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Hh pathway expression in human gut tissues and in inflammatory gut diseases

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Sonic hedgehog (Shh) directs early gut patterning via epithelial–mesenchymal signaling and remains expressed in endoderm-derived tissues into the adult period. In human adult gut epithelium *SHH*/SHH expression is strongest in basal layers, which suggests that SHH may function in the maintenance of gut epithelial stem or progenitor cells. Recent publications suggest a role for aberrant *SHH*/SHH expression in gut epithelial neoplasias. We hypothesized that the regenerating gut epithelium in inflammatory gut disorders would show an upregulation of *SHH*/SHH signaling and this abnormal signal may explain the increased incidence of neoplasia in these diseases. Archived healthy gut and inflammatory gut diseased tissues were analyzed by RNA *in situ* hybridization and immunohistochemistry to describe location and levels of SHH signaling. We show that *SHH*/SHH and its receptor *PTCH1*/PTCH1 expression is restricted to the glandular epithelium of the gut, in an antiluminal pattern (strongest in basal layers and weak to absent in luminal epithelium). Inflammatory diseases of the gut show dramatic increases in epithelial SHH signaling. Expression increases in inflamed glandular epithelium (including metaplastic glandular epithelium), losing its radial (crypt-villous) polarity, and expression appears upregulated and present in all epithelial cells. We also describe strong *SHH*/SHH and *PTCH1*/PTCH1 expression in intraepithelial and mucosal inflammatory cells. We suggest that SHH signaling in inflammatory diseases of the gut acts to ensure stem cell restitution of damaged mucosal epithelium. However, such signaling may also present a risk for neoplastic transformation.
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Hedgehog (Hh) signaling is a fundamental pathway directing patterning events during embryogenesis. The Hh signaling pathway plays a key role in important epithelial–mesenchymal interactions in many different anatomic sites including the gastrointestinal system (gut).[1–12] During gut development, Hh proteins are secreted by the epithelial cells throughout the gut and are believed to function in the underlying mesenchyme to activate molecules important in mesodermal differentiation.[1,2,5,13–15] In the developing vertebrate gut, two Hh proteins are expressed: Sonic Hh (Shh) and Indian Hh (Ihh), with Shh expressed in earlier developmental stages.[2,7] Expression of the receptors/effectors for Hh signaling, Patched (Ptc), Smoothened (Smo), and Gli denotes the location of action of the Hh signal.[16,17] These receptors and targets are expressed in the mesenchyme subjacent to the Hh expressing endoderm during early development.[15]

The Hh receptor is a complex of two proteins: Ptc and Smo. In the absence of Hh protein binding, Ptc (a membrane bound protein) inhibits Smo-directed activation of downstream targets. Hh binding to Ptc releases the antagonism of Smo and allows transcriptional activation of target genes (eg *Gli*, *Wnt*, and *Bmp*, see Cohen[18] for a review).

Shh as a signal to the mesoderm is evidenced by overexpression studies in which over/misexpression of *Shh* results in a mass-like hypertrophy of the gut mesoderm.[19] Shh signals must have an epithelial function as well, whether direct or indirect, as ectopic expression of *Shh* affects both the mesoderm and epithelium in the developing gut. For example, in the developing pancreas, *Shh*/Shh expression is absent in the endoderm that forms the pancreatic buds.[20] Ectopic expression of *Shh* in this

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endodermal region results in abnormal intestinal-like mesenchymal differentiation of the pancreas[13] and a preneoplastic intestinal alteration in the duct epithelium.[21]

Recently, there has been evidence that the Hh pathway functions in human adult gut epithelium. It has been shown that SHH protein expression correlates with the differentiation of gastric glands in *adult* human and rodent stomachs.[22] SHH protein expression is also detected in gastric metaplasias in the human adult gastrointestinal tract.[22] It has recently been reported that epithelial malignancies of the adult gut are associated with abnormal *SHH*/SHH expression.[21,23] These studies have suggested that SHH may function as a mediator of gut stem cell maintenance. An unregulated progenitor cell proliferation induced via abnormal SHH protein expression or pathway activation has been suggested to play a role in all cancers of the gastrointestinal tract.[21,23,24] The pathway activation has been described in a variety of epithelial carcinomas from other sites including lung and skin.[17,25–29] It is thought as well that Hh signaling ensures that a progenitor or stem cell population is retained in these tissues.[29–34]

Hh signaling may be important in other human diseases involving chronic tissue injury. Hh signaling has been shown to be upregulated in chronic pulmonary fibrosis, a non-neoplastic pulmonary disease.[35] Chronic pulmonary fibrosis is a fatal interstitial lung disease of multiple etiologies sharing destructive pulmonary fibrosis with associated interstitial inflammation.[36,37] The Hh signal upregulated in this disease was recently shown to be derived from the infiltrating lymphocytes but was also detected in the pulmonary epithelium in injured lung regions and in circulating T lymphocytes.[35] This is interesting as Hh signaling is active in the development of human lymphocytes.[38–42] It has been suggested that signaling between the CD4 expressing lymphocytes and the injured epithelium may be associated with either the disease process or the tissue-mediated recovery.[35]

These findings suggested to us that the Hh signaling pathway may be upregulated in other tissues disturbed by chronic inflammation including those of the gastrointestinal tract, and that they may play a role in epithelial regeneration. Chronically elevated Hh signaling may increase the risk for malignancy known to be associated with many of these disorders. We elected to examine the role of Hh signaling in chronic inflammatory disorders of the gastrointestinal tract, including foregut inflammatory conditions such as Barrett's esophagus and chronic gastritis, and hindgut inflammatory bowel diseases such as ulcerative colitis and Crohn's disease by analyzing the mRNA and protein expression patterns of SHH and PTCH1 (human Ptc) and comparing them with expression in normal healthy adult and fetal gut tissues.

Materials and methods

In Situ Hybridization and Immunohistochemistry

Cases and controls were pulled by diagnosis retrieval using SNOMED search criteria from the Department of Pathology archives at Massachusetts General Hospital and personal collections of GY, both with IRB approval. Only samples from 2000–2003 were used for this study. The diagnostic H&E slides were reviewed for confirmation of the diagnosis by GYL and DJR. The best representative slides from diseased and nondiseased tissue were chosen for each individual case. Nondiseased tissue sections were used as controls. In the case of ulcerative colitis, proximal small intestinal resection margins were available as normal control tissue. At least three cases and three controls were studied for each diagnosis and anatomic region. No other patient information was obtained (confidentiality was ensured). Paraffin-embedded tissue was gathered and sectioned at 3–6 μm onto coated slides (Fisher) and kept at 4°C until used. All slides were used within 3 months of sectioning.[43] *In situ* hybridization was performed in accordance with a published protocol for paraffin sections.[44] Probes for human *SHH* and *PTCH1* were obtained from C Tabin and have been published previously.[45,46] The signal was detected with BM Purple AP substrate following the manufacturer's recommendations (Roche). Immunohistochemistry studies followed standard protocol with minor changes. Antigen unmasking was achieved by boiling sections in 0.01 M sodium citrate, pH 6.0, for 10 min. Tissue was blocked in 10% sheep serum, and additional blocking of endogenous avidin/biotin binding sites was performed with an avidin/biotin blocking kit (Vector Labs). Antibodies were applied at the following dilutions: CD4 (Novocastra, 1:20), CD8 (DAKO, 1:100), CD20 (Neomarkers, 1:50), CD34 (Neomarkers, 1:50), PTCH1 (Santa Cruz, 1:50), SHH (Santa Cruz, 1:50). Protein was visualized with DAB chromagen (Sigma).

Results

All detected SHH and PTCH1 mRNA and protein expression patterns were cytoplasmic as has been described by others.[21,22,24]

SHH Expression is Present in Normal Adult Human Gut Epithelium

We have previously studied the expression of SHH (mRNA)/SHH (protein) in the normal adult human gut, where we described that SHH/SHH was not detected in the normal adult esophageal squamous epithelium or in the [corpus] or pyloric regions of the stomach. Herein, in resected surgical specimens we studied the expression of SHH/SHH in normal, noninflamed tissues from the same patients as the

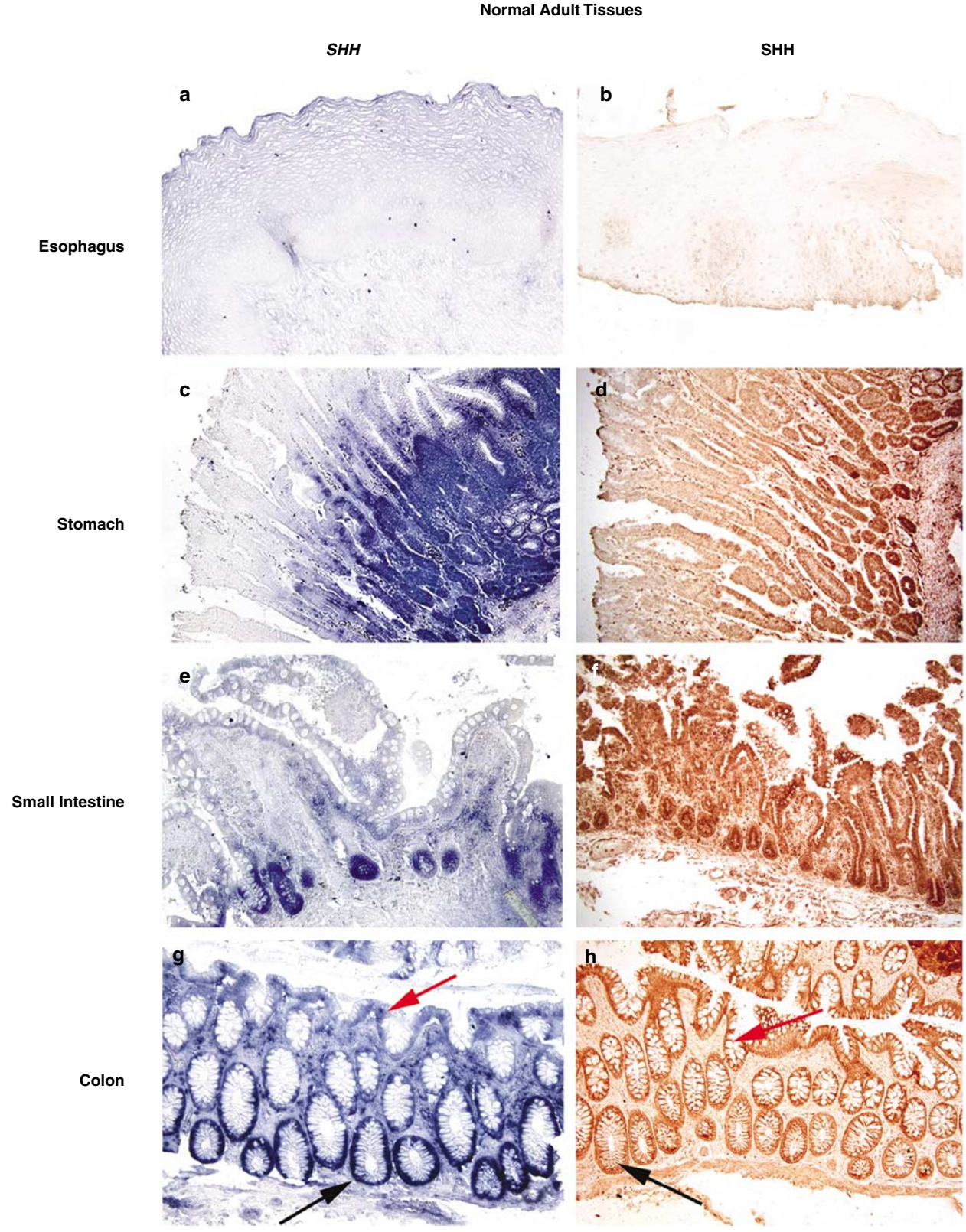


Figure 1 SHH expression in normal adult human gut. mRNA (**a**, **c**, **e**, **g**) and protein (**b**, **d**, **f**, **h**) expression of SHH in normal adult human gut tissues. Expression is not detected in the normal (**a**, **b**) esophageal squamous epithelium. The normal stomach expresses SHH in the glands (**c**, **d**). Normal small intestine (duodenum, jejunum, and ileum) expresses *SHH* strongly at the base of the villi (arrow in **e**) without detectable *SHH* expression in the villous tips (arrowhead in **e**). SHH is present in both villous base and villous tip epithelial sites (**f**) although stronger in the base of the villi. Healthy adult colon expresses *SHH* and SHH in the crypts (black arrows in **h** and **g**) with weak focal protein expression in the luminal epithelium (**h** and **g**, red arrows).

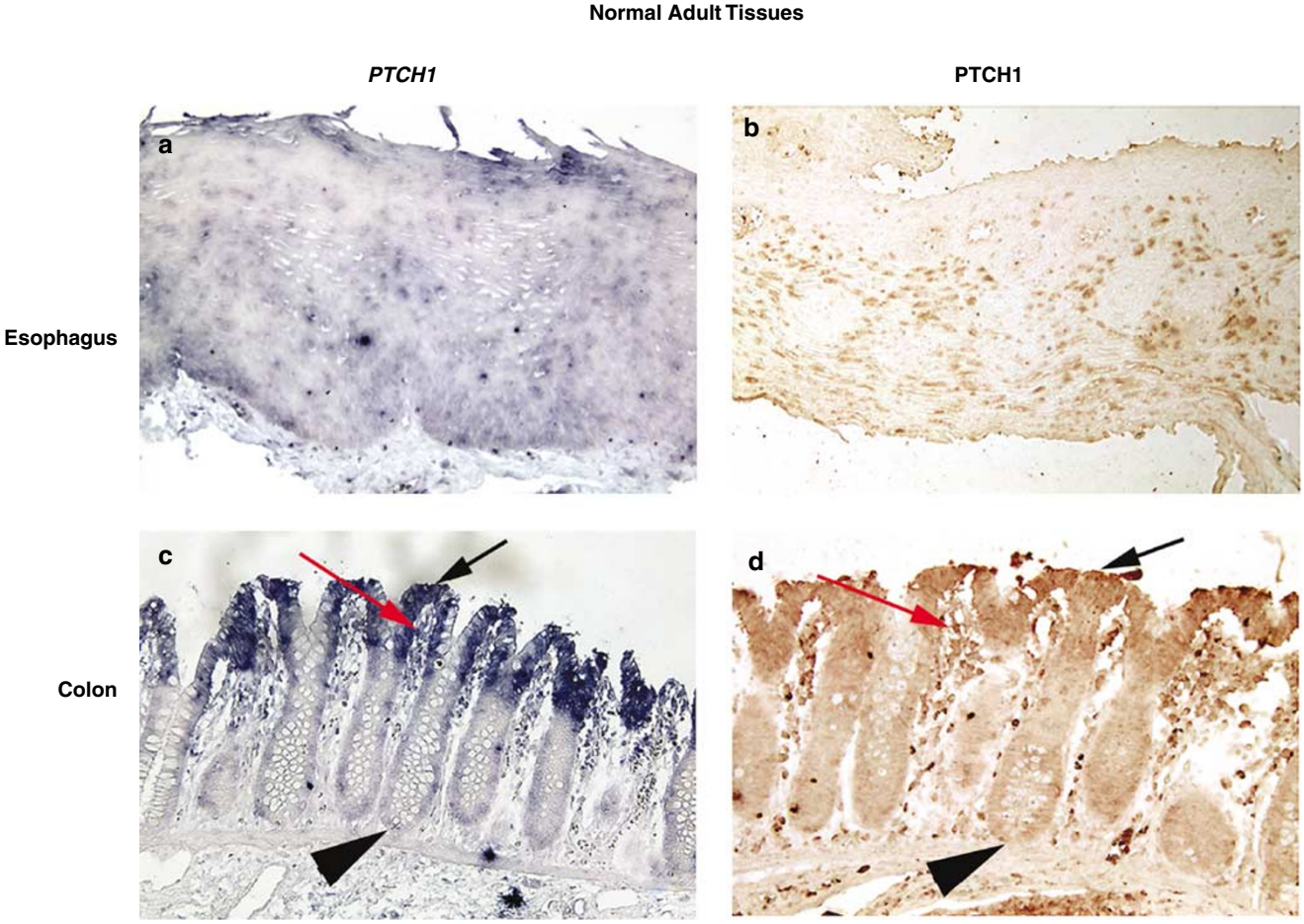


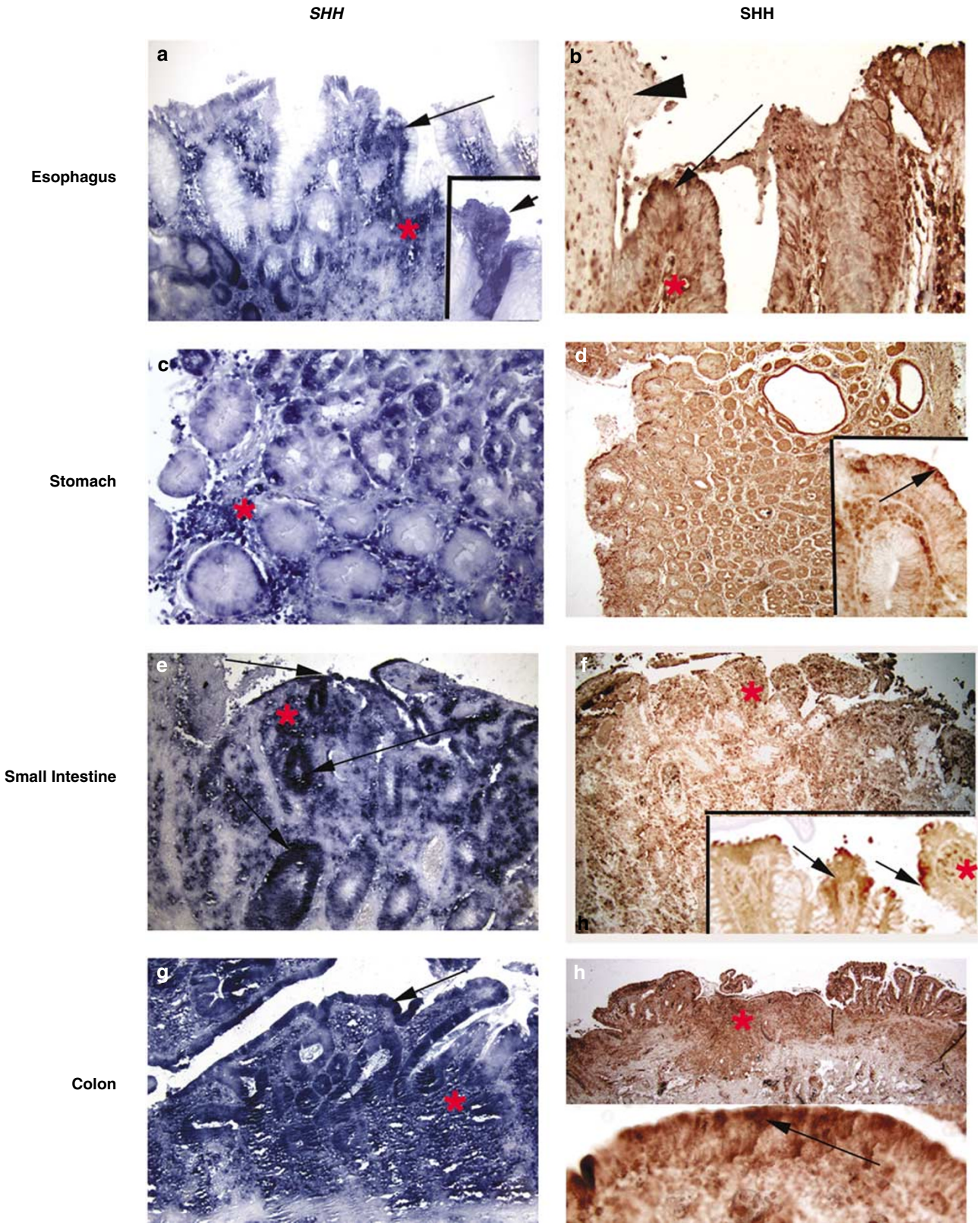
Figure 2 PTCH1 is expressed in normal adult gut. The expression of *PTCH1*/PTCH1 (mRNA, **a** and **c**; protein, **b** and **d**) is similar to *SHH*/SHH in normal adult gut and in inflammatory gut in all regions except for the colon. Only background expression is detected in the normal (**a**, **b**) esophageal squamous epithelium. The normal stomach expresses PTCH1 in the glands as seen in SHH (data not shown). We did not detect *PTCH1*/PTCH1 in the lamina propria of any region of the human stomach. Normal small intestine (duodenum, jejunum, and ileum) expresses *PTCH1*/PTCH1 in the epithelium at base of the villi and in the lamina propria without detectable *PTCH1* expression in the villous tips but PTCH1 is present in both epithelial sites (as seen with SHH expression, data not shown). Healthy adult colon expresses *PTCH1*/PTCH1 in the luminal epithelium (**c** and **d**, black arrows) and in the subjacent lamina propria (red arrows) with no expression detected in the crypts (arrowheads). Figure illustrations are limited so as to provide optimal resolution for images shown.

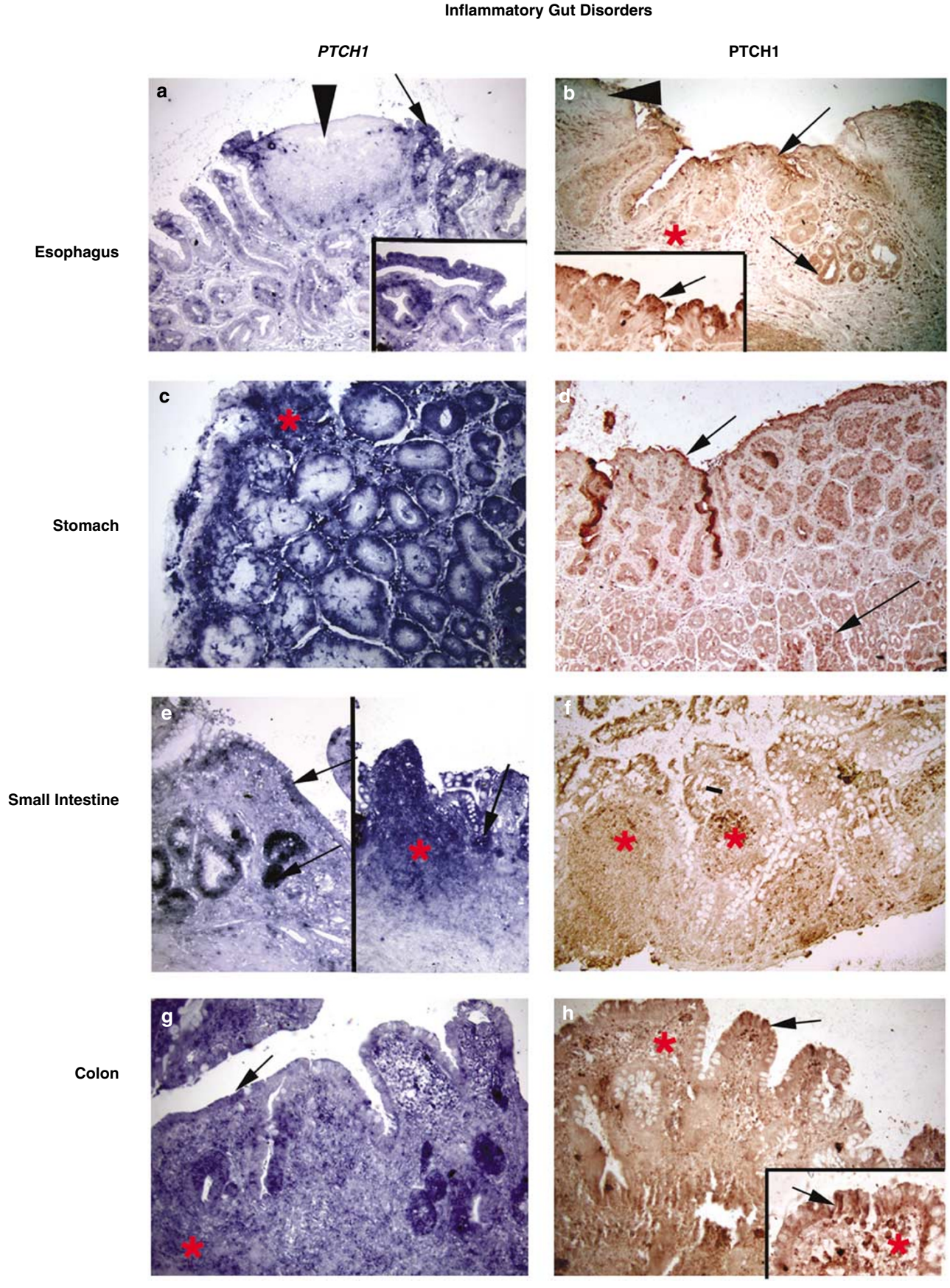
inflamed tissues. We also studied histologically normal biopsies taken from 10 different patients, who were shown not to have inflammatory disease at the time of endoscopy or colonoscopy.

This study confirms our previous report, and shows that the mRNA and protein expression of SHH/SHH are similar. No mRNA or protein expression is detectable in normal squamous epithelium of the esophagus (Figure 1a and b). In the stomach, mRNA and protein (are expressed strongly), and both are restricted to the fundic glands (Figure 1c and d). In the small intestine, SHH mRNA expression is detected only in the epithelium at the villus base (Figure 1e) and in the Brunner's glands of the duodenum (data not shown). However, weak protein expression can be detected in all small intestinal epithelial cells, but is most robust in the epithelium at the base of villi (Figure 1f). Colonic mRNA expression is nearly restricted to the crypts (Figure 1g). Protein expression is strongest in the crypt epithelium, but can also be detected weakly and focally in the surface epithelium (Figure 1h).

Figure 3 Expression of *SHH*/SHH in inflammatory gut diseases. Strong expression of both mRNA and protein is present in glandular metaplasias in Barrett's esophagitis (arrows in **a** and **b**, and inset **a**) with adjacent squamous epithelium negative (arrowhead in **b**). All epithelium gastritis mucosa strongly expresses *SHH*/SHH (**c** and **d**, inset **d** shows superficial expression, arrow). Similarly all epithelium expresses *SHH*/SHH in Crohn's disease of the small bowl (**e** and **f**, inset **f** arrows show surface expression) and in both colonic Crohn's disease (data not shown) and ulcerative colitis (**g** and two panels in **h**). Arrow in **g** and **h** highlight luminal epithelial expression. We detect strong expression in inflammatory cells in all inflamed mucosa (red asterisks).

Inflammatory Gut Disorders




Inflammatory Gut Disorders
PTCH1
PTCH1
Esophagus
Stomach
Small Intestine
Colon
a
b
c
d
e
f
g
h

Hence, SHH mRNA expression is spatially restricted within the gut epithelium, sparing the most luminal regions and being present only in glandular (not squamous) epithelium. SHH protein expression follows mRNA expression in the stomach, but differs somewhat in the intestines, with expression in the more superficial epithelium as well. All tissues showed expression in the ganglia of the enteric nervous system (data not shown) and in scattered inflammatory cells in the lamina propria (see below).

PTCH1/PTCH1 Expression in Normal Adult Human Gut

Similar to *SHH*/SHH, we did not detect *PTCH1*/PTCH1 expression in the esophageal squamous epithelium (Figure 2a and b). *PTCH1*/PTCH1 expression is present in the fundic glandular epithelium of the stomach and in epithelium in the base of the villi of the small intestine (data not shown). *PTCH1*/PTCH1 expression is also present in the stromal cells of the lamina propria in both the small intestine and colon with strongest expression in the lamina propria nearest the lumen (red arrows in Figure 2) and in scattered inflammatory cells in the mucosa (see below). The expression of *PTCH1*/PTCH1 differs from *SHH*/SHH in the colonic epithelium. We find both the mRNA and protein expression of PTCH1 restricted in the epithelium to the luminal epithelium (Figure 2c and d) whereas *SHH* is expressed weakly in the crypt epithelium and only weakly and patchy in the luminal epithelium (Figure 1g and h). In all gut regions, we detect *PTCH1*/PTCH1 expression in the enteric nervous system ganglia (data not shown).

SHH Expression in Inflammatory Conditions of the Adult Human Gut

The *SHH*/SHH expression pattern is altered in human inflammatory gastrointestinal disorders (Figure 3). The normal radial (crypt-villous) pattern of *SHH*/SHH expression is disturbed in inflamed epithelium. Normally, *SHH*/SHH expression can be detected most strongly and often only in the basal layers of each region of the gut (that thought to be the germative layer, see Figure 1). In the inflamed gut regions, with the exception of the esophageal squamous epithelium, we detect *SHH*/SHH strong expression throughout the epithelium from the base to the lumen. *SHH*/SHH expression is strongly detected in the metaplastic and inflamed glandular epithelium (both gastric and intestinal metaplasias) in nine of nine cases from different patients with Barrett's esophagus (four associated with adenocarcinoma—resections and biopsies, five with reflux esophagitis—biopsies) (Figure 3a and b). We do not detect *SHH*/SHH in any of the patients' uninflamed normal squamous esophageal epithelium (data not shown). In all conditions studied, *SHH*/SHH was detected in the inflammatory cells present in the mucosa (Figure 1, red asterisks).

We found the epithelial expression of *SHH*/SHH is diffuse in the other inflammatory conditions studied, including all five separate cases of 'gastritis' (one gastrectomy with gastric adenocarcinoma—atrophic, three biopsies: one with *H. pylori*, one 'chemical', one reflux and one atrophic; Figure 3c and d), three of three separate cases of Crohn's disease of the small intestine (Figure 3e and f), 11 of 11 separate cases of ulcerative colitis (Figure 3g and h), and five of five separate cases of Crohn's disease of the colon (data not shown). All small intestine and colon tissues were obtained from colectomy specimens. In all cases with regions of unaffected small intestine or colon, the normal pattern of *SHH*/SHH expression was demonstrated (Figure 1 and some data not shown).

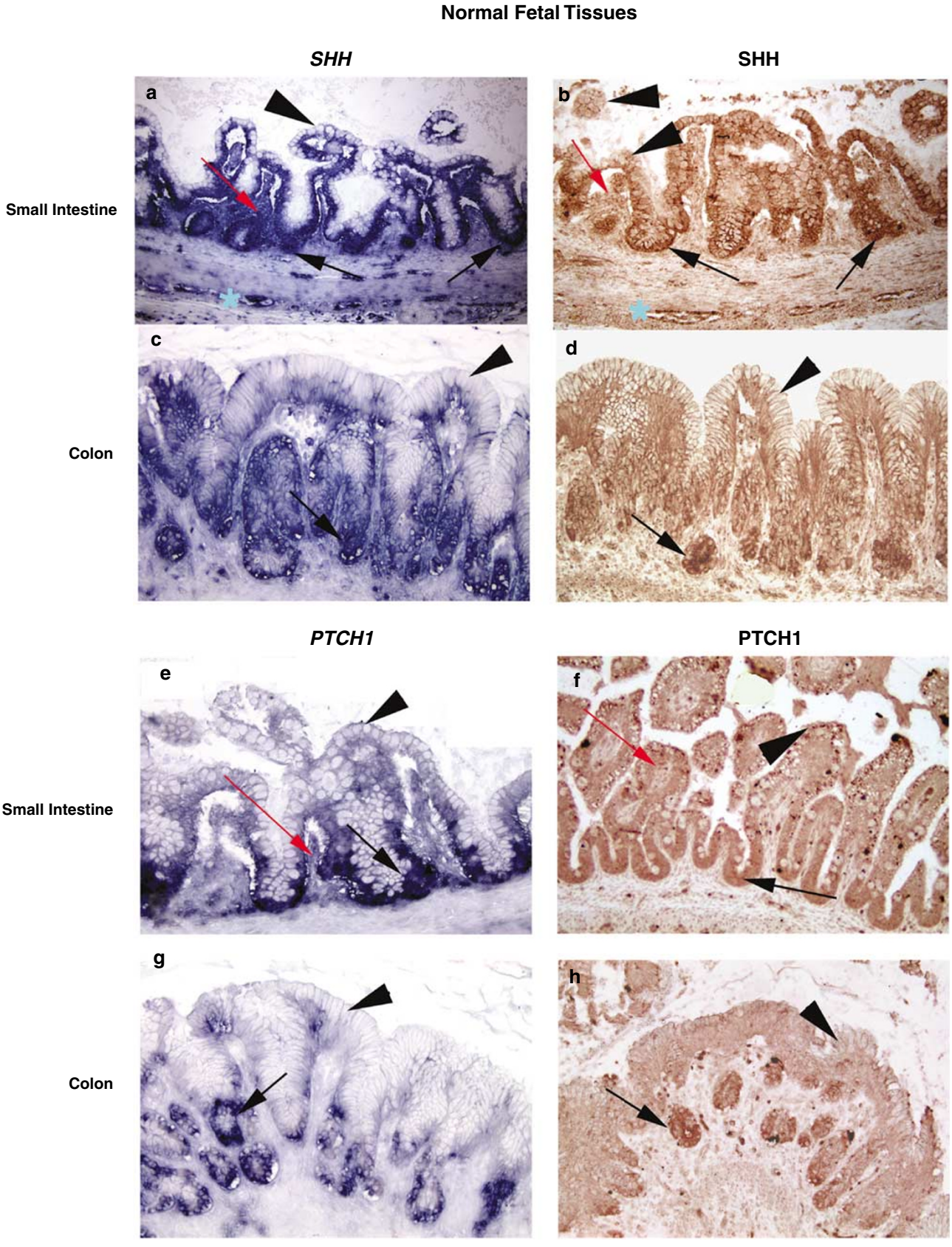
PTCH1/PTCH1 Expression in Inflamed Tissues of the Gut

PTCH1/PTCH1 is present in metaplastic and regenerating epithelial cells of inflammatory disorders of the gut including the esophagus, stomach, and small intestine (Figure 4a–f). In ulcerative colitis (Figure 4g and h) and colonic Crohn's disease (data not shown), we do not detect PTCH1 mRNA, and only focally detect PTCH1 protein (arrows in Figure 4h) in the surface (luminal-most) epithelium. Both PTCH1 mRNA and protein are strongly detected in the crypts. In all regions, *PTCH1*/PTCH1 is strongly detected in the inflammatory cells in the lamina propria and epithelium (red asterisks in Figure 4).

SHH/SHH and *PTCH1*/PTCH1 in Midgestation Human Fetal Intestine and Colon

SHH/SHH expression is present in all epithelial cells of the fetal small intestine but levels are strongest in the base of the villi (arrows in Figure

Figure 4 Expression of *PTCH1*/PTCH1 in inflammatory gut diseases. Expression of *PTCH1*/PTCH1 mirrors that of *SHH*/SHH in all regions of the gut affected with inflammatory diseases. Barrett's esophagitis expressed *PTCH1*/PTCH1 in all metaplastic glandular epithelium (Figure 1**a** and **b**, arrows), sparing squamous epithelium (arrowheads). Inset **a** and **b** shows higher power of luminal glandular epithelium positive for *PTCH1*/PTCH1. Gastritis (**c** and **d**) and Crohn's disease of the small intestine (**e** and **f**) show strong expression in all levels of epithelium (arrows). In all cases of colitis studied, we detect *PTCH1*/PTCH1 in nonsurface epithelium and only patchy expression on the luminal epithelium (ulcerative colitis in **g**, **h**, and inset **h**, with positive expression highlighted by black arrows). All inflamed mucosa show strong expression of *PTCH1*/PTCH1 in the inflammatory cells.


Normal Fetal Tissues
SHH
SHH
Small Intestine
a
b
Colon
c
d
PTCH1
PTCH1
Small Intestine
e
f
Colon
g
h

5a and b). We also detect strong mRNA (Figure 5a, red arrow) and weaker protein levels (Figure 5b, red arrows) in the small intestinal lamina proprial stroma. Colonic epithelial mRNA expression is restricted to the epithelial crypts as in adults (black arrows Figure 5c) and protein expression is much more restricted to the crypt epithelium than seen in adults (Figure 5d). Expression is present in both colon and small intestine enteric nervous system (eg see blue asterisks in Figure 5a and b).

PTCH1 expression mirrored *SHH* in both the small and large intestine (Figure 5e–h). Protein expression of PTCH1 is not detected in the lamina propria at either site.

We also detect mRNA and protein for *PTCH1*/PTCH1 and *SHH*/SHH in the fetal thymus (data not shown).

Gut Mucosal Inflammatory Cells in Inflammatory Diseases Coexpress *SHH* and CD4

In cells expressing *SHH*, B-cell markers CD20 and CD34 are not detected (data not shown). Conversely, T-cell antigen CD4 (helper/inducer T-cell marker) is strongly coexpressed in *SHH* expressing inflammatory cells (Figure 6). We do not detect CD8 in *SHH*-expressing cells (data not shown).

Discussion

The role of the Hh signaling pathway in adult human gut tissues is not well understood. In other vertebrates, study has focused on its patterning function and the earliest times of gut development. During the early organogenesis period, Shh signaling has been demonstrated to be from endoderm to mesoderm.[1,2] We show that in human fetal gut at midgestation (much later in development than equivalent chick or murine expression studies), the pathway is primarily active in the epithelium as both ligand and receptor proteins are expressed there (Figure 5). This suggests that there is a change in pathway signaling from epithelial to mesenchymal in early development to a predominantly autocrine signaling within the epithelium in later developmental stages. In adult gut, there may be both as *PTCH1*/PTCH1 is expressed in the epithelium and the lamina propria (Figure 2).

In intestinal inflammatory gut conditions, expression of these factors is disturbed with a loss of the normal restricted pattern in the radial (crypt-villous) axis. The inflammatory cells in the epithelium and lamina propria express both *SHH*/SHH and *PTCH1*/PTCH1 (Figures 3 and 4). These cells are CD4 + lymphocytes (Figure 6). This finding suggests that some of the Hh signaling may derive from the inflammatory infiltrate in these disorders. Stewart *et al*[35] also found PTCH1 expression in the interstitial infiltrate in a patient with chronic pulmonary fibrosis (see Figure 4e in Stewart *et al*[35]) and found SHH expressed in the affected metaplastic respiratory epithelial cells. They also describe upregulation of Shh expression in a murine model of chronic pulmonary fibrosis and suggest that Shh is required for epithelial repair and maintenance of the inflammatory infiltrate. Whether the signaling is epithelial to inflammatory cell (suggested by Steward *et al*[35]) or inflammatory cell to epithelium needs to be further investigated. It may be that the expression of Hh proteins outside the epithelium is unrelated to the epithelial expression or its increased expression in injured epithelium. Studies in immunocompromised patients or animal models would be interesting to study this question.

We suggest that the surface SHH protein-expressing cells represent an upregulation of the SHH protein-expressing stem cells, which normally are spatially restricted to the base of the epithelium. This expression suggests that Hh signaling may play a role in the repopulation of the damaged/altered mucosa by either expansion of the stem cell compartment or inhibition (or delay) of epithelial cell differentiation. This theory provides a logical hypothesis for the neoplastic risk many of these diseases carry. The persistence of stem or undifferentiated cells and abnormal spatial distribution of Hh signaling may predispose the epithelium to malignant transformation, as has been suggested by others.[29,47–51] Recent publications show strong expression of *SHH*/SHH in malignancies of the gastrointestinal tract including the pancreas,[21,23] correlating well with our findings of upregulation in preneoplastic diseases of the gut.

While we and others could not detect expression or SHH in neoplastic colonic epithelium,[23,52] Oniscu *et al*[24] finds expression upregulated in progression of colonic neoplasia. Herein we describe normal

Figure 5 Expression of *SHH*/SHH and *PTCH1*/PTCH1 in midgestation human fetal gut tissues. *SHH* expression is present in the epithelium of the small intestine (**a**) and colon (**c**), strongest at the base of the villi or crypts (compare black arrows with arrowheads in **a** and **c**). Expression is also present in the lamina proprial cells in the small intestine (red arrow, **a**). SHH expression mirrors that of *SHH* (**b**, **d**). Expression is also detected in the enteric nervous system (see ganglia marked with blue asterisk in **a**, **b**). *PTCH1* expression is the same as *SHH* in the human fetal small intestine and colon, with strongest epithelial expression in the epithelium at the base of the villi (black arrow in **e**) or crypts (black arrow in **g**). The arrowhead shows background expression in the luminal epithelium (**e**, **g**). *PTCH1*/PTCH1 expression is also present in the lamina propria of the small intestine (red arrows in **e** and **f**). PTCH1 expression mirrors *PTCH1* expression (**f**, **g**) and is also present in the ganglia of the enteric nervous system (data not shown). In the small intestine, PTCH1 expression is also present in the luminal epithelium (arrowhead in **f**) but not in the luminal epithelium of the colon (arrowhead in **h**).

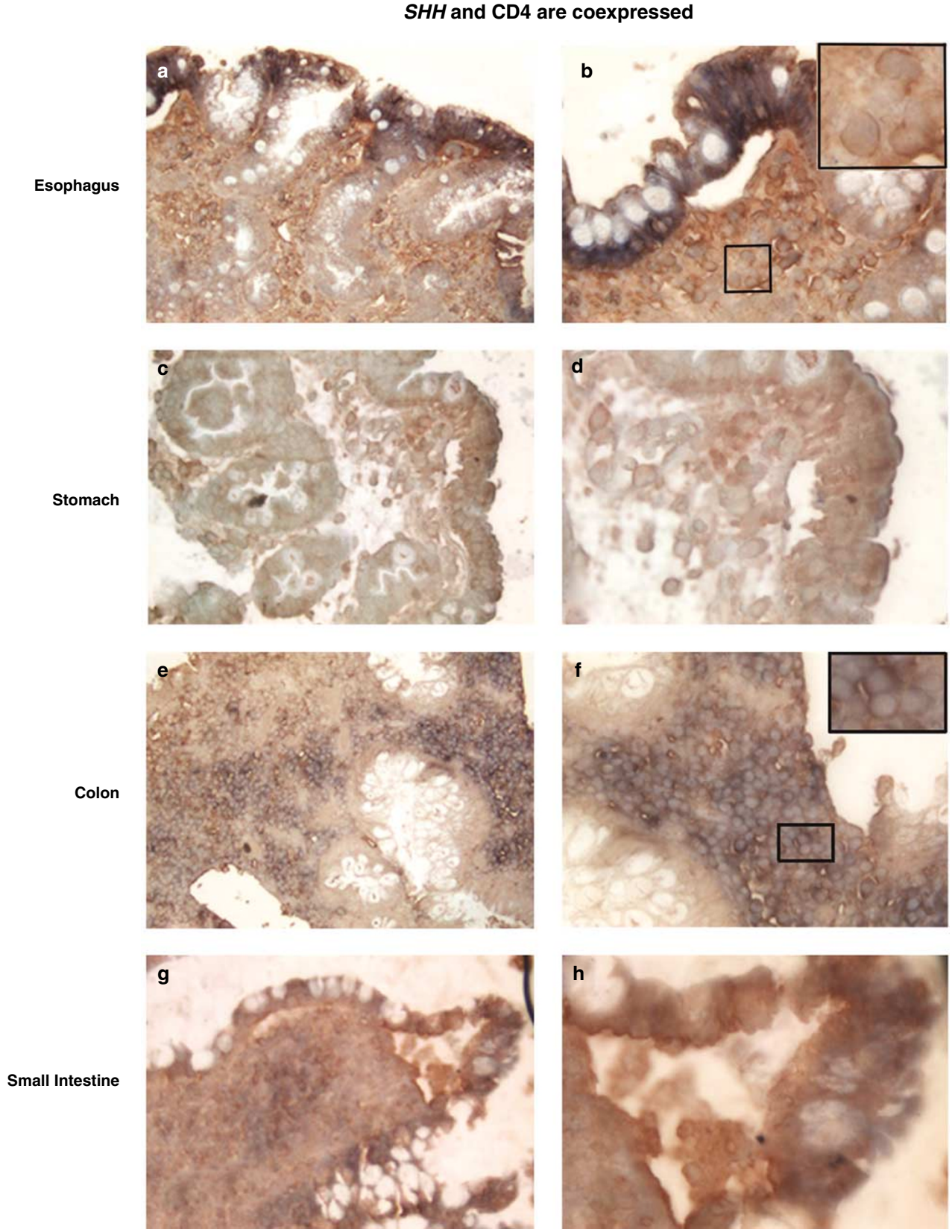


Figure 6 Cells in inflamed gut tissue coexpress SHH and the CD4 antigen. *SHH* and CD4 expression in metaplastic mucosa of Barrett's esophagitis (**a**, **b**), gastritis (**c**, **d**), ulcerative colitis (**e**, **f**), and Crohn's disease of the ileum (**g**, **h**) and colon (data not shown). Brown staining shows CD4 positivity. Insets show blue staining of *SHH* in CD4-positive cells.

colonic epithelial expression of SHH mRNA restricted to the crypts (Figure 1) but show that protein expression can be focally (weakly) detected in epithelium even at the surface (luminal). Oniscu *et al* shows expression only at the lumen by immunohistochemistry for both SHH and PTCH1 (Figure 1a in Oniscu *et al*[24]) but find mRNA expression in crypts (Figure 2 in Oniscu *et al*[24]). These discrepancies should be studied further and may only represent different techniques of detection or perhaps different patient ages or regions of the colon.

We do not detect *PTCH1*/PTCH1 in inflamed colonic surface epithelium and only weakly detect SHH protein. The luminal epithelial location of *PTCH1*/PTCH1 expression in the normal, uninflamed, adult colon also contrasts with its expression elsewhere in the gut and constrasts with the crypt location of *SHH*/SHH expression. These findings suggest that Hh signaling may have a different role in colon compared with other regions of the gastrointestinal tract. We have noted expression of *IHH*/IHH in the colonic surface most luminal epithelium (similar to that of *PTCH1*/PTCH1) and therefore IHH may be the functional Hh protein in the human adult colon. Hh signaling in the colon may be more directed towards gut epithelial differentiation as apposed stem cell maintenance elsewhere in the gut.[52,53] Support for the role of SHH in gut epithelial stem cell maintenance was recently demonstrated when SHH expression was noted to be associated with neoplastic gut diseases.[21,23] These authors describe SHH expression (protein and mRNA) in epithelial malignancies of the gut and pancreas (but not the colon) in animal models, cell lines, and on human pancreatic tissue sections demonstrating the progression of pancreatic malignancies from dysplasia (*in situ*) to frank carcinoma. The authors (and we herein) suggest that the abnormal persistence or ectopic expression of SHH may stimulate an abnormal stem cell hyperplasia, suggested to be a critical step in the neoplastic transition of these tissues.

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