Hedgehog signaling in homeostasis of the gastrointestinal tract
van Dop, W.A.

Citation for published version (APA):
van Dop, W. A. (2011). Hedgehog signaling in homeostasis of the gastrointestinal tract
Chapter 4

Hedgehog signaling stimulates precursor cell accumulation and impairs epithelial maturation in the murine esophagus

Willemijn A. van Dop, Sanne L. Rosekrans, Anja Uhmann, Viljar Jaks, G. Johan Offerhaus, Marius A. van den Bergh Weerman, Jarom Heijmans, James C. Hardwick, Daan W. Hommes, Rune Toftgård, Heidi Hahn, Gijs R. van den Brink

Manuscript in preparation
**Background & aims:** Morphogens pattern tissues during development and maintain tissue architecture in rapidly renewing adult organs such as the gastrointestinal tract. In the intestine Hedgehog (Hh) signaling is from epithelium to mesenchyme and negatively regulates epithelial precursor cell fate. The role of Hh signaling in the esophagus has not been studied *in vivo*. Here we examined the role of Hh signaling in epithelial homeostasis of the mouse esophagus.

**Methods:** We used two different strategies for conditionally activating the Hh pathway in adult mice. We used a mouse in which the Hh receptor Patched1 (Ptch1) could be conditionally inactivated in a body wide manner and a mouse in which Gli1 could be induced specifically in the epithelium of the skin and esophagus. Effects on epithelial homeostasis of the esophagus were examined using immunohistochemistry, in situ hybridization, transmission electron microscopy, and real-time polymerase chain reaction.

**Results:** Sonic Hedgehog was the only Hh homologue produced in the esophagus. The Hh signaling receptor Smoothened and transcriptional targets Gli1 and Hhip were expressed by the epithelial precursor cells in the basal layer. In both mouse models we observed an expansion of the epithelial precursor cell compartment and failure of epithelial maturation and migration.

**Conclusions:** In contrast to the intestine, Hh signaling in the esophageal epithelium of the adult mouse occurs in the epithelium. It is a positive regulator of the precursor cell compartment and a negative regulator of epithelial maturation and migration.
Introduction

The esophagus is lined by a stratified squamous epithelium which is composed of several cell layers. The proliferating cells of the basal layer align the basal membrane and differentiate as they move towards the lumen. In mice the epithelial layer keratinizes at the luminal surface whereas the human epithelium is non-keratinized. The esophageal epithelium is rapidly renewed from the precursor cells at the basal layer. Proliferating epithelial cells are shed from the epithelium in less than 48 hours (see results).

The mechanisms that are in place to regulate epithelial homeostasis in the esophagus are relatively poorly characterized. It is becoming increasingly clear that morphogens not only act to lay down patterns of cellular differentiation during organogenesis but play a critical role in the maintenance of homeostatic equilibria of rapidly renewing epithelial tissues such as those of the gastrointestinal tract. For example the Wnt, Hedgehog (Hh) and Bone Morphogenetic Protein (Bmp) pathways play an important role in epithelial homeostasis in the intestine but their role in the adult esophagus is much less well established.

Three Hhs are present in humans and mice, Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh). These Hhs bind to a common receptor Patched which subsequently loses its repressive activity towards the Hh signaling receptor Smoothened (Smo). Smo signals through the Gli family (Gli1-3) of transcription factors to induce transcriptional targets many of which are components of the pathway such as \textit{Ptch1}, \textit{Hedgehog interacting protein} (Hhip) and \textit{Gli1}. Hh signaling plays a critical role in the development of the normal esophagus.\(^1\) Signaling is from endoderm to mesoderm during development. Shh is produced by the endoderm\(^2\), \(^3\) whereas Gli1-3, the transcription factors that mediate Hh signaling, are expressed in the mesoderm.\(^4\) Mice with mutations in either \textit{Shh} or a combination of \textit{Gli2} and \textit{Gli3} display a growth defect of the foregut mesenchyme. This mesenchymal growth defect results in incomplete tracheoesophageal separation and the development of a single tube that connects both the airways and the stomach, a pathology that is thought to resemble tracheo-esophageal fistula in human patients.\(^5\) Conflicting data have been published on the possible expression of Hh pathway components in the adult esophagus. Isohata \textit{et al}.\(^6\) found expression of both SHH and Gli1 in the basal layer using immunohistochemistry. The data suggested autocrine SHH signaling in the basal layer of the esophageal epithelium. This would be an important difference with Hh signaling in the columnar epithelium of the intestine and stomach where signaling is uniquely from the epithelium to the underlying mesenchyme.\(^7\), \(^8\) In the intestine the Hh pathway does not act as a pro-proliferative pathway as in many other tissues but controls an as yet unidentified mesenchymal factor which negatively regulates intestinal epithelial precursor cells.\(^8\) However, using a different antibody Wang \textit{et al}. found no expression of SHH in the normal esophagus or LacZ expression in a
*Ptch1-LacZ* reporter mouse and concluded that there was no Hh signaling in the normal adult esophagus.

No functional experiments have been performed to address the potential role of Hh signaling in the esophagus *in vivo*. It is important to carefully evaluate a possible role for Hh signaling in the epithelium of the esophagus. The esophagus shows remarkable similarity to the skin and Hh signaling plays an important role in the development of basal cell carcinoma in the skin where the first promising results have now been obtained in a clinical trial using a Hh antagonist. If there is Hh signaling in the precursor cells of the basal layer of the esophagus the Hh pathway may act as an oncogenic pathway in esophageal carcinogenesis and be a target for therapy. Here we examine two distinct mouse models in which activation of the Hh pathway can be induced to examine the role of Hh signaling in the adult mouse esophagus.

**Material and Methods**

**Mice**

*Ptch1* mutant mice

We used previously generated *Ptch1*\textsuperscript{loxP/lox}\textsuperscript{10} and *Rosa26CreERT2*\textsuperscript{11} mouse lines. The *Ptch1*\textsuperscript{loxP/lox} mice had a *loxP* site inserted into intron 7 and intron 9 of the *Ptch1* gene. The *Rosa26CreERT2* mutant mouse strain expressed a fusion gene encoding Cre recombinase and a modified ligand-binding domain for the estrogen receptor under control of the endogenous *Rosa26* promoter. The two mouse lines were crossed to obtain *Ptch1*\textsuperscript{loxP/lox} CreERT2\textsuperscript{-/-} mice. Eight-week-old mice were injected intra-peritoneally with 1 mg tamoxifen on 5 consecutive days to induce the *Ptch1*\textsuperscript{del} mutation to get *Ptch1* mutant mice (n=19). Both *Ptch1*\textsuperscript{loxP/lox} CreERT2\textsuperscript{-/-} mice treated with tamoxifen and vehicle treated *Ptch1*\textsuperscript{loxP/lox} CreERT2\textsuperscript{-/-} mice were used as controls (n=19). 19 days after the first day of tamoxifen application mice were sacrificed and esophagi were collected for examination. Recombination efficiency was determined by real-time PCR on genomic DNA (supplementary methods). The study was approved by the Institutional Animal Care and Use Committee of the University of Göttingen.

*Gli1*-inducible mice

For induction of *Gli1* mice with a Tet-Off transactivator placed after the keratin 5 promoter (*K5-tTA* mouse)\textsuperscript{12} were crossed with *Tre-Gli1* mice.\textsuperscript{13} Doxycycline was delivered by addition of 2 mg/ml of doxycycline to the drinking water. *Gli1* was induced by withdrawal of doxycycline and mice were examined two weeks after withdrawal of doxycycline. *Tre-Gli1* mice and wild-type mice treated with doxycycline were used as
controls. The study was approved by the Institutional Animal Care and Use Committee of the Karolinska Institute.

**Gli1-reporter mice**

Six Gli1-CreERT214 were crossed with Rosa26-Stop^{fl/fl}-LacZ15 mice in which LacZ is separated from the ubiquitously expressed Rosa26 locus by a floxed stop codon. The mice were induced with an oral dose of 10 mg tamoxifen per mouse for two days in a row. An X-gal staining was performed on esophageal tissue following standard procedures. See supplementary methods.

**Immunohistochemistry and immunofluorescence**

Immunohistochemistry and immunofluorescence were performed using standard protocols. For a detailed description of the protocols and antibodies used see supplementary methods.

**Probe generation and In Situ Hybridization**

Generation of probes and in situ hybridization were performed as previously described.16 A detailed protocol of the procedure is available in the supplementary methods.

**Transmission Electron Microscopy**

The esophagi were dissected and fixed in Karnovsky fixative. After fixation, the material was post fixed in 1% osmiumtetroxide, block-staining with 1% uranylacetate, one-step dehydration in dimethoxypropane and embedding in epoxysin LX-112. Sections were stained with tannic acid, uranylacetate and lead citrate and examined in a transmission electron microscope (FEI/Philips CM10).

**RNA isolation, complementary DNA synthesis and Quantitative RT-PCR**

For isolation of RNA a piece of esophageal tissue was collected from control and mutant mice. A detailed description of RNA isolation, complementary DNA synthesis and the Quantitative RT-PCR protocol as well as primer information can all be found in the supplementary methods. Concentrations were divided by the amount of *Gapdh* in each sample. *Gapdh* was used as household gene and its expression was equally distributed between the wild-types and the *Ptch1* mutant mice. For a detailed protocol see supplementary methods.

**Cell culture experiments**

Het1A cells were cultured in DMEM (Gibco 42430), replenished with 5% Fetal Calf Serum and 1% penicillin/streptomycin. Upon reaching 80% confluency they were treated with 1 μg/ml recombinant BMP7 (R&D, 354-BP-010) for 24 hours. RNA was obtained using a standard Trizol and Chloroform extraction method. Complementary DNA synthesis and quantitative RT-PCR were performed as described above. The experiments
shown here were representatives of at least three different experiments, all performed in triplicate.

**Statistics**

Statistical analysis was performed with Prism 5.0 (GraphPad Software). All values were represented as the mean ± standard error of the mean (SEM). Samples were analyzed using a student’s t-test. Differences were considered statistically significant at $P < 0.05$.

**Results**

**Localization of Hh pathway components in the adult mouse esophagus and effective recombination of Ptch1**

In the developing mouse esophagus $Shh$ is expressed by the endoderm. It is crucial for the separation of the developing trachea from the developing esophagus. $^2$ Signaling is paracrine, from endoderm to mesoderm, as Hh targets $Ptch$ and $Gli1-3$ are expressed by the mesenchyme. $^2, 4$ We examined expression levels of the three Hh homologues $Shh$, $Ihh$, and $Dhh$ in the mouse esophagus by quantitative RT-PCR. We have previously shown that in the colonic epithelium $Ihh$ is the main Hh homologue expressed. $^8$ We now found that $Shh$ is the only Hh expressed at detectable levels in the esophagus. We used the colon as a negative control for $Shh$ (Figure 1A, $n=5$ for both groups, $P=0.0005$), and the colon and testis as a positive control for $Ihh$ and $Dhh$ respectively (Figure 1A, $n=5$ for the esophagus, $n=5$ for colon and $n=2$ for testis, $P=0.0002$ and $P=0.0096$ for $Ihh$ and $Dhh$ respectively). To identify the Hh target cells we investigated the esophageal expression pattern of $Smo$, $Hhip$ and $Gli1$ in the adult mouse by in situ hybridization (Figure 1B). We found that $Smo$, $Hhip$ and $Gli1$ were all expressed by the basal cells of the esophageal epithelium. Expression of $Gli1$ by the epithelial cells of the esophagus was further confirmed by crossing $Gli1CreERT2$ mice with $Rosa26-Stop{^\beta}\beta-LacZ$ mice inducing them with 2 oral doses of tamoxifen. X-gal staining of the esophageal tissue demonstrated that $Gli1$ expression was confined to the basal epithelial cells (Figure 1C), though in a less extensive manner than the $Gli1$ in situ hybridization. Since Hh target expression was very different from what has been described during development of the esophagus we used the same in situ probes and protocol to examine expression in the developing esophagus. The results showed the expected mesenchymal expression pattern for $Gli1$ and $Hhip$ (Supplementary Figure 1), confirming the specificity of the probes and in situ protocol.

Successful Cre-mediated recombination of $Pch1{^\beta}\beta CreERT2$ mice was confirmed by real-time PCR on genomic DNA (Figure 2A). Recombination efficiency at the $Ptch1^{flox}$ locus in the esophagus was 95% ± 4.3%. Induction of Hh target genes $Gli1$, $Hhip$ and $Ptch1$ was studied by quantitative RT-PCR. Although in the $Ptch1$ mutant mice exons 8 and 9 were deleted upon injections with tamoxifen, the mutant mRNA was still expressed and could still be induced by increased Hh signaling as we have previously shown in the
Conditional deletion of exons 8 and 9 of *Ptch1* resulted in up-regulation of Hh target genes (Figure 2B, n=5 for the control group, n≥3 for the mutant group, P=0.0012, P=0.0025, P=0.005 for *Gli1*, *Hhip* and *Ptch1* respectively). In situ hybridization for *Smo*, *Hhip* and *Gli1*. The dashed red line indicates the border between mesenchyme and epithelium. (C) X-gal staining of the esophagus of *Gli1-CreERT2 x Rosa26-Stop^fl/fl^-LacZ* mice induced with tamoxifen. Arrows in the right panel indicate cells that express LacZ. Original Magnifications: 400x.

These results show that tamoxifen induced deletion of exons 8 and 9 of *Ptch1* was successful...
and resulted in increased Hh signaling in the epithelium of *Ptch1* mutant mice. The *Ptch1* mutant mice can be examined until three weeks after recombination as the mice then develop systemic signs of illness.

**Loss of polarization and impaired epithelial maturation in Ptch1 and K5tTA-TreGlI1 mutant mice.**

The normal wild-type esophagus in mice is lined with stratified keratinizing squamous epithelium. H&E stainings of both the *K5tTA-TreGlI1* mutant mice and the *Ptch1* mutant mice showed several changes in the esophageal epithelial layer. The basal cells, which are normally positioned in a well organized single layer of round-shaped cells, as was seen in the control mice (Figure 3A,B, left panel), formed multiple disorganized layers in the mutant mice. They were positioned in an irregular pattern and their shape had changed. The basal cells had become oval-shaped and were palisading (Figure 3A,B, middle panel), a situation where the basal cells have a longitudinal orientation, a feature also often seen in basal cell carcinomas (BCC). In addition there were places where the
Hedgehog signaling in the murine esophagus was protruding into the underlying mesenchyme, forming small buds (Figure 3A, B right panel). The disorganized cell layers, palisading of basal cells and budding are all signs of loss of polarity. Furthermore signs of impaired maturation were observed in the H&E stainings. The keratin layer, which lines the esophageal epithelium, was clearly visible in the control mice, whereas in the mutant mice this layer was very thin (Figure 2A, B). The keratohyalin granules (Figure 2A, B, closed arrow in left panel), which play a role in the keratinization of the differentiating epithelial cells, were strongly reduced in the mutant mice. Another sign of impaired maturation was the presence of parakeratosis in the mutant mice, where the nuclei of the keratinocytes persisted as they migrated into the upper most layers of the epithelium (open arrow Figure 3A, middle panel). In addition to the H&E stainings, transmission electron microscopy on esophageal epithelium was performed to examine changes in cell orientation and morphology at the ultra-structural level (Figure 3C). Also by electron microscopy palisading of the basal cells was observed.

**Figure 3.** Loss of polarity and impaired maturation upon increased Hh signaling. (A) H&E stainings of $\text{Ptch1}^{+/\text{mut}}$ mutant mice and (B) $\text{K5tTA- TreGli1}$ mice, both with a control shown in the left panel. Closed arrows in the left panels indicate the keratohyalin granules, which are lost in the mutant mice. Open arrows in the middle panels indicate parakeratosis. (C) Electron microscopy of a control and $\text{Ptch1}^{+/\text{mut}}$ mutant mouse. The left two panels show the basal layer, the right two panels show the suprabasal layers of the esophageal epithelium. Original magnifications (A, B): 400x, (C): 2600x.
and there was a lack of stratification in the mutant mice. In the superficial layer of the epithelium more nucleated cells with high nuclear to cytoplasmic ratios were observed, signs of incomplete differentiation. Both loss of polarization and impaired maturation are features found during development of dysplasia.

**Increased epithelial proliferation and impaired migration in Ptch1 mutant mice**

We examined proliferation by performing immunohistochemistry for proliferating cell nuclear antigen (Pcna) (Figure 4A). In control mice Pcna staining was positive in the majority of basal cells and only few cells in the suprabasal layers. In the mutant mice Pcna staining was also positive in the majority of basal cells, but an increased amount of proliferating cells could be observed in the suprabasal layers (Figure 4B, \( P=0.0025 \))

![Image](image.png)

**Figure 4.** Increased proliferation of precursor cells and retention of differentiating cells in the epithelial layer. (A) Immunohistochemistry for Pcna in control mice (left panel) and mutant mice (right panel). (B) Counting of Pcna positive cells per 500 \( \mu m \) in the basal epithelial layer and each layer above. (C) Immunohistochemical staining for BrdU positive cells 48 hours after a BrdU pulse. (D) Counting of BrdU positive cells per transverse section. (E) Immunofluorescent double staining of Ki67 (red) and BrdU (green) in the BrdU pulsed mutant mice. Original magnifications: 400x (A,E), 200x (C).
for the first suprabasal layer, \( P = 0.0012 \) for the second suprabasal layer and \( P = 0.0445 \) for the layers above that, \( n=8 \) for both control and mutant group). To examine whether impaired maturation resulted in retention of differentiating cells in the epithelium mice were pulse-labeled with BrdU 48 hours before sacrifice. BrdU positive cells were stained by immunohistochemistry (Figure 4C). In control mice only a few cells in the basal layer had retained BrdU 48 hours after injection, indicating that the normal rate of turnover of the proliferating cells is less than 2 days in the mouse esophagus. In the mutant mice an increased amount of BrdU positive cells was observed, mostly located in the suprabasal layers demonstrating that impaired maturation results in retention of cells in the epithelial layer. Figure 4D shows the increased amount of BrdU positive cells per transverse section (\( n=3 \) and \( n=4 \) for the control group and the mutant group respectively, \( P = 0.0176 \)). We performed an immunofluorescent double staining for BrdU and Ki67 to test whether the BrdU positive cells that were retained in the epithelial layer were still proliferating 48 hours after pulse-labeling (Figure 4E). We found that the BrdU positive cells that had migrated upwards, out of the basal layer, were all Ki67 negative. Apparently these cells had undergone cell cycle exit and were differentiating cells showing impaired migration towards the lumen.

**Expansion of the precursor cell compartment and diminished epithelial differentiation in Ptch1 mutant mice.**

To examine the apparent defect in epithelial maturation that was observed at histological examination of the mutant mice in more detail, we examined the expression of markers of basal cells and differentiating esophageal epithelial cells. The expression of basal cell markers p63 and keratin 14 was examined by immunohistochemistry and quantitative RT-PCR (Figure 5A,B). The p63 staining showed an increased number of positive cells in the mutant mice (Figure 5A left graph, \( n=6 \) per group, \( P < 0.0001 \)). This increase in p63 expression was corroborated by quantitative RT-PCR (Figure 5A right graph, \( n=5 \) per group, \( P = 0.0066 \)). Both immunohistochemistry and quantitative RT-PCR for keratin 14 confirmed this expansion of the precursor cell compartment of the basal layer in the mutant mice (Figure 5B, \( n=3 \) per group, \( P = 0.0002 \)). Furthermore, cells retained keratin 14 expression as they moved out of the expanded basal layer upwards towards the lumen (Figure 5B, left) indicating a defect in epithelial maturation. Loricrin, involucrin and keratin 13 were examined as markers of differentiation. These makers are expressed by the differentiated epithelium between the basal layer and the stratum corneum. Using these markers we observed thinning of the layer of differentiated cells (Figure 5C-E) which showed clear signs of parakeratosis (Figure 5E). Together these markers suggest that activation of Hh signaling in the esophageal epithelium expands the precursor cell compartment and diminishes epithelial differentiation.

**Induction of Klf4 in the Ptch1 mutant mice.**

Recently Tetrault et al examined the role of Krüppel-Like Factor 4 (Klf4) in the esophagus using mice with a deletion of Klf4 in the esophageal epithelium.\(^{18}\) The phenotype of the
Klf4 mutant esophagus showed similarities to the phenotype we observed in our Ptch1 mutant mice. The authors described histological changes that resemble the changes observed in our Ptch1 mutant and Gli1 induced mice and found a similar expansion of the precursor cell compartment and impaired epithelial maturation. Because of the very similar phenotypes we examined if expression of Klf4 might be lost in our mice. We found expression of Klf4 in the suprabasal layer of the esophageal epithelium and in contrast to what we expected we observed an up-regulation of Klf4 mRNA and protein in the mutant mice (n=5 per group, P=0.0085).

Figure 5. Increased expression of basal cell markers and decreased expression of differentiation markers of the esophageal epithelium of Ptch1Δβ mutant mice. (A) Immunohistochemical staining for p63, a basal cell marker (left). Counting of p63 positive cells per 100 μm in control and Ptch1Δβ mutant mice (middle) and quantitative RT-PCR for p63 (right). (B) Immunohistochemical staining and quantitative RT-PCR for keratin 14, another basal cell marker. (C-E) Immunohistochemical staining for the differentiation markers loricrin (C), keratin 13 (D) and involucrin (E). The red arrow in D indicates the presence of an unfragmented nucleus in the keratin layer (‘parakeratosis’). Original magnifications: 400x (A).
Bmps have been described as Hh signaling targets in development and are known to play a role in differentiation of intestinal epithelial cells. We therefore examined expression of Bmp 2, 4, 5 and 7 in the control and Ptch1 mutant mice. Of the different Bmps we screened Bmp 2, 4 and 5 were expressed at very low levels relative to Gapdh (not shown). However, Bmp7 was more abundantly expressed and up-regulated in the mutant mice (P=0.0005, n=5 and n=8 for control and mutant group respectively, Figure 7A). To study the expression pattern of Bmp7 we performed in situ hybridization for Bmp7 and found that it was expressed in the basal layer (Figure 7A). We examined the phosphorylation status of the Bmp signaling specific Smads 1, 5, and 8 by immunohistochemistry using a phospho-specific antibody (pSmad1,5,8), to examine which cells were responding to the increased levels of Bmp7. Control mice were negative for pSmad1,5,8 in the basal layer and weakly positive in the suprabasal cells. In the mutant mice staining in the suprabasal cells was more intense, consistent with increased Bmp signaling (Figure 7B). To examine the effect of recombinant BMP7 in vitro we treated Het1A cells with recombinant BMP7 and performed quantitative RT-PCR for BMP targets ID2 and ID4 and for the differentiation marker involucrin (n\geq 3 per group, Figure 7C). The significant increase in both ID2 (P=0.025) and ID4 (P=0.023) demonstrated that Het1A cells respond to BMP7. Involucrin was significantly increased upon treatment with BMP7 (P=0.0066), suggesting a role for BMP signaling in differentiation of esophageal epithelial cells. Treatment with BMP7 did not affect levels of KLF4 in Het1A cells (Figure 7C).
Figure 7. (A) Quantitative RT-PCR demonstrates significant upregulation of Bmp7 in the Ptch1<sup>fl/fl</sup> mutant mice. In situ hybridization shows that Bmp7 is expressed by the basal cells of the epithelium. (B) Immunohistochemistry for mediators of Bmp signaling phospho-Smad1,5,8 shows up-regulation in the Ptch1<sup>fl/fl</sup> mutant mice. (C) Treatment of Het1A cells with recombinant BMP7 demonstrates up-regulation of BMP targets ID2 and ID4 and of differentiation marker involucrin. No effect was observed on expression of KLF4. Original magnifications: 400x.
Hedgehog signaling in the murine esophagus

Discussion

Hhs are expressed throughout the adult gastrointestinal tract. Conflicting results have been published on the potential expression of Hh pathway components in the normal adult esophagus. No functional experiments had been performed to examine the role of Hh signaling in the esophagus in vivo. Here we show that Shh is the only Hh homologue expressed in the esophagus. Hh signaling is active in the epithelium where Hh receptor Smo and Hh signaling targets Gli1 and Hhip are expressed. Conditional body wide deletion of Ptch1 and inducible epithelial activation of Gli1 lead to a similar histological phenotype with features of epithelial dysplasia. Careful examination of the Ptch1 mutant mice shows expansion of the precursor cell compartment of the basal layer and impaired maturation and migration of the esophageal cells.

Using immunohistochemistry different groups have obtained different results regarding the possible expression of Hhs in the esophagus. Isohata et al. described SHH expression in the basal layer and DHH in the differentiated epithelial cells. In contrast Wang et al. failed to detect any SHH using a different antibody. In our hands only Shh mRNA is detectable in the mouse esophagus. The results indicate that Shh is the main Hh in the esophagus but expression may be low and therefore difficult to detect using immunohistochemistry. During development of the gut Hh signaling is paracrine, from endoderm to mesoderm, throughout the gastrointestinal tract. This paracrine epithelial to mesenchymal Hh signaling is maintained in the adult stomach and intestine. Here we find that in contrast to the columnar epithelium of the rest of the gastrointestinal tract, the squamous epithelium of the adult esophagus is a direct target of Hh signaling. Several independent lines of evidence from our experiments indicate that Hh signaling occurs primarily in the basal layer of the epithelium in the esophagus. First, in situ hybridization showed that Hh signaling receptor Smo, transcription factor Gli1 and transcriptional targets Gli1 and Hhip all localize to the basal layer. To corroborate the specificity of our in situ probe and protocol we used a mouse embryo to examine expression of Gli1 and Hhip, and observed expression of these Hh targets in the mesenchyme, in agreement with the literature. Second, the specificity of this staining was further established by the finding that Gli1 was specifically upregulated in the basal layer in the Ptch1 mutant mice. Finally a functional experiment in which Gli1 expression cells can be conditionally marked upon an injection with tamoxifen in Gli1CreERT2-Rosa26-Stopβ-LacZ reporter mice showed exclusive marking of cells in the basal layer of the epithelium. In our current study we have only examined induction of Hh signaling. It is of course possible that under physiological conditions expression levels of Shh and its targets are low and restricted to few cells. To further study the importance of Hh signaling in esophageal homeostasis it would be valuable to also examine the effect of absence of Hh signaling from the adult esophageal epithelium.
The mouse esophageal epithelium has many similarities with the skin epidermis both at the histological and at the molecular level. For example the precursor cells of the basal layer of both esophagus and skin are marked by expression of keratin 5, keratin 14 and p63. Upon differentiation, cells of both tissues lose the expression of basal cell markers and express loricrin and involucrin and keratin 13, markers of early terminal differentiation. Further maturation is characterized by the formation of granular layer with the keratohyalin granules, and by fragmentation of nuclei and development of the cornified layer. Histological examination of the esophagus of \textit{Ptch1} mutant and \textit{Gli1} induced mice showed and expansion of the basal layer and altered organization of the cells which showed so called nuclear palisading and budding into the mesenchyme. The increased number of precursor cells was confirmed by the finding that the normally single layer of Pcna and p63 positive cells of the basal layer was extended to several cell layers in the \textit{Ptch1} mutant mouse. The expression of basal cell marker keratin 14 was maintained in the differentiating cells suggesting that epithelial differentiation was incomplete in the \textit{Ptch1} mutant mice. Indeed, inspection of the H&E staining showed a decrease in keratohyalin granules, presence of parakeratosis and a thin cornified layer in the mutant mice, all signs of defective epithelial maturation. This was further confirmed using keratin 13, involucrin and loricrin as markers of esophageal epithelial differentiation. The accumulation and palisading of precursor cells and impaired terminal differentiation with marked parakeratosis are aspects of epithelial dysplasia. Even though these (pre)dysplastic features are present in our \textit{Ptch1} mutant and \textit{Gli1} induced mice, we did not have a chance to follow the mice in time to see whether more marked dysplasia or cancer would develop due to the fact that Hh pathway activation is not esophagus specific and the mice develop systemic signs of disease at two (\textit{Gli1} inducible) and three (\textit{Ptch1} mutant) weeks after induction.

Tetrault \textit{et al.} recently described a mouse model with some noticeable similarities to our findings in the \textit{Ptch1} mutant mice. The authors examined the effect of loss of Klf4 on the esophagus. Klf4 is a DNA-binding transcriptional regulator, expressed by the suprabasal cells. Even though Klf4 is not expressed by the basal cells, the Klf4 mutant mice showed important changes in the basal layer that were similar to changes observed by us. Thus Klf4 likely controls the expression of a paracrine signal from the suprabasal cells to the basal layer that reduces proliferation and stimulates exit of cells from the basal layer and may therefore act as a negative feedback loop. To examine a possible link between Hh and Klf4, we performed IHC and quantitative RT-PCR for Klf4. Both techniques showed a clear up-regulation of Klf4 in the \textit{Ptch1} mutant mice. The upregulation of Klf4 in response to the impaired maturation and accumulation of precursor cells in the mutant mice seems to be in accordance with a role for Klf4 as part of a negative feedback signaling circuit from the suprabasal to the basal cells.

The Hh signaling pathway interacts with the Bmp pathway during development and in the adult gastrointestinal tract. We examined different Bmps in the esophagus and found
that Bmp7 is expressed in the basal layer and induced in the mutant mice. Expression of
the active (phosphorylated) form of Bmp specific Smads 1, 5 and 8 showed that Bmp
signaling was to the suprabasal cells. Bmp7 signaling is therefore paracrine from the
basal layer to the suprabasal layer. In vitro experiments demonstrate that increased BMP
signaling can induce expression of involucrin, suggesting a role for BMP in differentiation.
Bmp7 signaling may therefore be involved in maintaining a balance between the size of
the precursor cell compartment and cellular differentiation in the normal esophagus.
Since both Klf4 and Bmp7 seem to be involved in paracrine signaling between the basal
and suprabasal layer we examined if Bmp7 may regulate Klf4. We found no evidence of
regulation of KLF4 by BMP7 at least in vitro in transformed human esophageal cells.

In summary, we show that in contrast to the rest of the gastrointestinal tract the
precursor cells of the esophageal squamous epithelium are direct targets of Hh signaling.
Our data provide genetic evidence that Hh signaling positively regulates precursor cell
fate in the esophageal epithelium of the adult mouse and impairs epithelial maturation
and migration. The fact that Hh signaling in the esophagus stimulates the development
of dysplastic features suggests Hh may function as an oncogene in the esophagus, which
could have important implications for the use of Hh inhibitors in the treatment of
esophageal cancer.
Reference List

1. van den Brink GR. Hedgehog signaling in development and homeostasis of the gastrointestinal tract. Physiol Rev 2007;87:1343-1375.


Supplementary Methods

Detection and quantification of CreERT2-mediated recombination at the Ptch\textsuperscript{flox} locus

Four tamoxifen-treated Ptch\textsuperscript{flox/flox}ERT2\textsuperscript{+/–} mice were killed at day 19 after the first injection and genomic DNA was isolated from the esophagus. Recombination efficiency was quantified on an ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA) by real-time PCR using forward primers hybridizing to intron 7 and intron 9, and a common reverse primer hybridizing immediately downstream of the second \textit{loxP} site in intron 9. A FAM-labeled probe was used for detection of the deleted Ptch\textsubscript{del} allele, whereas a Yakima Yellow–labeled probe detected the Ptch\textsubscript{flox} allele. A pelota gene-specific quantitative PCR assay was used for data normalization. Data were processed using the standard curve method for relative quantification. The deletion efficiency was calculated as the ratio of the values for the deleted allele to the total value from the Ptch\textsubscript{flox} allele plus the deleted allele, and is expressed in percentage.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5’-3’ orientation)</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPTCNx_f</td>
<td>TGG TAA TTC TGG GCT CCC GT</td>
<td>Genotyping of Ptch\textsubscript{flox} mice (for primer combinations see text)</td>
</tr>
<tr>
<td>mPTCdelNx_f</td>
<td>TTC ATT GAA CCT TGG GGA ACA TT</td>
<td></td>
</tr>
<tr>
<td>mPTCNx_r</td>
<td>CCG GTA GAA TTA GCT TGA AGT TCC T</td>
<td></td>
</tr>
<tr>
<td>mPTCNx_S2</td>
<td>YYE[1] ~ TTG GTT TGT AAT TTT ACT TTG ACG GTA CCT CGA ~ BHQ2a~Q[2]</td>
<td>Quantification of recombination at the genomic Ptch\textsubscript{flox} locus. Probes mPTCNx_S2 and mPTCdelNx_S1 recognize the Ptch\textsubscript{flox} and Ptch\textsubscript{del} alleles, respectively.</td>
</tr>
<tr>
<td>mPTCdelNx_S1</td>
<td>FAM[2] ~ CAC ACC AGA CCA GCT TGC AAA GAG ATC ~ BHQ1[3]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(in combination with primers mPTCdelNx_f / mPTCNx_r; see above)</td>
<td></td>
</tr>
<tr>
<td>Pelo-F1</td>
<td>CGG TCT GAG TGC TGG TAG GGA</td>
<td>Quantification of genomic Pelota DNA</td>
</tr>
<tr>
<td>Pelo-R</td>
<td>TCT GCA CCT TAG CGT GAA GCC</td>
<td></td>
</tr>
</tbody>
</table>


Immunohistochemistry

Slides were deparaffinized, dehydrated and immersed in 1.5% H\textsubscript{2}O\textsubscript{2} in Phosphate-Buffered Saline (PBS) for 30 minutes. Slides were cooked at 100°C for 10 minutes in 0.1 M sodium citrate (pH 6) and blocked with Teng-T (10 mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25% gelatin, 0.05% [vol/vol] Tween-20, pH 8.0) for 30 minutes, followed by incubation overnight at 4°C with the primary antibody in PBS with 0.1% Triton X-100 and 1% bovine serum albumin. For the BrdU pulse experiment mice were injected once with 100 mg/kg BrdU (Sigma) intraperitoneally 48 hours before sacrifice. Pretreatment for the BrdU staining was different. DNA was denaturated in 2N HCl for 30 minutes at 37°C.
Slides were prerinsed in 0.1M Na₂B₄O₇ (Borax) 3 times for 5 minutes and subsequently thoroughly rinsed in PBS. Enzymatic pretreatment was performed by incubating slides in 0.4% (w/v) pepsin in 0.01N HCl for 30 minutes at 37°C. For the involucrin staining a different method of antigen retrieval was performed. Slides were treated with trypsin at 1mg/ml for 5 minutes at 37°C. The Pcna antibody (1:5000) was from Santa Cruz (sc-56) the BrdU antibody (1:500) was from Dako Sigma (B2531), the Ki67 antibody (1:30) was from Dako (M7249), the p63 antibody (1:50) was from Santa Cruz (sc8431), the keratin 14 antibody (1:500) was from Abcam (ab49806-200), the involucrin antibody (1:25) was from Imgenex (80192) and the keratin 13 antibody (1:50) was from Abcam (ab90096), the phospho-Smad1,5,8 antibody (1:200) was from Cell Signaling (9511). For detection of the primary antibody an avidin-biotin detection system was used. All secondary antibodies were from Dako (1:200) (anti-rat: E0468, anti-goat: E0466, anti-rabbit: E0432, anti-Mouse: E0433), diluted in PBS with 10% human serum and incubated for one hour at room temperature (RT). Slides were then incubated for one hour with horseradish peroxidase-conjugated avidin-biotin complex (DAKO K0355). Peroxidase activity was detected with Sigma Fast 3,3-diaminobenzidine Tablets (Sigma, D4293). Sections were counterstained with Haematoxylin, dehydrated and mounted with Pertex (Histolab 00801).

**Immunofluorescent staining**

For immunofluorescent double staining slides were pretreated as for immunohistochemistry, see above. Antibodies were diluted in PBS with 0.1% Triton X-100 and 1% bovine serum albumin and slides were incubated o/n. The next day slides were washed in PBS and incubated for 2 hours in goat-anti-mouse 488 and goat-anti-rat 568 (Invitrogen, A-11001 and A-11077 respectively) diluted 1:1000 in PBS with 10% serum at RT. Slides were washed and mounted with Vectashield (Brunswick, H-1000) with DAPI (Roche, 10236276001).

**LacZ staining**

The esophagus was collected and prefixed in cold 1% PFH in PBS for 2 hours. The tissue was washed 3 times for 15 minutes in PBS and incubated in an X-gal containing solution (5 mM Potassium Ferricyanide Crystalline, 5mM Potassium Ferricyanide Trihydrate and 2 mM Magnesium Chloride in 50 ml PBS with 1.25 ml of the X-gal stock solution (20 mg/ml X-gal in dimethylformamide, Qiagen, 129931) for 3 hours at 37°C, then o/n at RT shaking in the dark. The tissue was washed in PBS and fixed o/n in 4% PFH.

**Generation of Dig-labeled probes.**

For mRNA in situ hybridization all probes were generated by subcloning mouse partial cDNA into a vector. One mg of linearized plasmid DNA was transcribed in vitro using either T3 (Roche 1103163001) or T7 (Roche 10881767001) RNA polymerase in the presence of digoxin-labeled uridine 5’-triphosphate. All cDNA fragments were sequenced to verify the identity. Partial cDNA of Shh (NM_009179, bp 440-1084) was subcloned
into the pBluescript II SK and linearized with HindIII (antisense) and XbaI (sense). Partial cDNA of Smo (NM_176996, 2234-2827 bp) was subcloned into the pBluescript vector and linearized with HindIII (antisense) and Not1 (sense). Partial cDNA of Gli1 (NM_010296, 1241-2513 bp) was subcloned into a pBluescript vector and linearized with Not1 (antisense) and HindIII (sense). Partial cDNA of Bmp7 (NM_007557, bp 15-657) was subcloned in a pGEM-7z vector and linearized with Xba1. Partial cDNA of Hhip (NM_020259, 5-526) was subcloned into the T-easy vector and linearized with Spel (antisense) and NCO1 (sense). In vitro transcription was performed following the protocol of the commercially available RNA DIG labeling mix (Roche 1277073). The RNA pellet was dissolved in 100 ml of Depc-treated water and 100 ml of formamide and stored at -80°C.

In situ hybridization

dH2O and all solutions used in the in situ protocol were treated with 300 ml/l diethylpyrocarbonate (DEPC, Brunschwig chemie, #18835) and autoclaved before use. Paraffin embedded tissue was sectioned (8 μm) and mounted on SuperFrost Plus microscope slides (Menzel-Gläser, J1800AMNZ). Tissue slides were deparaffinated, rehydrated and post-fixed in 4% paraformaldehyde in PBS on ice for 20 minutes. After rinsing the slides twice in PBS for 5 minutes, sections were overlayed with 500 ml of 40 μg/ml proteinase K (Roche 161519) in 50 mM Tris HCl (pH 8.0), 5 mM EDTA and incubated for 7.5 minutes. Slides were washed in PBS for 5 minutes and refixed in 4% paraformaldehyde in PBS on ice for 5 minutes. Slides were washed shortly in PBS and dH2O and placed in 0.1 M triethanolamine (Fluka, 90279) on a magnetic stirrer. While stirring 625 μl of acetic anhydride (Sigma A-6404) was added and left for 10 minutes. Slides were washed with PBS and saline for 5 minutes each. Slides were dehydrated and air dried. Probe stocks were diluted 100x in hybridization mixture (see above) and heated for 2 minutes at 80°C and placed on ice. The probe mix was brought to RT and spread evenly over the tissues and covered with a coverslip. As negative controls, sense probes were applied. Slides were incubated overnight in a hybridization oven at 55°C together with tissues soaked in 50% formamide and 5x SSC (43.8 g NaCl, 22 g sodium citrate in 1 liter H2O, pH 6.4) to prevent dehydration of the tissues. The next day slides were washed in 5x SSC at RT, followed by a high stringency wash in 50% formamide, 2x SSC at 55°C. Subsequently slides were washed in STE (0.5 M NaCl, 10 mM Tris HCl (pH 8), 5 mM EDTA) 1 time at RT and 3 times at 37°C for 10 minutes each. Slides were treated with 20 μg/ml RNase (Sigma R6513) in 50 ml STE at 37°C for 30 minutes, washed with STE at 37°C for 15 minutes, followed by another high stringency wash in 50% formamide, 2x SSC at 70°C for 30 minutes. Slides were washed with 2x SSC at RT for 15 minutes. After this step slides treated with the radioactive probes were dehydrated, air-dried and exposed to hyperfilm (Amersham Biosciences RPN6K) to determine the exposure time to LM-1 solution. For autoradiography slides were treated with hypercoat LM-1 emulsion (Amersham RPN40) according to manufacturer’s instructions. Slides treated with DIG-labeled probes were incubated in NT-buffer (0.15 M NaCl, 0.1 M Tris (pH 7.5)) for 10
minutes at RT, followed by washing 3 times 5 minutes in MBST+T (100 mM maleic Acid, 150 mM NaCl, 0.1% Tween-20 (pH 7.5) + 2 mM tetramisole (Sigma T-1512)). Tissues were blocked in MBST + 2% blocking reagent (Roche 1096176) and 10% heat inactivated sheep serum (Sigma S2263) for 1 hour. Meanwhile 3 mg embryo-powder was dissolved in 0.5 ml MBST with 2% BMB and rotated in an oven at 70ºC for 30 minutes and subsequently shaken at RT for 10 minutes. The solution was cooled on ice and 5 µl of heat inactivated sheep serum and 1 µl of anti-digoxin-AP Fab fragments (Roche 1093274) was added. After rotating at 4ºC for 1 hour the solution was spunned and supernatant was taken off and diluted with MBST, 2% blocking reagent and 1% HISS to make 3 ml of anti-digoxin-AP solution. This was added to the slides and slides were incubated overnight at 4ºC. On the third day slides were washed in MBST for 15 minutes 6 times, 3 times for 5 minutes in NTMT+T (2 ml 5 M NaCl, 5 ml 2 M Tris (pH 9.5), 5 ml 1 M MgCl2, 0.1 ml tween-20 + tetramisole) and enough BM Purple was added onto the slides to cover the tissue-section. Slides were incubated in BM purple AP substrate (Roche 1093274) in a humidified chamber in the dark until staining became visible. Staining was stopped with TE (pH 8.0), slides were fixed in 4% paraformaldehyde/0.2% glutaraldehyde in PBS for 1 hour at RT. After washing the slides in PBS twice for 5 minutes slides were mounted in glycer-gel mounting medium (Dako, C0563) under a coverslip.

**RNA isolation and complementary DNA synthesis**

For isolation of RNA from the esophagi an RNeasy kit from Qiagen (74104) was used. The tissue was homogenized in buffer RLT using a rotor-stator homogenizer. Homogenates were spunned at 14000 RPM and supernatant was used for RNA isolation, following the Qiagen-protocol. Obtained RNA was treated with DNAse-I (Promega M610A) in 10x DNAse buffer (Promega M198A) for 30 minutes at 37ºC. The reaction was stopped by adding Stop Solution (Promega M199A) and incubating the solution for 5 minutes at 65ºC. Complementary DNA was synthesized from a mixture of 100 ng/ml RNA, First strand buffer (Invitrogen y02321), dNTPs (Invitrogen 18427-013), random Hexamers (Promega #C1181), RNase inhibitor (Promega 23766810), Superscript II Reverse Transcriptase (Invitrogen 18064-014). This mixture was incubated at 25ºC for 7 minutes, 42ºC for 60 minutes, 95ºC for 3 minutes and cooled to 10ºC.

**Quantitative RT-PCR**

Quantitative RT-PCR was performed using a mix of 12.5 µl Quantifast Sybr Green (Qiagen, #204052), 5 µl H2O, 2.5 µl primermix and 5 µl diluted cDNA (1:4). Most primers were pre-optimized primers from Qiagen: Bmp2 (QT01054277), Bmp4 (QT00111174), Bmp5 (QT00132041), Bmp7 (QT00096026), Dhh (QT00170114), Gli1 (QT00173537), Hhip (QT00147518), Ihh (QT00096215), Klf4, (QT00104174) Ptc1 (QT00149135), p63 (QT00197904), Shh (QT00122479). Other primers used are listed in the table below. A Bio-Rad iCycler IQ5 was used for the quantitative reverse-transcription PCR assay
Supplementary Figure 1. Expression of Shh and Hh targets Gli1 and Hhip in the mouse embryo (E13.5) examined by in situ hybridization. During development Gli1 and Hhip are expressed by the mesenchyme. The dashed red line indicates the border between epithelium and mesenchyme in the esophagus. Original magnifications: 200x.