Homeostasis of the esophageal epithelium: A quest for the stem cell
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Citation for published version (APA):

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Chapter 3

Hedgehog signalling stimulates precursor cell accumulation and impairs epithelial maturation in the murine oesophagus


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Gut. 2013 Mar;62(3):348-57
ABSTRACT

Objective: In the intestine Hedgehog (Hh) signalling is directed from epithelium to mesenchyme and negatively regulates epithelial precursor cell fate. The role of Hh signalling in the oesophagus has not been studied in vivo. Here we examined the role of Hh signalling in epithelial homeostasis of oesophagus.

Design: We used transgenic mice in which the Hh receptor Patched1 (Ptch1) could be conditionally inactivated in a body wide manner and mice in which Gli1 could be induced specifically in the epithelium of the skin and oesophagus. Effects on epithelial homeostasis of the oesophagus were examined using immunohistochemistry, in situ hybridization, transmission electron microscopy, and real-time polymerase chain reaction. Hedgehog signalling was examined in patients with oesophageal squamous cell carcinoma (SSC) by qRT-PCR.

Results: Sonic Hedgehog signalled in an autocrine manner in the basal layer of the oesophagus. Activation of Hh signalling resulted in an expansion of the epithelial precursor cell compartment and failure of epithelial maturation and migration. Levels of Hh targets GLI1, HHIP and PTCH1 were increased in SSC compared to normal tissue from the same patients.

Conclusion: Here we find that Hedgehog signalling positively regulates the precursor cell compartment in the oesophageal epithelium in an autocrine manner. Since Hedgehog signalling targets precursor cells in the oesophageal epithelium and signalling is increased in squamous cell carcinomas, Hedgehog signalling may be involved in oesophageal squamous cell carcinoma formation.
What is already known about this subject?
• Hedgehog signalling plays a key role in epithelial homeostasis in the intestine
• In the intestine Hedgehog signalling is exclusively to the mesenchyme
• Signalling by Sonic Hedgehog plays a key role in the development of the oesophagus
• The oesophagus shows remarkable similarity to the skin and Hedgehog signalling plays an important role in the development of basal cell carcinoma in the skin

What are the new findings?
• Sonic Hedgehog is the only Hedgehog homologue produced in the adult oesophagus
• The epithelial precursor cells in the basal layer are the target of Sonic Hedgehog signalling
• Increased Hedgehog signalling in the mouse oesophagus leads to an expansion of the epithelial precursor cell compartment and failure of epithelial maturation and migration
• In conclusion, we describe a novel pathway that promotes basal cell fate in the oesophageal epithelium

How might it impact on clinical practice in the foreseeable future?
• Our data provide genetic evidence that Hh signalling positively regulates precursor cell fate in the oesophageal epithelium of the adult mouse and impairs epithelial maturation and migration. Our data suggest that the Hh pathway may act as an oncogenic pathway in oesophageal carcinogenesis and therefore be a novel target for therapy
INTRODUCTION

The oesophagus 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 oesophageal 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 oesophagus 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 oesophagus is much less well established.

Three Hh homologues are present in mammals, Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh). These Hhs bind to a common receptor Patched (Ptch) which subsequently loses its repressive activity towards the Hh signalling receptor Smoothened (Smo). Smo signals through the Gli family (Gli1-3) of transcription factors. Many of the transcriptional targets are components of the pathway such as Ptch1, Hedgehog interacting protein (Hhip) and Gli1. Hh signalling plays a critical role in the development of the normal oesophagus. Signalling is directed from endoderm to mesoderm during development. Sonic Hedgehog (Shh) is produced by the endoderm whereas Gli1-3, the transcription factors that mediate Hh signalling, are expressed in the mesoderm. Mice with mutations in either Shh or a combination of Gli2 and Gli3 display a growth defect of the foregut mesenchyme. This mesenchymal growth defect results in incomplete tracheo-oesophageal separation and the development of a single tube that connects both the airways and the stomach a pathology that is thought to resemble tracheo-oesophageal fistula in human patients. Conflicting data have been published on the possible expression of Hh pathway components in the adult oesophagus. Isohata et al. demonstrated expression of both SHH and multiple components of the Hh pathway in the basal layer using immunohistochemistry and RT-PCR and found evidence for a role for Hh signalling in oesophageal epithelial differentiation in vitro.

Autocrine Hh signalling in the basal layer of the oesophagus would be an important difference with Hh signalling in the columnar epithelium of the intestine and stomach where signalling is paracrine, from epithelium to the underlying mesenchyme. 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.\textsuperscript{6} However, using a different antibody Wang et al.\textsuperscript{9} found no expression of Shh in the normal oesophagus or LacZ expression in a \textit{Ptch1-LacZ} reporter mouse and concluded that there was no Hh signalling in the normal adult oesophagus.

Several studies have suggested that Hh signalling may play a role in oesophageal squamous cell carcinoma (SCC). In a study using immunohistochemistry, high GLI1 expression was detected in 50\% of patients with oesophageal SCC and correlated with poor prognosis and lymph node metastasis.\textsuperscript{10} Isohata et al. examined tissue from patients with oesophageal SCC by RT-PCR and found \textit{GLI1} or \textit{GLI2} to be expressed in 41 out of 42 patients but no quantitative comparison was made with normal oesophageal tissue.\textsuperscript{6} In a smaller study which included material from 15 patients it was suggested that Hedgehog pathway components were upregulated in human SCC compared to normal tissue but most data are not shown.\textsuperscript{11}

No functional experiments have been performed to address the potential role of Hh signalling in the adult oesophagus \textit{in vivo}. It is important to carefully evaluate a possible role for Hh signalling in the epithelium of the oesophagus. The oesophagus shows remarkable similarity to the skin and Hh signalling 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 an Hh antagonist.\textsuperscript{12} If Hh signalling plays a role in the precursor cells of the basal layer of the oesophagus, the Hh pathway may act as an oncogenic pathway in oesophageal carcinogenesis and be a target for therapy.

Here we use \textit{in situ} hybridization, an inducible reporter mouse and two distinct mouse models in which activation of the Hh pathway can be induced to examine the role of Hh signalling in the adult mouse oesophagus. Additionally we examine resection material from patients with oesophageal SCC by qRT-PCR.

**METHODS**

**Ethics approval**

**Mice.** All mouse experimental protocols were either approved by the Institutional Animal Care and Use Committee of the University of Göttingen or approved by the Institutional Animal Care and Use Committee of the Karolinska Institute.

**\textit{Ptch1} mutant mice.** We used previously generated \textit{Ptch1\textsuperscript{lox/lox}} and \textit{Rosa26CreERT2}\textsuperscript{14} mouse lines. The \textit{Ptch1\textsuperscript{lox/lox}} mice had a \textit{loxP} site inserted into intron 7 and intron 9 of the \textit{Ptch1} gene. The \textit{Rosa26CreERT2} mutant mouse strain expressed a fusion gene encoding Cre recombinase and a modified ligand-binding domain for the oestrogen receptor under control of the endogenous \textit{Rosa26} promoter. The two mouse lines
were crossed to obtain $Ptch_1^{floxflox CreERT2^{+/\text{--}}}$ mice. Eight-week-old mice were injected intra-peritoneally with 1 mg tamoxifen on 5 consecutive days to induce the $Ptch_1^{del}$ mutation to get $Ptch_1$ mutant mice (n=19). Both $Ptch_1^{floxflox CreERT2^{+/\text{--}}}$ mice treated with tamoxifen and vehicle treated $Ptch_1^{floxflox CreERT2^{+/\text{--}}}$ mice were used as controls (n=19). Three weeks after recombination mice were sacrificed and oesophagi 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 ($K5-tTA$ mice) were crossed with Tre-Gli1 mice. 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-CreERT2 were crossed with Rosa26-Stop$^{\beta\text{Gal}}$.LacZ 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 oesophageal tissue following standard procedures. See supplementary methods.

**Immunohistochemistry, immunofluorescence and Transmission Electron Microscopy.** Immunohistochemistry, immunofluorescence and Transmission Electron Microscopy 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. A detailed protocol of the procedure is available in the supplementary methods.

**Patient material**
Twenty patients with oesophageal squamous cell carcinoma (SCC) were selected from a tissue biobank of the Leiden University Medical Center (LUMC). Tissue stored in the biobank has been collected from excess material from patients operated for gastrointestinal cancer. The study on human material was performed according to the instructions and guidelines of the LUMC Medical Ethics Committee and in compliance with the Helsinki Declaration. All tumours were examined by the pathology department.
of the LUMC and were confirmed as oesophageal SCC. Both normal and tumour tissue from patients was used for experiments.

RNA isolation, complementary DNA synthesis and Quantitative RT-PCR
For isolation of RNA a piece of oesophageal tissue was collected from either control and mutant mice or resection material from patients with oesophageal squamous cell carcinoma. 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 or GAPDH in each sample. Gapdh or GAPDH was used as housekeeping gene and its expression was equally distributed between the samples. For a detailed protocol see supplementary methods.

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. Statistical analysis on human oesophageal SSC material was performed using a paired t-test. Differences were considered statistically significant at \( P<0.05 \).

RESULTS

Localization of Hh pathway components in the adult mouse oesophagus and effective recombination of Ptch1
In the developing mouse oesophagus Shh is expressed by the endoderm. It is crucial for the separation of the developing trachea from the developing oesophagus.\(^2\) Signalling is paracrine, from endoderm to mesoderm, as Hh targets Ptch and Gli1-3 are expressed in the mesenchyme.\(^2,4\) We examined expression levels of the three Hh homologues Shh, Ihh, and Dhh in the mouse oesophagus by quantitative RT-PCR. We previously found that Ihh is the main Hh homologue expressed in the intestine.\(^8\) In contrast, Shh is the only Hh expressed at detectable levels in the oesophagus. The colon was used as a negative control for Shh (Figure 1A, \( n=5 \) for both groups, \( P=0.0005 \)), and the colon and testis were used as a positive control for Ihh and Dhh respectively (Figure 1A, \( n=5 \) for the oesophagus, \( n=5 \) for colon and \( n=2 \) for testis, \( P=0.0002 \) and \( P=0.0096 \) for Ihh and Dhh respectively). To determine the expression of the different Hh homologues in human oesophagus we performed qRT-PCR on oesophageal samples and compared them to ileum and testis. SHH was found to be equally expressed in the human oesophagus and the ileum (Figure 1B, \( n=6 \) for oesophagus and \( n=3 \) for ileum, \( P=0.9 \)). The detectable
levels of SHH in human but not mouse intestine correlate well with the fact that we have previously been able to detect SHH in human\textsuperscript{20} but not mouse intestine\textsuperscript{8} by in situ hybridization.

Both IHH and DHH were found to be absent in the human oesophagus compared to their positive controls (Figure 1B, \(n=6\) for the oesophagus, \(n=3\) for ileum and \(n=2\) for testis, \(P=0.0001\) and \(P=<0.0001\) for IHH and DHH respectively). By in situ hybridization (ISH) we localized the mRNA for Shh to be present in the basal layer of the epithelium of the oesophagus (Figure 1C). To identify the Hh target cells we investigated the oesophageal expression pattern of Smo, Hhip and Gli1 in the adult mouse by ISH (Figure 1C). We found that Smo, Hhip and Gli1 were all expressed by the basal cells of the oesophageal epithelium. Expression of Gli1 by the epithelial cells of the oesophagus was further confirmed by crossing Gli1\textsuperscript{CreERT2} mice with Rosa26-LacZ reporter mice. In these mice Cre is expressed under the Gli1 promoter. Upon induction with tamoxifen Cre+ cells stain blue. Analysis of the oesophageal tissue demonstrated that Gli1 expression was confined to the basal epithelial cells (Figure 1D), though in a less extensive manner than the Gli1 ISH. Since Hh target expression is very different from what has been described during development of the oesophagus we used the same ISH probes and protocol to examine expression in the developing oesophagus. The results showed the expected\textsuperscript{4} endodermal expression of Shh and a mesodermal expression pattern for Gli1 and Hhip (Supplementary Figure 1), confirming the specificity of the probes and ISH protocol.

Successful Cre-mediated recombination of Ptch1\textsuperscript{flox/flox}CreERT2\textsuperscript{+/\textminus} mice was confirmed by real-time PCR on genomic DNA (Figure 2A). Recombination efficiency at the Ptch1\textsuperscript{flox} locus in the oesophagus 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 signalling as we have previously shown in the colon.\textsuperscript{8} 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, \(n\geq3\) for the mutant group, \(P=0.0012\), \(P=0.0025\), \(P=0.005\) for Gli1, Hhip and Ptch1 respectively). ISH for Gli1 on control mice and mutant mice (Figure 2C) demonstrated up-regulation of Gli1 in the basal layer of the mutant mice. The specific upregulation of Gli1 in the basal layer of Ptch1 mutant mice is further confirmation of the activity of the Hh pathway in these cells. These results show that tamoxifen induced deletion of exons 8 and 9 of Ptch1 was successful and resulted in increased Hh signalling 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.\textsuperscript{13}
Hedgehog signalling stimulates precursor cell accumulation

**Figure 1 | Localization of Hh pathway components in the adult oesophagus.** (A) Quantitative RT-PCR for the different Hh homologues Shh, Ihh and Dhh on mouse tissue and (B) human tissue. (C) In situ hybridization for Shh, Smo, Gli1 and Hhip on wild type mouse tissue. The dashed red line indicates the border between mesenchyme and epithelium. (D) X-gal staining of the oesophagus of Gli1-CreERT2 x Rosa26-Stopfl/fl-LacZ mice induced with tamoxifen. Arrows in the right panel indicate cells that express LacZ. Original Magnifications: 400x.
Loss of polarization and impaired epithelial maturation in *Ptch1* and *K5tTA-TreGli1* mutant mice

The normal wild-type oesophagus in mice is lined with stratified keratinizing squamous epithelium. H&E stainings of both the *K5tTA-TreGli1* mutant mice and the *Ptch1*<sup>flox/flox</sup>*CreERT2</sup><sup>+</sup> mutant mice showed several changes in the epithelium of the oesophagus. 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 mouse. They were positioned in an irregular pattern and their shape had changed. The basal cells had become oval-shaped and were palisading (Figure 3A, middle panel), a situation where the basal cells have a longitudinal orientation, a feature also often seen in basal cell
Hedgehog signalling stimulates precursor cell accumulation

Figure 3 | Loss of polarity and impaired maturation upon increased Hh signalling. (A) H&E stainings of \(\text{Ptch}^{1\text{floxed}}\text{CreERT2}^-\) mice (lower panel in A are magnifications of indicated area in upper row) and (B) \(\text{K5T}^{-}\text{TetGli1}\) mice, both with a control shown in the left panel. Closed arrows in the left panel indicate the keratohyalin granules, which are lost in the mutant mice. Open arrows in the middle panels indicate parakeratosis. (C,D) Electron microscopy of a \(\text{Ptch}^{1\text{floxed}}\text{CreERT2}^-\) control and mutant mouse. The left two panels show the basal layer (C), the right two panels show the superficial layers (D) of the oesophageal epithelium. Original magnifications (A): 400x upper row and 800x for lower row. (B): 400x.

carcinomas (BCC). In addition, there were places where the epithelium was protruding into the underlying mesenchyme, forming small buds (Figure 3A, right panel). The disorganized cell layers, palisading of basal cells and budding are all signs of loss of polarity. The keratin layer, which lines the oesophageal epithelium, was clearly visible in
the control mice, whereas in the mutant mice this layer became very thin (Figure 3A,B). The keratohyalin granules (Figure 3A,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 oesophageal epithelium was performed to examine changes in cell orientation and morphology at the ultra-structural level (Figure 3C,D). Electron microscopy clearly showed the palisading of the basal cells (Figure 3C closed arrow in second panel). In the superficial layer of the epithelium (Figure 3D) we observed more nucleated cells with high nuclear to cytoplasmic ratios. This indicates an incomplete differentiation. Both loss of polarization and impaired maturation are features found during development of dysplasia.

Increased epithelial proliferation and impaired migration in Ptc1 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, \( P=0.0025 \) 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 oesophagus. 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 in which migration towards the lumen is impaired.
Hedgehog signalling stimulates precursor cell accumulation

A

control

Ptch1 mutant

B

Pcna

control

mutant

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 mm 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).

Expansion of the precursor cell compartment and diminished epithelial differentiation in Ptch1 and K5tTA-TreGli1 mutant mice

To examine the apparent defect in epithelial maturation that was observed at histological examination of in the mutant mice in more detail, we performed immunohistochemistry for basal cell markers p63 and keratin 14. The p63 staining showed an increased number of positive cells in the mutant mice (Figure 5A,6A). Immunohistochemistry for keratin 14 confirmed this expansion of the precursor cell compartment of the basal layer in the mutant mice.
Figure 5 | Increased expression of basal cell markers and decreased expression of differentiation markers of the oesophageal epithelium in Ptch1 mutant mice. (A) Immunohistochemical staining for P63, a basal cell marker (left). (B) Immunohistochemical staining for keratin 14, another basal cell marker. (C-D) Immunohistochemical staining for the differentiation markers loricrin (C), keratin 13 (D). Original magnifications: 400x (A-D).
Figure 6 | Increased expression of basal cell markers and decreased expression of differentiation markers of the oesophageal epithelium in K5tTA-TreGli1 mutant mice. (A) Immunohistochemical staining for P63, a basal cell marker (left). (B) Immunohistochemical staining for keratin 14, another basal cell marker. (C,D) Immunohistochemical staining for the differentiation markers loricrin (C) and keratin 13 (D). Original magnifications: 400x (A-D).
Furthermore, cells retained keratin 14 expression as they moved out of the expanded basal layer upwards towards the lumen (Figure 5B,6B) indicating a defect in epithelial maturation. Loricrin and keratin 13 were used as markers of differentiation. These markers are expressed by the differentiated epithelium between the basal layer and the stratum corneum. By means of these markers we observed a clear thinning of the layer of differentiated cells (Figure 5C-D,6C-D) which showed evident signs of parakeratosis. Together these findings suggest that activation of Hh signalling in the oesophageal epithelium expands the precursor cell compartment and diminishes epithelial differentiation.

Upregulation of targets of the Hedgehog pathway in patients with squamous cell carcinoma

Previously it has been shown that several components of the Hedgehog pathway are expressed in oesophageal SCC.\textsuperscript{6,10,11} To compare quantitative levels of Hh signalling targets between normal tissue and SCC derived from the same patient we examined the respective resection material from twenty patients with oesophageal SCC. By qRT-PCR we determined relative expression levels of three different targets of the Hedgehog pathway $GLI1$ (Figure 7A $p=0.0238$), $HHIP$ (Figure 7B $p=0.027$) and $PTCH$ (Figure 7C $p=0.015$). All three are significantly upregulated in tumour tissue compared to normal tissue of the same patients. This suggests that Hh signalling is increased in SCC compared to normal oesophagus.

![Figure 7](image)

**Figure 7** | Hedgehog targets are increased in squamous cell carcinoma compared to normal tissue of the same patients. Quantitative RT-PCRs for Hh targets $Gli1$ (A), $Hhip$ (B) and $Ptch1$ (C) on resection material of patients with oesophageal SCC.
DISCUSSION

Here we show that Shh is the only Hh homologue present in the oesophagus and is expressed by the cells in the basal layer. Hh signalling is active in the epithelium where Hh receptor Smo and Hh signalling targets Gli1 and Hhip are expressed. Conditional body wide deletion of Ptc1 and inducible epithelial activation of Gli1 lead to a similar histological phenotype with features that are also seen in epithelial dysplasia in humans. Careful examination of the Ptc1 mutant and Gli1 induced mice shows expansion of the precursor cell compartment of the basal layer and impaired maturation and migration of the oesophageal cells.

Using immunohistochemistry different groups have obtained different results regarding the possible expression of Hhs in the oesophagus. Isohata et al. described SHH expression in the basal layer of the human oesophagus, in contrast Wang et al. failed to detect any SHH using a different antibody. In our hands SHH is the only detectable Hh in the human and mouse oesophagus and in situ hybridization shows Shh expression in the basal layer of the oesophageal epithelium in mice. Levels of Shh in the normal oesophagus seem to be low, especially compared to the embryonal oesophageal specimens we used as a positive control for the in situ hybridization. Isohata et al. also detected DHH in the differentiated epithelial cells by immunohistochemistry. However, there are no Hh signalling receptors or targets that are expressed in the superficial epithelium and we fail to detect appreciable levels of DHH expression in either human or mouse oesophagus by qRT-PCR. We therefore tentatively conclude that the staining observed with the anti-DHH antibody in the study by Isohata et al. may be the result of non specific staining. Thus, in contrast to the small intestine and colon where Ihh is the major Hh that is expressed, Shh is the major Hh expressed in the adult oesophagus.

Several independent lines of evidence from our experiments indicate that Hh signalling occurs primarily in the basal layer of the epithelium in the oesophagus and signals in an autocrine manner. First, ISH showed that Hh homologue Shh, Hh signalling receptor Smo, transcription factor Gli1 and transcriptional targets Gli1 and Hhip all localize to the basal layer. Second, the specificity of this staining was further established by the finding that Gli1 was specifically upregulated in the basal layer in the Ptc1 mutant mice. Finally a functional experiment in which Gli1 expressing cells can be conditionally marked upon an injection with tamoxifen in Gli1CreERT2-Rosa26-Stopfl/fl-LacZ reporter mice showed exclusive marking of cells in the basal layer of the epithelium. Multiple independent experiments by both Isohata and ourselves indicate that there is active Hedgehog signalling in the basal layer of the normal oesophagus albeit at low levels. We therefore conclude that the Ptc1-LacZ mouse that was used by Wang et al may not be able to detect low levels of Hedgehog signalling.
The mouse oesophageal 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 oesophagus and skin are marked by expression of keratin 5, keratin 14 and p63. Upon differentiating cells of both tissues lose the expression of basal cell markers and express loricrin 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.\textsuperscript{22,23}

Histological examination of the oesophagus of \textit{Ptch1} mutant mouse showed an 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 and \textit{Gli1} induced mice. The expression of basal cell marker keratin 14 was maintained in the differentiating cells suggesting that epithelial differentiation was incomplete in the both 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 and loricrin as markers of oesophageal epithelial differentiation. The accumulation and palisading of precursor cells and impaired terminal differentiation with marked parakeratosis are aspects of epithelial dysplasia. Even though these 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 oesophagus specific and the mice develop systemic signs of disease at six (\textit{Gli1} inducible) and three (\textit{Ptch1} mutant) weeks after induction.

In summary, we show that in contrast to the rest of the gastrointestinal tract the epithelial precursor cells of the squamous epithelium of the oesophagus are direct targets of Hh signalling produced in the epithelium. Our data provide genetic evidence that Hh signalling positively regulates the precursor cell compartment in the oesophageal epithelium in an autocrine manner. Furthermore we see an upregulation of Hedgehog targets in human oesophageal squamous cell carcinoma. Taken together Hedgehog signalling may be involved in oesophageal squamous cell carcinoma formation.

\textbf{Conflicts of interest.} No conflicts of interest exist for any of the authors

\textbf{Grant support.} The research leading to these results has received funding from the European Research Council under the European Community’s Seventh Framework Program (FP7/2007-2013)/European Research Council grant agreement number 241344 (GvdB) and a Vidi grant from the Netherlands Organisation for Scientific Research (NWO) (GvdB).
REFERENCES

1. van den Brink GR. Hedgehog signaling in development and homeostasis of the gastrointestinal tract. Physiol Rev. 2007;87(4):1343-75.


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 oesophagus. Original magnifications: 200x.