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Inhibition of Hedgehog Signaling in the Gastrointestinal Tract: Targeting the Cancer Microenvironment

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Abstract

This review summarizes emerging information regarding the Hedgehog (Hh) signaling pathway during neoplastic transformation in the gastrointestinal tract. Although there is a role for the well-established canonical pathway in which Hedgehog ligands interact with their receptor Patched, there is sufficient evidence that downstream components of the Hh pathway, e.g., Gli1, are hijacked by non-Hh signaling pathways to promote the conversion of the epithelium to dysplasia and carcinoma. We review the canonical pathway and involvement of primary cilia, and then focus on current evidence for Hh signaling in luminal bowel cancers as well as accessory organs, i.e., liver, pancreas and biliary ducts. We conclude that targeting the Hh pathway with small molecules, nutraceuticals and other mechanisms will likely require a combination of inhibitors that target Gli transcription factors in addition to canonical modulators such as *Smoothened*.

Keywords

primary cilia; stroma; MDSCs; Gli1; stomach

Introduction

During the 1970s, mutagenesis screens in *Drosophila* uncovered a number of important developmental signaling pathways that have since been applicable to mechanisms for both mammalian development and cancer ¹. The Hedgehog (Hh) signal transduction pathway is one such pathway. The initial discovery uncovered a gene locus that when mutated induced the abnormal development of hair-like projections on flies (denticles) such that its physical appearance was reminiscent of the porcupine-like mammal called hedgehog. The Hh locus was found to encode a 50 kDa precursor protein autocatalytically processed to the 19-20 kDa functional protein that was later discovered to be the ligand for a receptor encoded by another locus called *Patched* (*Ptch*). Hh ligand binding to its receptor *Ptch* relieves the inhibition exerted on a vast signaling cascade, which includes *Smoothened* (*Smo*), and a repressor complex (Costal 2, Fused kinase, Suppressor of Fused) that regulates the availability of the transcriptional regulator *Cubitus Interruptus* (*Ci*) ². Details of the *Drosophila* pathway have been extensively reviewed elsewhere ³⁻⁵. Moreover since this review focuses on Hh signaling in the mammalian gastrointestinal tract during

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transformation, key components of the mammalian Hh pathway and what is known regarding canonical Hh signal transduction will be briefly summarized first for several tissue types ⁶⁻⁹. Second, the role of Hh signaling during neoplastic transformation for individual cancers of the gastrointestinal (GI) tract will be described with an emphasis on the response by stromal cells. Stromal cells have typically been the targets of small molecule development and natural product inhibitors (nutraceuticals) with the expectation that they will serve as adjuvant therapies for Hh responsive cancers ^{10, 11}. The final section will discuss specific examples of how Hh signaling contributes to transformation by activating tumor associated mesenchymal and immune cell types. Investigations into the mechanism of Hh signaling has led to the discovery that some cancers are ligand independent (non-canonical signaling) ¹² and will require examination of therapies that inhibit downstream signaling components such as the mammalian homolog of Ci called glioma-associated protein 1 (Gli1) ^{13, 14}. In addition, one might query whether components of the Hh signaling pathway can be used as biomarkers ¹⁵. Interestingly, it has recently been reported that Shh circulates in plasma raising the possibility of using the ligand as a biomarker in some cancers ¹⁶.

Overview of the Hh Signaling Pathway in Mammalian Cells

Canonical Hh signaling involves *epithelial* expression of the ligands, which subsequently bind the 12-pass transmembrane receptors Ptch1 and Ptch2 to relieve their inhibitory influence on an adjacent 7-pass transmembrane Hh activator called Smo. Smo is located on ectodermal (neural) or mesodermal-derived cell types that respond to the Hh ligands (Fig. 1). Co-receptors that bind ligand and cooperate with Ptch to modulate the cellular response such as proliferation include members of the immunoglobulin superfamily, Growth arrest specific 1 (Gas1), CAM-related/down-regulated by oncogenes (Cdo) and brother of Cdo (Boc) ¹⁷⁻¹⁹. Mammalian cells express three Hh ligands--Sonic Hh (Shh), Indian Hh (Ihh) and Desert Hh (Dhh) that bind the Ptch receptor with apparently the same affinity ⁶ (Table 1). In the gastrointestinal tract, the major ligands expressed are Shh in the proximal gut (esophagus, stomach, liver and pancreas) and Ihh in the midgut and hindgut (small intestine and colon) ²⁰. However, Dhh expression appears to be tissue-restricted to the nervous system and testes ²¹. Some tissues exhibit differential potency amongst the ligand paralogs with respect to Hh signaling (Shh>>>Ihh>Dhh). Although the luminal GI tract (stomach, small intestine and colon) constitutively expresses Hh ligands during development and after birth, parenchymal organs (liver and pancreas) express the ligands only in mature tissues and when injured ²²⁻²⁶.

Unlike Ptch, Smo is a heterotrimeric guanine nucleotide binding-protein coupled receptor (GPCR) that can activate at least two intracellular signaling cascades-- a non-canonical, ligand-independent pathway that ultimately modulates the actin cytoskeleton by activating Rac1 and Rho1 GTPases ^{27,28} or a canonical, ligand-dependent pathway via Gli2 activation. Canonical signaling through Smo involves intracellular activation of Gli2 by limited proteolysis. Full-length Gli2 resides in the cell cytoplasm coupled to a suppressor complex comprised of Fused kinase (Fu), Suppressor of Fused (SuFu) and Costal2 (Cos2). Gli2 repression of target genes occurs after a series of phosphorylation steps by protein kinase A (PKA), glycogen synthase 3 (GSK3) and casein kinase I (CK1), which directs Gli2 to the proteasome for limited protein degradation after ubiquitination by the Skip-Cullin-F-box (SCF) protein/ Transducin repeat Containing Protein (TrCP) complex (Fig. 2). Limited degradation of phosphorylated Gli2 removes the C-terminal activation domain uncovering the N-terminal repressor domain. The partially degraded DNA binding protein then translocates to the nucleus and forms a complex with co-repressors such as HDAC-Sin3A to block target gene activation ^{29, 30}. Smo activation releases Gli2 from the suppressor complex and the transcription factor preventing phosphorylation by PKA, GSK3 and CK1. Limited

proteasomal degradation in the absence of specific phosphorylation removes the N-terminal repressor domain producing the Gli2 activator version of the protein, which translocates to the nucleus to bind the promoters of genes induced by Hh signaling. Examples of genes induced by Gli2-mediated Hh signaling include its receptor *Ptch*, *Hedgehog interacting protein (Hhip)* and the transcription factor *Gli1*²⁹. Thus *Gli1*, *Ptch* and *Hhip* are general transcriptional targets of canonical Hh signaling activity. Gli1 mediates transcriptional activation; whereas *Ptch* and *Hhip* are transmembrane receptors that block the pathway, albeit by different mechanisms. *Hhip* binds and sequesters ligand; whereas, ligand binding to *Ptch* relieves its inhibition on Smo de-repressing Hh signaling. Examples of numerous tissue-restricted genes regulated by Hh signaling include Vascular Endothelial Growth Factors (VEGF), Angiopoietin-1 and *Angiopoietin-2* in endothelial cells^{31, 32}. *Snail*, *Zeb1,2*, *Twist2*, *FoxF1* in fibroblasts³³⁻³⁵ -smooth muscle actin, vimentin and IL-6 in myofibroblasts^{36, 37}, *N-myc* in neurons regulating development and myelination^{38, 39}, *BMI*, *Sox2*, *Nanog* in cancer stem cells^{7, 40} (Table 1).

The Hh pathway has evolved to represent crosstalk between the endodermally-derived epithelium that produces the ligand and responding cells derived from ectoderm (neural cells) and mesoderm (fibroblasts, immune cells, myocytes) (Fig. 1). In the adult gastrointestinal tract, it is the stromal cells derived from meso- or ectodermal cell layers that typically express the Hh signaling receptor and transcription factors. However, immunohistochemical staining of adenocarcinomas from the gut and other cancer tissue types suggest that these epithelial-derived human tumors are capable of both ligand and receptor expression²⁶. This observation implies autocrine regulation of neoplastic cell growth that emerges during transformation since *Ptch*, *Smo* and *Gli1*, *Gli2*, *Gli3* mRNA expression is essentially never observed in the normal gut epithelium⁴¹.

In addition to expression of specific signaling components, some Hh responding cells also produce an extracellular organelle called the primary cilium, which is required to properly transduce canonical Hh signaling^{42, 43}. Primary cilia are collections of acetylated -tubulin arranged as nine dimers in a cylindrical configuration with (9+2) or without a central dimer (9+0) encased within a plasma membrane sheath. Regardless of the arrangement, primary cilia are motile or non-motile sensory organelles. Although some chemosensory cells such as tuft cells contain bundles of acetylated -tubulin at the cell apex, these bundles do not associate with basal bodies and therefore are not considered primary cilia. A notable characteristic of primary cilia is that there is only one cilium per cell⁴⁴. Acetylated -tubulin dimers form tracks that move diverse signaling molecules, e.g., Smo, Gli2 or Gli3, bidirectionally via the ciliary transport complex comprised of proteins affected in the human obesity-prone Bardet-Biedl syndrome (BBS), intraflagellar transport proteins (IFT), kinesin (Kif) and dynein^{45, 46} (Fig. 2). All cells are generally thought to produce primary cilia. Nevertheless, these extracellular organelles have been best characterized for a subset of stromal cells, e.g., fibroblasts, myofibroblasts, myocytes, T lymphocytes and neurons. Some lamina propria-residing stromal cells throughout the entire gastrointestinal tract produce primary cilia, as well as a subset of epithelial cells lining the stomach and biliary ducts; whereas intestinal and colonic epithelial cells do not⁴⁷ (Fig. 3). Moreover, the gastric epithelial cells expressing primary cilia are mostly endocrine cells that secrete gastrin, ghrelin or somatostatin⁴⁷. Pancreatic duct cells and endocrine cells in the islet express primary cilia, but normal acinar cells do not⁴⁸⁻⁵⁰. Bile duct cholangiocytes express primary cilia but hepatocytes and endothelial cells lining hepatic sinusoids do not^{51, 52}. The canals of Hering are lined by hepatocytes that segue into cilia-expressing cholangiocytes as they carry bile from the central portion of the hepatic lobule to the terminal bile ducts in the periphery⁵³. The canals of Hering are distinguished by characteristic immunohistochemical staining for biliary cytokeratins 19 and 7. Both Hh ligands and Hh-regulated transcription factors are expressed in the canals of Hering, which coincides with the location of liver

progenitor cells, suggesting possible autocrine Hh regulation within this distinct site. This observation also raises the possibility that if the hepatic progenitor cell produces a primary cilium that it could be regulated by autocrine Hh signaling ⁵².

Hedgehog in GI Cancers

Hedgehog signaling initiates cancer in some organ systems, e.g., skin (basal cell), brain (medulloblastoma) ^{54, 55}, but a clear etiologic role has not been described for gastrointestinal cancers in the luminal GI tract such as esophagus, colon and stomach ⁵⁶. Nevertheless since there are several Hh signaling inhibitors currently used in clinical trials, it will be important to better define whether and how they might be used to treat gastrointestinal cancers, all of which have a poor prognosis if diagnosed in the late stages. Beachy and coworkers were the first to demonstrate widespread expression of Hh signaling proteins in gastrointestinal tract tumors and implicate their role in cancer progression ⁵⁷. Subsequent studies confirmed the expression of Hh pathway components in cancer tissue and cell lines, putative cancer stem cells and the stroma ^{26, 58-60}. The pattern of Hh ligand expression and its signaling components have been used to determine the direction of signaling with implications for developing anti-cancer strategies ⁶¹. Typically canonical Hh signaling indicates that the ligand is produced in an epithelial cell and received by a stromal cell (Fig. 1). However there are several other signaling mechanisms that neoplastic cells use to promote unregulated growth ^{8, 62}. During neoplastic transformation, canonical signaling indicates ligand expression from the adenocarcinoma to the supporting stroma (paracrine signaling). However, reverse paracrine signaling suggests ligand expression by stromal cells that regulate epithelial-derived adenocarcinomas. Autocrine signaling has been described for both epithelial-derived cancer cells as well as cells within the tumor microenvironment, with the obvious phenotype being expansion of either the glandular adenocarcinoma cell compartment or the stromal cell compartment ⁶³. Finally, a number of reports have implicated a role for non-ligand dependent tumor regulation in which mutant Ptch (Gorlin's syndrome) and SuFu or constitutively active Smo and Gli proteins initiate the cancer phenotype ⁶⁴⁻⁶⁷. Smo and Gli proteins are druggable targets within the Hh pathway currently in clinical trials ⁶⁸⁻⁷¹. More recent observations show that tumor resistance emerges in the presence of Smo inhibition underscoring the importance of better understanding non-canonical regulation, especially through Gli1 activation ⁷². It was previously reported that both the TGF and Ras-MAP kinase pathways directly activate Gli1 independent of Smo ^{73, 74}. Moreover, it is now evident that cytokines activate Gli1 ⁷⁵. Das et al. reported that the inflammatory cytokine osteopontin (OPN) secreted by epithelial cancers into the tumor microenvironment modulates AKT-GSK3 signaling ⁶⁴. Gli1 binds to the OPN promoter to induce its expression, while OPN, a phosphoglycoprotein inhibits GSK3, which allows Gli1 to accumulate in the nucleus. cMyc binding and induction of Gli1 in Burkitt lymphoma cell lines represents another example of non-canonical Gli1 transcriptional activation. The authors found that the Gli inhibitor GANT 61 increases apoptosis and reduces the viability of Burkitt lymphoma cell lines, while exposure to Hh ligands or the Smo inhibitor cyclopamine had no effect ¹³.

Esophageal cancers

Although squamous cell carcinoma is the most frequent type of esophageal cancer, the prevalence of adenocarcinoma of the esophagus is rapidly increasing, especially in developed countries where the 5 year survival ranges from 15 to 25 percent ⁷⁶. Obesity and esophageal reflux disease are the major risk factors for Barrett's esophagus, a precursor lesion and risk factor for adenocarcinoma of the esophagus especially if features of intestinal metaplasia and dysplasia are present. By contrast, cigarette smoke is the major risk factor for squamous cell carcinoma, suggesting that there are genetic differences that distinguish these two histologic subtypes. Gene amplification events in basal progenitor cells of stratified

epithelium were frequently observed for squamous cell cancer compared to adenocarcinoma at gene loci such as paired homeobox 9 (PAX9) on chromosome 14 and Sry-related HMG box 2 (Sox2) ^{77, 78}. Downstream regulators of Hh signaling are overexpressed in both adenocarcinoma and squamous esophageal cancers ⁷⁹. However, the most consistent observation was the overexpression of Gli1 in Barrett's and adenocarcinoma of the esophagus ^{80, 81}. Cdx2 is a transcription factor and marker for intestinal metaplasia and cell proliferation in gastric lesions and Barrett's esophagus ⁸²⁻⁸⁵. Using chromatin immunoprecipitation, Rizvi et al. showed that Gli1 binds to the Cdx2 promoter in Barrett's-associated adenocarcinoma cell lines (SKGT4 and FLO-1) and that the activation occurs independently of Smo ⁸¹. Thus non-canonical signaling appears to mediate transition from squamous to columnar epithelium creating an environment suitable for Barrett's intestinal metaplasia ⁸⁶.

Recently, two genome-wide analyses of esophageal adenocarcinomas revealed polymorphisms at several gene loci including TP53, CDKN2A, SMAD4, the Major Histocompatibility Complex locus and chromosome 16q24.1, which associates with predisposition to Barrett's esophagus ^{86, 87}. Chromosome 16q24.1 is closest to the locus for FOXF1, which is an Hh signaling target gene. The Forkhead homeobox (FOX) transcription factor gene cluster (*FOXF1*, *FOXC2*, *FOXL1*, *MTHFS*) has been implicated in foregut development (lung, esophagus and stomach) but also micro-deletions in the *FOX* locus generate a phenotype resembling VACTERL (vertebral anomalies, gastrointestinal atresias, congenital heart malformations, urinary tract malformations, lung anomalies and alveolar capillary dysplasia) ⁸⁸. At present, it is not clear whether the polymorphisms found in esophageal adenocarcinomas result in elevated or reduced expression of FOX transcription factors. However, prior work from Kaestner and co-workers showed that the *FOXF1* and *FOXL1* homeobox genes in stromal cells mediate Hh ligand-mediated suppression of Wnt signaling. More recently, *Ihh* regulation of *Foxf2* was shown to inhibit intestinal polyp development, consistent with the prior observation that canonical activation of these Forkhead homeobox genes in stromal cells suppresses epithelial proliferation ^{89, 90}. Collectively, these studies suggest that FOX transcription factors activate a program of genes in stromal cells that inhibits epithelial proliferation.

Gastric cancer

Gastric cancers are the 2nd leading cause of cancer deaths worldwide (www.globocan.iarc.fr) and like esophageal cancer, the 5 year survival rate is about 20 percent ⁹¹. Most gastric cancers are intestinal-type or diffuse adenocarcinomas that arise from the epithelium. Compared to the intestinal-type, diffuse gastric cancers are characterized by rapidly proliferating epithelial cells that do not form glandular structures and are highly invasive. By contrast, gastrointestinal stromal tumors (GIST), which arise from the interstitial cells of Cajal are positive for tyrosine kinase receptors, e.g., cKit + (CD117) inhibited by imatinib or the platelet-derived growth factor (PDGF) receptor alpha+ ^{92, 93}; while diffuse gastric cancers exhibit inactivating mutations in the *E-cadherin* gene (*CDH1*), an inhibitor of the pro-proliferative Wnt- catenin pathway ⁹⁴. Until the late 1980s, gastric adenocarcinomas typically arose in the distal stomach (antrum). Interestingly, a switch in the primary cancer site from distal to proximal stomach has coincided with the increased use of gastric acid blocking agents or proton pump inhibitors. Using the SEER (Surveillance Epidemiology & End Results) database (www.seer.cancer.gov), Camargo et al. reported that cancers in the distal stomach (antrum) have decreased, while more proximal gastric cancers in the corpus and cardia have increased ⁹⁵. Extrapolating from these epidemiologic trends, adenocarcinomas of the stomach appear to exhibit a different behavior depending on their location suggesting that different molecular mechanisms regulate neoplastic transformation in the proximal and distal gastric sub-sites ^{95, 96}.

Over the past several years, we have characterized a substantive role for Hh signaling in the homeostasis of the stomach^{6, 97-99}. Apparently a substantial proportion of Shh ligand in circulation during *Helicobacter* infection originates from the gastric parietal cells and functions as a chemoattractant for myeloid cells¹⁰⁰. Gastric cancer initiated by chronic inflammation, e.g., *Helicobacter* infection, typically progresses to atrophic gastritis, metaplasia, dysplasia then adenocarcinoma¹⁰¹. Recently, we reported that Gli1 is required for the transition from chronic gastritis to corpus metaplasia or spasmolytic polypeptide-expressing metaplasia (SPEM) 6 months after *Helicobacter* infection in mice¹⁰². However, ectopic expression of Shh ligand in the corpus was not sufficient to induce metaplasia unless infected with *Helicobacter*. Although super-physiologic levels of Shh expression increased the intensity of the inflammation and induced metaplasia, our study underscores the importance of Gli1+ inflammatory cells in initiating preneoplastic changes. Treating the macrophage-like RAW 264.7 cells with Shh ligand did not induce Gli1 expression. This result was consistent with prior studies suggesting that mature myeloid cells do not respond to Hh ligand despite the expression of *Ptch1* and *Smo* mRNA^{103, 104}. Indeed, mutations in *Ptch1* and *Smo* are infrequent in gastric cancers¹⁰⁵. Acutely, Shh ligand acts as a chemoattractant recruiting inflammatory cells to the stomach¹⁰⁰, but chronically myeloid cells do not appear to be responsive to canonical signaling indicating that non-canonical Hh signaling modulates Gli1 activity during chronic gastritis^{6, 75, 102}. Thus the use of Hh inhibitors in human subjects chronically infected with *Helicobacter* might effectively prevent pre-neoplastic mucosal changes, i.e., SPEM, from which gastric adenocarcinomas can arise¹⁰⁶.

In contrast to inflammation-dependent regulation of *Gli1* in the corpus¹⁰², we found using LacZ reporter mice that *Gli2* but not *Gli1* expression increased in hyperplastic antrums of gastrin null mice⁷⁵. Loss of gastrin predisposes to tumor formation in the antrum but not the corpus¹⁰⁷. Further, the pro-inflammatory cytokine IL-1 stimulates expression of *Gli2* instead of *Gli1* in the AGS gastric cell line. Thus, the Hh-related transcription factor in the antrum initiating preneoplastic changes appears to be epithelial *Gli2* and not *Gli1*, whose expression remained exclusively in the stroma⁷⁵. This study uncovered the possibility that transformation in the gut can be mediated by epithelial expression of Gli2 independent of Hh ligand.

Colon Cancer

In the colon, several studies have implicated Hh signaling in proliferation and neoplastic transformation. Qualtrough et al. examined the expression of Hh signaling components in colorectal cell lines and found expression of Shh and Ihh ligands as well as Ptch, Smo and Gli1¹⁰⁸. Since cell lines expressed both ligands and signaling components, the authors concluded that Hh-mediated tumorigenesis in the colon is autocrine. Studies by Varnat et al. showed by lineage-tracing with the *Shh*Cre;*ROSA26*^{LacZ} reporter mice that there is strong *Shh* gene expression in the epithelium of adult mouse colons¹⁰⁹. Conditional deletion of *Smo* (*Smo*^{FL/FL}) decreased polyp formation in *APC*^{FL/FL}; *Actin*CreERT2 chimeric mice¹⁰⁹. Clinically, this result might be related to non-canonical activation of Smo due to a *Ptch* mutation (Gorlin's syndrome) or Erk activation of *Gli1* via growth factor induction of Ras. Indeed, Houghton and coworkers showed that the Gli inhibitor GANT61 inhibits colon cell line proliferation^{110, 111}. Vitamin D, a hydroxysteroid, inhibits Hh signaling by facilitating the interaction of Ptch with Smo^{112, 113} and is likely to have relevance since the steroid exhibits efficacy against colon cancer¹¹⁴. Moreover, Vitamin D suppression of Hh signaling has been recently shown to be an effective treatment for clear cell renal and basal cell carcinomas^{115, 116}. Thus the current evidence supports a significant role for Hh signaling-mediated tumorigenesis in the colon through both the canonical and non-canonical pathways.

Pancreatic Cancer

Pancreatic cancer is the 4th leading cause of cancer deaths in the US primarily due to the lack of early symptoms, biomarkers and advanced stage once discovered ¹¹⁷. The 5-year survival rate is less than 5 percent if un-resected. Hh signaling is required for pancreatic development and re-emerges in the adult pancreas when tissue regeneration is required, such as after an inflammatory or toxic injury ¹¹⁸. Hh ligand expression increases during pancreatic tumorigenesis suggesting that canonical Hh signaling mediates tumor progression. Pancreatic cancer generates a robust fibrotic response that has recently been shown to be the result of epithelial to mesenchymal transition (EMT) ¹¹⁹. Using lineage tracing in genetically-engineered mouse models, Stanger and coworkers showed that pancreatic duct epithelial cells exhibit a mesenchymal phenotype before they enter the circulation and seed the liver. This invasive phenotype was accelerated by inflammation. Although the authors did not examine Hh signaling directly in their report, pancreatic duct cells are known to express Shh ligand when inflamed or with transformation ¹⁶. Recent studies show that curcumin-mediated inhibition of Shh-Gli signaling will block EMT ¹²⁰. Moreover, Gli1 deletion in a mouse model of pancreatic cancer generated by expressing oncogenic KRAS impedes cancer progression ²². Although Shh levels in the pre-neoplastic pancreas increase locally, we observed a decrease rather than an increase in circulating levels of the ligand, which we concluded correlated with the increase in fibrosis accompanying cells expressing the Ptch receptor ¹⁶. Thus both canonical and non-canonical signaling appear to participate in the progression of pancreatic cancer. However, the robust stromal response is apparently related to ligand- and Smo-independent components of the Hh pathway ²².

Hepatocellular carcinoma

Hepatocellular carcinoma or hepatomas arise within a chronically damaged organ primarily in response to viral hepatitis or toxins such as alcohol ¹²¹. Alcohol-induced liver cancer primarily proceeds through an intermediate lesion called cirrhosis in which stromal cells proliferate and secrete collagen replacing the normal hepatocyte. By contrast, viral hepatitis can either result in carcinogenesis in the hepatocyte or trigger the cirrhotic response prior to transformation. Low levels of Hh signaling are observed in the normal liver due to overexpression of HhIP, which can prevent any ligand from binding Ptch ⁵². In addition, Hh signaling components decrease as the liver matures ⁵². Additional evidence of Hh signaling in the liver include ciliopathies that result in cystic malformations of the intrahepatic biliary tree and liver fibrosis ⁴³. However, *Ptch* and *Smo* become overexpressed in human hepatomas ¹²²⁻¹²⁴, suggesting a ramping-up of Hh signaling with injury and transformation. Injured hepatocytes release Hh ligands as they undergo apoptosis coincident with a decrease in *HhIP* expression. Hh ligands are then able to bind Ptch to initiate canonical signaling of resident quiescent cells, e.g., hepatic stellate cells, immune cells and myofibroblasts. These stromal cells then release factors such as TGF and PDGF that potentiate Hh signaling via Smo-independent activation ⁵². Diehl and coworkers have nicely shown that induction of Hh ligand expression in hepatocytes stimulates the accumulation of Hh-responsive myofibroblasts, which produce lactate and fuels the proliferation of adjacent malignant hepatocytes ³⁷. Clinical trials in which Gli1 is targeted by a nanoparticle-encapsulated Gli1 inhibitor are underway ¹²⁵. Moreover, inhibition of Hh signaling by the Smo inhibitor GDC-0449 in a mouse model of liver fibrosis and cancer reduced liver tumors and metastatic lesions ¹²⁶. Thus once fibrosis has been initiated, it will likely be important to inhibit Smo independent regulators, i.e., Gli1.

Cholangiocarcinoma

As in the pancreas and liver, Hh signaling in the epithelial cells lining of the biliary tree known as cholangiocytes is primarily active during development ^{127, 128}. However, recent

studies by LaRusso and colleagues have demonstrated that the loss of primary cilia contributes to aberrant activation of both Erk and Hh signaling pathways ¹²⁹. Apparently, the mechano-shear forces of bile flow in the duct are sensed by primary cilia on the cholangiocytes, which in turn regulate normal functions, such as proliferation and transport ¹³⁰. It is known that protrusion of the cilia varies inversely with cell proliferation ^{131, 132}. Consistent with this concept, the authors found that loss of primary cilia by HDAC6 overexpression or preventing cilia regeneration with IFT88-shRNA induces cell proliferation, anchorage-independent growth and activation of the Hh pathway ¹²⁹. By contrast, inhibition of HDAC6 expression by shRNA or by tubastatin-A treatment decreased cholangiocarcinoma tumor growth in a xenograft mouse model. Although the authors do not examine whether activation of Hh signaling in the cholangiocytes is Hh ligand-dependent, the observation that Erks are activated suggests non-canonical signaling activated by loss of primary cilia. This concept is consistent with the observation that primary cilia suppress Hh signaling in cartilage tumors ¹³³.

Hedgehog Signaling in the Mesenchyme

Adenocarcinoma development is a sequential process (metaplasia, dysplasia and neoplasia) generated by the accumulation of genomic aberrations in the epithelial cell and sustained by different immune and stromal cell types in the tumor microenvironment. A prominent feature of the tumor microenvironment for example in breast, lung and pancreatic cancers is the recruitment of cancer associated fibroblasts (CAFs), tumor-associated myeloid and T regulatory cells (Tregs) that retard the access of cytotoxic killer cells to the tumor but also secrete cytokines and growth factors that amplify the fibrotic (stromal) reaction in the tumor ^{134, 135}. In pancreatic cancer, this “stromal reaction” is a prominent feature of cancer development and significantly contributes to the difficulty in developing effective treatments for this deadly cancer. Hedgehog inhibitors targeting Smo decrease proliferation of CAFs in pancreatic cancer and cholangiocarcinomas ^{136, 137}. Moreover, CAFs orchestrate tumor-promoting inflammation ¹³⁸ and produce the oncogenic cytokine IL-6 in gastric cancer ¹³⁹. Myeloid-derived suppressor cells (MDSCs) are a subset of immune regulatory cells that play a prominent role in modulating the tumor microenvironment ¹⁴⁰. Hallmarks of these cells include expression of the CD11b and Gr1 surface markers and their ability to inhibit T cell proliferation ¹⁴¹⁻¹⁴³. In inflammation-driven gastric cancer, cells in the tumor microenvironment include MDSCs and CAFs, which represent a major source of pro-inflammatory cytokines ¹⁴⁴. TAMs and M1/M2-polarized macrophages are also an essential component of the tumor microenvironment ^{145, 146}, which have not been thoroughly investigated in the stomach ^{147, 148}. A prior study demonstrated that MDSCs induce gastric carcinoma on a murine *Rag2*^{-/-} background in the absence of T-cells ¹⁴⁴. This result suggested that MDSCs promote gastric cancer in a T-cell independent manner. Therefore MDSCs might play a dual role by promoting pro-tumorigenic cytokines to initiate carcinogenesis, but also mediate cytotoxic T-cell tolerance of tumor cells ¹⁴⁹. Collectively these immune cell types likely exhibit some phenotypic overlap, and therefore constitute an attractive target for therapy. However, until now the pathways driving emergence of these cell types have not been defined.

To study the role of Hh signaling during *Helicobacter*-induced gastritis, we infected C57BL/6 wild type and *Gli1*^{-/-} mice for two and six months ¹⁰². Chronic gastritis was observed in both groups. However, only wild type mice developed SPEM at 6 months whereas, the infected *Gli1* null mice did not progress to exhibit the metaplastic changes (Fig. 4). Moreover, we found that *Helicobacter* infection induced a subset of myeloid cells expressing CD45⁺MHCII⁺CD11b⁺CD11c⁺ surface markers suggestive of the MDSC phenotype ¹⁰². A microarray analysis of *Gli1*-dependent genes up-regulated during *Helicobacter*-induced metaplasia revealed several genes that regulate myeloid cell recruitment and differentiation,

e.g., serum amyloid protein A3 (*SAA3*), indoleamine 2,3 dioxygenase 1 (*Ido1*), *Schlafen-2* and *Schlafen-4* (*Slfn-2*, *Slfn-4*). *SAA3* regulates myeloid cell recruitment¹⁵⁰. *Ido1* initiates tryptophan catabolism and produces bioactive kynurenine metabolites¹⁵¹. Elevated levels of *Ido1* in colon cancers predict a poor prognosis. Moreover, *Ido1* and subsequently an increase in the kynurenine/tryptophan ratio favors suppression of effector T cell function and differentiation of regulatory T cells, which in turn facilitate immune escape by the cancer¹⁵². *Slfn 2* and *4* are myeloid differentiation factors¹⁵³ that were found to mark the myeloid subpopulation exhibiting MDSC-like surface markers (*CD45*⁺*MHCII*⁺*CD11b*⁺*CD11c*⁺)¹⁰². The MDSC population we identified was not present in the bone marrow and only appeared in the gastric microenvironment in response to chronic inflammation from the *Helicobacter* infection at 6-month suggesting that these cells were “educated” in the inflamed gastric microenvironment (Fig. 4). Despite the robust inflammation in the mouse corpus from *Helicobacter felis* infection, the antrum remained unaffected, consistent with the notion that the corpus and antrum respond differently to inflammation.

We characterized the mechanism by which IL-1 induces mucous cell metaplasia (SPEM) and found that IL-6 was expressed in proliferating mucous neck epithelial cells in the gastric corpus but was absent in the *Gli1*^{-/-}-infected mice (Fig. 4). This result was in contrast to reports that IL-6 is secreted primarily from fibroblasts and myeloid cells¹⁵⁴. Correlating with the increased IL-6 production by mucous cells, SPEM cells expressed high levels of pSTAT3 and were Ki67+. SPEM was rarely observed in the *Shh*-overexpressing mice unless the mice were infected with *Helicobacter*. Therefore, we concluded that *Shh* is not sufficient to drive gastric metaplasia in the absence of chronic inflammation. However, the acute inflammatory response initiated by *Helicobacter* infection was diminished with gastric epithelial deletion of the *Shh* ligand or *Smo* loci demonstrating a role for canonical signaling during acute gastric inflammation¹⁰⁰. In summary, our study shows that Hedgehog-dependent gastric metaplasia depends on a MDSC-like population (Fig. 4). Thus therapies targeting this aberrant myeloid-derived cell population could be essential in preventing the irreversible progression to dysplasia not only for gastric cancer but also for other epithelial-derived neoplasia triggered by chronic inflammation.

Concluding Remarks

In summary, there are several consistent patterns regarding the Hh signaling pathway and carcinogenesis that continue to emerge. First, non-canonical signaling appears to be a significant signaling mechanism used by a variety of tumors to uncouple expression of proliferative signals from the normal regulatory mechanisms. Both *Gli1* and *Gli2* appear to be important targets of pro-proliferative pathways independent of *Smo* as well as Hh ligands (non-canonical Hh signaling). Second, multiple components of the Hh signaling pathway will need to be targeted to effectively block neoplastic transformation¹⁵⁵. Third, the signaling pathways capable of activating non-canonical *Gli1* signaling is ever expanding and includes TGF β , growth factor activation of the Ras-ERK pathway and more recently inflammatory cytokines. Finally, what will this mean for biomarker and small molecule development? The *Shh* ligand clearly circulates in human plasma, but whether it is a reliable marker of tumorigenesis or merely increases its abundance in the setting of receptor uncoupling remains to be determined. A corollary to this concept is the notion that while targeting the ligand with humanized antibodies, nutraceuticals or small molecules is accessible, resistance is likely to emerge since *Gli* proteins appear to be regulated by multiple pathways.

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Figure 1. Canonical Hedgehog Signal Transduction

Canonical Hedgehog signal transduction implies that the ligand, e.g., sonic hedgehog (Shh) is secreted by an epithelial cell and forms a gradient as it diffuses away from the cell. The ligand is sensed by cells in the stroma, which express Hh signaling components and primary cilia (ciliated versus non-ciliated).

Figure 2. Primary cilia and Hedgehog signal transduction

Sonic Hedgehog (Shh) ligand binds its receptor Patch (Ptch), which subsequently releases its inhibitory effect on quiescent Smoothened (Smo) contained in cytoplasmic vesicles. Activated Smo moves *antegrade* into the cilia along tracks comprised of acetylated tubulin coupled to ciliary proteins Bardet-Biedl syndrome proteins (BBS), intraflagellar transport protein (IFT) and *kinesin* proteins. At the vesicle tip activated Smo induces the release of Gli2 from the cilia. Ciliary proteins (BBS and IFT) plus *dynein* move cargo proteins *retrograde* into the cytoplasm. Once cytoplasmic, limited degradation directs the transcription factor to the nucleus to bind and induce Hh-regulated genes Gli1, Ptch and Hedgehog interacting protein (HhIP).

Figure 3. Primary cilia in the luminal gastrointestinal tract

Schematic depiction of cells lining the esophagus, stomach, intestine and colon. The cells in the esophagus are squamous with the exception of the basal cell layer, which are cuboidal. The cuboidal cells of the basal cell layer produce primary cilia. The stomach, small intestine and colon all have columnar epithelia that sit on a basement membrane. Only the stomach contains a few epithelial cells that express primary cilia. Most of these are enteroendocrine cells. None of the epithelial cells in the intestine or colon express primary cilia. There are several cell types within the lamina propria including neurons, fibroblast, immune cells and myocytes.

Figure 4. Paradigm for gastritis-induced pre-neoplastic changes

Shh secreted by gastric parietal cells recruits inflammatory cells to the stomach during the acute phase of *Helicobacter* infection. By 6 months, there is a switch in the quality of myeloid cells present in the stomach that is mediated by Gli1 target genes, e.g., Ido1, Schlafen 2 and Schlafen 4. These cells also express MHCII CD45, Cd11b and Cd11c surface markers suggestive of an myeloid-derived suppressor cell type (MDSC). The cells expressing additional targets of Gli1, i.e., Slfn 2, Slfn4 also produce proinflammatory cytokines IL-1 and TNF. IL-1 stimulates epithelial mucous neck cells to produce IL-6 and the proto-oncogene STAT3 coincident with the development of SP-expressing metaplasia (SP-EM). The Slfn2+, Slfn4+ cells apparently develop within the microenvironment of the inflamed gastric mucosa since these markers were not observed in the bone marrow.

Major components of the Hedgehog signaling pathway

Each column represents a different component of the canonical Hh signaling pathway from ligand to transmembrane receptors to signaling components and tissue specific gene targets. Thus although there are only three Hh ligands, the myriad of genes encoding modulating factors will influence which tissue-specific gene targets become activated.

Table 1

Ligands	Trans-membrane Receptors	Signaling Modulators	Signaling Adaptors	Transcrip-tion Factors	Regulatory Kinases	Gene Targets
Sonic Hedgehog	Patched 1 (Pchl) 9q22.3	Dispatched-multimerization of ligand	Costal2 (COS2)	Gli1	Casein kinase1	General: Pch, HhP, Gli1, cyclinD
Indian Hedgehog	Patched 2 (Pch2) 1p34	Skinny Hedgehog (Skn)-lipid modification of ligand	Fused (FU)	Gli2	Protein kinase A	Endothelial: VEGF, Ang1,2
Desert Hedgehog	Smoothened (SMO)	ADAM proteases	Suppressor of Fused (SuFu)	Gli3	Glycogen-S Kinase 3b	Bone: PTHrP, Igf1,2
	Hedgehog Interacting Peptide (HhIP)		-transducin repeat-containing protein (TrCP)			Neural: N-myc
	Growth Arrest Specific (GAS)1		Skip/Cullin1/F-box (SCF) E3 ubiquitin ligase			Gut: Lasp-1, H,K-ATPase, Gastrin
	CAM-related Down-regulated by Oncogenes (CDO)					Fibroblast: FOXL1, SFRP, SNAIL
	Brother of CDO					Stem Cells: Bmi1, Sox2, Nanog