Morphostasis of the adult gastrointestinal tract
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CHAPTER 6

Sonic Hedgehog regulates gastric gland morphogenesis in man and mouse

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Abstract

Background & Aims
In contrast to most of the intestine, gastric epithelial renewal is an asymmetric process. A stem cell located halfway up the tubular unit gives rise to both a basal gland region and a luminal pit compartment, but the mechanisms responsible for the maintenance of this asymmetry are obscure. Here we investigated if Sonic hedgehog (Shh), an established polarizing signal protein during development, is expressed and functional in the adult human and murine stomach.

Methods
Expression of Shh was investigated with immunoblot and immunohistochemistry and compared with a linear system of differentiation, the small intestine. Hereafter mice were treated with the Shh inhibitor cycloamine and we investigated both expression of downstream Shh targets and proliferation of gastric epithelial cells.

Results
Shh was expressed in the stomach whereas no Shh was detected in the small intestine. In cycloamine treated mice we observed decreased expression of HNF3β, Islet (Isl)-1 and BMP4, three Shh target genes. Inhibition of Shh markedly enhanced gastric epithelial proliferation and affected the cell cycle of gastric epithelial gland cells, whereas pit cells remained unaffected.

Conclusions
Shh controls the expression of at least three factors important for epithelial differentiation and is a negative regulator of gastric gland cell proliferation. Shh is a candidate polarizing-signal in the maintenance of gastric pit-gland asymmetry.
Introduction

During organogenesis, intricate patterns of differentiated cells are formed. The mechanisms that govern subsequent pattern maintenance, or morphostasis, in the adult organism have been given relatively little attention. Generally speaking a variety of differentiated cell types originate from a single stem cell in a series of tightly controlled differentiation events. This is especially clear in the rapidly renewing epithelial cell population of the gut, where a clear topographical division exists between the regenerating unit of proliferative cells containing one or several stem cells, and the location of differentiated cells. The molecular mechanisms, which determine cell fate specification along the adult gastrointestinal tract, are still poorly understood. Along most of the intestinal mucosa, epithelial cells follow a unidirectional linear path from the proliferating to the differentiated compartment. In the small intestine and most of the colon, proliferating cells are located at the base of tubular invaginations, the crypts of Lieberkühn. With the exception of the Paneth cell, epithelial cells move towards the intestinal lumen. In the small intestine, differentiated cells migrate towards the villus tip, where they are shed into the intestinal lumen or undergo apoptosis. In the colon this occurs at the luminal end of the crypts and the superficial epithelium between crypts, the intercrypt tables (for review, see reference 1).

In contrast to this linear process, the differentiation of the glandular epithelium of the stomach is bi-directional. Just as in the intestine, the epithelial cells of the gastric mucosa are organized in vertical tubular units. These consist of an apical pit region, and an isthmus just below the pit, whereas the actual gland region forms the lower part of the vertical unit. The gland consists of a neck and a base. However, the life cycle of the various epithelial cells is not a unidirectional process. The progenitor cell of the gastric unit is located somewhere in the region of the isthmus, in the middle of the vertical tubular unit and gives rise to all gastric epithelial cells that migrate either up or down from this point.2

The glandular epithelium of the stomach is divided into a proximal zymogenic zone (corpus) and a distal mucous zone (antrum).3 This paper focuses on the organization of the gastric units in the zymogenic region (see figure 1). The units contain a small pit zone and a relatively large glandular part. A single precursor cell can give rise to all epithelial cell types (see reference 4 for discussion). The mucus producing pit cells migrate up from the progenitor cell towards the gastric lumen.4 The mucous neck cells migrate down towards the base of the gland where they give rise to the pepsinogen producing zymogenic cell (or chief cell).5 The acid secreting parietal cell and the endocrine cells migrate both ways although most are found in the gland region (see Figure 1).6,7

The different epithelial cell types are found in gradients,6-9 which are most pronounced in the human stomach. The parietal cells are found in high numbers in the neck region whereas zymogenic and endocrine cells pre-
dominate at the base of the gland. Thus the stem cell in the gastric unit gives rise to two compartments and several functional cell types present in quantitative gradients (see Figure 1).

Although these compartments and gradients are well described in histomorphological terms the mechanisms that may underlie this complex patterning have been given little attention. However, compartmentalization and polarization are phenomena that are well studied in the embryo. A small number of gene families are used repeatedly throughout development in a wide range of organ systems. In most patterning events studied to date, the Hedgehog, fibroblast growth factor (Fgf), Wnt and bone morphogenetic protein (Bmp) families of proteins play a key role. Although some plasticity seems to exist between species in the use of this system (see 10 for review), its players seem to be conserved from fly to human and recently the term “morphogenetic code” was coined by Hogan.11

Members of the hedgehog family of proteins are prime regulators in many patterning processes throughout both vertebrate and invertebrate development.12 After the identification of Drosophila hedgehog in a genetic screen for segment polarity genes13 several hedgehog homologues have been identified in vertebrates. Of these Sonic hedgehog (Shh) remains the most thoroughly studied to date. Shh is produced as a 47-49 kDa precursor protein. After the covalent attachment of a cholesterol moiety to the precursor protein, a 19 kDa signaling protein is cleaved from the precursor by autoproteolysis. Whereas the remaining 29-31 kDa carboxy-terminal frag-
ment can freely diffuse from the cell, the signaling peptide remains tethered to the cell membrane by virtue of its cholesterol modification. Additional levels of control seem to determine if Shh acts short-range or is released from the cell and acts more distally. Limited information is available about the role of Shh in the development of the human gastrointestinal tract. However, in chicken and mice Shh has been shown to perform an important signaling function in both regionalization along the longitudinal axis of the gastrointestinal tract and radial patterning of the intestinal tube and interestingly, Shh null mice fail to develop gastric epithelium. Although it has been shown by northern blot that the Shh receptor “patched” (Ptc) is expressed in a variety of adult organs in the mouse, and that hedgehog family member Indian hedgehog (Ihh) is expressed in the adult human liver and kidney, a possible role for hedgehog signaling in the maintenance of the complex organization of the adult gastrointestinal epithelium has so far not been examined.

Here we show that Shh is expressed in the epithelium of the adult human and murine stomach and that Shh controls gastric epithelial proliferation in a compartmentalized fashion in the mouse. Hence Shh is a candidate polarizing-signal in the maintenance of pit-gland asymmetry in the adult stomach.

Materials & Methods

Antibodies

The antibodies used are listed below, concentrations given in normal font are all for immunohistochemistry, concentrations in italic are as used for immunoblot. A goat polyclonal α-Shh raised against and affinity purified with E.coli-derived recombinant mouse Shh N-terminal peptide (1:1000) a mouse monoclonal α-bone morphogenetic protein (BMP)2 (mAb 355, 1:1000, 1:2000), and a mouse monoclonal α-BMP4 (mAb 757, 1:500, 1:2000), were obtained from R&D systems (Minneapolis, MN). A goat polyclonal α-Shh antibody (N-19) produced by immunizing with an amino acid sequence mapping to the amino terminus of the murine Shh precursor (1:200, 1:2000), a goat polyclonal α-Ihh (1:19, 1:1000), a goat polyclonal α-Ptc (C-20, 1:50), a goat polyclonal α-HNF3β (P-19, 1:500) and a goat polyclonal α-βActin (1-19, 1:1000) were all from Santa Cruz (Santa Cruz, CA). A rabbit polyclonal α-Ptc (1:200), was a kind gift of Dr R. Töftgard, specificity of this antibody was confirmed in mice over-expressing GLI-1. A mouse monoclonal α-H+/K+-ATPase (1:6000) was from Affinity bioreagents (Golden, CO), a mouse monoclonal α-IsIl-1 (40.2D6, 1:2000) and a mouse monoclonal α-HNF3β (4C7, 1:10, 1:1000), both developed by Dr J.M. Jessell’s lab, were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Mouse monoclonal antibody 45M1 (1:100, Novocastra, Newcastle upon Tyne, England) was used to detect MUC5AC. A mouse monoclonal α-proliferating cell nuclear antigen (PCNA, 1:2000) and a mouse monoclonal α-5-bromo-2'-deoxyuridine (BrdU, 1:100) were from Roche (Almere,
the Netherlands) Specificity of all antibodies used in immunohistochemistry was confirmed on immunoblot.

**Immunoblotting**

Murine stomach and small intestine were homogenized in lysis buffer (300 mM NaCl, 30 mM Tris, 2 mM MgCl$_2$, 2 mM CaCl$_2$, 1% Triton X-100, pH 7.4, supplemented with one tablet of protease inhibitor -Roche- per 50 ml). Protein concentration was measured using the Bradford method. Lysates were diluted 1:3 in protein sample buffer (125 mM Tris/HCl, pH 6.8; 4% SDS; 2% β-mercaptoethanol; 20% glycerol, 1 mg bromophenol blue) and 100-200 μg of homogenate was loaded per lane on a SDS-PAGE gel. After protein separation the proteins were blotted on to a PVDF membrane (Millipore, Bedford, MA). Membranes were blocked with 2% protifar (Nutricia, Zoetermeer, The Netherlands) in PBS, supplemented with 0.1% Tween-20 for 1 hour at room temperature. After a brief wash in washing buffer (0.2% protifar; 0.1% Tween-20), membranes were incubated overnight at 4°C with antibody diluted in washing buffer at the indicated concentration. The following day, membranes were washed three times for 5 min, and subsequently incubated with a secondary horseradish peroxidase (HRP)-conjugated antibody in a 1:2000 dilution. After enhanced chemoluminescence using Lumilight$^+$ substrate (Roche, Mannheim, Germany), antibody binding was visualized using a Lumi-Imager (Boehringer Mannheim).

**Immunohistochemistry**

Formalin fixed paraffin embedded human biopsy specimens of uninflamed gastric and small intestinal mucosa, were obtained from the archives of the pathology department of the Academic Medical Center. The gastric specimens were from patients that were *Helicobacter pylori* negative both by culture and histopathology. Mouse material was obtained and processed as described below. Immunohistochemistry was performed as described in detail previously.$^{27,28}$ Briefly, sections (4μm) were dewaxed and rehydrated in graded alcohols. Endogenous peroxidase activity was quenched, antigen retrieval was performed by heating for 10 min at 100°C in 0.01 M sodium citrate, and non-specific staining was reduced by a blocking step. For BrdU visualization sections were incubated in 2N HCl at 37°C for 60 min and thereafter washed in Boric acid pH 8.5 before the blocking step. The primary antibody was applied in PBS containing 1% bovine serum albumin and 0.1% Triton and incubated overnight at 4°C. The following day, for single staining, a three-step detection method was used as previously described, using a biotinylated goat α-rabbit Ig (DAKO, 1:500) α-mouse Ig (DAKO, 1:200) or rabbit α-goat Ig (DAKO, 1:250) antibody. Detection was performed either with HRP or β-galactosidase as an enzyme. Where HRP was used, sections were incubated with HRP conjugated ABcomplex (DAKO) for 60 min and peroxidase activity was detected with diaminobenzidine (fast DAB, Sigma, St. Louis, MO) used according to the manufacturers instruc-
tions, resulting in the formation of a brown reaction product. Alternatively, sections were incubated with streptavidin β-galactosidase (1:40, Boehringer Mannheim) at RT for 30 min. The streptavidin β-galactosidase was detected using 1% X-gal (DAKO) in iron phosphate buffer (0.02% MgCl$_2$•6H$_2$O, 0.099% potassium ferricyanide, 0.127% potassium ferrocyanide) at 37°C for 15 min, resulting in a blue color. If indicated, mucins were stained by PAS stain to visualize the different epithelial cell types by incubating 10 min in 1% periodic acid, briefly washing with water, a subsequent 10 min incubation with Schiff’s reagent (Merck, Darmstadt, Germany), and hereafter a 10 min wash in distilled water. Sections were briefly counterstained with hematoxylline, dehydrated in graded alcohols and mounted. Specificity of Shh staining was confirmed with a blocking peptide (Santa Cruz). Further controls consisted of omitting the primary and secondary antibody and use of an appropriate Ig control.

**Double staining**

**Double staining of MUC5AC and Shh.**
After overnight incubation with both primary antibodies, sections were incubated for 60 min with a biotinylated rabbit α-goat Ig for detection of Shh and the blue β-galactosidase staining was developed as described above. Hereafter, sections were incubated for 60 min with a HRP-conjugated goat α-mouse Ig (1:100) to detect MUC5AC. The brown HRP precipitate was developed as described above.

**Double staining of the H$^+$K$^+$ ATPase and Shh.**
After overnight incubation with both primary antibodies, sections were incubated for 60 min with a biotinylated swine α-goat Ig (BioSource, Nivelles, Belgium, 1:200). After developing the blue β-galactosidase staining, a HRP-conjugated rabbit α-mouse Ig (DAKO, 1:200) was applied and staining performed as described. All double stainings were preceded and confirmed by staining of adjacent sections.

**Double staining of the H$^+$K$^+$ ATPase and HNF3β.**
After overnight incubation with the monoclonal α-HNF3β antibody, the sections were incubated for 60 min with a biotinylated goat α-mouse Ig (1:100), washed, incubated 60 min with HRP conjugated Abcomplex, and the HRP precipitate was developed as described above. Hereafter the sections were heated to 100°C for 5 min in 0.01M sodium citrate to remove antibodies. Sections were blocked and the α- H$^+$K$^+$ ATPase antibody was applied overnight. The following day the β-galactosidase three-step detection method was used to visualize H$^+$K$^+$ ATPase expression as described above.

**In vivo administration of Cyclopamine**
To assess a possible role of hedgehog signaling in the life cycle of gastric epithelial cells, mice were treated with daily injections of cyclopamine, a
potent hedgehog signaling inhibitor\textsuperscript{29-31} that inhibits hedgehog signaling somewhere downstream of patched and upstream of the transcriptional effectors of the GlI family, most likely at the level of Smoothed.\textsuperscript{31} The cyclopamine was a kind gift of Dr W. Gaffield. The study protocol was approved by the animal ethics review board of the University of Amsterdam. Cyclopamine was administered complexed with 2-hydroxypropyl-\(\beta\)-cyclodextrin (HBC, Sigma). A Cyclopamine-HBC stock solution was produced by suspending 1 mg cyclopamine per ml of 45\% HBC in sterile PBS and stirring for 60 min at 65\(^\circ\)C. The cyclopamine-HBC was stored at -20\(^\circ\)C until administration. A total of eight, seven week old, female C57BL/6 mice were given daily intraperitoneal injections of 2 mg/kg Cyclopamine-HBC for 14 days. Eight mice received solvent only as a control. After 14 days mice were given a single intraperitoneal injection of 150 mg/kg BrdU to label cells in S-phase. One hour after BrdU administration mice were sacrificed by cervical dislocation. To allow optimal orientation of the gastric tissue, flat stomachs were prepared according to the method described by Lee et al.\textsuperscript{3} and fixed in this position with needles. The stomach was transected along the longitudinal axis, half of the stomach was homogenized and gastric lysates were produced as described above. Hereafter the remaining stomach half and small intestine were fixed in 4\% paraformaldehyde and embedded in paraffin.

**Scoring of BrdU/PCNA positive nuclei**
To assess proliferation of gastrointestinal epithelial cells in the cyclopamine treated mice, gastric and small intestinal specimens were stained with antibodies against BrdU and PCNA. Two pictures of each section were taken at 100x magnification and positive nuclei counted, blind to the treatment, in each microscope field with the use of an image analysis program (EFM Software, Rotterdam, The Netherlands). In each field, 5 well-oriented vertical units were counted for the PCNA stain and 10 for the BrdU stain (this needs more counted crypts because of the low amount of BrdU labeled cells). The average number of positive nuclei per vertical unit was compared between groups. To be able to compare the results between animals, it was ensured that all sections visualized the entire axis from the superficial epithelium to the muscularis mucosa.

**Statistical analysis**
Data are presented as mean \(\pm\) SEM. Comparisons between groups of data were made using the Student’s \(t\)-test. \(P\) values < 0.05 were considered statistically significant.

**Results**

**Sonic Hedgehog expression in the adult murine and human stomach**
The continuing regeneration of the adult gastric glands is a process with two compartments and several gradients. We investigated if the Shh signal pro-
Shh in gastric gland morphogenesis

Figure 2. Shh is expressed in murine stomach. Immunoblot, demonstrating that both Shh precursor (A, Santa Cruz antibody) and cleaved amino terminal Shh signal protein (B, R&D antibody) are found in the murine stomach but not in the duodenum. Ihh (C) is expressed in both kidney and liver, but not in either stomach or duodenum. Both liver and kidney were Shh negative (R&D antibody).

Shh, involved in many patterning processes during development, is expressed in this system. The small intestine, as a model of an intestinal linear differentiation process, was used for comparison. As shown in figure 2, both Shh precursor protein (Figure 2A) and large amounts of the cleaved amino terminal protein (Figure 2B) are present in the murine stomach when assessed by immunoblot. In contrast, no Shh protein was detected in the murine duodenum. In accordance with previous findings by Marigo et.al. Indian Hedgehog (Ihh) expression was found in both adult liver and kidney. However, no Ihh was detected in either stomach or duodenum.

Identification of Shh expressing cell types

As outlined in the introduction, the tubular units of the stomach consist of two compartments relative to the position of the epithelial stem cell. The pit region consists of cells migrating towards the gastric lumen whereas the gland proper is made up of cells that migrate in the opposite direction. As a biochemical marker, a periodic acid Schiff (PAS) stain for mucins can be used to discriminate between both compartments. PAS stains pit cell mucins dark purple, mucous neck cells pink, and zymogenic cells slightly grey/blue whereas parietal cells clearly stand out because of the lack of stain. We have shown previously that gastric mucins, the most important structural component of mucus, can be used as genetic markers of either compartment. MUC5AC expression is a marker for gastric pit cells, whereas gastric
Figure 3. Compartmentalized Shh expression in the stomach. The Santa Cruz α-Shh precursor antibody was used for all immunohistochemistry. Human stomach (A,B,E-G,I,J). Murine stomach (C,D,H). Shh precursor protein is expressed in both human and murine stomach (brown precipitate, A,C) whereas no expression was found in the small intestine of either species (B,D). A Shh blocking peptide competed efficiently with binding of the α-Shh antibody (α-Shh without blocking peptide in E versus α-Shh plus a 50 fold excess blocking peptide in F). Use of a PAS stain that stains the pit cells dark purple combined with a Shh stain demonstrates that Shh is exclusively expressed in the gland compartment in both human (Shh stained blue, G) and murine (Shh stained brown, H) gastric units. The expression of the pit cell marker MUC5AC (brown color, I,J) demonstrates that Shh expression (blue in I and J) shows no overlap with MUC5AC and is expressed exclusively in the gland region. Note the graded Shh expression in the human stomach (A,J) from high at the pit-gland transition (arrows) to low deeper in the gland (asterisks). Original magnifications: A, 25x; B-D, 50x; E,F, 40x; G,H, 200x; I, 25x; J, 100x.
gland cells produce MUC6. Using both methods to discriminate between pit and gland cells, it was found that Shh expression is restricted to the gland compartment in both human and murine stomach (Figure 3G–I). In the human stomach, highest expression of Shh is clearly found at the pit-gland transition, whereas the staining intensity gradually diminishes towards the base of the gland (Figure 3A and J).

Morphologically the Shh seemed restricted to the large triangular parietal cells in humans, and this observation was confirmed by double staining for Shh and a parietal cell marker, the H+/K+-ATPase (proton pump). As can be seen clearly from figure 4A–C, a complete overlap was found between Shh expression and H+/K+-ATPase staining, with high Shh expression in the parietal cells close to the pit-gland transition (Figure 4B), and low Shh expression in the parietal cells near the base of the gland (Figure 4C). A difference in Shh distribution was found between human and murine glands. In the mouse, parietal cells where Shh positive but the most intense immunoreactivity was observed in the secretory granula of the zymogenetic cells (Figure 4D,E) and its precursor cell, the mucous neck cell (Figure 4F). Although the staining intensity of the parietal cells in the murine stomach was lower than that of the mucous neck cells and zymogenetic cells we feel that the staining is specific since the antibody detected a single band on immunoblot. Additionally no difference in specificity was found with the blocking peptide since all staining disappeared with similar efficiency (not shown). Hence, we feel that the differences in staining intensity reflect differences in Shh expression rather than the recognition of different epitopes by the antibody. Thus Shh is expressed in both human and murine gastric gland cells, with a clearly graded expression pattern in the human stomach.

**Shh targets**

To establish the nature of the Shh target cell in the human and murine stomach we examined the expression of several indicators of Shh activity. The specificity of all antibodies used was confirmed on immunoblot (not shown). We began by examining the expression of the Shh receptor, Ptc, was examined to determine candidate Shh-responsive cells. Since Ptc is both Shh receptor and a transcriptional target of Shh, expression of Ptc indicates both Shh receptiveness and active Shh signaling. Staining patterns of two different α-Ptc antibodies were identical. In both the human and murine stomach Ptc is expressed on the epithelial cells of the gastric gland region and some of the interstitial cells (Figure 5E,F,H,J). Whereas pit cells were Ptc negative, parietal cells in both human and mouse expressed Ptc (Figure 5E,H). In addition to the parietal cell staining, intense staining was also observed of the epithelial cells at the base of the glands in both humans and mice (Figure 5E,H). Thus Shh signaling in the gastric epithelium seems to be partially autocrine.
Figure 4. Identification of Shh expressing cell type. Human stomach (A-C). Double stain of Shh (blue) and parietal cell marker H+K+-ATPase (brown), performed as described in the method section, showing complete overlap. Again a clear expression gradient is observed, parietal cells close to the pit express high Shh (arrows, B, blow-up of area boxed with continuous line in A), whereas this expression gradually diminishes towards the gland base (asterisks, C, blow-up of area boxed with striped line in A). Murine stomach (D-F). Serial sections showing expression of Shh (brown, D) and the H+K+-ATPase (brown, E). In the murine gland Shh is expressed by parietal cells (arrows) and zymogenic cells (asterisks). As can be seen in the combined Shh (brown)/PAS (pink) stain in F, Shh is also expressed in mucous neck cells. Arrows in F denote cells positive for both Shh and the PAS reaction. Original magnifications: A, 25x; B,C 100x; D,E, 200x; F, 1000x.

We then examined the expression of three putative targets of Shh signaling, the TGFβ family members BMP2 and BMP4,\(^{17,33}\) and the transcription factor HNF3β. Whereas BMP2 was expressed in the epithelial cells (Figure 5 C,L), BMP4 expression was restricted to interstitial myofibroblast like cells in both human and mouse stomachs (Figure 5 K, human not shown). The expression of HNF3β was investigated using two different antibodies. The monoclonal antibody 4C7 stains nuclei of gastric epithelial cells of both humans and mice with highest expression in the parietal cells, as can be seen in double staining of HNF3β and the H+K+-ATPase (Figure 5A,B,G,J). In addition to this nuclear epithelial staining we observed cytoplasmic staining of interstitial myofibroblast-like cells. This staining is most likely an artifact because HNF3β is normally only found in endodermally derived cells and has previously only been detected in the epithelial cells of the murine stomach by in situ hybridization.\(^{34}\) The epithelial cell expression was confirmed with a polyclonal α-HNF3β antibody (not shown). This antibody stained the parietal cells of both humans and mice and did not react with any epitope on interstitial cells suggesting that the interstitial cell staining with the monoclonal antibody is indeed aspecific. Hence a variety of different putative Shh targets are expressed in both epithelial and interstitial cells of the human and murine stomach.

Cyclopamine decreases Shh target gene expression in vivo

Further confirmation of the function of the Shh signal in the adult stomach was obtained by experiments in which mice were treated with the hedgehog inhibitor cyclopamine for 14 days. Cyclopamine is a veratrum alkaloid that was suspected to be a Shh inhibitor because the birth defects that occurred in animals that ingested the lily Veratrum Californicum were identical to the holencephalocoele observed in humans and mice with Shh mutations.\(^{35,36}\) Subsequently, cyclopamine has indeed been shown to interfere with Shh
CHAPTER 6

HNF3β H'K'-ATPase

BMP2

H'K'-ATPase

Patched

H'K'-ATPase

HNF3β H'K'-ATPase

Patched

H'K'-ATPase

HNF3β H'K'-ATPase

BMP4

BMP2
Figure 5. Expression of Shh transcriptional targets. Human stomach (A-F). Expression of HNF3β (A,B) in parietal cells is evident in a double stain of HNF3β (brown) and parietal cells (blue) performed as described in the methods section (arrows in B denote positive cells). BMP2 expression (C) and Ptc expression (antibody from Dr. Töftgard, E) are both confined to the gland region as shown by parietal cell staining in an adjacent sections (D,F), which help indicate the gland region. Murine stomach (G-L). Expression of HNF3β (G,J, arrows denote positive cells) in parietal cells is identical to the human stomach using the same double staining method as in A and B. Expression of Ptc (H), and the parietal cell marker H+/K+-ATPase (I). Ptc is mainly expressed in parietal cells (arrows), and zymogenic cells (asterisk). The expression pattern of BMP4 (K) and BMP2 (L) shows localization of BMP4 to stromal cells (arrows in K) whereas BMP2 is expressed in the epithelial cells (arrows in L). Original magnification: A, 200x; B, 1000x; C-I, 200x; J, 1000x; K,L, 200x.

signaling downstream of the Shh receptor complex. After 14 days treatment with daily intraperitoneal injections of 2mg/kg Cyclopamine-HBC, the expression of four putative Shh target genes was studied in gastric lysates by immunoblot. As shown in figure 6, the expression of HNF3β, Isl-1, and BMP4 was significantly diminished in cyclopamine treated mice whereas we were unable to detect any decrease in BMP2 expression. This validates both the activity of Shh signaling and the fact that HNF3β, Isl-1 and BMP4 are indeed downstream targets of Shh in the stomach.

Figure 6. Reduced expression of HNF3β, Isl-1 and BMP4 in cyclopamine treated mice. Immunoblots showing HNF3β, Isl-1, BMP2, BMP4 and Actin expression (A) in whole gastric lysates from 7 control (lane 1-7) and 7 cycloamine treated (lane 8-14) mice. Signals where quantified with a PhosphorImager, corrected for the β-Actin signal, and expressed in relative units in figure B. Values are means plus standard error. Asterisks denote statistically significant differences between control and cycloamine treated animals (p = 0.005 for HNF3β, p = 0.006 for Isl-1, p = 0.001 for BMP4, student’s t-test).
**Figure 7.** Inhibition of Shh increases gland cell proliferation in the murine stomach. Panel 1. BrdU stain (A,B), arrows denote BrdU positive cells. Compared to controls (A) BrdU incorporation is substantially increased in the precursor cells in the isthmus in cyclopamine treated mice (B). PCNA expression (C-H). Whereas PCNA staining in the pit cells remained unaltered in treated animals (E) compared to control (C), gland cell expression increased considerably (D and F, control and treated respectively). As can be seen at higher magnification (G,H), expression increased especially in zymogenic cells (see H versus control in G). Panel 2. Graphs depicting the mean number of BrdU and PCNA positive nuclei per crypt/gland in the stomach and duodenum of cyclopamine treated mice (open bars, n=8) and controls (black bars, n=8). Error bars indicate the standard error. Values in treated mice were significantly different from controls (stomach: p = 0.2 for BrdU, p < 0.001 for PCNA; duodenum: p = 0.02 for BrdU, p = 0.03 for PCNA, student's t-test). Original magnification: A,B, 200x; C,D, 250x; E,F, 400x.

**Shh controls gastric epithelial cell proliferation**

Since Shh and its downstream target BMP4 are likely to affect the life cycle of receptive cells, epithelial proliferation was studied in the cyclopamine
treated mice. We compared proliferation in the Shh expressing gastric gland with that in the crypts of the duodenum. Murine stomach and small intestine were examined for PCNA expression and BrdU incorporation as two different measures of epithelial proliferation. Epithelial proliferation was significantly altered compared to control. Interestingly, while in the crypts of the small intestine proliferation was approximately 10% lower in cyclopamine treated mice than in controls (11% as judged by PCNA stain, p = 0.02, and 9% as determined by BrdU incorporation, p = 0.03, see Figure 7 panel B), gastric epithelial proliferation increased considerably in the treated animals. Proliferation of gastric epithelial cells was 169% of control as assessed by PCNA stain (p = 0.0002, Figure 7C-H and panel B) and 164% with BrdU incorporation as a measure (p = 0.025, Figure 7A,B and panel B). BrdU only incorporates in cells in S phase but PCNA is a proliferation marker that is more broadly expressed throughout the cell cycle and can thus be used to study the effect of cyclopamine on (partly) differentiated cells. We observed in our experiment that PCNA expression in the pit cells did not change (Figure 7C,E) but was enhanced in the gland cells especially the zymogenic and parietal cells (Figure 7D,F). Thus inhibition of Shh increases gastric epithelial cell cycling in the gland compartment. We concluded that both Shh expression and its activity are restricted to the gland compartment of both the human and murine gastric tubular units, indicating that Shh is indeed involved in compartmentalization in the adult.

Discussion
The mechanisms that maintain intricate patterns of differentiation in the adult organism have been given little consideration. We set out to investigate whether the signaling modules used during morphogenesis of the stomach remain active in morphostasis throughout life. Here it is shown that Shh may play a role in this process in the human and murine gastric epithelium. A first indication of active Shh signaling is the expression of Shh itself and Ptc, BMP2, BMP4, and HNF3β, four established transcriptional targets of Shh signal transduction in the stomach of both humans and mice. We investigated whether the signal is functional by blocking Shh in vivo with cyclopamine in mice. We found decreased expression of the Shh transcriptional targets HNF3β, Isl-1 and BMP4 and markedly enhanced proliferation in the gland compartment of the gastric units in these mice. Hence we propose that hedgehog signaling is not only important in gastric organogenesis but remains important for organ structure throughout life. Interestingly, careful examination of the gastrointestinal phenotype of Shh null mice revealed that these animals do not develop gastric epithelium. The stomachs of these mice contain intestinal epithelium. Apparently, Shh is essential in the induction of the epithelial phenotype during gastric organogenesis. Thus, since Shh seems necessary for gastric epithelial differentiation during organogenesis, our finding that Shh is still expressed and functional
in adult gastric epithelium is interesting and, although our data offer no direct evidence for such a role, we speculate that Shh may play a role in epithelial differentiation in the adult.

In the mouse, Shh, Ptc, HNF3β, and BMP2 are all expressed in the epithelial cells of the gland. BMP4 is expressed in interstitial myofibroblast like cells. In the human stomach a similar distribution pattern is observed for all these molecules. The main difference between the expression patterns found in mice and humans is that Shh is more restricted in the human stomach, with exclusive expression in the parietal cells. This kind of difference in cell type specific expression between humans and rodents is not uncommon; for example, intrinsic factor is exclusively expressed and produced in parietal cells in humans but mainly produced by zymogenic cells in rats and mice.

Treatment of mice with the hedgehog inhibitor cyclopamine demonstrates that HNF3β, Isl-1 and BMP4 expression are regulated by Shh in the gastric glands because all three putative targets were found to be decreased in treated mice. BMP2 expression remained unaltered in treated mice. Thus it may be that BMP2 and BMP4 are differentially regulated in the adult stomach. Indeed some data in the literature suggest that these two BMPs are regulated independently to a certain extent. In the vertebrate limb bud for example, BMP2 but not BMP4 can be induced by ectopic expression of Shh, whereas BMP4 can be induced by Shh in gut mesenchyme.

The increase in BrdU incorporation and enhanced PCNA expression found in treated mice suggests that Shh is a negative regulator of gastric gland cell proliferation, whereas proliferation in the crypts of the duodenum is diminished by inhibition of Shh. The strong stimulation of gastric proliferation observed upon Shh inhibition by cyclopamine is somewhat surprising since Shh usually stimulates epithelial proliferation. Activating mutations in almost any part of the Shh signaling machinery have been found to have an oncogenic effect in skin and neural cells (see reference 40 for review). Also, overexpression of Shh has been shown to oppose cell cycle arrest in human epidermis grafted on to immune-compromised mice. The enhanced proliferation observed upon Shh inhibition is therefore likely to be a downstream effect and possibly caused by the decreased expression of BMP4, which is downstream of Shh in the stomach. Bone morphogenetic proteins are well known to inhibit cellular proliferation and stimulate differentiation and apoptosis (see reference 33), over expression of BMP4 leads to inhibition of epithelial proliferation in the lung. Also, in mice with a targeted mutation in the transcription factor Fkh6, decreased expression of BMP2 and BMP4 correlates well with increased proliferation in the gastrointestinal tract. BrdU incorporation only occurs in the undifferentiated precursor cells in S phase in the region of the isthmus, thus the enhanced BrdU labeling found in this study suggests that Shh directly or indirectly negatively regulates precursor cell proliferation. PCNA expression was enhanced in both zymogenic and parietal cells of the gland compartment, indicating that Shh negatively regulates the cell cycle of these cells. PCNA expression of the pit com-
partment was not affected, thus the effect of Shh seems compartmental, and this is in line with a possible function for Shh as a morphostat. Although the Shh null mouse shows no alteration in proliferation of the epithelial cells in its stomach, it is hard to extrapolate from the findings in the Shh null mouse. Since this mouse never develops gastric epithelium it is difficult to tell from this animal what the effect of Shh on fully differentiated gastric epithelial cells could be.

The small but significant decrease in proliferation in the duodenum is intriguing since we did not detect any Shh or Ihh in the duodenum either by western blot analysis or immunohistochemistry. Although we cannot exclude that the decreased proliferation observed in the duodenum is due to a specific effects of the cyclopamine, an alternative explanation may be that Sonic Hedgehog and/or Indian Hedgehog expression, in an organ other than the duodenum, controls the production and/or release of one or more of the many endocrine factors that are known to influence intestinal proliferation. Several other possibilities exist, such as duodenal expression of smoothened and its activation by a ligand other than Sonic or Indian hedgehog. Again, we cannot exclude that the effect on the duodenum is aspecific, however we hope to address these possibilities in future experiments.

A function of Shh as a morphostat is further supported by the graded hedgehog signal that is especially clear in the human stomach. Highest Shh expression was detected in the parietal cells at the gland-pit boundary, with gradually declining expression along the axis of the gland. This is reminiscent of the graded Shh signaling during organogenesis, when cells determine their fate based on their position relative to organizing centers. These organizing centers function by secreting diffusible signals that form a concentration gradient. Interestingly, previous experiments in mice by the laboratory of Dr. J.I. Gordon have suggested that parietal cells indeed function as an organizing center. In these experiments parietal cell ablation was achieved by expressing a toxin behind a parietal cell specific promoter. In these mice, mucous neck precursor cells accumulated and failed to mature into differentiated neck and zymogenic cells. Also a large increase was seen in the proliferation of all types of precursor cells, suggesting that parietal cells produce or induce a negative regulator of gastric precursor cell proliferation. The results of this experiment are mimicked in patients with autoimmune gastritis, who produce autoantibodies against parietal cells. Although the autoantibodies are exclusively directed toward parietal cells, zymogenic cells are also depleted together with the parietal cells, and both cell types are replaced by cells with an intestinal phenotype, causing intestinal metaplasia (see reference for review). Thus parietal cells seem to determine cell fate of the other gland cell types and have organizer center properties in the adult.

In conclusion, Shh hedgehog and its target genes are expressed in a compartmentalized fashion in the adult human and rodent stomach. Shh positively regulates expression of BMP4, HNF3β, and Isl-1. Shh is a negative regulator of precursor cell and gland cell proliferation. Our data support the idea
that the "morphogenetic code" is utilized not only during organogenesis of the stomach but remains important to maintain complex tissue organization, or morphostasis, in the adult.

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