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# Indian Hedgehog Mediates Gastrin-Induced Proliferation in Stomach of Adult Mice

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## Abstract



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**Background & Aims—**Loss of expression of sonic hedgehog (SHH) from parietal cells results in hypergastrinemia in mice, accompanied by increased expression of indian hedgehog (IHH) and hyperproliferation of surface mucous cells. We investigated whether hypergastrinemia induces gastric epithelial proliferation by activating IHH signaling in mice.

**Methods—**We studied mice with parietal cell-specific deletion of *Shh* (PC-$Shh^{KO}$) and hypergastrinemia, crossed with gastrin-deficient (GKO) mice (PC-$Shh^{KO}$/GKO). When mice were 3–4 months old, gastric tissues were collected and analyzed by histology, for incorporation of bromodeoxyuridine (BrdU), and for expression of the surface mucous cell marker ulex europaeus. PC-$Shh^{KO}$/GKO mice were given gastrin infusions for 7 days; gastric surface epithelium was collected and expression of IHH was quantified by laser capture microdissection followed by quantitative reverse transcriptase PCR. Mouse stomach-derived organoids were incubated with or without inhibitors of WNT (DKK1) or smoothened (vismodegib) and then co-cultured with immortalized stomach mesenchymal cells, to assess proliferative responses to gastrin.

**Results—**Gastric tissues from PC-$Shh^{KO}$/GKO mice with hypergastrinemia had an expanded surface pit epithelium, indicated by a significant increase in numbers of BrdU- and ulex europaeus-positive cells, but there was no evidence for hyperproliferation. Gastrin infusion of PC PC-$Shh^{KO}$/GKO mice increased expression of *Ihh* and proliferation within the surface epithelium, compared with mice given infusions of saline. In gastric organoids co-cultured with immortalized stomach mesenchymal cells, antagonists of WNT and smoothened inhibited gastrin-induced proliferation and WNT activity. Activity of WNT in media collected from immortalized stomach mesenchymal cells correlated with increased expression of *Gli1*, and was inhibited by DKK1 or vismodegib.

**Conclusion—**IHH signaling mediates gastrin-induced proliferation of epithelial cells in stomachs of adult mice.

### Keywords

development; gastric epithelium; signal transduction; tissue regeneration

## INTRODUCTION

The Hedgehog (Hh) genes Sonic Hedgehog (Shh) and Indian Hedgehog (Ihh) are well known for their roles in governing gastric tissue patterning during embryonic development [1, 2]. In the adult stomach, Shh signaling maintains gastric acid secretion and regulates multiple molecular processes such as cell differentiation, proliferation and tissue regeneration [1, 3–6]. However, the role of Ihh within the adult stomach is largely unknown. In the adult stomach, Shh ligand is predominantly expressed and secreted from parietal cells whereas Ihh is expressed primarily in the surface epithelium [4, 7]. Deletion of Shh specifically in the murine parietal cells induces expression of Ihh. However, induction of Ihh does not compensate for the loss of Shh which leads to disrupted zymogen cells differentiation [4]. This raises the possibility that Ihh plays a different role in adult stomach.

Based on our published study [4], parietal cell-specific deletion of Shh (PC-$Shh^{KO}$) mice develop elevated plasma gastrin (hypergastrinemia), increased Ihh expression and hyperproliferation of the surface epithelium. Suppression of hypergastrinemia by

somatostatin analogue octreotide reverses all these changes and thus indicates that these observations are largely due to elevated plasma gastrin. We postulate that hypergastrinemia results in the upregulation of Ihh within the surface mucous epithelium that subsequently results in hyperproliferation that was originally reported in the PC-Shh$^{KO}$ mouse stomachs. Gastrin has been shown to induce Shh production, processing and activity that is dependent on acid secretion [8, 9]. However, there is little information regarding the direct regulatory function of gastrin on Ihh in the stomach.

In addition to its known role in acid secretion, gastrin also stimulates and maintains the proliferation of the gastric epithelium (reviewed in [10])[4, 11–13]. However, the molecular mechanisms that mediate these actions have not yet been identified. The current study tests the hypothesis that Ihh acts as a mediator of gastrin-induced proliferation in the adult stomach. In the current study, PC-Shh$^{KO}$ mice were crossed with the gastrin-deficient (GKO) mouse model (PC-Shh$^{KO}$/GKO) [14]. Using the PC-Shh$^{KO}$/GKO mouse model, the current study demonstrates that Ihh signaling mediates gastrin-induced proliferation of the surface mucous epithelium. These findings provide a critical link towards understanding the molecular mechanisms that control gastric epithelial proliferation in the adult stomach.

## MATERIALS AND METHODS

### Animals

A mouse model expressing a parietal cell-specific constitutive deletion of Shh (PC-Shh$^{KO}$) was generated and used based on our pulished studies by Xiao et al [4]. PC-Shh$^{KO}$ were crossed with gastrin deficient mice (GKO backcrossed onto a C57BL/6 background were the gift of Dr. Linda Samuelson, University of Michigan) [14]. Control (C57BL/6 or HKCre), gastrin-deficient (GKO), PC-Shh$^{KO}$ and PC-Shh$^{KO}$/GKO were analyzed at 3 months of age. To visualize Shh ligand expression we used a mouse model that expresses Shh fused to green fluorescent protein in place of wild-type Shh (Shh::GFP mice, The Jackson Laboratory, stock number 08466). GFP is inserted into the Shh protein such that secreted ligand retains both GFP and lipid modifications required for signaling. Genotyping was based on polymerase chain reaction primers and protocols described in Clausen *et al.* [15]. All mouse studies were approved by the University of Cincinnati Institutional Animal Care and Use Committee that maintains an American Association of Assessment and Accreditation of Laboratory Animal Care facility.

### Generation of gastric organoids

Gastric fundic organoids were prepared based on the protocol by Barker *et al.* [16] and our recently reported protocol [17]. Primary epithelial cells from adult mice were cultured as 3-dimensional structures called organoids. We dissected stomachs from PC-Shh$^{KO}$/GKO mice, made an incission along the greater curvature and washed in ice-cold $Ca^{2+}$/$Mg^{2+}$-free Dulbecco's Phosphate Buffered Saline (DPBS). The stomach was separated from loose mesenchymal tissue and visible blood vessels. Gastric fundus was further separated and cut into 2–3 pieces. Tissue was incubated in 5 ml 5mM EDTA for 2 hours at 4°C with gentle shaking. Fluid was replaced with 5ml chelation agent. Next tissue was shaken vigorously for approximately 2 minutes to dissociate glands. Dissociated glands were centrifuged at 150 g

for 5 minutes then were embedded in Matrigel (BD Biosciences) supplemented with Advanced DMEM/F12 medium (Invitrogen), Wnt conditioned medium, R-spondin conditioned medium supplemented with gastric growth factors including bone morphogenetic protein inhibitor, Noggin (PeproTech), Gastrin (Sigma), Epidermal grow factor (EGF, PeproTech) and Fibroblast growth factor 10 (FGF-10, PeproTech) as previously described [16]. Glands matured into organoids by 1–2 days. Gastric organoids were passaged every 12 days. Organoids were removed from matrigel using ice-cold DPBS, and broken up by pipetting. Fractions were spun down at 150 g for 5 minutes, and the pellet re-suspended in matrigel followed by the addition of medium supplemented with growth factors.

For detailed methods please refer to Supplemental Materials and Methods section

## RESULTS

### Hypergastrinemia induces proliferation of the surface mucous pit epithelium in PC-Shh$^{KO}$ mice

To identify the role of hypergastrinemia as a cause of the expansion in the surface epithelium, we developed a mouse model expressing a parietal cell-specific deletion of Shh on a gastrin-null background (PC-Shh$^{KO}$/GKO). Gastrin-expressing G-cell numbers and circulating plasma concentrations were measured in controls, PC-Shh$^{KO}$, gastrin-deficient (GKO) and PC-Shh$^{KO}$/GKO mice (Supplemental Figure 1A, B). Morphometric analysis showed significantly greater numbers gastrin-expressing cells in the antrums of PC-Shh$^{KO}$ mice compared to the control group (Supplemental Figure 1A). Increased gastrin cell numbers correlated with severe hypergastrinemia in the PC-Shh$^{KO}$ mice when compared to the controls (Supplemental Figure 1B), consistent with previous reports [4]. Gastrin-expressing cells and circulating gastrin concentrations were not detected in GKO and PC-Shh$^{KO}$/GKO mice (Supplemental Figure 1A, B).

Histological evaluation showed that compared to the control mouse stomachs, foveolar hyperplasia (a cystic expansion of surface epithelium into gland and base region of the stomach) was significantly greater in the PC-Shh$^{KO}$ mice (Supplemental Figure 2A, B). Foveolar hyperplasia was not observed in the GKO (Supplemental Figure 2A, B) or in the PC-Shh$^{KO}$/GKO (Supplemental Figure 2A, B) mouse stomachs. Consistent with our previous report was the lack of inflammation and atrophy within the stomachs of the PC-Shh$^{KO}$ mice (Supplemental Figure 2C, D) [4]. While inflammation and atrophy was documented in the stomachs of the GKO mice [18] (Supplemental Figure 2C, D), PC-Shh$^{KO}$/GKO animals did not develop inflammation (Supplemental Figure 2C, D). These data demonstrated that expansion of the surface pit epithelium previously observed in the PC-Shh$^{KO}$ mouse model [4] may be explained by the hypergastrinemia generated as a result of loss of Shh.

### Expansion of the surface mucous pit epithelium correlates with increased proliferation

To qualitatively compare the surface epithelial cell census, stomachs collected from controls, GKO, PC-Shh$^{KO}$ and PC-Shh$^{KO}$/GKO mice were stained with mucous pit cell

marker UEAI (Figure 1A, B). Compared to the control, PC-Shh$^{KO}$ mice clearly showed an expansion in the surface epithelium into the base of the gland (Figure 1A). Both GKO and PC-Shh$^{KO}$/GKO mice had surface epithelium that was similar to the control. Based on the number of BrdU-labeled/UEAI+ cells, PC-Shh$^{KO}$ had significantly increased proliferation compared to controls (Figure 1B). However, PC-Shh$^{KO}$/GKO mice showed decreased proliferation when compared to the PC-Shh$^{KO}$ animals (Figure 1B). Consistent with previous reports [18], GKO mice had increased proliferation when compared to the control group (Figure 1B). Consistent with the histological evaluation, immunofluorescence staining revealed significant increase in the number of UEAI+ stained surface pit cells in hypergastrinemic PC-Shh$^{KO}$ mice (Figure 1C, D). These data demonstrated that expansion of the surface pit epithelium previously observed in the PC-Shh$^{KO}$ mouse model [4] may be explained by the hypergastrinemia generated as a result of loss of Shh.

Also, consistent with the histological evaluation was the observation that PC-Shh$^{KO}$ mice did not exhibit diminished parietal cell numbers as indicated by immunofluorescence using an antibody specific for $H^+$, $K^+$-ATPase (Figure 1C, D). In contrast to the PC-Shh$^{KO}$ mice, GKO mice had significantly fewer parietal cells within the gastric mucosa (Figure 1C, D). Morphometric analysis using a Chromogranin A (CgA)-specific antibody revealed a significant increase in the number of ECL-cells in the hypergastrinemic PC-Shh$^{KO}$ mice relative to controls (Figure 1C, D). However, there was no detectable difference in the ECL-cells numbers in the GKO or PC-Shh$^{KO}$/GKO mouse stomachs (Figure 1C, D). Thus, while hypergastrinemia resulted in increased ECL-cells, the lack of gastrin did not reduce the total number of ECL-cells. This was consistent with previous studies showing that, gastrin deficiency causes ECL-cell functional impairment but does not alter overall cell number [14].

### Expression pattern of Shh and Ihh in the stomach

The expression patterns of Shh and Ihh were investigated using stomach sections collected from a mouse model that expressed Shh fused to green fluorescent protein in place of wild-type Shh (Shh::GFP mice) that were co-stained for Ihh and UEAI. Ihh was prodominantly expressed in the pit region and colocalized with the expression of lectin UEAI (Figure 2A, B). While we also observed expression of GFP, indicating Shh ligand expression, at the surface pit region, GFP was predominantly expressed within parietal cells of the fundic mucosa (Figure 2A, B).

Immunofluorescence staining was confirmed by qRT-PCR performed on cells captured by LCM from the pit, neck and base regions of the gastric mucosa of control, GKO, PC-Shh$^{KO}$ and PC-Shh$^{KO}$/GKO mice (Supplemental Figure 3A). The purity of each region captured was analyzed by qRT-PCR for specific genes known to be restricted to the pit, neck and based and included: ATP4α (parietal cells), MUC5AC (surface pit cells) and MUC6 (neck cells) and PgC (zymogen cells). As expected, the expression of ATP4α (parietal cells marker) and MUC6 (a mucous neck cell marker) were higher in the neck region compared to pit cells, while expression of MUC5AC (a surface mucous cell marker) was higher in pit than neck and base cells (Supplemental Figure 3B, C). Cells collected by LCM from the pit, neck and base of the gastric tissue all expressed the CCK-BR (Supplemental Figure 3C).

Relative to total epithelium collected from control mice, approximately 80% of Shh gene expression was observed in the neck region (Figure 2C). Relative to total epithelium collected from control mice, the overall total Shh expression was significantly decreased in the stomachs of PC-Shh$^{KO}$, GKO and PC-Shh$^{KO}$/GKO mice (Figure 2C). In addition, relative to total epithelium collected from control mice Shh expression was significantly decreased in the neck region of PC-Shh$^{KO}$, GKO and PC-Shh$^{KO}$/GKO mouse stomachs (Figure 2C). Relative to total epithelium collected from control mice, approximately 20% of Shh was expressed within the pit region of control mouse stomachs (Figure 2C). Pit cell-expressed Shh was similar among all genotypes (Figure 2C). Thus, most of Shh is expressed within the cells of the neck region of the stomach that is significantly reduced in the PC-Shh$^{KO}$, GKO and PC-Shh$^{KO}$/GKO mice.

Relative to total epithelium collected from control mice, approximately 80–90% of Ihh was expressed within the pit region of the stomach (Figure 2D). Relative to total epithelium collected from control mice, the overall total Ihh expression was significantly increased in the stomachs of PC-Shh$^{KO}$ mice that was accounted for in the pit region (Figure 2D) and as previously reported [4]. Gastrin deficiency in both GKO and PC-Shh$^{KO}$/GKO mice correlated with significantly decreased Ihh expression in the total and pit epithelium compared to expression measured in control animals (Figure 2D). These data suggest that hypergastrinemia may induce Ihh expression at the surface epithelium.

## Gastrin stimulates proliferation and Ihh expression in the surface pit epithelium

To identify the direct role of gastrin as a regulator of Ihh expression, PC-Shh$^{KO}$/GKO mice were implanted with a minipump and infused with gastrin. Changes in gastric epithelial proliferation were measured by BrdU incorporation and gene expression by the surface epithelium measured by qRT-PCR (Figure 3). Gastrin radioimmunoassay confirmed a significant increase in circulating gastrin concentrations during the 7-day infusion (Figure 3A). Gastrin induced a significant increase both Ihh and Gli1 in the cells collected from the pit region of the gastric tissue. (Figure 3B). The increase of Ihh and Gli1 expression correlated with a significant increase in cell proliferation indicated by BrdU incorporation and UEAI labeled surface pit cells numbers in PC-Shh$^{KO}$/GKO mice stomachs (Figure 3C, D, E). To identify whether gastrin-induced cell proliferation was mediated by Hedgehog signaling, gastrin-infused PC-Shh$^{KO}$/GKO mice were treated with cyclopamine. Cyclopamine treatment significantly suppressed Hedgehog target gene Gli1 (Figure 3B), gastrin-induced cell proliferation (Figure 3D) and surface pit cell numbers (Figure 3E) in PC-Shh$^{KO}$/GKO mice. These data show that gastrin induces Ihh expression within the surface epithelium of the adult stomach. Subsequently the proliferative response to gastrin is mediated by Hedgehog signaling.

## Ihh signals via the mesenchyme when mediating gastrin-induced proliferation

To identify whether Ihh signals via an epithelial-mesenchymal crosstalk as part of the regulation of gastrin-induced proliferation, epithelial and mesenchymal components of 7 day gastrin-infused PC-Shh$^{KO}$/GKO mouse stomachs were separated (Figure 4A). The purity of each fraction was analyzed by qRT-PCR for specific genes known to be restricted to the mesenchyme (vimentin, Actg2 and Madcam1) (Figure 4B) or epithelium (keratin 20 and

MUC5AC) (Figure 4C). We found that Ihh expression was restricted to the epithelium in the adult stomach (Figure 4C). In gastrin-infused PC-Shh$^{KO}$/GKO mouse stomachs, gastrin induced Ihh expression predominantly in epithelium and this increase was abrogated by the gastrin receptor (CCKBR) antagonist L-365, 260 (Figure 4D). In contrast to Ihh expression, gastrin induced Gli1 expression within the mesenchyme (Figure 4E) suggesting that Ihh may mediate gastrin-induced proliferation via an epithelial-mesenchymal crosstalk.

## Ihh mediates gastrin-induced proliferation in a culture of primary fundic epithelial 3-D organoids

To investigate whether epithelial-mesenchymal crosstalk is involved in the regulation of epithelial proliferation we used a co-culture system of gastric organoids derived from the PC-Shh$^{KO}$/GKO mouse fundus and an immortalized stomach mesenchymal cell line (ISMC) [19]. Supplemental Figure 4A demonstrates our ability to maintain an organoid culture system derived from the fundic region of the stomach based on our protocol [17] modified from Barker *et al.* [16]. The gastric glands rapidly formed small cyst-like structures that were visible within 4 days of culture. While gastric organoids expressed the mesenchymal marker alpha smooth muscle actin (αSMA), the expression levels decreased with increased length of time in culture in parallel with decreased expression of Gli1 (Supplemental Figure 4B). Based on declining Gli1 expression, it was not surprising to observe decreased proliferative capacity in response to gastrin in organoids kept in culture both for 12 and 21 days compared to the 7 day-old cultures (Supplemental Figure 4C). This demonstrated that gastric organoid cultures lack a mesenchymal compartment which is a limitation when studying epithelial-mesenchymal crosstalk. This limitation was overcome by co-culturing the fundic gastric organoids with a mouse-derived immortalized stomach mesenchymal cell line (ISMCs). At 10 days of co-culture we observed that the fundic organoids expressed all the major cell lineages of the stomach as determined by qRT-PCR (Supplemental Figure 4D) and flow cytometry (Supplemental Figure 4E, F). In particular, gastrin expression was absent and Shh expression reduced from the fundic organoids generated from PC-Shh$^{KO}$/GKO mice (Supplemental Figure 4D). Therefore, for subsequent experiments we used fundic organoids derived from the stomachs of PC-Shh$^{KO}$/GKO mice co-cultured with ISMCs.

Based on our work in the PC-Shh$^{KO}$ mice, loss of Shh triggers a number of molecular events that lead to hyperproliferation [4]. Activation of the Wnt pathway is known to result in β-catenin nuclear accumulation [20], and experiments using rat kidney epithelial cells (RK3E cells) show that Gli induces transcription of Wnt [21]. Therefore, we hypothesized that Gli1 activation, via gastrin-induced Ihh, signals via Wnt to induce proliferation within the organoids. We first assessed the proliferative response to gastrin with or without Wnt/β-catenin signaling antagonist recombinant Dkk1 or smoothened inhibitor vismodegib pretreatment (Figure 5A). Immunofluorescence staining of proliferative marker EdU revealed a significant induction in proliferation within cultured organoids in response to 100 nM gastrin compared to cultures depleted of gastrin (Figure 5B). Both Dkk1 and vismodegib pretreatment significantly blocked the proliferative response to gastrin in the cultured organoids (Figure 5B).

Gastrin-induced proliferation correlated with a significant induction in Wnt activity as measured by the TOPflash assay using conditioned medium collected from the ISMCs cultured compartment of the transwell (Figure 5C). Dkk1 and vismodegib pre-treatment significantly inhibited gastrin-induced Wnt activity (Figure 5C). The Wnt activity in the medium collected from the ISMCs correlated with a significant increase in Gli1 expression by ISMCs that was inhibited by either Dkk1 and vismodegib pretreatment (Figure 5D). Gli1 was not expressed in the organoids after culture with ISMCs (data not shown). Ihh secretion (Figure 5E) and expression (Figure 5F) was significantly induced in response to gastrin, a response that was not inhibited by either Dkk1 or vismodegib pretreatment. Ihh was not expressed in the ISMCs after culture with organoids (data not shown). All experiments were repeated using Wnt antagonist FH535 and Smoothened inhibitor itraconazole (Supplemental Figure 6A–F). All responses to FH535 and itraconazole pretreatments were similar to those reported in response to Dkk1 and vismodegib (Supplemental Figure 6A–F). Therefore, gastrin-induced proliferation of fundic gastric organoids was mediated by Hedgehog signaling and likely the release of Wnt ligand from the ISMCs.

To confirm that the epithelial proliferative response to gastrin was indeed mediated by the CCK-B receptor, fundic organoids were generated from the stomachs of CCK-B receptor-deficient (CCK-BR KO) mice. Interestingly, we observed that the average sizes of the organoids derived from CCK-BR KO mice were smaller and less proliferative compared to PC-Shh$^{KO}$/GKO organoids (Figure 5B). Loss of gastrin-induced proliferation was observed in the CCK-BR KO mouse-derived organoids (Figure 5B). Consistent with the loss of gastrin-induced proliferation in CCK-BR KO organoids, Wnt activity (Figure 5C), Gli1 expression in the ISMCs (Figure 5D) and Ihh expression in organoids (Figure 5E, F) was also lost in response to gastrin. These data show that the CCK-BR mediates the epithelial proliferative response to gastrin observed in the organoid cultures.

## ISMC conditioned medium collected after Ihh treatment induced proliferation in the organoids

To identify the role of Ihh signaling in Wnt-mediated proliferation organoids were treated with conditioned media collected from 6 treatment groups (Figure 6A). ISMCs were transduced to express empty vector (ISMC$^{EV}$) or a knockdown of smoothened (ISMC$^{SmoKD}$) that was confirmed by western blot analysis (Figure 6A). ISMC$^{EV}$ and ISMC$^{SmoKD}$ cells were treated with either PBS (treatments 1 and 3) or recombinant Ihh (rIhh, treatments 2 and 4) (Figure 6A). With Smo intact in ISMC$^{EV}$ cells, rIhh treatment resulted in a significant increase in Gli1 gene expression from the cells (Figure 6B) and elevated Wnt activity in the conditioned medium (Figure 6C) compared to PBS treated groups. In contrast to the ISMC$^{EV}$ cells, rIhh did not induce Gli gene expression (Figure 6B) or Wnt activity in the conditioned medium (Figure 6C) collected from the ISMC$^{SmoKD}$ cells. Importantly, conditioned medium collected from ISMC$^{EV}$ cells treated with rIhh induced a significant increase in proliferation in the organoid cultures (Figure 6D, E). However, the conditioned medium collected from ISMC$^{SmoKD}$ cells treated with rIhh did not induce a proliferative response in the organoids (Figure 6D, E). Furthermore, conditioned medium collected from ISMC$^{EV}$ cells treated with rIhh was pretreated with Wnt antagonist Dkk1. While Gli1 was still induced in the ISMC$^{EV}$ cells (Figure 6B), Dkk1 abolished the Wnt

activity within conditioned medium (Figure 6C), and the proliferative response within the organoids treated with conditioned medium collected from $ISMC^{EV}$ cells treated with rIhh (Figure 6D, E). Taken together, these results showed that Ihh induces Wnt-mediated epithelial cell proliferation and thus supporting a role of soluble Wnts in this proliferative response.

## DISCUSSION

Within the adult stomach Ihh acts as a mediator of epithelial proliferation based on the evidence that: 1) *in vivo* PC-$Shh^{KO}$/GKO mice that have loss of gastrin and Ihh expression exhibit decreased proliferation compared to the hypergastrinemic PC-$Shh^{KO}$ mice, 2) *in vivo* gastrin-induced proliferation in PC-$Shh^{KO}$/GKO mice is blocked by Hedgehog signaling inhibitor cyclopamine, and 3) *in vitro* gastrin-induced proliferation of fundic organoids derived from PC-$Shh^{KO}$/GKO mouse stomach, is blocked by a smoothened inhibitor. Previous studies, including those from our laboratory, have clearly demonstrated that hypergastrinemia correlates with hyperproliferation and foveolar hyperplasia [4, 11–13]. However, none of these studies investigated the role of Ihh as a mediator of this proliferative response. Based on our published study [4], PC-$Shh^{KO}$ mice develop hypergastrinemia with increased Ihh expression and hyperproliferation of the surface epithelium. Here we extend these findings by demonstrating that hypergastrinemia results in the upregulation of Ihh within the surface mucous epithelium that subsequently results in hyperproliferation that was originally reported in the PC-$Shh^{KO}$ mice.

We show that when hypergastrinemic PC-$Shh^{KO}$ mice are crossed onto a GKO background, there was a significant reduction in Ihh expression that correlated with decreased proliferation within the surface epithelium. In support of our data, the function of gastrin as a regulator of gastric proliferation is well accepted. In rats, increased gastric proliferation is observed after ingestion of a meal. Gastrin immunoneutralization inhibits this meal-induced proliferation and thus demonstrates a direct role of gastrin as a regulator of gastric proliferation [12]. Furthermore increased circulating gastrin concentrations (hypergastrinemia), as a consequence of treatment with acid blockers, gastrin-secreting tumors, or transgenic mice over-expressing gastrin, leads to increased gastric proliferation [13,22]. Here we advance these findings by demonstrating that Ihh mediates gastric-induced proliferation.

*In vivo*, gastrin stimulated proliferation by inducing the expression of Ihh specifically within the surface pit epithelium. We know from studies using the GKO mouse model and cultured canine parietal cells that gastrin induces Shh expression and processing. Moreover, in the adult stomach hormonally-regulated Shh processing is acid-dependent and is mediated by the aspartic protease pepsin A [9, 23]. Other studies using isolated canine parietal cells and mouse organ cultures demonstrate that gastrin regulates Shh gene expression by increasing intracellular calcium release and protein kinase (PKC) activation [8]. The functions of gastrin are mediated by the G-protein coupled CCK-B receptor. Binding gastrin with the CCK-B receptor activates phospholipase C via stimulating PKC and increasing intracellular calcium [10]. Similar to Shh regulation, we may speculate that gastrin regulates Ihh expression by increasing intracellular calcium release and PKC activation, but this required further

investigation. Our *in vitro* studies using organoids derived from the CCK-B receptor deficient mouse stomachs also supported the role of gastrin as an inducer of the epithelial proliferative response. In the mouse, in situ hybridization indicates that gastrin directly stimulates the growth of the pit cell lineage by inducing the CCK-B receptor in pit cell precursors [24]. Collectively this evidence supports an interaction between gastrin and the surface pit epithelium via the CCK-B receptor.

Gastrin induced an increase in epithelial Ihh expression that correlated with elevated Gli1 expression within the mesenchyme *in vivo*. These data suggested that Ihh may mediate gastrin-induced proliferation via an epithelial-mesenchymal crosstalk. To investigate whether epithelial-mesenchymal crosstalk was involved in the regulation of epithelial proliferation we used a co-culture system of mouse-derived fundic gastric organoids and an immortalized stomach mesenchymal cell line (ISMC) [19]. We hypothesized that Gli1 activation, via gastrin-induced Ihh, signals via Wnt to induce proliferation within the organoids. Our first observation was that gastrin-induced proliferation was significantly inhibited by either Wnt antagonist Dkk1 and smoothened inhibitor vismodegib thus supporting a necessary role of both Wnt and Hedgehog signalling in gastrin-induced proliferation. Inhibition of epithelial proliferation correlated with a significant reduction in gastrin-induced Wnt activity as measured using medium collected from the ISMCs cultured compartment of the transwell. Our second observation was that the induction of Wnt activity in response to gastrin correlated with a significant increase in Ihh release from the gastric epithelial organoids and a significant increase in Gli1 expression that was inhibited by either Dkk1 or vismodegib. These data support the conclusion that gastrin-induced proliferation of fundic gastric organoids was mediated by Hedgehog signaling and likely the release of Wnt ligand from the ISMCs.

Importantly, conditioned medium collected from $ISMC^{EV}$ cells treated with rIhh induced a significant increase in proliferation in the organoid cultures. However, the conditioned medium collected from $ISMC^{SmoKD}$ cells treated with rIhh did not induce a proliferative response in the organoids. Thus supporting the role of soluble Wnts as mediators of Ihh-induced proliferation. Our findings support a signaling pathway involving the crosstalk between epithelial Ihh signaling to Gli1 then relaying to an intermediate mediator to induce proliferation. In support of this notion, based on our work in the PC-$Shh^{KO}$ mice, loss of Shh triggers a number of molecular events that lead to hyperproliferation [4]. Activation of the Wnt pathway is known to result in β-catenin nuclear accumulation [20], and experiments using rat kidney epithelial cells (RK3E cells) show that Gli induces transcription of Wnt [21]. Therefore, we may conclude that Gli1 activation may signal via Wnt to induce proliferation within the surface epithelium (Figure 7). Collectively, these findings provide a critical link towards understanding the molecular mechanisms that control gastric epithelial proliferation in the adult stomach.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

| | |
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| **Hh** | Hedgehog |
| **Shh** | Sonic Hedgehog |
| **Ihh** | Indian Hedgehog |
| **Gli** | Gli1, glioma-associated oncogene homolog 1 |
| **PgC** | pepsinogen |
| **Ptch** | patched receptor |
| **Smo** | Smoothened |
| **UEA1** | *Ulex europaeus* 1 |
| **Atp4a** | $H^+$,$K^+$-ATPase |
| **MUC5AC** | mucin 5AC |
| **MUC6** | mucin 6 |
| **CCK-BR** | cholecystokinin/gastrin-B receptor |
| **GKO** | gastrin-deficient |
| **PC-Shh$^{KO}$** | mice expressing a parietal cell-specific deletion of Shh |
| **PC-Shh$^{KO}$/GKO** | PC-Shh$^{KO}$ mice on a gastrin-deficient background |

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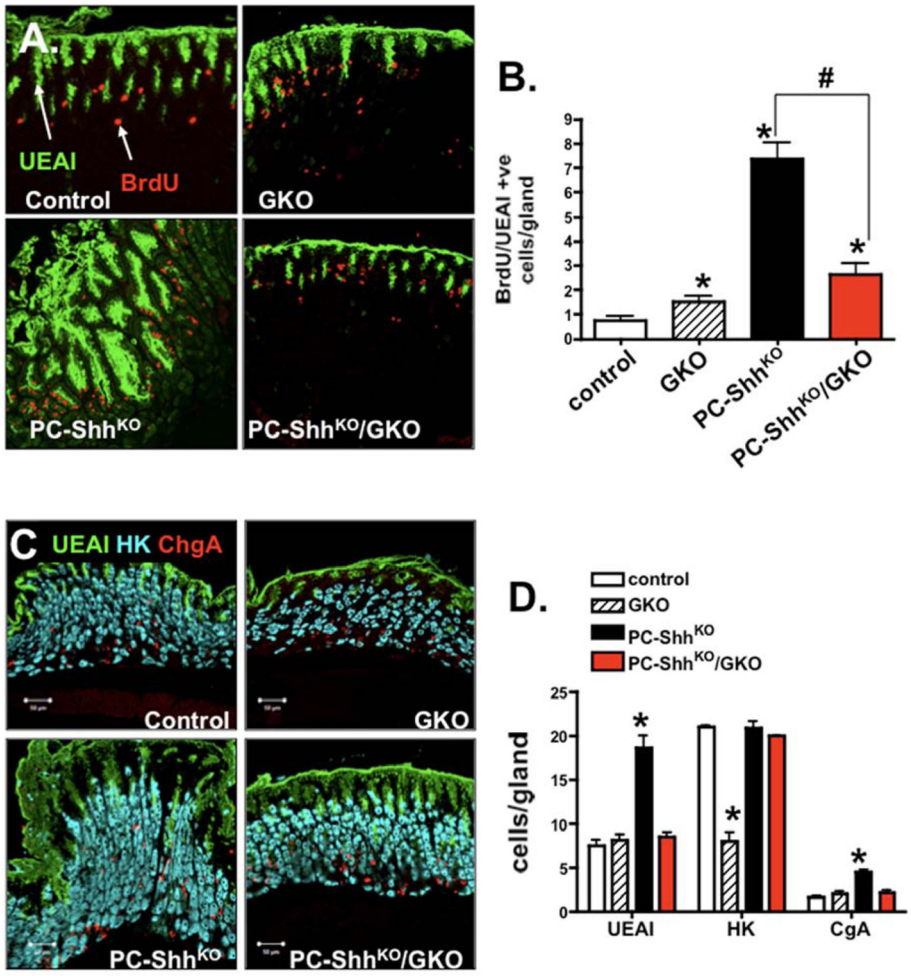



**Figure 1. Quantification of epithelial cells and surface pit mucous-proliferating cells in control, GKO, PC-Shh$^{KO}$ and PC-Shh$^{KO}$/GKO mouse stomachs**

**(A)** Stomach sections collected from BrdU-injected mice were immunostained for UEAI (green) and BrdU (red) in 3–4 month old control, GKO, PC-Shh$^{KO}$ and PC-Shh$^{KO}$/GKO mice. (**B**) BrdU-labeled nuclei were counted and expressed as BrdU positive cells/gland. **(C)** Stomach sections collected from control, GKO, PC-Shh$^{KO}$ and PC-Shh$^{KO}$/GKO were immunostained for surface mucous pit cells (UEAI, green), parietal cells (HK, blue) and chromagranin A (Cg A, red). **(D)** The number of UEAI+, HK+ and CgA+ cells were quantified. Representative of n = 4 mice per group. Data is expressed as the mean ± SEM. *$P < 0.05$ compared to control mice, #P<0.05 compared to PC-Shh$^{KO}$ mice as analyzed by one way ANOVA.

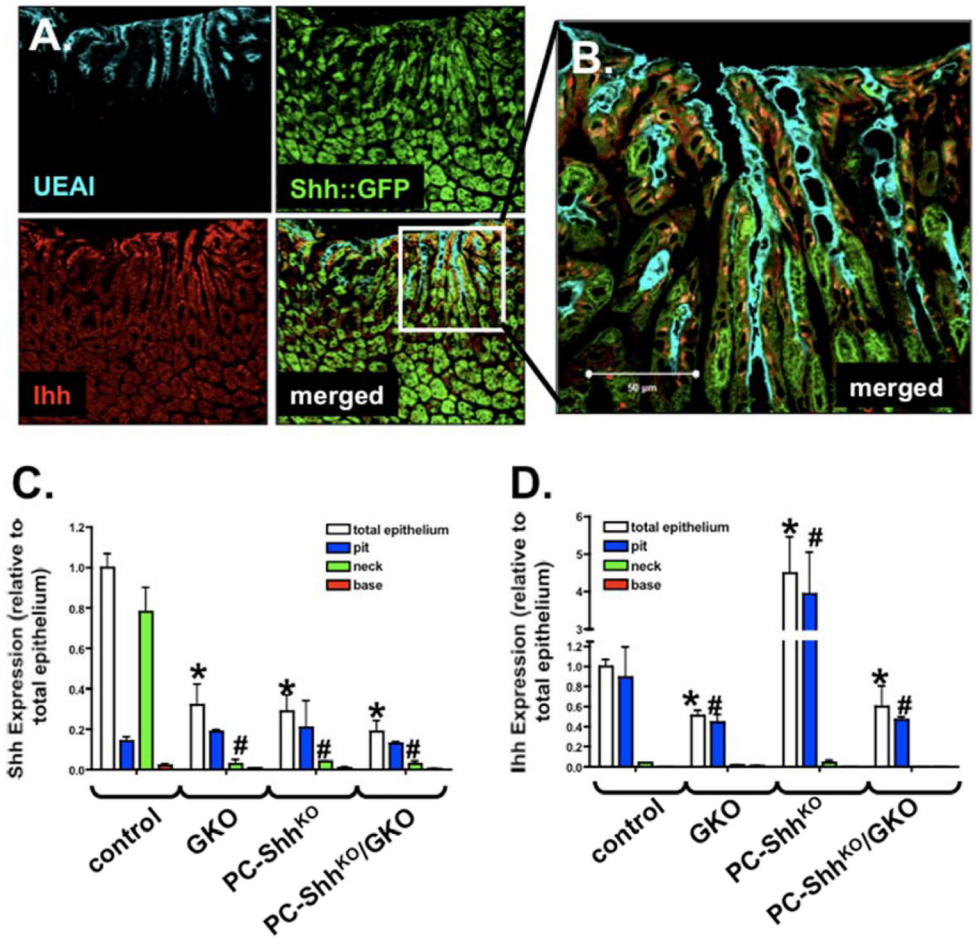



**Figure 2. Expression pattern of Shh and Ihh in fundic gastric mucosa**

**(A)** Fundic sections were collected from stomachs of Shh::GFP mice were immunostained using antibodies specific for UEAI (blue), GFP (Shh-expressing cells, green) and Ihh (red). Representative of n=4 mice. Higher magnification is shown in **(B)**. Quantitative RT-PCR was performed on RNA prepared from total epithelium, surface pit epithelium, neck, and base collected from control, GKO, PC-Shh$^{KO}$ and PC-Shh$^{KO}$/GKO mice by LCM. Shown is the expression of Shh **(C)** and Ihh **(D)** mRNA relative to total epithelium collected from the control group. Data is expressed as the mean ± SEM. *$P < 0.05$ compared to control total epithelium, #P<0.05 compared to control neck or pit as analyzed by one way ANOVA.

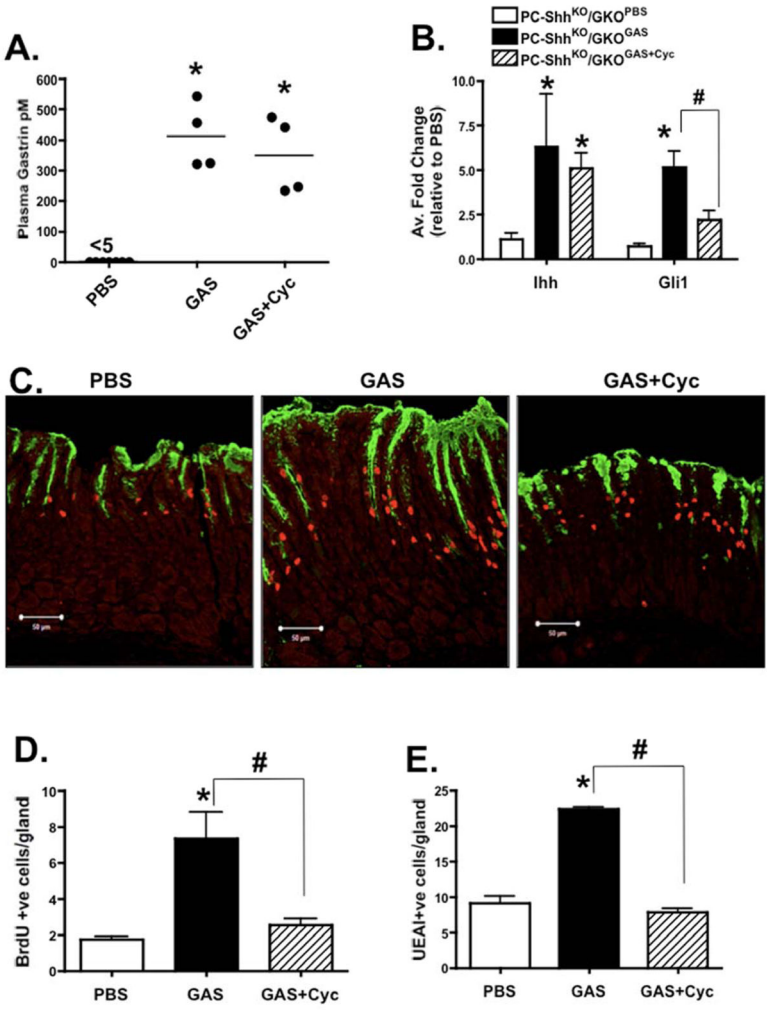



**Figure 3. Epithelial proliferation in response to gastrin and cyclopamine treatments**

(**A**) Circulating gastrin concentrations were measured in plasma collected from PC-Shh$^{KO}$/GKO mice infused with PBS, gastrin (GAS) alone or GAS + cyclopamine (Cyc). Each data point represents the concentration of plasma gastrin (pM) from an individual animal. (**B**) Quantitative RT-PCR was performed on stomach RNA prepared from gastric mucosa collected from PC-Shh$^{KO}$/GKO mice infused with PBS, GAS alone or GAS + Cyc. Shown is the expression of Ihh and Gli1 mRNA relative to PBS infused mice. **(C)** Stomach sections were immunostained for UEAI (green) and BrdU (red) in PC-Shh$^{KO}$/GKO mice infused with PBS, gastrin (GAS) alone or GAS + cyclopamine (Cyc). **(D)** BrdU-labeled nuclei were counted from PC-Shh$^{KO}$/GKO mouse stomachs and expressed as BrdU positive cells/gland. **(E)** The number of surface pit cells were counted and expressed as UEAI positive cells/gland. Data is expressed as the mean ± SEM. *P < 0.05 compared to PBS treated group as analyzed by one way ANOVA, #P<0.05 compared to gastrin infused group. ND: not detected, n = 3–6 animals/group.

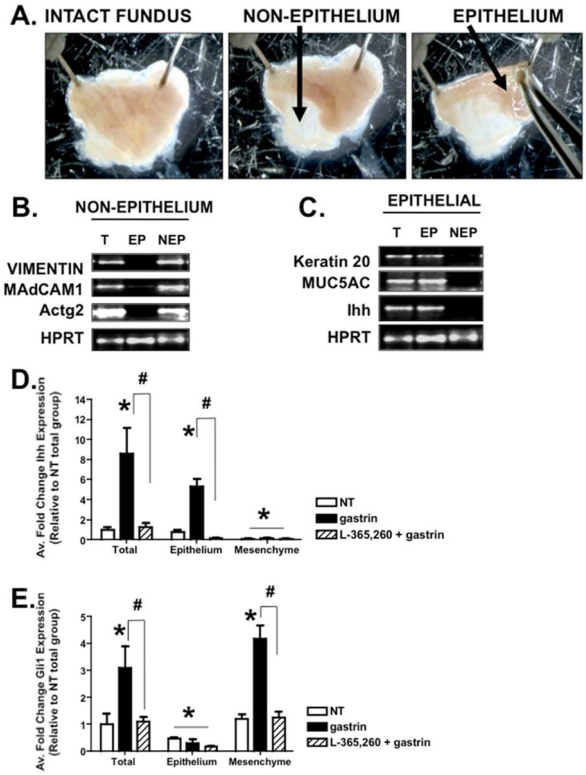



**Figure 4. Epithelial and non-epithelial Ihh and Gli1 expression**

**(A)** Dissection of total stomach tissue (T), epithelium (EP) and non-epithelium (NEP) from PC-Shh$^{KO}$/GKO mouse stomach. Quantitative RT-PCR analysis of known **(B)** mesenchymal (vimentin, MAdCAM1 and Actg2) and **(C)** epithelial (keratin 20 and Muc5ac) markers. Representative of three independently isolated fractions from total stomach. Quantitative RT-PCR was performed on stomach RNA prepared from total, epithelial and non-epithelial fractions collected from untreated (NT), gastrin infused and L-365, 260 + gastrin infused mice. Shown is the expression of (**D**) Ihh and (**E**) Gli1 mRNA relative to untreated total stomach tissue. Data were normalized to HPRT expression and presented as the mean ± SEM. * P<0.05 vs. NT group, # P<0.05 vs. gastrin infused group, n = 4 mice/ group.

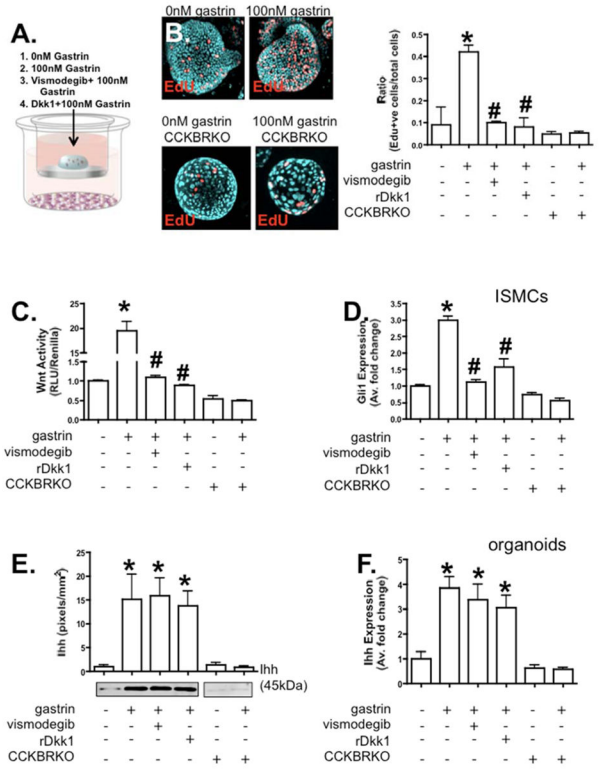
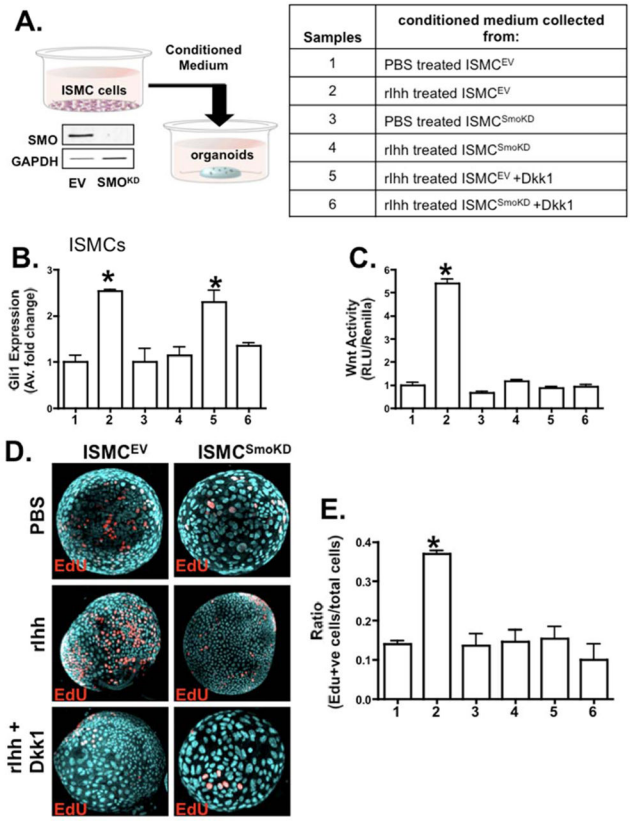



**Figure 5. Gastrin-induced proliferation in gastric organoids/ISMCs co-cultures**

**(A)** Fundic gastric organoids derived from PC-Shh$^{KO}$/GKO or CCK-BRKO mouse stomachs were co-cultured on transwell membranes with immortalized stomach mesenchymal cells (ISMCs) and treated with either 0nM gastrin, 100 nM gastrin with or without Wnt antagonist (recombinant Dkk1) or smoothened inhibitor (Vismodgib). **(B)** Organoids were immunostained for EdU (red) and nucleus (blue). Representative of n = 4 mice individual organoid cultures per group. Total cell number and EdU-labeled nuclei were counted from the four groups and expressed as EdU positive cells/total cells ratio. **(C)** Wnt activity was measured by a TOPflash assay using medium collected from the lower chamber of ISMC cultures. **(D)** qRT-PCR was used to measure Gli1 expression using RNA collected from cultured ISMCs. **(E)** Medium was collected from the top chamber of organoids cultures, immunoprecipitated and changes in Ihh protein expression measured by western blot. **(F)** Ihh expression was measured in organoids collected from co-cultured by qRT-PCR. *P<0.05 compared to 0nM gastrin group. #P<0.05 compared to 100nM gastrin group. n = 4 individual cultures per group.




| Samples | conditioned medium collected from: |
|---|---|
| 1 | PBS treated ISMC$^{EV}$ |
| 2 | rIhh treated ISMC$^{EV}$ |
| 3 | PBS treated ISMC$^{SmoKD}$ |
| 4 | rIhh treated ISMC$^{SmoKD}$ |
| 5 | rIhh treated ISMC$^{EV}$ +Dkk1 |
| 6 | rIhh treated ISMC$^{SmoKD}$ +Dkk1 |

**Figure 6. Organoid proliferation in response to ISMC conditioned medium**

**(A)** ISMCs were transduced to express empty vector (ISMC$^{EV}$) or a knockdown of smoothened (ISMC$^{SmoKD}$) that was confirmed by western blot analysis. ISMC$^{EV}$ and ISMC$^{SmoKD}$ cells were treated with either PBS (treatments 1 and 3), recombinant Ihh (rIhh, treatments 2 and 4) or recombinant Ihh plus Dkk1 (treatments 5 and 6). **(B)** qRT-PCR was used to measure Gli1 expression using RNA collected from cultured ISMCs. **(C)** Wnt activity was measured by a TOPflash assay using medium collected from the conditioned medium of ISMC cultures. **(D)** Organoids were immunostained for EdU (red) and nucleus (blue). Representative of n = 4 mice individual organoid cultures per group. **(E)** Total cell number and EdU-labeled nuclei were counted from the six groups and expressed as EdU positive cells/total cells ratio. *P<0.05 compared to PBS treated group. n = 4 individual cultures per group.

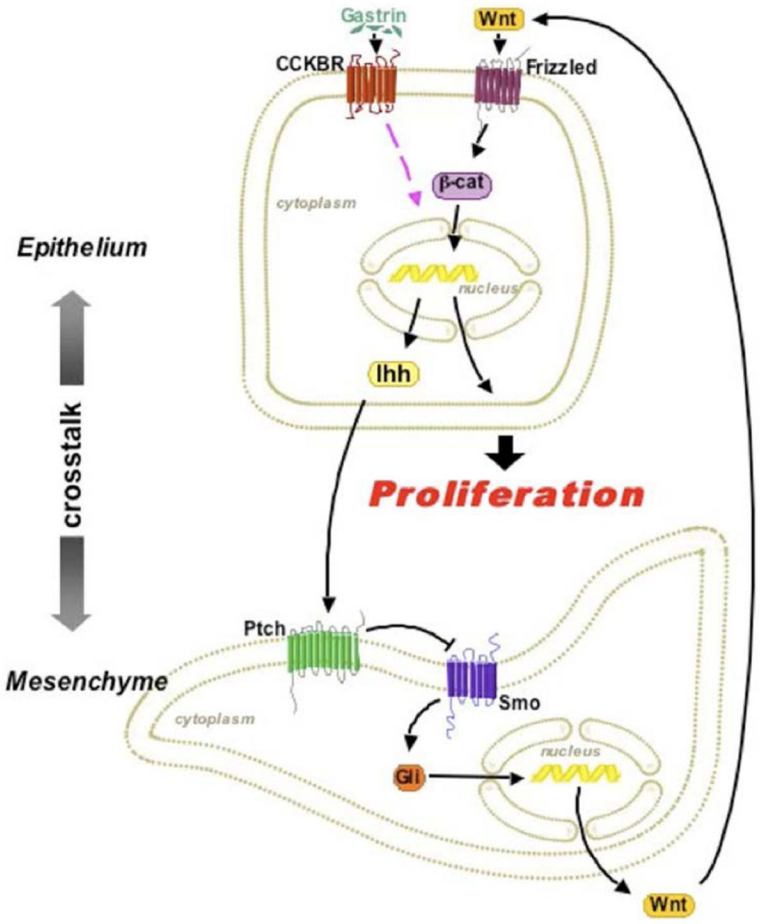



**Figure 7. Proposed model of gastrin-induced epithelial proliferation**

We propose a signaling pathway involving the crosstalk between epithelial Ihh signaling to Gli1 within the mesenchyme, then relaying to an intermediate mediator, such as Wnt, to induce proliferation. Gli1 activation within the mesenchyme may signal via Wnt to induce proliferation within the surface epithelium.