Combined activities of hedgehog signaling inhibitors regulate pancreas development

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Summary

Hedgehog signaling is known to regulate tissue morphogenesis and cell differentiation in a dose-dependent manner. Loss of Indian hedgehog (*Ihh*) results in reduction in pancreas size, indicating a requirement for hedgehog signaling during pancreas development. By contrast, ectopic expression of sonic hedgehog (*Shh*) inhibits pancreatic marker expression and results in transformation of pancreatic mesenchyme into duodenal mesoderm. These observations suggest that hedgehog signaling activity has to be regulated tightly to ensure proper pancreas development. We have analyzed the function of two hedgehog inhibitors, Hhip and patched 1 (Ptch), during pancreas formation. Our results indicated that loss of *Hhip* results in increased hedgehog signaling within the pancreas anlage. Pancreas morphogenesis, islet formation and endocrine cell proliferation is impaired in *Hhip* mutant embryos. Additional loss of one *Ptch* allele in *Hhip*$^{-/-}$*Ptch*$^{+/-}$ embryos further impairs pancreatic growth and endodermal cell differentiation. These results demonstrate combined requirements for Hhip and Ptch during pancreas development and point to a dose-dependent response to hedgehog signaling within pancreatic tissue. Reduction of *Fgf10* expression in *Hhip* homozygous mutants suggests that at least some of the observed phenotypes result from hedgehog-mediated inhibition of Fgf signaling at early stages.

Key words: Pancreas, Islets, Hedgehog signaling, Hhip, Patched

Introduction

Hedgehog signaling regulates various aspects of morphogenesis, including cell proliferation and differentiation in embryonic tissues (McMahon et al., 2003). The mammalian hedgehog (Hh) genes, sonic (*Shh*), Indian (*Ihh*) and desert hedgehog (*Dhh*), encode secreted proteins that elicit concentration-dependent responses from target cells (Ingham and McMahon, 2001). In contrast to its inductive activities during the development of other organs, Shh inhibits pancreas morphogenesis and cell differentiation (Hebrok, 2003). *Shh* is excluded from developing pancreatic tissue (Apelqvist et al., 1997; Hebrok et al., 2000) and ectopic expression of *Shh* in embryonic pancreatic epithelium disrupts expression of pancreatic marker genes and pancreas morphogenesis (Apelqvist et al., 1997; Hebrok et al., 1998). Inhibition of Hh signaling with cyclopamine, a plant-derived steroidal alkaloid that specifically blocks smoothened (Smo) function (Chen et al., 2002), leads to ectopic budding of pancreatic structures and expression of pancreatic markers in stomach and duodenum (Kim and Melton, 1998). These results indicate that Shh functions to inhibit ectopic pancreas formation in the fore-midgut area that includes stomach, duodenum and liver, thereby negatively regulating the growth and size of the developing pancreas. While *Shh* is excluded from developing and mature pancreas, the other two family members, *Dhh* and *Ihh* as well as the receptor patched 1 (*Ptch*), are detected within pancreatic tissue (Hebrok et al., 2000; Thomas et al., 2000). Evidence for a functional requirement of Hh signaling within pancreatic cells comes from studies of cultured β-cell lines in which Hh ligands induce expression of insulin and pancreatic and duodenal homeobox gene 1 (*Pdx1*; Ipf1 – Mouse Genome Informatics), a homeobox transcription factor required for proper β-cell differentiation and function in mice and humans (Jonsson et al., 1994; Offield et al., 1996; Stoffers et al., 1997; Thomas et al., 2001; Thomas et al., 2000). In addition, pancreas size is reduced in *Ihh* mutant embryos, suggesting that Hh signaling is required for proper organ development (Hebrok et al., 2000). These studies indicate that tight regulation of the activity of pancreatic hedgehog signaling is essential for proper organogenesis as elevated or reduced Hh levels result in developmental defects.

Regulation of Hh signaling activity is essential for embryogenesis as different levels of hedgehog signaling have been shown to specify different cell types. In the neural tube, specification of ventral cell types is regulated via graded levels of Hh signaling (Chiang et al., 1996; Ericson et al., 1997; Marti et al., 1995; Roelink et al., 1995). Extensive feedback mechanisms have evolved to ensure that hedgehog gradients are established within responding tissues, in part through the expression of cell surface proteins that bind and thus limit the diffusion of Hh ligands. All three Hh proteins bind to Ptch (Marigo et al., 1996; Stone et al., 1996), a transmembrane

receptor expressed in hedgehog target cells (Goodrich et al., 1996). *Ptch* is a transcriptional target of hedgehog signaling (Ingham and McMahon, 2001) and by increasing the level of Ptch protein in responding cells, Hh signaling attenuates its own activity in a negative feedback loop. Similarly, another hedgehog binding protein, Hhip (previously known as Hip1), has been identified (Chuang and McMahon, 1999). Biochemical studies have shown that *Hhip* binds to all three Hh ligands with affinities similar to Ptch (Chuang and McMahon, 1999). Ectopic expression of *Hhip* in transgenic animals inhibits hedgehog function (Chuang and McMahon, 1999; Treier et al., 2001), while loss of Hhip function results in increased Hh signaling (Chuang et al., 2003). Combined activities of Hhip and Ptch have been reported during lung development (Chuang et al., 2003), demonstrating that the function of these regulatory proteins during normal embryogenesis is to restrict overt Hh signaling by sequestering of Hh ligands.

We have tested the requirement of Hhip and Ptch during pancreas formation. We show that *Hhip* is co-expressed with *Ptch* at high levels in stomach and duodenum, while only low levels of *Hhip* expression are detectable in pancreatic tissue. Targeted deletion of *Hhip* results in increased hedgehog signaling activity in pancreatic tissue, reduction of pancreas mass, endocrine cell numbers and smaller islets of Langerhans. The additional loss of one *Ptch* allele in *Hhip*$^{-/-}$ embryos further compromises pancreas morphogenesis and endocrine cell differentiation, demonstrating that Hhip and Ptch function jointly to restrict hedgehog signaling activity during pancreas organogenesis. Thus, as it has been shown for other tissues, pancreatic tissue responds to hedgehog signaling in a dose-dependent manner.

Materials and methods

Mice

Mice used in these studies were maintained in the barrier facility according to protocols approved by the Committee on Animal Research at the University of California, San Francisco. *Ptch* mutant mice were kindly provided by Dr Matthew Scott (Stanford University).

Tissue preparation, immunohistochemistry and microscopy

The lower trunk of E12.5 (embryonic day 12.5) embryos or isolated pancreas at E18.5 were fixed in 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 1 or 4 hours at 4°C, respectively. Histological analysis, quantification of the tissue area and counting of cells were performed as previously described (Hebrok et al., 2000). Hematoxylin/Eosin staining, immunohistochemical and immunofluorescence analyses were performed on paraffin wax-embedded sections as described previously (Kim et al., 1997). The following primary antibodies were used: guinea pig anti-insulin diluted 1:500 (Linco); rabbit anti-glucagon diluted 1:500 (Linco); rabbit Pdx1 diluted 1:3000 (gift from Dr Michael German); rabbit anti-Glut2 diluted 1:1000 (Chemicon); rabbit anti-amylase diluted 1:750 (Sigma); mouse Cy3-conjugated anti-α-smooth muscle actin diluted 1:200 (Sigma); rabbit anti-Ki-67 diluted 1:200 (Novocastra Laboratories) and mouse anti-Isl-1 diluted 1:100 (Developmental Studies Hybridoma Bank).

For immunohistochemistry, the biotinylated anti-mouse IgG (Vector) was used as secondary antibodies at a 1:200 dilution. Staining for diaminobenzidine (DAB) was performed with the ABC Elite immunoperoxidase system (Vector). The following secondary antibodies were used for immunofluorescence: FITC-conjugated anti-guinea pig 1:750 (Molecular Probes), Cy3-conjugated anti-rabbit diluted 1:750 (Molecular Probes). Fluorescence was visualized and photographed with a Zeiss Axiphoto2 plus microscope.

Staining for β-galactosidase activity and whole mount in situ hybridization

Upper abdominal organs isolated from heterozygous *Hhip* mutant mice, in which the *lacZ* gene was inserted into the *Hhip* locus, were fixed for 15 minutes at 4°C in 4% PFA and then incubated overnight in phosphate-buffered saline (PBS) supplemented with 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal; 400 μg/ml) at 4°C. For whole-mount in situ hybridyzation, gastrointestinal tract including lung, stomach, pancreas and duodenum were dissected and fixed in 4% PFA overnight at 4°C. Whole-mount in situ hybridization with digoxigenin-labeled *Fgf10* riboprobe was performed as previously described (Chuang et al., 2003). Stained tissues were photographed on a Leica MZ FL3 equipped with a Leica IM500 system.

RNA preparation and RT-PCR analysis

Dissected embryonic pancreas rudiments were dissolved in Trizol (Gibco-BRL) and total RNA was prepared according to the manufacturer's methods. RT-PCR was performed as described elsewhere (Wilson and Melton, 1994). *Hhip* PCR was performed under the following conditions: 1 cycle of 94°C for 2 minutes; 60°C for 1 minute; 72°C for 1.5 minutes followed by 35 cycles of 94°C for 1 minute; 60°C for 1 minute; 72°C for 1.5 minutes. Mouse ribosomal protein L19 was used as the internal control. Forward and reverse primer sequences used are listed 5′ to 3′.

Hhip: AATTGCCAAGTGTGAGCCAG and TGCCCACTGGAA-AGATAGAC

L19: CTGAAGGTCAAAGGGAATGTG and GGACAGAGTCT-TGATGATCTC

SYBR Green real-time quantitative PCR

PCR amplifications were performed using an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). Reactions were performed in a reaction mixture consisting of a 50 μl volume solution containing 1×SYBR Green PCR master mix (Applied Biosystems) and 300 nM of each primer. Amplification was performed by initial polymerase activation for 10 minutes at 95°C, and 40 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 40 seconds and elongation for 1 minute at 72°C. To exclude contamination with nonspecific PCR products such as primer dimers, melting curve analysis was applied to all final PCR products after the cycling protocol. Forward and reverse primer sequences used are listed 5′ to 3′.

Actin: ATGACGATATCGCTGCGCTGGT and ATAGGAGTCC-TTCTGACCCATTCC

Gli: TTGTCCAGCTTGGATGAAGG and CCCAGACGGCGA-GACAC

Morphometric quantification of pancreatic tissues and cell counting

To obtain representative results, the whole pancreas was used for quantification. The first three or five consecutive sections of E12.5 or E18.5 pancreatic tissue, respectively, were mounted on the first of a series of three or five microscope slides, followed by the next three or five sections placed on the second slide. A total of three individual slides (1a-3a) were filled with consecutive sections for E12.5 embryos, and a total of five individual slides (1a-5a) for E18.5 specimen. When necessary, additional series of three (1b-3b, etc) or five (1b-5b, etc) slides were prepared until all pancreatic sections were mounted.

After Hematoxylin/Eosin staining or immunohistochemistry, pancreatic epithelial areas were outlined and measured with the OpenLab software. Insulin- and glucagons-positive areas of the E18.5

pancreas were measured on every 25th section (every 150 μm) from one set of slides (1a-1e). Ki-67-, Isl1-, insulin- and glucagons-positive cells were counted using one series of the slides at E12.5 or E18.5 (e.g. 1a-c). The widest region of the posterior stomach at E12.5 was used to measure thickness of epithelium or mesenchyme. Data analysis was performed with Minitab (version 13, State College, PA). Statistical significance was assessed by employing Mann-Whitney test, except the result of Isl1-expressing cell numbers in Fig. 6L, which was analyzed using Student's *t*-test because of similar standard deviations.

Results

Low-level *Hhip* expression in developing and mature pancreas

Two hedgehog ligands, *Ihh* and *Dhh*, as well as the ligand binding protein *Ptch*, are expressed at low levels in developing and mature pancreatic tissue (Hebrok et al., 2000; Thomas et al., 2000). Previous studies have shown that *Hhip* is a transcriptional target of hedgehog signaling and that its expression colocalizes with *Ptch* in tissues immediately adjacent to areas of hedgehog ligand expression (Chuang and McMahon, 1999). However, careful examination of the expression of *Hhip* in the pancreas anlage had not been performed. We have used heterozygous *Hhip-lacZ* knock-in animals that express *lacZ* under control of the endogenous *Hhip* promoter to determine the expression of *Hhip* in the fore-midgut area by staining for β-galactosidase activity. Similar to *Ptch*, *Hhip* expression was found in tissues adjacent to the pancreas, including stomach and duodenum; however, macroscopic analysis did not reveal any β-galactosidase activity in embryonic or adult pancreatic tissue (Fig. 1A-E). Analysis of sectioned adult tissue showed low level β-galactosidase activity within pancreatic islets (Fig. 1F). A more sensitive RT-PCR analysis revealed low levels of *Hhip* expression throughout pancreas development and in mature tissue (Fig. 1G), a result similar to that of other components in the hedgehog signaling pathway reported previously (Hebrok et al., 2000).

Defects in pancreas and spleen formation in *Hhip*$^{-/-}$ embryos

Previous studies have demonstrated that elevated levels of hedgehog signaling block pancreas formation (Apelqvist et al., 1997; Hebrok et al., 1998). Ectopic expression of *Hhip* has been shown to inhibit hedgehog signaling in bone and pituitary (Chuang and McMahon, 1999; Treier et al., 2001), suggesting that *Hhip* normally functions to limit the level of hedgehog signaling. To determine if *Hhip* blocks Hh signaling in the fore-midgut region, we performed two sets of experiments. First, we analyzed β-galactosidase activity in heterozygous and homozygous *Hhip* mutants (Fig. 2A,B). In *Hhip*$^{-/-}$ embryos β-galactosidase activity was significantly increased when compared with *Hip1*$^{+/-}$ mutants, suggesting that loss of *Hhip* resulted in elevated Hh levels. Second, to obtain quantitative information about the increase in hedgehog signaling, we used 'real time' PCR to measure the increase in expression of *Gli*, a transcription factor and target gene of Hh signaling (Hynes et al., 1997; Lee et al., 1997; Marigo et al., 1996). Comparison of *Gli* mRNA levels in wild type, heterozygous and homozygous *Hhip* mutant pancreatic tissue revealed that loss of *Hhip* alleles leads to gradual increase of Hh signaling (Fig. 2C). Thus, *Hhip* regulates the level of Hh signaling during pancreas organogenesis.

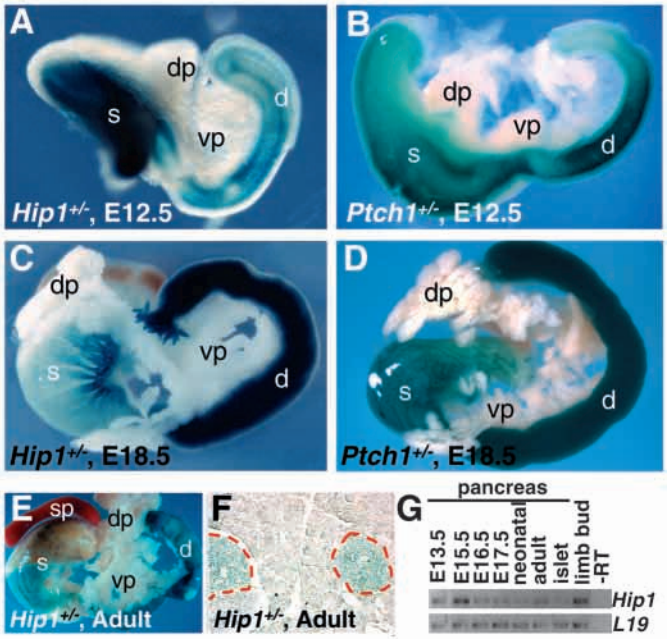

Fig. 1. Hhip (Hip1 in figure) is expressed at low levels in developing and mature pancreas. Staining for β-galactosidase activity in heterozygous *Hhip* mice that carry the *lacZ* gene under control of the endogenous Hhip promoter reveals that high level expression within the fore-midgut region is confined to stomach and duodenum, but is excluded from pancreatic tissue (A,C,E). Staining for β-galactosidase activity in *Ptch-lacZ* knock-in embryos (B,D; *Ptch1* in figure). Low level of β-galactosidase activity is observed in adult islets of *Hhip-lacZ* knock-in mice (F). RT-PCR analysis of *Hhip* gene expression of pancreatic tissue at different stages reveals low-level expression (G). Top lane, Hhip expression; bottom lane, mouse ribosomal protein L19 expression as an internal control. d, duodenum; dp, dorsal pancreas; s, stomach; sp, spleen; vp, ventral pancreas.

Analysis of organ formation in the fore-midgut area demonstrates that loss of *Hhip* function disrupts pancreas morphogenesis. In the most severe cases (Fig. 2F,G), the dorsal and ventral parts of the pancreas fuse to form a compact mass of tissue that is reduced in size (Fig. 2F, Fig. 4C). Ventral pancreas tissue, normally attached to the dorsal region of the duodenum, extends ventrally towards the mediolateral area of the duodenum. Formation of ectopic pancreas is observed in some mutants in which patches of pancreatic tissue become incorporated into the duodenum (Fig. 2H-K). In addition, the spleen, an organ derived from the posterior stomach mesenchyme, is deformed and reduced in mass (Fig. 2D,F). These results show that *Hhip* function is required for proper spleen and pancreas morphogenesis; however, potential defects in adult tissue could not be analyzed as homozygous *Hhip* mutants die shortly after birth (Chuang et al., 2003).

Mesenchymal and acinar cell differentiation is unperturbed in *Hhip*$^{-/-}$ mutant embryos

The vast majority of pancreatic tissue consists of enzyme producing acinar cells. The loss of pancreatic mass (Fig. 2F, Fig. 4C) prompted us to determine if exocrine differentiation was affected in *Hhip* mutant embryos. Histological analysis

of pancreatic sections revealed normal architecture of acinar cells (Fig. 3A,B), a finding that was confirmed by immunofluorescent staining with antibodies directed against amylase, a digestive enzyme produced by acinar cells (Fig. 3C,D). By contrast, the islets of Langerhans, marked by insulin- and glucagon-expressing cells, appeared disorganized and reduced in size (Fig. 3A-D). Forced expression of *Shh* under control of the *Pdx* promoter results in transdifferentiation of pancreatic mesoderm into duodenal mesenchyme, a process that is marked by the expression of *smooth muscle actin* (*sma*) (Apelqvist et al., 1997). To determine if loss of *Hhip* activity was sufficient to change the differentiation path of pancreatic mesenchymal cell, we performed immunohistochemistry against *sma* (Fig. 3E,F). Although *sma* is readily detectable in smooth muscle cells surrounding blood vessels (Skalli et al., 1986), no expression was detected within other regions of the wild-type or *Hhip* mutant pancreas. Thus, loss of *Hhip* activity does not affect acinar cell differentiation or result in transformation of pancreatic mesoderm.

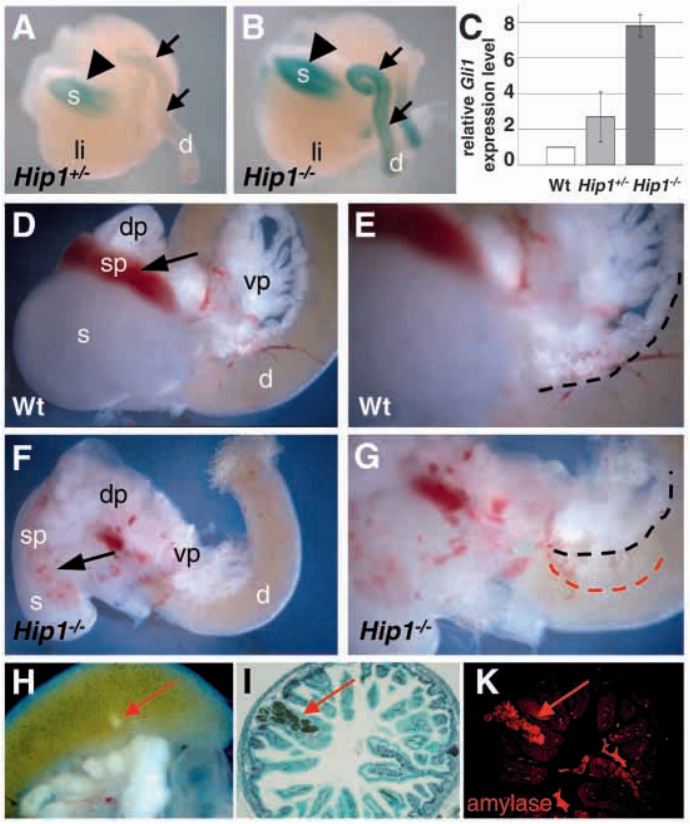

Fig. 2. Hhip (Hip1 in figure) function is required for inhibition of Hh signaling and proper pancreas morphogenesis. Staining for *Hhip* promoter regulated β-galactosidase activity in heterozygous and homozygous embryos (A,B; E12.5). β-galactosidase activity in stomach (arrowhead) and duodenum (arrows) is significantly increased in $Hhip^{-/-}$ embryos. 'Real time' PCR analysis of *Gli* expression in E17.5 pancreas (C). *Gli* expression levels are shown relative to the level of actin mRNA. To facilitate comparison, expression in wild-type pancreas (white bar) has been adjusted to '1'. $Hhip^{+/-}$ (2.7±1.4; light-gray bar) and $Hhip^{-/-}$ mutant pancreas (7.8±0.6; dark-gray bar). Error bars shown are ±s.d. Pancreas and spleen (arrows) are deformed in $Hhip^{-/-}$ embryo at E18.5 (F,G) compared with wild type (D,E). Higher magnification reveals that the connection between ventral pancreas and duodenum is confined to the dorsal region of the duodenum (E, broken line) in wild-type embryos but extends laterally in $Hhip^{-/-}$ embryos (G, broken red line). (H-K) In some cases, ectopic pieces of pancreas are integrated within the duodenum (red arrows), as shown by Feulgen staining (I) and staining for amylase (K), a marker of pancreatic exocrine cells. d, duodenum; dp, dorsal pancreas; li, liver; s, stomach; sp, spleen; vp, ventral pancreas.

Hhip function is required for proper islet morphogenesis

The expression of Hh signaling components in pancreatic endocrine cells suggests that their activity is required for some aspects of cell differentiation and organ function (Hebrok et al., 2000; Thomas et al., 2000). Cell culture studies have shown that hedgehog signaling activates *Pdx1* expression, a transcription factor essential for β-cell differentiation and function (Ahlgren et al., 1998; Jonsson et al., 1994; Offield et al., 1996; Thomas et al., 2001). Islets start to form at the end of gestation when the four different endocrine cell types aggregate as cell clusters (Kim and Hebrok, 2001). Wild-type islets form discrete clusters of cells characterized by a central core of insulin-producing β-cells surrounded by glucagon-positive α-cells (Fig. 4A). In *Hhip* mutants, endocrine cells still clustered into islet like structures; however, the size of the aggregates was significantly reduced (Fig. 4B). Morphometric analysis revealed that the overall islet area was diminished by 45% after adjustment for changes in body mass (Fig. 4C). The reduction in endocrine cells was due to the loss of larger islets

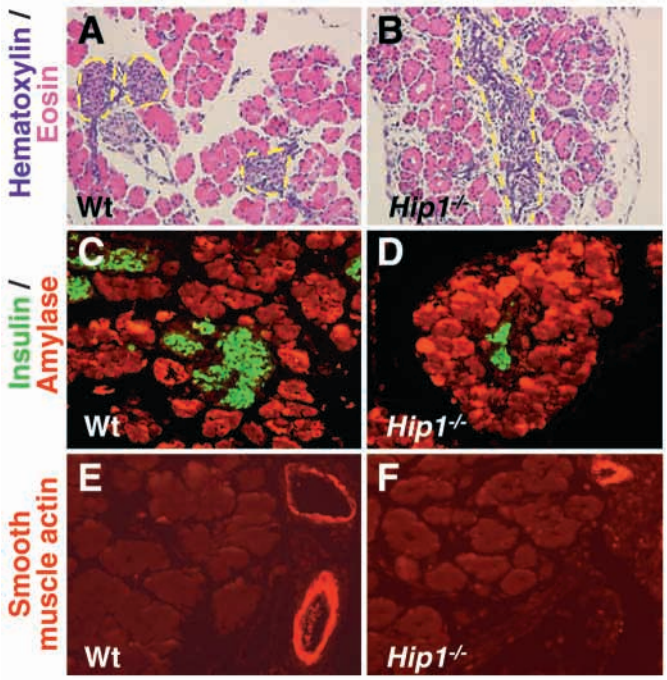

Fig. 3. Histological analysis of pancreatic tissue at E18.5. Hematoxylin/Eosin (A,B) and amylase (red; C,D) staining of E18.5 pancreatic tissue shows normal exocrine architecture in $Hhip^{-/-}$ (Hip1 in figure; B,D). By contrast, clustering (A,B; broken yellow lines) and size of islets as shown by insulin staining (green; C,D) is impaired in $Hhip^{-/-}$ embryos (B,D). Expression of smooth muscle actin is confined to blood vessels in mutants and wild type (E,F), indicating that loss of Hhip function does not lead to transformation of pancreatic mesenchyme into duodenal mesoderm.

(>4000 μm^2), while the number of small islets (<4000 μm^2) was maintained (Fig. 4D). Ki-67 staining was performed to determine if the loss of β-cells was due to a decrease in proliferation. Insulin/Ki-67 double-positive cells were observed in both wild-type and *Hhip* mutants and quantitative analysis revealed a slight, but significant decrease in the proliferation activity of β-cells (Fig. 4E).

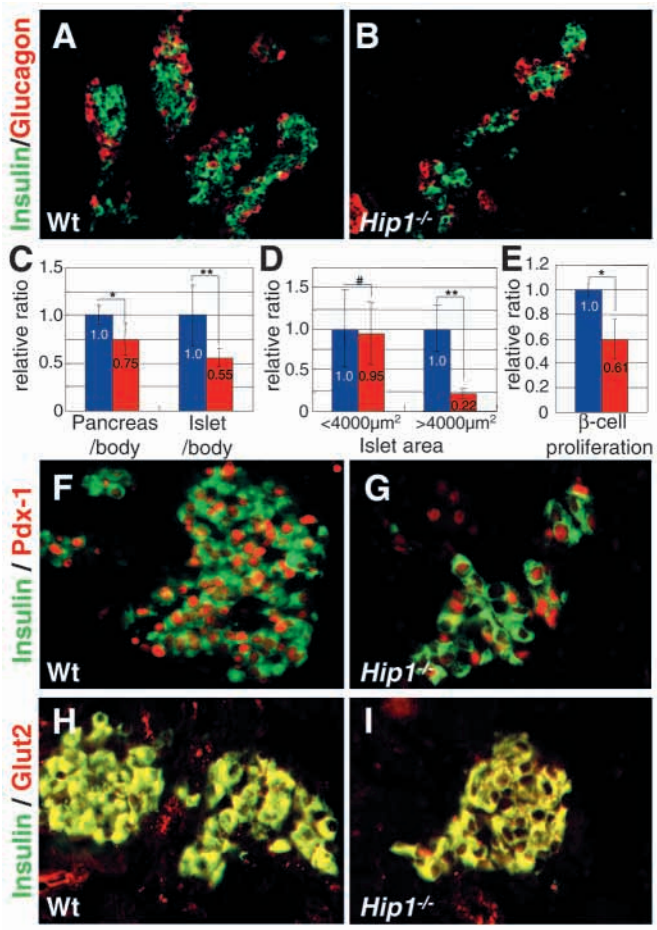

Fig. 4. Islet mass and β-cell proliferation is decreased in *Hhip*$^{-/-}$ embryos (*Hip1* in figure). Islets were stained with antibodies directed against centrally located insulin (green) and marginally located glucagon producing cells (red). Hhip function is required for normal islet morphogenesis (A,B). Clusters of insulin- and glucagon-positive cells form but are significantly smaller than the ones in control embryos (wild-type or heterozygotes, compare B with A). To adjust for differences in body mass, pancreas weight and islet area were divided by body weight. (C, blue, control, n=6; red, *Hhip*$^{-/-}$, n=5; *P<0.05, **P<0.01). Quantification of islet areas revealed a 45% reduction that is more pronounced than the general loss of pancreatic tissue (C, blue, control; red, *Hhip*$^{-/-}$). The reduction in islet mass is due to the loss of larger islets (>4000 μm^2) while the number of smaller islets (>4000 μm^2) is maintained in Hhip mutants (D, blue, control, n=5; red, *Hhip*$^{-/-}$, n=5; #no significant difference, **P<0.01). Staining for the nuclear marker Ki-67 showed that proliferation of β-cells at E18.5 is reduced by 39% (E, blue, control, n=3; red, *Hhip*$^{-/-}$, n=5; *P<0.05). Changes in islet morphogenesis and β-cell proliferation are not due to incomplete cell differentiation (F-I). β-cells express mature markers, including Pdx1 (F,G; insulin, green; Pdx1, red) and glucose transporter 2 (H,I; insulin, green; Glut2, red). Error bars shown are ±s.d.

To understand if increased hedgehog signaling influences β-cell differentiation, we examined the expression of mature β-cell markers, including Pdx1 and glucose transporter 2 (Glut2; Slc2a2 – Mouse Genome Informatics), a low-affinity transporter present in the plasma membrane of pancreatic β-cells, in *Hhip* mutant embryos (Fig. 4F-I). Expression of these markers is unchanged in *Hhip* mutant β-cells, indicating that elevated hedgehog signaling impairs β-cell proliferation but not differentiation.

Fgf10 expressions in pancreatic mesenchyme are affected in *Hhip* mutants

The relative loss of pancreatic mass and endocrine cell numbers is reminiscent of defects reported in mice lacking *Fgf10* (Bhushan et al., 2001). Transient expression of *Fgf10* in pancreatic mesenchyme between E9.5 to E11.5 is essential for proliferation of Pdx1-positive epithelial progenitor cells. We performed in situ hybridization to test if pancreatic *Fgf10* expression is affected by increased hedgehog signaling in *Hhip* homozygous mutants. Although *Fgf10* expression is rapidly detectable in control pancreatic and lung buds at E10.5, *Fgf10* transcripts are significantly reduced in *Hhip*$^{-/-}$ tissue (Fig. 5A,B). However, *Fgf10* expression is not completely abolished, as similar levels of expression are detected in control and *Hhip* mutant pancreas at E11.5 (Fig. 5C,D). The partial reduction of *Fgf10* expression is likely to account for some of the pancreatic defects observed in *Hhip* mutant mice.

Combined activities of *Hhip* and *Ptch* govern early pancreas formation

Previous studies have suggested that *Hhip* and *Ptch* both function as negative regulators of the hedgehog signaling pathway and that they share redundant roles during mammalian embryogenesis. This is supported by the recent finding that loss of one *Ptch* allele in *Hhip*$^{-/-}$ mice leads to accelerated lethality before E13 and that internal organs in *Hhip*$^{-/-}$;*Ptch*$^{+/-}$ mice

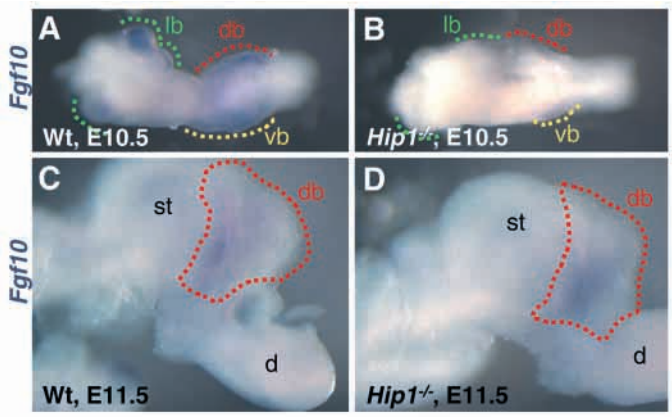

Fig. 5. Reduction of *Fgf10* expression in *Hhip* (Hip1 in figure) mutant pancreas. Whole-mount in situ hybridization of *Fgf10* expression in E10.5 (A,B) and E11.5 (C,D) pancreatic tissue in control (A,C) and *Hhip*$^{-/-}$ embryos (B,D). *Fgf10* expression (blue) was attenuated in *Hhip*$^{-/-}$ mutant pancreas bud at E10.5 (B); however, *Fgf10* levels were recovered at E11.5 (D). Staining of control and mutant tissues was performed in parallel to detect quantitative differences in *Fgf10* expression. d, duodenum; db, dorsal pancreas bud; lb, lung bud; st, stomach; vb, ventral pancreas bud.

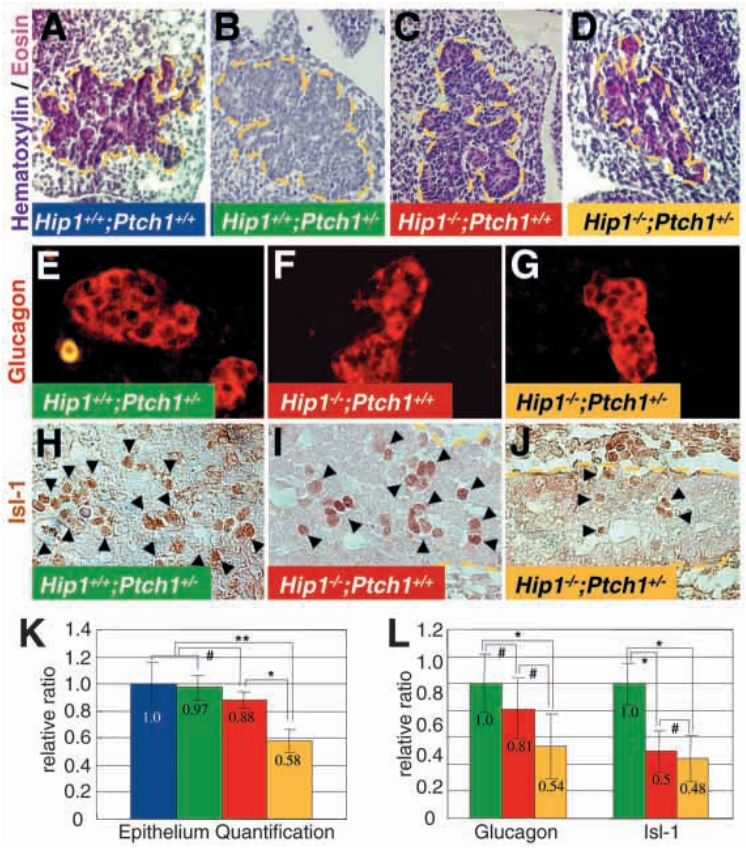

Fig. 6. Combined activities of Hhip and Ptch during pancreas development and endocrine cell development. Hematoxylin/Eosin staining (A-D) shows a stepwise decline in pancreatic epithelial size and branching associated with the loss of *Hhip* (*Hip1* in figure) and *Ptch* (*Ptch1* in figure) alleles (throughout the figure dorsal is towards the top and anterior towards the left). Morphometric analysis was used to outline and quantify pancreatic epithelium (A-D,K, blue $Hhip^{+/+};Ptch^{+/+}$, n=5; green, $Hhip^{+/+};Ptch^{+/-}$, n=3; red, $Hhip^{-/-};Ptch^{+/+}$, n=3; yellow, $Hhip^{-/-};Ptch^{+/-}$; n=4; #no significant difference, *P<0.05, **P<0.01). Staining for glucagon- (red; E-G) and Isl1- (brown; H-J) expressing cells within the pancreatic epithelium and quantification of both cell types (L, green, $Hhip^{+/+};Ptch^{+/-}$ n=3; red, $Hhip^{-/-};Ptch^{+/+}$ n=3; yellow, $Hhip^{-/-};Ptch^{+/-}$ n=3; #no significant difference, *P<0.05; Glucagon, Mann-Whitney test; Isl1, Student's t-test) reveals additive requirements of Hhip and Ptch for endocrine cell differentiation. Arrowheads indicate Isl1-positive cells within pancreatic epithelium. Yellow cell in E correspond to autofluorescing erythrocytes.

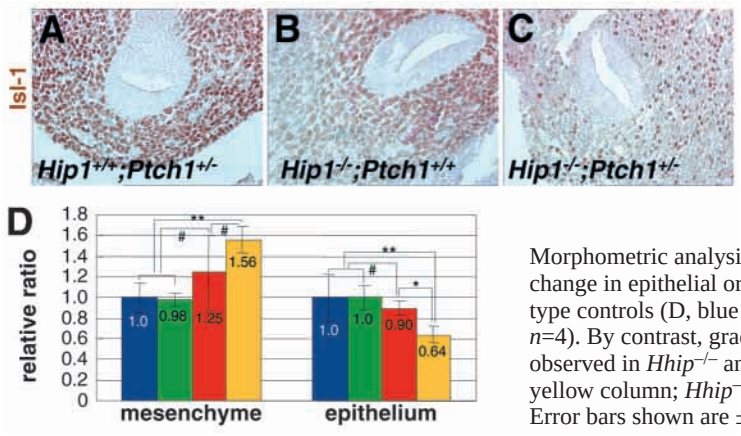

exhibit more severe defects than those found in $Hhip^{-/-}$ mutant mice (Chuang et al., 2003). To test if *Hhip* and *Ptch* act jointly during pancreas formation, we analyzed $Hhip^{-/-};Ptch^{+/-}$ embryos at E12.5. Histological analysis revealed that pancreatic buds develop in $Hhip^{-/-};Ptch^{+/-}$ mutants; however, more severe defects in pancreas morphogenesis and cell differentiation were observed in these embryos because of the additional loss of one *Ptch* allele (Fig. 6A-D,K). At E12.5, pancreatic epithelium has started to branch into the surrounding mesenchyme, a process that was apparent in wild type (Fig. 6A), $Hhip^{+/+};Ptch^{+/-}$ (Fig. 6B) and $Hhip^{-/-};Ptch^{+/+}$ (Fig. 6C) mice. However, epithelial branching was visibly impaired in $Hhip^{-/-};Ptch^{+/-}$ embryos (Fig. 6D), indicating that functional interactions between both proteins are required for proper pancreas formation. Quantitative measurements of the pancreatic epithelial area revealed a slight, albeit statistical insignificant, reduction in *Hhip* mutant embryos (Fig. 6K). By contrast, loss of an additional *Ptch* allele significantly reduced the pancreatic epithelium (Fig. 6K), further suggesting a requirement to tightly control the levels of hedgehog signaling during pancreas formation. Similar results were obtained when mutant epithelium was analyzed for the expression of endocrine cell markers glucagon and islet 1 (Isl1), a homeobox transcription factor expressed in pancreatic endocrine cells (Fig. 6E-J,L) (Ahlgren et al., 1997). The number of cells expressing either of these markers in epithelial cells decreases in a stepwise manner, in correlation with the progressive loss of *Hhip* and *Ptch* alleles. In the case of islet 1-positive cells, loss of *Hhip* is sufficient to cause a statistically significant reduction, while additional loss of one *Ptch* allele does not further decreases the number of Isl1-positive cells.

Defects in organ formation at the fore-midgut area in $Hhip^{-/-}Ptch^{+/-}$ mutants

In addition to defects in pancreas development, $Hhip^{-/-}$ mutants also showed structural abnormalities in stomach and spleen (Figs 2, 7) (Apelqvist et al., 1997). In control embryos, wild-type and $Hhip^{+/-}$, the proximal third of the stomach (fore-stomach) is lined by a squamous epithelium, while the distal two-thirds are covered by a glandular epithelium. In *Hhip* mutants, there was a tendency that the thickness of the posterior epithelium was reduced. Further reduction was observed in

Fig. 7. Hhip and Ptch (Hip1 and Ptch1 in figure) regulate mesenchymal-epithelial interaction in posterior stomach. Stomach morphology and gene expression in mutant embryos were analyzed at E12.5. Strong expression of Isl1 is observed in posterior stomach of $Hhip^{+/+};Ptch^{+/-}$ mice (A). Homozygous *Hhip* mutants display reduced Isl1 expression (B) that is further diminished in $Hhip^{-/-}Ptch^{+/-}$ embryos (C). Morphometric analysis of posterior stomach mesenchyme and epithelium reveals no change in epithelial or mesenchymal area in $Hhip^{+/+};Ptch^{+/-}$ mice compared with wild type controls (D, blue columns; $Hhip^{+/+};Ptch^{+/+}$, n=7; green columns; $Hhip^{+/+};Ptch^{+/-}$, n=4). By contrast, gradual epithelial thinning and mesenchymal thickening is in observed in $Hhip^{-/-}$ and $Hhip^{-/-};Ptch^{+/-}$ embryos (red columns; $Hhip^{-/-};Ptch^{+/+}$, n=4; yellow column; $Hhip^{-/-};Ptch^{+/-}$, n=4; #no significant difference, *P<0.05, **P<0.01). Error bars shown are ±s.d.

Hhip$^{-/-}$*Ptch*$^{+/-}$ embryos, indicating that combined activities of *Hhip* and *Ptch* are required for proper stomach development. In contrast to the epithelial reduction, a stepwise increase in posterior stomach mesenchyme was noted with decreasing levels of *Hhip* and *Ptch* (Fig. 7). These morphological changes are accompanied by molecular changes in marker gene expression, as demonstrated by changes in *Isl1* expression in stomach mesenchyme. *Isl1* is normally found in pancreatic and posterior stomach mesenchyme but is excluded from anterior stomach mesenchyme (Kim et al., 2000). In *Hhip*$^{-/-}$ embryos, and even more pronounced in *Hhip*$^{-/-}$*Ptch*$^{+/-}$ embryos, the expression in posterior stomach is reduced (Fig. 7A-C), suggesting that stepwise increase of Hh signaling leads to a gradual change in cell differentiation.

Discussion

Hhip is required for proper pancreas morphogenesis

The activity of the hedgehog proteins is partially controlled by ligand binding proteins that limit their diffusion (Ingham and McMahon, 2001). These binding proteins, including Hhip and Ptch, are transcriptional targets of hedgehog signaling and their expression is activated in cells that receive hedgehog signals. We have studied the requirement of *Hhip* and *Ptch* during pancreas formation. Our results indicate that *Hhip* is expressed during pancreas development and in mature pancreatic tissue, however the level of expression is too low for detection by staining for β-galactosidase activity in heterozygous *Hhip-lacZ* knock-in mice (Fig. 1). These results are consistent with previous studies demonstrating that hedgehog signaling is active at a low level within embryonic and mature pancreatic tissue (Hebrok et al., 2000; Thomas et al., 2001; Thomas et al., 2000).

Embryos carrying homozygous deletions of the *Hhip* gene develop without any obvious changes in gross morphology but newborn mice die shortly after birth because of deficiencies in lung development that result in respiratory failure (Chuang et al., 2003). Closer inspection of the developing lungs revealed elevated Hh levels as demonstrated by increased expression of the Hh target genes, *Ptch* and *Hhip* (elevated levels of *Hhip* were measured by comparing the intensities of β-galactosidase staining in *Hhip*$^{+/-}$ and *Hhip*$^{-/-}$ mutants). Similarly, *Hhip* also regulates hedgehog signaling in the pancreas anlage as the expression levels of the *lacZ* gene knocked into the *Hhip* locus was substantially higher in stomach and duodenum of E12.5 *Hhip*$^{-/-}$ embryos compared with that in *Hhip*$^{+/-}$ mutant embryos. The increase in β-galactosidase activity was significant, even when the differences in gene dosage of the *lacZ* gene in heterozygous and homozygous mutants was taken into account (Fig. 2A,B). Quantitative measurement of the hedgehog signaling activity with 'Real time' PCR detection of *Gli* transcripts revealed an approximately eightfold increase in homozygous *Hhip* mutants (Fig. 2C). The approximately threefold increase observed in heterozygous mutants indicates that loss of one *Hhip* allele is sufficient to substantially increase Hh signaling activity. Thus, these data demonstrate that *Hhip* functions as an inhibitor of Hh signaling within the developing organs of the fore-midgut region, including the pancreas proper.

Loss of *Hhip* function results in obvious changes in organ morphology in the pancreas anlage. These defects are reminiscent of, but more moderate than, changes found in transgenic mice ectopically expressing sonic hedgehog under control of the *Pdx1* promoter (*Pdx-Shh*) (Apelqvist et al., 1997). Although the spleen, an organ derived from posterior stomach mesenchyme, is missing in *Pdx-Shh* mice, it is misshapen and reduced in size in *Hhip*$^{-/-}$ embryos. In *Pdx-Shh* transgenics pancreatic mesenchyme transforms into duodenal mesoderm and clusters of pancreatic cells are dispersed in duodenal tissue. In *Hhip* mutants, ventral pancreatic tissue extends laterally towards the duodenum and in some cases small patches of ectopic pancreas are found within the gut (Fig. 2D-K). The border between the dorsal and ventral pancreas is diminished, leading to a contiguous mass of pancreatic tissue in severe cases (Fig. 3F,G). However, smooth muscle-like structures, indicative of transformation of pancreatic into duodenal tissue, were not observed in the pancreatic region of *Hhip*$^{-/-}$ mutants (Fig. 3E,F). Although the exocrine component appears normal, islet mass and architecture is significantly affected. Endocrine cells leave the pancreatic epithelium and aggregate in cell clusters but the total number of endocrine cells is reduced by 45%, a decrease predominately caused by the loss of larger islets (Fig. 4D). Thus, loss of *Hhip* function results in specific pancreatic phenotypes that are similar to, but less severe than, those observed in *Pdx-Shh* mice.

Level of hedgehog signaling regulates pancreas development

A possible explanation for the milder phenotype is that loss of *Hhip* might not increase hedgehog signaling to the level observed in *Pdx-Shh* transgenic mice. In addition to *Hhip*, *Ptch*, another attenuator of hedgehog signaling, is also expressed in pancreatic tissue and inactivation of *Ptch* has previously been shown to impair pancreatic marker expression (Hebrok et al., 2000). Thus, it is likely that loss of *Hhip* function is partially compensated for by the remaining function of *Ptch*. This hypothesis is supported by our studies of pancreas development in *Hhip*$^{-/-}$*;Ptch*$^{+/-}$ mice. Although the analysis of these compound mutant embryos is restricted to early developmental stages because of lethality before E13, pancreas development is more severely compromised compared with *Hhip*$^{-/-}$ mutants. Progressive loss of *Hhip* and *Ptch* alleles results in reduction of pancreas epithelium and endocrine marker genes in a graded fashion (Fig. 6). These results suggest that *Hhip* and *Ptch* function jointly during pancreas organogenesis. They also indicate that Hh signaling effects in the pancreas are concentration dependent, an observation that has previously been noted during the development of other organs, including limb bud and neural tube formation (Ericson et al., 1997).

Hhip function regulates endocrine cell mass

Our data indicate that moderate elevation of hedgehog signaling in the pancreas anlage interferes with proper organ formation (Apelqvist et al., 1997; Hebrok et al., 1998). General loss of *Hhip* and reduction of *Ptch* affects endocrine cell development and organ morphogenesis at early stages of pancreas organogenesis. In addition, proliferation of β-cells in E18.5 *Hhip*$^{-/-}$ embryos is significantly reduced, suggesting that elevated Hh levels throughout development are sufficient to impair expansion of β-cells. By contrast, treatment of established β-cell lines with hedgehog agonists has been shown

to activate *Pdx1* and insulin gene transcription and to stimulate insulin secretion (Thomas et al., 2001; Thomas et al., 2000), suggesting a requirement of Hh signaling in adult pancreas. Differences between developing and fully matured β-cells with regard to their response to Hh signaling could explain these discrepancies. Unfortunately, *Hhip*$^{-/-}$, *Ptch*$^{-/-}$ and *Hhip*$^{-/-}$;*Ptch*$^{+/-}$ mutants die early during embryogenesis or shortly after birth (Chuang et al., 2003; Goodrich et al., 1997), thereby preventing the analysis of their function in the adult pancreas. To address unequivocally the question of whether ectopic activation of this pathway affects glucose homeostasis in vivo, spatial and temporal deregulation of Hh signaling exclusively in the mature pancreas would be required.

Interaction between hedgehog and Fgf signaling pathways

How does elevated hedgehog signaling affect pancreas morphogenesis and endocrine cell proliferation? Previous studies have shown that hedgehog signaling attenuates Fgf signaling during lung development (Bellusci et al., 1997; Litingtung et al., 1998; Pepicelli et al., 1998). Sonic hedgehog-mediated repression of *Fgf10* expression within localized areas of the lung mesenchyme is required for proper branching morphogenesis. Uniform elevation of sonic hedgehog activity in *Hhip*$^{-/-}$ lung epithelium results in general inhibition of *Fgf10* expression and disruption of secondary branching (Chuang et al., 2003). Our results suggest a similar relationship between hedgehog and Fgf10 signaling during pancreas development (Fig. 5). Loss of *Fgf10* function reduces proliferation of *Pdx1*-positive epithelial cells, thereby impairing pancreas growth and expansion of endocrine cell types (Bhushan et al., 2001). Pancreatic defects in *Hhip* homozygous mutant mice are less severe than those found in *Fgf10* mutant embryos, a difference that might be explained by residual *Fgf10* activity that is maintained in *Hhip* mutants (Fig. 5). Nonetheless, these data suggest that pancreatic organ size and endocrine cell proliferation could potentially be controlled by the interaction between hedgehog and Fgf signaling during early stages of pancreas formation. Inhibition of Fgf signaling mediated through FGF receptor 1c has been shown to impair mature β-cell function (Hart et al., 2000). Hedgehog signaling is active in adult islets and future studies will address if this activity controls expression of other Fgf ligands within islets and if interactions between these pathways affect mature endocrine functions.

Combined activities of *Hhip* and *Ptch* regulate stomach development

Hedgehog signaling is essential for proper development of other intestinal organs within the pancreas anlage, including stomach and spleen (Harmon et al., 2002; Ramalho-Santos et al., 2000; Sukegawa et al., 2000). The mammalian stomach is patterned along its AP axis and posterior stomach contains mucin-negative vacuoles in columnar epithelium, adjacent to a thick mesenchymal layer, whereas anterior stomach epithelium is normally squamous and nonvacuolated (Larsson et al., 1996). During embryogenesis, *Shh* becomes restricted to the anterior stomach epithelium, while *Ihh* is mainly expressed in the posterior compartment (Aubin et al., 2002; Bitgood and McMahon, 1995; Ramalho-Santos et al., 2000). In addition, *Hhip* and *Ptch* are found within the surrounding stomach mesenchyme (Fig. 1) (Ramalho-Santos et al., 2000), indicating that Hh signaling is active in these tissues. Loss of *Shh* leads to stomach epithelial overgrowth and increased numbers of glucagon producing endocrine cells (Hebrok et al., 2000; Ramalho-Santos et al., 2000). By contrast, reduction of activin signaling or loss of the transcription factor *Hox5a*, disturbs mesenchymal-epithelial patterning of posterior stomach regions, most probably because of an increase in Hh signaling (Aubin et al., 2002; Kim et al., 2000). Our results support this hypothesis as we observe more severe defects in posterior stomach morphology and marker expression in correlation with the progressive loss of *Hhip* and *Ptch* alleles (Fig. 7). Posterior epithelial thickness increases gradually while mesenchymal thickness decreases in response to the additive loss of *Hhip* and *Ptch* alleles. The morphological changes are reflected in the decrease of Isl1 expression in posterior mesenchyme, indicating a possible anterior transformation of this tissue. In addition, *Hhip*$^{-/-}$ mutants display a severe reduction in spleen size (Fig. 2), a phenotype that might be explained by alterations in epithelial-mesenchymal interactions and marker expression in posterior stomach mesenchyme.

In summary, this study demonstrates that *Hhip* and *Ptch* act jointly to control Hh signaling within the embryonic fore-midgut region. One potential implication of these findings is that hypomorphic mutations in Hh inhibitors could affect adult pancreatic functions. Although adult heterozygous *Ptc1*$^{+/-}$ mice display the inability to maintain glucose homeostasis after injection of a concentrated glucose solution (Hebrok et al., 2000), adult *Hhip*$^{+/-}$ mice are indistinguishable from wild-type littermates (data not shown). The different requirement for *Ptch* and *Hhip* function during maintenance of glucose homeostasis is in agreement with the severity of phenotypes in homozygous mutants. While *Hhip*$^{-/-}$ mice complete embryogenesis and only die shortly after birth, *Ptch*$^{-/-}$ embryos die before E10.5 (Goodrich et al., 1997), demonstrating different requirements for *Ptch* and *Hhip* function during embryonic development. Future studies might improve our mechanistic understanding of the different requirements for *Ptch* and *Hhip* in diverse tissues.

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