Molecular mechanisms in colon cancer

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Chapter IX

Indian Hedgehog is a novel regulator of colonic epithelial homeostasis in the adult rat and human

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Submitted
Abstract

**Background and aims:** In the colon, differentiated epithelial cells are continuously replaced from a pool of precursor cells at the base of the colonic crypts. We still have only limited insight into the lineage-instructive mechanisms that regulate differentiation of colonic epithelial precursor cell descendants. Here we investigate a possible role of Hedgehog signalling herein.

**Methods:** We examined expression of Indian Hedgehog (Ihh) in adult human and rodent colon and in HT-29 colon carcinoma cells. We treated rats with the hedgehog inhibitor Cyclopamine to examine the role of Hh signalling in the colon *in vivo*.

**Results:** We show that Ihh is expressed by the differentiated absorptive enterocytes of the adult colon. Ihh expression can be induced in HT-29 colon carcinoma cells upon differentiation with butyrate. Inhibition of Hh signalling *in vivo* identified several Hh regulated proteins and resulted in reduced expression of a marker of the absorptive enterocyte lineage and induction of the expression of a marker of the goblet cell lineage. Cyclopamine treatment increased precursor cell proliferation.

**Conclusions:** Ihh is a novel regulator of colonic epithelial homeostasis in the adult that differentially regulates cell lineage maturation and negatively regulates precursor cell proliferation.
Introduction

Continuous renewal of colonic epithelial cells in the adult occurs along a single vertical (or radial) axis. A common progenitor cell in the colonic crypt can give rise to a variety of epithelial cell types with digestive, absorptive, protective and endocrine functions.\(^{63}\)\(^{204}\)\(^{293}\)\(^{294}\) The epithelial cells differentiate as they move towards the intestinal lumen and undergo a death program thus maintaining homeostasis.\(^{205}\) The factors that regulate epithelial differentiation still remain to be identified.

Differentiation of the epithelial cells is a cell non-autonomous process that seems to be critically dependent on positional information along the vertical axis of renewal.\(^{295}\)\(^{296}\) Several tissue and cell lineage-specific transcription factors have been identified that regulate the expression of cell type specific markers of differentiation.\(^{204}\) However, the molecular mechanisms that time and direct the induction of these transcription factors at the appropriate position along the vertical axis remain largely unresolved. During embryogenesis cells receive the positional information that determines their developmental fate from their relation to gradients of secreted morphogens.\(^{68}\) We have hypothesized that morphogens play a similar role in the ongoing patterning events of GI epithelial homeostasis.\(^{275}\)

During development the GI tract is patterned through endodermal-mesenchymal interactions. In this interplay Sonic Hedgehog (Shh) and Indian Hedgehog (Ihh) are endodermally derived morphogens.\(^{293}\)\(^{297}\)\(^{298}\) Both have partially overlapping functions and act through the same receptor complex: Patched (Ptc), an Hh binding receptor and Smoothened (Smo) a receptor that signals through the Gli family of transcriptional effectors.\(^{299}\) Hh signalling plays an important role in the development of the hindgut and this role is conserved from fly to mice.\(^{67}\)\(^{245}\)\(^{300-303}\) We have previously shown that although a few cells at the base of some of the colonic crypts produce Shh mRNA, Shh protein is undetectable in the adult colon.\(^{304}\) However Ihh mRNA is produced in the colonic epithelium until at least one day prior to birth in mice\(^{67}\) and the adult colon has so far not been examined for expression of this Hh family member. Here we show (i) that Ihh is expressed by the absorptive enterocytes in the adult colon, (ii) provides a lineage-instructive signal to the differentiating epithelial cells and (iii) negatively regulates precursor cell proliferation. These results suggest that Ihh is an important factor in the maintenance of colonic epithelial homeostasis.
Materials and methods

Antibodies

Antibodies used are listed below, concentrations for immunohistochemistry are in normal font those used for immunoblot are italicized. An anti-BMP2 mouse monoclonal antibody (mAb) (355; 1:1000; 1:2000), and an anti-BMP4 mAb (757; 1:500; 1:2000) were from R&D systems (Minneapolis, MN). A goat polyclonal anti-Shh (N-19) that recognizes the Shh precursor protein \(^{275}\) (1:200; 1:2000), a goat polyclonal anti-Ihh that recognizes the 19 kDa active N-terminal peptide \(^{275}\) (1:19; 1:50; 1:500), a Rabbit polyclonal anti-GATA6 (H-29; 1:50; 1:500), a goat polyclonal anti-Villin (C-19; 1:1000) and a goat polyclonal anti-β-actin (1-19; 1:1000) were all from Santa Cruz (Santa Cruz, CA). An anti-HNF3β mAb (4C7; 1:10; 1:1000), and an anti-Engrailed-1 mAb, both developed by Dr J. M. Jessell's laboratory, were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). An anti-PCNA mAb (1:5000) and an anti-BrdU mAb (1:50) were from Roche (Almere, the Netherlands). An anti-cyclin D1 mAb (DCS6) was from Neomarkers (Fremont, CA). Two different anti-Ptc antibodies were used, a goat polyclonal anti-Ptc (C-20, 1:50) from Santa Cruz and A rabbit polyclonal anti-Ptc \(^{305}\) (1:200), a kind gift of Dr R. Tøftgard. A rabbit polyclonal anti-ITF was a kind gift of Dr D.K. Podolsky. Specificity of all antibodies used in immunohistochemistry was confirmed on immunoblot and in experiments using the appropriate control Ig or by omission of the primary antibody. Secondary antibodies used were all from Dako (Glostrup, Denmark).

Immunohistochemistry

Formalin fixed paraffin embedded human biopsy and resection specimens of uninflamed colonic mucosa, were obtained from the archives of the pathology department of the Academic Medical Center following institutional standards for human subject research. Immunohistochemistry was performed on 4μm sections using a three-step diaminobenzidine (DAB) detection method with antigen retrieval as described in detail previously.\(^{136}\) For BrdU visualization, sections were incubated in 2N HCl at 37°C for 60 minutes after deparaffinization and then washed in 0.1 M boric acid pH 8.5. Sections were counterstained with Mayer's hematoxylin, except when stained for HNF3β or engrailed-1 to allow optimal visualization of nuclear staining. Two different negative controls were used for the
immunohistochemical staining, omission of the primary antibody and use of an appropriate control Ig.

**In situ hybridisation**

Human Ihh cDNA, a gift of Dr C. Tabin, was used to transcribe a digoxigenin-labelled (Roche) mRNA probe according to Marigo et al. Following deparaffinization 4μm sections were treated with 10 mg/ml Proteinase K for 8 minutes and postfixed in 4% paraformaldehyde. Prior to application of the mRNA probe, sections were incubated in 0.1M triethanolamine/0.25% acetic anhydride and rinsed in 0.1M Tris-buffered glycine. Ihh probe hybridised at 70°C overnight. Post-hybridisation washes were carried out in (1) 50% formamide, 5X SSC, pH 4.5, 1% SDS, (2) 0.5M NaCl, 10mM TrisHCl, pH 7.5, 0.1% Tween-20, 10 mg/ml RnaseA, (3) 50% formamide, 2X SSC, pH 4.5, (4) TBST. Sections were blocked for 30 minutes RT in 5% sheep serum and incubated for 2 hours RT in antidigoxigenin (Roche) antibody solution. After additional washes in TBST, Ihh expression was detected using purple AP substrate (Roche, Mannheim, Germany). Tissue was mounted with Ultramount (DAKO).

**Cyclopamine treatment**

8-week-old female Wistar rats (n=7) were treated with daily intraperitoneal injections of 1 mg/kg of the Hh inhibitor Cyclopamine complexed with 2-hydroxypropyl-β-cyclodextrin (HBC; Sigma) as described. Control rats (n=7) received solvent only. After 14 days, rats were given a single intraperitoneal injection of 150 mg/kg BrdU one hour before being killed. The distal half of the colons was dissected along the longitudinal axis one half was fixed in paraformaldehyde and paraffin embedded the other half was homogenized to produce a protein lysate. The experiment was approved by the animal ethics review board of the University of Amsterdam.

**Scoring BrdU positive cells**

An investigator blind to the treatment performed BrdU scoring. To score BrdU positive cells in Cyclopamine treated rats and controls three pictures were taken of each rat colon at 100× magnification and positive nuclei were counted in each microscope field with
the use of an image analysis program (EFM Software, Rotterdam, The Netherlands). In each field, 5 well-oriented crypts were counted for the BrdU stain. The average numbers of positive nuclei per crypt were compared between groups. To enable comparison of the results between animals, all sections visualized the entire axis from the superficial epithelium to the muscularis mucosa.

**Immunoblot**

The distal colon was homogenized in lysis buffer (300 mmol/L NaCl, 30 mmol/L Tris, 2 mmol/L MgCl2, 2 mmol/L CaCl2, 1% Triton X-100, pH 7.4, supplemented with 1 tablet of protease inhibitor per 50 mL). Protein concentration was measured using the Bradford method. Lysates were diluted 1:3 in protein sample buffer (125 mmol/L Tris/HCl, pH 6.8; 4% sodium dodecyl sulfate; 2% β-mercaptoethanol; 20% glycerol, 1 mg bromophenol blue), and 100–200 μg of homogenate was loaded per lane on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. After protein separation, the proteins were blotted on to a PVDF membrane (Millipore, Bedford, MA). Membranes were blocked with 2% protifar (Nutricia, Zoetermeer, The Netherlands) in phosphate-buffered saline (PBS), supplemented with 0.1% Tween-20 for 1 hour at room temperature. After a brief wash in washing buffer (0.2% protifar; 0.1% Tween-20), membranes were incubated overnight at 4°C with antibody diluted in washing buffer at the indicated concentration. The next day, membranes were washed 3 times for 5 minutes each and subsequently incubated with a secondary horseradish peroxidase (HRP)–conjugated antibody in a 1:2000 dilution. After enhanced chemoluminescence using Lumilight+ substrate (Roche, Mannheim, Germany), antibody binding was visualized and relative expression levels quantified using a Lumi-Imager (Boehringer Mannheim, Mannheim, Germany).

**Cell culture**

HT 29 colon cancer cell lines were cultured according to routine procedures in the presence of 10% foetal calf serum (GIBCO). Butyrate (Sigma) was used at a 5mM concentration.
Results

Ihh is expressed in the adult colon

We first examined Ihh mRNA expression in the histologically normal human colon (Fig. 1a) and Ihh protein expression in humans, rats and mice (Fig. 1b and c). In all three species the terminally differentiated absorptive enterocytes expressed Ihh. No signal was detected when leaving out the primary antibody or when using control Ig. To see if Ihh correlated with the differentiation state in an in vitro model of enterocyte differentiation, we next examined Ihh expression in butyrate-treated HT29 cells.\textsuperscript{308} Induction of Ihh protein expression correlates well with expression of the differentiation marker Villin in this model (Fig. 1d).

Ihh regulates the expression of BMP-4 and transcription factors involved in tissue specific gene expression

To begin to understand the role of Hh signalling in the adult colon we focused on the vertebrate homologs of four Drosophila genes with an established role in hindgut formation. These are Dpp (vertebrate homologs BMP2 and BMP4), Fork Head (homolog HNF3β/FoxA2), Serpent (vertebrate GATA factors) and Engrailed (vertebrate Engrailed-1 and 2).\textsuperscript{301 302} We localized the expression of these proteins by immunohistochemistry and determined their relation to the Hh signal in vivo in the rat using the Hh inhibitor Cyclopamine. Cyclopamine is a potent Hh signalling inhibitor\textsuperscript{309} that inhibits Hh signalling at the level of Smo\textsuperscript{310}. Since only Ihh protein is detectable in the adult colon, we presume that the effects of Cyclopamine relate principally if not entirely to the inhibition of Ihh signalling.
Figure 1  Ihh is expressed by terminally differentiated enterocytes. (A) In situ hybridisation using an Ihh probe on normal human colon, detection with purple AP Substrate. The terminally differentiated enterocytes at the tips of the crypts (arrow) produce Ihh mRNA. (B,C) Immunohistochemistry on human (B) and rat (C) adult colon using the anti-Ihh antibody and DAB detection. Ihh protein is expressed by the terminally differentiated enterocytes in both species (arrows). (D) Western blot showing expression of Villin, Ihh and loading control β-Actin in HT-29 cells treated with 5 mM butyrate. Ihh expression is induced as the HT-29 cell differentiates. Original magnification: A-C: 80x.
Figure 2  Expression of Ptc, BMP2, BMP4, HNF3β and Engrailed-1. Immunohistochemistry on normal rat colon, using DAB as a substrate. Expression of (A) the Hh receptor Ptc is detected in the epithelial cells throughout the crypt and in several stromal cell types (arrows). (B) BMP-2 is expressed by the terminally differentiated enterocytes (arrow). (C,D) Myofibroblast-like cells (arrow, C) and some epithelial cells in the proximal colon with endocrine cell morphology (arrow, D) express BMP-4. (E) The transcription factor HNF3β is detected at highest levels in the nuclei of the epithelial cells at the base of the crypt and (E). Engrailed-1 is expressed by the epithelial cells and in lamina propria lymphocytes (F). Original magnification: A,B: 80x; C: 200x; D: 80x; E,F: 100x.

We used two different anti-patched antibodies with the same results. We found that the Hh binding receptor and transcriptional target Ptc was broadly expressed in the epithelial cells along the crypt axis and on mesenchymal cells (Fig. 2a). Thus Ihh may directly affect a wide range of target cells both within the epithelium and in the mesenchyme. *Hh* genes are often co-expressed with *BMP-2* and *BMP-4*. We found that the expression of both morphogens in the adult colon was similar to that found during embryonic and foetal development, BMP-2 is expressed by the differentiated enterocytes (Fig. 2b), whereas we detected BMP-4 in myofibroblast-like mesenchymal cells and in some epithelial cells in the proximal colon with endocrine cell morphology (Fig. 2c and d). Whereas no effect was found on the expression levels of BMP-2 upon Cyclopamine treatment, levels of BMP-4 were markedly induced in response to Hh inhibition. Since the colon homogenates were from the distal colon this result reflects an effect of Hh signalling on BMP-4 expressed by the mesenchymal cells and not the endocrine cells found in the proximal colon. This finding supports the known interaction between the Hh and BMP signalling pathways.

The transcription factor HNF3β is highly expressed in the epithelial cells at the base and middle of the crypt, but this expression is down regulated towards the Ihh expressing cells at the intercrypt tables (Fig. 2f). Engrailed expression was more generalized in the epithelial cells and also detected in lymphocytes in the lamina propria (Fig. 2e). Both HNF3β and engrailed-1 are dramatically upregulated in response to Cyclopamine treatment (Fig. 3). Of the GATA factors only GATA-6 has previously been found in colon cancer cells. In vivo,
we observed GATA-6 expression in the terminally differentiated enterocytes at the intercrypt tables (not shown). Upon Cyclopamine treatment GATA-6 was significantly downregulated (Fig. 3). Ihh signalling is not necessary for maintenance of its own expression (Fig. 3).

**Figure 3** The effect of Cyclopamine treatment on the expression of putative Hh targets. (A) Western blots showing protein levels of putative Hedgehog regulated proteins. The first seven lanes represent colonic homogenates of seven individual control animals whereas the seven lanes on the right are Cyclopamine treated animals. The molecular weight is indicated in kDa on the right of each blot. (B) Quantification of blots shown in (A), mean and standard error of the relative expression compared to the mean of the seven controls. *p* values (student’s t-test): Ihh, *p*=0.08; BMP-2, *p*=0.25; BMP-4, *p*=0.0001; HNF3β, *p*=0.001; En-1, *p*=0.002; GATA6, *p*=0.005.

**Cyclopamine treatment affects both differentiation and proliferation in vivo**

Since the Cyclopamine treatment altered the expression of several transcription factors involved in tissue specific gene expression, we examined the expression of two markers of the
main epithelial cell lineages of the colon, the enterocyte and the goblet cell. We observed a strong induction of Intestinal trefoil factor (ITF/TFF3), a goblet cell lineage marker (Fig. 4). In contrast, Cyclopamine treatment reduced the expression of Villin, a cytoskeletal protein that is specific for microvilli and is a marker of enterocyte differentiation. Inhibition of Hh signalling therefore seems to interfere with enterocyte differentiation in vivo, and promote the differentiation of the goblet cell lineage. Finally we used three markers of proliferation to assess the effect of Cyclopamine treatment on the precursor cell compartment. Cyclopamine treatment increased both the expression of the cyclin PCNA and cyclin D1 as assessed by western blot (Fig 4) and the number of 5-bromo-2'-deoxyuridine (BrdU) labelled epithelial precursor cells. These results show that Hh signalling may negatively regulate precursor cell proliferation in the adult colon.

![Western Blot](image)

**Figure 4** The effect of Cyclopamine treatment on proliferation and differentiation. (A) Western blots showing protein levels of markers of differentiation and proliferation. The first seven lanes represent colonic homogenates of seven individual control animals whereas the seven lanes on the right are Cyclopamine treated animals. The molecular weight is indicated in kDa on the right of each blot. (B) Quantification of blots shown in (A), mean and standard error of the relative expression compared to the mean of the seven controls. p values: Villin, p=0.02; ITF, p=0.008; Cyclin D1 p=0.01; PCNA, p<0.0001. (C) Graph showing the number of BrdU labelled cells per crypt in controls and Cyclopamine treated animals. Student’s t-test: p=0.036.
Chapter IX

Discussion

The mechanisms that determine position-dependent differentiation of colonic epithelial cells during their migration along the crypt axis remain among the least understood aspects of intestinal physiology. In the current study we show that Hh signalling stimulates differentiation of cells of the enterocyte lineage and negatively regulates precursor cell proliferation. We propose that a gradient of Ihh protein along the crypt axis may provide positional information for the differentiating epithelial cells.

Endodermal-mesenchymal interactions dictate the patterning of the developing gut tube and both Shh and Ihh have been shown to be critical endodermally derived factors in this cross-talk. Here we show that Ihh has a conserved expression pattern in the adult colon. Both Ihh mRNA and protein are expressed by the terminally differentiated enterocytes with highest expression in the most differentiated enterocytes and Ihh expression is induced in HT-29 cells during butyrate induced differentiation.

To examine the function of Ihh in the adult colon in vivo, we made use of the Hh signalling inhibitor Cyclopamine. Treatment of rats with Cyclopamine led to a substantially increased expression of HNF3β, preferentially expressed at the basal part of the crypt, and Engrailed-1. In contrast, GATA-6 expression by the differentiated enterocytes on the intercrypt table was decreased. Ihh therefore seems to differentially modulate the expression of transcription factors with a role in tissue specific gene expression.

Blocking Hh signalling increased PCNA and cyclin D1 expression and increased the number of epithelial precursor cells in S-phase. We previously noted a similar effect in the adult stomach. This contrasts with the proliferative effect of Shh observed on both keratinocyte and neural precursor cells and the role of activating mutations in the Hh pathway in tumours derived from these cell types. The role as a negative regulator of GI epithelial precursor cell proliferation has important implications since it suggests that Hh signalling may actually have a tumour suppressive action in the adult gut. The role of the Hh pathway in tumourigenesis may thus depend on the tissue context.

The Cyclopamine treatment had a differential effect on differentiation of the two main epithelial cell-lineages of the colon. Expression of the enterocyte differentiation marker villin was decreased whereas the expression of the goblet cell marker ITF was increased, suggesting that Ihh is a positive regulator of enterocyte differentiation and negative regulator of the Goblet cell lineage. Our results therefore suggest that Hh signalling provides lineage-specific instructive signals in the adult colon. Interestingly, an opposite effect has been described in
rats treated with recombinant Fgf-7 (keratinocyte growth factor),\textsuperscript{320} suggesting that both signals may have opposing effects on the regulation of cell fate in the colon.

Taken together, our data suggest that Ihh exerts an inhibitory influence on proliferation and differentiation of cells at the base of the crypt whereas it stimulates differentiation of cell on the intercrypt tables. The effects of Ihh therefore seem to be compartmental. In conclusion, we provide evidence that Ihh provides a cell-lineage specific instructive signal that is important for adult colonic enterocyte differentiation.

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