# Loss of Indian Hedgehog Activates Multiple Aspects of a Wound Healing Response in the Mouse Intestine

WILLEMIJN A. VAN DOP,[*,‡,§] JAROM HEIJMANS,[*] NIKÈ V. J. A. BÜLLER,[*] SUSANNE A. SNOEK,[‖] SANNE L. ROSEKRANS,[*] ELISABETH A. WASSENBERG,[‡] MARIUS A. VAN DEN BERGH WEERMAN,[¶] BEATE LANSKE,[#] ALAN R. CLARKE,[**] DOUGLAS J. WINTON,[‡‡] MARK WIJGERDE,[§§] G. JOHAN OFFERHAUS,[‖‖] DAAN W. HOMMES,[*] JAMES C. HARDWICK,[*] WOUTER J. DE JONGE,[‖] IZAK BIEMOND,[*] and GIJS R. VAN DEN BRINK[*]

*[*]Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, The Netherlands; [‡]Center for Experimental Molecular Medicine, [§]Department of Gastroenterology & Hepatology, [‖]Tytgat Institute for Liver and Intestinal Research, [¶]Department of Pathology, Academic Medical Center, Amsterdam, The Netherlands; [#]Department of Developmental Biology, Harvard School of Dental Medicine, Boston, Massachusetts; [**]School of Biosciences, University of Cardiff, Cardiff, United Kingdom; [‡‡]Cancer Research UK Department of Oncology, Cambridge Institute for Medical Research, Addenbrooke's Hospital, Cambridge, United Kingdom; [§§]Department of Reproduction and Development, Erasmus University Medical Center, Rotterdam, The Netherlands; and [‖‖]Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands*

**BACKGROUND & 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-CreIhh*$^{fl/fl}$ 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 bone morphogenetic protein 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 transforming growth factor-$\beta$ 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.

Differentiated cells in rapidly renewing tissues such as epithelia of the skin and the gastrointestinal tract are in a dynamic equilibrium with precursor cells 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. Indian Hedgehog (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 impaired.[1] In *Ihh*$^{-/-}$ mice we observed a failure of proliferating cells to differentiate and impaired crypt formation but these mice were not viable and died well before birth.[1] 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 Patch 1 (Ptch1; which acts by repressing the Hh signaling receptor Smoothened) resulted in inhibition of Wnt signaling and depletion of precursor cells that 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,

*Abbreviations used in this paper:* $\alpha$-Sma, $\alpha$-smooth muscle actin; Bmp, bone morphogenetic protein; BrdU, bromodeoxyuridine; Gli, glioma-associated oncogene; Hh, Hedgehog; Hhip, Hedgehog-interacting protein; HRP, horseradish peroxidase; Ihh, Indian hedgehog; Pai-1, plasminogen activator inhibitor-1; pSmad1,5,8, phosphorylated Smad 1, 5, and 8; pSmad2,3, pSmad2,3, phosphorylated Smad 2 and 3; Ptch1, Patched1; RT-PCR, reverse-transcription polymerase chain reaction; SSC, standard saline citrate; Tgf$\beta$, transforming growth factor-$\beta$.


0016-5085/$36.00
doi:10.1053/j.gastro.2010.07.045

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[3] it has been shown using a *Shh*$^{Green\ fluorescent\ protein}$ 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 Smads1, 5, and 8 from the top of the crypt toward the base of the crypt.[3] Because 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 3 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-β (Tgfβs) or Activins, which signal through Smad2 and 3, has not been examined.

The role of Hh signaling in the intestine may extend beyond negative regulation of epithelial precursor cells because 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 the Supplementary Materials and Methods section for details.

### *Mice*

The generation of *cytochrome P450-1a1Cre* (*Cyp1a1Cre*)[9] and *Ihh*$^{fl/fl}$[10] mice has been described previously. At 4 weeks of age, *Cyp1a1Cre-Ihh*$^{fl/fl}$ mice received intraperitoneal injections with either 80 mg/kg β-naphthoflavone (Sigma, St. Louis, MO) or vehicle (corn oil) for 5 days in a row. Mice were injected with 100 mg/kg bromodeoxyuridine (BrdU) (Sigma) intraperitoneally 1 hour before death and examined at 2 weeks, and at 1, 2, 4, and 6 months after recombination. Vehicle-injected *Cyp1a1Cre-Ihh*$^{fl/fl}$ and β-naphthoflavone–injected *Cyp1a1Cre-Ihh*$^{wt/wt}$ mice served as controls. For examination of recombination efficiency by Cre upon injections with β-naphthoflavone *Cyp1a1Cre* mice were crossed with *Rosa26Stop*$^{fl/fl}$*LacZ*[11] 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 the Supplementary Materials and Methods for more detail.

### *RNA Isolation, Complementary DNA Synthesis, and Quantitative Reverse-Transcription Polymerase Chain Reaction*

For isolation of RNA from the duodenum a small piece of proximal tissue was collected. A detailed description of RNA isolation and complementary DNA (cDNA) synthesis can be found in the Supplementary Materials and Methods section. Quantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed using Sybr Green (LightCycler 480 SYBR Green I Master, #04707516001; Roche, Basel, Switzerland) and pre-optimized primers from Qiagen (Hilden, Germany). *Glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) was used as household gene. *Gapdh* expression was distributed equally between the wild-type and the *Ihh* mutant mice.

### *Statistics*

Statistical analysis was performed with Prism 5.0 (GraphPad Software, La Jolla, CA). All values were represented as the mean ± standard error of the mean. Samples were analyzed using the Student *t* test. For multiple comparisons, a one-way analysis of variance was used followed by a Tukey post hoc test. Differences were considered statistically significant at a *P* value of less than .05.

## Results

### *Loss of Ihh Signaling in Adult β-Naphthoflavone–Injected Cyp1a1Cre-Ihh*$^{fl/fl}$ *Mice*

We examined expression of Ihh messenger RNA (mRNA) (Figure 1*A*) and protein (Figure 1*B*) in the small intestine of the mouse. Both Ihh protein and mRNA were expressed exclusively by the differentiated epithelial cells on the villi. To examine the role of Ihh signaling in the adult small intestinal mucosa we injected adult *Cyp1a1-Ihh*$^{fl/fl}$ mice and *Ihh*$^{fl/fl}$ control mice with β-naphthoflavone. This resulted in substantially reduced expression of Ihh protein (Figure1*B*, right panel). Quantitative RT-PCR showed sustained loss of *Ihh* expression at different time points after recombination (Figure 1*C*, $P < .001$ for all groups, $n \geq 4$/group). Loss of *Ihh* expression correlated with almost complete loss of Hh targets *Gli1* (86% re-