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BASIC—ALIMENTARY TRACT:

Loss of Parietal Cell Expression of Sonic Hedgehog Induces Hypergastrinemia and Hyperproliferation of Surface Mucous Cells

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Abstract

BACKGROUND & AIMS—Sonic Hedgehog (Shh) is expressed in the adult stomach, but its role as a gastric morphogen is unclear. We sought to identify mechanisms by which Shh might regulate gastric epithelial cell function and differentiation.

METHODS—Mice with a parietal cell–specific deletion of Shh (*HKCre/Shh^{KO}*) were created. Gastric morphology and function were studied in control and *HKCre/Shh^{KO}* mice between 1 and 8 months of age.

RESULTS—In contrast to control mice, *HKCre/Shh^{KO}* mice developed gastric hypochlorhydria, hypergastrinemia, and a phenotype that resembled foveolar hyperplasia. The fundic mucosa of *HKCre/Shh^{KO}* mice had an expanded surface pit cell lineage that was documented by increased incorporation of bromodeoxyuridine and was attributed to the hypergastrinemia. Compared with controls, numbers of total mucous neck and zymogen cells were significantly decreased in stomachs of *HKCre/Shh^{KO}* mice. In addition, zymogen and neck cell markers were coexpressed in the same cell populations, indicating disrupted differentiation of the zymogen cell lineage from the mucous neck cells in the stomachs of *HKCre/Shh^{KO}* mice. Laser capture microdissection of the surface epithelium, followed by quantitative reverse-transcription polymerase chain reaction, revealed a significant increase in expression of Indian Hedgehog, glioma-associated oncogene

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Supplementary Materials

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi:10.1053/j.gastro.2009.11.002.

Conflicts of interest

The authors disclose no conflicts.

homolog 1, Wnt, and cyclin D1. Laser capture microdissection analysis also showed a significant increase in Snail with a concomitant decrease in E-cadherin.

CONCLUSIONS—In the stomachs of adult mice, loss of Shh from parietal cells results in hypochlorhydria and hypergastrinemia. Hypergastrinemia might subsequently induce increased Hedgehog and Wnt signaling in the surface pit epithelium, resulting in hyperproliferation.

Sonic Hedgehog (Shh) is believed to regulate epithelial cell differentiation in the adult stomach, but its role as a morphogen is based on evidence that correlates the loss of Shh with inflammation of the gastric mucosa.¹ In a Mongolian gerbil model of *Helicobacter pylori* infection, bacterial colonization leads to down-regulation of Shh expression that is correlated with the development of atrophy and preneoplastic transformation.¹ In humans, loss of Shh is considered an early change that occurs in the gastric mucosa during *H pylori* infection but before neoplastic transformation.² However, in the absence of inflammation, the direct contribution of lost Shh expression to the disruption of epithelial cell differentiation has never been tested.

Binding of Hedgehog ligand to its receptor, Patched (Ptch), results in removal of the inhibition of Ptch on Smoothened (Smo). This removal of the inhibition on Smo subsequently results in the activation of the Gli family of Hedgehog transcription factors. Evidence from glioma-associated oncogene homolog 1 (Gli1) pathway studies in rat kidney epithelial cells (RK3E) shows that Gli1 induces the transcription of the zinc finger transcription factor, Snail.³ 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.^{4,5} In vitro data show that the Hedgehog signaling pathway is a key regulator of β -catenin,⁶ but whether Shh maintains the differentiated phenotype of the stomach by mediating Wnt pathway activation is unknown.

The purpose of this study was to identify the mechanism by which Shh acts as a regulator of gastric epithelial cell function and differentiation. Using a mouse model expressing a parietal cell–specific deletion of Shh (HKCre/Shh^{KO}), we show that loss of Shh results in hypochlorhydria and hypergastrinemia that results in hyperproliferation of the surface pit epithelium.

Materials and Methods

Parietal Cell–Specific Deletion of Shh

A mouse model expressing a parietal cell–specific deletion of Shh (HKCre/Shh^{KO}) was generated using transgenic animals bearing loxP sites flanking exon 2 of the Shh gene (Shh loxP, 129/Sv background) (kindly donated by Dr J. A. Whitsett, Department of Pediatrics, University of Cincinnati Children's Hospital Medical Center, Cincinnati, OH, with permission from Dr A. P. McMahon, Harvard University, Cambridge, MA) and mice expressing a Cre transgene under the control of the H⁺,K⁺–adenosine triphosphatase (ATPase) β subunit promoter (HKCre, C57Bl/6, FVB/N background, kindly donated by Dr J. Gordon, Washington University, St Louis, MO) (Supplementary Figure 1A). Genotyping

was based on polymerase chain reaction primers and protocols described in the Methods section of the report by Lewis et al⁷ (Supplementary Figure 1B). For primers and genotyping, see the supplementary information. Age-matched Shh loxP (homozygous for the loxP sites without the Cre transgene) and HKCre littermates were used as the control group. All mice were analyzed at 1, 2, 3, 4, and 8 months of age (n = 8 in each group). All mouse studies were approved by the University of Cincinnati Institutional Animal Care and Use Committee, which maintains an American Association of Assessment and Accreditation of Laboratory Animal Care facility.

β-Galactosidase (X-gal) Staining for Paraffin-Embedded Tissue Sections

To determine the efficiency of recombination, HKCre mice were crossed with Rosa26^{lacZ} reporter mice (purchased from The Jackson Laboratory, Bar Harbor, ME). Methods and reagents used for β-galactosidase (X-gal) staining are described in the supplementary information (Supplementary Figure 1C–H).

Octreotide Treatments

Control and HKCre/Shh^{KO} mice at 2.5 months of age were injected with 30 μg/kg intraperitoneal octreotide (Sigma–Aldrich, St. Louis, MO) or phosphate-buffered saline (PBS) each day for 1 month. Octreotide and PBS groups were then analyzed at 3.5 months of age. Plasma was collected for gastrin radioimmunoassay, and frozen stomach sections were collected for immunofluorescence, laser capture microdissection (LCM), and RNA extractions.

Histologic Evaluation

Histologic scores were recorded by a certified veterinary pathologist (Gregory Boivin, DVM, Boonshoft School of Medicine, Wright State University, Dayton, OH). Histologic score was graded on parietal cell loss (atrophy), inflammation (neutrophil and lymphocytic infiltration), foveolar hyperplasia, and intestinal metaplasia. A score of 1–4 was given based on the percentage of total mucosa exhibiting pathologic abnormalities (eg, 1 = 5%–25%, 2 = 26%–50%, 3 = 51%–75%, and 4 = 76%–100% of the total mucosa exhibiting pathologic characteristics; ie, for a mouse receiving a score of 4, 76%–100% of the total mucosa exhibits the pathologic abnormalities used in grading).

Immunohistochemistry and Immunofluorescence

Methods and reagents used for immunohistochemistry and immunofluorescence are described in the supplementary information.

Transmission Electron Microscopy

Transmission electron microscopy was performed according to a previously reported protocol.⁸ For details, refer to the supplementary information.

Gastric Acidity

Control and HKCre/Shh^{KO} mice were injected with PBS or histamine (stimulated group, 20 mg/kg in PBS, intraperitoneally), respectively, 30 minutes before analysis. Mouse stomachs

were then opened along the greater curvature and rinsed into 2 mL of normal saline (0.9% NaCl, pH 7.0). The supernatant was collected and titrated with 0.005N NaOH to determine the acid concentration.⁹ The gastric acidity was expressed as microequivalents and values were normalized to kilogram body weight.

LCM

LCM was used to determine the expression pattern of Shh, Indian Hedgehog (Ihh), Wnt3a, Wnt5A, cyclin D1, Gli1, Snail, E-cadherin, MUC5AC, Atp4a, MUC6, and pepsinogen (PgC) in the surface and neck regions of the gastric epithelium. For a detailed protocol, refer to the supplementary information.

Quantitative Reverse-Transcription Polymerase Chain Reaction

For a detailed protocol, refer to the supplementary information.

Western Blot Analysis

Refer to the supplementary information for protocol.

Gastrin Radioimmunoassay

Plasma samples were assayed for total amidated gastrin concentrations using antibody L2 (which reacts with G17 and G34, but not progastrin or Gly gastrins) as previously described.¹⁰

Statistical Analysis

The significance of the results was tested by unpaired *t* test using commercially available software (GraphPad Prism; GraphPad Software, San Diego, CA). A *P* value < .05 was considered significant.

Results

Shh Is Expressed Within Mouse Gastric Parietal Cells

Deletion of Shh within the parietal cells was corroborated at the gene level by LCM. Cells from the neck region of the stomach were sampled from 1-month-old control and HKCre/Shh^{KO} mouse stomachs. The expression of 4 marker genes, ATP4α (parietal cells), MUC5AC (surface pit cells), MUC6 (neck cells), and PgC (base and zymogen cells), was determined by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). The expression of ATP4α was higher in RNA collected from the neck region compared with MUC6, MUC5AC, and PgC messenger RNA (mRNA) expression in the same samples (Figure 1A). Compared with the control group, where Shh mRNA expression was detected in the neck region of the stomach, Shh expression was significantly decreased in the same region collected from HKCre/Shh^{KO} mice (Figure 1A). Although Ihh expression was detected in the neck region, expression was unchanged in the HKCre/Shh^{KO} mice relative to controls (Figure 1A).

The expression pattern of Shh was investigated further by immunofluorescence using antibodies specific for Shh and H⁺,K⁺-ATPase. Compared with the control group, where

H⁺,K⁺-ATPase staining (Figure 1B, green) colocalized with Shh (Figure 1B, red), Shh immunostaining was absent in the parietal cells of HKCre/Shh^{KO} mice (Figure 1C), verifying the successful deletion of Shh. Positive cells for the neck cell marker *Griffonia simplicifolia* II (GSII) lectin (Supplementary Figure 4A and B, green) localized to the neck region of the gastric unit and were devoid of Shh (Supplementary Figure 4A and B, red). Collectively, these data suggested that the Shh gene expression detected in the neck region of control animals was primarily expressed in parietal cells.

Ihh Is Expressed Primarily in the Surface Epithelium

To investigate the expression of Shh further, cells from the pit region of the stomach were sampled from 1-month-old control and HKCre/Shh^{KO} mouse stomachs by LCM and analyzed by qRT-PCR. The expression of MUC5AC was higher in RNA collected from the pit region compared with ATP4α, MUC6, and PgC mRNA expression in the same samples (Figure 1D). Ihh was expressed at higher levels in the HKCre/Shh^{KO} mice when compared with the control group (Figure 1D). There was a significantly higher mRNA expression of the marker MUC5AC in RNA collected from the HKCre/Shh^{KO} mouse pit region when compared with the controls (Figure 1D). Consistent with the significant increase in MUC5AC gene expression, there was an expansion in the expression of the gastric pit cell marker *Ulex europaeus* I (UEAI) in the HKCre/Shh^{KO} mice (Figure 1F) compared with controls (Figure 1E). Immunofluorescence shown in Figure 1E and F also showed a clear loss of Shh expression within the parietal cells, with expression in the pit region. Given that the antibody used to detect Shh (N-19; Santa Cruz Biotechnology, Santa Cruz, CA) is documented to cross-react with Ihh, positive immunofluorescence in the pit region may be attributed to the expression of Shh and Ihh.

HKCre/Shh^{KO} Mice Have an Expanded Surface Mucous Cell Epithelium

HKCre/Shh^{KO} mice developed a phenotype reminiscent of foveolar hyperplasia with an expansion of the surface epithelium into the gland and base regions of the stomach (Figure 2A and B) that was apparent following periodic acid–Schiff/Alcian blue staining (Figure 2C and D). Histologic score was graded on foveolar hyperplasia and intestinal metaplasia. A score of 1, 2, 3, and 4 was recorded based on whether 5%–25%, 26%–50%, 51%–75%, or 76%–100% of the total mucosa was affected, respectively. Histologic evaluation revealed that over a period from 1 to 8 months, HKCre/Shh^{KO} mice (Figure 2F) developed foveolar hyperplasia compared with the control animals (Figure 2E). Sections were also scored for the presence of parietal cell atrophy, inflammation, and intestinal metaplasia that were not observed in the HKCre/Shh^{KO} mice, and the higher histologic score in these animals was attributed primarily to hyperplasia.

Delayed Mucous Neck Cell to Zymogen Cell Differentiation in HKCre/Shh^{KO} Mice

To address whether loss of parietal cell–expressed Shh resulted in the disruption of the differentiation of mucous neck cells to zymogen cells, immunofluorescence with GSII lectin (mucous neck cell marker) and anti-intrinsic factor (IF; zymogen cell marker) antibody was used. Morphometric analysis showed a significant decrease in the number of GSII- and IF-positive cells in HKCre/Shh^{KO} mice compared with control mouse stomachs by 8 months of

age (Figure 3A). Disruption of zymogen cell differentiation was quantified by an increase in the number of prezymogenic cells that coexpress both GSII and IF markers.¹¹ In the control group, GSII (*red*, mucous neck cells) and IF (*green*, zymogen cells) clearly stained separate cells (Figure 3B). In contrast, in the HKCre/Shh^{KO} mouse gastric mucosa, there was an increase in the number of cells coexpressing GSII and IF cells at the base of the gland (Figure 3C). The decrease in the number of GSII- and IF-positive cells and increase in GSII/IF dual-labeled cells was age dependent, as shown in Supplementary Figure 5A–D. This result shows that Shh depletion induces a delay in the differentiation of zymogen cells. Morphometric analysis revealed that the total parietal cell number did not vary significantly between control and HKCre/Shh^{KO} mice (Figure 3A and Supplementary Figure 5A–D).

Expansion of the Surface Pit Mucous Cells in the HKCre/Shh^{KO} Stomachs and Nuclear Translocation of β -Catenin

Immunofluorescence using a lectin specific for surface mucous cells (UEAI) and bromodeoxyuridine (BrdU) incorporation were used to quantify the expansion of the pit region (Figure 4). Compared with the fundic mucosa of control mice, where DNA synthesis was detected between the isthmus and pit regions of the gland unit (Figure 4A), HKCre/Shh^{KO} animals had significantly greater numbers of BrdU-labeled nuclei found at the pit region (Figure 4B). Quantification of the UEAI-labeled cells (Figure 4C) and BrdU-labeled nuclei (Figure 4D) revealed a significant increase in DNA synthesis and expansion of the surface pit mucous cells between the ages of 3 and 8 months in HKCre/Shh^{KO} animals compared with controls.

Nuclear translocation of β -catenin induces cell proliferation.¹² To determine if such a mechanism could explain the phenotype observed in the HKCre/Shh^{KO} mice, Western blot analysis was used to determine the cellular location of β -catenin protein. At 2 months of age, β -catenin expression remained predominantly in the cytoplasm of cells from both control and HKCre/Shh^{KO} groups (Supplementary Figure 6A and B). In contrast, there was a significant translocation of β -catenin to the nucleus of cells within the gastric mucosa of the HKCre/Shh^{KO} animals by 4 months of age (Figure 4E and F).

Increased *Ihh*, *Wnt*, and *Cyclin D1* Expression in the Pit Region of HKCre/Shh^{KO} Mouse Stomachs

Translocation of β -catenin to the nucleus reflects the activation of the Wnt pathway and promotes the transcription of downstream target cyclin D1 that is associated with hyperproliferation.¹³ LCM and qRT-PCR were used to identify whether Wnt and cyclin D1 mRNA expressions were increased in the surface pit epithelium of the HKCre/Shh^{KO} mice. The expression of MUC5AC was significantly higher in RNA collected from the pit region of both compared with Atp4a, MUC6, and PgC (Supplementary Figure 7). At 2 months of age, cyclin D1 mRNA expression did not differ between the control and HKCre/Shh^{KO} mice (data not shown), consistent with the lack of hyperproliferation observed in the HKCre/Shh^{KO} group at this age (see Figure 4D). In contrast, the expression of cyclin D1, Wnt3a, and Wnt5a was significantly elevated in the HKCre/Shh^{KO} mice at 3 months of age relative to controls (Figure 5A). Given that Wnt signaling is a downstream effector of the Hedgehog signaling pathway,⁶ it was not surprising to see a significant increase in Gli1 mRNA

expression in the stomachs of the HKCre/Shh^{KO} mice (Figure 5A). The increase in Gli1 mRNA expression was consistent with a significant increase in Ihh in the pit cell area of the HKCre/Shh^{KO} mice (Figure 5A).

HKCre/Shh^{KO} Mouse Stomachs Have Increased Snail

Alterations in E-cadherin expression subsequently lead to the accumulation of cytoplasmic β -catenin that then translocates to the nucleus.⁶ Given that Snail is known to repress E-cadherin,^{4,6} expression of this transcription factor was studied in both control and HKCre/Shh^{KO} mouse stomachs using immunofluorescence. While expression was virtually absent in the control animals (Figure 5B and C), Snail was increased in the HKCre/Shh^{KO} mouse stomachs (Figure 5E and F). Counterstaining with UEAI lectin showed that this up-regulation occurred at the surface pit region and what appeared to be mesenchymal (Figure 5D and G). Changes in expression of both E-cadherin and Snail were confirmed by qRT-PCR using LCM of surface pit followed by qRT-PCR. Figure 5H shows a significant increase in Snail mRNA expression in the HKCre/Shh^{KO} mice compared with control animals that correlated with a significant decrease in E-cadherin expression. Immunofluorescence images show the transmembrane protein E-cadherin located on the cell membrane in the control mouse stomachs (Figure 5I and J, *arrow*). In the HKCre/Shh^{KO} mouse stomachs, E-cadherin expression was decreased and in some cells appeared within the cytoplasm (Figure 5K and L, *arrow*).

Loss of Normal Parietal Cell Function in the HKCre/Shh^{KO} Mice

From 2 months of age, HKCre/Shh^{KO} mice were unresponsive to the histamine treatment (Figure 6B), whereas gastric acidity increased in histamine-treated control animals in all age groups (Figure 6A). Morphometric analysis of immunostained stomach antrums revealed a significant increase in the number of gastrin-expressing (G) cells (Figure 6C) and a significant decrease in the number of somatostatin (D) cells (Figure 6D).

Transmission electron microscopy revealed secretory membrane changes in parietal cells with the deletion of Shh. The parietal cells of control animals exhibited canaliculi that appeared to be well organized within the cell with long microvilli (Figure 6E). Parietal cells within the HKCre/Shh^{KO} mouse stomach had a tendency to have disorganized and collapsed canaliculi and numerous tubulovesicles that appeared to be distorted and diffuse throughout the cytoplasm (Figure 6F). Collectively, these data showed that loss of parietal cell-expressed Shh results in hypochlorhydria, increased gastrin-expressing G cells with a concomitant decrease in somatostatin D cells.

Suppression of Hypergastrinemia Prevents the Development of Hyperproliferating Surface Mucous Cells in HKCre/Shh^{KO} Mice

HKCre/Shh^{KO} mice exhibited severe hypergastrinemia (Figure 7A). Thus, both control and HKCre/Shh^{KO} mice were treated with octreotide (somatostatin analogue) at 2.5 months of age for 1 month. Octreotide treatment for 1 month significantly reduced the hypergastrinemia that was observed in the HKCre/Shh^{KO} mice (Figure 7A) and circulating gastrin levels in the control group (Figure 7A). The numbers of surface mucous pit cells and BrdU-labeled cells observed in the octreotide-treated HKCre/Shh^{KO} group were down to

numbers equivalent to those counted in both treated and untreated control animals (Figure 7B).

The expression of MUC5AC was significantly greater in the HKCre/Shh^{KO} mice compared with the control animals (Supplementary Figure 8A) but decreased after octreotide treatment (Supplementary Figure 8B). Changes in the Hedgehog and Wnt pathways were measured by qRT-PCR. There was a significant decrease in *Ihh* and *Gli1* mRNA expression in the octreotide-treated HKCre/Shh^{KO} mice compared with the untreated group (Figure 7C and D). In addition, *Wnt3A*, *Wnt5A*, and cyclin D1 expression was also significantly decreased in HKCre/Shh^{KO} animals treated with octreotide (Figure 7D). Suppression of hypergastrinemia by octreotide resulted in increased numbers of GSII- and IF-positive cells that were accompanied by a decrease in GSII/IF dual-labeled cells in treated HKCre/Shh^{KO} mice (Figure 7F) compared with the untreated groups (Figure 7E). Collectively, the octreotide experiments show that somatostatin plays a crucial role in regulating hyperproliferation of the surface pit mucous cells in the HKCre/Shh^{KO} mice, probably via regulation of circulating gastrin concentrations.

Discussion

The HKCre/Shh^{KO} mouse model has allowed us to assay changes in gastric epithelial cell differentiation and function associated with loss of Shh and independent of inflammation. Compared with the normal gastric pathology of the control animals, every HKCre/Shh^{KO} mouse showed 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 overexpressing transforming growth factor (TGF)- α transgenic mice^{14–17} and in patients with Ménétrier's disease.^{18,19} Interestingly, TGF- α expression increased with loss of Shh and was localized along the foveolar region and base of the gastric gland in stomachs of HKCre/Shh^{KO} mice (Supplementary Figure 9), similar to that observed in the gastric fundus of patients with Ménétrier's disease.²⁰ However, unlike both patients with Ménétrier's disease and *H. pylori*-infected patients, the HKCre/Shh^{KO} mouse model did not develop parietal cell atrophy. Given that HKCre/Shh^{KO} mice also lacked inflammation, there may be a requirement for additional factors, such as inflammatory cytokines, for parietal cell atrophy to occur. Nevertheless, the role of TGF- α in the development of the hyperplasia observed in the HKCre/Shh^{KO} mice warrants further investigation.

Lack of acid secretion in the HKCre/Shh^{KO} mice was accompanied by significant hypergastrinemia. In addition to the current study, hypergastrinemia has been reported previously in a pharmacologic model of Hedgehog signaling inhibition using cyclopamine,²¹ and in a canine parietal cell in vitro model, Shh regulated H⁺,K⁺-ATPase expression.²² In fact, treatment of HKCre/Shh^{KO} mice with the somatostatin analogue octreotide significantly suppressed hypergastrinemia and subsequently hyperproliferation. Thus, the phenotype observed with loss of Shh may be attributed to increases in circulating gastrin concentrations due to loss of somatostatin. Our current understanding of hypergastrinemia is now extended because, 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 (Figure 8).

The HKCre/Shh^{KO} mice showed an age-dependent delay in the differentiation of zymogen cells from mucous neck cells. The overproduction of surface mucous cells often occurs at the expense of other cell lineages, such as the zymogen cells.^{14,15} As the mucous neck cells migrate toward the base of the gastric gland, these cells differentiate into the zymogen/chief cells.²³ The transition between mucous neck cells and zymogen cells is induced by the expression of the transcription factor Mist1.¹¹ The HKCre/Shh^{KO} mice exhibit a phenotype in contrast to models of parietal cell loss, where there is a complete disruption of terminal differentiation in zymogen cells.^{24,25} However, our findings are similar to the observations made in the Mist1-deficient mice, whereby zymogen cells located in the base of the gland frequently coexpress mucous neck cell markers.¹¹

We documented differences between the gene expression levels of Shh and Ihh in the surface pit and neck regions of the gastric epithelium. Our findings are 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 as opposed to Shh in the gland/neck region.²⁶ In the HKCre/Shh^{KO} mice, Ihh was significantly up-regulated. Given that octreotide treatment of the HKCre/Shh^{KO} mice resulted in decreased Ihh expression similar to levels measured in the control group, we propose here that the increased Ihh gene expression may be a consequence of hypergastrinemia. Further studies using the gastrin-deficient mouse model and the current HKCre/Shh^{KO} mice are required to test the direct role of gastrin as a direct regulator of Ihh.

Increased Ihh gene expression in the surface pit epithelium was accompanied by an approximate 40-fold increase in Snail and a significant decrease in E-cadherin expression in the same gastric region. This was an expected result given that Snail is known to repress E-cadherin.^{4,6} Decreased E-cadherin expression was accompanied by the translocation of β -catenin from the cytoplasm to the nucleus. It is known that E-cadherin expression negatively controls the transcriptional activity of β -catenin.²⁷ Accumulated β -catenin in the cytoplasm eventually translocates to the nucleus. Nuclear translocation of β -catenin binds to the DNA binding protein Tcf/Lef1 that subsequently regulates target genes including cyclin D1,¹² which plays an important role in proliferation.²⁸ Consistent with nuclear expression of β -catenin in the HKCre/Shh^{KO} mice, increased cyclin D1 and BrdU incorporation was reflective of increased cellular proliferation. Cytoplasmic accumulation and nuclear translocation of β -catenin was observed with increased canonical and noncanonical Wnt as well as Gli1 mRNA expression in the HKCre/Shh^{KO} mice. Activation of the Wnt pathway is known to result in β -catenin nuclear accumulation.¹² Experiments using rat kidney epithelial cells (RK3E cells) show that Gli induces transcription of Wnt.⁶ Increased Gli1 expression in the HKCre/Shh^{KO} mice was explained by the concomitant increase in Ihh mRNA expression. 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 of gastric epithelial cells³ (Figure 8).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

BrdU	bromodeoxyuridine
Gli1	glioma-associated oncogene homolog 1
GSII	<i>Griffonia simplicifolia II</i>
IF	intrinsic factor
Ihh	Indian Hedgehog
LCM	laser capture microdissection
PgC	pepsinogen
Ptch	Patched
qRT-PCR	quantitative reverse-transcription polymerase chain reaction
Shh	Sonic Hedgehog
Smo	Smoothed
TGF	transforming growth factor
UEAI	<i>Ulex europaeus I</i>

References

1. Nishizawa T, Suzuki H, Nakagawa I, et al. Early *Helicobacter pylori* eradication restores Sonic Hedgehog expression in the gastric mucosa of mongolian gerbils. *Digestion*. 2009; 79:99–108. [PubMed: 19295210]
2. Shiotani A, Iishi H, Uedo N, et al. Evidence that loss of Sonic Hedgehog is an indicator of *Helicobacter pylori*-induced atrophic gastritis progressing to gastric cancer. *Am J Gastroenterol*. 2005; 100:581–587. [PubMed: 15743355]

3. Li X, Deng W, Nail CD, et al. Snail induction is an early response to Gli1 that determines the efficiency of epithelial transformation. *Oncogene*. 2006; 25:609–621. [PubMed: 16158046]
4. Medici D, Hay E, Olsen BR. Snail and Slug promote epithelial-mesenchymal transition through beta-catenin-T-cell factor-4-dependent expression of transforming growth factor-beta3. *Mol Biol Cell*. 2008; 19:4875–4887. [PubMed: 18799618]
5. Tanaka M, Kitajima Y, Edakuni G, et al. Abnormal expression of E-cadherin and beta-catenin may be a molecular marker of sub-mucosal invasion and lymph node metastasis in early gastric cancer. *Br J Surg*. 2002; 89:236–244. [PubMed: 11856141]
6. Li X, Deng W, Lobo-Ruppert SM, et al. Gli1 acts through Snail and E-cadherin to promote nuclear signaling by beta-catenin. *Oncogene*. 2007; 26:4489–4498. [PubMed: 17297467]
7. Lewis PM, Dunn M, McMahon JA, et al. Cholesterol modification of Sonic Hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1. *Cell*. 2001; 105:599–612. [PubMed: 11389830]
8. Jain RN, Al-Menhali AA, Keeley TM, et al. Hip1r is expressed in gastric parietal cells and is required for tubulovesicle formation and cell survival in mice. *J Clin Invest*. 2008; 118:2459–2470. [PubMed: 18535670]
9. Zavros Y, Waghray M, Tessier A, et al. Reduced pepsin A processing of Sonic Hedgehog in parietal cells precedes gastric atrophy and transformation. *J Biol Chem*. 2007; 282:33265–33274. [PubMed: 17872943]
10. Dockray GJ, Hamer C, Evans D, et al. The secretory kinetics of the G cell in omeprazole-treated rats. *Gastroenterology*. 1991; 100:1187–1194. [PubMed: 2013368]
11. Ramsey VG, Doherty J, Chen CC, et al. The maturation of mucus-secreting gastric epithelial progenitors into digestive-enzyme secreting zymogenic cells requires Mist1. *Development*. 2007; 134:211–222. [PubMed: 17164426]
12. Nelson WJ, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science*. 2004; 303:1483–1487. [PubMed: 15001769]
13. Tomita H, Yamada Y, Oyama T, et al. Development of gastric tumors in Apc(Min/+) mice by the activation of the beta-catenin/Tcf signaling pathway. *Cancer Res*. 2007; 67:4079–4087. [PubMed: 17483318]
14. Bockman DE, Sharp R, Merlino G. Regulation of terminal differentiation of zymogenic cells by transforming growth factor α in transgenic mice. *Gastroenterology*. 1995; 108:447–454. [PubMed: 7835587]
15. Goldenring JR, Ray GS, Soroka CJ, et al. Overexpression of transforming growth factor-alpha alters differentiation of gastric cell lineages. *Dig Dis Sci*. 1996; 41:773–784. [PubMed: 8674399]
16. Sharp R, Babyatsky MW, Takagi H, et al. Transforming growth factor α disrupts the normal program of cellular differentiation in the gastric mucosa of transgenic mice. *Development*. 1995; 121:149–161. [PubMed: 7867496]
17. Nomura S, Settle SH, Leys CM, et al. Evidence for repatterning of the gastric fundic epithelium associated with Ménétrier's disease and TGF α overexpression. *Gastroenterology*. 2005; 125:1292–1305. [PubMed: 15887112]
18. Larsen B, Tarp U, Kristensen E. Familial giant hypertrophic gastritis (Menetrier's disease). *Gut*. 1987; 28:1517–1521. [PubMed: 3322957]
19. Wolfsen HC, Carpenter HA, Talley NJ. Menetrier's disease: a form of hypertrophic gastropathy or gastritis? *Gastroenterology*. 1993; 104:1310–1319. [PubMed: 8482445]
20. Bluth RF, Carpenter HA, Pittelkow MR, et al. Immunolocalization of transforming growth factor-alpha in normal and diseased human gastric mucosa. *Hum Pathol*. 1995; 26:1333–1340. [PubMed: 8522306]
21. El-Zaatari M, Grabowska A, McKenzie AJ, et al. Cyclopamine inhibition of the Sonic Hedgehog pathway in the stomach requires concomitant acid inhibition. *Regul Pept*. 2008; 146:131–139. [PubMed: 17996964]
22. Stepan V, Ramamoorthy S, Nitsche H, et al. Regulation and function of the Sonic Hedgehog signal transduction pathway in isolated gastric parietal cells. *J Biol Chem*. 2005; 280:15700–15708. [PubMed: 15691835]

23. Karam SM, Leblond CP. Dynamics of epithelial cells in the corpus of the mouse stomach. I. Identification of proliferative cell types and pinpointing of the stem cell. *Anat Rec.* 1993; 236:259–279. [PubMed: 8338232]
24. Bredemeyer AJ, Geahlen J, Weis VG, et al. The gastric epithelial progenitor cell niche and differentiation of the zymogenic (chief) cell lineage. *Dev Biol.* 2009; 325:211–224. [PubMed: 19013146]
25. Nozaki K, Ogawa M, Williams JA, et al. A molecular signature of gastric metaplasia arising in response to acute parietal cell loss. *Gastroenterology.* 2008; 134:511–522. [PubMed: 18242217]
26. Fukaya M, Isohata N, Ohta H, et al. Hedgehog signal activation in gastric pit cell and in diffuse-type gastric cancer. *Gastroenterology.* 2006; 131:14–29. [PubMed: 16831586]
27. Solanas G, Porta-de-la-Riva M, Agustí C, et al. E-cadherin controls beta-catenin and NF-kappaB transcriptional activity in mesenchymal gene expression. *J Cell Sci.* 2008; 121:2224–2234. [PubMed: 18565826]
28. Luo GQ, Li JH, Wen JF, et al. Effect and mechanism of the Twist gene on invasion and metastasis of gastric carcinoma cells. *World J Gastroenterol.* 2008; 14:2487–2493. [PubMed: 18442194]

Figure 1.

Expression pattern of Shh and cell lineage markers in the gastric mucosa. (A) Average fold change in gene expression of MUC5AC, Atp4a, MUC6, PgC, Shh, and Ihh in RNA collected from the neck region of both control and HKCre/Shh^{KO} mouse stomachs. Immunofluorescence of (B) control and (C) HKCre/Shh^{KO} mouse groups stained for Shh (red) together with H⁺,K⁺-ATPase (green). Merged images are shown (inset). Colocalization is indicated in yellow. Representative of n = 8. Original magnification 40×. (D) Average fold change in gene expression of MUC5AC, Atp4a, MUC6, PgC, Shh, and

Ihh in RNA collected from the pit region of both control and HKCre/Shh^{KO} mouse stomachs. Immunofluorescence of (E) control and (F) HKCre/Shh^{KO} mouse groups stained for Shh (red) together with UEAI (green). Merged images are shown (inset). Colocalization is indicated in yellow. Representative of n = 8. Data are presented as the mean ± SEM where **P* < .05 compared with either MUC5AC, Atp4α, MUC6, or PgC gene expression; #*P* < .05 compared with the control group; n = 3 mice in each group.

Figure 2.

Histologic evaluation of control and HKCre/Shh^{KO} mice. H&E staining of gastric tissue collected from 8-month-old (A) control and (B) HKCre/Shh^{KO} mice (original magnification 10×). Sections from (C) control and (D) HKCre/Shh^{KO} mice were stained with periodic acid-Schiff/Alcian blue. Original magnification 10×. Gastric sections collected from (E) control or (F) HKCre/Shh^{KO} groups were graded on the level of foveolar hyperplasia as detailed in Materials and Methods. Histologic score for tissues collected from 1-, 2-, 3-, 4-, and 8-month-old mice were recorded. *P < .05 compared with control, n = 8 mice per group.

Figure 3.

Quantification of parietal, mucous neck, and zymogen cells in control and HKCre/Shh^{KO} mice. (A) The number of parietal cells (H⁺,K⁺-ATPase β subunit), mucous neck cells (GSII), zymogen cells (IF), and dual-labeled GSII/IF cells in 8-month-old control and HKCre/Shh^{KO} mice was counted. Data are presented as the mean $\pm$ SEM where * P < .05 compared with the control animals, n = 8 mice in each group. Immunofluorescence of (B) control and (C) HKCre/Shh^{KO} mouse groups stained for IF (red) together with GSII (green). Merged

images are shown. Colocalization is indicated in *yellow*. Representative of n = 8. Original magnification 40×.

Figure 4.

Expression of surface mucous pit cells and BrdU-labeled nuclei in control and HKCre/Shh^{KO} mice. Immunofluorescence for UEAI (green) and BrdU (red) in 8-month-old (A) control and (B) HKCre/Shh^{KO} mice. Morphometric analysis of (C) UEAI-positive cells and (D) BrdU-labeled nuclei was counted from both control and HKCre/Shh^{KO} mice at 1, 2, 3, 4, and 8 months of age and expressed as UEAI or BrdU-positive cells per gland. (E) Immunoblots of cytoplasmic and nuclear β-catenin using protein extracts of 8-month-old control and HKCre/Shh^{KO} mice. (F) Cytoplasmic and nuclear β-catenin expression was

quantified and normalized for either glyceraldehyde-3-phosphate dehydrogenase (GAPDH; cytoplasmic protein) or Lamin B1 (nuclear protein). Data are expressed as the mean $\pm$ SEM, n = 4–8 mice per group where $*P < .05$ compared with control mice.

Figure 5.

Snail and E-cadherin expression in control and HKCre/Shh^{KO} mice. (A) Average fold change in gene expression of Shh, Ihh, Gli1, Wnt3a, Wnt5A, and cyclin D1 in RNA collected from the surface pit region of control and HKCre/Shh^{KO} mouse stomachs. Immunofluorescence of (B–D) control and (E–G) HKCre/Shh^{KO} stomachs immunostained for (B, C and E, F) Snail and nuclear marker TPORO or (D and G) Snail and UEA1. Snail staining is indicated (arrow). (H) Average fold change in gene expression of Snail and E-cadherin in RNA collected from the surface pit region of control and HKCre/Shh^{KO} mouse

stomachs. Immunofluorescence of (*I* and *J*) control and (*K* and *L*) HKCre/Shh^{KO} stomachs immunostained for E-cadherin. Membrane expressed E-cadherin (*arrow*) in the control mice and cytoplasmic expression (*arrow*) of E-cadherin in HKCre/Shh^{KO} mice. Original magnification: *I* and *K*, 20×; *J* and *L*, 40×. Representative figures of n = 8 per group at 4 months of age. Data are expressed as the mean ± SEM where **P* < .05 compared with the control animals, n = 3 mice in each group.

Figure 6.

Changes in gastric acidity and gastrin and somatostatin cell number in control and HKCre/Shh^{KO} mice. Gastric acid measurements (microequivalents H⁺/Kg) in PBS- or histamine (HIST)-treated (A) control and (B) HKCre/Shh^{KO} mice at 1–8 months of age. Morphometric analysis of (C) gastrin (G)- and (D) somatostatin (SOM)-expressing cells in antrums of control and HKCre/Shh^{KO} mice is shown. Electron micrographs of 4-month-old (E) control and (F) HKCre/Shh^{KO} mouse glandular stomach. CN, canalicular membrane; N, nucleus; M, mitochondria. Scale bar = 2 μm. Representative micrograph of 6 individual

animals. Data are expressed as the mean $\pm$ SEM where $*P < .05$ compared with control or PBS-treated group, n = 6–8 animals per group.

Figure 7.

Changes in plasma gastrin, proliferation, and surface pit, mucous neck, and zymogen cell numbers in control and HKCre/Shh^{KO} mice. (A) Plasma was collected from untreated and octreotide (Oct)-treated control and HKCre/Shh^{KO} mice and circulating gastrin concentrations measured by radioimmunoassay. (B) Morphometric analysis of UEAI-positive cells and BrdU-labeled nuclei was counted using gastric sections collected from untreated- and Oct-treated control and HKCre/Shh^{KO} mice and expressed as UEAI- or BrdU-positive cells per gland. (C) Average fold change gene expression of Shh and Ihh in

RNA collected from the surface pit region of control and HKCre/Shh^{KO} mice without Oct (W/O Oct) or with Oct (W/ Oct) treatment. (D) Average fold change in gene expression of Gli1, Wnt3a, Wnt5A, and cyclin D1 in RNA collected from the surface pit region of control and HKCre/Shh^{KO} mice W/O Oct or W/ Oct treatment. Quantification of mucous neck cells (GSII), zymogen cells (IF), and dual-labeled GSII/IF cells in control and HKCre/Shh^{KO} mice (E) W/O Oct or (F) W/ Oct treatment. Data are shown as the mean $\pm$ SEM where * P < .05 compared with the control animals, # P < .05 compared with HKCre/Shh^{KO} mice W/O Oct, n = 4 mice in each group.

Figure 8.

Proposed mechanism for the development of hyperproliferation in the surface mucous cells with loss of Shh. (A) In the epithelium, loss of Shh disrupts the inhibitory pathway between somatostatin and gastrin, thus inducing hypergastrinemia. Hypergastrinemia up-regulates the expression of Ihh within the epithelial surface mucous cells. (B) Upon binding to Ptch receptor in the mesenchyme, Ihh removes the inhibitory effect of Ptch on Smo and causes the translocation of Gli1 to the nucleus, where it induces expression of target gene Snail and

canonical Wnt. (C) Activation of the Wnt pathway within the epithelium targets cyclin D1, which promotes cell proliferation.