program of the antral and pyloric gastric mucosa cells. Strikingly, all mpS2$^{-/-}$ mice developed gastric adenoma, and 30% of them showed carcinoma. Thus, *mpS2* may function as a gastric-specific tumor suppressor gene. However, as only 30% of the pS2$^{-/-}$ mice developed carcinomas, the loss of mpS2 protein on its own is clearly not sufficient for malignancy. Additional genetic alteration may be required, as is the case for human colorectal tumorigenesis (*18*). Interestingly, whereas normal gastric tissues express large amounts of hpS2, about 50% of human gastric carcinomas have lost expression of hpS2 (*7*, *8*, *12*, *19*). No major alterations in *hpS2* have been found in genomic DNA extracted from gastric carcinomas (*19*). However, the presence of aberrant hpS2 transcripts has been reported (*7*), which suggests that subtle *hpS2* gene modifications (such as mutations leading to aberrant splicing events) might exist in some stomach carcinomas.

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Impaired Defense of Intestinal Mucosa in Mice Lacking Intestinal Trefoil Factor

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The mechanisms that maintain the epithelial integrity of the gastrointestinal tract remain largely undefined. The gene encoding intestinal trefoil factor (ITF), a protein secreted throughout the small intestine and colon, was rendered nonfunctional in mice by targeted disruption. Mice lacking ITF had impaired mucosal healing and died from extensive colitis after oral administration of dextran sulfate sodium, an agent that causes mild epithelial injury in wild-type mice. ITF-deficient mice manifested poor epithelial regeneration after injury. These findings reveal a central role for ITF in the maintenance and repair of the intestinal mucosa.

The gastrointestinal mucosa must maintain a barrier against the harsh luminal contents of acid, enzymes, bacteria, and toxins. Disruption of this barrier is the salient feature of a variety of common and important gastrointestinal disorders, including inflammatory bowel disease and peptic ulcers. Although general protective factors are thought to contribute to this barrier function, the role of specific mucosal surface proteins in sustaining mucosal integrity has not been defined.

The trefoil proteins are a family of proteins expressed specifically and abundantly at the mucosal surface of the gastrointestinal tract (*1*). They share a distinctive three-leafed secondary structure formed by intrachain disulfide bonds. These proteins appear to resist degradation by proteolytic enzymes and extremes of pH (*2*). Enhanced expression of trefoil proteins is observed after injury in both the proximal and distal gastrointestinal tract (*3*). These proteins are secreted onto the mucosal surface by goblet cells, and in vitro studies suggest that these proteins may promote maintenance of mucosal integrity. Addition of trefoil proteins to wounded intestinal epithelial monolayers increases the rate of restitution, the critical first phase of wound healing in which epithelial continuity is reestablished (*4*).

To explore the function of trefoil proteins in vivo, we produced mice unable to express ITF, one of the members of this protein family. We used rat ITF cDNA as a probe to isolate the murine *Itf* gene from a phage genomic library, and the identity of this gene was confirmed by nucleotide sequencing (*5*). A targeting vector for disrupting the gene by homologous recombination in embryonic stem (ES) cells was designed and constructed (Fig. 1A). This targeting vector replaces the entire second exon encoding the trefoil domain with the neomycin resistance gene and abolishes the ability of any resultant peptides to produce the loop structure characteristic of the trefoil proteins. Two independent lines of mice were generated from distinct ES cell clones. Disruption of the *Itf* gene in these mice was confirmed by Southern (DNA) blot analysis and the polymerase chain re-

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action (PCR) (6).

Northern (RNA) blot analysis of wild-type mice demonstrated a pattern of normal tissue expression of murine ITF similar to that of rat and human ITF, that is, in the small intestine and colon. Similar analysis of $Itf^{-/-}$ mice confirmed the lack of ITF expression in the gastrointestinal tract (Fig. 1B). In contrast, expression of the other trefoil proteins, SP and pS2, was unaltered in the gastrointestinal tract of $Itf^{-/-}$ mice. The lack of ITF protein in the colon and small intestine of $Itf^{-/-}$ mice was confirmed by immunohistochemical staining with rabbit polyclonal antibody raised against synthetic peptide from the predicted 18 COOH-terminal amino acids of murine ITF sequences (Fig. 2). The goblet cells of the $Itf^{-/-}$ colon lacked any detectable ITF but had preserved expression of colonic mucin. The epithelium of $Itf^{-/-}$ mice appeared morphologically comparable to that of wild-type mice.

$Itf^{-/-}$ mice derived from each ES clone appeared to develop normally and were grossly indistinguishable from their heterozygous and wild-type littermates. They manifested no evidence of growth retardation and reached adult maturity without evident diarrhea or occult fecal blood loss. Although $Itf^{-/-}$ mice exhibited normal mucosal architecture, their proliferative compartments were expanded [12.2 ± 1.7 bromodeoxyuridine (BrdU)–labeled nuclei per $Itf^{-/-}$ crypt, 8.6 ± 1.8 BrdU-labeled nuclei per wild-type crypt; $P = 0.001$]. The lack of ITF resulted in impaired physiological migration of epithelium to the mucosal surface, as evidenced by the virtual absence of labeled cells in the surface epithelium 3 days after the BrdU pulse (Fig. 3) compared with the high proportion of surface cells labeled in mucosa from wild-type mice.

ITF has been shown to promote epithelial migration into areas of wound in vitro, and we hypothesized that the colon of mice lacking ITF would be more prone to injury. Dextran sulfate sodium (DSS) administered in drinking water reproducibly creates mild colonic epithelial injury with ulceration in mice (7). After standardization of DSS effects in wild-type mouse cohorts, a group of 20 wild-type and 20 $Itf^{-/-}$ mice (littermates from heterozygous crosses, >20 g each) were treated with 2.5% DSS in their drinking water for 9 days. $Itf^{-/-}$ mice appeared markedly more sensitive to the injurious effects of DSS. Fifty percent of $Itf^{-/-}$ mice developed frankly bloody diarrhea and died (Fig. 4B); in contrast, only 10% of wild-type mice treated similarly exhibited bloody diarrhea and 5% died. Weight loss was also more pronounced in the $Itf^{-/-}$ mice than in wild-type mice receiving DSS (Fig. 4A).

Inspection of the colons of $Itf^{-/-}$ mice after DSS treatment demonstrated the presence of multiple sites of obvious ulceration and hemorrhage. In contrast, the colons of most of the DSS-treated wild-type mice were grossly indistinguishable from those of untreated mice. Histological examination of the treated $Itf^{-/-}$ colon confirmed the presence of multiple erosions and intense inflammatory changes, including crypt abscesses. Damage was more pronounced in the distal colon (descending colon, sigmoid, and rectum) of the $Itf^{-/-}$ mice; large, broad areas of mucosal ulceration were seen. Although wild-type mice exposed to DSS showed evidence of mucosal erosions upon microscopic inspection, most of these were small and exhibited the features typical of mucosal healing, with complete re-epithelialization of most lesions. In contrast, the colons of $Itf^{-/-}$ mice exposed to DSS had large stretches of denuded epithelia and lacked evidence of re-epithelialization (Fig. 4C).

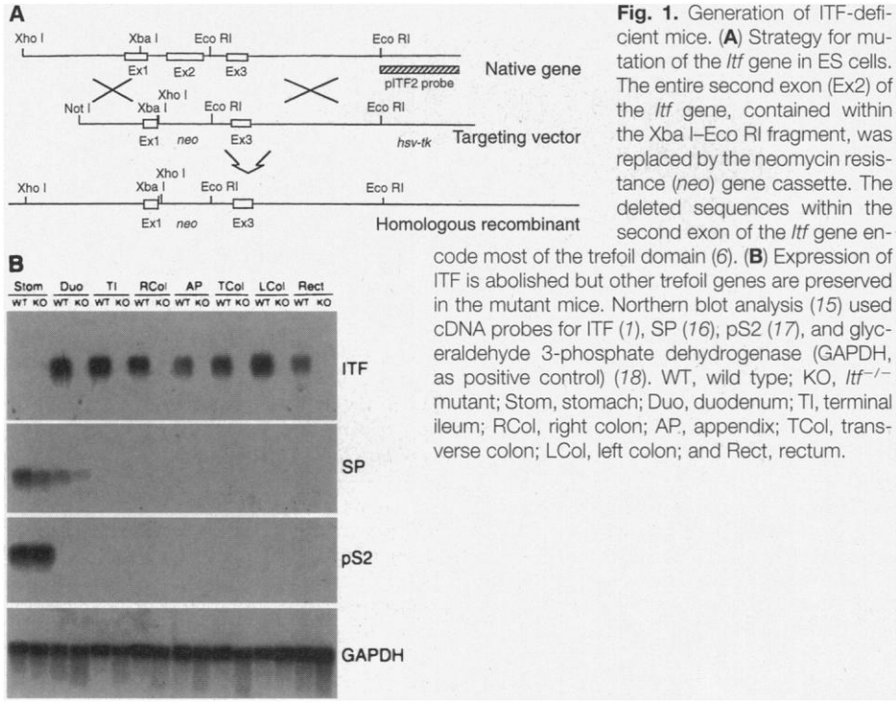

Fig. 1. Generation of ITF-deficient mice. (**A**) Strategy for mutation of the *Itf* gene in ES cells. The entire second exon (Ex2) of the *Itf* gene, contained within the Xba I–Eco RI fragment, was replaced by the neomycin resistance (*neo*) gene cassette. The deleted sequences within the second exon of the *Itf* gene encode most of the trefoil domain (6). (**B**) Expression of ITF is abolished but other trefoil genes are preserved in the mutant mice. Northern blot analysis (15) used cDNA probes for ITF (1), SP (16), pS2 (17), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, as positive control) (18). WT, wild type; KO, $Itf^{-/-}$ mutant; Stom, stomach; Duo, duodenum; TI, terminal ileum; RCol, right colon; AP, appendix; TCol, transverse colon; LCol, left colon; and Rect, rectum.

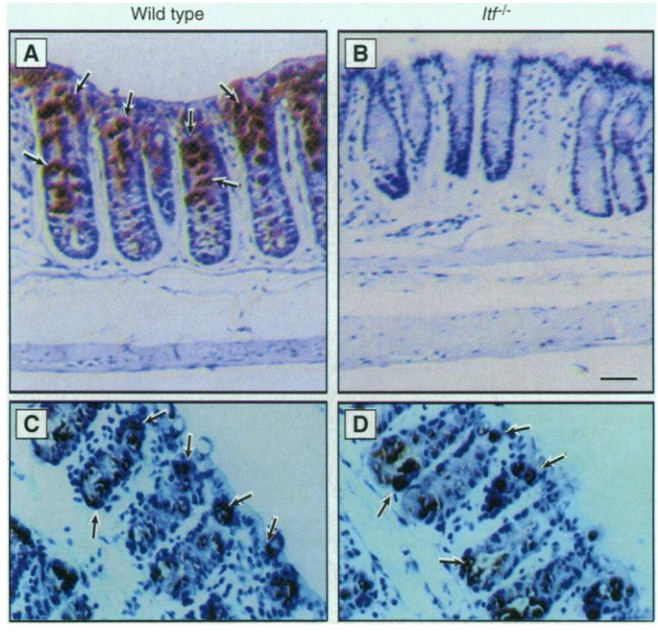

Fig. 2. ITF is not expressed in $Itf^{-/-}$ mice. (**A**) Wild-type mouse colon showing ITF-immunoreactive staining specific to the goblet cells (arrows). (**B**) $Itf^{-/-}$ mouse colon showing no ITF immunoreactivity. (**C**) Mucin stains in wild-type mouse (19) show expression within the goblet cells (arrows). (**D**) Preservation of mucin stains within $Itf^{-/-}$ colon (arrows). Scale bar, 50 μm.

To confirm that this failure of healing was secondary to the absence of ITF, we evaluated the ability of recombinant ITF to restore restitution after application of acetic acid, an agent that consistently induces focal mucosal injury in all animals (*8*). Repletion of ITF-deficient mice by luminal instillation of recombinant peptide resulted in reconstitution of normal healing with enhanced epithelial migration (Fig. 5) and marked attenuation of gross injury.

In vitro evidence suggests that trefoil proteins play a key role in reestablishing mucosal integrity after injury. Despite the normal restriction of SP and pS2 expression to the proximal gastrointestinal tract, these trefoil proteins and ITF are abundantly expressed at sites of colonic injury and repair. Addition of exogenous ITF to a confluent layer of intestinal epithelial cells causes increased migration of these cells into wounds (*4*). Addition of trefoil proteins protects human colonic cell lines in vitro from injury by toxins and ethanol (*9*). A parallel effect, protection against alcohol or nonsteroidal anti-inflammatory drug exposure, is conferred by prior topical administration of trefoil proteins in the rat stomach (*10*). Our results show that mice lacking ITF are markedly susceptible to mucosal injury and confirm a protective role of ITF in the maintenance of the mucosal barrier, and they also show that ITF has a role in the normal migration and turnover of the intestinal epithelium. Regulation of the constitutive migration mirrors the effects of trefoil peptide in promoting epithelial migration to reestablish continuity after mucosal injury.

Recent studies demonstrate the key role of a cytokine network in mucosal maintenance of the gastrointestinal tract. Gene-targeted deletion of cytokines that coordinate immune and inflammatory response results in colitis in mice (*8*, *11*). Although a cytokine network may coordinate response after injury, mediators that maintain the normal integrity of the mucosal barrier must be fundamental in preventing the activation of these mechanisms by digestive enzymes, bacteria, and toxins present in the gastrointestinal tract lumen. Our results with ITF-deficient mice demonstrate the

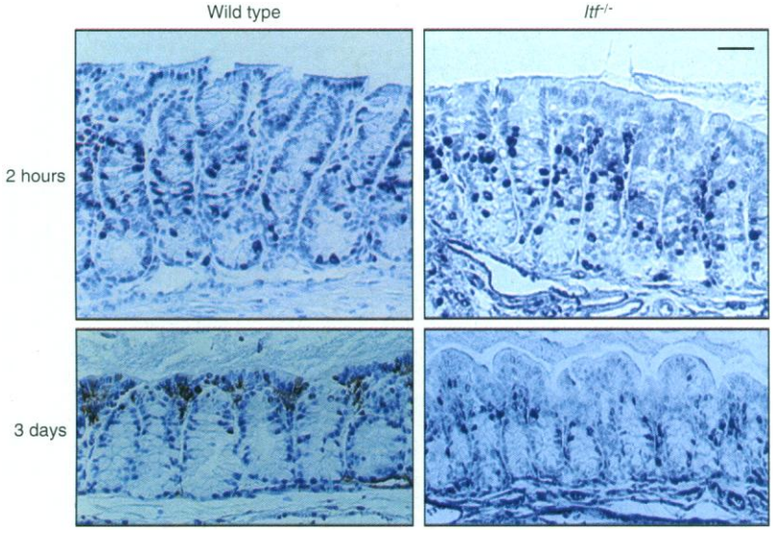

Fig. 3. Expansion of the proliferative compartment and impaired epithelial migration in *Itf*$^{-/-}$ mice. BrdU labeling (*20*) shows expansion of the proliferative compartment in *Itf*$^{-/-}$ colon at 2 hours. Migration of crypt cells was assessed 3 days after the BrdU pulse. The epithelium lining the lumen of *Itf*$^{-/-}$ mice is virtually devoid of BrdU-labeled cells, and residual labeled cells are seen in the crypts at 3 days after the pulse label. In contrast, in wild-type mice, most of the labeled cells have migrated out of the crypts and onto the lumenal surface by this time. Scale bar, 50 μm.

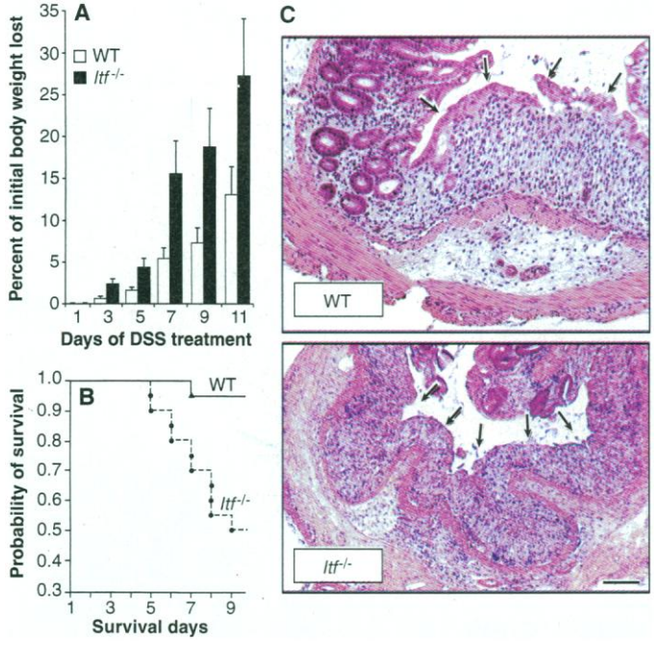

Fig. 4. Increased DSS-induced weight loss and death with severe colonic erosions in *Itf*$^{-/-}$ mice. (**A**) Weight loss (expressed as percent of initial body weight lost) is greater after DSS in *Itf*$^{-/-}$ mice (*21*). WT, wild type; bars represent SE. (**B**) Survival is diminished in *Itf*$^{-/-}$ mice, shown as Kaplan-Meier transform of probability versus days of DSS treatment. (**C**) Representative areas of healing ulceration in wild-type colon and nonhealing ulceration in *Itf*$^{-/-}$ colon. In the wild type, healing and re-epithelialization of ulcers are apparent (arrows) (*22*). These signs of healing are not present over the broad ulcer (arrows) in *Itf*$^{-/-}$ mice. Scale bar, 100 μm.

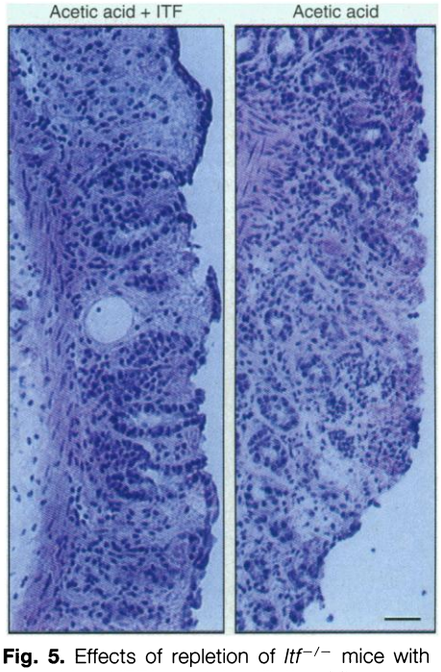

Fig. 5. Effects of repletion of *Itf*$^{-/-}$ mice with recombinant ITF by rectal instillation. Microscopic examination of ulcer borders from untreated and ITF-treated *Itf*$^{-/-}$ mice after acetic acid–induced injury (*23*) shows improved epithelialization over damaged mucosa in treated mice. Scale bar, 75 μm.

role of trefoil peptides (acting at the apical surface) in complementing the role of the cytokine network (acting within the lamina propria at the basolateral pole of the epithelium). Trefoil factors are unusual in their resistance to acid and proteolytic enzymes (*12*) and therefore have potential as an orally administered therapy for various forms of gastrointestinal tract injury, including inflammatory bowel diseases.

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20. Mice received a single intraperitoneal injection of BrdU (50 μg per gram of body weight) from a freshly made stock solution (5 mg/ml) dissolved in phosphate-buffered saline (PBS). The mice were killed 2 hours or 3 days later. At necropsy, a longitudinal 1-cm section of the ascending colon was taken. Samples were immediately placed in a cassette, fixed in Carnoy's fixative overnight, and embedded in paraffin wax. Immunohistochemical detection of BrdU was essentially as described [J. G. Fox *et al.*, *Gastroenterology* **110**, 155 (1996)] using monoclonal antibody to BrdU (Sigma), VectaStain kit, and hematoxylin counterstain. Only crypts longitudinally sectioned and visible in their entire length were analyzed for the number of labeled cells.
21. Mice were given 2.5% (w/v) DSS (molecular weight 40,000; ICN Biomedicals, Aurora, OH) in their drinking water for nine consecutive days and weighed every other day.
22. DSS-treated left colon transections were fixed in 4% paraformaldehyde, mounted in paraffin, and stained with hematoxylin and eosin. Mice were given 2.5% DSS for 9 days.
23. A solution of 4% acetic acid (pH 2.3) was slowly infused (5 μl/mg body weight) 3 cm into the rectal lumen of a lightly anesthetized mouse. After exposure for 30 s, excess fluid was withdrawn and the colon was flushed with 0.3 ml of PBS. After removal of excess fluid, the treatment group was infused (5 μl/mg body weight) with recombinant ITF (1 mg/ml), while the untreated group was infused (5 μl/mg body weight) with bovine serum albumin (BSA; 1 mg/ml). Mice were again infused with ITF (treatment group) or BSA (untreated group) after 12 hours. Mice were killed 30 hours after acid injury and the colons were inspected. Frozen sections of OCT (Miles)-embedded specimen were stained with hematoxylin and eosin.

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