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Hedgehog Signaling in the Stomach

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Abstract

The Hedgehog (Hh) signaling pathway not only plays a key part in controlling embryonic development, but in the adult stomach governs important cellular events such as epithelial cell differentiation, proliferation, gastric disease and regeneration. In particular, Sonic Hedgehog (Shh) signaling has been well studied for its role in gastric physiology and pathophysiology. Shh is secreted from the gastric parietal cells and contributes to the regeneration of the epithelium in response to injury, or the development of gastritis during *Helicobacter pylori* infection. Dysregulation of the Shh signaling pathway leads to the disruption of gastric differentiation, loss of gastric acid secretion and the development of cancer. In this chapter, we will review the most recent findings that reveal the role of Shh as a regulator of gastric physiology, regeneration and disease.

INTRODUCTION

Using a mutagenesis screen in *Drosophila*, Nusslein-Volhard and Wieschaus first described the Hedgehog (Hh) gene [1], and this was followed by the identification of three Hedgehog homologs that are greatly conserved from the fruit fly to humans. The three homologs are Sonic Hedgehog (Shh), Desert Hedgehog (Dhh), and Indian Hedgehog (Ihh). In the stomach, Hedgehog is known to control cellular functions including proliferation, differentiation and acid secretion. Therefore, identifying the role of Shh as a regulator of adult stomach physiology and differentiation is important for understanding the pathogenesis of gastric diseases. Shh also affects the division of adult stem cells. The regulation of adult stem cells through Shh signaling has linked this pathway in the development of cancer [2], of which gastric cancer is the focus of this review.

BIOCHEMISTRY OF THE HEDGEHOG SIGNALING PATHWAY

Initial studies of Hedgehog processing were performed in invertebrate systems, but Hedgehog processing in the mammalian system, in particular the stomach, has been shown

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to be protease- and acid-dependent [3]. Primary parietal cell cultures and tissue extracts from mouse and human stomachs, demonstrated that Shh is processed by pepsin A [3]. Interestingly, processed Shh in human gastric cancer tissue is absent as a consequence of hypochlorhydria due to the parietal cell atrophy [4]. The unprocessed full length Shh precursor protein is also biologically active [5]. Full length Shh binds to Hedgehog receptor Ptch and induces target gene expression in the developing chick neural tube and rescues the eye developmental defect in *Drosophila* Hedgehog mutants [5].

Shh processing is also regulated by gastrin [4,6]. The regulatory role of gastrin is indicated by studies using gastrin deficient mice demonstrating a significant decrease in Shh protein expression, and that the reinfusion of gastrin restores Shh expression [4]. In addition, using isolated canine parietal cell and mouse organ cultures to identify the mechanism that gastrin regulates Shh, El-Zaatari *et al.* demonstrated that intracellular calcium release and protein kinase C (PKC) activation stimulate Shh gene expression [6]. Based on previous reports that intracellular calcium release stimulates acid secretion in parietal cells, gadolinium-, thapsigargin-, and carbachol-mediated release of intracellular calcium also induced Shh expression [6].

Once Hedgehog protein has been secreted, it is believed to travel in a paracrine fashion towards the target cell [16]. Hedgehog then binds to, and stimulates the transmembrane receptor Patched 1 (Ptch1) [7], which causes a release of the inhibitory effect that Ptch1 has on G-protein coupled receptor Smoothened (Smo) [9]. Smo is then capable of travelling up the cilium tip via microtubular transport [10-11] where it promotes the dissociation of the glioma-associated oncogene homolog 1 (Gli1) [9,12]. The Gli family of transcription factor proteins (Gli1, Gli2, and Gli3) travel into the nucleus where they bind to the DNA consensus site (5′-GACCACCCA-3′) [12]. Gli1 is a powerful transcriptional activator, while Gli2 and Gli3 possess both transcriptional activation and repression capabilities [14]. The Gli transcription factor family effects the expression of genes that help control cell development and differentiation (cyclin D1, D2, N-myc, Wnt, PdgfRα, Igf2, FoxM1, Hes1), cell survival (Bcl2), self-renewal (Bmi1, Nanog), angiogenesis (Vegf), epithelial-mesenchymal transition (Snail1, Sip1, Elk1, Msx2), invasiveness and feedback inhibition or activation of the Hedgehog signaling cascade [14].

HEDGEHOG SIGNALING AND GASTRIC FUNCTION, DIFFERENTATION AND REGENERATION

The development of a mouse model expressing a parietal cell-specific deletion of Shh (HKCre/Shh^{KO} mice) has allowed us to assay changes in gastric epithelial cell differentiation, function and regeneration [15-18]. Compared to pathology of the control animals, HKCre/Shh^{KO} mice demonstrated an age-dependent increase in number of surface pit mucous cells reminiscent of foveolar hyperplasia [15]. Interestingly, the phenotype observed in HKCre/Shh^{KO} mouse was comparable to that observed in mice over-expressing the TGFα gene [19] and in patients with Menetrier's disease [20], without the development of parietal cell atrophy. Loss of parietal cell-expressed Shh was accompanied by hypergastrinemia and increased Ihh within the surface mucous pit epithelium. Somatostatin

is a known inhibitor of gastrin and when HKCre/Shh^{KO} mice were infused with the somatostatin analogue octreotide, circulating gastrin concentrations normalized and Ihh expression decreased [15]. Our existing understanding of hypergastrinemia is expanded because, besides the proposed role as a morphogen for the gastric epithelium, Shh may also be a regulator of the gastrin-gastric acid negative feedback mechanism (Figure 1).

We proposed that increased Ihh gene expression observed in HKCre/Shh^{KO} mice might be due to hypergastrinemia. We studied hypergastrinemic HKCre/Shh^{KO} mice bred with gastrin-deficient (GKO) mice (PC-Shh^{KO}/GKO) [16]. The PC-Shh^{KO}/GKO mice did not exhibit evidence of hyperproliferation, and gastrin infusion caused increased expression of Ihh and proliferation within the surface epithelium. *In vivo* gastrin-induced proliferation in PC-Shh^{KO}/GKO mice was blocked by Hedgehog signaling inhibitor cyclopamine [16]. In addition, *in vitro* gastrin-induced proliferation of fundic organoids derived from PC-Shh^{KO}/GKO mouse stomach, was blocked by a smoothened inhibitor [16]. We concluded that Ihh signaling regulates gastrin-induced proliferation of epithelial cells in stomachs of adult mice.

The HKCre/Shh^{KO} mice demonstrate that loss of Shh triggers a number of molecular changes [15] consistent with epithelial-to-mesenchymal transition (EMT) of the gastric epithelium [21]. This included loss of E cadherin expression and translocation of β-catenin to the nucleus [15]. After nuclear translocation, β-catenin binds to DNA-binding proteins Tcf/Lef1 which subsequently regulates target genes including Cyclin D1 [22] important in proliferation. Additionally, laser capture microdissection analysis of the gastric epithelium of HKCre/Shh^{KO} mice demonstrated a significant increase in Snail with a concurrent decrease in E-cadherin [15]. A study in rat kidney epithelial cells shows that Snail, a suppressor of E-cadherin, is a transcriptional target of Hedgehog signaling [23]. As discussed earlier, HKCre/Shh^{KO} mice exhibited hyperproliferation of surface mucous cells [15]. Hyperproliferation of surface mucous cells frequently disrupts differentiation of other cell lineages such as zymogen cells [24]. HKCre/Shh^{KO} mice have a delay in the differentiation of zymogen cells from mucous neck cells whereby zymogen cells located in the base of the gland frequently co-expressed mucous neck cell markers [15]. Aberrant E-cadherin expression causes changes in zymogenic cell morphology and differentiation [24]. We demonstrate that adherens-junction protein E-cadherin may be a downstream target of the Hedgehog signaling pathway [25]. The integrity of adherens- junctions is an important factor in determining epithelial morphology and cell differentiation [26], but also tumor growth, invasiveness and metastasis [27]. Understanding Hedgehog signaling as a regulator of the adherens-junctions advances our knowledge by which Shh may regulate gastric physiology.

Shh secreted from gastric parietal cells is important for epithelial repair following gastric injury. The HKCre/Shh^{KO} mice exhibit impaired gastric regeneration in response to injury. However, in parabiosis experiments a control mouse paired with a mouse expressing a parietal cell-specific deletion of Shh facilitates repair in the HKCre/Shh^{KO} mouse [17]. This suggested for the first time that parietal cell act as an endocrine source of Shh during gastric repair. In addition, the development of a tamoxifen-inducible mouse model expressing a parietal cell-specific deletion of Shh (PC-iShhKO) demonstrated that re-emergence of Shh

contributes to gastric regeneration [18]. Studies have indicated that downregulating Shh expression is a mechanism by which *Helicobacter pylori (H. pylori)* infection triggers gastric cancer, as the outcome of *H. pylori* infection is parietal cell atrophy and gastric hypochlorhydria. Intestinal metaplastic areas of *H. pylori*-positive human stomachs have decreased Shh expression [28]. Other studies suggest that early bacterial eradication restores the expression of Shh and the differentiation of the stomach [28-28]. Thus, studies using the PC-iShhKO mice may have clinical suggestions given that removal of *H. pylori* correlates with re-emergence of Shh and regeneration of the gastric epithelium.

HEDGEHOG SIGNALING AND *Helicobacter pylori (H. pylori)* INFECTION

The regulation of Shh during inflammation may be seen as a global increase or decrease in expression during the progression of inflammation to gastric cancer. Data suggests in the innate phase of the host immune response Shh is induced after activation of the NF-κB pathway [29]. During the early stages of infection and as a result of inflammation, pro-inflammatory cytokine interferon-γ (IFNγ) has been shown to stimulate Shh expression [30] (Figure 2). Macrophages recruited to the infection side secrete pro-inflammatory cytokines including TNFα and IL-1β which result in loss of Shh expression [31]. In an *in vivo* mouse model of *H. pylori* inflammation parietal cell-expressed Shh is induced within 2 days of infection [32]. *In vitro H. pylori* infection in gastric organoids also increased Shh expression; an effect that was blocked by inhibiting NFκB signaling. Thus, *H. pylori* results in increased Shh expression from parietal cells, via activation of the NFκB signaling pathway [33].

After induction of Shh during early stages of *H. pylori* infection, Shh then acts as a macrophage chemoattractant during early gastritis [32}. Bone marrow chimera experiments were achieved with mice that have myeloid cell-specific deletion of the Hedgehog signal transduction protein Smoothened (LysMCre/Smo^{KO}). Macrophage recruitment to the gastric epithelium was measured by fluorescence-activated cell sorting analysis. Control mice that received bone marrow transplants from control mice had an infiltration of macrophages to the gastric mucosa in response to *H. pylori* infection. In contrast, macrophage infiltration was not observed in *H. pylori*-infected mice that received bone marrow transplants from LysMCre/Smo^{KO} mice, suggesting that Shh signaling is crucial for macrophage infiltration [32]. Moreover, PC-Shh^{KO} mice (discussed earlier) did not develop gastritis, even after 6 months infection with *H. pylori*. However, control mice infected with *H. pylori* for 6 months experienced an inflammatory response characterized by infiltration of CD4(+) T cells and increased levels of IFNγ and IL-1β. These studies demonstrated that Shh acts as a macrophage chemoattractant during initiation of *H. pylori*-induced gastritis [32] (Figure 2). Decreases in the expression of Shh and Ptch1 have been observed in parietal cells during *H. pylori* infection of the gastric epithelium [34]. These finding represents changes during the early stages of *H. pylori* infection that takes place in gastric mucosal cells prior to neoplastic transformation [18].

HEDGEHOG SIGNALING AND GASTRIC CANCER

Gastric cancer is one of the foremost widespread types of cancer. In 2015, an estimated 24,590 people were diagnosed with stomach cancer and 10,720 people were predicted to succumb to this illness by the American Cancer Society. The five-year survival rate for people with gastric cancer was 28%. This percentage rate is low because most gastric cancer diagnoses are made after there has already been metastasis to surrounding organs. This is also indicative of the efficacy of current chemotherapeutics and the high recurrence rates, as well as metastasis events [38]. With regards to the role of the Hedgehog signaling pathway in gastric cancer development, high expression of Shh signaling components correlated with the development of gastrointestinal cancers [39] (Figure 2). Increased Ptch expression coincided with elevated Shh. *In vivo* data showed that increased Shh promoted cell proliferation, and that tumor growth regressed in a xenograft mouse model treated with the Hedgehog pathway inhibitor cyclopamine [39]. Further characterization of Ptch1 and Gli1 expression within the gastric tumor microenvironment was performed using a collection of human biopsies [40]. In these samples, elevated Shh corresponded to increased Ptch1 and Gli1 in cancerous tissue. Elevated Hedgehog pathway activation was most common in poorly differentiated and high-grade samples, implicating Shh as an inducer of an aggressive cancer phenotype [40].

Studies of both human intestinal- and diffuse-type gastric cancer were performed to localize Hedgehog signaling by cell type in the setting of tumor formation [41]. The intestinal phenotype expressed low mRNA abundance of Smo, Gli1 and Gli2, while the diffuse-type phenotype highly expressed Ptch, Smo, Gli1 and Gli2. Diffuse-type gastric cancer samples showed strong Ihh staining in epithelial cancer cells, while Shh was expressed in fibroblastic cells co-staining with vimentin, α-actin and desmin [41]. Immunostaining by our laboratory using tissue collected from a patient with diffuse-type gastric cancer and antibodies specific for Shh, Ptch and cancer stem cell marker CD44 demonstrated co-expression of Shh within CD44-expressing cells within the tumor (Figure 3). Interestingly, Ptch was expressed within the mesenchyme, but also co-localized to CD44+/Shh+cells within the tumor (Figure 3). These data are consistent with studies demonstrating that Hedgehog maintains the malignant transformation phenotype in CD44+ gastric cancer cells that promotes chemotherapy resistance [18]. A recent study by Syu *et al.* [42] further supports the role of Hedgehog signaling as a driver in the progression of gastric cancer. In this study the investigators developed a mouse model expressing Hedgehog pathway transcription factor GLI2 within Lgr5-expressing stem cells and their descendants in adult mice when treated with doxycycline. Mice developed gastric adenocarcinoma three weeks following induction of the Hedgehog pathway oncogene GLI2A. These data provided further evidence that dysregulated activation of Hedgehog/Gli2 signaling in Lgr5-positive stem cells drives the emergence of gastric adenocarcinoma in mice [42]. Therefore, in contrast to patients with atrophic gastritis that show loss of Shh protein expression, over-expression of the Hedgehog pathway is a driver and prognostic marker in the development of gastric carcinoma [42] (Figure 2).

The mechanism by which Hedgehog is elevated in gastric cancer and then able to act on cancer cells to induce their proliferation remains largely unknown. However, studies from

our laboratory suggest that mesenchymal stem cells (MSCs) may be a source of Hedgehog protein. The permanent engraftment of the bone marrow-derived MSCs in an IFNγ-rich tissue environment results in the differentiation of these cells into cells closely resembling stem cells, whereby they have acquired the capacity to self-renew and become incorporated in the developing tumor [43]. We first investigated the mechanism for recruitment of these MSCs into the gastric epithelium. The MSC population collected from bone marrow of control- or IFNγ-treated mice demonstrated that IFNγ significantly increased MSC proliferation, a response mediated by Hedgehog signaling. While MSC cell lines with intact Shh expression were recruited to the gastric mucosa in response to IFNγ, MSCs lacking expression of Shh were not. Thus, our data suggests that Hedgehog signaling is important in MSC proliferation and recruitment to the stomach in response to IFNγ [44]. Further studies from our laboratory using these 'transformed' MSCs secreted Shh responsible for providing a proliferative stimulation of the gastric epithelium which is associated with tumor development [45] (Figure 2).

HEDGEHOG SIGNALING AND POTENTIAL CANCER THERAPEUTICS

The aberrant activation of the Shh signaling pathway has been shown in different types of human cancers including gastric cancer, basal cell carcinoma, malignant gliomas, leukemias, breast, a n d pancreatic cancers [46-50]. Downstream players in the Shh pathway, smoothened (SMO) and glioma-associated oncogene homolog (GLI) family of zinc finger transcription factors, have been targeted for potential cancer therapeutics. Many efforts have been focused on pharmacologically targeting SMO, and to date, two SMO inhibitors (LDE225/Sonidegib and GDC-0449/Vismodegib) have received United States Food and Drug Administration (FDA) approval for treating basal cell carcinoma. Vismodegib is a second generation cyclopamine derivative developed by Roche/Genentech/Curis that binds directly to SMO to prevent GLI activation [80]. Vismodegib, approved by the FDA in January 2012, was the first drug targeting the Shh pathway approved for treating any cancer. Vismodegib is currently used to treat adults for metastatic basal cell carcinoma, or patients with recurrent basal cell carcinoma who are not candidates for surgery or radiation therapy [51]. In particular, GDC-0449/Vismodegib was also used in Phase II trials for treatment of metastatic gastric and esophageal cancers [52].

We also discussed above that the Hedgehog pathway maintains the malignant transformation phenotype in CD44+ gastric cancer cells that promotes chemotherapy resistance [9]. Compared to the CD44- cell population, CD44+ cells showed a resistance to 5-fluorouracil and cisplatin chemotherapy that was negated when Hedgehog signaling was inhibited using Smo shRNA or vismodegib [9]. Importantly, clinical tumor samples from a phase II trial of chemotherapy with or without Vismodegib for advanced gastric cancer were examined for CD44 expression. In the group that only received chemotherapy, increased CD44 expression was associated with a decrease in survival. In the chemotherapy plus Vismodegib group, high CD44 expression was associated with enhanced survival [9]. In another study, Xu *et al.* [53] showed that Shh and Gli1 signaling enhanced drug resistance in CD44(+)/Musashi-1(+) gastric cancer stem cells. These CD44(+)/Musashi-1(+) cells presented a high level of drug efflux activity. The cells were resistant to doxorubicin-induced apoptosis and also upregulated ATP-binding cassette sub-family G member 2 (ABCG2) expression. These

effects were reversed by treatment with Gli inhibitors GANT61 and GDC-0449 or by knocking down the expression of Gli1/Shh. The co-expression of CD44(+)/Musashi- 1(+) could be used as a method of detecting gastric cancer stem cells. Collectively, these studies demonstrate that gastric cancer is a heterogeneous and complicated disease. Thus, combining chemotherapy with Hedgehog inhibition may be effective only in tumors with high CD44 levels.

CONCLUSIONS

Shh is a parietal cell-secreted protein regulating gastric cell proliferation, differentiation, function and epithelial regeneration [7,18]. Intriguingly, Shh acts as a chemokine for monocyte recruitment during *H. pylori* infection and the development of gastritis [32]. Given the emerging roles of Shh as a regulator of the immune response, adult tissue physiology and regeneration and the development of gastric disease, this has broadened our understanding of this developmental morphogen in the stomach. Understanding the role of the Hedgehog pathway during tumorigenesis will allow for the development of effective therapies for the treatment of gastric disease.

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HIGHLIGHTS

- Hedgehog signaling regulates gastric physiology, regeneration and disease.
- Sonic Hedgehog is secreted from the gastric parietal cells.
- The dysregulation of Sonic Hedgehog signaling leads to the development of cancer

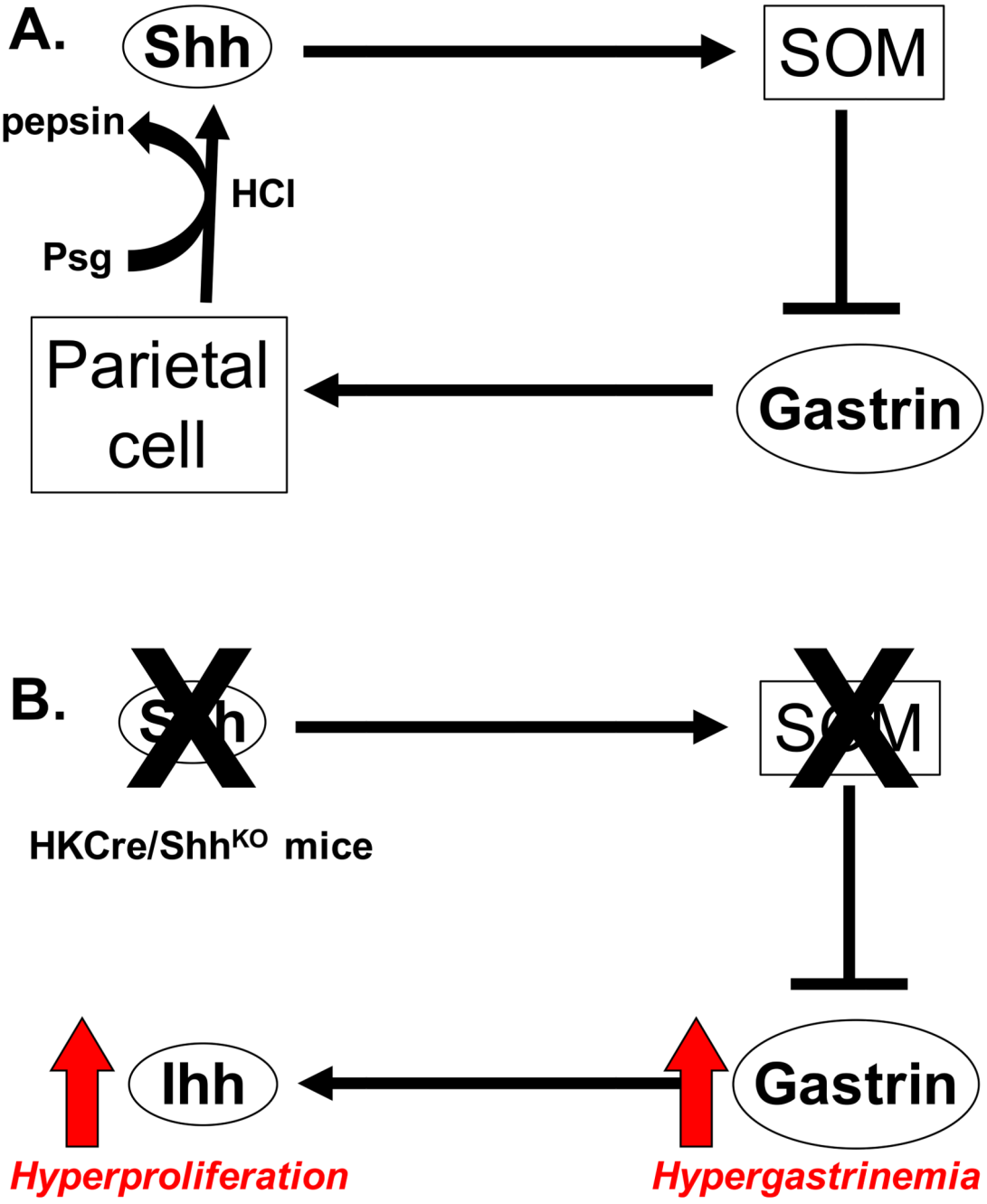

Figure 1. Shh as a regulator of gastric physiology and function

(**A**) Gastrin-induced acid secretion facilitates Shh processing and secretion that is protease-dependent. Shh induces somatostatin (SOM) secretion, which in turn further inhibits the gastrin production from G-cells and subsequent acid secretion from the parietal cells. (**B**) Loss of parietal cell-secreted Shh results in loss of the production of SOM and the subsequent increase in serum gastrin concentrations (hypergastrinemia). Hypergastrinemia induces Ihh expression in the stomach leading to hyperproliferation of the gastric epithelium.

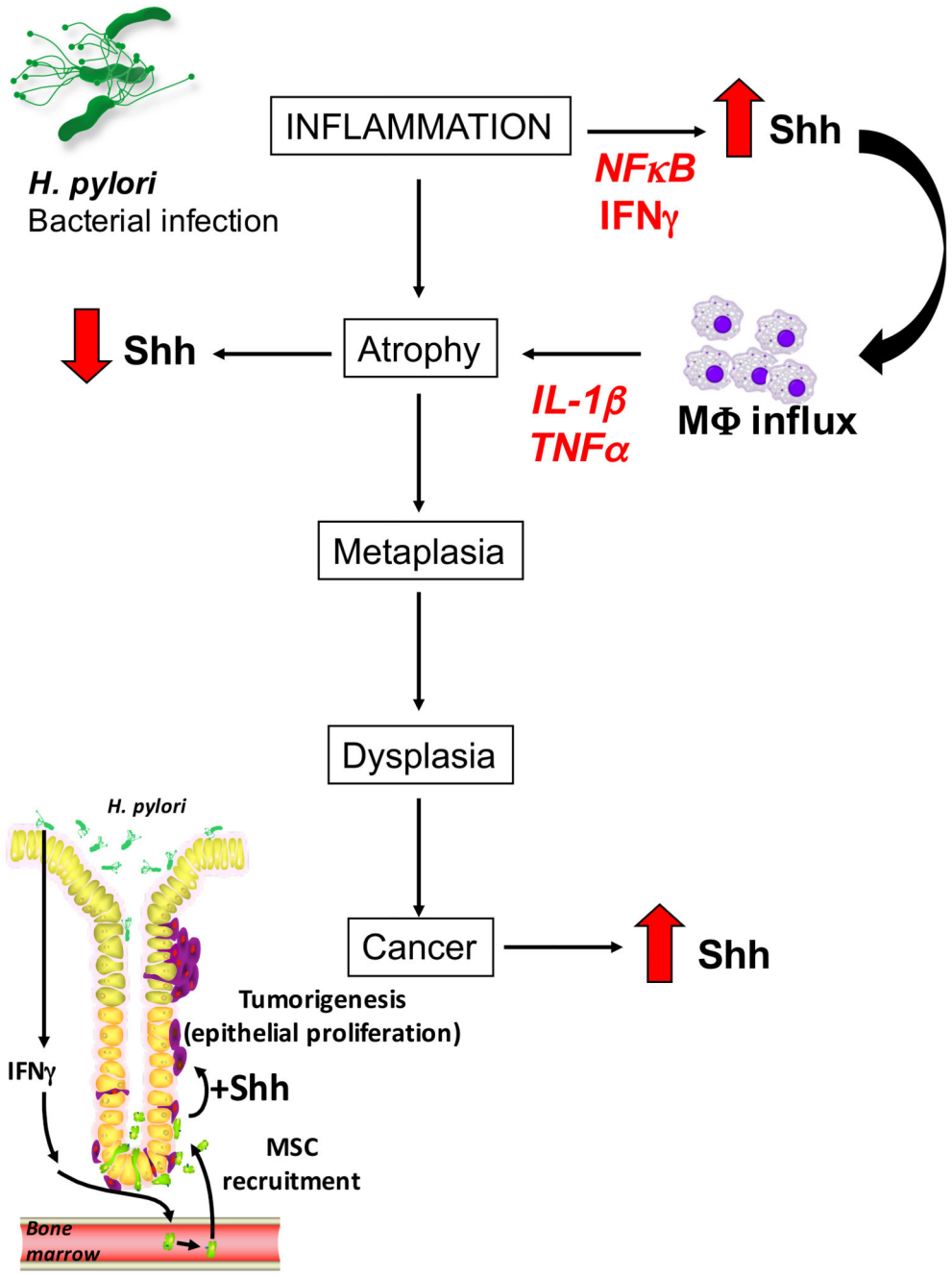

Figure 2. Changes in Shh expression during the progression from gastric inflammation to cancer

It is accepted that the major cause of chronic inflammation in the normal, acid-secreting stomach is *H. pylori* colonization. Upon *H. pylori* infection, Shh is induced by NFκB and IFNγ, and acts as a chemokine for the recruitment of macrophages to the stomach and the development of gastritis. Cytokines including IL- 1β and TNFα, leads to the loss of Shh, possibly as a consequence of parietal cell atrophy. Based on the Correa model for the development of gastric cancer [84], it is known that inflammation caused by *H. pylori* infection progresses through the development of atrophic gastritis, metaplasia, dysplasia and

eventually cancer. The Hedgehog signaling pathway is aberrantly activated during gastric cancer. MSCs recruited from the bone marrow to the tumor site are a major source of Shh and provide a proliferative stimulus for the epithelium.

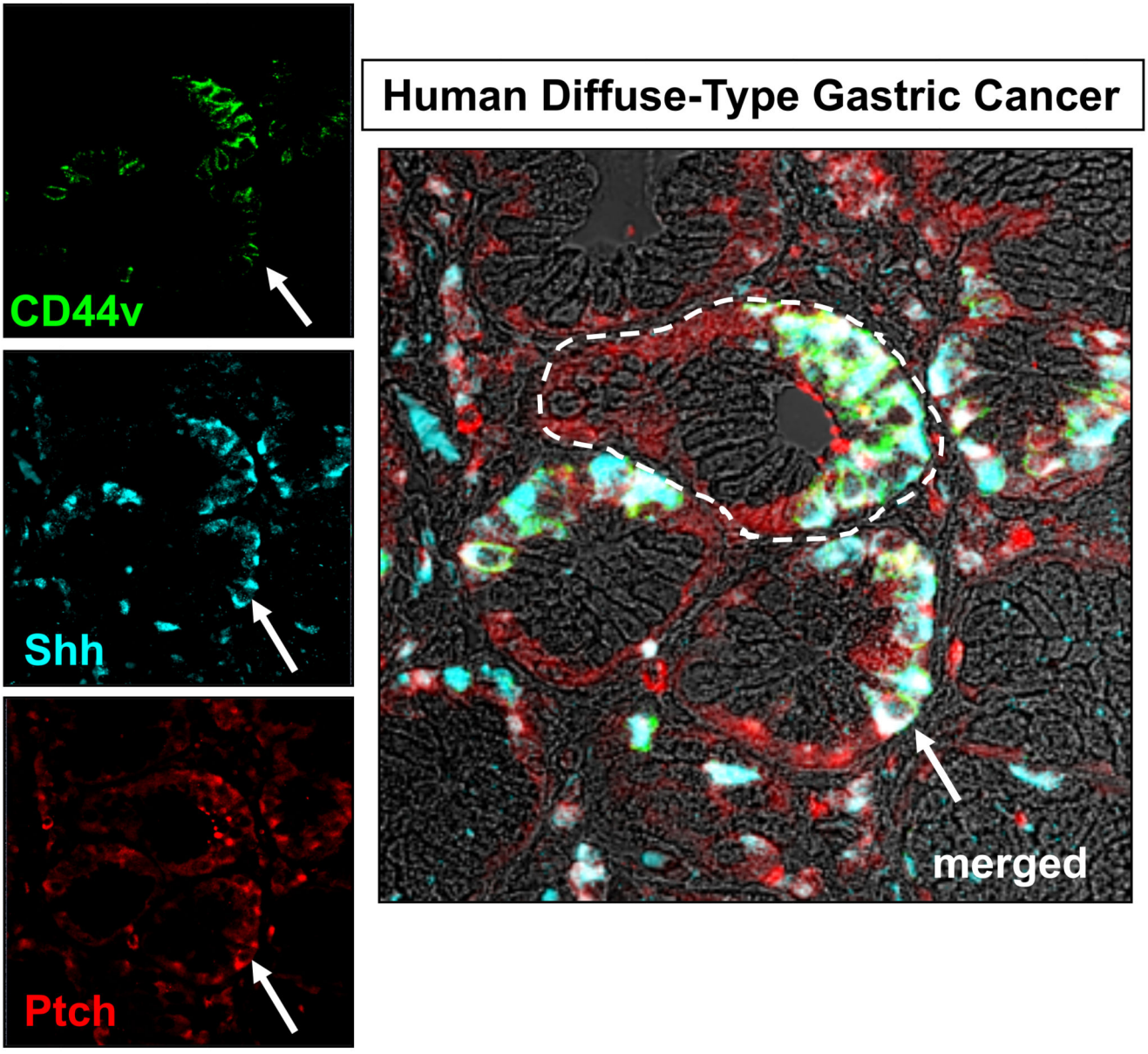

Figure 3. Expression of Shh, Ptch and CD44v in human diffuse-type gastric cancer

Immunostaining of tissue collected from a patient with diffuse-type gastric cancer using antibodies specific for CD44 variant isoform (CD44v, green), Shh (blue) and Ptch (red).