A close-up of colon cancer

Heijmans, Jarom

Citation for published version (APA):
Heijmans, J. (2013). A close-up of colon cancer

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Loss of Indian hedgehog activates multiple aspects of a wound healing response in the mouse intestine

Willemijn A. van Dop1,2,3, Jarom Heijmans1, Nikè V.J.A. Bülter1, Susanne A. Snoek1, Sanne L. Rosekrans1, Elisabeth A. Wassenberg2, Marius A. van den Bergh Weerman4, Beate Lanske5, Alan R. Clarke6, Douglas J Winton7, Mark Wijgerde8, G. Johan Offerhaus9, Daan W. Hommes1, James C. Hardwick1, Wouter J. de Jonge1, Izak Biemond1, Gijs R. van den Brink1

1 Department of Gastroenterology & Hepatology, Leiden University Medical Center, Leiden, The Netherlands
2 Center for Experimental Molecular Medicine, Academic Medical Center, Amsterdam, the Netherlands
3 Department of Gastroenterology & Hepatology, Academic Medical Center, Amsterdam, the Netherlands
4 Department of Pathology, Academic Medical Center, Amsterdam, The Netherlands
5 Department of Developmental Biology, Harvard School of Dental Medicine, Boston, USA
6 School of Biosciences, University of Cardiff, Cardiff, United Kingdom
7 Cancer Research UK Department of Oncology, Cambridge Institute for Medical Research, Addenbrooke’s Hospital, Hills Road, Cambridge, United Kingdom
8 Department of Reproduction and Development, Erasmus University Medical Center, Rotterdam, The Netherlands
9 Department of Pathology, University Medical Center Utrecht, Utrecht, the Netherlands

Gastroenterology 2010 Nov; 139(5):1665-1667
Abstract

Background and aims: Indian Hedgehog (Ihh) is expressed by the differentiated epithelial cells of the small intestine and signals to the mesenchyme where it induces unidentified factors that negatively regulate intestinal epithelial precursor cell fate. Recently genetic variants in the Hh pathway have been linked to the development of inflammatory bowel disease.

Methods: We deleted Ihh from the small intestinal epithelium in adult mice using Cyp1a1-Cre;Ihhfl/fl conditional Ihh mutant mice. Intestines were examined by immunohistochemistry, in situ hybridization and real-time polymerase chain reaction.

Results: Deletion of Ihh from the intestinal epithelium initially resulted in a proliferative response of the intestinal epithelium with lengthening and fissioning of crypts and increased Wnt signaling. The epithelial proliferative response was associated with loss of Bmp and Activin signaling from the epithelium of the villus and crypts respectively. At the same stage we observed a substantial influx of fibroblasts and macrophages into the villus core with increased mesenchymal Tgf-β signaling and deposition of extracellular matrix proteins. Prolonged loss of Ihh resulted in progressive leukocyte infiltration of the crypt area, blunting and loss of villi and the development of intestinal fibrosis.

Conclusions: Loss of Ihh initiates several events that are characteristic of an intestinal wound repair response. Prolonged loss resulted in progressive inflammation, mucosal damage and the development of intestinal fibrosis. Ihh is a signal derived from the superficial epithelial cells that may act as a critical indicator of epithelial integrity.

Keywords: Indian Hedgehog; intestine; regeneration; inflammation; wound healing response, fibrosis

Introduction

Differentiated cells in rapidly renewing tissues such as epithelia of the skin and the gastrointestinal tract are in a dynamic equilibrium with precursor cells in order to balance the rate of proliferation with cell loss at the epithelial surface. The balance between input and output in homeostatic dynamic equilibria depends on the presence of negative feedback loops. The fate and proliferation of intestinal precursor cells is regulated by Wnt signaling. Ihh is the major Hedgehog (Hh) expressed in the colon and it is secreted by the mature enterocytes at the top of the crypt. Treatment of rats with Hh inhibitor cyclopamine resulted in increased Wnt signaling and precursor cell proliferation whereas enterocyte differentiation was impaired1. In Ihh-/- mice we observed a failure of proliferating cells to differentiate and impaired crypt formation but these mice are not viable and died well before birth1. Inhibition of Hh signaling in the developing intestine by transgenic expression of Hh antagonist Hedgehog interacting protein (Hhip) similarly
resulted in accumulation of proliferating cells, some in ectopic foci on the small intestinal villi, and increased Wnt signaling\(^2\). Conversely, conditional activation of Hh signaling in the adult intestine by inducible deletion of Hh binding receptor Ptch1 (which acts by repressing the Hh signaling receptor Smoothened) resulted in inhibition of Wnt signaling and depletion of precursor cells which underwent premature differentiation into the enterocyte lineage\(^3\). Thus Hh signaling seems to act as a negative feedback signal that contributes to the dynamic equilibrium between epithelial precursor cells and enterocytes in the intestinal epithelium but several important questions remain. First, although a genetic loss of function experiment has been performed in the developing intestine\(^2\) a genetic loss of function experiment in the fully developed adult intestine still needs to be performed. Second, although we were unable to detect Shh in the mouse intestine by in situ hybridization\(^2\) it has been shown using a Shh\(^{Gfp}\) reporter mouse that low levels of Shh may be expressed by rare cells at the crypt base and thus the relative contribution of Shh and Ihh to Hh signaling in the adult intestine still needs to be addressed. Third, Hh signaling is exclusively from the epithelium to the mesenchyme\(^3,4\) and the mesenchymal factors that negatively regulate precursor cells in response to Hh signaling still await identification. Hh signaling regulates the expression of Bone Morphogenetic Proteins (Bmps) in the developing and adult intestine\(^1,3,5\) and increased Hh signaling in the adult extends the range of Bmp signaling through Smads 1, 5 and 8 from the top of the crypt towards the base of the crypt\(^3\). Since the Bmp pathway is not normally active at the base of the crypt it is unlikely that Bmps are the major negative regulators of Wnt signaling or precursor cell fate. Indeed, a transgenic mouse that overexpressed the Bmp antagonist noggin in the intestinal epithelium did not have a phenotype until three weeks after birth\(^6,7\) and these mice develop hamartomas, polyps that are characterized by abnormal growth of the mesenchyme rather than the epithelium. Possible Hh dependent expression of Transforming Growth Factor-\(\beta\) (Tgf\(\beta\)s) or Activins which signal through Smad 2 and 3 has not been examined.

The role of Hh signaling in the intestine may extend beyond negative regulation of epithelial precursor cells as a hypomorphic mutant of downstream transcription factor GLI1 has now been linked to the development of inflammatory bowel disease\(^8\). Here we examine the role of Ihh signaling in the adult small intestine using mice in which Ihh can be conditionally deleted from the epithelium of the small intestine.

**Materials and Methods (see supplementary methods for details)**

**Mice**

The generation of Cyp1a1-Cre\(^a\) and Ihh\(^{fl/fl}\) mice has previously been described. At four weeks of age, Cyp1a1Cre-Ihh\(^{fl/fl}\) mice received intraperitoneal injections with either 80 mg/kg \(\beta\)-naphthoflavone (Sigma) or vehicle (corn oil) for 5 days in a row. Mice were injected with 100 mg/kg BrdU (Sigma) intraperitoneally 1 hour before sacrifice and examined at 2 weeks, and 1,
2, 4 and 6 months after recombination. Vehicle injected Cyp1a1Cre-\(\text{Ihh}^{\text{fl/fl}}\) and \(\beta\)-naphthoflavone injected Cyp1a1Cre-\(\text{Ihh}^{\text{wt/wt}}\) mice served as controls. For examination of recombination efficiency by Cre upon injections with \(\beta\)-naphthoflavone Cyp1a1Cre mice were crossed with Rosa26Stop\(\text{LacZ}^{\text{fl/}}\) mice. The experiments were approved by the Institutional Animal Care and Use Committee of the University of Leiden and Amsterdam.

**Immunohistochemistry, LacZ staining and In Situ Hybridization**
Immunohistochemistry, LacZ staining, generation of probes and in situ hybridization were performed using standard protocols. See supplementary data.

**RNA isolation, complementary DNA synthesis and quantitative RT-PCR**
For isolation of RNA from the duodenum a small piece of proximal tissue was collected. A detailed description of RNA isolation and complementary DNA synthesis can be found in the supplementary methods. Quantitative RT-PCR was performed using Sybr Green (LightCycler 480 SYBR Green I Master, Roche, #04707516001) and pre-optimized primers from Qiagen. Gapdh was used as household gene. Gapdh expression was equally distributed between the wild-types and the \(\text{Ihh}\) mutant mice.

**Statistics**
Statistical analysis was performed with Prism 5.0 (GraphPad Software). All values were represented as the mean \(\pm\) standard error of the mean (SEM). Samples were analyzed using a student’s t-test. For multiple comparisons, a one-way ANOVA was utilized followed by a Tukey’s post-hoc test. Differences were considered statistically significant at \(P < 0.05\).

**Results**

**Loss of \(\text{Ihh}\) signaling in adult \(\beta\)-naphthoflavone injected Cyp1a1Cre-\(\text{Ihh}^{\text{fl/fl}}\) mice.**
We examined expression of \(\text{Ihh}\) mRNA (Figure 1A) and protein (Figure 1B) in the small intestine of the mouse. Both \(\text{Ihh}\) protein and mRNA were exclusively expressed by the differentiated epithelial cells on the villi. To examine the role of \(\text{Ihh}\) signaling in the adult small intestinal mucosa we injected adult Cyp1a1-\(\text{Ihh}^{\text{fl/}}\) mice and \(\text{Ihh}^{\text{fl/}}\) control mice with \(\beta\)-naphthoflavone. This resulted in substantially reduced expression of \(\text{Ihh}\) protein (Figure1B, right panel). Quantitative RT-PCR showed sustained loss of \(\text{Ihh}\) expression at different time points after recombination (Figure 1C, \(P<0.001\) for all groups, \(n \geq 4/\text{group}\)). Loss of \(\text{Ihh}\) expression correlated with almost complete loss of Hh targets Gli1 (86% reduced, \(P < 0.0001\)) and Hhip (94% reduced, \(P < 0.0001\)) but more modest reduction in Ptc1 (36% reduced, \(P = 0.03\)) and Ptc2 (65% reduced, \(P=0.03\)) expression two weeks after recombination (\(n = 8\) controls versus 6 \(\text{Ihh}\) mutants). Thus expression of Ptc1 and Ptc2 may only partially depend on Hh signaling. X-gal staining of the small intestines of
Loss of Indian hedgehog activates multiple aspects of a wound healing response in the mouse intestine (Figure 1E) as previously described.

**Figure 1.** Loss of Ihh signaling from the small intestine in β-naphthoflavone injected Cyp1a1-Cre-Ihh**fl/fl** adult mice. (A) In situ hybridization showed that Ihh mRNA was exclusively expressed by the epithelial cells on the villi. Expression was highest at the crypt villus junction (arrows in A) and diminished towards the villus tip. (B) Immunohistochemistry for Ihh showed expression of Ihh protein by the enterocytes on the villi in mice injected with solvent whereas β-naphthoflavone injected mice have lost Ihh expression at 2 weeks after treatment (B, right panel). Quantitative RT-PCR for Ihh at different time points (C) and Hh signaling targets Gli1, Hhip, Ptch1 and Ptch2 two weeks after recombination (D, black bars) on intestinal homogenates of β-naphthoflavone injected Ihh**fl/fl** control and Cyp1a1-Cre-Ihh**fl/fl** mice confirmed loss of Ihh expression. Original magnifications: 100x. (E) X-gal staining of the duodenum (dd) and ileum (il) of Cyp1A1-stop**fl/fl**LacZ mice injected with vehicle (control) and β-naphthoflavone (mutant).

**Loss of Ihh is sufficient to initiate an epithelial wound healing response.**

Intestinal epithelial wound healing is characterized by increased epithelial proliferation and lengthening of crypts which multiply by a process of budding and elongation termed crypt fissioning in order to replace lost crypts. We observed a strong increase in the rate of crypt fissioning in the Ihh mutant mice two weeks after recombination (Figure 2A,B, 3.3% in control...
Figure 2. Loss of Ihh is sufficient to initiate a regenerative response. 

(A, B) A strong increase in the rate of crypt fissioning was observed (arrows in A show fissioning crypts), which was maximal two weeks after recombination (B). (C) H&E staining of the duodenum demonstrated increased crypt density, deepening of crypts and lengthening of the villi in the Ihh mutant mice one month after injection with β-naphthoflavone. (D) Measurements of crypt density and crypt length confirmed an increase in the Ihh mutant mice one month and four months after recombination respectively. (E) Immunohistochemistry for BrdU at one month after recombination. (F) BrdU positive cells were counted and set out both as absolute number of positive cells per crypt and as labeling index, an indication for the percentage of cells per crypt that were positive for BrdU. Original magnifications: 100x (A left panel and C) and 200x (A right panel and E).
Figure 3. Increased Wnt signaling upon loss of Ihh.
(A) Immunohistochemistry for β-catenin. (B) An increase in the number of positive nuclei per crypt was observed in the Ihh mutant mice. In situ hybridization for Lgr5 (C) and Olfm4 (D) showed an increase in cells positive for these stem cell markers 1 month after recombination. (E) Incomplete formation of microvilli in the mutant mice one month after recombination. (F) Alkaline phosphatase was still present two months after recombination, but had disappeared from the remaining villi four months after recombination. Original Magnifications: 400x.
mice versus 13.7% in mutant mice $P<0.001$, n=5 per group). Two weeks later, when the crypts reached substantially increased density (1.7 and 3.0 crypts per 100 μm in the controls (n=8) versus $Ihh$ mutant mouse (n=7), $P<0.001$, Figure 2C), the rate of crypt fissioning returned to normal (3.3%, n=8, Figure 2B). Crypt fissioning returned again at 2 and 4 months in the context of chronic inflammation (see below). Crypts became progressively longer (from 66 μm in the controls to 154 μm in the $Ihh$ mutant mice at four months (n > 4/time point) $P<0.001$ for control vs 4 months) and a transient modest increase in villus length was observed (Supplementary Figure 1). Changes were measured in the duodenum but were similar in the rest of the small intestine (data not shown). $Ihh$ mutant mice showed an increase in BrdU positive cells (Figure 2E,F) with 6.9 BrdU positive cells per crypt in controls (n=5), 11.7 at two weeks (n=5, $P<0.01$) and 14.2 (n=7, $P<0.001$) at one month. Although the total number of BrdU positive cells per crypt increased, the relative number of positive cells per crypt cell (labeling index) remained stable. As we previously found an inhibitory role of Hh signaling on Wnt signaling1 3 we investigated the effect of loss of Ihh on Wnt signaling 1 months after recombination. The hallmark of activated Wnt signaling in intestinal epithelial cells is nuclear accumulation of β-catenin12 which was increased in mutant mice compared to controls (Figure 3A,B 1.9 positive nuclei in $Ihh$ mutants (n=6) versus 1.1 per crypt in controls (n=4), $P=0.01$). We examined the expression of targets of Wnt signaling, EphB2 and EphB3 and Cd4413 by immunohistochemistry and stem cell markers Lgr5 and Olfm4 by in situ hybridization. All were increased in the $Ihh$ mutant mice (Figure 3C,D and Supplementary Figure 2). We examined the four different epithelial cell lineages in the small intestine one month after recombination (Figure 3E and F and Supplementary Figure 3). We observed a modest increase in the numbers of Paneth cells at the base of the crypts (supplementary Figure 3A), which was stable in time. Electron microscopy showed incomplete microvillus formation on the enterocytes one month after recombination (Figure 3E), confirming our previous finding that Hh signaling is required for proper enterocyte differentiation1. A histochemical staining for alkaline phosphatase (AP) activity, a marker of differentiated epithelial cells, showed that the layer containing AP became slightly thinner over time and disappeared completely 4 months after recombination (Figure 3F).

**Figure 4.** Loss of Ihh signaling leads to reduced Bmp and Activin signaling in the epithelium of Ihh mutant mice and increases TGFβ signaling. (A) Immunohistochemistry for Bmp associated pSmad1,5,8 showed nuclear staining in the villi of control mice, whereas the crypts were negative. One month after recombination the staining was reduced in Ihh mutants. (B) Quantitative RT-PCR for Bmp2, Bmp4, Bmp7 and (C) targets Id1-4, demonstrated a significant reduction in the expression of Bmp4 which correlated with reduced Id1 and Id3. (D) Immunohistochemistry for TGFβ/Activin associated pSmad2,3 showed exclusive signaling by Smad 2 and 3 in the epithelial cells of the crypts. Upon loss of Hh signaling the staining in the crypts was lost. (E) Quantitative RT-PCR demonstrated a non-significant up-regulation of the TGFβs, mainly of TGFβ1, and a significant up-regulation of Pai-1, a target of TGFβ signaling. (F) Quantitative RT-PCR for the different Inhibins demonstrated that Inhibinα, which is needed to form Inhibins, was up-regulated, and that the different Inhibinβs (βa, ββ, βc and βe, which are subunits needed to form the Activins), were down-regulated. Original magnification: 100x (large panels in A and D) and 400x (enlargements in A and D).
Loss of Indian hedgehog activates multiple aspects of a wound healing response in the mouse intestine.
Loss of epithelial Smad signaling in Ihh mutant mice.

We examined Bmp signaling by immunohistochemistry using a phospho-specific antibody against the Bmp signaling specific Smads 1, 5 and 8 (pSmad1,5,8), by quantitative QT-PCR for Bmp2, 4 and 7 and of Bmp targets Id1-4. Nuclear pSmad1,5,8 staining was observed in the villi of control mice whereas the crypts were negative as described. Loss of Ihh expression lead to almost complete loss of pSmad1,5,8 (Figure 4A) which correlated with loss of Bmp4 expression (Figure 4B, P<0.0001, n=7/group) and reduced expression of Id1 and 3 (Figure 4C, P=0.04 and P=0.004 respectively, n=7/group). As pSmad1,5,8 signaling was restricted to differentiated cells, loss of Bmp signaling is no explanation for increased proliferation in the crypts of mutant mice. We therefore examined the localization of phosphorylated Smads 2 and 3 (pSmad2,3, Figure 4D) which mediate Tgfβ/Activin signaling. Intriguingly, we found that activity of Smad1,5,8 and Smad2,3 is mutually exclusive as the expression of pSmad2,3 was restricted to the epithelial cells in the crypt (Figure 4D). One month after recombination pSmad2,3 in the crypts was strongly reduced (Figure 4D). However, quantitative RT-PCR for Tgfβs and signaling target Plasminogen activator inhibitor-1 Pai-1 (Figure 4E) showed an increase in Tgfβ expression, although not significantly (P=0.17 for Tgfβ1, P=0.63 for Tgfβ2 and P=0.27 for Tgfβ3, n=7/group). Pai-1 expression was significantly increased (P=0.005, n=7/group). Immunohistochemistry for Pai-1 showed that up-regulation occurred mainly in the villus mesenchyme (Supplementary Figure 4) whereas no epithelial staining was observed. The lack of correlation between pSmad2,3 expression and Tgfβ signaling suggested that pSmad2,3 in the crypts may depend on Activin signaling. Activins are dimeric proteins and are composed of two Inhibinβ subunits, while Inhibins, antagonists of Activins, are composed of an Inhibinα and an Inhibinβ subunit. We examined the expression of different Inhibins and of Goosecoid, a conserved target of Activin signaling (Figure 4F). Two Goosecoid genes are present in mouse and man (Gsc and Gsc2). We found a non-significant up-regulation of the Inhibin specific Inhibinα (P=0.4) and a down-regulation of all Inhibinβs. (P=0.003 for Inhibinβa, P=0.022 for Inhibinβb, P=0.02 for Inhibinβc and P=0.31 for Inhibinβe, n=7/group). Expression of Inhibinβe was very low in both control and mutant mice. Expression of Activin target Gsc was reduced (P=0.01, n=7 for both groups), expression of Gsc2 was undetectable. Thus our data suggest that Ihh is required to maintain Bmp signaling in the differentiated epithelial cells on the villus and Activin signaling in the crypts.

Figure 5. Influx of macrophages and fibroblasts into the villus core.

(A) H&E stainings demonstrated increased cellularity of the villus core one month after recombination compared to the control group. An influx of macrophages (F4/80 positive cells, B), vimentin positive cells (C) and fibroblasts (S1004A positive cells, D) was observed at this time point. Counting of macrophages, vimentin positive cells and fibroblasts showed recruitment from the earliest time point examined (graph in B-D). (E) A double staining for vimentin (green) and Cd68 (red) showed no double staining one month after recombination. (F) A double staining for vimentin (red) and S1004A (green) demonstrated that part of the vimentin positive cells were also S1004A positive (white arrows in F). Original Magnifications 400x.
Loss of Indian hedgehog activates multiple aspects of a wound healing response in the mouse intestine
Loss of Ihh resulted in influx of macrophages and fibroblasts into the villus core. Since we observed an increased cellularity of the lamina propria of the villi after recombination (Figure 5A) we carefully examined the mesenchyme for inflammatory cells, markers of (myo) fibroblasts and smooth muscle cells. We observed a dramatic increase in the number of F4/80 positive macrophages in the villus core from 2 weeks after recombination which was stable in time (Figure 5B, 17 macrophages per villus in the control mice versus 39 in the mutant mice at two weeks, n=3 and n=4 respectively, P<0.01). Macrophages in the crypt area appeared at later stages (see below). In addition to the macrophages we observed a strong increase in the number of vimentin positive cells, mainly around the crypt villus junction at 2 weeks but throughout the crypt villus mesenchyme at 1 month (Figure 5C, from 10.7 positive cells in the controls (n=5) to 19.7 positive cells at two weeks (n=4, P<0.05) and 39 positive cells at 1 month (n=4, P<0.01) We examined whether these vimentin positive cells could be fibroblasts by staining for S100A4 (Fibroblast specific protein-1). The number of S100A4 positive cells was increased from the 2 week time point (Figure 5D, 0.8 S100A4 positive cells per villus in the control mice versus 10.2 in the mutant mice at two weeks, P<0.001). To further examine the nature of the vimentin positive cells we performed double stainings for vimentin and macrophage marker Cd68 and for vimentin and S100A4. The vimentin positive cells were negative for Cd68 (Figure 5E), but positive for fibroblast marker S100A4 (Figure 5F) indicating that most vimentin positive cells were fibroblasts. Thus loss of Ihh not only results in epithelial changes that are highly similar to a wound healing response but additionally to the recruitment of two of the principal cell types that play a role in wound healing, macrophages and fibroblasts.

Loss of smooth muscle cells in Ihh mutant mice
Analysis of the expression of α-smooth muscle actin (α-Sma) and desmin in the normal mouse showed mainly desmin single positive cells (smooth muscle cells) and desmin-α-Sma double positive cells (smooth muscle precursor cells) (Supplementary Figure 5). We observed only rare α-Sma single positive cells in the villus cores, as under normal circumstances myofibroblasts are mainly located in the peri-crypt areas[15]. Their number is increased during wound healing, in inflamed tissues and in tumor stroma[15, 16]. Ihh mutant mice showed a sequential and almost complete loss of the expression of α-Sma and desmin (Supplementary Figure 6A,B). First α-Sma expression was lost around the 1 month time point with only rare remaining cells in the villus core. Desmin expression was lost between 2 months and 4 months after recombination. We observed a substantial morphological change in the α-Sma positive and desmin positive cells before they lost expression of these markers. These cells normally have an elongated appearance but rolled up into rounded cells after loss of Ihh at the earliest time point examined (Supplementary Figure 6C,D). No apoptosis of mesenchymal cells was observed at any time point (2 weeks, 1, 2 and 4 months) after recombination using an active caspase-3 antibody and an appropriate positive control (data not shown) suggesting that smooth muscle cells are not lost by apoptosis.
Loss of Indian hedgehog activates multiple aspects of a wound healing response in the mouse intestine.

Prolonged loss of Ihh results in progressive leukocyte infiltration of the crypt area, villous atrophy and the development of intestinal fibrosis.

At 4 months after recombination Ihh mutant mice developed chronic enteritis (Figure 6A,B). The inflammation was characterized by a mixed infiltrate, consisting of macrophages neutrophils and T-cells and the presence of edema and blunting of small intestinal villi (Figure 7A). Macrophages increased from 1.3 F4/80 positive cells per inter crypt area in the control mice (n=3) to 16.3 positive cells four months after recombination (n=4, P<0.001). Neutrophils increased from 0
Ly6G positive cells per inter crypt area in control mice (n=5) to 8.8 positive cells four months after recombination (n=5, P<0.001). T-cells changed from 0 Cd3 positive cells per inter crypt area in the control mice (n=8) to 6.3 positive cells four months after recombination (n=4, P<0.001). Since profound changes in the composition and morphology of cells in the lamina propria preceded the development of inflammation, we examined the epithelial barrier function of the Ihh mutant mice at 1 month after recombination (before the first overt epithelial damage) using different techniques but found no defect in epithelial barrier function (Supplementary figure 7). Blunting of the villi was increasingly pronounced along the proximal-distal axis of the small intestine (Figure 6A,B) giving large parts of the small intestine the appearance of colonic mucosa. In contrast to the development of an inflammatory infiltrate in the crypt area (Figure 7A) we observed almost complete loss of Cd3 positive intraepithelial lymphocytes at four months and substantial loss of T cells from the villus core (Supplementary Figure 8). At 6 months after recombination the villous atrophy and inflammation was progressively worse. The structure of the few remaining villus cores was gone, and in most of the small intestine the villi were now completely gone. At some places erosions were observed (Figure 6E). The Ihh mutant animals had been growing less well than the control mice since the moment of recombination, and at 5 months after recombination they started losing weight, whereas the control mice kept gaining weight (Figure 6F). They weighed significantly less than the control group (P<0.001). Even though mutant mice were losing weight at this stage no blood loss or diarrhea was observed and their stools had a normal appearance.

Since the epithelial remodeling and influx of fibroblasts and macrophages are characteristic of a wound healing response we examined the mucosa for possible changes in the extra cellular matrix. A Sirius red stain for collagen showed progressive accumulation of collagen in the lamina propria from 2 weeks after recombination onwards ultimately resulting in an extensive accumulation in between the crypts (Figure 7B). The presence of intestinal fibrosis was further confirmed by immunohistochemistry for fibronectin which showed a similar progressive accumulation in the lamina propria (Figure 7C). Thus loss of Ihh results in the progressive development of intestinal fibrosis.

Discussion

We find that loss of Ihh results in an epithelial response that is characteristic of epithelial wound repair and is associated with loss of both Bmp and Activin signaling in the epithelial cells. In addition to the epithelial remodeling we observed substantial recruitment of macrophages and fibroblasts, two critical mesenchymal cell types involved in wound repair, into the villus core. Unresolved loss of Ihh ultimately results in loss of smooth muscle cells, the establishment of a pericryptal mixed inflammatory infiltrate, loss of villi, mucosal erosions and the development of extensive intestinal fibrosis.
Figure 7. Infiltration of inflammatory cells and development of intestinal fibrosis in the Ihh mutant mice. (A) Four months after recombination an inflammatory infiltrate appeared in the crypt area of the ihh mutant mice, consisting of macrophages (F4/80 positive), neutrophils (Ly6G positive) and T-cells (Cd3 positive). Progressive accumulation was observed of collagen (B) and fibronectin (C) in the lamina propria. Original magnifications 400x (A), 200x (B,C).
The most salient feature of a wound repair response in the Ihh mutant mice is the rapid increase in the rate of epithelial proliferation, crypt fissioning and lengthening of crypts. Although the increase in crypt length is progressive in time, the rate of crypt fissioning returns to control levels at 1 month after recombination when crypt density is markedly increased. This suggests that at this increased density an Ihh independent mechanism is activated that stops crypt fissioning. The epithelial changes observed in the Ihh mutant mouse are very similar to the adaptive response that is observed after massive small bowel resection\textsuperscript{17, 18}. It was previously found by others that expression of Ihh and Hh target genes are almost completely lost after massive small bowel resection in the mouse\textsuperscript{19}. Our data now show that this is in fact sufficient to initiate the adaptive response in the absence of loss of intestinal tissue or intestinal damage.

The epithelial remodeling events were associated with an almost complete loss of Smad signaling from the epithelium. Loss of phosphorylation of the Bmp specific Smads1,5,8 was associated with loss of Bmp4 expression which has previously been shown to be expressed in the small intestinal mesenchyme\textsuperscript{6, 7}. However, since pSmad1,5,8 is not normally observed in the crypt epithelial cells its loss cannot be directly responsible for the crypt budding and elongation phenotype in Ihh mutant mice. This suggests a role for one or more additional signaling pathways, acting directly on the epithelial cells of the crypt. We found that the key mediators of Tgf\beta and Activin signaling pSmad 2 and 3 were active in normal crypts. The activity of Smads1,5,8 and Smads2,3 is therefore mutually exclusive. Interestingly, it has previously been found that Smad1 can compete with Smads 2 and 3 for Smad4, the common mediator required for all Smad signaling to establish a bistable system (left versus right) in the establishment of left-right asymmetry in the early embryo\textsuperscript{20}. It will be intriguing to examine the role of the non-overlapping pattern of Smad activity along the crypt-villus axis. In the Ihh mutant mouse pSmad2,3 was lost from the crypt and this loss may be one of the factors that result in the increased crypt fissioning, Wnt signaling and epithelial proliferation. Since Tgf\beta signaling was up-regulated and Tgf\beta target Pai-1 localized exclusively to the villus mesenchyme, loss of pSmad2,3 from the crypt is unlikely to be related to changes in Tgf\beta signaling. Loss of pSmad2,3 was associated with a down-regulation of Inhibin\beta, which encode for the subunits that form the Activins. Thus our results suggest that Activins may be one of the mesenchymal signals that control crypt cell fate in response to Ihh derived from the differentiated cells.

The second important feature that is reminiscent of a wound healing response in the Ihh mutant mice is the substantial recruitment of both macrophages and fibroblasts to the lamina propria. Macrophages are a key component of the inflammatory phase of the wound healing response\textsuperscript{21}. A third feature of the Ihh mutant mouse that resembles wound healing is the deposition of extracellular matrix proteins in the lamina propria. We observed a progressive accumulation of both collagen and fibronectin from week 2 onwards. The fourth feature that is reminiscent of wound healing in the Ihh mutants is the apparent increase in Tgf-\beta signaling with appearance of Pai-1 positive cells in the mesenchyme.
Thus loss of *Ihh* results in several aspects of a wound healing response such as epithelial remodeling, recruitment of macrophages and fibroblasts and the deposition of extracellular matrix proteins. This suggests that the loss of *Ihh* protein expression that would be associated with loss of superficial intestinal epithelial cells is in itself sufficient to cause several key aspects of wound healing. In a normal wound the wound bed would soon be covered with fresh epithelial cells thus slowly restoring *Ihh* signaling to the underlying lamina propria cells. The later time points examined in the *Ihh* mutant mice may therefore represent an unresolved wound healing response. Indeed, prolonged loss of *Ihh* results in the progressive accumulation of inflammatory cells around the crypts, and the development of mucosal damage and extensive intestinal fibrosis. This is an intriguing finding in the light of the recent association between hypomorphic single nucleotide polymorphisms in the Hh transcription factor *GLI1* and the development of inflammatory bowel disease.

Since the loss of villi seems to occur concurrent with the complete loss of α-Sma and desmin positive cells it may be that the villi disintegrate due to the loss of these villus core cells that may act to anchor the villi into the intestinal mucosa. The villus atrophy shows an intriguing resemblance to the histology observed in patients with celiac disease. Even the ultrastructural changes of the microvilli on the enterocytes are typical for those observed in celiac disease. However, we find that there is loss of intraepithelial lymphocytes in the *Ihh* mutant mice rather than the accumulation that is one of the hallmarks of celiac disease. It is unlikely that loss of *Ihh* signaling plays a causal role in the development of celiac disease. It may be however that loss of epithelial *Ihh* expression results from the immunological response to gluten in patients with celiac disease and therefore does contribute to the development of villus atrophy.

Since the smooth muscle cells are at least numerically the most important Hh target cells in the intestinal mucosa loss of Hh signaling in these cells may result in the coordination of the multiple changes observed in the *Ihh* mutant mice. Experiments are currently underway in our laboratory to examine more precisely the molecular changes in the Hh target cells that seem to result in the activation of a wound healing-like mucosal response.

As we prepared a revised version of this manuscript a study with many similarities to our observations was published. In this study Hh antagonist Hhip was specifically expressed in the intestinal epithelium from around birth. Thus in this model Hhip is expressed during postnatal development of the intestine during which the intestine undergoes rapid growth and the crypt-villus system is established. The authors observe a similar proliferative response of the epithelium and loss of smooth muscle cells and a similar histology that is reminiscent of celiac disease at later stages. Our data show that the phenotype is not related to disruption of Hh during postnatal development of the intestine at which time the crypts are being formed and the villus core is still developing but is similar when Hh signaling is lost in a fully developed adult intestine.

In conclusion, we find that *Ihh* not only acts as a negative feedback regulator of the epithelial cells but that loss of expression is sufficient to cause several aspects of an intestinal wound healing response.
response. Unresolved loss of Ihh is ultimately detrimental to the intestine with the development of chronic enteritis, mucosal damage and intestinal fibrosis. Our data suggest that the Ihh signal derived from the superficial epithelium may act as a critical sensor of epithelial integrity in the intestine.

Acknowledgements

The research leading to these results has received funding from the European Research Council under the European Community’s Seventh Framework Programme (FP7/2007-2013)/ ERC Grant agreement n° [241344] and by a grant from the Dutch Digestive Foundation. Generation of floxed Ihh mice was funded by NIH AR050560 (BL).

Reference List


Supplementary figures

**Figure S1.** One month after recombination a mild transient increase in villus length was found (443 μm in the wild-type mice versus 525 μm in the mutant mice, P<0.05).
Figure S2. EphB2, EphB3 and Cd44, targets of Wnt signaling were all up regulated in the Ihh mutant mice one month after recombination. Original magnification: 400x.
Figure S3. (A) Immunohistochemistry for lysozyme, a Paneth cell marker, one month after recombination. A modest increase in lysozyme positive cells was observed at the base of the crypts of mutant mice one month after recombination. We found 3.2 positive cells per crypt (n=8) versus 4.1 positive cells per crypt in the Ihh mutant mice (n=5, P=0.015). (B) Immunohistochemistry for Alcian Blue, a Goblet cell marker, one month after recombination. There relative increase in Goblet cells was not significant (P=0.06, n=5 for both control and mutant group). (C) Immunohistochemistry for Chromogranine A, a marker for entero-endocrine cells, one month after recombination. No difference was found in relative amount of entero-endocrine cells between the control and the mutant mice (P=0.8, n=5 for both groups). Original magnification: 200x
Figure S4. Immunohistochemistry for Bmp signaling target Pai-1 showed increased levels of Pai-1 in the mesenchyme of mutant mice 1 month after recombination. Original magnification: 400x.

Figure S5. An immunofluorescent double staining for α-Sma and desmin in control mice. The double staining demonstrated that most cells in the villus core were either smooth muscle cells (desmin single positive cells, open arrows) or smooth muscle precursor cells (desmin-α-Sma double positive cells, closed arrows). The triple arrow indicates an α-Sma single positive cell. Original magnification: 400x.
Figure S6. Loss of smooth muscle cells from the villus core in Ihh mutant mice. α-Sma positive cells were reduced in number at one month after recombination and had disappeared at the two and four months time points (A). Desmin positive cells were present in the villi until two months after recombination and disappeared at four months after recombination (B). (C, D) α-Sma and desmin positive cells had an elongated appearance in the control mice. In the Ihh mutant mice, 1 month after recombination, the remaining α-Sma positive cells and the desmin positive cells had lost their elongated structure and seemed to roll up into a sphere like shape. Original magnifications: 200x (A, B) and 400x (C, D).
Loss of Indian hedgehog activates multiple aspects of a wound healing response in the mouse intestine.

Figure S7. No difference in barrier function between control mice and Ihh mutant mice one month after regeneration. (A) Ussing chamber experiment measuring the absolute amount of HRP passing through a piece of small intestine in 30, 60 and 90 minutes and (B) the average flux between the different time points. (C) Amount of HRP going from the luminal to the serosal side in an everted gut sac. (D) Levels of FITC-labeled dextran measured in the serum four hours after administration per gavage.

Figure S8. The number of Cd3 positive intraepithelial lymphocytes went down from 21.7 cells in control mice (n=8) to 3.5 cells in mutant mice four months after recombination (n=4, P<0.001) and the number of Cd3 positive cells in the villus core went down from 26.1 positive cells in control mice (n=8) to 9.3 positive cells in the mutant mice four months after recombination (n=4, P<0.001). Original magnifications: 400x (A) and 200x (B).