Loss of Smad5 leads to the disassembly of the apical junctional complex and increased susceptibility to experimental colitis

Joannie M. Allaire,1 Mathieu Darsigny,1 Sébastien S. Marcoux,1 Sébastien A. B. Roy,1 Jean-François Schmouth,1 Lieve Umans,2 An Zwijsen,2 François Boudreau,1 and Nathalie Perreault1

1Faculté de Médecine et des Sciences de la Santé, Département d’Anatomie et Biologie Cellulaire, Université de Sherbrooke, Sherbrooke, Quebec, Canada; and 2VIB K.U. Leuven, Department of Molecular and Developmental Genetics, Leuven, Belgium

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The intestinal epithelium represents a dynamic system with rapid cell turnover (14, 21). The adult intestinal mucosa is composed of undifferentiated pluripotent stem cells, located in the lower portion of the intestinal crypt, and differentiated functional epithelial cells distributed along the villus axis. The intestinal stem cell undergoes asymmetrical division to produce one stem cell, which remains multipotent and undifferentiated, and a daughter cell that is committed to differentiate (6, 14, 52). This committed daughter cell goes through a number of cell divisions while migrating from the proliferative crypt to the differentiated villus compartment, where it finally exfoliates. This renewal process occurs over ~2–3 days in the mouse small intestine. Therefore, the coordination between proliferation, migration, differentiation, and apoptosis is central for the establishment, morphogenesis, and maintenance of intestinal epithelial architecture.

The adhesive and migratory properties of epithelial cells are important for the establishment of the barrier function. Barrier integrity plays a critical role in normal development and is often compromised in a number of diseases, including inflammatory bowel disease (IBD) (7, 10). Tight junctions (TJ) and adherens junctions (AJ) regulate the barrier as well as cell-cell adhesion functions. Both TJ and AJ represent large complexes composed of transmembrane and cytosolic proteins (2, 25). The extracellular domain of the transmembrane proteins mediates homophilic interactions between adjacent cells, hence providing a mechanical link between the cell membranes that contribute to the physical barrier (2, 25). The intracellular function of these transmembrane proteins is mediated through the interaction of the actin cytoskeleton with cytosolic proteins. These junctions are positioned on the cellular apical side of the lateral cell membrane to form the apical junctional complex (AJC). This complex is a highly dynamic entity influencing cell polarization, proliferation, differentiation, migration, and paracellular permeability (57). For example, dysregulation of TJ protein expression, such as claudins, leads to dramatic changes in intestinal paracellular permeability and ion exchange (51, 55, 60). Importance of the E-cadherin AJ component during intestinal epithelial cell adhesion and migration was first demonstrated by the breakthrough chimeric-transgenic experiments performed by Hermiston’s group (29, 30). Perturbed E-cadherin function in the crypt epithelium in this context was associated with increased cell migration, cell proliferation, and apoptosis. The epithelial barrier of these mice was also disrupted and acquired the morphological characteristics of the epithelium of Crohn’s disease (CD) patients (29). E-cadherin has also been shown to be increasingly detected in the cytoplasm of patients with IBD (36). Recently, polymorphisms in the E-cadherin gene were linked to its increased cytoplasmic accumulation in Crohn’s samples, suggesting that a subtle defect in E-cadherin function or localization may contribute to intestinal inflammatory susceptibility (47). These combined experiments therefore reveal that defects in AJC assembly can correlate with a loss of barrier function and cell–cell contact leading to the acquisition of migratory cell potential in adult gut tissues. Internalization of AJC proteins...
appears to be a common mechanism to rapidly regulate epithelial barrier function and cell-cell adhesion allowing the remodeling of intercellular junctions (8, 33, 35, 47). However, the mechanisms by which AJC proteins are delocalized and endocytosed remain poorly understood (34).

Bone morphogenetic proteins (Bmps) are multifunctional growth factors belonging to the transforming growth factor-β (TGF-β) superfamily. Bmps play active roles in many developmental processes, in homeostasis as well as in various cellular functions in postnatal and adult animals (12, 43). Bmps signal through the serine/threonine kinase receptor subtypes I and II, where the type I receptor is activated upon Bmp-ligand binding and associates with the type II receptor. This activated receptor complex leads to the transphosphorylation of the Br-Smad proteins, which include Smad1, Smad5, and Smad8. These phosphorylated Br-Smads associate with the related protein Smad4 (Co-Smad), a shared partner of the TGF-β superfamily. The Br-Smad/Co-Smad complex then translates to the nucleus where it activates transcription of specific target genes. Individual Br-Smads share a close homology (44) with a distinct pattern of expression (3, 11, 56). Smad1 and Smad5 function cooperatively in the early embryo (3). Phenotypic differences observed in knockout (KO) mice suggest a significant specificity for these factors in vivo (4, 19, 42, 56, 63). As with the majority of KO mice for Bmp pathway effectors, Smad1 and Smad5 KO embryos are not viable and die at embryonic day 10.5 (11). In addition to the defects documented in angiogenesis and in extraembryonic tissues, Smad5 KO mice develop a vestigial gut lacking a foregut pocket and entrance to the hindgut diverticulum (11). This observation suggests a significant role for Smad5 in early intestinal organogenesis. Significant advancements in the understanding of the in vivo function of Bmp ligands and their receptors in the development and maintenance of the digestive tract have been achieved in recent years (15, 24, 26, 31). However, the specific roles played by the various Br-Smads in intestinal homeostasis and cellular functions are still of limited knowledge.

In the present study, we conditionally inactivated Smad5 in the mouse intestinal epithelium to delineate its function within the Bmp signaling cascade. Using this model, we uncovered an important role for Smad5 in intestinal epithelial cell migration. Our data indicate that loss of Smad5 promotes intestinal cell migration by disassembling the AJC through internalization of E-cadherin. We also demonstrate that the deficiency in epithelial barrier function and cell-cell adhesion allowing the remodeling of intercellular junctions (8, 33, 35, 47). However, the mechanisms by which AJC proteins are delocalized and endocytosed remain poorly understood (34).

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### MATERIALS AND METHODS

**Animals.** C57BL/6-Smad5ΔEΔC mice were provided by Dr. A. Zwijsen (58) while the C57BL/6 12.4KbVilCre transgenic line was provided by Dr. D. L. Gumucio (40). Genomic DNA was isolated using the Spin Doctor genomic DNA kit from Gerard Biotech according to the manufacturer’s protocol. All mutations were genotyped using protocols already published (40, 58). All experiments were approved by the animal research committee of the Faculty of Medicine and Health Sciences of the Université de Sherbrooke.

**Tissue preparation and histological staining.** Digestive tracts from 90- to 120-day-old control littermates and Smad5ΔEΔC mice were fixed, sectioned, and stained [hematoxylin and eosin (H&E)] as previously described (5).

**Tissue collection, RNA extraction, and gene expression analysis.** Total RNA was isolated and processed using the Totally RNA extraction kit (Ambion). Reverse-transcription PCR (RT-PCR) and quantitative real-time PCR were performed as described previously (5). PCR conditions and primer sequences are available upon request.

**Isolation of mouse adult intestinal epithelium.** RNA was isolated from pure epithelial intestinal fractions of adult mice by an adaptation of the MatriSperse dissociation method described previously for human intestine (49). Briefly, mice were killed, and the intestine was separated in sections of jejunum. Each section was opened longitudinally and rinsed with cold PBS. The sections were further cut in 5-mm pieces and incubated in 5 ml of cold MatriSperse (Becton-Dickinson) in 15-ml tubes at 4°C for 18–24 h. The epithelial layer was dissociated by gentle manual shaking. The epithelial suspension was collected, centrifuged, and washed with cold PBS. RNA extraction was performed as described above.

**Bromodeoxyuridine incorporation, transferase dUTP nick-end labeling assay, and immunofluorescence.** Mice were injected with 10 μl of bromodeoxyuridine (BrdU; Zymed) per gram of body weight 90 min (for the proliferation assay), 12 h (for the colonic migration assay), or 48 h (for the jejunum migration assay) before death. For the apoptosis assay, the transferase dUTP nick-end labeling (TUNEL) assay was performed following the manufacturer’s protocol (Roche Diagnostics). Immunofluorescence staining was performed as previously described (5). The following antibodies were used at the indicated dilutions: anti-BrdU (AB no. BMC 9318, 1:50; Roche Diagnostics), E-cadherin (AB no. C20820, 1:1,000; BD Transduction Laboratories), β-catenin (AB no. 9587, 1:1,000; Cell Signaling), claudin-1 (AB no. 51–9000, 1:500; Invitrogen), claudin-2 (AB no. 51–6100, 1:500; Invitrogen), FITC-conjugated anti-mouse IgG (1:200; Vector), and FITC-conjugated anti-rabbit IgG (1:200; Vector).

**Protein extraction and western blot analysis.** Total proteins were isolated from the intestinal mucosa of 90- to 120-day-old Smad5ΔEΔC mice and control littermates with RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% Triton X-100, 1 mM EDTA, 0.2% SDS, and 0.5% sodium deoxycholate) containing protease and phosphatase inhibitors (9). Twenty-five micrograms of protein extract was analyzed by 10% BisTris NuPAGE (Invitrogen) and transferred to a polyvinylidene difluoride blotting membrane (Roche Diagnostics). Western blotting was then performed as previously described (50). The following affinity-purified antibodies were used: E-cadherin mouse monoclonal antibody (1:5,000) and claudin-1 (1:500), claudin-2 (1:1,000), and occludin (1:500) rabbit polyclonal antibodies from Zymed Laboratories (Invitrogen); collagen (1:1,000), phosphorylated (p)-cofilin (1:1,000), and β-catenin (1:1,000) antibodies from Cell Signaling; and p-Smad5 (1:1,000) rabbit polyclonal antibodies from Abcam. For densitometry analyses, exposed films of Western blots were scanned, and images were analyzed using IMAGEJ (Rasband WS, ImageJ, United States National Institutes of Health, Bethesda, MD).

**CD and ulcerative colitis samples.** TissueScan Real-Time Crohn’s and colitis disease panels were purchased from Origene Technologies. Each panel was composed of total isolated RNA from 6 control samples, 21 CD samples, and 21 ulcerative colitis (UC) samples collected from different individuals. After resuspension of lyophilized cDNAs, qRT-PCR was performed as described above and was normalized against PBGD. PCR conditions and primers used are available upon request.

**Induction and assessment of dextran sulfate sodium-induced colitis and recovery.** Colitis was induced with dextran sulfate sodium (DSS) as previously described (48). Two independent groups were fed 3% (wt/vol) DSS water (4 controls and 4 Smad5ΔEΔC/group) ad libitum for 2, 4, and 7 days (molt wt 35,000–50,000; MP Biomedicals) as previously described (59). For water recovery experiments, two independent groups were fed 3% (wt/vol) DSS water (10 controls and 10 Smad5ΔEΔC/group) ad libitum for 7 days followed by a recovery period of 5 days with water only. One group of CD-1 mice was fed 4%
(wt/vol) DSS water ad libitum for 2, 4, and 7 days. One group of Smad5$	extsuperscript{-/-}$ mice compared with control mice and one group of 365-day-old Smad5$	extsuperscript{-/-}$ IEC mice compared with control mice were also used as water-only controls. Mice were killed on day 4 or 7 and assessed for disease activity on a 0–4 scale using the modified criteria from Cooper et al. (13). Scoring was as follows: weight loss (0, none; 1, 1–5%; 2, 6–10%; 3, 11–20%; 4, 21% or more), stool consistency (0, solid; 2, loose and adhering to anus; 4, severe diarrhea, often with emptied colon), fecal blood (0, none; 2, lightly colored; 4, heavily colored), rectal bleeding (0, none; 2, moderate; 4, heavy), and colon length (0, 100–96%; 1, 95–86%; 2, 85–76%; 3, 75–66%; 4, <66%) compared with nontreated mice. Disease activity index (DAI) represented the total of all measured criteria.

**Histological grading of colitis.** Colons were fixed in a Swiss-roll orientation in neutral buffered formalin followed by paraffin embedding. Histological scoring was compiled by two different individuals from 12 high-field images of H&E-stained sections taken blindly with a Leica DC300 camera on a DMLB2 microscope (Leica Microsystem Canada). Scoring, as validated by Dieleman et al. (16), was as follows: severity of inflammation (0, none; 1, mild; 2, moderate; 3, severe), extent of inflammation (0, none; 1, mucosa; 2, mucosa and submucosa; 3, transmural), crypt damage (0, none; 1, first 1/3 of crypt base; 2, 2/3 of crypt base; 3, only surface epithelium remaining; 4, no epithelium remaining). All scores were added to represent the histological colitis score.

**Electron microscopy.** Portions of mouse intestinal segments were rinsed with PBS, prefixed for 15 min with a 1:1 mixture of culture medium (Dulbecco’s modified Eagle’s medium) and freshly prepared 2.8% glutaraldehyde in cacodylate buffer (0.1 M cacodylate and 7.5% sucrose) and then fixed for 30 min with 2.8% glutaraldehyde at room temperature. After two rinses, specimens were postfixed for 1 h with 2% osmium tetroxide in cacodylate buffer. The tissues were then dehydrated using graded ethanol concentrations (40, 70, 90, 95, and 100%, three times each) and coated two times for 3 h with a thin layer of Araldite 502 resin (for ethanol substitution). Finally, the resin was allowed to polymerize at 60°C for 48 h. The specimens were detached from the plastic vessels, inverted in embedding molds, immersed in Araldite 502, and polymerized at 60°C for 48 h. Ultramicrotome-
prepared thin sections were contrasted with lead citrate and uranyl acetate and then observed on a Jeol 100 CX transmission electron microscope. All reagents were purchased from Electron Microscopy Sciences (Cedarlane, Hornby, ON, Canada).

**Quantification and statistical analyses.** All histological and cell count analyses or scores were performed using continuous sections from low-powered fields of well-oriented intestinal cross sections in a blind manner on an average of 10 independent fields/animal. The...
numbers of BrdU-labeled cells were quantified as described previously (5, 37). Briefly, proliferation was measured by counting the number of BrdU-labeled cells per crypt. Villus and crypt morphometry as well as rate of migration were determined using the MetaMorph software (Universal Imaging). Images were imported, and magnification was calibrated by comparison with a stage micrometer (graticules; Tonbridge, Kent, UK). Statistical analysis was performed using two-way ANOVA. Differences were considered significant with a P value of <0.05. For densitometry, qRT-PCR, and DAI score analyses, data were analyzed with the Student’s t-test for normal distribution data or using the Mann Whitney test for abnormal distribution. Two-way ANOVA test was used for statistical analysis of repeated measures. Differences were considered significant with a P value of <0.05. All statistical analyses were carried out using Graph Pad Prism 5 (Graph Pad, San Diego, CA).

RESULTS

Loss of epithelial intestinal Smad5 increases intestinal epithelial cell migration and length of the villus compartment. Homozygous floxed Smad5 mice (Smad5^fx/fx) (58) were crossed with the villin-Cre transgenic line, which exclusively directs expression of the transgene in all intestinal epithelial cells, including stem cells (40). Conditional KO mice for Smad5 (Smad5^IEC) were born at the expected Mendelian ratios and grew normally without obvious gross physical abnormalities. Because Smad5 is expressed in both epithelial and mesenchymal counterparts of the intestine, the nonenzymatic MatriSperse dissociation technique was used to separate the epithelium from the underlying mesenchyme (49) to evaluate epithelial expression levels of the Br-Smads in our mutant animals. Comparative analysis of Smad1, Smad5, and Smad8 mRNA expression by qPCR between control and Smad5^IEC mice confirmed a 97% epithelial loss of Smad5 expression in the intestine and colon with no compensatory changes in Smad1 or Smad8 expression in Smad5-deficient mice (Fig. 1A). Because the MatriSperse technique does not allow the exclusion of intraepithelial lymphocytes, this 3% residual expression is more likely to come from such cells still trapped in the epithelium.

To assess morphological changes occurring with the loss of the downstream signal mediator Smad5, histological analysis was performed with H&E staining of the small intestine in control and Smad5^IEC mice. The intestine of Smad5^IEC mice displayed abnormal epithelial intestinal morphology with elongated villi (Fig. 1C) compared with control littermates (Fig. 3).

Fig. 3. Ultrastructural evaluation of the apical junctional complex and immunolocalization of zonula occludens proteins in Smad5^IEC mice. Ultrastructural analysis exposed the presence of a less tight tight junction (TJ) and shallow adhesion plaques at the adherens junction (AJ) in Smad5^IEC mice in both jejunum (B) and colon (D) compared with controls (A and C, respectively). Immunostaining with a claudin-1 specific antibody showed that claudin-1 was diffused in the cytoplasm of jejunal and colonic epithelial cells in Smad5^IEC (F and H, respectively) compared with its localization at the apical membrane in control littermates (E and G, respectively). Immunostaining with a claudin-2 specific antibody showed that claudin-2 was localized normally at the apical membrane in both jejunal and colonic epithelial cells in Smad5^IEC (J) in control (I) littermates. Claudin-2 expression was found to be higher in the colonic gland of Smad5^IEC mice (L) compared with controls (K). Magnification ×40,000 (A–D) and ×40 (E–K). D, desmosomes.
1B); conversely, no difference in colonic gland morphology was observed in Smad5−/− mice (Fig. 1E) compared with controls (Fig. 1D). Measurement of crypt and villus length in Smad5−/− compared with control littermates revealed a significant 1.15-fold increase in jejunal villus length in Smad5−/− compared with control (Fig. 3, A and C). Statistical analysis of the number of positive BrdU cells in both tissues revealed no significant variation in proliferative cells between Smad5−/− and control mice (Fig. 3E). Migration assays with a 48-h BrdU pulse before death showed an increase in epithelial cell migration rate along the jejunal crypt/villus axis in Smad5−/− mice (Fig. 3G) compared with control littermates (Fig. 3F). Conversely, migration assays with a 12-h BrdU pulse before death revealed no modulation of the migration rate of colonic epithelial cells between Smad5−/− (Fig. 3F) and control mice (Fig. 3H). Statistical analysis of the distance of epithelial migration in Smad5−/− mice compared with control littermates showed a significant 1.22-fold increase in intestinal epithelial migration in Smad5−/− mice but no modulation in colonic glands (Fig. 2J). 

Apoptosis assays performed by TUNEL immunostaining on small intestine and colon revealed no significant differences in the number of apoptotic cells in the epithelium of either tissue in Smad5−/− mice (Fig. 2, L and N) compared with control (Fig. 2, K and M). Statistical analysis of the number of TUNEL-positive cells in both tissues corroborated the absence of modulation of apoptosis between Smad5−/− and control mice (Fig. 2O).

Deregulation of the epithelial AJC and dephosphorylation of cofilin in mice deficient for epithelial Smad5. Constant reorganization of TJ and AJ is a characteristic feature of processes involving epithelial cell migration and maintenance of epithelial barrier integrity in the gut (10, 33, 47, 57). These junctions are positioned on the apical side of the lateral cell membrane and form the AJC. To analyze the potential modification of the AJC in Smad5−/− mice, ultrastructural analysis was performed using electron microscopy. Ultrastructural analysis exposed the presence of a less tight TJ and shallow adhesion plaques at the AJ in Smad5−/− jejunum (Fig. 3B) and colon (Fig. 3D) comparatively to control mice (Fig. 3, A and C, respectively). Because it has been shown that alterations in expression or distribution of TJ proteins may impair the functionality of this junction (25, 51), immunostaining with occludin, claudin-1, claudin-2, and ZO-1 specific antibodies was performed on jejunal and colonic sections of control and mutant mice to analyze any modulation or mislocalization of proteins constituting the TJ. Analysis revealed a cytoplasmic relocalization for claudin-1 in Smad5−/− (Fig. 3, F and H) compared with control mice (Fig. 3, E and G), whereas a modest increase in claudin-2 expression was observed in both the jejenum and colon of mutant mice (Fig. 3, J and L) compared with controls (Fig. 3, I and K). Moreover, claudin-2 expression was found to be higher in the colonic gland of Smad5−/− (Fig. 3L) compared with its normal expression in controls (Fig. 3K). Finally, no modulation or mislocalization was observed for occludin or ZO-1 (data not shown).

The presence of shallow adhesion plaques at the AJ in Smad5−/− jejunum (Fig. 3B) suggested the presence of either low levels of E-cadherin in Smad5−/− mice or a mistargeting of the protein to the lateral membrane. To analyze the potential involvement of a mislocalization of E-cadherin at the lateral membrane of epithelial cells, immunostaining was performed...
on jejunal and colonic sections using an E-cadherin specific antibody. A cytoplasmic relocalization of E-cadherin was observed in Smad5<sup>ΔIEC</sup> mice (Fig. 4, B and D), whereas E-cadherin was found in the lateral membrane of control littermates (Fig. 4, A and C). Because β-catenin is known to be an E-cadherin cytoplasmic-associated protein, its localization was also investigated in our mutant mice by immunostaining with a β-catenin specific antibody on jejunal and colonic sections. Results also showed a moderate cytoplasmic relocalization for β-catenin in Smad5<sup>ΔIEC</sup> mice (Fig. 4, F and H), whereas β-catenin was found only in the lateral membrane in control animals (Fig. 4, E and G).

To evaluate the expression level of these AJC proteins, Western blot analysis was performed on total extracts of jejunal and colonic mucosa. Results revealed a significant reduction in claudin-1 and an increase in claudin-2 expression levels in Smad5<sup>ΔIEC</sup> mice compared with controls in both jejunum and colon (A and C, respectively). Claudin-3, claudin-4, occludin, E-cadherin, and β-catenin protein expression was not modulated by the loss of Smad5 in the intestinal epithelium (Fig. 5, A and B) and colonic mucosa (Fig. 5, C and D).

The dynamic turnover of the actin cytoskeleton has previously been shown to play a role in AJC disassembly and cell migration (34). Coflin is an actin-binding factor required for the polymerization of actin filaments. In its phosphorylated form, coflin is unable to bind actin, whereas dephosphorylation reactivates the actin-depolymerizing activity of coflin (1). The dephosphorylation of coflin has been shown to drive directional cell motility in epithelial cells (20). Moreover, dephosphorylation of coflin and subsequent activation of the actin-depolymerizing activity can lead to the disruption of intracellular contact with the result that E-cadherin becomes rapidly internalized to accumulate in subapical cytosolic compartments (34). To investigate the phosphorylation status of coflin-1 in Smad5<sup>ΔIEC</sup> mice, Western blot analysis was performed on total extracts of jejunal mucosa. A decrease in the phosphorylated status of coflin-1 was observed in Smad5<sup>ΔIEC</sup> mice compared with controls, a tendency that was not reflected on the total coflin-1 protein pool (Fig. 5, E and F).

Smad5 gene transcript expression is decreased in IBD patients and during mouse experimental colitis. Because the integrity of the AJC if often altered during IBD, we next investigated if a deregulation in the expression of Smad5 could be associated with the disease. A gene expression analysis was performed for Smad5 in intestinal samples collected from both CD and UC patients. The levels of Smad5 gene transcript were significantly decreased by 44% in CD samples and by 67% in UC samples compared with controls (Fig. 6A). We next inves-

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**Fig. 5.** Analysis of expression levels of apical junctional complex proteins and coflin-1 phosphorylation status in mice impaired for epithelial Smad5. Western blot analysis of total jejunal (A) and colonic (C) mucosal lysates isolated from control or Smad5<sup>ΔIEC</sup> mice revealed a significant reduction in claudin-1 and an increase in claudin-2 expression levels in Smad5<sup>ΔIEC</sup> mice in both jejunum and colon (A and C, respectively). Occludin, E-cadherin, and β-catenin protein levels were not modulated by the loss of Smad5 in either jejunal or colon (A and C, respectively). Occludin, E-cadherin, and β-catenin protein levels were not modulated by the loss of Smad5 in either jejunal or colon (A and C, respectively). Occludin, E-cadherin, and β-catenin protein levels were not modulated by the loss of Smad5 in either jejunal or colon (A and C, respectively). 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tigated whether Smad5 expression was modulated during DSS-induced colitis (48). As assessed by qPCR, Smad5 expression was decreased by 1.96-fold after 2 days and by 3.23-fold after 7 days of DSS treatment compared with untreated mice (Fig. 6B). Corroborating these data, Western blot analysis confirmed a similar reduction pattern of Smad5 protein in DSS-treated mice compared with untreated mice (Fig. 6C). However, reexpression of Smad5 protein was observed at 7 days of DSS treatment.

**Loss of intestinal epithelial Smad5 increases susceptibility to experimental colitis.** The severity of DSS-induced colitis was next assessed in Smad5−/− mice with control mice. Cohorts of Smad5−/− and control mice were provided with either water only or water containing 3% (wt/vol) DSS for 7 days (Fig. 7, A–H). Body weight change, stool consistency, and the presence of fecal blood were recorded and used to calculate a clinical score that reflected the overall disease activity index. Smad5−/− mice showed a more severe susceptibility (P < 0.05) to DSS-induced colitis at 4 days of treatment compared with control mice. By 7 days of treatment, Smad5−/− mice showed a DAI of 9.33 compared with 5.43 for control mice (Fig. 7J). Histological damage to the colonic mucosal glandular architecture was more severe in Smad5−/− mice (Fig. 7F) compared with control littermates (Fig. 7E). The severity of mucosal injury assessed microscopically revealed a histological score of 6.35 for Smad5−/− mice compared with 4.48 for control mice and correlated with the clinical score of the DAI (Fig. 7J).

Analyses of proliferation, migration, and apoptosis in the colon of DSS-treated mice revealed no significant modulation of these cell functions in mutant mice during DSS treatment (data not shown). To evaluate a possible role for Smad5 in the wound-healing processes, a DSS-water recovery treatment was performed where mutant and control mice were provided with water containing 3% (wt/vol) DSS for 7 days followed by a 5-day recovery phase with water only. At the outset, the DSS water recovery experiments in Smad5−/− mice led to a 33% death rate during the 5-day water recovery phase (Fig. 7K).

Analysis of the surviving animals following the completion of the recovery cycle showed a DAI of 2.4 for Smad5−/− mice compared with 0.83 for controls (Fig. 7J). The severity of the mucosal injury was also confirmed by a significant histological score of 5.78 for Smad5−/− mice compared with 4.90 for control mice (Fig. 7J) after 5 days of water recovery. Analysis of weight loss during the experiment demonstrated that Smad5−/− mice were not inclined to regain the weight during the recovery phase (Fig. 7K).

**DISCUSSION**

In recent years, the Bmp signaling pathway has been shown to play key crucial roles in gut morphogenesis, cell fate, and adult homeostasis (5, 24, 26). The wide range of biological responses obtained by Bmps is dependent on three downstream effectors, Smad1, Smad5, and Smad8. Despite increasing interest in gut Bmp signaling, very little is known of the specific roles played by individual Smads in intestinal epithelial cell functions and homeostasis. Herein, we revealed key roles for epithelial Smad5 within the Bmp signaling pathway in intestinal epithelial cell migration and AJC assembly. Of note, we observed a significant decrease in the level of Smad5 mRNA in samples from IBD patients and found that loss of Smad5 expression in the intestinal epithelium led to increased susceptibility to DSS-induced colitis in mice.

We and others have previously reported that loss of the upstream receptor bone morphogenetic protein receptor type IA (BmpR1a) leads to an increased lengthening of crypts and villi as well as an increase in the number of epithelial proliferating cells and crypt units per villus (5, 26). In the present study, we showed that loss of intestinal epithelial Smad5, one of the downstream effectors of the Bmp signaling pathway, led to a lengthening of the villi without any significant deregulation of crypt/villus architecture or the colonic gland. The loss of Smad5 also led to a deregulation of epithelial cell migration rate along the crypt/villus axis but did not alter epithelial cell proliferation. Epithelial migration rate in the colon on the other hand was not affected. This is in contrast to the BmpR1a epithelial KO phenotype where both cellular functions were found to be affected (5). These observations suggest that other Smad family members are likely involved or compensated for during Bmp-dependent epithelial cell proliferation.
The lengthening of the villi in the absence of either increased cell production in the crypt or decreased apoptosis as seen in Smad5\textsuperscript{AIEC} could be explained by the increased migration rate of epithelial cells along the crypt/villus axis without an accelerated extrusion rate of these cells from the apex of the villus (28). Therefore, based on the differential phenotypes observed between the upstream receptor BmpR1a (5) and the downstream effector Smad5, our findings indicate that epithelial Smad5 is crucial for Bmp-dependent regulation of intestinal epithelial cell migration but not for proliferation or apoptosis. This observation thus excludes Smad5 as a possible contributor to the repopulating of the epithelial cell pool following insult or injury to the intestinal epithelial sheet even if Bmp signaling is known to be involved in intestinal epithelial cell proliferation (5, 26). To our knowledge, this is the first report showing that
Smad5 is responsible for mediating some of the specific Bmp signals targeting intestinal epithelial cells.

The AJC consisting of the TJ and the AJ plays an active role during cell migration, polarization, and in the maintenance of epithelial barrier integrity (22, 23, 28, 30, 33, 57). The constant reorganization of these two junctional entities is a characteristic feature of processes involving epithelial cell migration in the gut (35). Moreover, due to its critical effects on epithelial intestinal cell migration, E-cadherin expression and localization have been shown to be tightly regulated in normal epithelial cells (22, 23, 28, 29, 36). Our analysis revealed that loss of Smad5 did not affect E-cadherin protein or mRNA expression but did lead to changes in relocalization of the protein from the lateral membrane to the cytoplasm of the cell. Interestingly, β-catenin, the intracellular partner of E-cadherin, followed the same localization pattern in mutant mice with no increase in its expression levels. This relocalization of E-cadherin/β-catenin suggests a possible defect in AJC assembly that could hypothetically lead to a loss of barrier function of cell-cell contact leading to the acquisition of migratory phenotypes. Of note, previous studies with intestinal epithelial cells have established a link between cytoskeleton turnover, AJC disassembly, and cell migration (27, 33–35). During this phenomenon, actin depolymerization factor (ADF/cofilin-1) plays an active role in F-actin polymerization. In its phosphorylated form, ADF/cofilin-1 is unable to bind to actin, whereas its dephosphorylation can reactivate the actin-depolymerizing activity of ADF/cofilin-1 (1). Dephosphorylation of ADF/cofilin is regulated by the slingshot (SSH) phosphatase and its phosphorylation by the LIM-kinase (32). Subsequently, depolymerization of the actin cytoskeleton leads to the disruption of intracellular contact and causes E-cadherin to become rapidly internalized (34). Our study revealed that Smad5IEC mice harbored a reduction in their intestinal phosphorylated ADF/cofilin-1 pool, suggesting an implication for a deregulation of actin depolymerization in E-cadherin internalization in Smad5IEC mice. This observation could also explain in part the increased migration rate seen in the intestinal epithelium of Smad5IEC mice. Moreover, this observation supports a mechanism explaining the decalocalization of E-cadherin into the cytoplasm of intestinal epithelial cells in the absence of Smad5. The basal level of ADF/cofilin-1 was not affected in Smad5IEC mice, thus supporting that this target was not transcriptionally dependent on Smad5. Nonetheless, it can be implied that the loss of Smad5 affects the dynamic turnover of the actin cytoskeleton through a deregulation of the phosphorylated status of ADF/cofilin-1, thus causing increased internalization of E-cadherin to the cytoplasm and leading to the disruption of the AJC and acquisition of migratory phenotypes. Similarly, activation of the Bmp signaling pathway by Bmp7 in culture neurons from Xenopus laevis growth cones has been shown to lead to decreased phosphorylation of ADF/cofilin-1 without a change in total ADF/cofilin protein thereby leading to the repulsive response of the Xenopus growth cones (62). In this latter study, Bmp7-treated cultures showed a marked attraction for the growth cones instead of the repulsion after introduction of a DN-SSH phosphatase. Whether Smad5 leads to transcriptional repression of SSH-phosphatase or transcriptional activation of the LIM-kinase in intestinal epithelial cells remains to be determined. Another important role for AJC consists in regulating gut permeability, which is dependent on the coordinated expression and interaction of proteins in the TJ (60, 61). Indeed, it has been shown that TJ proteins show alterations in distribution or expression in IBD (10). For example, claudin-1 protein has been reported to be decreased in IBD. In contrast to claudin-1, claudin-2 expression is increased in colonocytes of patients with active IBD (51). In addition to its role with the AJC, our study revealed that the colonic epithelium of Smad5IEC mice exhibited a reduction in claudin-1 and an increase in claudin-2 expression levels, thus suggesting an implication for Smad5 in the functionality of the TJ. Altogether, these observations strongly support a role for Smad5 in regulating the localization or expression of various AJC proteins and consequently participate in junctional functions such as gut permeability.

Damage and impairment of the intestinal epithelium can be observed following acute insult or in the presence of various diseases. Under normal conditions, the intestinal epithelium rapidly reestablishes itself by three successive mechanisms: restitution (migration and dedifferentiation of the epithelial cells), proliferation, and, finally, maturation and differentiation of the epithelial cells (17, 54). In chronic inflammation, disruption at various stages of this wound-healing process is often observed. Over the years, involvement of the TGF-β signaling pathway in IBD pathogenesis and the wound-healing process has been widely studied (18, 45, 46, 54). For example, prophylactic or therapeutic Bmp-7 treatment in TNBS-treated rats led to protection against inflammation and resulted in accelerated wound healing (41). However, the relationship between IBD and specific elements of Bmp signaling, such as Smad5, has not been reported. Our data suggest that Smad5 could play an essential role in regulating important components of the AJC and possibly in maintaining mucosal integrity of the intestine by controlling epithelial barrier permeability. Another
possibility may be that Smad5 influences the wound-healing process directly. The reexpression of Smad5 protein observed at 7 days in DSS-treated mice suggests a possible role for Smad5 in the restitution phase following injury. Indeed, loss of Smad5 could contribute to uncontrolled migration, hence inappropriately reestablishing the continuity of the surface epithelium after an extensive destruction. Under normal conditions, the redistribution of E-cadherin is not sufficient to influence intestinal epithelial polarization. However, following an inflammatory stress, the cytoplasmic redistribution of E-cadherin interferes with the normal function of the protein at the lateral membrane and affects the epithelial repolarization and differentiation phase of the wound-healing process, leading to a nonfunctional epithelium (47). In support of this, DSS water recovery experiments performed herein in Smad5−/−IEC mice led to a 53% death rate during the 5-day water recovery phase. Moreover, analysis of the surviving animals, after completion of the recovery cycle, revealed that mutant mice had not even begun to heal following the DSS insult, as observed by the presence of important areas denuded of epithelium in the colon.

Because Smad5 is expressed in both epithelial and mesenchymal counterparts of the intestine, it remains plausible that the reduction in Smad5 expression in samples from IBD patients may not only be restricted to the epithelial component. However, by taking advantage of genetic manipulation in mice to delete Smad5 exclusively in the epithelial compartment, we were able to assess its specific contribution during IBD pathogenesis. Specific epithelial deletion of Smad5 caused an increased susceptibility to DSS colitis, demonstrating that epithelial Smad5 is protective during gut inflammation. Globally, there are very few studies on the transcriptional regulating roles of Smad5 (38, 39, 53). One of the future challenges will be to define Smad5 gene targets during the intestinal inflammatory response.

In summary, the present data are the first to demonstrate that Smad5 is responsible for mediating some of the specific Bmp signals targeted at intestinal epithelial cells. More importantly, results demonstrate that the loss of Smad5 exclusively in the intestinal epithelial compartment promotes intestinal cell migration by disassembling the AIC through internalization of E-cadherin. This observed deficiency in epithelial Smad5 contributes to increased susceptibility to experimental colitis and impaired wound healing. Consequently, enhancing Bmp signaling through Smad5 may represent an attractive strategy for a protective role of this effector during IBD.

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DISCLOSURES

The authors make the statement that there is no conflict of interest to disclose.

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