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

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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. © 2001 Academic Press

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 (Parman-tier 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
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.

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 MgSO4, 2 mM CaCl2, 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 NotI, 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).
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 0.1x 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 0.1x 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, 5x SSC, 1% SDS, 1x Denhardt's reagent, 50 mM...
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 MgSO₄, 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-32P]dCTP (0.05 μCi) was used as a radiotracer, annealing tem-
perature was 60°C, and PCR products were resolved on 4% poly-
acrylamide gels. Primers (5' to 3'), sizes of the amplified products, 
and PCR cycle numbers were as follows: EF-1α (221 bp, 21 cycles) 
CCTGAATCACCAGGCGCATTAA and GAGGGTAGCTC-
GAAGCTTCCGCC; endoderm in (340 bp, 24 cycles) ATAT-
ACGTTCCCCCAACCCGGAAG and TGGGTTGCTGATGG-
GAATGT; intestinal fatty acid binding protein (IFABP; 145 bp, 24 
cycles) CAACTTCAACTGCAACCAC and CAACTTCACT-
CCAGGCCCCATCA; insulin (292 bp, 24 cycles) ATGGCTCAT-
ATGATGATG and AGAGAACATGTGCTGTGGCA; liver 
fatty acid binding protein (LFABP; 145 bp, 24 cycles) ACGAGAT-
TGAAACAGAATGG and CCTCCATGTTTACCACGGAC; orni-
thine decarboxylase (234 bp, 22 cycles) AATGGATTCCA-
GAGCCA and CCAAGGCTAAGATGGCA; and XIHbox8 (432 
bp, 30 cycles) CAACTTCACTCCAGCCCAATCA and TTTC-
CCTCCCCCTAATAACCCG.

In Situ Hybridization

mRNA in situ hybridization was performed as described previ-
ously (Kay and Peng, 1991), using 32P-radiolabeled sense and anti-
sense riboprobes corresponding to ~1 kb of the 3' end of Xenopus 
Ptc cDNA, isolated as above. Paraffin-embedded sections were 
deparaffinized, incubated in 4°C for 21 min, and prehybridized in 50%
formamide, 2× SSC, and 10% dextran sulfate in humidified chambers at 42°C 
for 4 h. 32P-labeled RNA probe (1× 106 cpm) and 50 μg of RNA 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) re-
vealed 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 (Muron et al., 1999), are 
particularly conserved between frog and man, with only one 
amino acid difference. The seventh transmembrane domain 
includes tryptophan at position 335 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 uncon-
trolled 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 neurops-
ithelium 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 ef-
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 neuropithelium 
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 un.injected 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

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(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 rhythm 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 by mesodermal cells that are initially positioned in the primitive gut tube (Chalmers and Slack, 1998, 2000), the intestinal phenotype is 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).

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 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 (Bltgood 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...
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. 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-overexpressing 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 yolk 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 un.injected 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.
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.

**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

<table>
<thead>
<tr>
<th>Injected RNA</th>
<th>Tier</th>
<th>Normal</th>
<th>Eye phenotype</th>
<th>Gut phenotype</th>
</tr>
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<tbody>
<tr>
<td>Smo (WT)</td>
<td>D</td>
<td>21</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Smo (WT)</td>
<td>Veg (8-cell)</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SmoM2</td>
<td>D</td>
<td>42</td>
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<td>1</td>
</tr>
<tr>
<td>SmoM2</td>
<td>Veg (8-cell)</td>
<td>2</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>SmoM2</td>
<td>Whole embryo (1-cell stage)</td>
<td>0</td>
<td>37</td>
<td>34</td>
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**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.

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.
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 (Außerheide and Ekblom, 1988; Golosow and Grobstein, 1962; Haffen et al., 1987; Immelryck 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 cytoidifferentiation (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.

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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.
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