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Hedgehog signals regulate multiple aspects of gastrointestinal development

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SUMMARY

The gastrointestinal tract develops from the embryonic gut, which is composed of an endodermally derived epithelium surrounded by cells of mesodermal origin. Cell signaling between these two tissue layers appears to play a critical role in coordinating patterning and organogenesis of the gut and its derivatives. We have assessed the function of *Sonic hedgehog* and *Indian hedgehog* genes, which encode members of the Hedgehog family of cell signals. Both are expressed in gut endoderm, whereas target genes are expressed in discrete layers in the mesenchyme. It was unclear whether functional redundancy between the two genes would preclude a genetic analysis of the roles of Hedgehog signaling in the mouse gut. We show here that the mouse gut has both common and separate requirements for *Sonic hedgehog* and *Indian hedgehog*. Both *Sonic hedgehog* and *Indian hedgehog* mutant mice show reduced smooth muscle, gut malrotation and annular pancreas. *Sonic hedgehog* mutants display intestinal transformation of the stomach, duodenal stenosis (obstruction), abnormal innervation of the gut and imperforate anus. *Indian hedgehog* mutants show reduced epithelial stem cell proliferation and differentiation, together with features typical of Hirschsprung's disease (aganglionic colon). These results show that Hedgehog signals are essential for organogenesis of the mammalian gastrointestinal tract and suggest that mutations in members of this signaling pathway may be involved in human gastrointestinal malformations.

Key words: Sonic Hedgehog, Indian Hedgehog, Gut development, Mouse, Patterning, Intestinal stem cells

INTRODUCTION

All metazoan embryos develop an inner gut cavity for the general purpose of nutrient absorption and gas exchange. The gut is lined by an endodermal epithelium and surrounded by mesenchymal cells in triploblastic animals, and has achieved a sophisticated level of patterning and cellular differentiation in vertebrates. Such sophistication includes regionalization of the gut into specialized compartments and development of accessory organs like the liver and the pancreas, that are derived from the endodermal epithelium. The endoderm is induced at gastrulation, and migrates to line the future inner surface of the embryo. Around 8.0 days post coitum (dpc) in the mouse the anterior- and posterior-most endoderm invaginates into the embryo, forming the foregut and hindgut pockets, respectively. These cylindrical pockets extend towards the midgut and eventually fuse, forming the gut tube. It is from the gut tube that throughout the rest of embryogenesis the respiratory and digestive systems arise, including the gastrointestinal tract.

The gastrointestinal tract is a complex organ system composed of esophagus, stomach, small intestine and colon, that performs essential functions of digestion, nutrient absorption and metabolic homeostasis. Three fundamental processes can be defined during embryonic development of this organ system from the primitive gut tube. The first is regionalization of the gut tube, such that distinct regions with differentiated functions are formed along the anterior-posterior axis. The second is radial patterning of the tube, in order to achieve proper placement of epithelium, connective tissue, muscle layers, nerve plexuses, vascular and lymphatic vessels and glands. The third is a continuous self-renewal of the gastrointestinal epithelium from stem cells, which persists into post-natal and adult life. Cell signaling events between the epithelial and mesenchymal layers are generally thought to play a major role in the coordination of these fundamental aspects of gastrointestinal tract development, but the molecular nature of the signaling mechanisms is poorly understood (Duluc et al., 1994; Kedinger et al., 1986; Wells and Melton, 1999; Yasugi, 1993).

The Hedgehog family of cell signals is known to participate in crucial developmental processes in both invertebrate and vertebrate species (Hammerschmidt et al., 1997; Johnston and Scott, 1998). Hedgehog genes have been shown to be expressed in the gut endoderm in all vertebrates examined (Bitgood and McMahon, 1995; Echelard et al., 1993; Ekker et al., 1995; Krauss et al., 1993; Roberts et al., 1995; Stolow and Shi, 1995). In mouse, two members of the Hedgehog family, *Sonic hedgehog* (*Shh*) and *Indian hedgehog* (*Ihh*) are co-expressed in the gut endoderm in partially overlapping patterns from early somite stages (Bitgood and McMahon, 1995; Echelard et al., 1993). Genetic analysis in the mouse has shown

that Hedgehog signaling plays important and diverse roles in morphogenesis of the limb, central nervous system (CNS), somite (Chiang et al., 1996), lung (Litingtung et al., 1998; Pepicelli et al., 1998), hair (St-Jacques et al., 1998), endochondral skeleton (St-Jacques et al., 1999) and testis (Bitgood et al., 1996). However, the possible roles of *Shh* and *Ihh* in gastrointestinal development had not yet been addressed genetically in the mouse.

Although no studies have clearly implicated Hedgehog signaling in human gastrointestinal development some indirect evidence for such a role is available in the literature. *Shh* mutations have been shown to cause holoprosencephaly in both mice (Chiang et al., 1996) and humans (Belloni et al., 1996; Roessler et al., 1996). Three gut abnormalities associated with holoprosencephaly in humans are gut malrotation, esophageal atresia and imperforate anus (Cohen, 1989). In addition, *GLI3* mutations in humans have been shown to cause Pallister-Hall syndrome (Kang et al., 1997) (*Gli* genes being transducers of Hedgehog signaling expressed in the gut – see Fig. 1), and a clinical manifestation of this syndrome is imperforate anus (Iafolla et al., 1989; Kang et al., 1997). Earlier studies in mouse have demonstrated that *Shh* is indeed essential for separation of the esophagus and trachea and branching of the lung (Litingtung et al., 1998; Pepicelli et al., 1998), a foregut derivative. Further, ectopic expression of *Shh* in the chick hindgut is able to alter expression of *Bmp4* and *Hox* genes (Roberts et al., 1998), which encode a signal and putative regional determinants, respectively. In addition, ectopic expression of *Shh* in the pancreatic region of the mouse gut disrupts pancreatic development and directs formation of smooth muscle, a tissue present in intestine but not pancreas (Apelqvist et al., 1997). Together these results suggested an involvement of Hedgehog signaling in vertebrate gastrointestinal development, but direct genetic evidence had been lacking. We have addressed the roles of *Hedgehog* genes in gastrointestinal organogenesis through detailed analysis of the gut phenotypes of *Shh* and *Ihh* mutant mice. Here we show that *Hedgehog* mutant mice have multiple gastrointestinal abnormalities, and that Hedgehog proteins play crucial roles in all three processes mentioned above, namely anterior-posterior patterning, radial patterning and epithelial stem cell proliferation and differentiation.

MATERIALS AND METHODS

Mice

Generation of $Shh^{+/-}$ and $Ihh^{+/-}$ mice has been described (St-Jacques et al., 1998, 1999). Heterozygous mice maintained on a mixed 129/BL6/CBA background were crossed to generate the desired genotypes. The morning of the appearance of vaginal plug was considered as 0.5 dpc. Mutant embryos were readily identified by their external appearance at 18.5 dpc.

In situ hybridization

Gastrointestinal tracts were dissected in ethanol:37% formaldehyde:acetic acid 6:3:1, fixed overnight in the same solution and processed for 6 μm paraffin wax sections using standard histology techniques. An adapted protocol for in situ hybridization using digoxigenin-labeled RNA probes in paraffin wax sections was used. In brief, rehydrated sections were treated with proteinase K (1 μg/ml) for 10 minutes and refixed in 4% paraformaldehyde in PBS for 20 minutes. Sections were washed in 2× SSC for 5 minutes and hybridization was performed overnight at 70°C with 1 μg/ml of digoxigenin-labeled probe in the following hybridization buffer: 50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 1 mg/ml yeast RNA, 200 mM NaCl, 1.1 mM Tris, 8.9 mM Tris-HCl, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 1.25 mM EDTA. Washes were done in 50% formamide, 1× SSC, 0.1% Tween 20 at 65°C, 3× 30 minutes, an then in MABT (100 mM maleic acid, 140 mM NaCl, 1% Tween 20, pH 7.5), 2× 30 minutes. Sections were incubated for 90 minutes in blocking buffer – 20% goat serum, 2% blocking reagent (Boehringer Mannheim) in MABT – and left overnight at room temperature in alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) at 1:2000 dilution in blocking buffer. Washes were done in MABT, 5× 30 minutes. Sections were then washed for 5 minutes in NTMT (100 mM NaCl, 100 mM Tris, 50 mM $MgCl_2$, 1% Tween 20, pH 9.5) and color reaction was performed in the dark in 4.5 μl/ml NBT (nitro blue tetrazolium), 3.5 μl/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate), 1 mM levamisole in NTMT.

Histochemistry

Wild-type and mutant gastrointestinal tracts were dissected in PBS, fixed overnight in 4% paraformaldehyde in PBS and processed for 6 μm paraffin wax sections. Hematoxylin and Eosin staining was performed using standard histology techniques. Primary antibodies used were against smooth muscle actin (Sigma, 1:2000 dilution), neural-specific β-tubulin (Sigma, 1:100), PCNA (S. Cruz, 1:100), Tcf4 (H. Clevers, University Hospital, Utrecht, 1:10), CCK (Peninsula, 1:800) and Pdx1 (C. Wright, Vanderbilt University, 1:1000). Signal amplification and detection was performed using a Cyanine-3 Tyramide Signal Amplification Kit (NEN) according to the manufacturer's instructions. Sections were counterstained to identify nuclei with Yo-pro-1 or To-pro-3 (Molecular Probes). Confocal microscopy was performed on a Zeiss LSM 410 microscope equipped with a krypton/argon laser. Alkaline phosphatase was detected by incubating rehydrated sections in BM Purple (Boehringer Mannheim) for 10 minutes. FITC-labeled *Wisteria floribunda* agglutinin (WFA) lectin (Sigma) was used according to the manufacturer's instructions.

Quantitative data

Smooth muscle was measured and PCNA/CCK-positive cells were counted on digital images of ≥5 sections of each of ≥3 embryos of each genotype captured through a cooled CCD camera connected to a Leitz epifluorescence microscope. Bar graphs of mean±standard error of the mean were plotted using Cricket Graph. All statistical data was found to be significant at $P<0.05$, as assessed by the Student's *t*-test.

RESULTS

Members of the Hedgehog pathway are expressed at 18.5 dpc in the mouse gut

The expression of *Shh* and *Ihh* in overlapping patterns in the mouse gut endoderm has been reported (Bitgood and McMahon, 1995; Echelard et al., 1993). Both genes are expressed in the gut endoderm from early somite stages (8.5 dpc), initially in two ventrolateral stripes (Echelard et al., 1993) (and data not shown). Expression is first detected in the caudal hindgut, then in the foregut pocket, and extends towards the midgut during gut closure. Broad expression persists in the gut tube, with the exception of the pancreatic buds (Apelqvist et al., 1997), until 10.5 dpc. Between 11.5 and 14.5 dpc *Shh* expression is downregulated in the hindstomach, jejunum and ileum, whereas *Ihh* is expressed from the hindstomach to the anus (Bitgood and McMahon, 1995). However, these previous

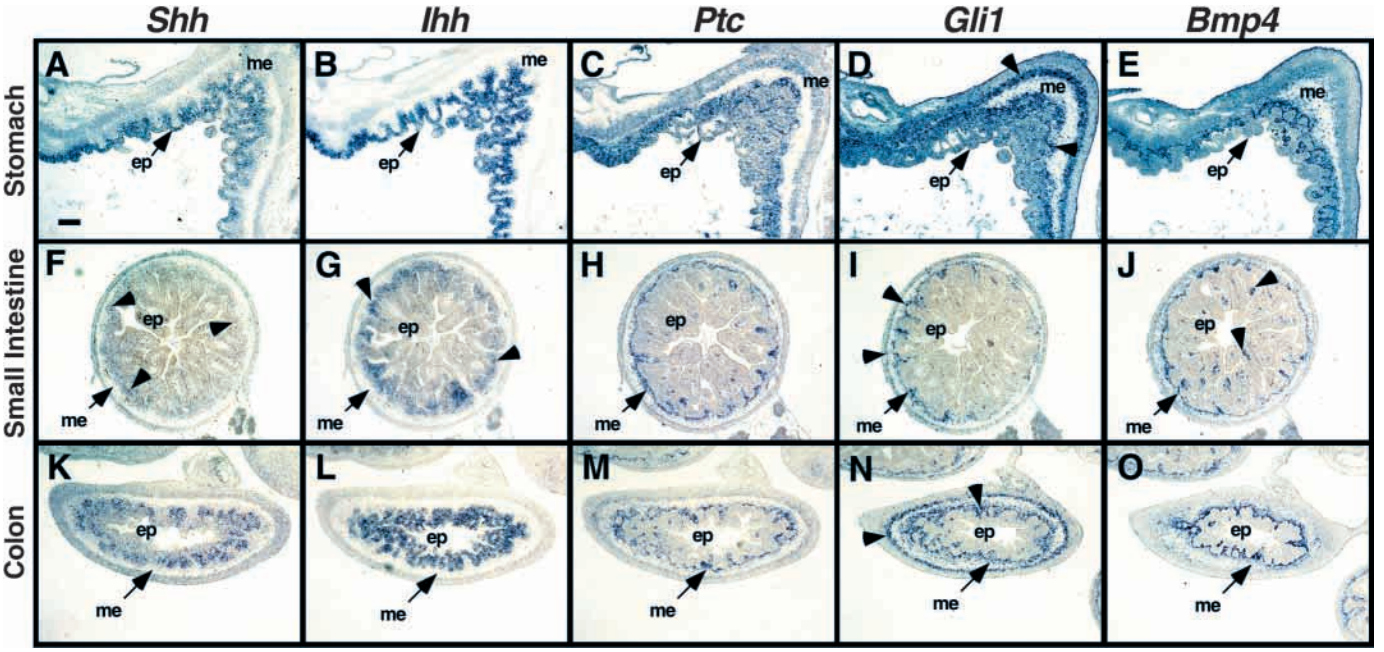

Fig. 1. Expression of components of the Hedgehog pathway in the gastrointestinal tract at 18.5 dpc. All panels show in situ hybridizations in sections of the 18.5 dpc gastrointestinal tract, namely glandular stomach (A-E), small intestine (F-J) and colon (K-O). Expression patterns of *Shh* (A,F,K), *Ihh* (B,G,L), *Ptc* (C,H,M), *Gli1* (D,I,N) and *Bmp4* (E,J,O) are shown. Arrowheads point to staining in particularly relevant cases (see text). ep, epithelium; me, mesenchyme. 100 μm for all panels.

studies did not address expression of the genes beyond 14.5 dpc. Major events of organogenesis and cytodifferentiation of the gastrointestinal tract occur after this stage, in preparation for birth (which occurs at about 19.5 dpc).

To address Hedgehog signaling after intestinal villi formation and epithelial differentiation we characterized the expression of members of this signaling pathway at 18.5 days post coitum (dpc) in the mouse by in situ hybridization. Fig. 1 shows the expression patterns of transcripts encoding the signals (*Shh* and *Ihh*), their receptor (*Ptc*), the transcriptional effector (*Gli1*) and a putative target (*Bmp4*). No expression was detected in the esophagus except at its very distal portion (data not shown) in patterns similar to the ones described below. *Shh* and *Ihh* are expressed in the epithelium of the glandular stomach (Fig. 1A,B), the small intestine (Fig. 1F,G) and the colon (Fig. 1K,L). Interestingly, in the small intestine both *Shh* (at a very low level) and *Ihh* are expressed at the base of villi (arrowheads in Fig. 1F,G), where epithelial stem cells are thought to reside (Klein, 1989, and see below). *Ihh* is expressed throughout the epithelium in the colon (Fig. 1L), whereas *Shh* is detected mostly in the crypts (Fig. 1K). *Ptc*, *Gli1* and *Bmp4*, which are presumed targets, are expressed in the mesenchyme throughout the gastrointestinal tract in two positions: just under the epithelium (in the submucosa) and within the muscle layers (best seen in the '*Gli1*' panels, arrowheads in Fig. 1D,I,N). *Ptc2* and *Gli2* are expressed at very low levels in patterns similar to those of *Ptc* and *Gli1*, respectively (data not shown). *Bmp4* is also expressed in the mesenchyme at the tips of the villi in the small intestine (arrowheads in Fig. 1J). This may be of particular relevance because epithelial cells, generated in the stem cell compartment, migrate up the villus and are eliminated at the tip by cell death (Klein, 1989; Stappenbeck et al., 1998), and *Bmp4* has been shown to induce apoptosis in both the limb (Zou and Niswander, 1996) and dorsal neural tube (Graham et al., 1994). Our results show that members of the Hedgehog pathway are expressed in the gastrointestinal tract at least until birth. It is likely that expression is maintained throughout life, since *Shh*, *Ptc* and *Ptc2* have been detected by RT-PCR in adult stomach and intestine (Motoyama et al., 1998).

Shh and *Ihh* mutant mice have multiple gastrointestinal defects

To assess functions of *Hedgehog* genes in gastrointestinal development, we analyzed and characterized gastrointestinal phenotypes in *Shh* and *Ihh* mutant mice, which had previously been generated (St-Jacques et al., 1998, 1999). *Shh* and *Ihh* mutants die at or shortly after birth, so we focussed our analysis

Table 1. Incidence of defects with similarities to human gut abnormalities in Hedgehog mutant mouse embryos

	Wild type	*Shh*$^{-/-}$	*Ihh*$^{-/-}$
Overt gut malrotation	0 (0/41)	100 (9/9)	100 (10/10)
Intestinal transformation of the stomach	0 (0/13)	100 (9/9)	0 (0/7)
Annular pancreas	0 (0/41)	85 (6/7)	43 (3/7)
Duodenal stenosis*	23 (3/13)	67 (6/9)	0 (0/7)
Hirschsprung's disease (aganglionic colon)	0 (0/8)	0 (0/9)	50 (5/10)
Imperforate anus	0 (0/41)	100 (9/9)	0 (0/7)

Note: values expressed as percentages, number of embryos in brackets. *In wild-type embryos a temporary occlusion of the small intestine by villi can be seen (here scored in 23% of the embryos), and is followed by recanalization just prior to birth. Such occlusion, present in some cases of duodenal stenosis (Riddlesberger, 1989), is significantly increased in *Shh*$^{-/-}$ (67%). All differences significant at $P<0.05$, as assessed by the Student's *t*-test.

at 18.5 dpc, one day prior to birth in the mouse. Our studies have concentrated on single mutants. Double mutants arrest at early somite stages (M. R.-S., D. A. M., A. P. M., unpublished observations) and are therefore not informative for these studies. Reducing the activity of either gene in the complete absence of activity of the other (i.e., in $Shh^{-/-}$;$Ihh^{+/-}$ or $Shh^{+/-}$;$Ihh^{-/-}$) does not appear to enhance single mutant phenotypes (data not shown).

Shh and *Ihh* mutant embryos have both common and distinct gastrointestinal defects (Table 1 and Fig. 2), indicating that despite an overlap in their expression patterns they are not playing completely redundant roles in gut development. The gastrointestinal tract of the mutants is smaller than wild-type, a reduction that correlates with the overall smaller size of the embryos. In common, the mutants display an overt malrotation of the gut, although no reversions in gut situs were detected, and annular pancreas with different penetrances (the pancreatic phenotypes of the mutants will be described elsewhere, M. Hebrok, S. Kim, B. St-Jacques, A. P. M., D. A. M.). Fig. 2 summarizes the histological defects of the mutants. *Shh* mutants display an overgrown stomach epithelium (Fig. 2E,H, see below) and occlusion of the duodenum by overgrown villi (Fig. 2K), reminiscent of duodenal stenosis (Gray and Skandalakis, 1972; Riddlesberger, 1989). *Shh* mutants also have imperforate anus, readily apparent upon dissection: the colon terminates in a blind dilation that is not fused to the surface ectoderm (inset in Fig. 2B). Particularly striking in *Ihh* mutants are the dilation of the small intestine and portions of the colon (Fig. 2L,O), and the reduction in size of the villi (Fig. 2L). The dilated colon with an abnormally thin wall observed in $Ihh^{-/-}$ (Fig. 2C,O) is reminiscent of Hirschsprung's disease (Gray and Skandalakis, 1972; Riddlesberger, 1989) (see below). Table 1 lists the incidence of defects in *Hedgehog* mutants with similarities to several important human gut abnormalities.

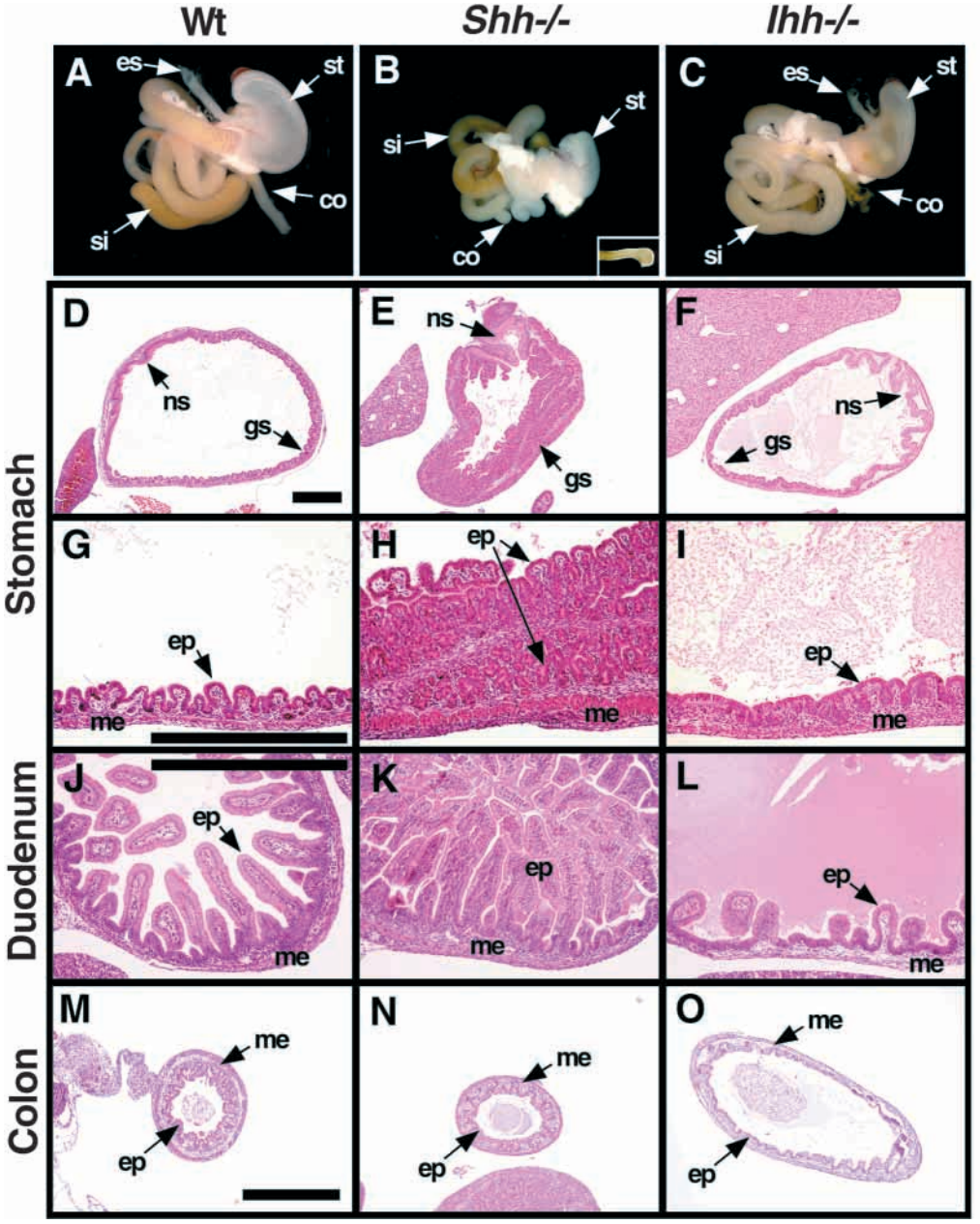

Fig. 2. Multiple gastrointestinal defects in *Hedgehog* mutants. (A-C) Ventral views of the 18.5 dpc gastrointestinal tract of wild-type (A), $Shh^{-/-}$ (B) and $Ihh^{-/-}$ (C) mice. The liver has been dissected away to reveal looping of the intestines. In $Shh^{-/-}$ (B), esophagus tissue is much reduced and fused to the trachea, and is not shown here. Inset in B shows the imperforate anus of $Shh^{-/-}$, hidden dorsally in this specimen. (D-O) Hematoxylin and Eosin stained histological sections of 18.5 dpc stomach (D-F, higher magnifications in G-I), duodenum (J-L) and colon (M-O) of wild-type (D,G,J,M), $Shh^{-/-}$ (E,H,K,N) and $Ihh^{-/-}$ (F,I,L,O). co, colon; ep, epithelium; es, esophagus; gs, glandular stomach; me, mesenchyme; ns, non-glandular stomach; si, small intestine; st, stomach. Scale bar, 0.5 mm.

Hedgehog mutants display smooth muscle defects

The expression of Hedgehog signal effectors and transcriptional targets in the mesenchyme (Fig. 1) suggests that mesenchymal cells are the primary target of epithelially derived Shh and Ihh. Consequently, we first characterized in detail mesenchymal defects in the mutants. The smooth muscle of the intestine is responsible for contractile movements during digestion, and is organized into two layers: an inner, circular

layer and an outer, longitudinal layer, both detected by the accumulation of smooth muscle α-actin (Fig. 3A). In the wild type at 18.5 dpc the circular layer comprises most of the smooth muscle of the gut (Fig. 3A). Both mutants show a significant reduction in thickness of the circular smooth muscle layer along the small intestine (Fig. 3A,C, quantified in the bar graph). In comparison to wild type (Fig. 3A), *Shh* mutants (Fig. 3B) show a reduction of 21%, and *Ihh* mutants (Fig. 3C) show a reduction of 34%. It is likely that the higher reduction in smooth muscle thickness in $Ihh^{-/-}$ is responsible for the dilation of the intestine observed only in these mutants and not in $Shh^{-/-}$ (see Fig. 2).

Hedgehog mutants have distinct enteric nervous system defects

The enteric nervous system (ENS) is essential for coordination of motility and glandular secretion in the gastrointestinal tract. It is composed of two main plexuses of ganglia: the muscular plexus and the submucosal plexus. The plexuses can be identified by the accumulation of neural-specific β-tubulin (Fig. 3D). Both of these two areas of neuronal differentiation in the gut may be targets of Hedgehog signaling (Fig. 1). We addressed the role of Hedgehog signaling in ENS development by staining for neural-specific β-tubulin in the mutants. Patterns of staining similar to those of neural-specific β-tubulin described below were observed for c-Ret, p75 and Neurofilament, other markers of enteric ganglia (data not shown). *Shh* mutants have a substantial number of neurons differentiating abnormally under the epithelium and into the villi (arrowheads in Fig. 3E, compare to wild-type in Fig. 3D). This phenotype is observed consistently throughout the small intestine, regardless of the neural marker used.

All the more surprising was the observation that *Ihh* mutants often lack neurons, which are completely absent along portions of the small intestine (Fig. 3F) and in the dilated regions of the colon (Fig. 3I), a phenotype very similar to Hirschsprung's disease (Gray and Skandalakis, 1972; Riddlesberger, 1989).

Shh mutants display intestinal transformation of the stomach

We also detected epithelial defects in the mutants, which are presumably due to an indirect effect on mesenchyme-to-epithelium signaling. *Shh* mutants (but not *Ihh* mutants) show an impressive overgrowth of the stomach epithelium (Fig. 2D,E,G,H), although normal rates of cell proliferation, as assessed by PCNA staining, were detected in the stomach at

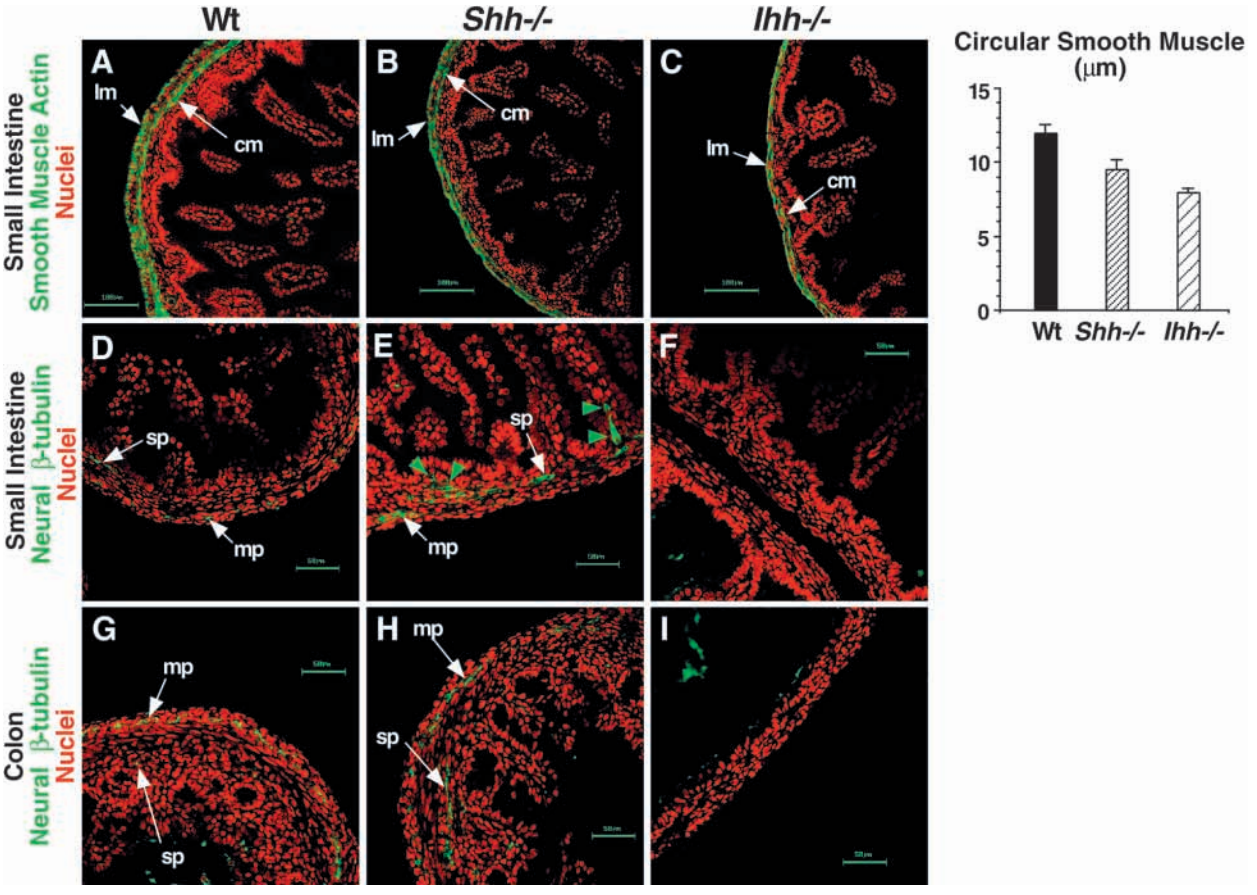

Fig. 3. Defective muscle and enteric nervous system development in *Hedgehog* mutants, including Hirschsprung's disease in $Ihh^{-/-}$. Immunohistochemistry using anti-smooth muscle α-actin (A-C) or anti-neural specific β-tubulin (D-I) antibodies (green) and nuclear counterstaining (red). Transverse sections of small intestine (A-F) and colon (G-I) of wild-type (A,D,G), $Shh^{-/-}$ (B,E,H) and $Ihh^{-/-}$ (C,F,I) are shown. Arrowheads in E point to excessive and abnormally located neurons in $Shh^{-/-}$. The bar graph plots the thickness of the circular smooth muscle layer in wild-type, $Shh^{-/-}$ and $Ihh^{-/-}$. Values, expressed in μm as mean±s.e.m., are: 12.0±0.6 for wild-type, 9.5±0.7 for $Shh^{-/-}$ (21% reduction) and 7.9±0.3 for $Ihh^{-/-}$ (34% reduction). cm, circular muscle; lm, longitudinal muscle; mp, muscular plexus; sp, submucosal plexus. Scale bars, 100 μm (A-C) and 50 μm (D-I).

18.5 dpc (data not shown). The epithelium seems to be correctly patterned into the glandular and non-glandular regions typical of the stomach (Fig. 2D,E). However, the histological appearance of the glandular epithelium is reminiscent of intestinal transformation (metaplasia), very frequently associated with gastric ulcer and cancer in humans (Slack, 1985; Stemmermann and Hayashi, 1968). One marker commonly used in clinical diagnosis of intestinal transformation of the stomach epithelium is alkaline phosphatase (Slack, 1985; Stemmermann and Hayashi, 1968), which is present in the intestine but absent in the stomach (Fig. 4A,C). Alkaline phosphatase activity is readily detected in both the intestine and in patches of the stomach of *Shh* mutants (Fig. 4B, arrowheads in D). Fig. 4D also illustrates the fact that even portions of the stomach epithelium not substantially overgrown display a transformed character. In addition, staining with WFA lectin, a marker of the brush border of enterocytes in the intestine (Falk et al., 1994) (Fig. 4E,F), is also indicative of an intestinal transformation of the stomach epithelium of *Shh* mutants (Fig. 4G,H).

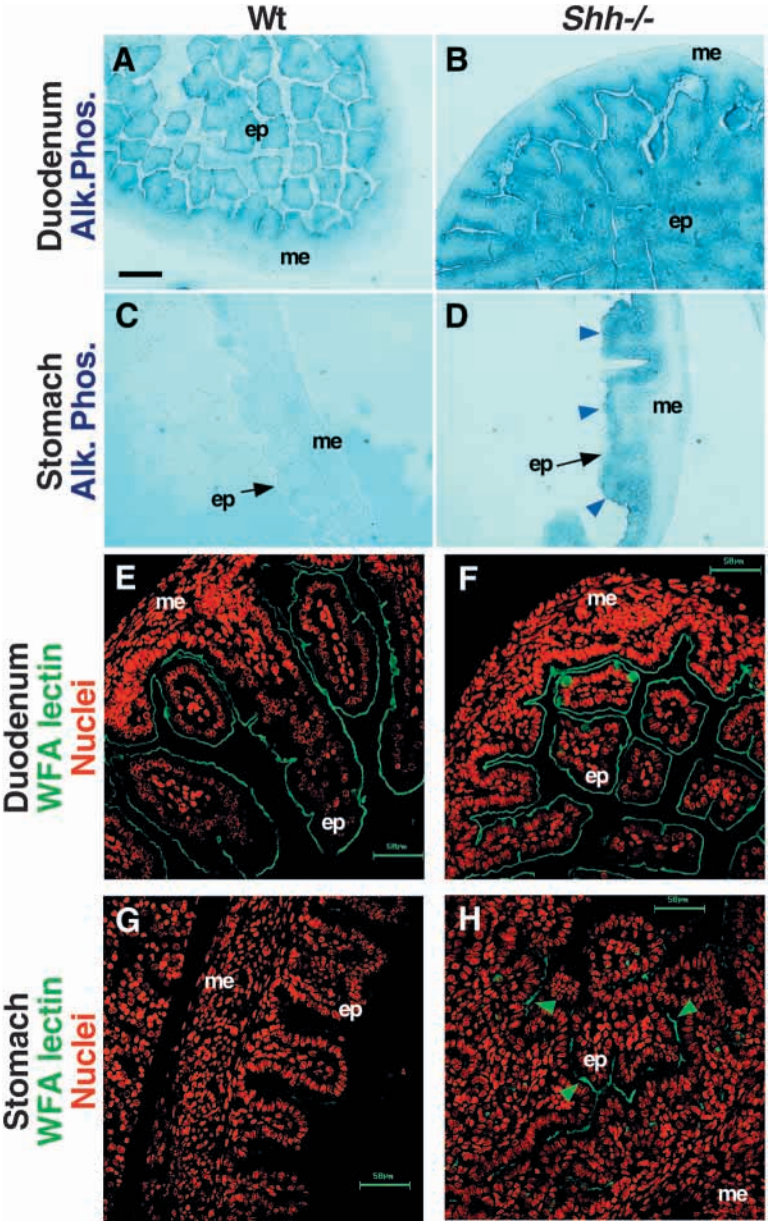

Fig. 4. Partial intestinal transformation of the stomach in *Shh* mutants. (A-D) Alkaline phosphatase staining in sections of the duodenum (A,B) and glandular stomach (C,D) of wild-type (A,C) and $Shh^{-/-}$ (B,D). (E-H) FITC-WFA lectin staining (green) with nuclear counterstaining (red) in sections of the duodenum (E,F) and glandular stomach (G,H) of wild-type (E,G) and $Shh^{-/-}$ (F,H). Arrowheads point to ectopic staining for alkaline phosphatase (D) and WFA lectin (H) in $Shh^{-/-}$ stomach. Scale bars, 100 μm (A-D) and 50 μm (E-H). ep, epithelium; me, mesenchyme.

Ihh mutants have defective stem cell proliferation and differentiation

In the small intestine the epithelium is arranged into villi, finger-like projections that maximize the absorption surface. Epithelial cells are continuously generated in a stem cell compartment located between and at the base of the villi at 18.5 dpc in the mouse (intervilli region) (Fig. 5A), which after birth resolves to depressions called crypts (Klein, 1989). The topic of intestinal stem cell proliferation has been the object of intense research for several decades, but to date very little is known about signaling molecules involved (Klein, 1989; Stappenbeck et al., 1998). *Ihh* is expressed in the intervilli region of the small intestine (Fig. 1G), and in $Ihh^{-/-}$ (but not $Shh^{-/-}$) mice there is a substantial reduction in size of the villi (Fig. 2L). These two results suggested to us that *Ihh* might be involved in regulating stem cell proliferation. Indeed, there is a 54% reduction in cell proliferation in the stem cell compartment of $Ihh^{-/-}$, as assessed by PCNA staining (arrowheads in Fig. 5A,B, quantified in the bar graph). Such a phenotype is similar to that of mice lacking Tcf4 (Korinek et al., 1998), an HMG transcription factor thought to be a downstream effector of Wnt signaling. However, we found that in $Ihh^{-/-}$ Tcf4 is still properly localized to the nucleus (arrowheads in Fig. 5D), indicating that the proliferation defect is not due to a loss of Tcf4.

Ihh mutants also show a reduction in the numbers of endocrine cells of the duodenum, such as cholecystokinin (CCK)-producing cells, which are reduced by 45% (arrowheads in Fig. 5E,F, quantified in the bar graph). A very similar reduction in endocrine cells is observed in the duodenum of mice lacking Pdx1 (Offield et al., 1996), a homeodomain transcription factor required for pancreatic (Jonsson et al., 1994; Offield et al., 1996) and proper duodenal development (Offield et al., 1996). Pdx1 protein, detected in the epithelium of wild-type embryos (arrowheads in Fig. 5G), is completely absent from the duodenum of $Ihh^{-/-}$ (Fig. 5H), but present in the pancreas (data not shown).

DISCUSSION

We have addressed the roles of Hedgehog signals in development of the mammalian gastrointestinal tract. *Shh* and *Ihh* homozygous mutant mice display multiple gut abnormalities, and our study confirms and

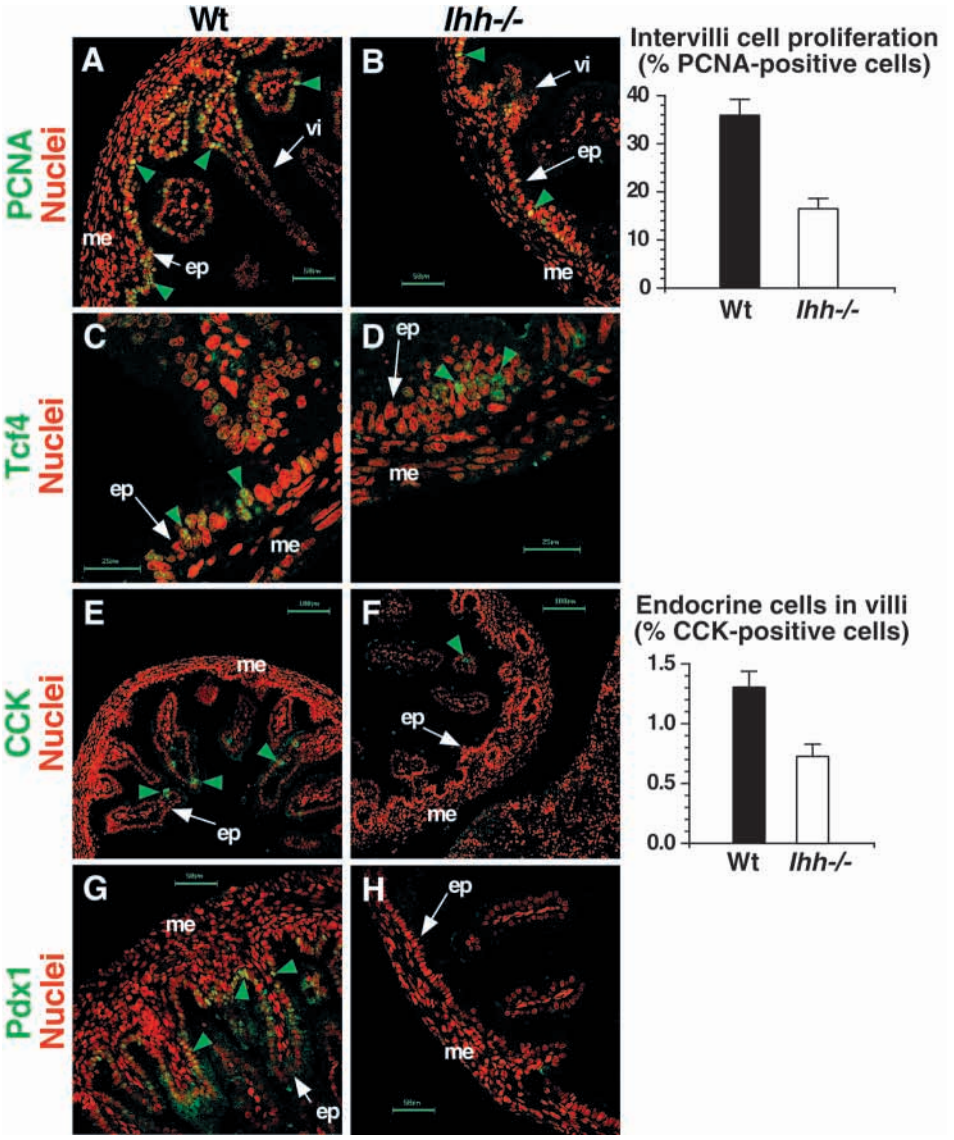

Fig. 5. Reduced epithelial stem cell proliferation and differentiation in *Ihh* mutants. Immunohistochemistry using anti-PCNA (A,B), anti-Tcf4 (C,D), anti-CCK (E,F) and anti-Pdx1 (G,H) antibodies (green) and nuclear counterstaining (red) in sections of the duodenum of wild-type (A,C,E,G) and $Ihh^{-/-}$ (B,D,F,H). Arrowheads point to antibody staining. The top bar graph plots intervilli cell proliferation in wild-type and $Ihh^{-/-}$. Values for percentage of PCNA-positive cells in the intervilli region are (mean±s.e.m.): 35.8±3.6 for wild-type and 16.4±2.1 for $Ihh^{-/-}$ (54% reduction). The bottom bar graph plots endocrine cells in villi in wild-type and $Ihh^{-/-}$. Values for percentage of CCK-positive cells in the villi are (mean±s.e.m.): 1.30±0.14 for wild-type and 0.72±0.11 for $Ihh^{-/-}$ (45% reduction). No significant differences from wild-type were detected in $Shh^{-/-}$ both for PCNA and CCK (not shown). ep, epithelium; me, mesenchyme; vi, villus. Scale bars, 50 μm (A,B,G,H), 25 μm (C,D) and 100 μm (E,F).

extends previous evidence suggestive of an involvement of *Hedgehog* mutations in some human gastrointestinal malformations. Detailed analyses of the mutants indicate that these genes are essential for several aspects of gut patterning and organogenesis.

Multiple gastrointestinal defects in *Hedgehog* mutant mice

We have described several defects with similarities to human gut abnormalities in *Hedgehog* mutants. Mutations in genes of the Hedgehog pathway in humans have been described and shown to cause holoprosencephaly, basal cell carcinoma, Greig syndrome, Pallister-Hall syndrome, and isolated postaxial polydactyly (Ming et al., 1998). Three gut abnormalities associated with holoprosencephaly in humans are gut malrotation, esophageal atresia and imperforate anus (Cohen, 1989), and we have described these exact features in *Shh* mutant mice. In addition, a clinical manifestation of Pallister-Hall syndrome, known to be caused by *GLI3* mutations, is imperforate anus (Iafolla et al., 1989; Kang et al., 1997). Curiously, in humans there is a rare association of tracheo-esophageal fistula (a previously described phenotype of *Shh* mutants; Litingtung et al., 1998; Pepicelli et al., 1998), duodenal stenosis or atresia and imperforate anus (described here), generally referred to as the VACTERL (Vertebral, Anal, Cardiovascular, Tracheo-esophageal, Radial and Limb) syndrome (Piekarski and Stephens, 1976). There is also a low association of annular pancreas and Hirschsprung's disease, two features of the phenotype of *Ihh* mutants, with trisomy 21 (Riddlesberger Jr., 1989). Our study shows that *Hedgehog* mutant mice have similarities to important human gut malformations and conditions, including intestinal transformation of the stomach, duodenal stenosis, gut malrotation, annular pancreas, Hirschsprung's disease and imperforate anus. We anticipate that lesions in *Hedgehog* genes or genes of the Hedgehog pathway may be implicated in forms of these conditions in humans.

Hedgehogs in smooth muscle development

Smooth muscle is significantly reduced in both *Shh* and *Ihh* mutants. We speculate that this reduction in smooth muscle may be related to the overt gut malrotation observed in the mutants. Most rotation of the gut occurs after initiation of smooth muscle development at 12.5 dpc (Takahashi et al., 1998), a time when essentially all the epithelium expresses *Shh* and/or *Ihh* (Bitgood and McMahon, 1995). *Shh* had been previously shown to be required for smooth muscle development in the lung (Pepicelli et al., 1998). In addition, misexpression of *Shh* in the pancreatic domain results in the formation of ectopic smooth muscle (Apelqvist et al., 1997). In agreement with these findings, our results show that *Shh* and *Ihh* regulate gut smooth muscle

development, possibly in a partially redundant way. Further studies should address whether complete blocking of Hedgehog signaling impairs smooth muscle development in the gut.

Shh and patterning of the enteric nervous system

Our results on the abnormal patterning of the ENS in *Shh* mutants are indicative of an inhibitory role for *Shh* in neural migration and/or differentiation in the gut, and are in agreement with recombination experiments in chick embryos. When the gut epithelium or *Shh*-expressing cells are grafted in the mesenchyme, neural differentiation is inhibited in the proximity (Sukegawa et al., 2000). Taken together these results suggest that *Shh* is essential for proper radial patterning of the ENS. In the neural tube, Shh appears to induce different ventral cell identities at distinct concentration thresholds (Marti et al., 1995; Roelink et al., 1995; Tanabe et al., 1995). As in the neural tube, it is possible that Shh acts in the radial axis of the gut as a morphogen, inducing different cell fates at different concentrations or distances from the source.

Ihh and development of the enteric nervous system

We have shown that Ihh is required for neural development in the gut. The fact that some neurons can be detected in a proper pattern in patches of the small intestine and non-dilated regions of the colon of *Ihh* mutants (data not shown) suggests to us that neural crest cells are able to migrate into the gut and differentiate, but locally fail to survive or proliferate in the absence of *Ihh*. Dilation of the colon and an associated lack of ganglia are typical of Hirschsprung's disease (or aganglionic megacolon), a major congenital birth defect in humans (1 in 5,000 births) (Gray and Skandalakis, 1972; Riddlesberger, 1989). Genes that have to date been implicated in regulation of enteric nervous system development, in mice and/or in humans, include members of the Ret pathway, endothelin pathway, *Sox10* and *Mash1* (Taraviras and Pachnis, 1999). Curiously, when human *GLI* gene was overexpressed in transgenic mice, a Hirschsprung's-like phenotype was observed (Yang et al., 1997), suggesting a role for the Hedgehog pathway in ENS development. The colon phenotype of $Ihh^{-/-}$ was observed with an incomplete penetrance of 50%, which suggests that interaction with other factors may be required for full expression of the phenotype. Nevertheless, our data show that *Ihh* is essential for development of the ENS.

It is possible that Hedgehog signals play several roles throughout ENS development. In this respect it is noteworthy that our expression data suggest that both enteric plexuses may still be targets of Hedgehog signaling at 18.5 dpc (Fig. 1). The intriguing observation that inactivation of two very similar signaling proteins, *Shh* and *Ihh* (with 90% amino acid identity over the signaling N-terminal peptide), results in opposite phenotypes raises the heretofore unappreciated possibility that they may have some distinct signaling properties. Alternatively, spatial differences in expression domains or, where both signals are co-expressed, the unequal contribution of either signal may account for these observations. We cannot at present distinguish between these possibilities.

Shh and patterning of the stomach epithelium

Our results indicate that the stomach epithelium of *Shh* mutants is at least partially transformed towards an intestinal character. At present it is unclear how the embryonic gut tube is regionalized at the molecular level. It is likely to rely on a complex interplay between *Hox* genes, *Parahox* genes (such as *Pdx1* and *Cdx*) and other transcription factors like members of the *Nkx* and *Sox* families (Wells and Melton, 1999). As mentioned above, *Shh* has been suggested to be involved in chick hindgut patterning (Roberts et al., 1998). Misexpression of *Shh* leads to ectopic expression of *Hoxd13* in the chick hindgut, but not in either the foregut or the midgut (Roberts et al., 1998). The results presented here are also consistent with a role for *Shh* in patterning of the stomach epithelium. It is likely that expression of *Shh* in the stomach is part of a regulatory network that induces and/or maintains the character of the epithelium as opposed to an intestinal character. Based on our evidence one is tempted to speculate that the intestinal character is developmentally a default state. This would be in agreement with the idea that the chordate intestine is an older structure from which the stomach later arose by regionalization (Coates and Cohn, 1998).

Our preliminary observations indicate that the pattern alteration in *Shh* mutants does not correlate with changes in expression of some genes implicated in regulating anterior/posterior patterning in the gut, including *Hoxd3*, *Hoxd4*, *Hoxd13* and *Cdx2* (data not shown). We have studied the expression of such putative patterning genes at 12.5 dpc, since it is a stage at which molecular boundaries have been shown to correlate with morphological landmarks in the gut (Sekimoto et al., 1998). It may be that the transformation of the stomach epithelium observed in *Shh* mutants does not depend on alterations in the expression of these genes, or that those alterations occur at a different stage. Further studies should elucidate the specific action of *Shh* in regional patterning of the gut, and assess whether perturbation of the Hedgehog pathway is involved in intestinal transformation and/or gastric ulcer and cancer in humans.

Ihh and intestinal stem cell proliferation and differentiation

We have shown that *Ihh* is required for intestinal stem cell proliferation. The Wnt pathway of cell signaling has equally been implicated in this process (Korinek et al., 1998). It is possible that, as in other systems (Tabata and Kornberg, 1994), a Hedgehog signal (Ihh) induces expression of as yet unknown *Wnt(s)*, which in turn promote cell proliferation. Further studies on the interplay between signaling pathways in the intestine should clarify these issues. In addition, our results also suggest that *Ihh* positively regulates *Pdx1* in duodenal endocrine cell differentiation. Interestingly, this finding is in striking contrast to early pancreas development, where Hedgehog signals negatively regulate *Pdx1* (Hebrok et al., 1998). The duodenum and the early pancreas thus interpret Hedgehog signaling in an opposite way, presumably reflecting distinct molecular complements of the responding cells.

The requirement for *Ihh* in intestinal cell proliferation and differentiation is likely to occur late in organogenesis, since proper villi formation and cytodifferentiation occur on or after 17.5 dpc. The expression of *Ihh* in the stem cell compartment at 18.5 dpc is consistent with this idea. We speculate that this role of *Ihh* may persist into postnatal life. Interestingly, a recent

study shows that signaling through the Rac1 GTPase, which is present in differentiated epithelial cells of the intestinal villi, acts to promote and/or maintain the differentiated state, without affecting proliferation (Stappenbeck and Gordon, 2000). We expect that the integration of signaling networks that regulate the precise balance between proliferation and differentiation in the intestinal epithelium will become increasingly clear in the coming years.

An ancestral role for *Hedgehogs* in the gut?

The results presented here show that *Hedgehog* genes coordinate several essential aspects of gastrointestinal development. Current models of gut tube patterning, muscle and ENS development and intestinal stem cell proliferation and differentiation must therefore take into account the roles of Shh and Ihh signals. Our study further underscores how the Hedgehog signaling pathway has been used in a wide variety of developmental processes during evolution. We propose that the expression and function of *Hedgehogs* in the gut endoderm is an ancestral character of chordates, based on two lines of evidence: first, the expression of *Hedgehogs* in the gut endoderm is conserved in all species studied, including in amphioxus, where *AmphiHh* is expressed in notochord, floor plate and gut endoderm (Shimeld, 1999). Second, the co-expression in the gut endoderm of two members of the *Hedgehog* family, *Shh* and *Ihh*, which likely arose by gene (or genome) duplication, may reflect a role of the ancestral gene (Holland, 1999). It is possible that this ancestral role in gut development extends back even further, as *Hh* has been shown to be expressed in the ectodermally derived epithelium of the *Drosophila* foregut and hindgut (Hoch and Pankratz, 1996; Pankratz and Hoch, 1995). Studies of the expression and function of Hedgehogs in more invertebrate animals, including ascidians (primitive chordates), acoel flatworms (primitive bilaterians) or cnidarians (diploblasts), should help clarify the evolution of the Hedgehog pathway in Metazoans.

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