Paracrine Hedgehog Signaling in Stomach and Intestine: New Roles for Hedgehog in Gastrointestinal Patterning

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BACKGROUND & AIMS: Hedgehog signaling is critical in gastrointestinal patterning. Mice deficient in Hedgehog signaling exhibit abnormalities that mirror deformities seen in the human VACTERL (vertebral, anal, cardiac, tracheal, esophageal, renal, limb) association. However, the direction of Hedgehog signal flow is controversial and the cellular targets of Hedgehog signaling change with time during development. We profiled cellular Hedgehog response patterns from embryonic day 10.5 (E10.5) to adult in murine antrum, pyloric region, small intestine, and colon. **METHODS:** Hedgehog signaling was profiled using Hedgehog pathway reporter mice and in situ hybridization. Cellular targets were identified by immunostaining. Ihh-overexpressing transgenic animals were generated and analyzed. **RESULTS:** Hedgehog signaling is strictly paracrine from antrum to colon throughout embryonic and adult life. Novel findings include the following: mesothelial cells of the serosa transduce Hedgehog signals in fetal life; the hindgut epithelium expresses Ptch but not Gli1 at E10.5; the 2 layers of the muscularis externa respond differently to Hedgehog signals; organogenesis of the pyloric sphincter is associated with robust Hedgehog signaling; dramatically different Hedgehog responses characterize stomach and intestine at E16; and after birth, the muscularis mucosa and villus smooth muscle consist primarily of Hedgehog-responsive cells and Hh levels actively modulate villus core smooth muscle. **CONCLUSIONS: These studies reveal a previously unrecognized association of paracrine Hedgehog signaling with several gastrointestinal patterning events involving the serosa, pylorus, and villus smooth muscle. The results may have implications for several human anomalies and could potentially expand the spectrum of the human VACTERL association.**

Organogenesis of the gut relies on soluble signals that pass bidirectionally between endodermal and mesodermal layers (reviewed in Roberts[1]). The Hedgehog (Hh) signaling pathway participates in this process at multiple sites along the developing gut.[2] Indeed, Hedgehog signaling is part of an ancient gut sculpting program, because components of this pathway in gut tissues of *Drosophila*,[3] *Amphioxus*,[4] leech,[5] sea urchin,[6] zebrafish,[7] *Xenopus*,[8] chicken,[9] and mouse[10,11] coordinate morphogenic patterning events that are specific to each regional address along the anterior/posterior axis of the gut tube.

In vertebrates, Hh ligands include Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh). All 3 are expressed in the developing gut tube. Shh and Ihh are epithelially expressed and do not overlap with Dhh, which is expressed in Schwann cells, peripheral nerves, and endothelial cells.[10] The 3 ligands bind to the receptors Patched (Ptch)-1 and Ptch-2. In the absence of ligand, the unoccupied Ptch receptor inhibits another membrane protein, Smoothened (Smo), deactivating the pathway. Hh ligand binding to Ptch relieves this repression, activating the pathway as measured by transcriptional modulation of target genes. The Gli transcription factors (Gli1, Gli2, and Gli3) represent the downstream effectors of Hh signaling in vertebrates (reviewed in Varjosalo and Taipale[12]). All 3 of these factors are expressed in the gastrointestinal tract.[13]

Significant gastrointestinal pathology results from reduction of Hh ligand levels. $Shh^{-/-}$ and $Ihh^{-/-}$ mice exhibit malrotation of the gastrointestinal tract, decreased muscularis propria, and enteric neuron abnormalities.[14,15] Other aspects of the phenotypes of these 2 ligand knockouts are distinct and include esophageal atresia with tracheal esophageal fistula, gastric overgrowth, and imperforate anus in Shh-deficient animals and Hirschsprung's-like dilation of the colon as well as epithelial stem cell defects in Ihh-null mice.[14,16] Reducing the combined (Shh + Ihh) Hh signal from the epithelium either by expression of a soluble form of the Hh inhibitor protein Hhip[17] or by injection of an anti-Hh

Abbreviations used in this paper: Dhh, Desert Hedgehog; E, embryonic day; Hh, Hedgehog; Ihh, Indian Hedgehog; ME, muscularis externa; MM, muscularis mucosa; Ptch, Patched; Shh, Sonic Hedgehog; SM, smooth muscle; SMA, smooth muscle actin; Smo, Smoothened; VACTERL, vertebral, anal, cardiac, tracheal, esophageal, renal, limb.

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antibody[18] results in a distinct phenotype that includes branched villi and vacuolated epithelium as well as disrupted mesenchymal patterning.

Loss of any of the Gli factors also has pathologic consequences. Gli2-null animals exhibit malformations of the esophagus and hindgut while Gli3-deficient mice present with anal stenosis and overgrowth of the distal stomach, without apparent small intestinal phenotype.[19] $Gli1^{-/-}$ mice show no apparent gut abnormalities,[20] but a full complement of Gli1 activity is important in coping with inflammatory stress; a Gli1 variant in the human population (E1100Q) is implicated in inflammatory bowel disease, and the $Gli1^{+/-}$ mouse is highly sensitive to chemically induced colitis.[21]

Likewise, in humans, perturbed Hh signaling is implicated in malformations of the gastrointestinal tract. Pallister–Hall syndrome, which includes limb defects, hypothalamic hamartomas, and imperforate anus, is due to a frameshift in the Gli3 protein.[22] In large part, however, the association of human deformities with the Hh pathway has been based on the similarity of these malformations to those described in mouse Hh pathway mutants; the human VACTERL (vertebral, anal, cardiac, tracheal, esophageal, renal, limb) association (which includes vertebral, cardiac, tracheoesophageal, and/or anorectal malformations) mirrors foregut and/or hindgut phenotypes seen in Shh-, Gli2-, or Gli3-null mice.[14,23]

Despite the importance of the Hh signaling program to gut development and disease, conflicting reports exist as to the direction of Hh signals. Some studies support a strictly paracrine Hh signal (from epithelium to mesenchyme),[10,14,17] while others suggest that the epithelium can also participate in an autocrine signaling program, especially during later development and adulthood.[24–26] Indeed, autocrine Hh signals have been proposed to mediate Paneth cell differentiation,[26] control colonic epithelial cell differentiation,[25] and promote epithelial regeneration in a setting of chronic inflammation.[24]

In this study, we mapped regional expression of Hh signaling molecules (Shh, Ihh, Ptch1, Gli1, Gli2, Gli3) throughout development in the antrum, small intestine, and large intestine. The data reveal that the Hh signaling pathway is strictly paracrine at all times. The dynamic expression domains of Hh pathway activators suggest that in addition to previously assigned roles, Hh signaling might be involved in formation of the pylorus, patterning of antral epithelium, emergence of intestinal cell identity, and development of the serosal layer. Analysis of transgenic mice that overexpress Ihh revealed that Hh levels control smooth muscle (SM) populations in the cores of the villi.

Materials and Methods

Mice

$Gli1^{+/LacZ}$, $Gli2^{+/LacZ}$, and $Ptch1^{+/LacZ}$ mice have been described.[20,27–29] $Shh^{+/LacZ}$ mice were used in a previous study[30]; their derivation will be described elsewhere (Gonzalez and Kottman, manuscript in preparation). Heterozygous mice were mated with C57Bl/6 mice, and the morning of vaginal plug was counted as embryonic day (E) 0.5. Genotyping was performed as previously described.[20,27–30] Protocols for X-gal staining, immunostaining, in situ hybridization, quantitative reverse-transcription polymerase chain reaction, and generation of Ihh transgenic mice are detailed in the Supplementary Methods.

Results

Although Gli1, Gli2, and Ptch1 are all components of the Hh pathway, they reveal different aspects of the Hh signaling network. Gli1 is a direct target of Hh, and its expression is dependent on active Hh signaling.[27] Ptch1 is also an Hh target gene, but its transcription is not solely dependent on Hh. Finally, Gli2 is an important mediator of activation,[27] but its expression is not transcriptionally regulated by Hh. Thus, $Ptch1^{LacZ/+}$ and $Gli2^{LacZ/+}$ mice indicate cells capable of responding to Hh signals, while $Gli1^{LacZ/+}$ mice reveal the cells that are actively responding. We used these reporters to map Hh pathway components in the developing and adult gastrointestinal tract. In situ hybridization was used to confirm the reporter findings and examine additional Hh pathway molecules (Gli3, Ptch2).

E10.5: Ptch1, but Not Gli1, Is Expressed in Hindgut Epithelium

Both Ihh and Shh are expressed robustly in the multilayered E10.5 endoderm throughout the midgut and hindgut as documented previously.[10] Ptch1 is expressed in mesenchymal cells of the presumptive antrum (data not shown) and midgut (Figure 1*A*). However, on the dorsal side of the hindgut epithelium and postanal portion of the tail gut, $Ptch1^{LacZ/+}$ staining is clearly seen in the epithelium (Figure 1*B* and *C*). In contrast, $Gli1^{LacZ/+}$ staining is strictly mesenchymal in all of these tissues (Figure 1*D–F*). Because Gli1 expression requires Hh ligands while Ptch1 may be expressed independently,[33,34] we conclude that Hh signaling is paracrine in these tissues at this time. Gli3 is also highly expressed in the early intestinal mesenchyme and is progressively down-regulated during fetal development (Supplementary Information 1).

E14.5: Novel Aspects of Hh Signaling in Stomach, Pyloric Sphincter, Muscularis Externa, Enteric Neurons, and Serosa

An opposing gradient of Shh and Ihh expression has been previously documented in E11 stomach.[10] We detected 3 different patterns of Hh expression in E14.5 stomach: in forestomach epithelium, Shh is expressed strongly while Ihh is undetectable; in distal stomach that will give rise to corpus and antrum, Ihh expression is