Regulation of pancreas development by hedgehog signaling

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SUMMARY

Pancreas organogenesis is regulated by the interaction of distinct signaling pathways that promote or restrict morphogenesis and cell differentiation. Previous work has shown that activin, a TGFβ signaling molecule, permits pancreas development by repressing expression of Sonic hedgehog (Shh), a member of the hedgehog family of signaling molecules that antagonize pancreas development. Here we show that Indian hedgehog (Ihh), another hedgehog family member, and Patched 1 (Ptci), a receptor and negative regulator of hedgehog activity, are expressed in pancreatic tissue. Targeted inactivation of Ihh in mice allows ectopic branching of ventral pancreatic tissue resulting in an annulus that encircles the duodenum, a phenotype frequently observed in humans suffering from a rare disorder known as annular pancreas. Shh−/− and Shh−/− Ihh+/− mutants have a threefold increase in pancreas mass, and a fourfold increase in pancreatic endocrine cell numbers. In contrast, mutations in Ptci reduce pancreas gene expression and impair glucose homeostasis. Thus, islet cell, pancreatic mass and pancreatic morphogenesis are regulated by hedgehog signaling molecules expressed within and adjacent to the embryonic pancreas. Defects in hedgehog signaling may lead to congenital pancreatic malformations and glucose intolerance.

Key words: Hedgehog, Patched, Pancreas, Annular pancreas, Organogenesis, Mouse

INTRODUCTION

Organ growth and function ordinarily match the size and physiologic demands of the host organism. Transplantation studies, for example, emphasize that renal, cardiac or pancreatic function is optimized when the mass of the donor organ fits the mass of the recipient. Mismatching organ and host size can be lethal, yet little is known about the mechanisms that achieve appropriate relative organ size during embryogenesis.

Members of the hedgehog family of secreted molecules, including Sonic hedgehog (Shh) and Indian hedgehog (Ihh), promote growth, differentiation and function of many organs in embryos and adults (Hammerschmidt et al., 1997). Sonic hedgehog functions to promote survival (Miao et al., 1997), proliferation (Duprez et al., 1998; Jensen and Wallace, 1997; Levine et al., 1997) and differentiation of distinct cell populations in ectoderm, mesoderm and endoderm-derived tissues (Duprez et al., 1999; Ericson et al., 1995; Roberts et al., 1995). Ihh is required for skeletal development regulating both the proliferation and differentiation of chondrocytes (Chuang and McMahon, 1999; St-Jacques et al., 1999; Karp et al., 2000). Defects in hedgehog signaling may lead to several congenital malformations of the foregut in humans, including esophageal atresia and stenosis, as well as tracheal and lung anomalies (Chiang et al., 1996; Litingtung et al., 1998; Motoyama et al., 1998; Pepicelli et al., 1998).

By contrast, in organs like the pancreas (Apelqvist et al., 1997; Hebrok et al., 1998) and pituitary (Treier et al., 1998), increased hedgehog signaling antagonizes organogenesis. Ectopic expression of Shh under the control of the pancreatic and duodenal homebox gene 1 (Pdx1) promoter disturbs pancreatic morphogenesis and transforms pancreatic mesoderm into intestinal mesenchyme. Islet architecture is affected; however, endocrine development is not completely abolished and the transgenic mice survive to adulthood (Apelqvist et al., 1997), indicating that ectopic hedgehog signaling in pancreas interferes with some but not all developmental aspects of organogenesis. Ectopic Shh expression is also observed in mice carrying targeted mutations in the two activin type II receptors, receptor IIA and IIB (Kim et al., 2000). Pancreas formation is severely impeded in these
animals. It includes a reduction in pancreas and islet size, as well as impaired pancreatic functions (Kim et al., 2000).

Loss of hedgehog signaling in chick embryos treated with cyclopamine, a steroid alkaloid similar to cholesterol (Cooper et al., 1998), leads to holoprosencephaly and ectopic pancreas formation (Kim and Melton, 1998). Holoprosencephaly is found in 5% of humans suffering from Smith-Lemli-Opitz syndrome (Kelley et al., 1996), a disorder characterized by a defect in cholesterol synthesis, and the appearance of nesidioblastosis and hypoglycemia (Lachman et al., 1991; McKeever and Young, 1990). These results suggest that regulation of hedgehog signaling is crucial for proper pancreatic organogenesis and indicate that misregulation could lead to changes in pancreatic function in humans.

Here, we demonstrate that Ihh and Ptc1, a transmembrane receptor protein that binds hedgehogs and antagonizes their signaling activities, are expressed at low levels in embryonic and adult pancreas. To investigate the functions of Shh, Ihh and Ptc1 in pancreas development, we have characterized mice with mutations in each gene. Loss of Shh and Ihh function leads to a relative increase in endocrine cell number and to malformations similar to a congenital human disorder, annular pancreas. In addition, pancreas size is relatively increased in Shh mutants. In contrast, mutations in Ptc1 interfere with embryonic pancreatic gene expression, and Ptc1<sup>+/−</sup> adults have impaired glucose tolerance. These results provide further evidence that hedgehog signaling regulates pancreatic size, morphogenesis and function, and suggest that errors in hedgehog signaling may promote human pancreatic disorders.

**MATERIALS AND METHODS**

**Mice**

The generation of Shh, Ihh, Ptc1 (St-Jacques et al., 1998, 1999; Goodrich et al., 1997) and Pdx1 (Ofield et al., 1996) mutant mice is described elsewhere. Shh and Ihh mutant animals were held in the 129 x C57Bl/6 x CBA inbred background. Heterozygotes were crossed to obtain homozygous and compound homo-heterozygous mutants and were also crossed with Pdx1<sup>+/−</sup>/lacZ animals that are kept in a Black Swiss x 129 x ICR genetic background.

**Immunohistochemistry, cell counting and microscopy**

7 μm paraffin sections of E18.5 mutant and wild-type tissue were stained by immunoperoxidase techniques (Kim et al., 1997) or fluorescence immunohistochemistry as described (Kim and Melton, 1998) except that fluorescein isothiocyanate was used at a 1:100 dilution. Primary antibodies were guinea pig anti-insulin (Dako), rabbit anti-glucagon (Chemicon) and anti-Pdx1 (generous gift from C. Wright). For glucagon staining on stomach tissue, guinea pig anti-glucagon was used with anti-guinea pig biotin antibody (1:200) and avidin coupled Cy3 (1:200).

For cell counting the embedded tissues were cut with a Zeiss microtome and the first four to five consecutive sections mounted on the first of a series of five microscope slides, followed by the next five sections placed on the second slide. A total of five individual slides (1a-5a) were filled with consecutive sections. When necessary another series of 5 slides (1b-5b) was used until the samples were cut completely. After antibody staining, insulin- and glucagon-positive cells were counted on every second section from one set of slides (1a,b,c), corresponding to a fifth of the original sample. Student’s t-test was performed to determine statistical significant differences between wild-type and mutant samples.

Mutant embryos containing the Pdx-lacZ or Ptc1-lacZ transgene were fixed for several hours at 4°C in 4% paraformaldehyde, washed 4-6 times in PBS and X-gal stained overnight at room temperature with Histomark X-gal solution (Histomark X-gal Substrate Set, Kirkegaard & Perry Lab, Gaithersburg, MA). Slides were photographed on a Zeiss Axioskop microscope, while embryos and dissected organs were photographed on a Leica WILD M10 microscope. For confocal analysis, a Zeiss Axiovert 100 TV equipped with a Zeiss Microscopes LSN 410 laser was used. Photos were scanned with a Kodak slide scanner and formatted with Adobe Photoshop.

**RNA preparation and RT-PCR analysis**

Dissected embryonic pancreatic rudiments were dissolved in Trizol (Gibco-BRL) and total RNA prepared according to the manufacturer’s methods. RT-PCR was performed as described in Wilson and Melton (1994). Primer pairs were used for PCR with the following conditions: 1 cycle of 94°C for 2 minutes; 58-60°C for 1 minutes; 72°C for 1.5 minutes followed by 25-35 cycles of 94°C for 1 minutes; 58-60°C for 1 minute; 72°C for 1.5 minutes. Primer sequences used are listed forward then reverse, 5’ to 3’. Ten of the PCR reaction volume was electrophoresed in a 5% polyacrylamide gel and processed as described by Wilson and Melton (1994).

Primers used for insulin (Gittes and Rutter, 1992); 25 cycles; annealing temperature, 60°C.

Shh: ATGCTGGCTCCTGGCCTGGTGG and TGGAGAAGTCGGCTGTCAGGC; 30-35 cycles; annealing temperature, 60°C.

Ihh: TCCTATGCCCTTCTCACAAG and TCTCTCTTAGCAAG-AGACC; 35 cycles; annealing temperature, 60°C.

Ptc: TGCTTGGTGCTGTTGCTGTG and TCTCTCACATT-CACGCTCGT; 30 cycles; annealing temperature, 58°C.

**Glucose tolerance tests**

Animals were fasted overnight and blood glucose level was measured with an automatic glucose monitor (‘Glucometer Elite’, Bayer, Elkhart, IN) from whole venous blood isolated from the tail vein. At time zero, mice were injected into the peritoneal cavity with a 200 mg/ml concentrated glucose solution for a final concentration of 2 g glucose/kg body weight. Glucose levels were determined at 30, 60 and 120 minutes after injection.

**RESULTS**

**Differential expression of hedgehog signaling components in embryonic and adult pancreas**

In the posterior foray of stage E12.5 mouse embryos, which contains the anlagen of the stomach, pancreas and duodenum, Shh expression was detected by in situ hybridization in rostral stomach and duodenal endoderm, but not in caudal stomach or pancreatic endoderm (Fig. 1A; Ahlgren et al., 1996; Apelquist et al., 1997). RT-PCR methods allowed detection of Shh expression in embryonic foregut tissues, including lung, and also demonstrated the absence of detectable Shh in embryonic pancreas or in mature pancreatic islets (Fig. 1D) (Ahlgren et al., 1997; Apelquist et al., 1997; Bitgood and McMahon, 1995; Hebrok et al., 1998). In situ hybridization studies with a probe specific for Ihh and Ptc1 revealed expression in embryonic intestinal endoderm and mesenchyme, respectively, but not in pancreas (Fig. 1B,C). However, RT-PCR assay showed expression of both genes in embryonic pancreas, and in isolated mature isolets (Fig. 1D). Thus, organ dissection and RT-PCR revealed the expression of Ihh and Ptc1 mRNA, and confirmed the absence of Shh expression, in embryonic and adult pancreas.
Hedgehog signaling and pancreas development

Fig. 1. Expression pattern of hedgehog signaling members in pancreas. (A-C) In situ hybridization of Shh (A), Ihh (B) and Ptc1 (C) in pancreas area of E12.5 mouse embryos. Expression is confined to intestine (arrowheads) and stomach (arrow) while no staining is observed in pancreas tissue. The pancreas is defined by morphology. The pancreatic epithelium has been outlined with a broken line to facilitate orientation. m, pancreatic mesenchyme; P, pancreas; S, stomach. (D) RT-PCR analysis of hedgehog and patched expression during pancreas development (E13.5-E17.5) and in mature tissue (neonatal, islet of Langerhans). Insulin was added as a pancreatic marker gene. Controls: E14.5 lung cDNA, insulin, Shh and Ptc; E14.5 stomach cDNA, Ihh.

Relative increase in pancreas size and endocrine cell number in Shh−/− and in Shh−/−Ihh−/− embryos

Hedgehog proteins are secreted molecules that affect differentiation of adjacent tissues in a concentration-dependent manner (Ericson et al., 1997; Yang et al., 1997). Therefore, a possible role for Shh expression in stomach and duodenum could be to limit the extent of pancreas growth. We have used Shh−/− mutants (St-Jacques et al., 1998) to test if loss of Shh function in stomach and duodenum expands pancreas size and increases endocrine development. Embryonic development is severely compromised in these mutants and their body weight is significantly reduced to 30% of wild-type littersmates (Fig. 2A,B; Table 1). The intestinal tract, including stomach and duodenum, is proportionally reduced in size and weight (Fig. 2C,D,E). However, compared to wild-type litter mates, the pancreas weight is maintained in Shh−/− embryos, such that the relative proportion of pancreas tissue in the mutants is significantly increased (Fig. 2C,D,E; Table 1). Analysis of pancreas gross morphology reveals that dorsal and ventral bud derivatives form properly, but that the mutant organ is more compact than wild-type tissue (Fig. 2C).

To investigate the effects of reduced Shh and Ihh activity on pancreas development, Shh−/− and Ihh−/− mutants were intercrossed. Shh−/−Ihh−/− embryos die at approximately E8.0, before pancreatic morphogenesis can be observed (Ramalho-Santos et al., 2000). Body size and the condensed appearance of the dorsal and ventral pancreas were indistinguishable in Shh−/− and Shh−/−Ihh−/− embryos (Fig. 2A,C). Pancreas mass is significantly increased relative to the embryonic body mass in both mutants, however the relative increase is more pronounced in Shh−/− than in Shh−/−Ihh−/− embryos (Fig. 2E, Table 1). Even though the difference in relative pancreas size between Shh−/−Ihh−/− mutants and Shh−/− is not significant (P>0.1), this finding shows that loss of one additional Ihh allele does not further increase pancreas size. In addition, pancreas and body mass are reduced proportionally in Ihh mutants (Table 1), indicating that Ihh stimulates embryonic organ growth.

To determine if Shh or Ihh mutations perturb cell differentiation and islet architecture, we investigated expression of endocrine and exocrine markers, including insulin, glucagon and carboxypeptidase A (Fig. 3A-C and data not shown). Histological examination showed that general pancreas architecture, including division into ducts, exocrine and endocrine structures, was maintained in Shh−/−Ihh−/− and Shh−/− embryos. However, pancreatic islets were clustered in mutants (data not shown), a variation that may result from general pancreas compaction in mutants. Islet architecture, with insulin-expressing cells in the center and glucagon positive cells confined to the outer layer is conserved in both mutants and wild-type littersmates (Fig. 3A-C). We did not

Table 1. Overview of pancreas weight and endocrine cell numbers in wild–type and hedgehog mutant mice

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>Shh−/−</th>
<th>Ihh−/−</th>
<th>Shh−/−Ihh−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>1.33±0.03</td>
<td>0.40±0.01**</td>
<td>0.89±0.02**</td>
<td>0.37±0.02**</td>
</tr>
<tr>
<td>Pancreas weight (mg)</td>
<td>10.2±0.73</td>
<td>10.45±0.66@</td>
<td>6.7±0.62###</td>
<td>8.2±0.96#</td>
</tr>
<tr>
<td>Pancreas/body</td>
<td>1.00±0.07</td>
<td>3.40±0.22**</td>
<td>0.99±0.09@</td>
<td>2.90±0.33***</td>
</tr>
<tr>
<td>Insulin-positive cells (×105)</td>
<td>6850±836</td>
<td>8580±632##</td>
<td>7907±1112#</td>
<td>9531±712##</td>
</tr>
<tr>
<td>Insulin/body</td>
<td>1.00±0.12</td>
<td>4.16±0.30**</td>
<td>1.71±0.28#</td>
<td>4.90±0.36**</td>
</tr>
<tr>
<td>Glucagon-positive cells</td>
<td>3415±309</td>
<td>4460±236###</td>
<td>4072±511#</td>
<td>4335±917#</td>
</tr>
<tr>
<td>Glucagon/body</td>
<td>1.00±0.09</td>
<td>4.33±0.23**</td>
<td>1.78±0.22#</td>
<td>4.50±0.96*</td>
</tr>
</tbody>
</table>

All results were obtained from E18.5 embryos. Body weight and pancreas weight are shown in g and mg, respectively. Numbers of insulin- and glucagon-positive cells represent one fifth of a whole pancreas. Relative values are italicized. Data are shown as means±s.e.m. Student’s t-test revealed identical values between wild-type and mutant samples marked with ‘@’. Insignificant to borderline significant statistical differences are marked with ‘#’ and highly significant differences are marked with ‘##’. P<0.05; **, P<0.005; *, P<0.001.
detecl any cells co-expressing insulin and glucagon, suggesting that endocrine differentiation is not affected. The absolute numbers of pancreatic β-cell and α-cells were similar in Shh−/−, Shh−/−/Ihh+/− and wild-type animals. Therefore, hedgehog mutants showed a significant relative increase in endocrine cells and pancreatic mass (Fig. 3D, Table 1), indicating that loss of hedgehog signaling expands pancreas development and cell differentiation relative to adjacent organs. In contrast, the moderate increase in endocrine cell numbers in Ihh−/− mutants was not significant, suggesting that

numbers are divided by body weight, a significant relative increase of endocrine cells is observed in hedgehog mutants (insulin: Wt 1±0.12; Shh−/− 4.16±0.30; Shh−/−/Ihh+/− 4.90±0.36; glucagon: Wt 1±0.09; Shh−/− 4.16±0.30; Shh−/−/Ihh+/− 4.50±0.96). (E) Increase of endocrine development in Shh−/− mutants is not only confined to pancreas tissue but can also be observed in organs that are severely affected by loss of Shh function, including the developing stomach. (F) At E18.5, more than twice as many glucagon-positive cells are found in mutant stomach when compared with Wt (Wt 1±0.11, n=4; Shh−/− 2.16±0.1, n=5). When divided by body weight to adjust for different embryo size an approximately sevenfold increase of endocrine cells are found in stomachs of Shh−/− mutant (Wt 1±0.11; Shh−/− 7.2±0.33). Data are shown as mean±s.e.m. Student’s t-test was performed to determine significant differences between wild-type and mutant samples. Identical values are marked with ‘@’ (P>0.8), insignificant statistical differences are marked with ‘#’ (P>0.1) and significant differences are marked with an asterisk (P<0.001).

Fig. 2. Pancreas size is relatively increased in Shh and Shh/Ihh mutants. (A,B) Size comparison of wild-type (Wt) and mutant embryos (Shh−/− and Shh−/−/Ihh+/−). At E18.5, Shh−/− and Shh−/−/Ihh+/− mutant embryos are indistinguishable and body weight is decreased to a third of Wt littermates (Wt 1±0.03, n=7; Shh−/− 0.3±0.01, n=5; Shh−/−/Ihh+/− 0.28±0.02, n=5, average weight of Wt samples was set to 1). Arrowhead points to the remnant of the head and arrows point to the remnants of the forelimbs. (C,D) In contrast to other organs of the intestinal tract, including stomach (s) and duodenum (d), pancreatic size is relatively unaffected. However, pancreas tissue is more compact in Shh−/− and Shh−/−/Ihh+/− embryos and isolated pancreas from Shh−/− mutants weighs as much as Wt pancreas (WT 1±0.07, n=5; Shh−/− 1.02±0.3, n=8), whereas pancreas in Shh−/−/Ihh+/− embryos is lighter (Shh−/−/Ihh+/− 0.73±0.6, n=3). Arrowheads point to dorsal pancreas and arrows to ventral pancreas. (E) Pancreas weight is relatively increased in Shh−/− and Shh−/−/Ihh+/− mutants when divided by body weight (Wt 1±0.07; Shh−/− 3±0.22; Shh−/−/Ihh+/− 2.9±0.33). In contrast, the stomach weight is proportionally reduced to a third in Shh−/− embryos (Wt 1±0.04, n=7; Shh−/− 0.32±0.02, n=6) and unchanged when divided by body weight (1.07±0.07). Data are shown as mean±s.e.m. Student’s t-test was performed to determine significant differences between wild-type and mutant samples. Identical values are marked with ‘@’ (P>0.8), insignificant statistical differences are marked with ‘#’ (P>0.1) and significant differences are marked with an asterisk (P<0.001).

Fig. 3. Relative increase of endocrine cells in Shh−/− and Shh−/−/Ihh+/− mutants. (A-C) Confocal analysis of pancreas islets stained with antibodies specific for insulin (green) and glucagon (red). High magnification reveals normal architecture of islets in hedgehog mutants with insulin-positive cells located in the center surrounded by glucagon-positive cells (A-C). (D) Counting insulin- and glucagon-positive cells as markers for endocrine development revealed a small absolute increase of endocrine cells in Shh−/− and Shh−/−/Ihh+/− mutants at E18.5 (insulin: Wt 1±0.12, n=3; Shh−/− 1.25±0.09, n=4; Shh−/−/Ihh+/− 1.39±0.1, n=3; glucagon: Wt 1±0.09, n=5; Shh−/− 1.3±0.07, n=5; Shh−/−/Ihh+/− 1.27±0.27, n=3). When absolute numbers are divided by body weight, a significant relative increase of endocrine cells is observed in hedgehog mutants (insulin: Wt 1±0.12; Shh−/− 4.16±0.30; Shh−/−/Ihh+/− 4.90±0.36; glucagon: Wt 1±0.09; Shh−/− 4.16±0.30; Shh−/−/Ihh+/− 4.50±0.96). (E) Increase of endocrine development in Shh−/− mutants is not only confined to pancreas tissue but can also be observed in organs that are severely affected by loss of Shh function, including the developing stomach. (F) At E18.5, more than twice as many glucagon-positive cells are found in mutant stomach when compared with Wt (Wt 1±0.11, n=4; Shh−/− 2.16±0.1, n=5). When divided by body weight to adjust for different embryo size an approximately sevenfold increase of endocrine cells are found in stomachs of Shh−/− mutant (Wt 1±0.11; Shh−/− 7.2±0.33). Data are shown as mean±s.e.m. Significant or borderline significant statistical differences are marked with ‘#’ and highly significant differences are marked with and asterisk. #, P>0.1; #, P<0.1; ###, P<0.05; *, P<0.005; **, P<0.001.
Ihh does not block endocrine differentiation as efficiently as Shh (Table 1).

To test if Shh interferes with endocrine development in non-pancreatic tissue we investigated endocrine cell differentiation in organs depending on hedgehog signaling. Stomachs in Shh mutants were reduced in size (Fig. 2C,D). However histological analysis revealed an increased number of glucagon-positive cells in mutant stomachs (Fig. 3E). Compared with wild-type stomach, twice as many glucagon-positive cells were detected in Shhh−/− embryos, a significant sevenfold increase in glucagon-expressing stomach epithelial cells when adjusted to body weight (Fig. 3F).

**Annular pancreas in hedgehog mutants**

Misregulation of hedgehog signaling in the foregut region has been associated with the formation of congenital malformations (Litingtung et al., 1998). In this study we report that Hedgehog signaling is upregulated in glucagon-expressing stomach epithelial cells when adjusted to body weight (Fig. 3F).

**Impaired pancreas development in Ptc mutants**

Human disorders can also be caused by ectopic expression of hedgehog signals and upregulation of hedgehog signaling is observed in mice carrying targeted mutations in *Patched 1*, which encodes the hedgehog receptor (Goodrich et al., 1997). To study the function of Ptc1 in pancreas development and function, we examined mice with Ptc1 mutations for pancreatic defects. Homozygous Ptc1 mutants die early during development thereby limiting the number of pancreatic marker genes available to determine the extent of pancreas development (Goodrich et al., 1997). However, at E9-9.5 *Pdx1* and glucagon were found in forming pancreas buds of wild-type embryos (Fig. 5A,C), while both markers were absent in Ptc1−/− mutant embryos (Fig. 5A,C). As shown in Fig. 4E-G, analysis of these mice revealed that only the ventral bud morphogenesis appeared abnormal. There were at least two distinct ventral pancreatic malformations associated with annular pancreas in Shhh−/− mutants. At E12.5 *Pdx1* expression was detectable in pancreas, stomach and duodenum. At this stage gut rotation took place and the ventral pancreatic bud had already turned towards the dorsal bud (Fig. 4E). In some cases, branching of the ventral duct leading towards the main pancreatic duct occurred (Fig. 4F). In other cases the already lobulated ventral bud split to give rise to yet another ventral bud that would form an annular pancreas (Fig. 4G). Thus, our data show that the constricting annulus is exclusively derived from ventral pancreatic tissue. While a second ventral pancreatic primordium has not been reported in mice, our results support previous hypotheses, indicating that similar mechanisms might lead to annular pancreas in humans and mice.

**Fig. 4.** Annular pancreas formation in *Ihh*−/− mutant embryos. (A) At E12.5, the pancreas in Wt embryos can be divided into the four pancreatic buds, including the hypotrophy of both ventral and dorsal bud, fixation of the tip of the ventral bud prior to rotation or persistence of the left bud of the paired ventral primordium (Hill and Lebenthal, 1993). Delineation of pancreas bud anatomy can be easily observed in mice carrying the *Pdx1* promoter (Offield et al., 1996). We have crossed these transgenic animals with *Ihh* mutants to generate *Ihh*−/−-*Pdx1-lacZ* mice. As shown in Fig. 4E-G, analysis of these mice revealed that only the ventral bud morphogenesis appeared abnormal. There were at least two distinct ventral pancreatic malformations associated with annular pancreas in *Ihh*−/− mutants. At E12.5 *Pdx1* expression was detectable in pancreas, stomach and duodenum. At this stage gut rotation took place and the ventral pancreatic bud had already turned towards the dorsal bud (Fig. 4E). In some cases, branching of the ventral duct leading towards the main pancreatic duct occurred (Fig. 4F). In other cases the already lobulated ventral bud split to give rise to yet another ventral bud that would form an annular pancreas (Fig. 4G). Thus, our data show that the constricting annulus is exclusively derived from ventral pancreatic tissue. While a second ventral pancreatic primordium has not been reported in mice, our results support previous hypotheses, indicating that similar mechanisms might lead to annular pancreas in humans and mice.
from developing Ptc1<sup>−/−</sup> mutants (Fig. 5B,D), arguing for a requirement of Ptc1 function during early pancreas development. Heterozygous Ptc1<sup>−/+</sup> mutants survive to adulthood and can be used for studies on pancreas function. Expression of Ptc1 in adult pancreas was confined to ducts and islets of Langerhans where it overlapped with insulin expression (Fig. 5E), raising the possibility that Ptc1 is required to maintain glucose homeostasis. A simple test of endocrine function is to perform glucose tolerance tests. Heterozygote animals show normal glucose levels after overnight fasting, indicating an average potential to deal with physiological levels of glucose. However, a significant increase of blood sugar was observed after glucose injection when compared with wild-type animals (Fig. 5F). Changes in glucose tolerance were only observed in male mice, while female animals behaved as wild-type. A sex-specific difference in glucose homeostasis has been demonstrated before in mice heterozygous for mutations in the insulin receptor and insulin receptor substrate genes (Bruning et al., 1997). Thus, male Ptc1 mutants have impaired glucose tolerance, a disorder also observed in pre-diabetic humans.

DISCUSSION

Requirement for hedgehog signaling during pancreas development
This study demonstrates that pancreas development is disrupted by the inactivation of hedgehog signaling components expressed within or adjacent to the pancreas anlage. We and others have shown previously that Shh is excluded from developing pancreas tissue in chick and mice (Fig. 1A,B) (Ahlgren et al., 1997; Apelqvist et al., 1997; Bitgood and McMahon, 1995; Hebrok et al., 1998). Here we report the detection of low level expression of Ihh and Ptc1 in pancreas by RT-PCR, and pancreatic defects in mice with mutations in Ihh and Ptc1. Thus, hedgehog signaling may regulate pancreas development in at least two ways. First, Shh expression in intestinal and stomach tissues that border the pancreas may limit the extent of pancreas expression. Second, Ihh expression in pancreas may regulate the extent and pattern of pancreatic growth.

To test the individual and combined functions of Shh and Ihh we investigated pancreas formation in embryos carrying targeted mutations in both genes. Similar phenotypes are observed in Shh<sup>−/−</sup> and Shh<sup>−/−</sup> Ihh<sup>−/−</sup> mutants. Embryonic development is severely compromised and the embryos are reduced to a third of wild-type littermates. Organs of the intestinal tract are reduced proportionally, with the exception of the pancreas, suggesting that loss of hedgehog signaling does not affect pancreas growth. We observe moderate changes in pancreas morphology, including density and shape of islets, in both mutants, together with a significant relative increase of endocrine cell types (Figs 2, 3; Table 1). Similar observations have been made in chick embryos treated with cyclopamine, a
chemical compound similar to cholesterol (Kim and Melton, 1998). However, the defects observed in treated chick embryos were more pronounced, including ectopic pancreas formation in stomach and duodenum, malformations that were not detected in $Shh^{-/-}$ and $Shh^{+/+}$ mouse embryos. Presumably, these differences result from the ability of cyclopamine to block all hedgehog signaling, while at least one functional $Ihh$ allele was still present in the mouse mutants described here.

Our results also indicate that Ihh and Shh have distinct effects in pancreatic organogenesis and that Ihh does not mimic Shh function to repress pancreas growth and endocrine differentiation, but is required for pancreas organogenesis. First, the loss of one $Ihh$ allele in addition to both $Shh$ alleles does not lead to an increase in endocrine development or pancreas size. Second, pancreas and body mass in $Ihh$ single mutants are proportionally reduced and only a small, statistically insignificant increase in endocrine cell number is observed (Table 1). These results suggest that the restriction of pancreas size during normal development is mainly caused by Shh produced in stomach and duodenum. Long-range effects of Shh have been reported previously for other tissues interactions (Fan et al., 1995; Fan and Tessier-Lavigne, 1994), and their ability to regulate pancreas size is supported by findings in mice mutant for activin receptor type II A and B. In these mutants, a posterior shift of $Shh$ expression towards the pancreas anlage leads to a decrease in pancreas size and function (Kim et al., 2000). Therefore, our results suggest that pancreas formation is regulated by the combined action of endogenous $Ihh$ produced in pancreatic tissue and $Shh$ expressed adjacent to pancreas tissue.

To optimize normal functional demands, organ mass must be matched to the mass of the host organism (see Slack, 1995; Bonner-Weir et al., 1989). Our data show that Shh inactivation does not significantly change absolute pancreas mass, but it is perhaps more pertinent that the relative pancreatic mass in these mutant animals is abnormally increased. In addition to the data presented here, previous studies suggest that Shh restricts pancreatic development. Ectopic expression of $Shh$ in pancreatic tissue transforms pancreatic mesenchyme into duodenal mesoderm and disturbs islet differentiation (Apelqvist et al., 1997). Blocking of $Shh$ signaling induces pancreatic marker gene expression in isolated chick endoderm (Hebrosk et al., 1998) and increases the number of glucagon-positive cells in stomachs of $Shh^{-/-}$ embryos (Fig. 3E,F), an organ that depends on Shh function during development (Fig. 2, Ramalho-Santos et al., 2000). Finally, depending on the genetic background, $Shh$ mutants display an annular pancreas (Ramalho-Santos et al., 2000), indicating that proper pancreas morphogenesis depends on accurately regulated $Shh$ signaling in the pancreas anlage.

Annular pancreas linked to hedgehog signaling

Interference of hedgehog signaling is associated with several human disorders, including holoprosencephaly (Belloni et al., 1996; Chiang et al., 1996) and Gorlin syndrome (Hahn et al., 1996; Johnson et al., 1996). In this study we have investigated whether mice carrying mutations in the $Ihh$ or $Shh$ genes develop pancreatic malformations similar to human disorders. Interestingly, in 42% of $Ihh$ mutants we detect changes in ventral pancreas bud morphology, similar to a human disorder known as annular pancreas (Hill and Lebenthal, 1993). Most often, a band of pancreatic tissue completely encircles the duodenum. However, we have not yet found any pancreas cells interspersed in duodenal muscularis, a change in gut morphology frequently observed in humans (Hill and Lebenthal, 1993).

Analysis of $Ihh$ mutants has revealed that defects in ventral pancreatic morphogenesis during early developmental stages may result in annular pancreas. Annular pancreas in humans often leads to duodenal atresia and stenosis after birth and the onset of symptoms can vary from neonates to adults (Gross and Chisholm, 1944). Thus, while $Ihh$ mutants may allow studies of earlier stages in the pathogenesis of this condition, the embryonic lethality of $Ihh$ mutants limits their use as a model system for later complications of annular pancreas.

An interesting and poorly understood aspect of annular pancreas is the association of this disorder with other congenital malformations, including intestinal malrotation and imperforate anus in humans (Kiernan et al., 1980). Depending on the genetic background, these disorders are frequently found in combination with annular pancreases in $Shh$ mutants (Ramalho-Santos et al., 2000). Therefore, our studies, together with reports describing familial annular pancreas (Hill and Lebenthal, 1993), suggest a genetic transmission and point to changes in hedgehog signaling as a possible contributory factor for this anomaly. In addition, annular pancreas is among the most frequently associated findings in individuals with Down syndrome (Kallen et al., 1996; Levy, 1991). Interestingly, $Shh$ is not expressed in pancreatic tissue and therefore we cannot exclude that development of this disorder is regulated by hedgehog signals originating from the duodenum, as both $Shh$ and $Ihh$ are expressed in the gut adjacent to the ventral pancreas (Bitgood and McMahon, 1995). Furthermore, the observation that annular pancreas formation in $Shh$ mutants depends on the genetic background indicates that genetic modifiers may play an important role during formation of this disorder.

Negative regulation of hedgehog expression in pancreas

A well-known modifier of hedgehog signaling is Ptc1, a transmembrane receptor known to bind all members of the hedgehog family (Carpenter et al., 1998; Chuang and McMahon, 1999). The identification of Ptc-transcripts throughout pancreas development and in islets of Langerhans (Figs 1D, 5E) led us to test whether Ptc1-function was required for pancreas development. Loss of Ptc1-function leads to impaired expression of two early pancreas marker genes (Fig. 5A-D), indicating that pancreas development is disturbed or delayed. Homozygous Ptc1 mutants die very early during development (Goodrich et al., 1997), thereby restricting analysis to the initial stages of pancreas organogenesis. However, heterozygous animals survive to adulthood and can be used to study physiological changes in blood glucose homeostasis. While regulation of glucose levels is unchanged under normal conditions, Ptc1 heterozygotes are unable to efficiently adjust their blood sugar levels when challenged by peritoneal injection of concentrated glucose solutions (Fig. 5F). Previous studies have shown that Ptc1 mutants show an increase in body weight when compared with wild-type littermates (Goodrich et al., 1999). Although we have used age-and weight-matched animals for our studies, further
experiments are needed to determine if the observed changes in glucose tolerance result from alterations in pancreas function, or from disturbances of glucose metabolism in peripheral organs.

In summary, results presented here have shown that individual hedgehog genes have independent and overlapping functions during pancreas development. Loss of Ihh or Shh genes increase endocrine development and can lead to the development of pancreatic malformations, similar to a human disorder known as annular pancreas. However, Shh functions independently to restrict pancreas growth while Ihh is required for proper pancreas growth. Mutations in Ptc1, an inhibitor of hedgehog signaling, block pancreas marker expression and impair glucose homeostasis. Further studies will show if regulation of blood glucose levels in humans is also controlled in part by hedgehog signaling and if mutations lead to pancreatic disorders.

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