Developmental Biology **229,** 188–202 (2001)
doi:10.1006/dbio.2000.9953, available online at http://www.idealibrary.com on IDEAL®

Downregulation of Hedgehog Signaling Is Required for Organogenesis of the Small Intestine in *Xenopus*

Jian Zhang,* Arnon Rosenthal,* Frederic J. de Sauvage,* and Ramesh A. Shivdasani†

**Department of Molecular Oncology, Genentech, Inc., South San Francisco, California 94080; and †Department of Adult Oncology and Department of Cancer Biology, Dana-Farber Cancer Institute, and Department of Medicine, Brigham & Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115*

Hedgehog ligands interact with receptor complexes containing Patched (PTC) and Smoothened (SMO) proteins to regulate many aspects of development. The mutation W535L (SmoM2) in human *Smo* is associated with basal cell skin cancers, causes constitutive, ligand-independent signaling through the Hedgehog pathway, and provides a powerful means to test effects of unregulated Hedgehog signaling. Expression of SmoM2 in *Xenopus* embryos leads to developmental anomalies that are consistent with known requirements for regulated Hedgehog signaling in the eye and pancreas. Additionally, it results in failure of midgut epithelial cytodifferentiation and of the intestine to lengthen and coil. The midgut mesenchyme shows increased cell numbers and attenuated expression of the differentiation marker smooth muscle actin. With the exception of the pancreas, differentiation of foregut and hindgut derivatives is unaffected. The intestinal epithelial abnormalities are reproduced in embryos or organ explants treated directly with active recombinant hedgehog protein. *Ptc* mRNA, a principal target of Hedgehog signaling, is maximally expressed at stages corresponding to the onset of the intestinal defects. In advanced embryos expressing SmoM2, *Ptc* expression is remarkably confined to the intestinal wall. Considered together, these findings suggest that the splanchnic mesoderm responds to endodermal Hedgehog signals by inhibiting the transition of midgut endoderm into intestinal epithelium and that attenuation of this feedback is required for normal development of the vertebrate intestine.

Key Words: **Hedgehog; intestine development; organogenesis; Smoothened; SmoM2; gut endoderm.**

INTRODUCTION

The extracellular signaling molecule Hedgehog (Hh) was originally identified as the product of a segment polarity gene in *Drosophila* (Nusslein-Volhard and Wieschaus, 1980), and its several vertebrate homologues are involved in a variety of patterning processes during development. Mammalian homologues include Indian hedgehog (Ihh), which regulates chondrocyte (Vortkamp *et al.,* 1996) and peripheral nerve (Parmantier *et al.,* 1999) development, and Desert hedgehog, whose activity is restricted to germ cell development (Bitgood *et al.,* 1996). The best characterized to date, Sonic hedgehog (Shh), is expressed in various organizing centers, plays a key role in dorsoventral neural patterning (Echelard *et al.,* 1993; Roelink *et al.,* 1995), and regulates anteroposterior patterning of the developing limb (Riddle *et al.,* 1993). Additional Hh proteins identified in *Xenopus* display partially overlapping expression patterns and have less well characterized functions, although it is notable that their mRNA levels peak during neural induction and early organogenesis (Ekker *et al.,* 1995).

Additional recognized functions of Hh proteins include mediating epithelial–mesenchymal interactions that are required for late development of the aerodigestive tract. Shh is expressed in the epithelium of the developing mouse lung, while its receptor, Patched, is expressed in the adjacent mesenchyme, and ectopic expression of Shh provides evidence that it regulates lung mesenchymal cell proliferation *in vivo* (Bellusci *et al.,* 1997; Pepicelli *et al.,* 1998). Shh may also act at an earlier stage of pulmonary development, as formation of lung buds is severely attenuated in Shh-deficient mice (Litingtung *et al.,* 1998; Pepicelli *et al.,* 1998). Shh is expressed almost throughout the gut endoderm at developmental stages corresponding to formation of the gut tube. In chick embryos

0012-1606/01 $35.00

it elicits restricted hindgut expression of bone morphogenetic protein (BMP)-4 and Hoxd13 (Roberts *et al.*, 1995, 1998), and each of these factors displays patterning activities. In contrast, Shh expression is specifically absent in the nascent pancreatic endoderm (Hebrok *et al.*, 1998), where ectopic Shh expression in transgenic mice leads to differentiation of the overlying mesoderm into gut- rather than pancreas-associated mesenchyme (Apelqvist *et al.*, 1997). Finally, mice lacking either Shh or Ihh show morphologic and histologic abnormalities of the intestine in late gestation, after regional specification of the various gut tube derivatives (Ramalho-Santos *et al.*, 2000). Thus, several independent studies point to a role for Hh ligands in organogenesis of the vertebrate aerodigestive tract.

Candidate Hh receptors include Patched (PTC), a protein with 12 transmembrane domains that functions as a negative regulator of the Hh signal, and Smoothened (SMO), a seven-transmembrane protein structurally related to G-protein-coupled receptors (Alcedo *et al.*, 1996; Hooper and Scott, 1989; van den Heuvel and Ingham, 1996). In epistasis analysis in *Drosophila*, *Smo* acts downstream of *Ptc*. Biochemical evidence indicates that Hh can bind directly to PTC but not to SMO and that PTC and SMO can coimmunoprecipitate, suggesting that they form a receptor complex. The prevailing model hence suggests that in the absence of Hh, PTC binds to SMO and inhibits its signaling; upon binding of Hh to PTC, this inhibition is relieved and SMO is allowed to transmit the Hh signal (Chen and Struhl, 1996; Marigo *et al.*, 1996; Stone *et al.*, 1996). In agreement with this model, loss-of-function mutations of *Ptc* have been identified in patients with the nevoid basal cell carcinoma (Gorlin) syndrome and in sporadic human basal cell carcinoma (BCC) and gain-of-function mutations of *Smo* in sporadic BCC (Chidambaram *et al.*, 1996; Gailani *et al.*, 1996; Hahn *et al.*, 1996; Johnson *et al.*, 1996; Unden *et al.*, 1996; Wicking *et al.*, 1997; Xie *et al.*, 1998). These observations highlight the notion that human cancers often result from disruption of developmental regulatory pathways.

Expression of the constitutively active mutant SmoM2 constitutes a powerful and convenient way to deregulate the Hh signaling pathway in cells. To further investigate the requirements and functions of Hh signaling in development, we therefore tested the effects of expressing SmoM2 in *Xenopus* embryos. This leads to dramatic developmental consequences, including disruption of cellular organization in the eye, absence of muscle development in the coelomic body wall, absence of the pancreas, and arrested cytodifferentiation in the midgut epithelium. Here we report detailed analysis of the specific consequences on intestinal organogenesis. Our results suggest a heretofore unappreciated role for downregulation of Hh signaling activity in proper cytodifferentiation during the transformation of primitive endoderm into intestinal epithelium.

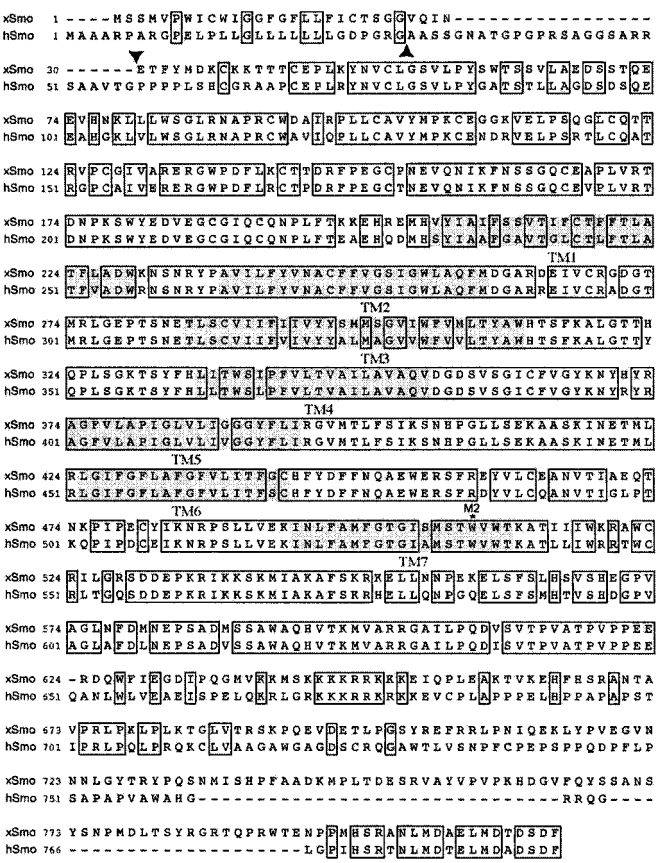


FIG. 1. Comparison of the deduced amino acid sequences of *Xenopus* (x) and human *Smo*. Identity between amino acids in the two sequences is boxed and the seven regions of hydrophobic residues representing putative transmembrane (TM) domains are shaded. The location of the tryptophan (W) residue at positions 535 and 508 of the human and *Xenopus* sequences, respectively, is marked by an asterisk and designated M2.

MATERIALS AND METHODS

Embryo Manipulations and RNA Microinjections

Xenopus laevis embryos were obtained by artificial insemination after induction of females with 600 U human chorionic gonadotropin and staged according to Nieuwkoop and Faber (1967). After removal of the jelly with 2% cysteine (pH 7.9), embryos were washed and cultured in 0.1× MMR (1× MMR is 0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM Hepes, and 0.1 mM EDTA). Embryos were washed briefly in 0.5× MMR and 3% Ficoll before injection. cDNAs encoding human Smo and SmoM2 were cloned in pCS2, the constructs were linearized with *Not*I, and capped mRNAs were synthesized using the mMessage mMachine kit (Ambion) according to the manufacturer's instructions. Between 100 pg and 2 ng RNA was microinjected into one- to two-cell stage embryos (2 ng in most experiments), or 500 pg was injected into each of four selected blastomeres (Table 1), as defined previously (Moody, 1987).

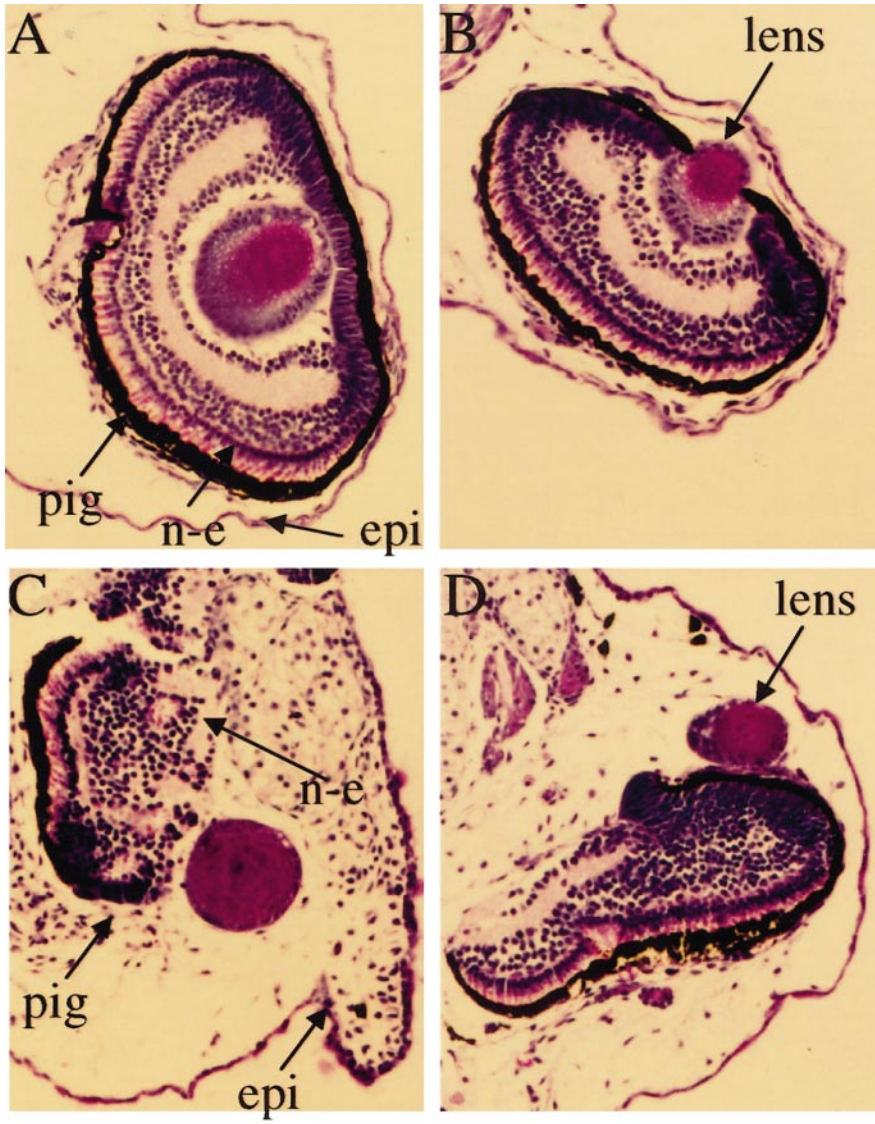


FIG. 2. Histology of eye defects seen with overexpression of SmoM2. In comparison to the normal organization of epithelia in control tadpoles injected with wild-type Smo (A, B), the eye fields of SmoM2-injected tadpoles (C, D) show a normal-appearing pigmented epithelium (pig) but a highly disorganized neuroepithelium (n-e), displaced but developed lens, and considerable distance from the epidermal surface of the skin (epi).

To determine effects of direct Hh stimulation, *Xenopus* tadpoles were harvested at Nieuwkoop–Faber Stage 41 and maintained over the next 16–18 h at 21°C in 0.1× MMR in the presence of 0, 1, 5, or 10 μg/ml recombinant amino-terminal Shh (N-Shh). Hexahistidine-tagged recombinant N-Shh was expressed in bacteria and purified by nickel chromatography. In parallel experiments, the intestines were isolated from about 20 tadpoles per group by dissection under the microscope and cultured for the next 16–18 h at 21°C in 0.1× MMR supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 0, 1, 5, or 10 μg/ml recombinant N-Shh. Tadpoles and organ explants were subsequently fixed and processed for histologic examination as outlined below.

cDNA Library and Screening

Xenopus Smo and *Ptc* cDNA clones were isolated by screening a stage 20 cDNA library and a testis cDNA library with human *Smo* and rat *Ptc* cDNA probes, respectively, at low stringency (30% formamide, 5× SSC, 1% SDS, 1× Denhardt's reagent, 50 mM

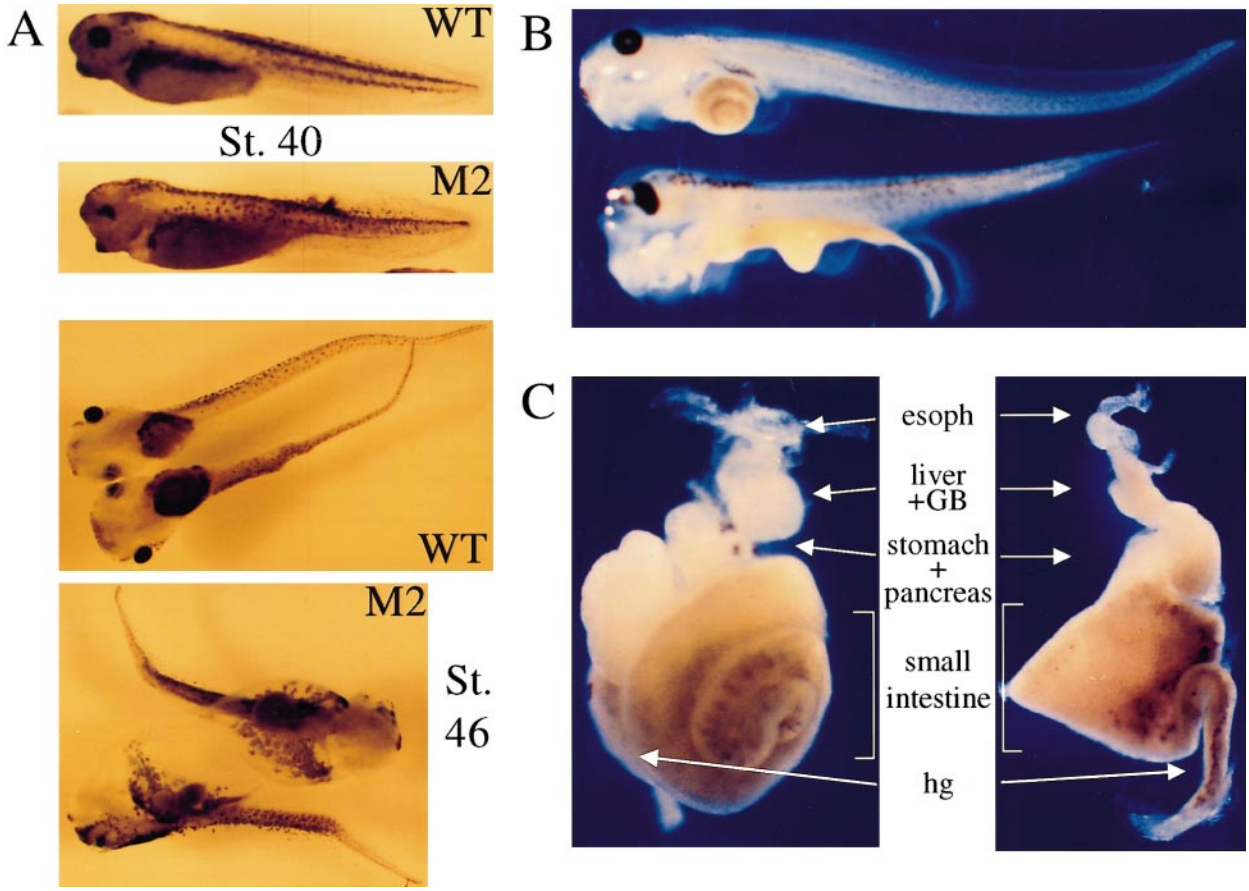


FIG. 3. Gross phenotype and abnormal intestine of tadpoles expressing SmoM2. (A) At Nieuwkoop–Faber Stage 40 (top two images), defects in embryos overexpressing SmoM2 (M2) are limited to the eye fields and subtle abnormalities in ventral morphology, compared to control embryos injected with wild-type Smo RNA (WT). By Stage 46 (bottom two), these embryos are smaller than the controls and show dramatic abnormalities in the intestine and ventral epidermis. Embryos are shown here with the ventral surface up to reveal these abnormalities. (B) Appearance of Smo- (control, top) and SmoM2-injected (bottom) embryos at Nieuwkoop–Faber Stage 45/46 after removal of the skin, highlighting the absence of intestinal coiling. (C) Morphology of the isolated digestive tract from Smo- (left) and SmoM2-injected (right) embryos, with indication of the approximate levels of the esophagus (esoph), liver and gall bladder (GB), stomach and pancreas, small intestine, and hindgut (hg). Gross abnormalities are restricted to the midgut.

sodium phosphate, pH 6.5, 0.1% sodium pyrophosphate) at 42°C. Recovered clones were sequenced on both strands.

Histology and Immunohistochemistry

Embryos at different stages were fixed for 1–4 h in MEMFA (0.1 M Mops, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde), stored briefly in methanol, and embedded in paraffin. Paraffin blocks were sectioned at a thickness of 5 or 10 μm, and slides were stained with hematoxylin and eosin. For immunohistochemistry, formalin-fixed, paraffin-embedded sections were incubated with 4 μg/ml monoclonal antibody against smooth muscle actin (clone 1A4; DAKO Corp., Carpinteria, CA) or an irrelevant isotype control antibody overnight at room temperature. Sections were then washed and incubated serially with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA), horseradish peroxidase-conjugated avidin (Vector Laboratories), and diaminobenzidine. Sections were counterstained with Mayer's hematoxylin for 1 min. Staining with the isotype control was consistently negative (data not shown). Whole-mount staining of isolated intestines at Stage 45 for alkaline phosphatase activity was done exactly as described previously (Chalmers and Slack, 1998).

Reverse Transcriptase (RT)-PCR

Intestines were isolated from tadpoles at Nieuwkoop–Faber Stages 42 and 45, and total RNA was extracted using RNAzol B (Tel-Test, Friendswood, TX) and reverse transcribed with oligo(dT) primers. Aliquots of this reaction were used as templates for PCRs that were always performed within the linear range of amplification to ensure optimal detection of differences in mRNA levels. [α-^{32}P]dCTP (0.05 μCi) was used as a radiotracer, annealing tem-

perature was 60°C, and PCR products were resolved on 4% polyacrylamide gels. Primers (5′ to 3′), sizes of the amplified products, and PCR cycle numbers were as follows: EF-1α (221 bp, 21 cycles) CCTGAATCACCCAGGCCAGATTAA and GAGGGTAGTCTGAGAAGCTCTCCACG; endodermin (340 bp, 24 cycles) ATAACGTTCCCCACCCCAAAGA and TTGGGTTGCTGATGGGAATGT; intestinal fatty acid binding protein (IFABP; 299 bp, 24 cycles) CAAGTTTACCCTTGCACAACCC and CAACTTCATCCCAGCCCAATCA; insulin (292 bp, 24 cycles) ATGGCTCTATGGATGCAGTG and AGAGAACATGTGCTGTGGCA; liver fatty acid binding protein (LFABP; 145 bp, 24 cycles) ACCGAGATTGAACAGAATGG and CCTCCATGTTTACCACGGAC; ornithine decarboxylase (234 bp, 22 cycles) AATGGATTTCAGAGACCA and CCAAGGCTAAAGTTGCAG; and XlHbox8 (432 bp, 30 cycles) CAACTTCATCCCAGCCCAATCA and TTTCCCTTCCCCTAATAACCCG.

In Situ Hybridization

mRNA *in situ* hybridization was performed as described previously (Kay and Peng, 1991), using ^{33}P-radiolabeled sense and antisense riboprobes corresponding to ~1 kb of the 3′ end of *Xenopus Ptc* cDNA, isolated as above. Paraffin-embedded sections were deparaffinized, incubated in 4 μg/ml proteinase K at 37°C for 30 min, and prehybridized in 50% formamide, 2× SSC, and 10% dextran sulfate in humidified chambers at 42°C for 4 h. ^{33}P-labeled RNA probe (1×10^6 cpm) and 50 μg of tRNA were then added and the slides incubated overnight at 55°C. Slides were then washed briefly in 2× SSC, 1 mM EDTA, treated with 20 μg/ml RNase A at 37°C for 30 min, washed further to a final stringency of 0.5× SSC at 55°C, dehydrated, and exposed to NTB2 emulsion (Kodak) for 30 days at 4°C.

RESULTS

Isolation of a Xenopus Smoothened cDNA

Although many components of the Hh signaling pathway are encoded by members of multigene families (reviewed in Ingham, 1998), only a single *Smo* gene has been identified to date in *Drosophila* or in mammals (Alcedo *et al.*, 1996; Stone *et al.*, 1996; van den Heuvel and Ingham, 1996). We used cross-species hybridization to isolate the *Xenopus* homologue of *Smoothened*. Sequence analysis (Fig. 1) revealed 32% homology with the *Drosophila* protein and 70% homology with the mouse and human sequences. The third cytoplasmic loop and seventh transmembrane domain, regions critical for the activation of downstream effectors of the Hh pathway (Murone *et al.*, 1999), are particularly conserved between frog and man, with only one amino acid difference. The seventh transmembrane domain includes tryptophan at position 535 of the human sequence, which is conserved in *Xenopus* (Fig. 1). Human SMO protein carrying the amino acid substitution W535L was originally identified in tumor samples from patients with sporadic BCC (Reifenberger *et al.*, 1998; Xie *et al.*, 1998). This mutant protein, designated SmoM2, triggers uncontrolled SMO signaling in cultured cells and transgenic mice (Xie *et al.*, 1998) and transduces Hh signals constitutively, in the absence of a potential ligand and free from repression by PTC (Hynes *et al.*, 2000; Murone *et al.*, 1999).

Effects of Constitutive Hedgehog Signaling

To better understand the spectrum of developmental regulation by Hh proteins, we forced expression of human SmoM2 in early *Xenopus* embryos. Notable among the ensuing abnormalities was the appearance of the eye fields, characterized grossly by a limited zone of pigmentation, displacement of the lens, and an internal location relative to the skin surface (Figs. 2 and 3). Whereas the pigmented retinal epithelium differentiated normally, the neuroepithelium was highly disorganized and many cells were located outside the optic cup. Although lens fiber cells appeared to differentiate normally, the intact lens was rarely observed in its normal location within the optic cup, presumably as a consequence of disorganization of the retinal epithelium. These results demonstrate the late effects of early SmoM2 injection in a known site of Hh signaling and provide *in vivo* support for one proposed function of Hh in vertebrate retinal development, based on *in vitro* culture of murine retinal epithelial cells (Jensen and Wallace, 1997).

Cutaneous expression of SmoM2 in transgenic mice results in hyperproliferation reminiscent of the human syndrome of nevoid BCC (Xie *et al.*, 1998). After Stage 41 (Nieuwkoop and Faber, 1967), SmoM2-injected tadpoles also displayed a greatly expanded and redundant epidermis, which was especially apparent ventrally (Fig. 3). Histologic inspection revealed a single-cell-thick epidermis in SmoM2-injected tadpoles, compared to multiple layers in controls (Figs. 4F and 4G). This appears in part to reflect a stretching effect, because the total number of epidermal cells, counted in comparable histologic sections, was nearly identical in control and SmoM2-injected embryos (data not shown). Moreover, the body wall is uniformly missing a layer of subepidermal muscle whose contractile function presumably maintains body wall tension and integrity (Figs. 4F and 4G). These findings suggest a role for regulated Hh signaling in differentiation of the retinal neuroepithelium and in assembly of the body wall in *Xenopus*.

Constitutive Hedgehog Signaling Results in Abnormal Development of the Gut

The most dramatic effect of SmoM2 expression was observed in the digestive tract of almost every injected embryo. Up to about Stage 40, SmoM2-injected tadpoles were similar to uninjected or control-injected embryos in gross appearance, except for the ocular abnormalities and perhaps subtle differences in ventral morphology (Fig. 3A). Thereafter, development of the intestine in control embryos was characterized by elongation and progressive coiling of the gut tube, according to a defined pattern

(Chalmers and Slack, 1998), whereas the intestine failed to elongate or coil in tadpoles injected with SmoM2 (Figs. 3A and 3B). This defect was associated with a shortened anterior–posterior axis, particularly in the tail (Fig. 3B), and displayed a linear response to the dose of injected SmoM2 mRNA. In one representative experiment, the intestinal phenotype was observed in 14 of 32 embryos injected with 100 pg SmoM2 RNA, in 26 of 32 embryos injected with 200 pg RNA, and in 34 of 37 embryos injected with 2 ng RNA; severity of the phenotype also increased with increasing dose of injected RNA. The gills, somites, muscles, and blood appeared to develop normally. Although the heart showed normal morphology and rhythmic contractions, it was reduced in size compared to controls (data not shown). Injection of wild-type Smo RNA, used as a specificity control in all experiments, did not result in any of the abnormalities seen with SmoM2 (Figs. 2–4).

Lengthening of the gut tube in *Xenopus* embryos is accompanied by cytodifferentiation of endodermal cells into a characteristic epithelium and by radial intercalation of the many endodermal cells that are initially positioned ventrally in the primitive gut tube (Chalmers and Slack, 1998, 2000). The intestinal morphology of SmoM2-injected embryos (Figs. 3B and 3C) suggests a failure in each of these processes. The transition from endoderm to epithelium occurs in waves that spread inward from the rostral and caudal ends of the gut tube and converge in the midgut (Nieuwkoop and Faber, 1967). SmoM2-injected tadpoles showed apparently normal epithelial differentiation of the foregut (esophagus, stomach, and proximal small intestine), hindgut, and proctodeum, but virtual absence of epithelial cytodifferentiation throughout the midgut (Figs. 4A–4D). Whereas control tadpoles showed significant replacement of the yolky endoderm by a maturing intestinal epithelium by Stage 45, midgut histology in SmoM2-injected tadpoles was dominated by the persistence of yolk platelets and virtual absence of epithelial cells (Figs. 4B and 4D) or a brush border; the primitive gut tube remained short and thickened. These abnormalities do not simply reflect a developmental delay because there was no progression when the tadpoles were observed up to 1 day after clear recognition of the phenotype; after this time, most SmoM2-injected tadpoles died.

Despite the marked absence of epithelial cytodifferentiation, the intestines of SmoM2-injected tadpoles did not contain reduced levels of the mRNA encoding the IFABP (Shi and Hayes, 1994) or the transcription factor GATA-5 (Jiang and Evans, 1996), either early in gut organogenesis (Stage 42) or at Stage 45/46 (Fig. 5). Similarly, the level of endodermin mRNA, a marker of immature endoderm (Sasai *et al.*, 1996) that is downregulated with advanced intestinal development (Chalmers and Slack, 1998), was not appreciably increased (Fig. 5), and alkaline phosphatase staining in whole mounts of isolated intestines at Stage 45/46 was no different between SmoM2- and control-injected embryos (data not shown). Thus, SmoM2 induces a profound block in midgut epithelial cytodifferentiation without concomitant alteration in expression of selected differentiation markers.

The liver and pancreas are derived from foregut endoderm. Development of the pancreas in chick embryos occurs over a small region notable for absence of Hh mRNA (Hebrok *et al.*, 1998; Kim *et al.*, 1997a), and misdirection of Hh signaling in the prospective pancreatic endoderm in mice results in intestinal metaplasia (Apelqvist *et al.*, 1997). SmoM2-injected tadpoles revealed complete histologic absence of the pancreas at Stage 45 (data not shown) but no apparent abnormalities in cytodifferentiation of the liver (Fig. 4E). There was no significant change in RNA levels of tissue-specific markers of these organs, insulin and the LFABP, or of the duodenopancreatic marker XlHbox8 (Gamer and Wright, 1995) (Fig. 5). Metaplasia of the pancreatic endoderm and mesoderm into intestinal cell types in transgenic mice expressing ectopic Shh is similarly restricted to cell differentiation and glandular organization, with persistent expression of lineage markers and viability of the animals (Apelqvist *et al.*, 1997). Our findings are thus consistent with the prevailing view that Hh signaling abrogates pancreas development (Hebrok *et al.*, 1998) and further demonstrate a selective effect of constitutive Hh signaling on the developing small intestine.

The Intestinal Developmental Abnormalities Likely Reflect Disruption of Epithelial–Mesenchymal Interactions and Direct Effects of Constitutive Hh Signaling

The endoderm-derived gut epithelium is surrounded by smooth muscle and connective tissue that originate in mesoderm and are contiguous with the mesentery. It is worth noting that prior to metamorphosis, the mesodermal layer in amphibians contains only a single layer of muscle and very little connective tissue and is hence much thinner than in avian or mammalian embryos. Experimental evidence strongly favors the notion that gut epithelial differentiation is modulated by signals originating within the surrounding mesenchyme (Immergluck *et al.*, 1990; Kedinger *et al.*, 1986, 1998; Panganiban *et al.*, 1990; Wessells and Cohen, 1967). Detailed studies in several species, including *Xenopus*, chicken, and mouse, have established that Hh family members are expressed in the undifferentiated gut endoderm (Bitgood and McMahon, 1995; Echelard *et al.*, 1993; Ekker *et al.*, 1995; Hebrok *et al.*, 1998; Roberts *et al.*, 1998), while their receptors are expressed in the adjacent mesenchyme (Hebrok *et al.*, 1998; Platt *et al.*, 1997; Ramalho-Santos *et al.*, 2000; Sukegawa *et al.*, 2000). This mesenchyme is therefore the likely target of constitutive Hh signaling induced by expression of SmoM2. Indeed, inspection of the intestinal mesenchyme in SmoM2-injected tadpoles revealed areas of thickening that contain

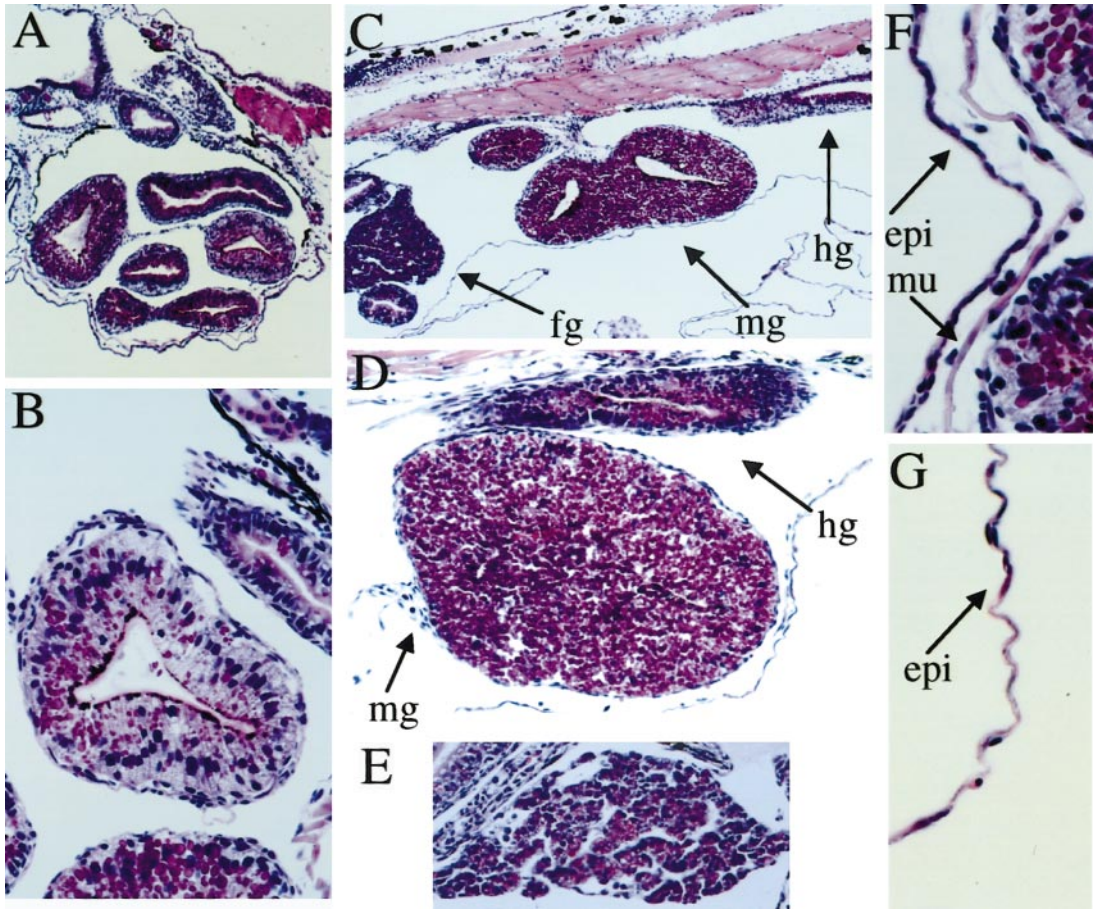


FIG. 4. Histology of the gastrointestinal tract and ventral epidermis in embryos expressing SmoM2. Coiling of multiple intestinal loops joined by a thin mesentery is evident in sagittal sections of the abdomen of control (Smo-injected) embryos (A), whereas SmoM2-injected embryos (C) show a virtually linear arrangement of foregut (fg), midgut (mg) and hindgut (hg) derivatives. In contrast to the mature intestinal epithelium seen in many loops of control-injected embryos (B), the mg derivatives of SmoM2-overexpressing embryos (D) remain filled with yolky material and fail to develop an organized epithelium, while the hg displays a maturing epithelial lining. (E) Differentiation of the liver appears normal by histologic criteria in embryos expressing SmoM2. The ventral epidermis (epi) of uninjected or Smo-injected embryos (F) has a continuous layer of underlying striated muscle (mu), which maintains the skin in close apposition to the developing intestine (seen to the right). In contrast, the epidermis of embryos expressing SmoM2 (G) is stretched and completely lacks underlying muscle.

several cell layers instead of two (Figs. 6A and 6B) and uniformly reduced levels of the differentiation marker smooth muscle actin (Figs. 6C and 6D). The thickening was nonuniform and hence does not correspond spatially to the global absence of epithelial cytodifferentiation. Unregulated Hh signaling thus results in improper development of the intestinal mesenchyme, which is the likely basis for the lack of epithelial differentiation.

Independently to assess the site of SmoM2 action that results in a discrete midgut phenotype, we targeted this mRNA into different regions of *Xenopus* embryos, and Table 1 shows data representative of three independent experiments. All aspects of development were normal when SmoM2 mRNA was injected into the 32-cell stage D tier, which is fated almost exclusively to form endoderm (Dale and Slack, 1987; Moody, 1987). In contrast, injection into vegetal blastomeres at the 8-cell stage, the prospective source of both mesoderm and endoderm prior to separation of the C and D tiers, resulted in the same gut phenotype observed with whole embryo injections, but eye development was normal. These data indicate that the lack of midgut epithelial differentiation in the setting of constitutive Hh signaling results from defects in a mesoderm-derived tissue.

These late effects of early SmoM2 RNA injection in *Xenopus* embryos could potentially reflect either a direct

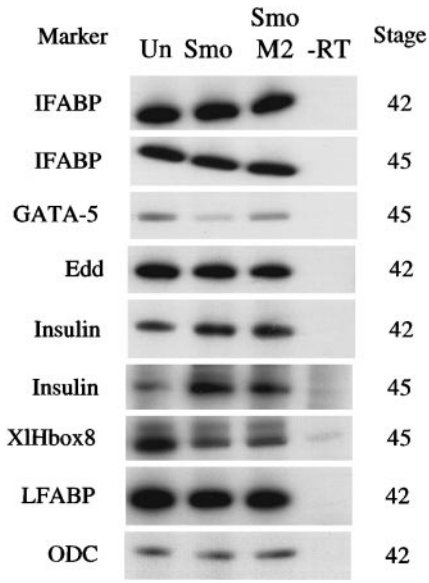


FIG. 5. Molecular correlates of gut abnormalities seen with expression of SmoM2. RT-PCR analysis of differentiation markers of gut derivatives in isolated digestive tract tissues at Nieuwkoop–Faber Stages 42 and 45. Comparison is made between mRNA levels in untreated (Un) embryos and embryos injected with either wild-type Smo or SmoM2. PCR was carried out in the linear range of amplification for each marker, samples treated without reverse transcriptase (−RT) were included as controls, and PCR for ornithine decarboxylase (ODC) and elongation factor-1α (data not shown) confirm equal loading. IFABP, intestinal fatty acid binding protein; Edd, endodermin; LFABP, liver fatty acid binding protein.

consequence of constitutive Hh signaling in the gut or indirect effects of other disturbances. We therefore treated *Xenopus* tadpoles and intestinal explants with a recombinant amino-terminal fragment of Shh (N-Shh) during early feeding stages 41–43. As expected, these embryos did not show developmental abnormalities of the eye or body wall (Fig. 7A), and the pancreas was normal (data not shown). Forty whole embryos treated with 10 μg/ml N-Shh did not develop grossly abnormal intestines, but epithelial cytodifferentiation was much reduced in all 18 embryos examined histologically, very similar to our observation in SmoM2-injected tadpoles (Figs. 7B and 7C). Although the 60 explanted guts differentiated poorly *ex vivo*, the presence of epithelial cells was much more evident in 20 control explants than in the same number of explants treated with 5 (data not shown) or 10 μg/ml recombinant N-Shh (Figs. 7D and 7E). These results strongly suggest that the intestinal defects observed upon overexpression of SmoM2 result directly from Hh overactivity.

Localized Hh Signaling in the Gut during Normal Development

Prior to metamorphosis, expression of mRNA encoding the Sonic-like Hh in *Xenopus* embryos peaks between Stages 33 and 40 and declines thereafter (Stolow and Shi, 1995). This temporal expression pattern is consistent with our results, wherein SmoM2-mediated persistence of Hh signaling produces an intestinal phenotype only after Stage 40. To further establish the molecular basis of this phenotype, we used whole-mount *in situ* hybridization to demonstrate diffuse expression of the Hh target gene *Ptc* (Goodrich *et al.*, 1996; Marigo and Tabin, 1996) and hence confirmed the persistent activity of SmoM2 in tadpoles beyond Stage 35. Highest levels of *Ptc* activity in untreated control embryos were observed between Stages 40 and 42 (Fig. 8A), coincident with the onset of intestinal abnormalities in SmoM2-injected tadpoles. Unlike control embryos, which showed much lower *Ptc* expression by Stage 45, embryos injected with SmoM2 showed persistent expression of *Ptc* mRNA at this stage, and the signal was remarkably localized to the wall of the intestine (Fig. 8A). To confirm this finding, we performed *in situ* hybridization on cut sections of the developing *Xenopus* intestine at Stage 42 and observed *Ptc* mRNA to be localized exclusively in mesoderm-derived cells in the periphery in both control and SmoM2-injected tadpoles (Fig. 8B). These results strongly suggest that constitutive Hh signaling in the midgut mesenchyme is responsible for the failure of epithelial cytodifferentiation and implicate downregulation of this activity as a requirement for normal intestinal development.

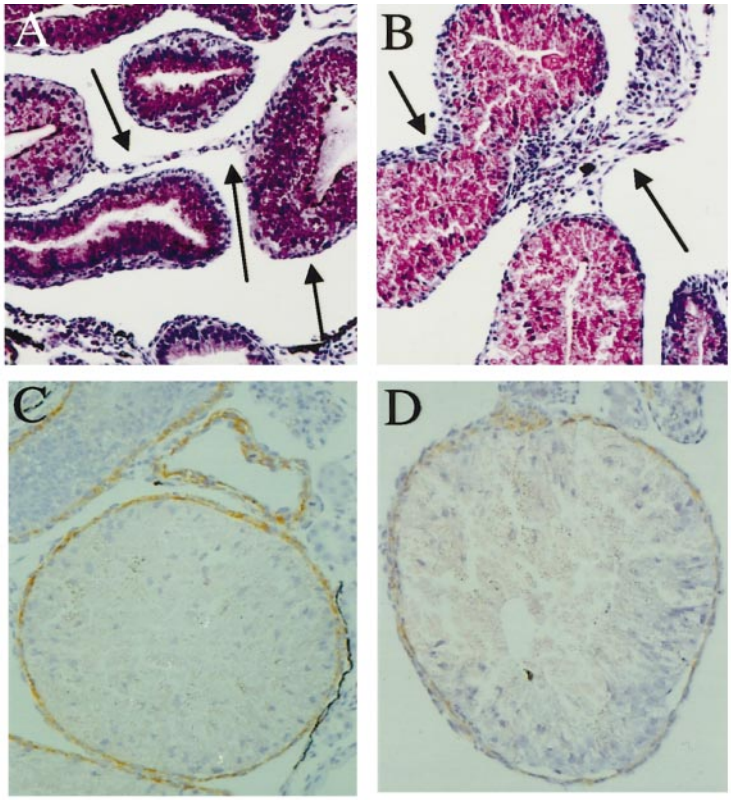
DISCUSSION

We have used a constitutively active mutant of *Smoothened* (SmoM2; Xie *et al.*, 1998), the signal-transducing component of the Hh receptor, to uncover critical roles of regulated Hh signaling in development of the vertebrate eye, body wall, and midgut. These findings indicate that

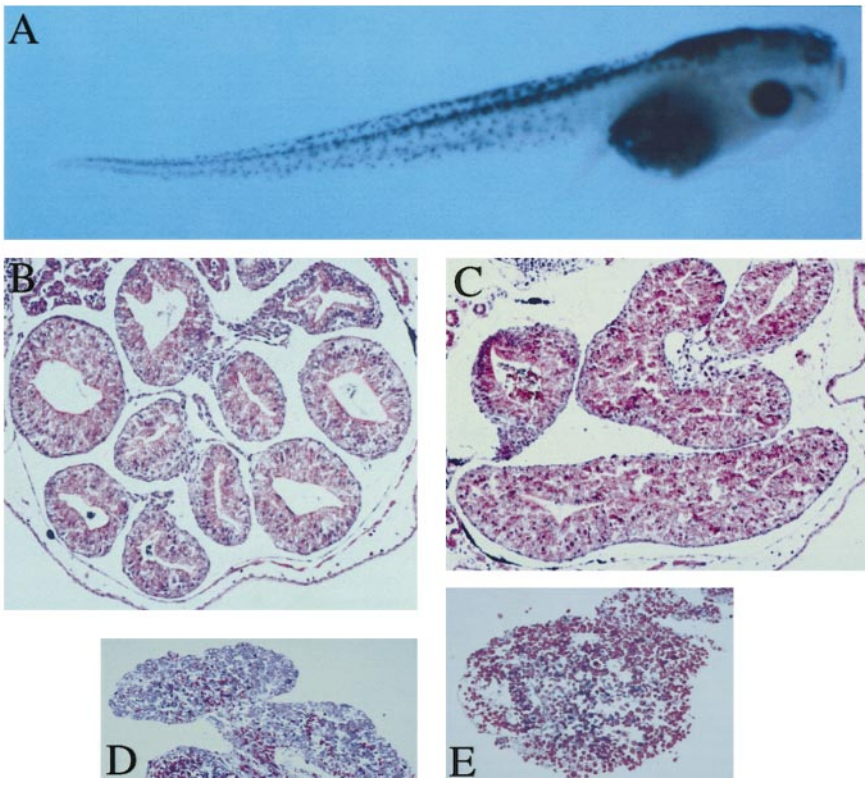
TABLE 1

Phenotypes of *Xenopus* Embryos When Overexpression of SmoM2 Is Targeted to Selected Regions

Injected RNA	Tier	No. of embryos		
		Normal	Eye phenotype	Gut phenotype
Smo (WT)	D	21	0	0
Smo (WT)	Veg (8-cell)	15	0	0
SmoM2	D	42	0	1
SmoM2	Veg (8-cell)	2	0	21
SmoM2	Whole embryo (1-cell stage)	0	37	34



6



7

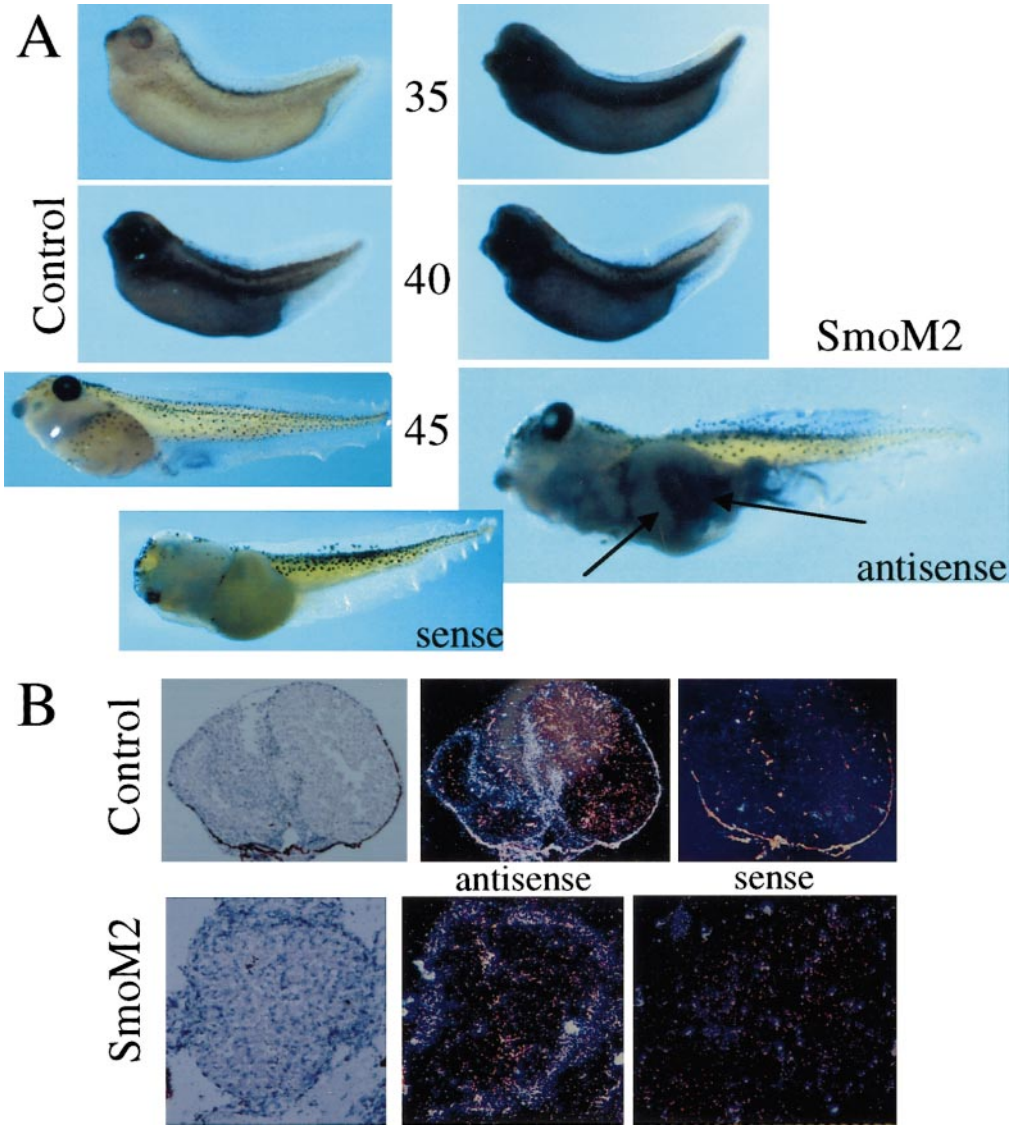


FIG. 8. *Patched* (*Ptc*) mRNA expression in normal development and with expression of SmoM2. (A) Whole-mount *in situ* hybridization of uninjected (left) and SmoM2-injected (right) embryos at Nieuwkoop–Faber Stages 35, 40, and 45 using an antisense *Xenopus Ptc* riboprobe. In normal development, activity of the Hh pathway, mirrored in *Ptc* mRNA expression, is highest around Stage 40, whereas constitutive activation through SmoM2 results in *Ptc* overexpression at earlier stages. By Stage 45, *Ptc* expression is barely detectable in control embryos and largely localized to the intestinal wall (arrows) in embryos overexpressing SmoM2. Staining with a sense probe was consistently negative, as illustrated in a single representative of Stage 45 SmoM2 embryos. (B) *In situ* hybridization of sections of the intestine of untreated (top) and Smo M2-injected (bottom) tadpoles at Stage 42, using radiolabeled *Ptc* antisense (center) or sense (right) riboprobes, showing restriction of *Ptc* mRNA expression to the splanchnic mesoderm. Corresponding bright-field images are shown on the left.

FIG. 6. Features of the mesentery with expression of SmoM2. (A) Histological analysis shows a two-cell layer thick mesentery in control embryos that is contiguous with the wall of the gut (arrows). (B) In SmoM2-expressing embryos at Nieuwkoop–Faber Stage 45/46, this mesentery shows abnormal thickening in many areas (arrows). (C) The mesenchyme in Smo-injected embryos shows intense staining with an antibody directed against smooth muscle actin. (D) Embryos expressing SmoM2 show much weaker staining.

FIG. 7. Direct effects of Hh activity in the developing *Xenopus* midgut. (A) Representative whole embryo treated with 10 μg/ml recombinant N-Shh. Histology of the midgut from whole tadpoles (B, C) and isolated intestinal explants (D, E) cultured between Nieuwkoop–Faber Stages 41 and 43 in the absence (B, D) or presence (C, E) of 10 μg/ml recombinant N-Shh.

appropriate downregulation of Hh signaling is essential for selected processes in development and organogenesis. The eye defects and absence of a pancreas are consistent with known aspects of Hh signaling in development (Dominguez and Hafen, 1997; Heberlein *et al.*, 1993; Hebrok *et al.*, 1998; Jensen and Wallace, 1997; Kim *et al.*, 1997a), whereas the abnormalities in the body wall and midgut suggest previously unrecognized functions for regulated Hh activity. Our results illustrate the principle that morphogens like Hh function in the context of exquisite spatial and temporal control to govern the outcomes of dynamic tissue interactions during development. Interestingly, injection of RNA encoding *Xenopus* Hh in early embryos does not result in the same spectrum of anomalies but only in an ectopic cement gland (Ekker *et al.*, 1995), which we never observed. While there are many possible reasons for this apparent discrepancy, including differential RNA or protein stability, the explanation is most likely to reside in the intrinsic properties of SmoM2, which cannot be repressed by PTC (Hynes *et al.*, 2000; Murone *et al.*, 1999). In contrast, overexpression of Hh would be expected to upregulate PTC, which would bind and neutralize hedgehog and limit the developmental effects. This also explains the lack of any phenotype upon overexpression of wild-type Smo, which was used as a control in all the present experiments, in this and other studies (Hynes *et al.*, 2000; Murone *et al.*, 1999).

The vertebrate embryonic digestive tract is divided into three regions: foregut, which gives rise to the esophagus, stomach, proximal small intestine, liver, and pancreas; midgut, which develops into most of the small intestine; and hindgut, from which the colon and rectum are derived. Endodermal cells, the source of all gut epithelium, initially recruit a surrounding splanchnic mesoderm that subsequently specializes into gut-associated smooth muscle and connective tissue. Hereafter, cell differentiation in these tissues is inextricably linked and probably co-dependent (Aufderheide and Ekblom, 1988; Golosow and Grobstein, 1962; Haffen *et al.*, 1987; Immergluck *et al.*, 1990; Kedinger *et al.*, 1986; Panganiban *et al.*, 1990; Wessells and Cohen, 1967), and absence of selected signals results in specific defects in intestinal organogenesis. For example, mice deficient in BMP-4 show incomplete ventral closure of the midgut mesoderm (Winnier *et al.*, 1995), and exposure to retinoic acid (Zeynali and Dixon, 1998) or expression of a dominant inhibitory fibroblast growth factor (FGF) receptor in *Xenopus* embryos (Saint-Jeannet *et al.*, 1994) inhibits proper development of the gastrointestinal tract. Our findings address the specific role of Hh signaling in early development of the vertebrate gut.

Hh mRNAs are expressed at high levels almost throughout the gut endoderm (Bitgood and McMahon, 1995; Echelard *et al.*, 1993; Ekker *et al.*, 1995), except for a small region fated to develop into the pancreas; here, absence of Shh expression in chick embryos is critical for specification of pancreatic cell fate (Hebrok *et al.*, 1998; Kim *et al.*, 1997b). Ectopic expression of Shh in the pancreatic endoderm in transgenic mice results in differentiation of a surrounding mesoderm with properties of intestinal smooth muscle instead of pancreatic mesenchyme (Apelqvist *et al.*, 1997) and directly implicates endodermal Shh in aspects of differentiation of the gut-associated mesoderm. Also in chick embryos, BMP-4 in the hindgut mesoderm appears to be a target of endodermal Shh signaling that shows a pattern of regional restriction (Roberts *et al.*, 1995, 1998) and regulates cell differentiation. In mice, various components of the Hh signaling pathway are essential in development of the aerodigestive tract. $Shh^{-/-}$ mice develop with esophageal atresia or stenosis and tracheoesophageal fistulas (Litingtung *et al.*, 1998); similar foregut defects are observed in the absence of the Hh transcriptional effectors Gli2 and Gli3, with the most severe phenotype (complete absence of esophagus, trachea, and lung) manifested in $Gli2^{-/-}$; $Gli3^{-/-}$ compound homozygotes (Motoyama *et al.*, 1998). Each of these knockout phenotypes is best interpreted as reflecting disrupted signaling between the endoderm and the splanchnic mesoderm. Accordingly, inducible expression of human Gli in adult transgenic mice has the most obvious effect on the gut mesoderm, with smooth muscle attenuation and a defective enteric nervous system (Yang *et al.*, 1997). Considered in the light of our findings, the latter result also implicates attenuation of Hh signaling as a requirement for intestinal organogenesis.

Our observations hence suggest that the response to Hh signaling in the developing gut is manifested in part in the same cells that produce Hh ligands, the gut endoderm, according to the feedback signaling model shown in Fig. 9. Early in gut development, we infer that the splanchnic mesoderm responds to Hh signals by actively inhibiting epithelial differentiation. Later, Hh signaling must be attenuated to allow proper midgut development, including elongation, coiling, radial intercalation of ventral endodermal cells (Chalmers and Slack, 2000), and ordered differentiation of both the epithelium and the surrounding mesenchyme. Constitutive signaling through SmoM2 is principally activated in the gut mesoderm, where morphologic correlates include patchy thickening and reduced expression of the differentiation marker smooth muscle actin (Fig. 6). Although failure of the gut tube to elongate and coil is also likely related to mesenchymal defects, the major consequence is absence of midgut epithelial cytodifferentiation (Fig. 4). Since effectors of Hh signaling localize to the gut mesoderm and not the endoderm (Grindley *et al.*, 1997; Platt *et al.*, 1997; and Fig. 8), we interpret this to represent a secondary effect reflecting a requirement for regulated attenuation of Hh activity in the differentiation of the intestinal epithelium. The previously reported decline in Hh mRNA levels after Stage 40 in *Xenopus* development (Stolow and Shi, 1995) is consistent with this model.

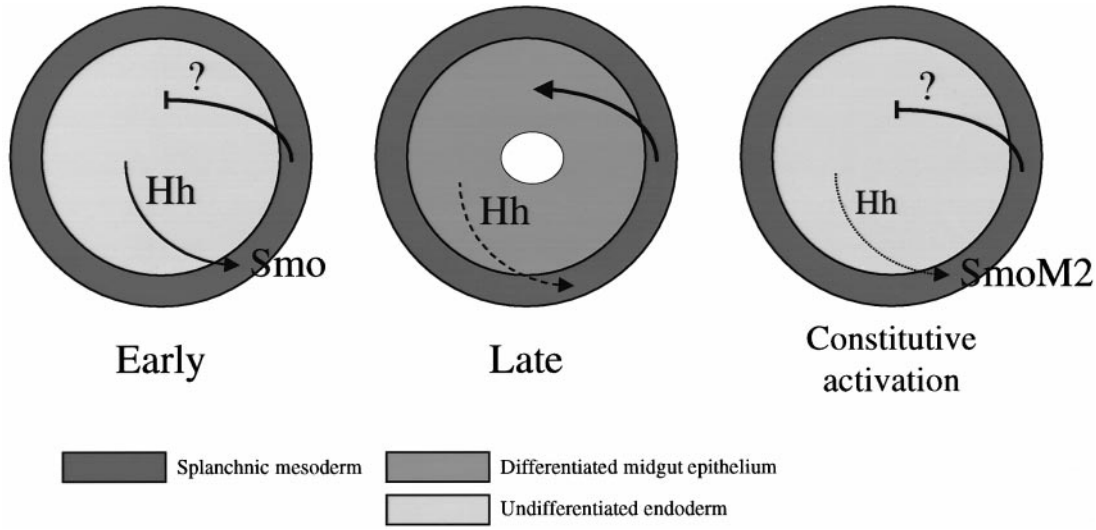


FIG. 9. Model for the role of regulated Hh signaling in differentiation of the midgut epithelium. Early in gut development (before Nieuwkoop–Faber Stage 40–42 in *Xenopus* development), the Smo receptor expressed in the splanchnic mesoderm is inferred to respond to Hh signaling by inhibiting progression of undifferentiated endoderm into intestinal epithelium via an unknown mediator (?). Later (between Nieuwkoop–Faber Stages 42 and 45), the Hh signal is naturally attenuated and inhibition of epithelial differentiation is lifted. When Hh signaling is activated artificially via expression of SmoM2, the status of the Hh ligand is irrelevant, and cytodifferentiation of the midgut epithelium is constitutively inhibited.

However, the identity of the reverse signal is not known. BMP-4 is one important target of Hh signaling in the hindgut of chick embryos (Roberts *et al.*, 1995), and the *Drosophila* homologue *decapentaplegic* mediates signaling between splanchnic mesoderm and endoderm in flies (Immergluck *et al.*, 1990; Panganiban *et al.*, 1990). Factors of this class are hence candidate mediators of the isolated midgut phenotype observed in SmoM2-expressing *Xenopus* embryos. Treatment of *Xenopus* embryos with retinoic acid (Zeynali and Dixon, 1998) or injection of a truncated, dominant inhibitory FGF receptor (Saint-Jeannet *et al.*, 1994) results in intestinal developmental abnormalities partially resembling those seen with expression of SmoM2. In particular, FGF receptor signaling is required for synchronized differentiation of intestinal smooth muscle (Saint-Jeannet *et al.*, 1994). Thus, a number of known signaling pathways appear to function in concert to effect intestinal organogenesis during vertebrate development.

The work of several other groups suggests that Hh ligands play additional roles in differentiation of the gastrointestinal epithelium at later stages. Expression of a Sonic-like Hh mRNA is reactivated in the gut epithelium during metamorphosis in *Xenopus,* when important morphogenetic changes occur (Stolow and Shi, 1995). Knockout mice lacking either Shh or Ihh show a variety of late gut developmental defects, including reduced smooth muscle, intestinal metaplasia of the stomach lining, reduced stem cell numbers, and an abnormal enteric nervous system (Ramalho-Santos *et al.*, 2000). Epithelium-derived Shh also regulates the topographical arrangement of concentric cell layers in the chicken gut mesenchyme (Sukegawa *et al.*, 2000). In contrast to each of these studies, our findings in *Xenopus* embryos address the role of temporally regulated Hh signaling at a considerably earlier point in gut development, during the transition from undifferentiated endoderm to a primitive epithelium. The sum of these observations hence illustrates the dynamic aspects of expression and regulation of Hh signaling during the development of a single organ.

There is a strong parallel between our proposed model of intestinal organogenesis and current concepts of morphogenesis in the lung. Early in development of the mouse lung *Shh* mRNA is expressed in the epithelium of terminal buds, while *Ptc* mRNA is detected in the adjacent mesenchyme; the levels of each of these as well as of *Gli* mRNAs decline in mid–late gestation (Bellusci *et al.*, 1997; Grindley *et al.*, 1997). Overexpression of *Shh* throughout the lung epithelium results in mesenchymal hyperproliferation, disruption of branching, and presumptive secondary (feedback) effects on neighboring epithelial cells (Bellusci *et al.*, 1997; Pepicelli *et al.*, 1998). Independently, increased respiratory mesenchymal cell mass is also associated with inhibition of epithelial differentiation (Zhou *et al.*, 1996). These observations support the notion that proper differentiation of endoderm-derived epithelia depends upon regulated attenuation of Hh signaling.

ACKNOWLEDGMENTS

We thank James Lee for help with library construction, Gretchen Frantz for help with *in situ* hybridization, Hartmut Koeppen and Massimo Loda for assistance with interpretation of pathology, Jeremy Green for helpful discussions, and members of the Genentech DNA sequencing lab. R.A.S. was supported in part by the Cancer Research Fund of the Damon Runyon–Walter Winchell Foundation.

REFERENCES

Alcedo, J., Ayzenzon, M., Von Ohlen, T., Noll, M., and Hooper, J. E. (1996). The Drosophila smoothened gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. *Cell* **86,** 221–232.

Apelqvist, A., Ahlgren, U., and Edlund, H. (1997). Sonic hedgehog directs specialized mesoderm differentiation in the intestine and pancreas. *Curr. Biol.* **7,** 801–804.

Aufderheide, E., and Ekblom, P. (1988). Tenascin during gut development: Appearance in the mesenchyme, shift in molecular forms, and dependence on epithelial–mesenchymal interactions. *J. Cell Biol.* **107,** 2341–2349.

Bellusci, S., Furuta, Y., Rush, M. G., Henderson, R., Winnier, G., and Hogan, B. L. (1997). Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. *Development* **124,** 53–63.

Bitgood, M. J., and McMahon, A. P. (1995). Hedgehog and Bmp genes are coexpressed at many diverse sites of cell–cell interaction in the mouse embryo. *Dev. Biol.* **172,** 126–138.

Bitgood, M. J., Shen, L., and McMahon, A. P. (1996). Sertoli cell signaling by Desert hedgehog regulates the male germline. *Curr. Biol.* **6,** 298–304.

Chalmers, A. D., and Slack, J. M. (1998). Development of the gut in Xenopus laevis. *Dev. Dyn.* **212,** 509–521.

Chalmers, A. D., and Slack, J. M. (2000). The Xenopus tadpole gut: Fate maps and morphogenetic movements. *Development* **127,** 381–392.

Chen, Y., and Struhl, G. (1996). Dual roles for patched in sequestering and transducing Hedgehog. *Cell* **87,** 553–563.

Chidambaram, A., Goldstein, A. M., Gailani, M. R., Gerrard, B., Bale, S. J., DiGiovanna, J. J., Bale, A. E., and Dean, M. (1996). Mutations in the human homologue of the Drosophila patched gene in Caucasian and African-American nevoid basal cell carcinoma syndrome patients. *Cancer Res.* **56,** 4599–4601.

Dale, L., and Slack, J. M. (1987). Fate map for the 32-cell stage of Xenopus laevis. *Development* **99,** 527–551.

Dominguez, M., and Hafen, E. (1997). Hedgehog directly controls initiation and propagation of retinal differentiation in the Drosophila eye. *Genes Dev.* **11,** 3254–3264.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A., and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75,** 1417–1430.

Ekker, S. C., McGrew, L. L., Lai, C. J., Lee, J. J., von Kessler, D. P., Moon, R. T., and Beachy, P. A. (1995). Distinct expression and shared activities of members of the hedgehog gene family of Xenopus laevis. *Development* **121,** 2337–2347.

Gailani, M. R., Stahle-Backdahl, M., Leffell, D. J., Glynn, M., Zaphiropoulos, P. G., Pressman, C., Unden, A. B., Dean, M., Brash, D. E., Bale, A. E., and Toftgard, R. (1996). The role of the human homologue of Drosophila patched in sporadic basal cell carcinomas. *Nat. Genet.* **14,** 78–81.

Gamer, L. W., and Wright, C. V. E. (1995). Autonomous endodermal determination in *Xenopus:* Regulation of expression of the pancreatic gene XlHbox 8. *Dev. Biol.* **171,** 240–251.

Golosow, N., and Grobstein, C. (1962). Epitheliomesenchymal interaction in pancreatic morphogenesis. *Dev. Biol.* **4,** 242–255.

Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. A., and Scott, M. P. (1996). Conservation of the hedgehog/patched signaling pathway from flies to mice: Induction of a mouse patched gene by Hedgehog. *Genes Dev.* **10,** 301–312.

Grindley, J. C., Bellusci, S., Perkins, D., and Hogan, B. L. (1997). Evidence for the involvement of the Gli gene family in embryonic mouse lung development. *Dev. Biol.* **188,** 337–348.

Haffen, K., Kedinger, M., and Simon-Assmann, P. (1987). Mesenchyme-dependent differentiation of epithelial progenitor cells in the gut. *J. Pediatr. Gastroenterol. Nutr.* **6,** 14–23.

Hahn, H., Wicking, C., Zaphiropoulous, P. G., Gailani, M. R., Shanley, S., Chidambaram, A., Vorechovsky, I., Holmberg, E., Unden, A. B., Gillies, S., Negus, K., Smyth, I., Pressman, C., Leffell, D. J., Gerrard, B., Goldstein, A. M., Dean, M., Toftgard, R., Chenevix-Trench, G., Wainwright, B., and Bale, A. E. (1996). Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. *Cell* **85,** 841–851.

Heberlein, U., Wolff, T., and Rubin, G. M. (1993). The TGF beta homolog dpp and the segment polarity gene hedgehog are required for propagation of a morphogenetic wave in the Drosophila retina. *Cell* **75,** 913–926.

Hebrok, M., Kim, S. K., and Melton, D. A. (1998). Notochord repression of endodermal Sonic hedgehog permits pancreas development. *Genes Dev.* **12,** 1705–1713.

Hooper, J. E., and Scott, M. P. (1989). The Drosophila patched gene encodes a putative membrane protein required for segmental patterning. *Cell* **59,** 751–765.

Hynes, M., Ye, W., Wang, K., Stone, D., Murone, M., de Sauvage, F., and Rosenthal, A. (2000). The seven-transmembrane receptor Smoothened cell-autonomously induces multiple ventral cell types. *Nat. Neurosci.* **3,** 41–46.

Immergluck, K., Lawrence, P. A., and Bienz, M. (1990). Induction across germ layers in Drosophila mediated by a genetic cascade. *Cell* **62,** 261–268.

Ingham, P. W. (1998). Transducing Hedgehog: The story so far. *EMBO J.* **17,** 3505–3511.

Jensen, A. M., and Wallace, V. A. (1997). Expression of Sonic hedgehog and its putative role as a precursor cell mitogen in the developing mouse retina. *Development* **124,** 363–371.

Jiang, Y., and Evans, T. (1996). The *Xenopus* GATA-4/5/6 genes are associated with cardiac specification and can regulate cardiac-specific transcription during embryogenesis. *Dev. Biol.* **174,** 258–270.

Johnson, R. L., Rothman, A. L., Xie, J., Goodrich, L. V., Bare, J. W., Bonifas, J. M., Quinn, A. G., Myers, R. M., Cox, D. R., Epstein, E. H., Jr., and Scott, M. P. (1996). Human homolog of patched, a candidate gene for the basal cell nevus syndrome. *Science* **272,** 1668–1671.

Kay, B. K., and Peng, H. B. (1991). "*Xenopus laevis:* Practical Uses in Cell and Molecular Biology." Academic Press, San Diego.

Kedinger, M., Duluc, I., Fritsch, C., Lorentz, O., Plateroti, M., and Freund, J. N. (1998). Intestinal epithelial–mesenchymal cell interactions. *Ann. N. Y. Acad. Sci.* **859,** 1–17.

Kedinger, M., Simon-Assmann, P. M., Lacroix, B., Marxer, A., Hauri, H. P., and Haffen, K. (1986). Fetal gut mesenchyme induces differentiation of cultured intestinal endodermal and crypt cells. *Dev. Biol.* **113,** 474–483.

Kim, S. K., Hebrok, M., and Melton, D. A. (1997a). Notochord to endoderm signaling is required for pancreas development. *Development* **124,** 4243–4252.

Kim, S. K., Hebrok, M., and Melton, D. A. (1997b). Pancreas development in the chick embryo. *Cold Spring Harbor Symp. Quant. Biol.* **62,** 377–383.

Litingtung, Y., Lei, L., Westphal, H., and Chiang, C. (1998). Sonic hedgehog is essential to foregut development. *Nat. Genet.* **20,** 58–61.

Marigo, V., Davey, R. A., Zuo, Y., Cunningham, J. M., and Tabin, C. J. (1996). Biochemical evidence that patched is the Hedgehog receptor. *Nature* **384,** 176–179.

Marigo, V., and Tabin, C. J. (1996). Regulation of patched by sonic hedgehog in the developing neural tube. *Proc. Natl. Acad. Sci. USA* **93,** 9346–9351.

Moody, S. A. (1987). Fates of the blastomeres of the 32-cell-stage *Xenopus* embryo. *Dev. Biol.* **122,** 300–319.

Motoyama, J., Liu, J., Mo, R., Ding, Q., Post, M., and Hui, C. C. (1998). Essential function of Gli2 and Gli3 in the formation of lung, trachea and oesophagus. *Nat. Genet.* **20,** 54–57.

Murone, M., Rosenthal, A., and de Sauvage, F. J. (1999). Sonic hedgehog signaling by the patched–smoothened receptor complex. *Curr. Biol.* **9,** 76–84.

Nieuwkoop, P. D., and Faber, J. (1967). "Normal Table of *Xenopus laevis*." North-Holland, Amsterdam.

Nusslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. *Nature* **287,** 795–801.

Panganiban, G. E. F., Reuter, R., Scott, M. P., and Hoffmann, F. M. (1990). A Drosophila growth factor homolog, decapentaplegic, regulates homeotic gene expression within and across germ layers during midgut morphogenesis. *Development* **110,** 1041–1050.

Parmantier, E., Lynn, B., Lawson, D., Turmaine, M., Namini, S. S., Chakrabarti, L., McMahon, A. P., Jessen, K. R., and Mirsky, R. (1999). Schwann cell-derived Desert hedgehog controls the development of peripheral nerve sheaths. *Neuron* **23,** 713–724.

Pepicelli, C. V., Lewis, P. M., and McMahon, A. P. (1998). Sonic hedgehog regulates branching morphogenesis in the mammalian lung. *Curr. Biol.* **8,** 1083–1086.

Platt, K. A., Michaud, J., and Joyner, A. L. (1997). Expression of the mouse Gli and Ptc genes is adjacent to embryonic sources of hedgehog signals suggesting a conservation of pathways between flies and mice. *Mech. Dev.* **62,** 121–135.

Ramalho-Santos, M., Melton, D. A., and McMahon, A. P. (2000). Hedgehog signals regulate multiple aspects of gastrointestinal development. *Development* **127,** 2763–2772.

Reifenberger, J., Wolter, M., Weber, R. G., Megahed, M., Ruzicka, T., Lichter, P., and Reifenberger, G. (1998). Missense mutations in SMOH in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. *Cancer Res.* **58,** 1798–1803.

Riddle, R. D., Johnson, R. L., Laufer, E., and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75,** 1401–1416.

Roberts, D. J., Johnson, R. L., Burke, A. C., Nelson, C. E., Morgan, B. A., and Tabin, C. (1995). Sonic hedgehog is an endodermal signal inducing Bmp-4 and Hox genes during induction and regionalization of the chick hindgut. *Development* **121,** 3163–3174.

Roberts, D. J., Smith, D. M., Goff, D. J., and Tabin, C. J. (1998). Epithelial–mesenchymal signaling during the regionalization of the chick gut. *Development* **125,** 2791–2801.

Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A., and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell* **81,** 445–455.

Saint-Jeannet, J. P., Thiery, J. P., and Koteliansky, V. E. (1994). Effect of an inhibitory mutant of the FGF receptor on mesoderm-derived α-smooth muscle actin-expressing cells in *Xenopus* embryo. *Dev. Biol.* **164,** 374–382.

Sasai, Y., Lu, B., Piccolo, S., and De Robertis, E. M. (1996). Endoderm induction by the organizer-secreted factors chordin and noggin in Xenopus animal caps. *EMBO J.* **15,** 4547–4555.

Shi, Y. B., and Hayes, W. P. (1994). Thyroid hormone-dependent regulation of the intestinal fatty acid-binding protein gene during amphibian metamorphosis. *Dev. Biol.* **161,** 48–58.

Stolow, M. A., and Shi, Y. B. (1995). Xenopus sonic hedgehog as a potential morphogen during embryogenesis and thyroid hormone-dependent metamorphosis. *Nucleic Acids Res.* **23,** 2555–2562.

Stone, D. M., Hynes, M., Armanini, M., Swanson, T. A., Gu, Q., Johnson, R. L., Scott, M. P., Pennica, D., Goddard, A., Phillips, H., Noll, M., Hooper, J. E., de Sauvage, F., and Rosenthal, A. (1996). The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. *Nature* **384,** 129–134.

Sukegawa, A., Narita, T., Kameda, T., Saitoh, K., Nohno, T., Iba, H., Yasugi, S., and Fukuda, K. (2000). The concentric structure of the developing gut is regulated by Sonic hedgehog derived from endodermal epithelium. *Development* **127,** 1971–1980.

Unden, A. B., Holmberg, E., Lundh-Rozell, B., Stahle-Backdahl, M., Zaphiropoulos, P. G., Toftgard, R., and Vorechovsky, I. (1996). Mutations in the human homologue of Drosophila patched (PTCH) in basal cell carcinomas and the Gorlin syndrome: Different in vivo mechanisms of PTCH inactivation. *Cancer Res.* **56,** 4562–4565.

van den Heuvel, M., and Ingham, P. W. (1996). smoothened encodes a receptor-like serpentine protein required for hedgehog signalling. *Nature* **382,** 547–551.

Vortkamp, A., Lee, K., Lanske, B., Segre, G. V., Kronenberg, H. M., and Tabin, C. J. (1996). Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* **273,** 613–622.

Wessells, N. K., and Cohen, J. H. (1967). Early pancreas organogenesis: Morphogenesis, tissue interactions, and mass effects. *Dev. Biol.* **15,** 237–270.

Wicking, C., Gillies, S., Smyth, I., Shanley, S., Fowles, L., Ratcliffe, J., Wainwright, B., and Chenevix-Trench, G. (1997). De novo mutations of the Patched gene in nevoid basal cell carcinoma syndrome help to define the clinical phenotype. *Am. J. Med. Genet.* **73,** 304–307.

Winnier, G., Blessing, M., Labosky, P. A., and Hogan, B. L. (1995). Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev.* **9,** 2105–2116.

Xie, J., Murone, M., Luoh, S. M., Ryan, A., Gu, Q., Zhang, C., Bonifas, J. M., Lam, C. W., Hynes, M., Goddard, A., Rosenthal, A., Epstein, E. H., Jr., and de Sauvage, F. J. (1998). Activating Smoothened mutations in sporadic basal-cell carcinoma. *Nature* **391,** 90–92.

Yang, J. T., Liu, C. Z., Villavicencio, E. H., Yoon, J. W., Walterhouse, D., and Iannaccone, P. M. (1997). Expression of human GLI in mice results in failure to thrive, early death, and patchy Hirschsprung-like gastrointestinal dilatation. *Mol. Med.* **3,** 826–835.

Zeynali, B., and Dixon, K. E. (1998). Effects of retinoic acid on the endoderm in Xenopus embryos. *Dev. Genes Evol.* **208,** 318–326.

Zhou, L., Dey, C. R., Wert, S. E., and Whitsett, J. A. (1996). Arrested lung morphogenesis in transgenic mice bearing an SP-C-TGF-beta 1 chimeric gene. *Dev. Biol.* **175,** 227–238.

Received for publication April 4, 2000
Revised September 26, 2000
Accepted September 26, 2000
Published online November 28, 2000