (Figure 2i) and, in addition, explants exposed to Shh exhibited distinct peristaltic movements, with 5–6 contractions per minute (see Supplementary material for a movie of the contractions).

The *Ipf1/Pdx1–Shh* transgenic pancreatic epithelium shows a mixed pancreatic–duodenal phenotype

The conversion of pancreatic mesoderm into intestinal mesenchyme prompted us to investigate the fate of the pancreatic endoderm in the *Ipf1/Pdx1–Shh* transgenic mice. Cells expressing glucagon (Figure 3a,b), insulin (Figure 3c,d) and amylase (Figure 3e,f) were still present in transgenic pancreatic buds, but endocrine cells failed to form organised islets and exocrine cells similarly did not exhibit

Figure 3



The *Ipf1/Pdx1–Shh* transgenic pancreatic epithelium shows a mixed pancreatic–duodenal phenotype. Immunohistochemical analysis of pancreatic markers in (a,c,e) wild-type and (b,d,f) transgenic pancreas. Both exocrine and endocrine cells differentiate in the transgenic pancreas as shown by the appearance of (a,b) glucagon$^+$, (c,d) insulin$^+$ and (e,f) amylase$^+$ cells. **(g–i)** Periodic acid-Schiff reaction (PAS) staining of the (g,h) pancreas and (i) duodenum derived from (g,i) wild-type or (h) transgenic mice show the presence of basic mucin$^+$ cells (indicated by black arrowheads) in the transgenic pancreas and wild-type duodenum. The scale bar in (a–f) corresponds to 20 μm, and in (g–i) 50 μm.

distinct acinar structures (Figure 3a–i and data not shown). The transgenic pancreatic buds also had an abnormal, duodenal-like organisation of the epithelium (Figure 3g–i and data not shown) and contained cells that were positive for basic mucins (Figure 3h), which are normally present in the duodenum (Figure 3i) but not in the pancreas (Figure 3g).

Differentiation of pancreatic cell types is dependent on the presence of mesoderm [5,6,20]. The appearance of pancreatic cell types in the *Ipf1/Pdx1–Shh* transgenic mice under conditions in which the pancreatic mesoderm is converted into intestinal mesenchyme implies either that the conversion occurs only gradually, or that, despite the conversion, the mesenchyme still can promote differentiation of pancreatic epithelial cells. The appearance of duodenal cell types in the transgenic pancreatic buds suggests that Shh acts either directly on the endoderm to induce duodenal differentiation, or indirectly through the induction of instructive signals in the converted mesoderm. The latter hypothesis would agree with earlier results showing that intestinal mesenchyme has instructive properties that are capable of partially redirecting the development of the receiving endoderm [1,2].

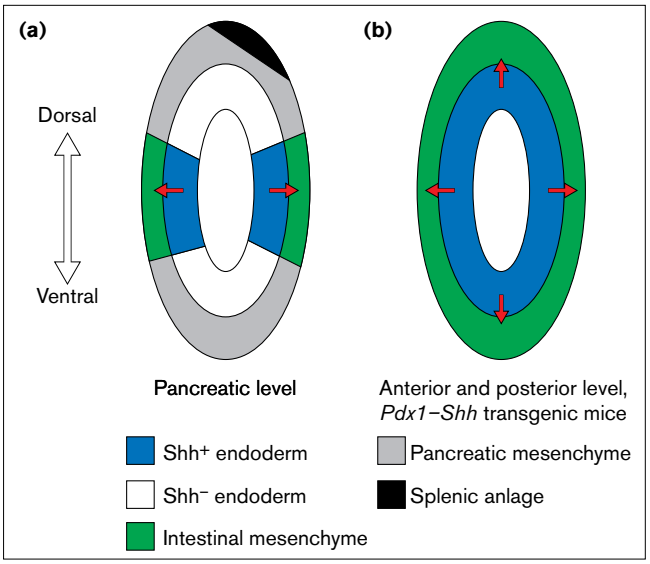
Taken together, these findings provide evidence that Shh-mediated signalling from the endoderm directs the developmental potential and/or differentiation of the adjacent gut mesoderm, and that the spatial restriction in *Shh* expression observed at different anteroposterior levels generates distinct mesodermal derivatives. At the pancreatic level of the gut tube, the absence of *Shh* expression permits the differentiation of mesoderm into pancreatic mesenchyme and spleen, suggesting that expression of *Shh* in the duodenal endoderm either directly or indirectly induces gut-specific mesodermal differentiation of intestinal smooth muscle cells and ICCs (Figure 4). Thus, the regional differentiation of the gut depends on the restriction of *Shh* expression along the anteroposterior axis of the endoderm. The control of early anteroposterior patterning in the endoderm by establishing different domains of *Shh* expression therefore has a critical influence on later aspects of organogenesis within the embryonic gut tube.

Materials and methods

Preparation of construct for transgenic mice

A 4.5 kb *Not*I–*Nae*I genomic fragment located immediately upstream of the *Pdx1* gene was subcloned into a vector carrying a 2.6 kb *Xho*I fragment of full-length rat *Shh* cDNA and a SV40 polyA site. A 7.4 kb

Figure 4



Model for the role of Shh in the differentiation of the gut mesoderm. **(a)** At the pancreatic anterior–posterior (A–P) level, expression of *Shh* in the lateral endoderm instructs (red arrows) the adjacent lateral mesoderm to adopt an intestinal fate including smooth muscle and ICCs. In contrast, *Shh* is not expressed in the dorsal and ventral gut endoderm at this A–P level, permitting the adjacent mesoderm to differentiate into pancreatic mesenchyme and spleen. **(b)** At A–P levels anterior and posterior of the pancreas and at the pancreatic A–P level in *Ipf1/Pdx1–Shh* transgenic mice, *Shh* is expressed throughout the endoderm, resulting in an intestinal fate of the surrounding mesoderm.

expression cassette was excised using *Not*I and *Bam*HI and transgenic mice were generated by pronuclear injection of the purified fragment (1.8 ng/ml) into F2 hybrid oocytes from B6/CBA parents as described [21]. The genotype was determined by PCR analysis of genomic DNA extracted from tail biopsies. The primers used were: 5′-TAGC-GAGGGGGAAGAGGAGAT-3′ (*Pdx1*-primer for 5′) and 5′-CAGTG-GATGCGAGCTTTGGAT-3′ (*rShh* for 3′).

In situ *hybridization and immunohistochemistry*

In situ hybridization using a full-length rat *Shh* probe, kindly provided by T.M. Jessel, and a partial *Ptc* probe, kindly provided by M. Scott, was carried out as described [11]. Immunohistochemical localization of antigens, double-label immunohistochemistry and *in situ* hybridization were carried out as described [11]. The periodic acid-Schiff reaction was carried out using standard procedures. Primary antibodies used were: rabbit anti-Pdx1 [9], GP anti-rat insulin C-peptide serum (Linco), GP anti-glucagon serum (Linco), rabbit anti-human-α-amylase (Sigma), Cy3-conjugated mouse anti-α-smooth muscle actin (Sigma) and rabbit anti-human c-kit (C-19) (Santa Cruz Biotech). Secondary antibodies used were: Cy3-conjugated goat anti-rabbit IgG (Jackson), fluorescein (DTAF)-conjugated goat anti-GP IgG.

Isolation and cultivation of pancreatic explants

The dorsal pancreatic bud from e10 wild-type embryos were isolated and cultured with or without 1×10^{-8} M soluble Shh [19] (kindly provided by T.M. Jessell) for 7 days as described [11], with the modification that the medium was supplemented with 10% Fe^{2+}-supplemented fetal calf serum (Hyclone).

Supplementary material

A movie showing the contractions of a pancreatic explant exposed to soluble Shh and a supplementary figure showing *lacZ* expression driven by the *Ipf1/Pdx*1 promoter in the pancreatic buds are published with this paper on the internet.

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