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# The Hedgehog Signaling Network and the Development of Gastric Cancer

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## 1. Introduction

The Correa model of gastric cancer reported that atrophy (parietal cell loss) was one of several significant changes that occurred after chronic inflammation (Correa et al., 1975). We now understand that the major cause of chronic inflammation in the normal, acid-secreting stomach is *Helicobacter pylori (H. pylori)* bacterial colonization. It is widely accepted that inflammation that is caused by *H. pylori* infection is a trigger for the development of gastric cancer. An explanation for the causal role of *H. pylori* infection in the pathogenesis of gastric cancer has been described by disruption of differentiation of epithelia as a consequence of elevated pro-inflammatory cytokines such as IFNγ, TNFα and IL-1β (Moss et al., 1994; Padol IT, 2004; Sawai et al., 1999; Smythies et al., 2000; Zavros et al., 2003). However, the question of the mechanism by which inflammatory cytokines induce mucosal damage remains unanswered. Since stomach secretes numerous factors such as TGFβ, Wnt, FGFs and Hedgehog proteins that are known to be responsible for the differentiation of the gastric epithelium, one favored explanation linking inflammation and progression to cancer is due to the loss of these factors (reviewed in (Katoh Y, 2006)). During the progression from inflammation to metaplasia and cancer the cell composition of the stomach changes. In particular, loss of the acid-secreting parietal cells (atrophy) leads to alterations in the cell lineages with the expansion of metaplastic mucous cells.

Emerging evidence shows that Sonic Hedgehog (Shh) signaling is expressed in acid-secreting parietal cells within the adult stomach (van den Brink et al., 2002; Zavros et al., 2008). Since studies have suggested that Shh acts as a morphogen in the adult stomach (Shiotani et al., 2005a; van den Brink et al., 2002), an important hypothesis is that loss of Shh expression during gastric inflammation results in the disruption of epithelial cell differentiation and function leading to cancer. During *H. pylori* infection, the site of chronic inflammation coincides with the secretion of IFNγ and the engraftment of bone marrow-derived mesenchymal stem cells (BM-MSCs) whose progeny populate gastric tumors (Houghton et al., 2004). The mechanism that regulates BM-MSC proliferation and cellular engraftment with host cells during chronic inflammation is unknown.

The permanent engraftment of the BM-MSCs in an area of an IFNγ-rich and abnormal tissue environment results in differentiation of these cells through stages of metaplasia and dysplasia (Li et al., 2006). For this reason these cells behave much like cancer stem cells whereby they have acquired the ability to self-renew and become incorporated into the developing tumor (Li et al., 2006). The mechanism that regulates BM-MSC proliferation and cellular engraftment with host cells during chronic inflammation is unknown, but based on our studies these cells secrete Shh that is responsible for the proliferation and thus may contribute to the differentiation into tumors. In pathological conditions in which immune cells have been implicated, the Hedgehog signaling pathway mediates IFNγ-induced tumor development (Stewart et al., 2003; Wang et al., 2003; Zavros et al., 2005). Given that chronic gastritis is associated with elevated IFNγ expression and the development of cancer (Zavros et al., 2005) a similar mechanism may be occurring in the stomach. *The current chapter focuses on the Shh signaling pathway and its role in the development of gastric cancer, specifically in response to Helicobacter pylori infection. In particular, the chapter presents a comprehensive discussion of the role of the Hedgehog signaling network as a regulatory mechanism within the BM-MSC compartment during the development of gastric cancer.*

## 2. Role of Shh as a regulator of gastric tissue homeostasis and disease

### 2.1 Discovery, processing and signaling

Using a saturation mutagenesis screen performed to study the effect of mutations on the patterning of segmented *Drosophila* embryos, Nüsslein-Volhard and Wieschaus first discovered Hedgehog (Nüsslein-Volhard & Wieschaus, 1980). As a result of the mutagenesis screen, Nüsslein-Volhard & Wieschaus identified a group of *Drosophila* mutants that remained covered entirely with denticles (Nüsslein-Volhard & Wieschaus, 1980). The inspiration for the name Hedgehog came from the "spiny" phenotype of the embryos, which resembled a hedgehog. Since the identification of the Hedgehog mutant, three vertebrate Hedgehog homologs have been identified that include Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh). Of the Hedgehog homologs, Shh has been the most studied in terms of the Hedgehog signaling pathway in vertebrates and in particular gastric function and disease.

In *Drosophila* or zebrafish models (Porter et al., 1995), Shh is synthesized as a 45-kDa precursor protein. The full-length protein subsequently undergoes an autocatalytic cleavage to yield a 26-kDa carboxy-terminal fragment and a 19-kDa amino-terminal fragment (ShhN). ShhC is responsible for catalyzing cleavage of the 45-kDa precursor protein while ShhN is the active signaling fragment. Concomitant with cleavage, ShhC acts as a cholesterol transferase covalently linking a cholesterol moiety to the carboxy-terminus of the 19-kDa fragment (ShhN) (Goetz et al., 2006). The 19-kDa fragment (ShhN) is further modified by a membrane bound O-acyltransferase commonly known as Skinny hedgehog (Ski), which covalently links a molecule of palmitate to the 19- kDa fragment (ShhNp) (Mann et al., 2004; Torroja et al., 2005). The phenotypes of *Drosophila* lacking Ski resemble those of *Drosophila* with Shh knocked out and thus demonstrating the importance of palmitoylation for Shh signaling (Chamoun et al., 2001; Pepinsky et al., 1998). ShhNp can remain anchored to the cell membrane or form secreted, soluble and freely diffusible multimeric units (Goetz et al., 2006). Both the cell-retained and secreted Shh protein fragments are able to activate hedgehog signaling through the hedgehog receptor Ptch (Goetz et al., 2006) (Figure 1A). However, recently it is reported that the full-length precursor Shh protein may also bind to

Ptch and exhibits biological activity (Tokhunts et al., 2009). In an in vivo assay using the developing chick neural tube, full-length Shh induced activation of Shh-dependent *luciferase* reporter gene (Tokhunts et al., 2009). Such findings are relevant when considering the biological activity of Shh with regards to gastric cancer. Prior studies using xenografts of human gastric cancer cell lines show that Hedgehog signaling is required for cancer cell growth. It has been assumed that the Shh ligand mediating the activation was the processed ShhN protein, but the form of Shh produced by the xenografts was not evaluated directly in these studies (Berman et al., 2003). In another study using human gastric tumor samples it was observed that the major form of the Shh ligand present is the 45-kDa peptide (Zavros et al., 2007). However, it remains to be determined whether full-length Shh has any biologic activity and acts as a regulator of tumorigenesis that differs from the processed 19-kDa form.

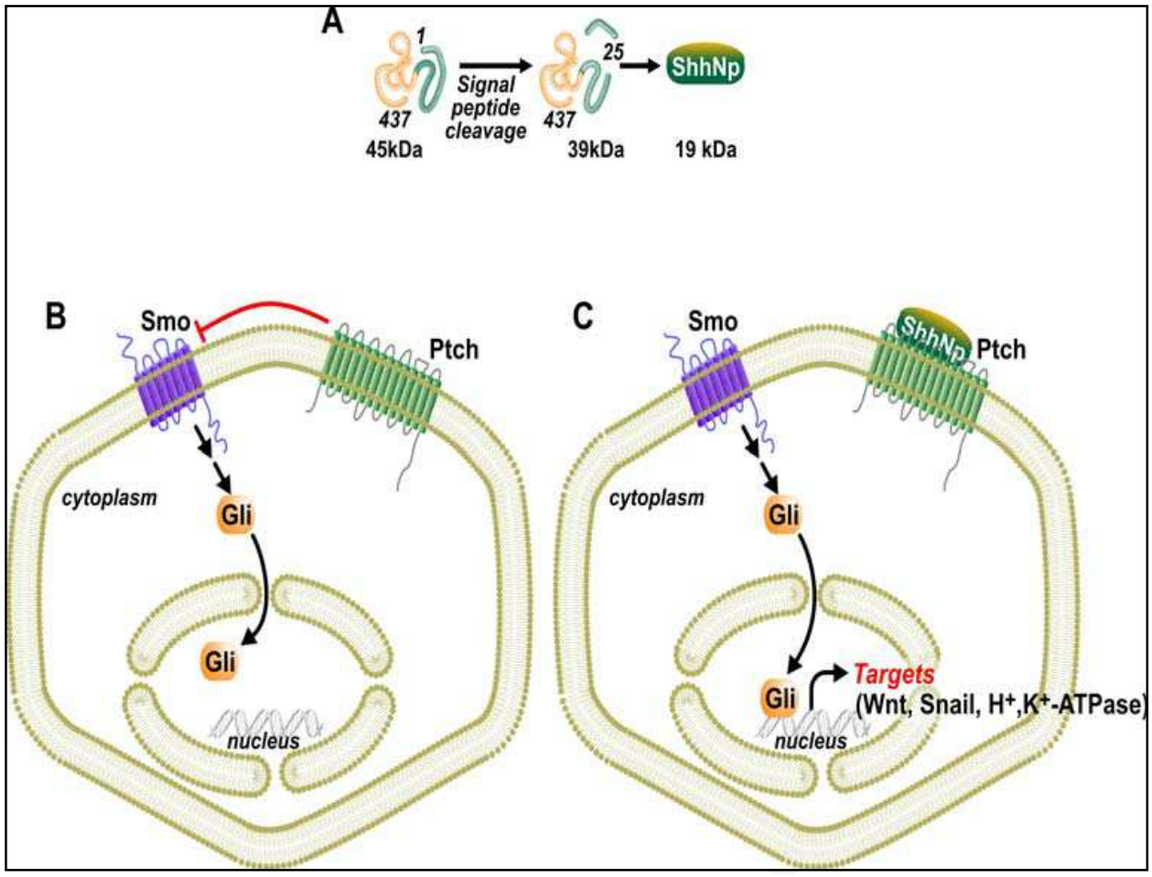


Fig. 1. Schematic diagram of Shh processing and signaling. (A) The cleavage of the 45-kDa full-length precursor generates the signal peptide (39kDa). Autocatalytic or protease-dependent cleavage yields a secreted 19-kDa fragment and a 26-KDa cholesterol modified cell bound protein (ShhNp). (B) In the absence of Shh ligand or unstimulated cells, the activity of the transmembrane protein Smo is suppressed by the Hedgehog receptor Ptch. (C) Binding of Shh to Ptch results in the removal of the inhibitory restraint of Ptch on Smo, consequently activating Smo. Smo then transduces the Shh signal into the cytoplasm. Transduction of the Shh signal into the cytoplasm leads to activation of the Glioblastoma (Gli) family of transcription factors and activation of downstream targets

Shh processing within the gastric mucosa appears to have diverged from the autocatalytic processing originally reported in *Drosophila* and zebrafish models (Porter et al., 1995; Zavros et al., 2007; Zavros et al., 2008). In the mammalian stomach, Shh processing is hormonally regulated and acid dependent (Zavros et al., 2007; Zavros et al., 2008). Changes in acid secretion, that is stimulated by both histamine and gastrin, induces Shh expression and processing (Zavros et al., 2007; Zavros et al., 2008). In particular, intracellular calcium release and protein kinase C activation stimulate Shh gene expression during gastric acid secretion (El-Zaatari et al., 2010). Subsequently, within the acidic environment pepsin A cleaves the 45-kDa precursor protein into the biologically active 19-kDa protein (Zavros et al., 2007). These studies indicate that although autocatalytic processing of Shh may occur in the gastric mucosa, processing of the 45-kDa Shh precursor may predominantly require acidic conditions and the acid-activated protease pepsin A.

Shh signaling in vertebrates is mediated by the seven-span transmembrane receptor Smoothened (Smo) (Goodrich et al., 1996; Taipale et al., 2002). Shh indirectly controls the activity of Smo through binding to the Patched (Ptch) receptor (Goodrich et al., 1996; Taipale et al., 2002). Ptch is a twelve-span transmembrane receptor that catalytically inhibits signaling through Smo in the absence of Hedgehog (Goodrich et al., 1996; Taipale et al., 2002) (Figure 1B). Binding of Hedgehog to Ptch relieves the inhibitory effect of Ptch on Smo and consequently activates Smo (Goodrich et al., 1996; Taipale et al., 2002) (Figure 1C). Transduction of the Hedgehog signal into the cytoplasm leads to activation of the Glioblastoma (Gli) family of transcription factors and target genes that are known to regulate cell cycle, proliferation and differentiation (Hui CC, 1994).

### 2.2 The role of Shh as a regulator of gastric tissue homeostasis

Evidence for the crucial role of Shh as a regulator of gastrointestinal development comes from the Shh null mouse models ($Shh^{-/-}$ mice), whereby Shh-/- mouse stomachs exhibit an intestinal rather than gastric-type mucosa (Kim et al., 2005; Ramalho-Santos et al., 2000). It is only recently that the direct role of Shh in the adult stomach has been investigated (Waghray et al., 2010; Xiao et al., 2010; Zavros et al., 2008). Shh is believed to regulate epithelial cell differentiation, but its role as a morphogen is based on evidence that correlates the loss of Shh with inflammation of the adult stomach (Shiotani et al., 2005a; Suzuki et al., 2005; van den Brink et al., 2002). In the absence of inflammation, the direct contribution of reduced Shh expression to the disruption of epithelial cell differentiation and cancer progression had never been tested. We have made significant contributions that have advanced the current understanding of not only the role of Hedgehog in the adult stomach, but also the mechanism regulating Shh secretion (Xiao et al., 2010; Zavros et al., 2008). Our laboratory is responsible for discovering that in the mammalian system, Shh secretion from parietal cells is acid- and hormonally-regulated (Zavros, 2007; Zavros et al., 2008). Moreover, we show that Shh from this acid-secreting single cell type has significant biological activity, regulating the differentiation of cell lineages and controlling gastric physiological function (Xiao et al., 2010).

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 and function in the adult stomach (Xiao et al., 2010). The HKCre/$Shh^{KO}$ mouse demonstrated an age-dependent increase in the number of surface pit mucous cells. The surface mucous cell expansion that was observed in the HKCre/$Shh^{KO}$ mice was reminiscent of foveolar hyperplasia observed in the over-expressing TGFα transgenic mice

(Bockman et al., 1995; Goldenring et al., 1996; Nomura S, 2005) and in patients with Menetrier's disease (Larsen et al., 1987; Wolfsen et al., 1993). However, unlike Menetrier's disease and *H. pylori* infected patients, the HKCre/Shh$^{KO}$ mouse model did not develop loss of parietal cells (atrophy). Given that HKCre/Shh$^{KO}$ mice lacked inflammation, this suggests a requirement for additional factors, such as inflammatory cytokines, for parietal cell atrophy to occur. The overproduction of surface mucous cells often occurs at the expense of other cell lineages such as the zymogen cells (Bockman et al., 1995; Goldenring et al., 1996). Consistent with this notion, we observed that the HKCre/Shh$^{KO}$ mice also had delayed differentiation of the zymogen cell lineage from the mucous neck cells in the stomachs of HKCre/Shh$^{KO}$ mice (Xiao et al., 2010). Although we have acquired new knowledge of Hedgehog signaling and gastric differentiation and function, the HKCre/Shh$^{KO}$ mice have experimental limitations.

The HKCre/Shh$^{KO}$ mouse is a constitutive model of parietal cell-expressed Shh. Thus, Shh is deleted during development when the $H^+$,$K^+$-ATPase is expressed and is not re-expressed in the gastric parietal cell. In mice, this means Shh would be deleted on embryonic day 19 when H+,K+-ATPase has developed and is expressed within parietal cells (Pettitt et al., 1992). We have created a complimentary experimental approach by developing an advanced mouse model expressing a tamoxifen-inducible parietal cell-specific deletion of Shh (HKCre$^{ERT2}$/Shh$^{KO}$). There are two major advantages to using the inducible HKCre$^{ERT2}$/Shh$^{KO}$ mice for the proposed studies and these include: 1) the inducible model will give us the ability to identify the role of Shh signaling in the adult stomach in a fully differentiated epithelium, and 2) the inducible HKCre$^{ERT2}$/Shh$^{KO}$ mice is an approach that will allow us to assay changes in epithelial cell differentiation and function in relation to the loss and gain of Shh expression, independent of inflammation. Clinically this is important given that re-expression of Shh in *H. pylori*-infected patients after eradication of bacterial infection results in regeneration of the gastric epithelium and ulcer healing (Kang et al., 2009; Shiotani et al., 2005a; Suzuki et al., 2005).

Observations made in the HKCre/Shh$^{KO}$ mice have allowed us to formulate hypotheses explaining the role of Hedgehog signaling in the stomach. The HKCre/Shh$^{KO}$ mice lacked the ability to secrete acid in response to histamine that was accompanied by severe hypergastrinemia and decreased somatostatin expression (Xiao et al., 2010). Hypergastrinemia was associated with increased Indian Hedgehog (Ihh) consistent with observations made in human stomach where Ihh was predominantly expressed in the pit cells where it induces pit cell differentiation in primary mouse gastric cells (Fukaya et al., 2006). Thus, the phenotype observed with loss of Shh may be attributed to increases in circulating gastrin concentrations due to loss of somatostatin. Besides the proposed role as a morphogen for the gastric epithelium, Shh may also be a fundamental regulator of the gastrin-gastric acid negative feedback mechanism.

Loss of Shh is accompanied by increased Ihh gene expression in the surface pit epithelium (Xiao et al., 2010). As shown in Figure 1, binding of Hedgehog ligand to its receptor Ptch results in removal of the inhibition of Ptch on Smo, and this removal of the inhibition on Smo subsequently results in the activation of the Gli-family of Hedgehog transcription factors. Evidence from Gli1 pathway studies in rat kidney epithelial cells (RK3E) show that Gli1 induces the transcription of the zinc-finger transcription factor, Snail (Li et al., 2006). Snail inhibits transcription of E-cadherin, an integral cell-adhesion protein known to associate with β-catenin at the cell membrane. Suppression of E-cadherin expression is implicated with increased nuclear β-catenin and activation of Wnt pathway targets such as

CD44, MMP-7, c-Myc and Cyclin D1 that have been associated with the progression of gastric cancer (Tanaka M, 2002). In vitro data shows that the Hedgehog signaling pathway is a key regulator of β-catenin (Li et al., 2007), but whether Shh maintains the differentiated phenotype of the stomach by mediating Wnt pathway activation is unknown. Collectively, loss of Shh triggers a number of molecular events, including increased Snail and loss of E cadherin expression, translocation of β catenin and activation of the Wnt pathway, that are consistent with epithelial-to-mesenchymal transition (EMT) of gastric epithelial cells (Li et al., 2006).

Evidence from the HKCre/Shh$^{KO}$ mice demonstrated that loss of Shh triggers epithelial changes consistent with EMT, that included increased Snail accompanied by loss of E cadherin expression (Xiao et al., 2010). While bringing to light that loss of Shh in the stomach contributes to the development of EMT, we turned our attention to tight junctions as a marker of epithelial integrity. In the stomach, the expression pattern of tight-junction scaffolding protein ZO-1 determines epithelial cell organization, differentiation and function, in particular the zymogen cell lineage (Zhu et al., 2009). Disruption of the tight-junction complex is characteristic of a number of diseases including *H. pylori* gastritis (Amieva et al., 2003; Krueger S, 2007). Evidence collected from studies using primary mouse epithelial cell cultures over-expressing Snail demonstrates that Snail directly represses gene expression of claudins/occludin (Ikenouchi et al., 2003). Snail also causes translocation of ZO-1 from the membrane to the cytoplasm in the same isolated mouse epithelial cell cultures (Ikenouchi et al., 2003). In addition, the HKCre/Shh$^{KO}$ mice develop severe hypergastrinemia (Xiao et al., 2010). Besides the contribution of Snail to the disruption of tight-junctions in the stomach, the hypergastrinemia induced in the HKCre/Shh$^{KO}$ mice may also explain the disrupted ZO-1 expression. In support of this notion, progastrin causes the dissociation of tight-junctions by delocalizing ZO-1 and occludin from the membrane to the cytoplasm in IMGE-5 cells (Hollande et al., 2003). Collectively, these studies support that deletion EMT of the gastric epithelium contributes to the dissociation of tight-junction protein ZO-1 and warrants further investigation.

Aside from its role as a regulator of gastric epithelial cell differentiation, Shh may also act to regulate the physiological secretion of acid from the parietal cells. In response to EGF, parietal cells express Shh which positively regulates the expression of the $H^+$,$K^+$-ATPase (Stepan et al., 2005). Emerging studies using mouse models in which Shh signaling or expression have been pharmacologically or genetically inhibited suggest that Shh may directly and/or indirectly act as a regulator of the gastrin-gastric acid negative feedback mechanism regulating acid secretion (El-Zaatari et al., 2008; El-Zaatari et al., 2007; El-Zaatari et al., 2010; Xiao et al., 2010). Treatment of mice with cyclopamine, an inhibitor of Hedgehog signaling receptor Smo, results in elevated circulating gastrin levels (El-Zaatari et al., 2008). Loss of Shh may impair acid secretion by decreasing the activity or expression of parietal cell $H^+$-$K^+$-ATPase. The reduction in acid secretion would reduce somatostatin release from D-cells of the stomach thus removing the somatostatin-inhibitory effect on gastrin secretion. In support of this hypothesis, we observed that the lack of acid secretion in the HKCre/Shh$^{KO}$ mice was accompanied by significant hypergastrinemia. Treatment of HKCre/Shh$^{KO}$ mice, with the somatostatin analogue octreotide, significantly suppressed hypergastrinemia and subsequently restored differentiation of the zymogen cell lineage and parietal cell function (Xiao et al., 2010). Given that gastrin promotes the growth of gastric adenocarcinomas, the role of Shh as a regulator of gastrin and somatostatin secretion has important implications for the study of gastric cancer.

### 2.3 Decreased Shh expression during *Helicobacter pylori* infection

*Helicobacter pylori (H. pylori)* colonizes the stomachs of half the world's population (Bergman et al., 2005). Chronic inflammation caused by persistent *H. pylori* infection is the most consistent lesion that causes the development of gastric cancer (Correa et al., 1975; Correa P, 2007). The gastric mucosal changes of *H. pylori* infection begin with chronic inflammation followed by hyperproliferation, parietal cell atrophy, and metaplastic cell lineage changes including spasmolytic polypeptide-expressing metaplasia (SPEM), intestinal metaplasia and antralization of glands that then proceeds with dysplasia and eventually cancer (Correa P, 2007) (Goldenring JR, 2006). Loss of mature parietal cells from the gastric glands of the stomach plays a central role in the progression of these gastric alterations. Atrophy leads to alterations in the cell lineages with the expansion of metaplastic mucous cells. Since stomach secretes numerous factors such as TGFβ, Wnt, FGFs and including Hedgehog proteins that are responsible for the differentiation of the gastric epithelium, one favored explanation linking inflammation and progression to cancer is due to the loss of Hedgehog as result parietal cell atrophy (reviewed in (Katoh & Katoh, 2006)). In conditions such as gastric atrophy and intestinal metaplasia, where normal gastric morphogenesis is lost, Shh is reduced or absent (Dimmler et al., 2003; Shiotani et al., 2005a; Shiotani et al., 2005b; Suzuki et al., 2005; van den Brink et al., 2002; Van Den Brink et al., 2001). In support of this, in Mongolian gerbils infected with *H. pylori* loss of Shh expression correlates with loss of parietal cells, impaired maturation of the zymogenic chief cells in gastric glands, and intestinal metaplasia (Suzuki et al., 2005). Therefore, loss of Shh signaling may address the impairment of chief cell differentiation and the development of intestinal metaplasia found in late stage *H. pylori* associated gastritis.

It is only until recently that the mechanism responsible for the loss of Shh expression during *H. pylori* infection has been elucidated in vivo (Minegishi Y, 2007; Waghray et al., 2010). As reviewed, acid secretion plays an important role in maintaining Shh expression and secretion in the adult stomach (Minegishi Y, 2007; Waghray et al., 2010; Zavros, 2007; Zavros et al., 2008). Experiments using models of parietal cell dysfunction such as the histamine H(2) receptor-knockout mice in vivo (Minegishi Y, 2007) and isolated rabbit gastric glands and canine parietal cells treated with $H^+$,$K^+$-ATPase blocker omeprazole (Zavros, 2007; Zavros et al., 2008), demonstrate that in the absence of acid secretion Shh expression is significantly reduced. Thus hypoacidity would induce the loss of Shh typically found in *H. pylori* infection. However, another group of potential candidates that may inhibit Shh expression are the inflammatory cytokines released in response to *H. pylori* colonization. For example, exogenous infusion of interferon-γ (IFN-γ) alone is sufficient to induce hypergastrinemia and metaplasia in mice, but very little is known about the regulation of Shh by pro-inflammatory cytokines (Zavros et al., 2003). Alternatively, IL-1β correlates with gastric atrophy and gastric cancer (El-Omar et al., 2000; El-Omar et al., 2001) and is a potent inhibitor of gastric acid secretion (El-Omar et al., 2003) making this cytokine also a strong candidate for the causal role of Shh expression. A recent study using Shh-LacZ reporter mice demonstrates that IL-1β produced during *Helicobacter* infection inhibited gastric acid and subsequently Shh expression through IL-1 receptor activation (Waghray et al., 2010). The investigators concluded from this study that proinflammatory cytokine IL-1β reduces Shh expression and function in the gastric mucosa by reducing acid secretion from parietal cells (Waghray et al., 2010). Since Shh induces $H^+$,$K^+$-ATPase gene expression in isolated canine parietal cells (Stepan et al., 2005), the investigators rationalized that chronically suppressed

levels of Shh may eventually reduce enzyme expression that is sufficient to induce gastric atrophy and thus, inhibit Shh expression in parietal cells (Waghray et al., 2010). Our study using the HKCre/Shh$^{KO}$ mouse model demonstrates that in the absence of inflammation, although Shh induces foveolar hyperplasia and hypochlorhydria, this was not sufficient to induce atrophy (Xiao et al., 2010). Therefore, there may be a requirement for additional factors, such as inflammatory cytokines, for parietal cell atrophy to develop.

## 3. Over-expression of sonic hedgehog signaling in gastrointestinal cancers: The role of Shh within the tumor microenvironment

### 3.1 Over-expression of sonic hedgehog in cancer

The over-expression of Shh signaling components in correlation with the development of gastrointestinal cancers was first recognized through investigation of mRNA expression of Shh and Ihh in tumors throughout the gastrointestinal tract (Berman et al., 2003). Ptch and Gli mRNA transcript levels were measured as indicators of Hedgehog pathway activity, whereby increased Ptch expression was coincident with elevated Shh. In vivo data suggested that the effect of increased Shh was to promote aberrant cell proliferation as tumor growth regressed with treatment of tumor-bearing mice with the Hedgehog pathway inhibitor cyclopamine. These data also confirmed that the tumor growth was indeed stimulated by the Hedgehog autonomous signaling network rather than a result of mutation. Further characterization of Ptch1 and Gli1 expression within the gastric tumor microenvironment was performed using a collection of human biopsies representing matched normal tissue as compared to inflamed tissue, tubular adenocarcinoma, papillary adenocarcinoma and signet-ring cell carcinomas from a series of patients (Ma et al., 2005). In these human samples, elevated Shh corresponded to increased Ptch1 and Gli1 only in cancerous tissue and not in the surrounding normal tissue. Elevated Hedgehog pathway activation was most common in poorly differentiated and high-grade samples, implicating Shh as an inducer of an aggressive phenotype able to evade normal cell cycle control (Ma et al., 2005).

Further work with both intestinal and diffuse gastric cancer-derived cell lines and corresponding human samples, compared to intestinal metaplasias, were used to localize Shh signaling by cell type in the setting of tumor formation and included an examination of the role of Ihh and Dhh (Fukaya et al., 2006). In samples collected from patients with intestinal metaplasia, the mRNA level of Hedgehog signaling pathway components were weakly expressed, while in contrast both Ihh and Shh were increased. Interestingly, the intestinal phenotype expressed low mRNA levels of the downstream targets Smo, Gli1 and Gli2 while the diffuse-type phenotype highly expressed Ptch, Smo, Gli1 and Gli2. The complementary immunohistochemical evaluation of the cells expressing these proteins was crucial, revealing that very little expression of any of the Hedgehog signaling components were detectable in any cell type in intestinal type cancers. However, the diffuse-type samples showed strong Ihh staining throughout the epithelial cancer cells while Shh was expressed in fibroblastic cells co-staining with the markers vimentin, α-actin and desmin (Fukaya et al., 2006). Gastric cancer cell lines used in proliferation assays with cyclopamine treatment confirmed these results implicating Hedgehog activation in increased cell proliferation in cancers representative of the diffuse-type development with high expression of Smo. In contrast to patients with atrophic gastritis that show loss of Shh protein expression, over-expression of Shh appears in gastric carcinoma. The mechanism by which

Hedgehog is first elevated in cancer and then able to act on cancer cells to induce their proliferation remains largely unknown.

### 3.2 Hedgehog signaling regulates cell-cycle progression in gastric cancer cells

There is overwhelming evidence showing that elevated Hedgehog is capable of promoting cancer cell proliferation and evasion of apoptosis, and recent work has begun to unravel the mechanistic details behind this finding. The treatment of the gastric cancer cell line, SNU16, with cyclopamine consistently induces cell apoptosis and arrest of cells in the $G_0/G_1$ phase of the cell cycle (Han et al., 2009). Cytochrome c staining within the mitochondria of cyclopamine-treated cells was characteristic of cells entering the apoptotic pathway, with diffuse staining throughout the cell, while in control cells cytochrome c staining co-localized with a marker for mitochondria only. The Bcl-2 protein is one of the key proteins regulating the release of cytochrome c from mitochondria, and the cyclopamine-treated cells exhibited significant decreases in both Bcl-2 and Gli1 by immunoblot. Collectively, these data suggest that the Shh signaling pathway is important in maintaining the level of anti-apoptotic proteins within cancer cells (Han et al., 2009). This study was limited by the use of only one type of gastric cancer cell line, however, a recent study performed a similar analysis on the SNU-16 cell line as well as the AGS, KATO-III, SNU-5, SNU-601 and SNU-638 cell lines (Lee et al., 2010). Cells either over-expressing Shh or having Shh knocked-down were co-cultured with *H. pylori* as the factor initiating cell apoptosis. Serial passage of one of the AGS/N-Shh over-expressing clones exposed to *H. pylori* showed adopted resistance to *H. pylori*-associated apoptosis concomitant with elevations in all Hedgehog signaling components, therefore cell-cycle protein activation was further characterized in this cell line. An immunoblot showed an absence of Bcl-2, confirming the results of Han, et al. (Han et al., 2009), as well as an increase in Cyclin D1 (Lee et al., 2010). These studies demonstrate that the reactivation of the Shh signaling pathway in response to infectious or inflammatory stimuli is critical to the inhibition of programmed cell death. It also provides an interesting hypothesis that mutated gastric cells evade apoptosis and with a proliferative stimulus may repopulate the epithelium and lead to tumor development.

Another interesting update to the role of Hedgehog signaling in the regulation of the cell-cycle comes from a biochemical analysis that demonstrates a direct physical interaction between Ptch and the cyclins (Barnes et al., 2001). Cyclin B1 is a critical regulator of mitotic cell division. During G2 phase, cyclin B1 accumulates in the nucleus as a part of cyclin dependent kinase 1 (CDK1) protein complex and plays a critical role during the G2/M phase transition of the cell cycle (Jenkins, 2009). Interestingly, Shh and Ptch participate in the G2/M phase checkpoint in a 'non-canonical' pathway that may be independent of Smo and Gli (Barnes et al., 2001). In the absence of Hedgehog ligand Ptch1 binds to cyclin B1 and inhibits the translocation to the nucleus. In the presence of Shh ligand, Ptch1 dissociates from cyclin B1 and cyclin B1 is translocated to the nucleus and promotes cell cycle progression.). Given that Ptch functions as a 'tumor suppressor', it is almost intuitive to hypothesize that within the tumor microenvironment, where Shh is elevated, increased proliferation is expected. In support of this hypothesis, mutations in Ptch have been linked to both cancers such as basal cell carcinomas and medulloblastomas (Ruiz i Altaba et al., 2002). Although these examples provide a firm genetic link between mutations of the Hedgehog signaling pathway and the incidence of cancer, there is little biological evidence of the underlying mechanisms linking Hedgehog signaling, cell-cycle progression and its relevance to gastric cancer progression.

## 4. The role of Bone-marrow derived mesenchymal stem cells (BM-MSCs) in promoting gastric cancer progression

### 4.1 Bone marrow-derived mesenchymal stem cell (BM-MSCs) recruitment to areas of chronic inflammation

Migration and differentiation of stem or progenitor cells within the stem cell niche of tissues are appropriately regulated to maintain normal organ structure and function. Although stem cells are critical to gastrointestinal development, tissue repair and normal function, the malignant transformation of these cells is critical for initiation of cancer including stomach, colon, liver and pancreas (reviewed in (Merchant & Matsui, 2010)). Traditionally, cancer has been viewed as a disease in which environmental factors induced mutations in critical oncogenes and tumor suppressor genes within a normal cell leading to cancer development. Recently, interest in cancer stem cells has arisen, and evidence has emerged demonstrating that cancer originates from the transformation of tissue stem cells induced by regulatory signals generated within the tumor microenvironment. Such regulatory signals contribute to the cancer stem cell niche by promoting growth of developing tumors through stimulating angiogenesis and the evasion of normal cell death. The intrinsic malignant transformation of stem cells occurs at an accelerated rate under certain environmental pressures that include injury and inflammatory cytokines.

Recruitment of bone marrow-derived mesenchymal stem cells (BM-MSCs) to the local tissue environment is a phenomenon that is traditionally related to the development of an inflammatory response during tumor development in a variety of organs throughout the body (Anjos-Afonso F, 2004; Coffelt et al., 2009; Kidd et al., 2009; Santamaria-Martínez et al., 2009; Shinagawa et al., 2010). The mechanism by which BM-MSCs alter the tissues to which they are recruited is unknown. With chronic inflammation of the stomach bone marrow-derived cells are recruited to the epithelium where, as the inflammatory response progresses, these cells repopulate entire glands and with tumor formation comprise part of the stroma (Houghton J, 2004). Further investigation then demonstrated that mesenchymal stem cells (MSCs) co-expressed gastric markers suggesting they could become incorporated within the gastric epithelium upon recruitment and contribute to the tumor stroma (Houghton J, 2004). Recently, this has been confirmed through an examination of MSC-like cells isolated from human gastric cancer samples which were shown to share many of the same properties as bone marrow-derived MSCs (Cao et al., 2009). To expand on these findings, a further analysis was performed that compared the MSC-like cells isolated from cancer to the same cells from non-cancerous tissue within the same patient, revealing that both populations express similar cells surface markers and genes characteristics of pluriopotent stem cells, mesenchymal cells and factors related to angiogenesis (Xu et al., 2011). Cell cycle analysis also revealed that there was significantly more cancer derived MSC-like cells within the S phase of the cell cycle as compared to the non-cancerous MSC-like cells isolated and normal BM-MSCs (Xu et al., 2011) suggesting that these cells are actively proliferating within the inflamed environment.

To understand how MSCs become carcinogenic, isolated normal BM-MSCs were serially passaged in vitro for over a year and monitored at several stages for malignant transformation by assaying colony formation, growth in soft agar and tumor development in xenografts established in immunocompetent mice (Li et al., 2007). After 12 months of continuous culture, carcinogenic potential was exhibited based on the results of each of these assays and the MSCs were termed “spontaneously transformed”, or stMSCs (Li H,

2007). We have extended these findings by demonstrating that proliferation of stMSCs is dependent on Hedgehog signaling (Figure 2). To examine the role of Hedgehog signaling in stMSC growth in vivo, subcutaneous xenografts using MSCs were established in C57Bl/6 mice. Mice injected with culture media alone were used as controls. Tumors approximately 100-200 $mm^3$ were measured in mice within 7 days of injection. Media controls showed no tumor growth. After the tumors had grown to approximately 100-200 $mm^3$, mice bearing these tumors were injected daily with the hedgehog signaling inhibitor cyclopamine ($MSCs^{Cyclopamine}$) or vehicle ($MSCs^{Vehicle}$). While the tumors in the vehicle treated mice ($MSCs^{Vehicle}$) continued to grow over the next 6 days (Figure 2A), the cyclopamine treated animals ($MSCs^{Cyclopamine}$) ceased to grow and began to regress in size (Figure 2A). Interestingly, mice injected with stMSCs expressing knockdown of Shh ($MSC^{ShhKO}$) cells showed delayed tumor growth and in some animals no tumors at all (Figure 2A). These data show that the Hedgehog signaling pathway is a key component for the growth and proliferation of stMSCs in vivo.

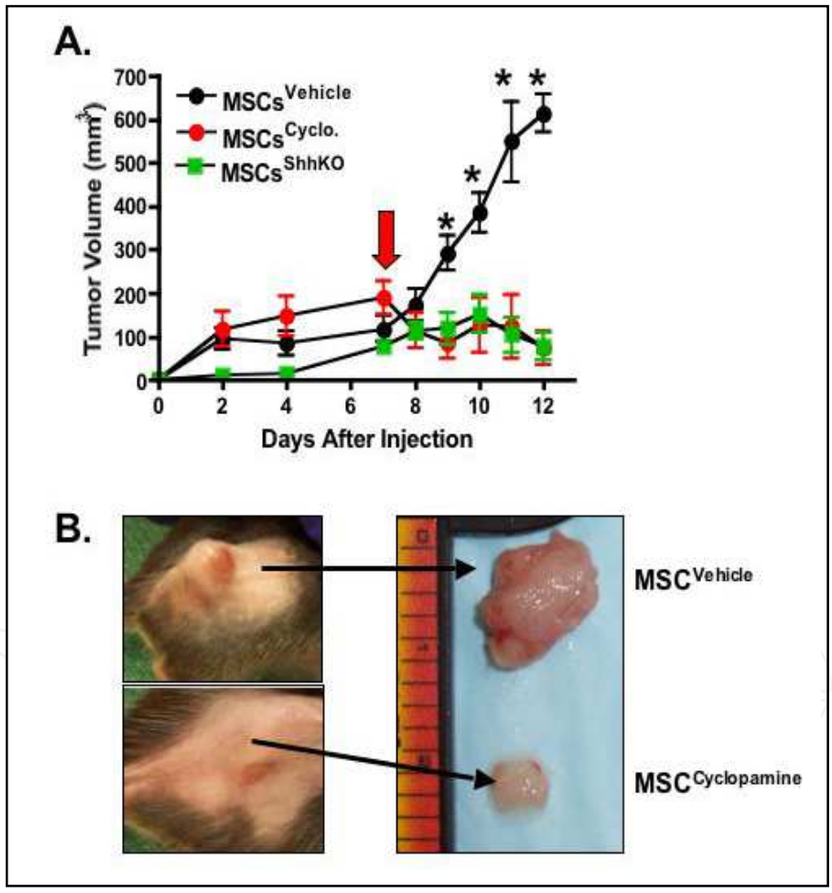


Fig. 2. Hedgehog pathway activity and requirement for growth of stMSCs in vivo. (A) Change in tumor volume ($mm^3$) in response to either vehicle or cyclopamine treatment, or transduced $MSC^{ShhKO59}$ cells over the 12 day experiment. $P<0.05$ compared to $MSC^{Vehicle}$, n = 3-6 mice per group, data shown as mean ± SEM. Arrow shows start of cyclopamine treatment. (B) Changes in tumor sizes dissected from vehicle-treated and cyclopamine-treated $stMSC^{WT}$ injected mice 12 days after xenograft

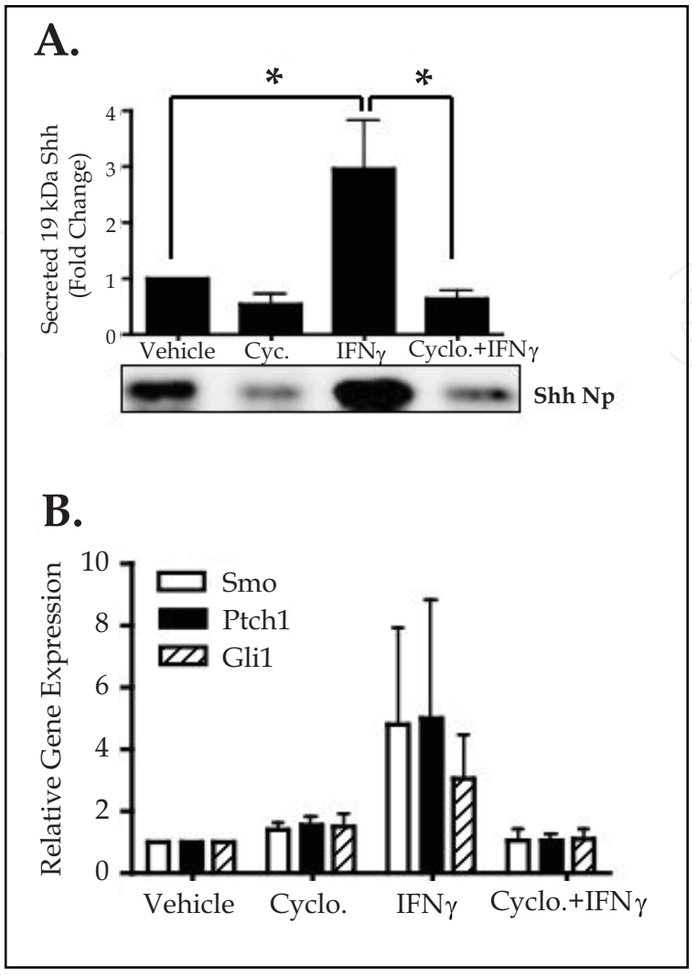


Fig. 3. Changes in Shh secretion and gene expression from IFNγ-treated stMSCs. (A) Western blot analysis of changes in secreted Shh protein (19-kDa, ShhNp) in media collected from stMSCs treated with vehicle, cyclopamine (Cyc.), IFNγ or Cyc. Plus IFNγ. (B) RNA was extracted from stMSCs treated with vehicle, cyclopamine (Cyc.), IFNγ or Cyc. Plus IFNγ and Smo, Ptch and Gli gene expression analyzed by quantitative real-time PCR. *P<0.05 compared to vehicle treated cells, n = 4 individual experiments

The gene expression profile of these cells was compared to early passage BM-MSCs and MSCs isolated from naturally occurring tumors in both aged mice and humans. Carcinogenic MSCs of each type showed uniform increases in expression of factors important in pluripotency and matrix remodeling and metastasis while harboring clinically relevant p53 mutations in addition to alterations in other tumor suppressor genes (Li et al., 2007). These results have since been confirmed in a study of in vivo transformation of MSCs, using BM-MSCs isolated from 2, 8 and 26 month old C57Bl/6 mice in which each set of cell populations were grown in vitro to homogeneity then gene expression compared using the Affymetrix Mouse Genome 430 2.0 GeneChip Array. Of particular interest, between the 8 and 26 month old groups, MSCs displayed significant decreases (> 2 fold) in p53, Cdkn1a, CHEK2 and p21 gene expression, among other apopotic pathway genes (Wilson et al., 2010). Immunoblot in 26 month old MSCs indicated p53 protein expression was essentially absent (Wilson et al.,

2010). While spontaneous transformation of human MSCs (hMSCs) has been more controversial, it has been shown in similar studies that they do undergo transformation in vitro. In fact, data indicate that hMSCs adopt carcinogenic properties as early as 25 days after their initial isolation (Røsland et al., 2009). While these data suggest that the natural process of aging results in mutation and evasion of normal cell death in BM-MSCs, it may not answer the question of what these cells are doing once recruited to a site of inflammation and whether inflammatory insults can also result in modulation of MSC phenotype or transformation.

### 4.2 Bone marrow-derived mesenchymal stem cells (BM-MSCs) express sonic hedgehog

While there is no doubt that increased Hedgehog is apparent in cancer and plays a critical role in driving cancer progression, the source of its increased expression has yet to be identified. One hypothesis is that remodeling at the DNA level is responsible for increased Shh expression in gastric cancer cells. In studies looking at human gastric cancer samples representative of each clinical stage of gastric cancer progression, elevated Shh was correlated with both loss of methylation within the Shh promoter region and hypermethylation of the promoter for Hedgehog interacting protein, an antagonist of the Hedgehog signaling pathway, changes which synergize to produce more Shh gene transcription (Taniguchi et al., 2007). During gastric cancer development it is shown that both Ihh and Shh are increasing within the cancer promoting cells of the epithelium and within a population of cells recruited to the mesenchyme of the stomach, respectively. An alternative hypothesis may be that in cases in which organs of the gastrointestinal tract are acutely injured or have developed carcinoma, there may be an intimate relationship between the inflammatory response and stimulation of Hedgehog secretion.

There is evidence in medulloblastomas of the cerebellum that expression of the cytokine interferon-gamma (IFNγ) during the immune response leads to a direct elevation of Shh protein (Lin et al., 2004; Sun L, 2010; Wang J, 2004; Wang J, 2003). Our laboratory has investigated this relationship within stMSCs harboring p53 mutations that are aggressively carcinogenic (Houghton et al., 2010; Li H, 2007). Bone marrow-derived mesenchymal stem cells (BM-MSCs) were first recognized as regulators of gastric carcinogenesis with the observation that they are recruited to the site of tumor formation in mice infected with *Helicobacter felis* (*H. felis*) and comprise part of the stroma of developing tumors (Houghton J, 2004). We have observed that treatment of these cells in vitro with recombinant IFN□ induces a two-fold increase in Shh secretion (Figure 3A) and gene expression of Hedgehog signaling components Smo, Ptch and Gli (Figure 3B). An interesting observation that was made from these data is that cyclopamine pre-treatment of IFNγ-treated cells resulted in an inhibition of Shh secretion compared to the IFNγ treatment alone (Figure 3A). These data would suggest that there is an autocrine feedback mechanism regulating Shh production from the stMSCs in response to IFNγ. Collectively, these data provide compelling evidence that the recruited stMSCs are in fact the source of local Shh, and may be a first step in identifying the historically elusive source of Shh in advanced gastrointestinal tumors.

### 4.3 Bone marrow-derived mesenchymal stem cells (BM-MSCs) as regulators of cancer stem cells

Recent work with stMSCs using a mouse model of breast cancer has defined the importance of aberrant immune responses in the in vivo transformation and maintenance of these cells

(Houghton et al., 2010). The local tissue environment may be considered carcinogenic, whereby with the ablation of tumor necrosis factor α (TNFα), the progression of neoplasia stimulated by recruited MSCs within epithelia can be halted (Houghton et al., 2010). A study using human MSCs transduced to express TNF-related apoptosis-inducing ligand (TRAIL), are recruited to the site of tumor formation where they produce significant cancer cell apoptosis in a model of squamous and lung cancer cells, enhancing the effects of chemotherapeutic agents (Loebinger et al., 2010). Based on the stromal cell phenotype, investigators suggest that recruited MSCs take on the phenotype of cancer-associated fibroblasts (CAF) (Quante et al., 2011). In IL-1β and *H. felis*-infected mouse models, MSCs are recruited to the gastric epithelium and adopt expression of α–smooth muscle actin, vimentin and FSP1 similar to CAFs (Quante et al., 2011). Differentiated CAFs also express high levels of the chemokines and cytokines such as IL-6, TGF-β, TNFα, and SDF-1α, as compared to wild-type gastric myofibroblasts (Quante et al., 2011). This work suggests several possible roles for these cells in cancer progression, in that they may be involved in regulating the local environment to form a niche for cancer stem cells, impacting the inflammatory response through release of soluble factors and cell-cell interaction or behaving as cancer stem cells themselves.

CD44 is an adhesion molecule expressed in cancer stem cells (Takaishi et al., 2009). The characterization of the resident stem cells of the gastric epithelium is a constantly evolving field of study within gastrointestinal research. While the identification of the CD44 positive cells is unknown, the activation and proliferation within a chronic inflammatory response has indicated that these cells may be the source of the cancer stem cell in the stomach (Ishimoto et al., 2011; Takaishi et al., 2009). The CD44 positive cell population isolated from several human gastric cancer cell lines by flow cytometry displays the phenotype of a cancer stem cell when assayed for spheroid colony formation and in vivo tumorigenicity (Takaishi et al., 2009). Higher CD44 expression was correlated with more aggressive tumor formation when cell lines were transplanted into the skin and stomach of immunodeficient mice, an effect that could be ablated by lentiviral knockdown using shRNA against the CD44 gene (Takaishi et al., 2009). Studies of specific CD44 variants produced by alternative splicing indicate that *H. pylori* infection and inflammation result in upregulation of CD44v6 and CD44v9, while CD44v6 is expressed in the normal gastric mucosa (Fan et al., 1996). Given the recently reported role of Shh as one of the primary stimuli in the induction of cancer stem cell proliferation (Song et al., 2011), we sought to identify the role of Hedgehog as a mediator of stMSC-induced proliferation of CD44 positive cancer stem cells. Figure 4 shows stomach sections collected from mice that were transplanted with $stMSC^{Vect}$ cells tagged with red fluorescent protein (RFP) and injected with either phosphate buffered saline (control, PBS) or IFNγ for 21 days. Stomach sections were collected and immunostained for proliferation marker bromodeoxyuridine (BrdU) and RFP. RFP-tagged $stMSC^{Vect}$ cells were recruited to the gastric mucosa of mice injected with IFNγ(Figure 4B, E) compared to the absence of RFP-tagged $MSC^{Vect}$ cells in the stomachs of PBS-injected mice (Figure 4A). Although IFNγ-treatment appeared to increase the number of proliferating cells within the gastric mucosa (Figure 4B) compared to the PBS-injected mice (Figure 4A), RFP-tagged $MSC^{vect}$ cells stained negative for BrdU (Figure 4B, E). Interestingly, when the same section were immunostained for BrdU and cancer stem cell marker CD44, it appeared as though the proliferating cells were in fact CD44 positive. These results may suggest that BM-MSCs, harboring mutations, are recruited to the sites of inflammation and drive cancer progression through the elevated production of Shh protein that may subsequently induce proliferation of the cancer stem cells.

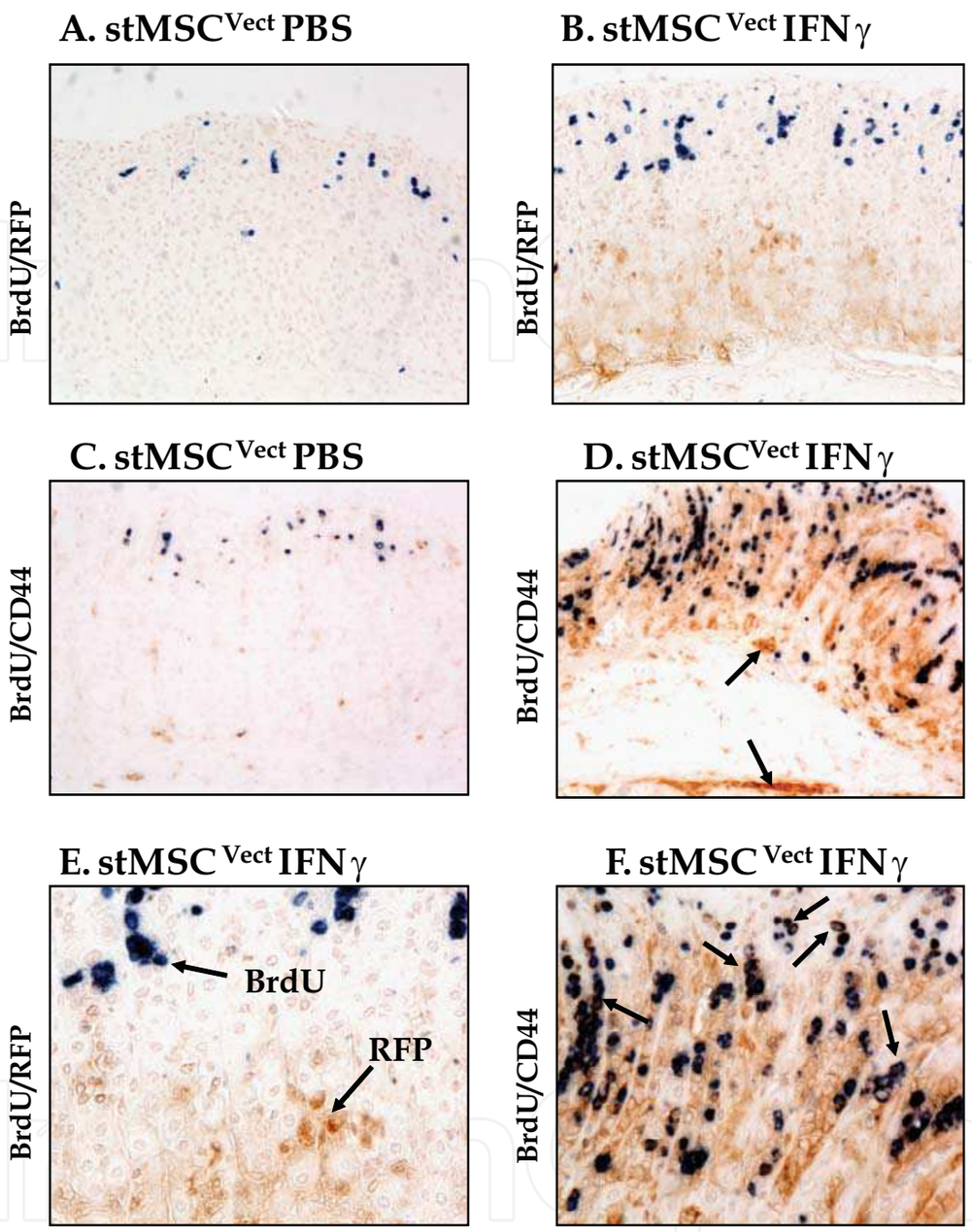


Fig. 4. Proliferation cells within the gastric mucosa of IFNγ-treated mice. Gastric mucosa collected from mice transplanted with stMSC$^{vect}$ cells injected with (A) PBS or (B, E) IFNγ were BrdU labeled (blue) and co-stained with anti-RFP antibody (brown). Higher magnification of image in (B) is shown in (E) where arrows show separate BrdU positive proliferating cells and RFP-tagged stMSCs. Gastric mucosa collected from mice transplanted with stMSC$^{vect}$ cells injected with (C) PBS or (D, F) IFNγ were BrdU labeled (blue) and co-stained with anti-CD44 antibody (brown). Higher magnification of image in (D) is shown in (F) where arrows show BrdU positive proliferating cells co-expressing gastric cancer cell maker CD44. Arrows shown in (D) indicate the expression of CD44 positive cells that are not proliferating. Representative of n = 4-6 mice per group

## 5. Conclusion: The hedgehog signaling network and the cancer stem cell compartment

While loss of Shh is associated with gastric atrophy, the reemergence and over-expression of Shh protein in gastric cancer is an observation that is well established (Berman et al., 2003). The underlying mechanism(s) regulating Shh re-expression and over-expression in malignant

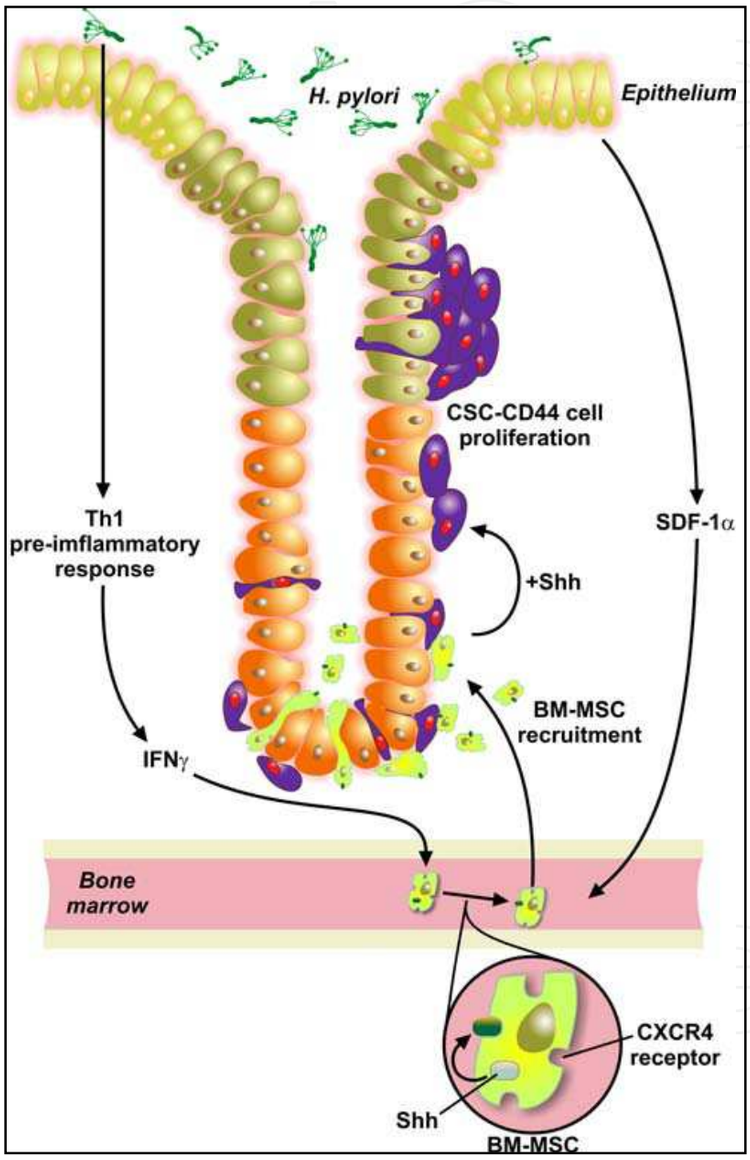


Fig. 5. Proposed model for the role of Shh in the development of gastric cancer. Th1 proinflammatory cytokine IFNg induces Shh expression and secretion from BM-MSCs within the bone marrow compartment. Shh regulates the expression of the CXCR4 that is critical for the recruitment of BM-MSCs to the stomach in response to SDF-1a. The recruitment of BM-MSCs expressing and actively secreting Shh in an environment rich in IFNγ repopulate the damaged gastric epithelium. Shh then induces proliferation of gastric cancer stem cells

that the molecular events begin in the bone marrow compartment in response to inflammation whereby in the stomach is induced by *H. pylori* infection. Several groups have implicated the CXCR4/SDF-1 axis in the recruitment of mesenchymal stem cells to sites of injury as well as to areas of developing carcinoma/tumor stroma (Haider et al., 2008; Kyriakou et al., 2008). What emerges from this body of work is a plausible mechanism for the recruitment of MSCs to the site of developing carcinoma. SDF-1α, that is secreted from the infected epithelium then signals to the BM-MSCs to initiate recruitment to the stomach. The recruitment of BM-MSCs expressing and actively secreting Shh in an environment rich in inflammatory cytokines including IFNγ repopulate the damaged gastric epithelium. Shh then acts on the gastric cancer stem cells to induce proliferation and eventually tumor development (Figure 5). BM-MSCs play a multifaceted role contributing to the cancer stem cell niche but also promote growth of developing tumors through stimulating angiogenesis and the evasion of normal cell death within the cancer stem cell population. Therefore, it is critical to define the mechanisms by which BM-MSCs support these alterations in the setting of cancer development in order to create new therapeutic approaches. The intrinsic transformation into malignant cells, which can occur at an accelerated rate under certain environmental pressures, only highlights the need for more advanced studies.

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**Cancer Stem Cells - The Cutting Edge**

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Over the last thirty years, the foremost inspiration for research on metastasis, cancer recurrence, and increased resistance to chemo- and radiotherapy has been the notion of cancer stem cells.The twenty-eight chapters assembled in Cancer Stem Cells - The Cutting Edge summarize the work of cancer researchers and oncologists at leading universities and hospitals around the world on every aspect of cancer stem cells, from theory and models to specific applications (glioma), from laboratory research on signal pathways to clinical trials of bio-therapies using a host of devices, from solutions to laboratory problems to speculation on cancersâ€™ stem cellsâ€™ evolution. Cancer stem cells may or may not be a subset of slowly dividing cancer cells that both disseminate cancers and defy oncotoxic drugs and radiation directed at rapidly dividing bulk cancer cells, but research on cancer stem cells has paid dividends for cancer prevention, detection, targeted treatment, and improved prognosis.

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