

## 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 TGF $\beta$  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* (*Ptc1*), 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*<sup>−/−</sup> and *Shh*<sup>−/−</sup> *Ihh*<sup>+/−</sup> mutants have a threefold increase in pancreas mass, and a fourfold increase in pancreatic endocrine cell numbers. In contrast, mutations in *Ptc1* 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; Kim and Melton, 1998) and pituitary (Treier et al., 1998), increased hedgehog signaling antagonizes organogenesis. Ectopic expression of *Shh* under the control of the *pancreatic and duodenal homeobox 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* (Offield et al., 1996) mutant mice is described elsewhere. *Shh* and *Ihh* mutant animals were held in the 129 × C57Bl/6 × 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 × 129 × 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 Axiophot 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 Microsystems 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 pancreas 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'. One tenth 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*: ATGCTGGCTCGCCTGGCTGTGGAA and TGAGGAAGT-CGCTGTACAGCA; 30-35 cycles; annealing temperature, 60°C.

*Ihh*: TCCTATGCTCCTCTCACAAG and TCTCTTCTAGCAAG-AGACGC; 35 cycles; annealing temperature, 60°C.

*Ptc*: TGCTTCGGTGACTGTTGCTGTG and TCTCCTCACATT-CCACGTCCTG; 30 cycles; annealing temperature, 58°C.

### Glucose tolerance tests

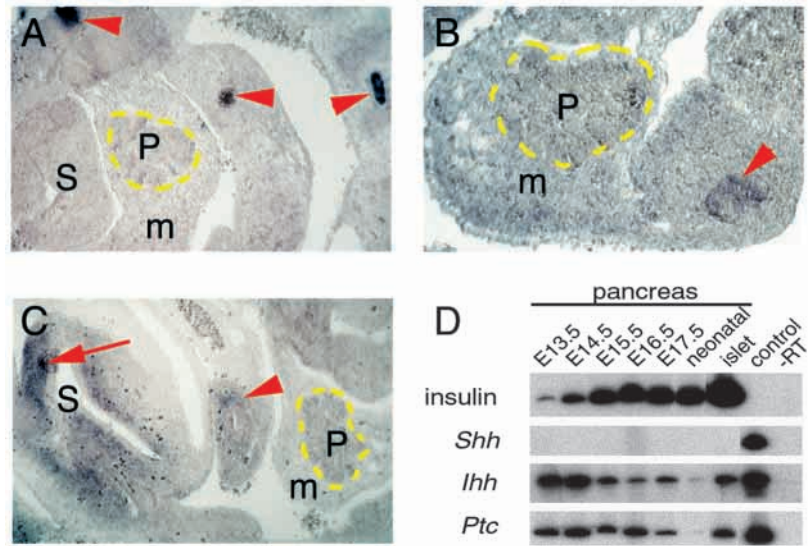
Animals were fasted overnight and blood glucose level was measured with an automatic glucose monitor ('Glucometer Elite', Bayer, Elkhart, IN) from whole venous blood isolated from the tail vein. At time zero, mice were injected into the peritoneal cavity with a 200 mg/ml concentrated glucose solution 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 foregut 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; Apelqvist 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; Apelqvist 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 islets (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.

**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*<sup>−/−</sup> and in *Shh*<sup>−/−</sup>*Ihh*<sup>+/-</sup> 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*<sup>−/−</sup> 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 littermates (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*<sup>−/−</sup> 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*<sup>−/−</sup> *Ihh*<sup>−/−</sup> 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*<sup>−/−</sup> and *Shh*<sup>−/−</sup>*Ihh*<sup>+/-</sup> 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*<sup>−/−</sup> than in *Shh*<sup>−/−</sup>*Ihh*<sup>+/-</sup> embryos (Fig. 2E, Table 1). Even though the difference in relative pancreas size between *Shh*<sup>−/−</sup> *Ihh*<sup>+/-</sup> mutants and *Shh*<sup>−/−</sup> 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*<sup>−/−</sup> *Ihh*<sup>+/-</sup> and *Shh*<sup>−/−</sup> 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 littermates (Fig. 3A–C). We did not

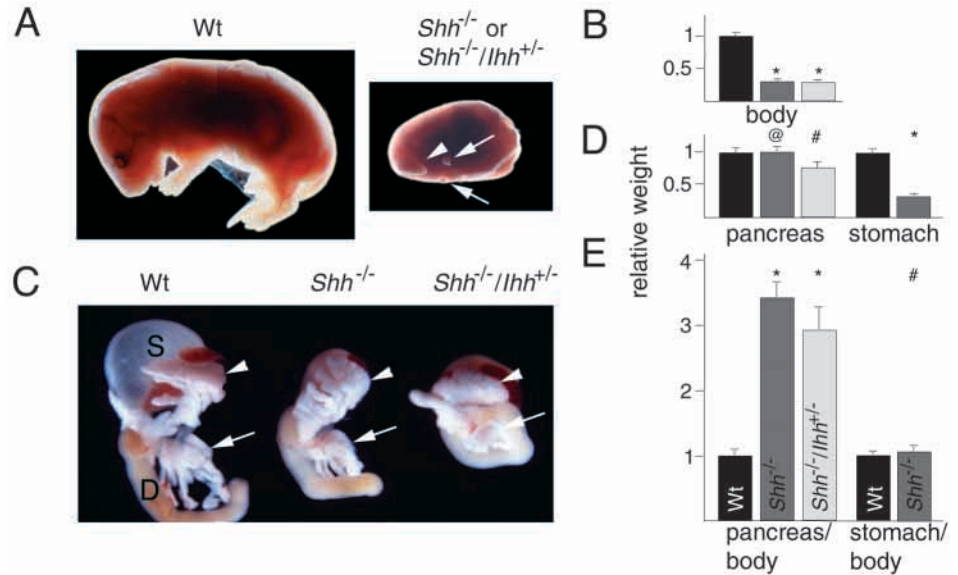
**Table 1. Overview of pancreas weight and endocrine cell numbers in wild-type and hedgehog mutant mice**

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

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 mean±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 an asterisk. @,  $P>0.8$ ; #,  $P>0.1$ ; ##,  $P<0.1$ ; ###,  $P<0.05$ ; \*,  $P<0.005$ ; \*\*,  $P<0.001$ .



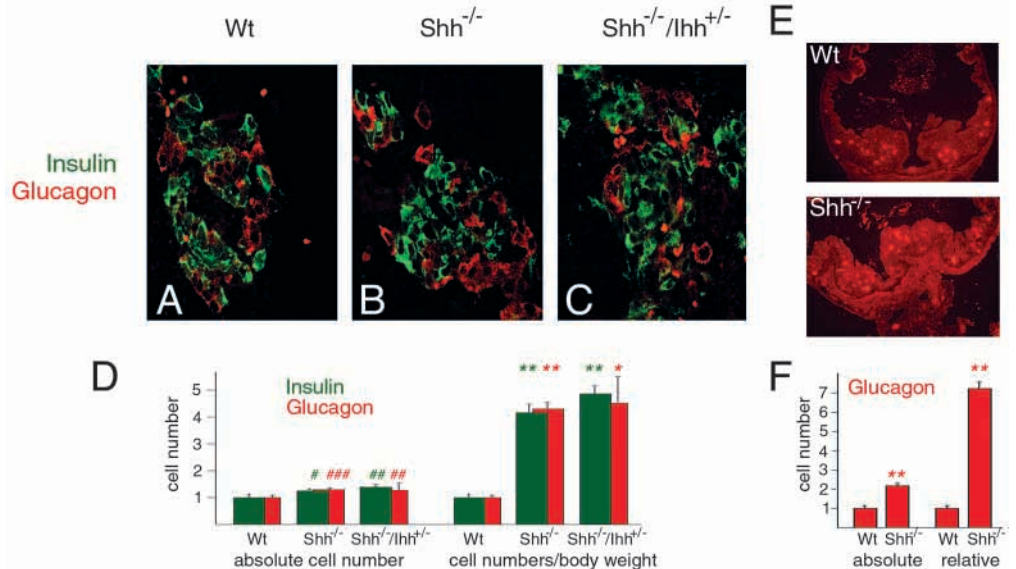
**Fig. 2.** Pancreas size is relatively increased in *Shh* and *Shh/Ihh* mutants. (A,B) Size comparison of wild-type (Wt) and mutant embryos (*Shh*<sup>-/-</sup> and *Shh*<sup>-/-</sup> *Ihh*<sup>+/-</sup>). At E18.5, *Shh*<sup>-/-</sup> and *Shh*<sup>-/-</sup> *Ihh*<sup>+/-</sup> mutant embryos are indistinguishable and body weight is decreased to a third of Wt littermates (Wt 1±0.03, n=7; *Shh*<sup>-/-</sup> 0.3±0.01, n=5; *Shh*<sup>-/-</sup> *Ihh*<sup>+/-</sup> 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*<sup>-/-</sup> and *Shh*<sup>-/-</sup> *Ihh*<sup>+/-</sup> embryos and isolated pancreas from *Shh*<sup>-/-</sup> mutants weighs as much as Wt pancreas (Wt 1±0.07, n=5; *Shh*<sup>-/-</sup> 1.02±6.3, n=8), whereas pancreas in *Shh*<sup>-/-</sup> *Ihh*<sup>+/-</sup> embryos is lighter (*Shh*<sup>-/-</sup> *Ihh*<sup>+/-</sup> 0.73±8.6, n=3). Arrowheads point to dorsal pancreas and arrows to ventral pancreas. (E) Pancreas weight is relatively increased in *Shh*<sup>-/-</sup> and *Shh*<sup>-/-</sup> *Ihh*<sup>+/-</sup> mutants when divided by body weight (Wt 1±0.07; *Shh*<sup>-/-</sup> 3.4±0.22; *Shh*<sup>-/-</sup> *Ihh*<sup>+/-</sup> 2.9 ±/− 0.33). (D,E) In contrast, the stomach weight is proportionally reduced to a third in *Shh*<sup>-/-</sup> embryos (Wt 1±0.04, n=7; *Shh*<sup>-/-</sup> 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).



detect any cells co-expressing insulin and glucagon, suggesting that endocrine differentiation is not affected. The absolute numbers of pancreatic  $\beta$ -cell and  $\alpha$ -cells were similar in *Shh*<sup>-/-</sup>, *Shh*<sup>-/-</sup> *Ihh*<sup>+/-</sup> 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*<sup>-/-</sup> mutants was not significant, suggesting that

**Fig. 3.** Relative increase of endocrine cells in *Shh*<sup>-/-</sup> and *Shh*<sup>-/-</sup> *Ihh*<sup>+/-</sup> 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*<sup>-/-</sup> and *Shh*<sup>-/-</sup> *Ihh*<sup>+/-</sup> mutant embryos at E18.5 (insulin: Wt 1±0.12, n=3; *Shh*<sup>-/-</sup> 1.25±0.09, n=4; *Shh*<sup>-/-</sup> *Ihh*<sup>+/-</sup> 1.39±0.1, n=3; glucagon: Wt 1±0.09, n=5; *Shh*<sup>-/-</sup> 1.3±0.07, n=5; *Shh*<sup>-/-</sup> *Ihh*<sup>+/-</sup> 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*<sup>-/-</sup> 4.16±0.30; *Shh*<sup>-/-</sup> *Ihh*<sup>+/-</sup> 4.90±0.36; glucagon: Wt 1±0.09; *Shh*<sup>-/-</sup> 4.16±0.30; *Shh*<sup>-/-</sup> *Ihh*<sup>+/-</sup> 4.50±0.96). (E) Increase of endocrine development in *Shh*<sup>-/-</sup> 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*<sup>-/-</sup> 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*<sup>-/-</sup> mutant (Wt 1±0.11; *Shh*<sup>-/-</sup> 7.2±0.33). Data are shown as mean±s.e.m. Insignificant or borderline significant statistical differences are marked with '#' and highly significant differences are marked with 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 *Shh*<sup>-/-</sup> embryos, a significant sevenfold increase in glucagon-expressing stomach epithelial cells when adjusted to body weight (Fig. 3F).

### Annular pancreas in hedgehog mutants

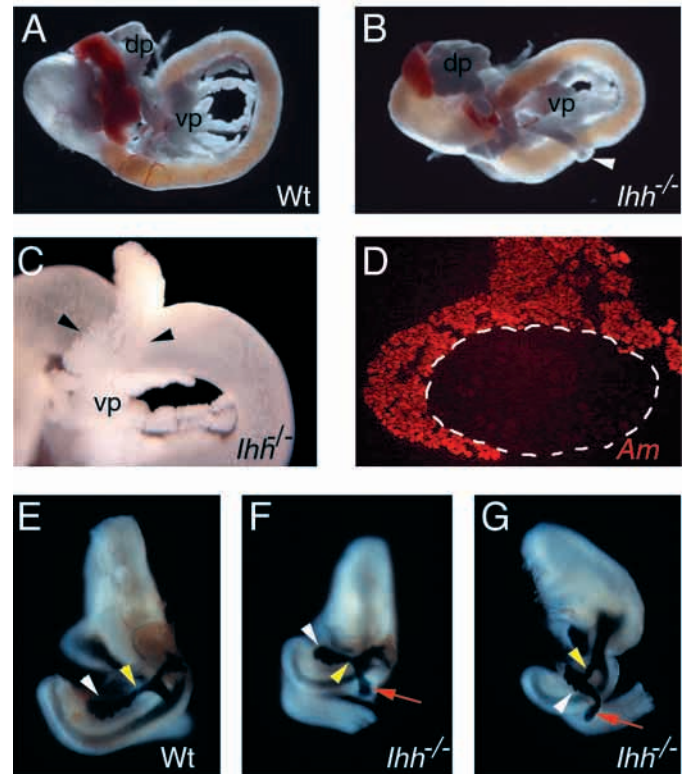
Misregulation of hedgehog signaling in the foregut region has been associated with the formation of congenital malformations in humans (Litingtung et al., 1998). In this study we report that in 42% of all *Ihh*<sup>-/-</sup> embryos ( $n=12$ ; E18.5) an extension of the ventral pancreas formed an annulus around the duodenum (Fig. 4B), a phenotype similar to a rare human disorder known as annular pancreas (Hill and Lebenthal, 1993). As in humans, the pancreas tissue often completely encircled the duodenum (Fig. 4C), while continuing to express pancreas markers, including amylase (Fig. 4D). In contrast, no ventral pancreas extensions were found in *Shh*<sup>-/-</sup>*Ihh*<sup>+/-</sup> mutants ( $n>20$ ). Only once have we observed an annular pancreas in *Shh* mutants ( $n=17$ ), although mouse strain background appears to modulate the frequency of annular pancreas in *Shh*<sup>-/-</sup> embryos (Ramalho-Santos et al., 2000). These data suggest that disruption of hedgehog signaling may contribute to development of annular pancreas in humans, which has a familial genetic transmission (Hill and Lebenthal, 1993) and is associated with Down syndrome (Kallen et al., 1996; Levy, 1991).

Several theories have previously been posited to explain the formation of an annular pancreas, including the hypertrophy 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 *lacZ* gene under control of the *Pdx1* promoter (Offield et al., 1996). We have crossed these transgenic animals with *Ihh* mutants to generate *Ihh*<sup>-/-</sup>*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*<sup>-/-</sup> 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.

### Impaired pancreas development in *Ptc* mutants

Human disorders can also be caused by ectopic expression of

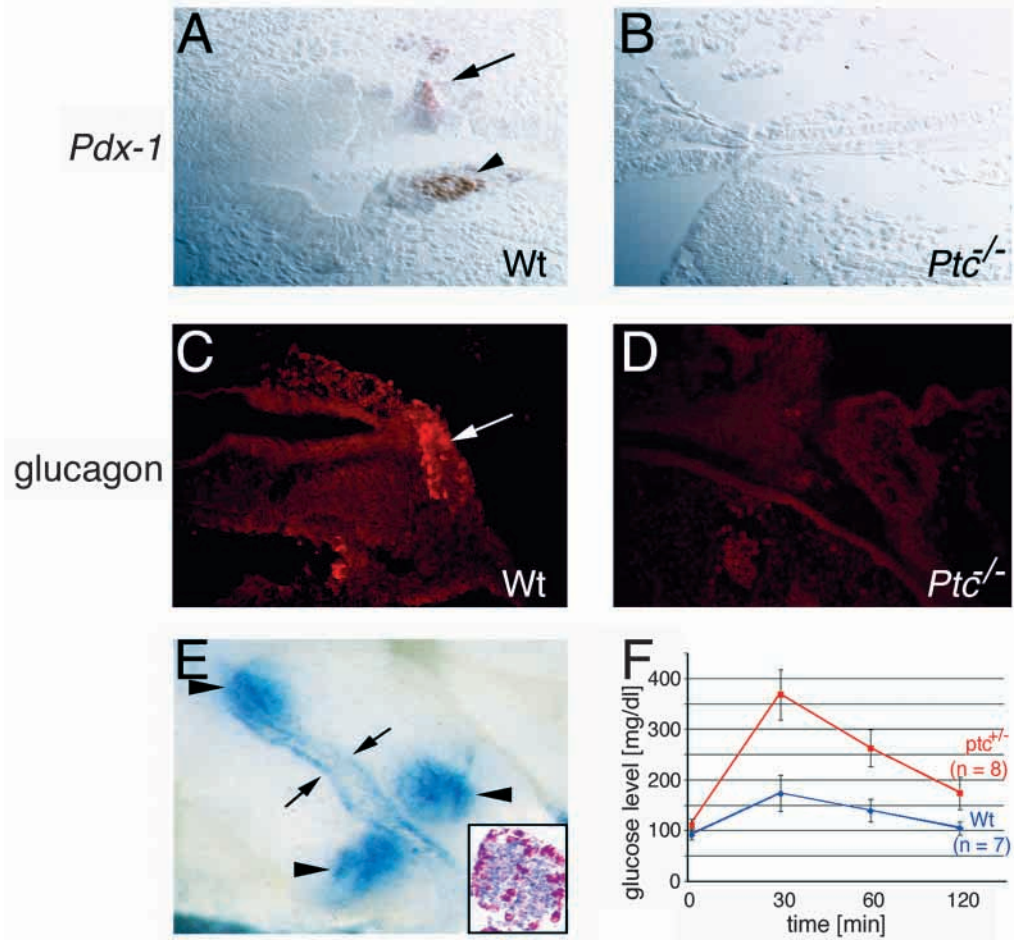
hedgehog signals and upregulation of hedgehog signaling is observed in mice carrying targeted mutations in *Patched 1*, which encodes the hedgehog receptor (Goodrich et al., 1997). To study the function of *Ptc1* in pancreas development and function, we examined mice with *Ptc1* mutations for pancreatic 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



**Fig. 4.** Annular pancreas formation in *Ihh*<sup>-/-</sup> mutant embryos. (A) At E18.5, the pancreas in Wt embryos can be divided into the dorsal part adjacent to the stomach and the ventral part close to the first duodenal loop. (B) Gross morphology in *Ihh* mutants is similar to Wt. However, in 42% of mutants, an extension of the ventral pancreas bud (arrowhead) wraps around the duodenum. (C) A close-up of a ventral bud protrusion shows the complete encircling of the duodenum (arrowheads). dp, dorsal pancreas; vp, ventral pancreas. (D) Transverse section through annular pancreas and duodenum immunostained with an antibody directed against amylase (Am), a digestive enzyme produced and secreted from pancreas tissue. The duodenum is outlined by a broken line and does not stain for amylase. (E-G) A mouse strain carrying the *lacZ* gene under the control of the *Pdx1* promoter was crossed with *Ihh* mutant animals to visualize *Pdx1* expression. (E) *Pdx1* expression in Wt embryos at E12.5 is confined to duodenum, dorsal and ventral pancreas (white arrowhead), including the connecting duct (yellow arrowhead) that links the ventral bud with the main pancreatic duct. In *Ihh*<sup>-/-</sup> embryos two distinct mechanisms of annular pancreas formation were observed (F,G). (F) Formation of a separate ventral bud (red arrow) which branches from the connecting duct (yellow arrowhead) while the original ventral bud (white arrowhead) forms normally. (G) Branching of the already lobulated ventral bud (red arrow) without a change of duct morphology (yellow arrowhead).



**Fig. 5.** Pancreas development and function is affected in *Ptc1* mutants. (A–D) Expression of *Pdx1* and glucagon, two early pancreatic markers, can be easily detected in E9.5 Wt embryos in dorsal (arrows) and ventral pancreas bud (arrowhead) (A,C), but are absent in *Ptc<sup>-/-</sup>* embryos (B,D). (E) Staining for  $\beta$ -galactosidase activity of pancreas isolated from a mouse heterozygous for the patched mutations. The *lacZ* gene is under the control of the *Ptc* promoter and  $\beta$ -galactosidase activity is present in pancreatic ducts (arrows) and islets (arrowheads). The inset presents a close-up of an islet stained for *Ptc* expression (blue) and glucagon (magenta) showing that *Ptc* expression is confined to the center of the islets, the region occupied by insulin-positive cells. (F) Glucose tolerance tests of 8–10 week old, male *Ptc<sup>+/-</sup>* and Wt littermates. Patched heterozygote animals display elevated levels of blood glucose when injected with a high-glucose solution. Note that blood glucose levels in mutants after overnight fasting are normal, indicating that the animals can maintain glucose homeostasis under normal circumstances.



from developing *Ptc1<sup>-/-</sup>* mutants (Fig. 5B,D), arguing for a requirement of *Ptc1* function during early pancreas development. Heterozygous *Ptc1* 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*<sup>-/-</sup> and *Shh*<sup>-/-</sup> *Ihh*<sup>+/-</sup> 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 (Hebrok et al., 1998) and increases the number of glucagon-positive cells in stomachs of *Shh*<sup>-/-</sup> 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 pancreas 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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