Hedgehog Signaling Links Chronic Inflammation to Gastric Cancer Precursor Lesions

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SUMMARY

Hedgehog signaling plays an essential role in gastric development, homeostasis, and neoplastic transformation. This article reviews the evidence for its role in the initiation of gastric inflammation due to Helicobacter infection but then chronically polarizes myeloid cells into myeloid-derived suppressor cells creating a microenvironment favoring cancer development.

Since its initial discovery in Drosophila, Hedgehog (HH) signaling has long been associated with foregut development. The mammalian genome expresses 3 HH ligands, with sonic hedgehog (SHH) levels highest in the mucosa of the embryonic foregut. More recently, interest in the pathway has shifted to improving our understanding of its role in gastrointestinal cancers. The use of reporter mice proved instrumental in our ability to probe the expression pattern of SHH ligand and the cell types responding to canonical HH signaling during homeostasis, inflammation, and neoplastic transformation. SHH is highly expressed in parietal cells and is required for these cells to produce gastric acid. Furthermore, myofibroblasts are the predominant cell type responding to HH ligand in the uninfected stomach. Chronic infection caused by Helicobacter pylori and associated inflammation induces parietal cell atrophy and the expansion of metaplastic cell types, a precursor to gastric cancer in human subjects. During Helicobacter infection in mice, canonical HH signaling is required for inflammatory cells to be recruited from the bone marrow to the stomach and for metaplastic development. Specifically, polarization of the invading myeloid cells to myeloid-derived suppressor cells requires the HH-regulated transcription factor GLI1, thereby creating a microenvironment favoring wound healing and neoplastic transformation. In mice, GLI1 mediates the phenotypic shift to gastric myeloid-derived suppressor cells by directly inducing Schlafen 4 (slfn4). However, the human homologs of SLFN4, designated SLFN5 and SLFN12L, also correlate with intestinal metaplasia and could be used as biomarkers to predict the subset of individuals who might progress to gastric cancer and benefit from treatment with HH antagonists. (Cell Mol Gastroenterol Hepatol 2017;3:201–210; http://dx.doi.org/10.1016/j.jcmgh.2017.01.004)

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Hedgehog (HH) signaling initiates cancer in several organ systems, but a clear etiologic role has not been shown for this pathway in gastric cancer. Because HH inhibitors currently are undergoing clinical trials for different types of cancer, understanding the role of HH signaling in regulating the tumor microenvironment becomes an important target to consider. Based on prior mouse studies of increased HH signaling in preneoplastic lesions, we have suggested that the use of HH inhibitors in human subjects chronically infected with Helicobacter might prevent progression of chronic atrophic gastritis to mucous gland metaplasias, a sentinel lesion that increases the likelihood of gastric cancer. Thus, the focus of the current review is to understand the basis for HH signaling in normal adult stomach and how this developmental pathway might play a role in neoplastic transformation. Because our current understanding of HH signaling in the stomach arises from transgenic mouse models, the information presented refers to the mouse except when information from human studies exists.

Role of Hedgehog Signaling in Gastric Homeostasis

To date, there are 3 known mammalian genes encoding the hedgehog ligands: Sonic hedgehog (SHH), Indian hedgehog, and Desert hedgehog. During embryonic development, SHH is expressed throughout the gut and in other foregut-derived organs (eg, lung, pancreas). Although its function in mature gastric epithelium was not initially studied in adult mammals, it became apparent that SHH remains highly expressed in the stomach once expression in...
the intestine diminishes.\textsuperscript{17,18} Subsequently, it was reported that SHH regulates epithelial cell maturation and differentiation in the adult stomach.\textsuperscript{19,20} Normally, SHH is expressed in mature acid-secreting glands of the adult mouse and human stomachs, primarily within parietal cells\textsuperscript{19,21–23} (Figure 1). During progression from the inflamed stomach to gastric cancer, the acid-producing parietal cells fail to produce acid and eventually are replaced by mucous-secreting cells that express spasmolytic polypeptide (SP) or trefoil factor 2.\textsuperscript{7,24} Mostly in mice, but also in human subjects, SP-expressing mucosa (SPEM) is a type of oxyntic gland atrophy.\textsuperscript{25,26} In concert with parietal cell atrophy, SHH expression in these acid-producing cells also is lost.\textsuperscript{23,27} Although SHH expression diminishes along with loss of parietal cells, the expanding mucous cell compartment or SPEM continues to produce acid and eventually are replaced by mucous-secreting cells that express spasmyloytic polypeptide (SP) or trefoil factor 2.\textsuperscript{7,24} In mice, but also in human subjects, SP-expressing mucosa (SPEM) is a type of oxyntic gland atrophy.\textsuperscript{25,26} In concert with parietal cell atrophy, SHH expression in these acid-producing cells also is lost.\textsuperscript{23,27} Although SHH expression diminishes along with loss of parietal cells, the expanding mucous cell compartment or SPEM continues to produce SHH in both human subjects\textsuperscript{20,23} and rodents,\textsuperscript{4,27} but remains unprocessed, maintaining the full-length 45-kilodalton form\textsuperscript{28} (Figure 1). Surprisingly, even unprocessed Hedgehog protein (\textit{Drosophila}) shows activity where it traffics to the cell membrane to participate in cell–cell signaling.\textsuperscript{29} This result suggests that aberrant HH signaling in cancer might function as an autocrine or paracrine regulator, especially in the stem cell niche.\textsuperscript{30–32}

Processing of SHH to its active form (19 kilodaltons) in parietal cells becomes compromised in the absence of gastric acid.\textsuperscript{28} Atrophy of parietal and zymogenic (chief cell) lineages result in hypochlorhydria and reduced serum pepsinogen I (A) levels compared with pepsinogen II (C).\textsuperscript{33–39} These zymogens are proteins encoded by different gene loci that are used clinically to indicate preneoplastic changes in the stomach.\textsuperscript{38,39} Pepsinogens A and C are converted to the enzymatically active aspartic proteinases, pepsin A and pepsin C, through intramolecular self-cleavage.\textsuperscript{39,40} We showed previously that pepsinogen A is produced primarily in the mouse corpus by parietal cells, whereas pepsinogen C is produced primarily by both mucous neck and chief cells throughout the stomach.\textsuperscript{28} This result is consistent with the exclusive expression of pepsinogen A in the human corpus and not the antrum, whereas pepsinogen C marks mucous cells of both the antrum and corpus (\textit{www.proteinatlas.org}). Pepsin A prefers to cleave proteins at hydrophobic and aromatic residues, particularly at phenylalanine (F) when the pH is less than 2. By contrast, pepsin C recognizes a broader consensus site and uses a wider pH spectrum than pepsin A.\textsuperscript{40,41} Specifically, we showed using site-directed

\textbf{Figure 1.} SHH expression in the stomach corpus of wild-type (WT) and \textit{Gli1}\textsuperscript{−/−} mice. Shown is the co-localization of SHH with E-cadherin, F4/80 (macrophage/myeloid marker), or α-smooth muscle actin (SMA) (myofibroblasts) protein markers in the absence or presence of \textit{Helicobacter felis} infection. 4′,6-diamidino-2-phenylindole (DAPI) indicates cell nuclei. Arrows indicate the presence of SPEM. Reprinted with permission from El-Zaatari et al.\textsuperscript{4} Scale bars = 100 μm.
mutagenesis that pepsin A cleaves the nascent 45-kilodalton SHH polypeptide at residue 200 (SGGC\textsuperscript{200}P) to generate the active 19-kilodalton form, whereas pepsin C does not cleave SHH peptide\textsuperscript{28} (Figure 2).

**Processing of Sonic Hedgehog**

Perhaps because of difficulties in measuring SHH protein during development, most studies primarily have relied on messenger RNA (mRNA) levels and not protein to study SHH expression. Nevertheless, prior studies examining generation of SHH protein showed that the protein undergoes a complex series of processing steps that includes the initial generation of a 45-kilodalton precursor polypeptide, subsequent removal of the N-terminal 24 amino acid residue signal peptide, and then cleavage of the amino terminus to generate a 19-kilodalton protein that can be modified post-translationally by palmitate and cholesterol\textsuperscript{42-45} (Figure 2). These studies performed initially in *Drosophila* showed that cholesterol transferase activity resides in the C-terminal portion of the Hedgehog molecule such that esterification of cysteine 198 by the transferase results in intramolecular autocatalytic cleavage of the 45-kilodalton precursor\textsuperscript{46} (Figure 2). Additional studies have shown that the extent of lipid modification modulates SHH diffusion away from the cell of origin. Apparently, a shorter range of diffusion correlates with a higher degree of lipid (palmitate) modification and membrane association.\textsuperscript{47,48}
By contrast, we showed in the adult mouse and human stomach that both SHH processing and gene expression are linked to acid secretion. Specifically, infusion of the hormone gastrin over 2 weeks using osmotic pumps stimulates SHH gene expression in a hypochlorhydric gastrin-deficient mouse in concert with re-establishing acid secretion. Moreover, post-translational processing of SHH precursor to its secreted form depends on cleavage by the acid-activated protease pepsin A generated from pepsinogen A. Thus, we concluded that generation of the biologically active form of SHH in the stomach is regulated. If gastric acidity is reduced, as a result of inhibition of acid secretion (omeprazole therapy) or loss of the parietal cell (atrophy), then pepsin A is not activated and most of the precursor SHH protein produced is not cleaved into its functionally active 19-kilodalton form. These observations are consistent with the finding that oxyntic gland atrophy (specifically loss of the parietal cell) correlates with reduced pepsinogen A to C ratios. More importantly, these observations suggest that SHH is processed within parietal cells. SHH co-localizes to the tubulovesicle fraction with the H^+_K^+-adenosine triphosphatase (ATPase) enzyme. With the addition of a secretogogue, movement of the SHH precursor to the canalicular membrane coincides with insertion of the proton pump into the apical membrane. This thesis would account for the ability of SHH processing and secretion to coincide with the production of gastric acid. Moreover, this mechanism would predict that a significant amount of SHH would be less lipid-modified and capable of diffusing both basolaterally and apically throughout the gastric gland and into the circulation. Indeed, we and others have found that blood levels of SHH peptide originate in part from the parietal cell and can be detected in the circulation of human volunteers.

Hedgehog Signaling in the Adult Stomach

Canonical HH signaling involves epithelial expression of ligand (typically SHH in the stomach), which subsequently binds to its receptor Patched (PTCH) and relieves its inhibitory influence on an adjacent transmembrane HH activator called Smoothened (SMO). Once SMO inhibition is relieved, glioma-associated protein 2 (GLI2) is processed to an activator form, translocates to the nucleus, and then binds to the promoters of HH effectors including PTCH, hedgehog-interacting protein (HHIP), and GLI1. Thus, GLI1, PTCH, and HHIP are transcriptional read-outs of canonical HH signaling activity. The extracellular signals regulating shh gene expression in the stomach are not well defined but might correlate with those reported in other tissues. For example, during pancreatic development, shh expression appears to be regulated by activin A. During limb bud development, fibroblast growth factors and bone morphogenetic proteins regulate shh expression. Shh null (shh−/−) mice do not survive past postnatal day 1. However, the stomachs of these mice were hyperplastic and further underscored that loss of HH signaling shows important functional consequences. Subsequent studies have been performed in adult mice using a H^+,K^+-ATPase-Cre transgene to delete the shh gene locus only in parietal cells. Conditional deletion of the shh gene resulted in parietal cell atrophy and foveolar hyperplasia. Indeed, SHH signaling is required for optimal H^+,K^+-ATPase expression. In addition, a prior study in a gastric cancer cell line showed that increased gastric acidity stimulates shh expression. Accordingly, modulators of gastric acid such as gastrin and somatostatin (SST) have been shown to modulate SHH levels and HH signaling.

Inflammation Regulates Gastric Acid Secretion and SHH

We previously examined modulation of gastric acid secretion by proinflammatory cytokines and reported that both gastrin and SST are regulated, albeit in a reciprocal manner, by cytokines in vivo and in primary cell cultures. Interferon γ (IFNγ), a T1-helper cytokine, stimulated gastrin and inhibited SST, and interleukin-4 (IL4), a T2-helper cytokine, stimulated SST and inhibited gastrin. Thus, similar to the negative feedback regulation known to exist for gastrin and SST, immune modulators impart parallel control of these peptides and therefore acid secretion. Teleologically, it makes sense that the innate immune system regulates gastric acid because acid is one of the first defense mechanisms that is activated by the gastrointestinal tract to combat invading organisms. However, prior studies by Beales and other investigators have shown by using a rabbit primary culture system that either IL1β or tumor necrosis factor-α infusion into rodents suppresses acid secretion. Subsequently, more ominous implications became attributed to cytokine suppression of acid secretion when El-Omar et al and other investigators showed that IL1β, but not tumor necrosis factor-α polymorphisms, predispose human subjects to gastric atrophy and gastric cancer. Testing the significance of the polymorphism, Tu et al reported that transgenic overexpression of IL1β in mouse parietal cells induced gastric inflammation and dysplasia. By contrast, IFNγ polymorphisms do not appear to correlate with gastric atrophy. Indeed, we reported that proinflammatory cytokines show differential effects on SHH expression with IFNγ stimulating SHH expression and IL1β inhibiting expression when added acutely (6 h) to parietal cell cultures. It generally has been assumed that all proinflammatory cytokines generated during bacterial colonization exert the same effect on gastric cells. However, our results in primary parietal cell cultures coupled with differences in the association of cytokine polymorphisms support the likelihood that the effects of these proinflammatory cytokines on parietal cells are distinct.

SHH Regulates Gastrin and Gastric Acidity

To examine the impact of HH signaling in vivo, we generated a transgenic mouse that secreted the natural inhibitor of HH ligands called HHIP expressed from the...
cell-specific \( H^+,K^+\text{-ATPase} \) \( \beta \) subunit promoter.\(^6\) Our results showed that loss of HH signaling in parietal cells, caused by the production of secreted HHIP, reduced \( H^+,K^+\text{-ATPase} \) gene expression and gastric acid.\(^6\) Normally, hypochlorhydria stimulates gastrin gene expression through a decrease in SST.\(^7\) Accordingly, we found coincident with increased plasma gastrin occurring in the \( hh\) transgenic mice that \( st \) gene expression also decreased. This result showed that modulation of HH signaling in parietal cells is sufficient to activate the normal feedback mechanisms typically attributed to gastrin and SST. Indeed, we reported that both antral G and D cells possess primary cilia, organelles protruding from the plasma membrane, which transduce HH signaling.\(^7,7^6\) Therefore, gastric endocrine cells are capable of responding directly to the SHH ligand. We showed that transgenic overexpression of GLI2 suppresses gastrin gene expression.\(^5\) Thus, gastrin stimulates gastric acid and SHH expression whereas HH signaling suppresses gastrin expression.\(^5\) Taken together, the production of SHH by parietal cells and the ability of gastric endocrine cells to sense the ligand through primary cilia are consistent with a central role for HH signaling in the feedback regulation of gastric acidity.

**Cross-Talk Between Gastric Epithelium and Mesenchyme**

Canonical HH signaling typically involves cross-talk between epithelial cells that produce the ligands, for example, SHH, Indian hedgehog, and cells that express the receptor-signaling complexes, such as PTCH, SMO, HHIP, and transcription factors GLI1, GLI2, and GLI3. Therefore, to identify gastric cells that respond to HH ligands in the absence and presence of *Helicobacter*, we used the Gli-LacZ reporter mouse. The \( \beta \)-galactosidase complementary DNA was recombined into the \( gli1 \) gene locus to create heterozygous or homozygous genotypes.\(^53\) In the absence of a *Helicobacter* infection, we found that \( \alpha \)-smooth muscle actin–positive myofibroblasts were the major population expressing the LacZ reporter.\(^4\) However, during a *Helicobacter* infection, the LacZ\(^+\) cells infiltrating the gastric mesenchyme were myeloid cells and correlated with parietal cell atrophy and the emergence of SPEM.\(^5\) Strikingly, when either the \( \text{Gli}^{1\text{lacZ}^+/+} \) or \( \text{Gli}^{1\text{lacZ}/\text{lacZ}} \) mice were infected, they did not develop SPEM. This result showed that canonical HH signaling was required for SPEM.\(^5\) Use of microarray analysis to identify GLI1 target genes showed that induction of a myeloid differentiation factor called Schlafen 4 (SLFN4) contributed to the GLI1-dependent development of SPEM. However, the induction of \( slfn4 \) mRNA coincident with SPEM was time-dependent, and required 6 months to be expressed in wild-type mice or 4 months in the presence of constitutively elevated levels of SHH ligand (\( pCMVShh \)).\(^4\) Overexpression of SHH accelerated the development of SPEM, but only in the presence of a *Helicobacter* infection.\(^5\) Therefore, both SPEM and the appearance of SLFN4\(^+\) myeloid cells required GLI1. We concluded that HH signaling is required to polarize a subset of myeloid cells and transition gastric mucosa from a proinflammatory to a preneoplasia state, but only in the presence of the bacterial infection (Figure 3).

The heterogeneous populations of myeloid cells that emerge during chronic atrophic gastritis are phenotypically myeloid-derived suppressor cells (MDSCs). MDSCs show both monocyte (Mo-MDSC) and granulocytic (Gr-MDSC) features, suggesting that they might represent the reprogramming of monocytes and neutrophils recruited to the tissue.\(^6\) MDSCs suppress T-cell function by consuming L-arginine through arginase 1 and inducible nitric oxide synthase activated to generate reactive oxygen species. L-arginine is required for T-cell proliferation and its ability to block cancer growth.\(^9,7^4\) Therefore, the presence of MDSCs has been strongly linked to tumor promotion, owing to their immunosuppressive role once cancer has emerged.\(^8,7^2\) By contrast, our recent studies strongly have suggested that a subset of MDSCs appear during the preneoplastic phase of cancer development and require HH signaling.\(^5^1\)

Schlafen are a family of molecules strongly induced by type 1 IFNs (IFN\(\alpha\)), and their expression typically correlates with immune cell quiescence.\(^9,4,4^\) Specifically, SLFN4 modulates myelopoiesis.\(^8^5\) Coincident with the apoptosis of parietal cells, tissue levels of damage-associated molecular patterns (DAMPs) accumulate, culminating in increasing IFN\(\alpha\) secretion from plasmacytoid-derived dendritic cells.\(^5^1,8^6\) Toll-like receptors (TLRs) 3, 7, 8, and 9 are the intracellular TLRs that recognize DAMPs by initiating a complex cascade of signaling molecules (eg, myeloid differentiation primary response gene 88, interferon factor regulatory transcriptions, and signal transducer and activator of transcriptions) that ultimately induce expression of type 1 interferons (IFN\(\alpha\) and IFN\(\beta\)) (Figure 3). Recent studies have indicated that chronic *Helicobacter* infection in both mice and human beings induces TLR9 expression.\(^8\) Apparently, DAMPs and their subsequent activation of TLR9 are associated with immune suppression.\(^9,7^9,9^0\) Moreover, there is an increased incidence of gastric neoplasia in subjects with TLR9 polymorphisms and *H pylori* infection.\(^9,7^2\) Increased tissue levels of type I interferons can suppress inflammation.\(^8^9\) In addition, DAMPs have been implicated in the reprogramming of mononuclear cells to become Mo-MDSCs.\(^8^0\) In addition, our results show that DAMP signals also polarize Gr-MDSCs as observed for Mo-MDSCs (Figure 3). In particular, the novelty of this observation is that Gr-MDSCs appear during the metaplastic phase of the transforming gastric mucosa before frank cancer emerges.

Collectively, the emergence of SLFN4\(^+\) MDSCs might be cognate biomarkers because the \( slfn4 \) promoter remains quiescent until both transcriptional regulators—one constitutive (GLI1) and one inducible (IRFs/STAT)—engage the promoter (Figure 3). In this way, immune suppressor function only becomes active under the appropriate conditions (ie, to dampen the chronic gastritis initiated by *Helicobacter*). The T-cell suppressor activity exerted by immature myeloid cells occurs because they restrict T-cell access to L-arginine, a substrate for the MDSC enzymes arginase 1 and inducible nitric oxide synthase.\(^8^1\) We showed previously that small interfering RNA knockdown of \( slfn4 \)
significantly reduces arg1 and inos mRNA in SLFN4+ MDSCs, suggesting that SLFN4 is required for myeloid cells to acquire their immune-suppressor function. Because GLI1 gene expression blocks maturation of an immature myeloid cell subpopulation, creating a gastric microenvironment favorable for metaplasia and transformation, we examined the pattern for SLFN4 homologs in human subjects. Human SLFN 5, SLFN12, and SLFN12L show the closest homology to mouse SLFN4 protein (the slfn4 gene does not exist in the human genome). Consistent with the mouse model, we recently reported in a 13-year follow-up study that SLFN5 is increased most significantly in those subjects with intestinal metaplasia whose lesions progressed to gastric cancer. Although SLFN5 is expressed in myeloid cells, we found that its expression also occurred primarily in T cells. When we examined the expression pattern of SLFN12L, we found its expression correlated with the human surface markers for Gr-MDSCs. Therefore, human myeloid cells express SLFN12L as observed for SLFN4 in mice. Furthermore, we would predict that increased SLFN12L levels, like SLFN5, might predict those individuals with metaplasia who are more likely to develop gastric cancer.

Summary

HH signaling in the stomach plays a significant role in gastric development, homeostasis, and neoplastic transformation. Although extensive developmental literature on SHH protein and downstream targets exists, essentially none of the information was applied to the stomach, despite the evidence that SHH is highly expressed in gastric cancer cell lines. Although increased levels of SHH have been reported in gastric cancers, its specific role in gastric transformation remains elusive but carries significance because of the availability of HH antagonists. Here, we reviewed the role of HH signaling in normal gastric homeostasis, inflammation, and transformation. In particular, we highlighted our studies showing that the phenotype of infiltrating myeloid cells changes over time to become MDSCs and that the polarization requires HH signaling. More importantly, expression of GLI1, which targets SLFN4
(mice) and SLFN12L and SLFN5 (human beings), is an early indicator that the myeloid cells recruited during chronic inflammation have become polarized toward Gr-MDSCs, a cell type that appears to favor neoplastic development. In addition to MDSCs, other bone marrow–derived cells are recruited to the stomach and have been implicated in facilitating gastric transformation.\textsuperscript{24,94} The ability to track these cell types in the preneoplastic state broadens options for more effective screening of subjects predisposed to eventually develop gastric cancer as well as to expand options for prophylactic therapy once atrophic gastritis develops, including antagonists of mTOR (mechanistic antagonist of rapamycin).\textsuperscript{97,98,99} Although Hedgehog antagonists have been used for other cancer types, their use in clinical trials for gastric cancer is still in its infancy.\textsuperscript{1,31} Where initiated, those trials have focused on targeting CD44-positive gastric stem cells to treat metastatic disease.\textsuperscript{100}

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