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Epithelial Cell-Intrinsic Notch Signaling Plays an Essential Role in the Maintenance of Gut Immune Homeostasis

Yuuki Obata,*† Daisuke Takahashi,†‡ Masashi Ebisawa,†‡ Kisa Kakiguchi,§ Shigenobu Yonemura,§ Toshi Jinnohara,†‡ Takashi Kanaya,† Yumiko Fujimura,† Masumi Ohmae,† Koji Hase,†‡,※ and Hiroshi Ohno*,†‡

Intestinal epithelial cells (IECs) have important functions as the first line of defense against diverse microorganisms on the luminal surface. Impaired integrity of IEC has been implicated in increasing the risk for inflammatory disorders in the gut. Notch signaling plays a critical role in the maintenance of epithelial integrity by regulating the balance of secretory and absorptive cell lineages, and also by facilitating epithelial cell proliferation. We show in this article that mice harboring IEC-specific deletion of Rbpj (RBP-J^ΔIEC), a transcription factor that mediates signaling through Notch receptors, spontaneously develop chronic colitis characterized by the accumulation of Th17 cells in colonic lamina propria. Intestinal bacteria are responsible for the development of colitis, because their depletion with antibiotics prevented the development of colitis in RBP-J^ΔIEC mice. Furthermore, bacterial translocation was evident in the colonic mucosa of RBP-J^ΔIEC mice before the onset of colitis, suggesting attenuated epithelial barrier functions in these mice. Indeed, RBP-J^ΔIEC mice displayed increased intestinal permeability after rectal administration of FITC-dextran. In addition to the defect in physical barrier, loss of Notch signaling led to arrest of epithelial cell turnover caused by downregulation of Hes1, a transcriptional repres sor of p27^Kip1 and p57^Kip2. Thus, epithelial cell-intrinsic Notch signaling ensures integrity and homeostasis of IEC, and this mechanism is required for containment of intestinal inflammation. The Journal of Immunology, 2012, 188: 2427–2436.

The mucosal surface of the gastrointestinal tract is continuously exposed to a profusion of foreign Ags including food Ags, food-borne pathogens, and commensal bacteria. A single layer of columnar epithelial cells covering the intestinal mucosa serves as a barrier to protect the host from invasion by potential pathogens, as well as a sentinel to warn of their presence. Epithelial stem cells located at the crypt bottom continuously divide to produce transit-amplifying cells, which can then divide several times before creating terminally differentiated epithelial cell lineages: absorptive enterocytes and three types of secretory epithelial cells, namely, goblet cells, enteroendocrine cells, and Paneth cells. Under physiological conditions, the cellular composition of the intestinal epithelium is kept in balance; however, disruption of this homeostatic state has been associated with various gastrointestinal diseases including ulcerative colitis (UC) (1), experimental colitis induced by dextran sodium sulfate (DSS) (2), and an infection model with Citrobacter rodentium (3, 4). These findings raise the possibility that the balanced composition of the four types of intestinal epithelial cells (IECs) is essential for the maintenance of intestinal homeostasis and for host defense functions. Notch functions as receptors for transmembrane ligands, Jagged and Delta-like proteins, to regulate a broad spectrum of cell fate decisions. Upon ligand activation, Notch receptors undergo γ-secretase–mediated proteolytic cleavage to release the Notch intracellular domain (NICD). The liberated NICD translocates into the nucleus, where it forms a transcriptional activator complex with recombination signal binding protein for Ig κ J region (RBP-J) to regulate the Notch signaling pathway (5). In the intestinal epithelium, this complex activates Notch target genes such as Hes1, which, in turn, represses the expression of Math1, a master regulator for differentiation of secretory cell lineages (6, 7). Consequently, activation of epithelial Notch signaling suppresses the differentiation of secretory cell lineages (8), and thus plays a central role in the binary cell fate decision of intestinal progenitors between the secretory and absorptive lineages.

Previous studies with a γ-secretase inhibitor and transient loss of function of RBP-J have demonstrated that inactivation of Notch signaling results in conversion of proliferating progenitors into postmigratory goblet cells (9). Conversely, transgenic expression of NICD inhibits secretory cell differentiation with a reciprocal increase in immature progenitors (8). These observations support the biological significance of Notch signaling not only in the binary cell fate decision but also in the maintenance of the proliferating progenitors in the crypts of the intestinal epithelium.

By contrast, the contribution of Notch signaling to epithelial defense functions remains elusive. Notch signaling within the
intestinal epithelium is upregulated in the inflamed mucosa of UC patients and in DSS-treated mice. This is thought to be important for epithelial regeneration in these diseases (10), and inhibition of Notch activation at the late stage of colitis development in the DSS model exacerbates disease by impairing epithelial regeneration (10). In contrast, inhibition of Notch signaling in early-stage DSS colitis alleviates acute inflammation by preventing the typical decrease in the number of goblet cells seen in this model (11). Thus, it remains controversial whether Notch signaling participates in the development or prevention of colitis.

To better understand the role of Notch signaling in epithelial defense functions and gut immune homeostasis, we have generated mice in which there is an IEC-specific deletion of the Rhpj gene (RBP-J^{flox}). In this article, we report that RBP-J^{flox} mice spontaneously develop Th17-dominant chronic colitis. Bacterial translocation into colonic mucosa preceded the development of colitis, suggesting an impaired epithelial defense against bacteria on the colonic mucosa of these mice. This defect was remarkable in the crypt region, where there was goblet-cell hyperplasia and epithelial turnover was significantly retarded by the absence of Notch signaling. Our findings underscore the importance of Notch signaling in epithelial defense functions by securing the epithelial integrity and rapid turnover that is required for the maintenance of gut immune homeostasis.

Materials and Methods

Animal experiments

Mice carrying a floxed Rhpj allele (RBP-J^{flox}) (12) were obtained from the RIKEN BioResource Center. To generate RBP-J^{flox} mice, we crossed RBP-J^{flox} mice with Villin-Cre transgenic mice obtained from The Jackson Laboratory. RBP-J^{flox} mice and control RBP-J^{flox} littermates were maintained under specific pathogen-free (SPF) or conventional (CV) conditions at the RIKEN or Yokohama City University animal facilities, respectively. To deplete commensal bacteria, we maintained 12- to 14-wk-old RBP-J^{flox} and RBP-J^{flox} mice under CV conditions, and they received a 4-mM acetic acid solution containing 1 g/l ampicillin sodium, 0.5 g/l vancomycin hydrochloride, 1 g/l neomycin sulfate, and 1 g/l metronidazole (all from Wako Pure Chemical Industries) as drinking water for 5 wk. All studies were approved by the Animal Research Committees of RIKEN Yokohama Research Institute and Yokohama City University.

Histology

Prefixed colonic tissue sections were deparaffinized, rehydrated, and stained with either H&E or Alcian blue-nuclear fast red. The specimens were histologically examined for scoring the degree of colitis based on the following criteria: inflammatory infiltrates, mucosal hyperplasia, mucosal erosion and/or ulceration, and loss of goblet cells.

Immunostaining

Frozen sections of colonic tissue were fixed with cold acetone for 10 min. Background signals were blocked by incubating the sections in 5% normal serum in PBS from the same species as the secondary Abs. The sections were then incubated overnight at 4˚C with anti-CD4 (H129.19), anti-CD11c (HL3), biotinylated anti-CD11b (M1/70; all from BD Biosciences); anti–myeloperoxidase (anti-MPO; Abcam) and anti-lysozyme (DAKO Cytomation) polyclonal Abs or an identical concentration of control rat IgG overnight at 4˚C. The binding of primary Ab was detected with biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch) followed by streptavidin-HRP (ABC Elite; Vector Laboratories), and was visualized with 3,3’-diaminobenzidine (Dako Cytomation). The tissue sections were counterstained with hematoxylin.

For lysozyme and chromogranin A immunostaining, prefixed small and large intestinal sections were treated and analyzed as described earlier, except that rabbit anti-lysozyme (Dako Cytomation) and anti-chromogranin A (ImmunoStar) polyclonal Abs or an identical concentration of control rat IgG were used as primary reagents.

Preparation of lamina propria mononuclear cells

Colonic tissue was fragmented into 2- to 3-cm segments and then incubated in HBSS containing 20 mM EDTA and 2% FCS at 37˚C for 20 min to remove epithelial cells. The segments were further minced and dissociated with RPMI 1640 medium containing 20 mM HEPES (pH 7.4), 2% FCS, 0.5 mg/ml collagenase (Wako Pure Chemical Industries), 250 U/ml Dispase (BD Biosciences), and 0.5 mg/ml DNase I (Roche) at 37˚C for 30 min. After this incubation, lamina propria mononuclear cells (LPMCs) were fractionated on discontinuous Percoll gradients as described previously (14).

Flow cytometry

LPMCs were preincubated with anti-CD16/CD32 mAb (93; eBioscience) for FcγR blocking, followed by incubation with FITC-anti-CD11b (M1/
70), PE–anti-CD11c (HL3), V500–anti-CD3ε (500A2), and allophycocyanin–anti-CD4 (GK1.5; eBioscience) mAbs.

For intracellular cytokine staining, LPMCs were cultured for 6 h in complete medium (RPMI 1640 containing 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 55 µM mercaptoethanol, and 20 mM HEPES (pH 7.2)) supplemented with 50 ng/ml PMA, 500 ng/ml ionomycin, and GolgiPlug (BD Pharmingen) in 12-well plates. The LPMCs were then stained with V500–anti-CD3ε (500A2), Pacific blue–anti-CD4 (RM4-5), and PE–anti-IL-17A (TC11-18H10), PE–Cy7–anti–IL-4 (BVD6-24G2; eBioscience) mAbs. All mAbs were obtained from BD Biosciences unless specified otherwise.

The stained samples were analyzed using a FACS CantoII (BD Biosciences), and the obtained raw data were further analyzed using FlowJo software version 8.8.7 (Tony Digital Biology).

Quantitative PCR
Total RNA was isolated from colonic tissues with the RNeasy mini kit (Qiagen) and was subjected to reverse transcription using the ReverTra Ace kit (Toyobo), following the manufacturer’s instructions. The cDNA samples were amplified with the SYBR premix Ex Taq (Takara Bio) and the primer sets specific for mouse genes. The sequences of the primer sets are available upon request. Target gene expression was assessed by a comparative cycling threshold method, using expression of Actb as the control, according to the manufacturer’s protocol (Thermal Cycler Dice Real Time System; Takara Bio).

Bacterial genomic DNA was isolated as described previously with minor modifications (15). In brief, fecal samples were freeze-dried, homogenized, and disrupted using 0.1 mm Zirconia/Silica Beads, and were extracted with 5 mM Tris–HCl buffer (pH 7.2) solution containing 5% SDS and 5 mM EDTA. Bacterial genomic DNA was purified from the fecal extracts by a phenol/chloroform/isoamyl alcohol method. Bacterial genomic DNA samples were amplified with a Cycler Dice Real Time System using the SYBR premix Ex Taq (Takara Bio) and the primer sets specific for bacterial 16S rDNA (16).

Fluorescence in situ hybridization
Fluorescence in situ hybridization (FISH) analysis for bacterial 16S rRNA was performed as described previously (17). In brief, frozen sections of colonic tissues were fixed in 4% paraformaldehyde and then incubated in 50 µl of 30% formamid hybridization buffer containing 25 ng of 5′-Cy3-labeled oligonucleotide probes for universal eubacterial EUB338 (5′-gct ggc tcc cgt agg agt-3′) or control NONEB (5′-act cct cag gga ggc acg-3′) for 90 min at 46°C. After washing at the same stringency for 20 min at 48°C, the specimens were then mounted and analyzed using a BX51 fluorescence microscope (Olympus).

Transmission electron microscopy
Colonic tissues were fixed with 2% fresh formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature. After washing with 0.1 M cacodylate buffer (pH 7.4) three times (5 min each), they were postfixed with ice-cold 1% OsO4 in the same buffer for 2 h. The postfixed samples were rinsed with distilled water, stained with 0.5% aqueous uranyl acetate for 2 h or overnight at room temperature, dehydrated with ethanol, and embedded in Poly/Bed 812. Ultrathin sections were doubly stained with 4% uranyl acetate and 0.1% lead citrate, and viewed with a JEM 1010 transmission electron microscope (JEOL) at an accelerating voltage of 100 kV.

**FIGURE 2.** RBP-JΔIEC mice spontaneously develop colitis. (A–C) Mice were maintained under SPF conditions for 20–26 wk after birth (A, B), or SPF conditions for 12–14 wk followed by CV conditions for 2 wk (C). Macroscopic view of rectal prolapse in RBP-JΔIEC mice (B). Colonic tissue sections were stained with H&E for histological examination (A, C, upper panels) or with Alcian blue–nuclear fast red for detection of mucus (turquoise)-producing goblet cells (A, C, middle panels). Immunofluorescence staining of the distal colon from colitis-positive SPF RBP-JΔIEC mice (A) and CV RBP-JΔIEC mice (C) was performed using polyclonal Abs against MPO (green), and nuclei were counterstained with DAPI (blue) (A, C, lower panels). Scale bars, 200 µm. Data are representative of three independent experiments. (D) The number of MPO⁺ cells per mucosal area (1 mm²) was quantified. Values are mean ± SD (n = 3). *p < 0.05 (Student t test). (E) The histological colitis score was calculated based on the criteria described in Materials and Methods. Values are mean ± SD of 28 (SPF) and 8 (CV) mice. *p < 0.05, **p < 0.01 (Mann–Whitney U test).
**In vivo epithelial barrier permeability**

The in vivo assay of barrier function was performed using a FITC-labeled dextran method. In brief, 12-wk-old SPF mice fasted for 4 h were rectally administered with 4 kDa FITC-dextran (1 mg/g body weight) as a permeability tracer. Blood was collected after 1 h, and serum fluorescence intensity was measured using a Fusion-α microplate analyzer (Packard BioScience). Concentrations of FITC-dextran were calculated from standard curves generated by serial dilution of FITC-dextran.

**EdU-labeling study**

A 5-ethyl-uridine (EdU)-labeling study was performed using Click-iT EdU Imaging Kits (Invitrogen), following the manufacturer’s instructions. RBP-JΔIEC and RBP-JF/F mice were sacrificed at 4, 96, and 144 h after i.p. injection of 1 mg/mouse EdU. Frozen sections of colonic tissue were fixed with 4% paraformaldehyde for 15 min at room temperature. After washing with 3% BSA in PBS, the colonic tissue sections were incubated with 0.5% Triton X-100 in PBS for 20 min at room temperature. EdU-labeled cells were visualized using Click-iT reaction cocktails (Invitrogen). The specimens were incubated with Hoechst 33342 to visualize nuclei, then mounted in VectorShield (Vector Laboratories) and analyzed using a BX51 fluorescence microscope (Olympus).

**Statistical analysis**

Differences between two groups were analyzed by Student t test. When variances were not homogeneous, the data were analyzed by Mann–Whitney U test. Differences among more than two groups were analyzed by one-way ANOVA followed by Dunnett test. When variances were not homogeneous, the data were analyzed by Kruskal–Wallis test.

**Results**

**RBP-JΔIEC mice spontaneously develop colitis**

To directly assess the role of epithelial Notch signaling in intestinal homeostasis, we generated RBP-JΔIEC mice and maintained them under SPF conditions. These mice were born at the expected Mendelian ratio. Histological analysis of 6-wk-old RBP-JΔIEC mice demonstrated that the number of secretory cell lineages was increased in both small and large intestines (Fig. 1A, 1B), an observation consistent with a previous observation in mice with inducible RBP-J deficiency (9). Goblet cell hyperplasia was particularly obvious in the colon of RBP-JΔIEC mice (Fig. 1B). In association with these anomalies, 20- to 26-wk-old RBP-JΔIEC mice had an aberrant accumulation of mucus under the tunica serosa (Fig. 1C, arrowheads). Furthermore, nearly half of the RBP-JΔIEC mice spontaneously developed colitis characterized by thickening of the colonic wall (Fig. 2A) and rectal prolapse (Fig. 2B). Immunofluorescent staining of the colonic tissue demonstrated infiltration of MPO⁺ cells in RBP-JΔIEC mice, but not in control RBP-JF/F mice (Fig. 2A, lower panels). These data indi-

![FIGURE 3. Expression of cytokine mRNA in colonic tissue. Q-PCR analysis of colonic tissue from 14- to 18-wk-old CV RBP-JΔIEC mice and control RBP-JF/F littermates was performed using specific primers for the indicated genes. Data were normalized to expression of Actb (encoding B-actin). Values are mean ± SD (n = 5). *p < 0.05 (Student t or Mann–Whitney U test).](http://www.jimmunol.org/)

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cate that RBP-J^ΔIEC mice have a defect not only in regulation of IEC differentiation, but also in maintenance of gut homeostasis.

In multiple experimental models of colitis and human inflammatory bowel diseases (IBDs), environmental factors such as the microbiota have been shown to be involved in the development of inflammatory response in the colon (18, 19). To explore the potential role of the intestinal microbiota in colitis development in RBP-J^ΔIEC mice, we maintained them under CV condition. Strikingly, all of the CV RBP-J^ΔIEC mice developed spontaneous colitis. Histological analysis of these mice showed a loss of goblet cells and a massive cellular infiltration into the colonic mucosa and submucosa (Fig. 2C, upper and middle panels). Immunofluorescent staining of the colonic tissue demonstrated massive infiltration of MPO^+ cells in RBP-J^ΔIEC mice, but not in control littermates (Fig. 2C, lower panels). Based on histological scores, RBP-J^ΔIEC mice developed more severe colitis in CV than in SPF conditions (Fig. 2D, 2E). Hereafter, we mainly sought to characterize the immunopathogenesis of spontaneous colitis in CV RBP-J^ΔIEC mice.

**CD11b^+ cells and CD4^+ T cells accumulate in the colonic lamina propria of RBP-J^ΔIEC mice**

For the initial characterization of the inflammatory response in the colonic tissue of RBP-J^ΔIEC mice, we measured cytokine expression profiles. Quantitative real-time PCR (Q-PCR) analysis demonstrated that transcripts of proinflammatory cytokine genes Il1b, Il6, Il17a, Tnfsf1a, and Ifng were significantly upregulated in the RBP-J^ΔIEC mice compared with control RBP-J^F/F littermates (Fig. 3). Of note, these proinflammatory cytokine genes, except Ifng, were also significantly upregulated in colitis-positive SPF RBP-J^ΔIEC mice (Supplemental Fig. 1). Moreover, the expression of inducible NO synthase, which has been shown to be increased in the mucosa of IBD patients (20), was also highly upregulated in the RBP-J^ΔIEC mice. These data confirmed that ablation of epithelial Notch signaling leads to chronic inflammation in the colon.

We subsequently examined the cellular composition of the inflammatory infiltrates in the colonic tissue. Immunofluorescent staining of the colonic tissue demonstrated massive infiltration of CD4^+ T cells, CD11c^+ dendritic cells (DCs), and CD11b^+ cells in RBP-J^ΔIEC mice, but not in control mice (Fig. 4A). FACS analysis also confirmed a marked increase in the total number of CD4^+ T cells, CD11c^+ DCs, and CD11b^+ cells (Fig. 4B), as well as in the percentage of CD11b^+ cells and CD4^+ T cells (Supplemental Fig. 2A, 2B). In contrast, the proportion of B220^+ B cells in the lamina propria of RBP-J^ΔIEC mice was decreased (Supplemental Fig. 2A, 2B). To determine the phenotype of these CD4^+ T cells, we performed intracellular staining of representative T cell cytokines IL-17A, IFN-γ, TNF-α, and IL-4. The frequency of IL-17A–expressing CD4^+ T cells was increased >2-fold in the RBP-J^ΔIEC mice compared with littermate controls (Supplemental Fig. 2C, 2D), whereas the frequency of cells producing the other cytokines was unchanged. These results indicate that RBP-J^ΔIEC mice develop Th17-dominant chronic colitis.

**Translocation of commensal microbiota into the colonic mucosa causes chronic inflammation in RBP-J^ΔIEC mice**

Previous studies have shown that human IBDs result from inappropriate and ongoing activation of the mucosal immune system driven by the commensal microbiota (21). To test this possibility, we initially compared the composition of fecal bacteria between RBP-J^ΔIEC mice and control littermates housed together under CV condition. Q-PCR analysis using primer sets for specific groups of commensal bacteria, such as SFB, Bacteroides, Clostridiales, and Lactobacillaceae, revealed the existence of comparable levels of these bacterial groups in RBP-J^ΔIEC and littermate controls (Supplementary Fig. 3), suggesting that the development of spontaneous colitis is not attributed to the alteration of intestinal microbiota. Therefore, we next analyzed the localization of commensal bacteria by FISH in colitis-free 12- to 14-wk-old CV RBP-J^ΔIEC mice and control RBP-J^F/F littermates was performed using mAbs against CD4 (green), CD11b (red), and CD11c (green). Nuclei were counterstained with DAPI (blue). Scale bars, 200 μm. Data are representative of three independent experiments. (B) The total number of colonic lamina propria-infiltrating CD4^+ T cells, CD11c^+ DCs, and CD11b^+ cells of CV RBP-J^ΔIEC mice and control RBP-J^F/F littermates was quantified by FACS analysis. Values are mean ± SD (n = 5). *p < 0.05 (Student t test).

**FIGURE 4.** Characterization of infiltrating cells in the colonic lamina propria. (A) Immunofluorescence staining of the distal colon from 14- to 18-wk-old CV RBP-J^ΔIEC mice and control RBP-J^F/F littermates was performed using mAbs against CD4 (green), CD11b (red), and CD11c (green). Nuclei were counterstained with DAPI (blue). Scale bars, 200 μm. Data are representative of three independent experiments. (B) The total number of colonic lamina propria-infiltrating CD4^+ T cells, CD11c^+ DCs, and CD11b^+ cells of CV RBP-J^ΔIEC mice and control RBP-J^F/F littermates was quantified by FACS analysis. Values are mean ± SD (n = 5). *p < 0.05 (Student t test).
directly, we gave RBP-J$_{ΔIEC}$ mice under CV condition a combination of broad-spectrum antibiotics via their drinking water for 5 wk. Histological analysis indicated that the depletion of commensal microbiota alleviated the intestinal inflammation (Fig. 5B, 5C). Infiltration of CD4+, CD11b+, CD11c+, and MPO+ cells in the colonic lamina propria was also markedly decreased by the antibiotic treatment (Fig. 5D–F). These observations suggest that the aberrant bacterial translocation into the colonic mucosa leads to recruitment and activation of inflammatory cells, resulting in chronic inflammation of the colon in RBP-J$_{ΔIEC}$ mice.

A physical barrier is defective in RBP-J$_{ΔIEC}$ mice

The results presented earlier raise the question as to how luminal bacteria translocate into the colonic mucosa. We hypothesized that RBP-J$_{ΔIEC}$ mice have a defect in epithelial-intrinsic barrier functions, such as formation of physical barrier or production of antimicrobial proteins. To test these possibilities, we initially analyzed the formation of intercellular junctional complexes, namely, tight junctions (TJs) and adherens junctions (AJs), which are responsible for the physical barrier. Because inflammatory stimuli frequently induce physical barrier dysfunction (22), we analyzed colonic epithelial cells from 12- to 14-wk-old SPF RBP-J$_{ΔIEC}$ and control RBP-J$_{f/f}$ littermates by immunofluorescent staining and transmission electron microscopy (TEM). Immunofluorescent staining of the distal colon was performed using polyclonal Abs against MPO (green), and nuclei were counterstained with DAPI (blue). The number of MPO+ cells per mucosal area (1 mm$^2$) was quantified. Values are mean ± SD ($n$ = 3). *$p < 0.05$ (Student $t$ test). Immunostaining of the distal colonic sections from the same mice as (B). (F) The total number of colonic lamina propria-infiltrating CD4$^+$ T cells, CD11c$^+$ cells, and CD11b$^+$ cells of CV RBP-J$_{ΔIEC}$ mice and control RBP-J$_{f/f}$ littermates was quantified by FACS analysis. Values are mean ± SD ($n$ = 5). *$p < 0.05$, **$p < 0.01$ (Student $t$ test). Scale bars, 100 μm (A, B, right panel, and E) and 200 μm (B, left panel).
control mice. To further examine the functional integrity of the colonic epithelial barrier, we analyzed control and RBP-JΔIEC mice for systemic translocation of rectally administered, FITC-labeled dextran. We observed significant increase in serum levels of FITC-labeled dextran in RBP-JΔIEC mice compared with control littermates, indicative of increased colonic permeability (Fig. 6C). Together, these data indicate that deficiency of RBP-J impairs epithelial barrier functions conceivably by establishment of leaky junctions.

We subsequently examined the expression of antimicrobial proteins in colonic epithelium. The expression of the genes encoding β-defensins, cathelicidin, angiogenins, CCL6, RegIIIα, and RegIIIβ in the colonic epithelium of RBP-JΔIEC mice was normal (Supplemental Fig. 4A). Interestingly, the expression of α-defensins, lysozyme-1, and phospholipase A2, group IIA, all of which are known to be secreted by Paneth cells (23, 24), was remarkably upregulated in the colonic epithelium of RBP-JΔIEC mice (Supplemental Fig. 4B). Although Paneth cells exclusively localize in the small intestine and not the colon (25), our immunostaining of lysozyme identified Paneth-like cells in colonic epithelium of RBP-JΔIEC mice (Supplemental Fig. 4C). The ectopic appearance of Paneth-like cells in the colon of these mice is similar to the pathological change called Paneth cell metaplasia seen in patients with UC (26). The expression of antimicrobial proteins in small intestinal epithelium of RBP-JΔIEC mice was also normal, although α-defensin expression was upregulated in RBP-JΔIEC mice (Supplemental Fig. 4D). It is plausible that upregulation of α-defensins results from Paneth cell hyperplasia in the small intestine (Fig. 1A, middle panels). Collectively, ablation of RBP-J unlikely attenuates the production of antimicrobial proteins by IECs.

**RBP-JΔIEC mice have a defect in epithelial regeneration**

The rapid turnover of IECs because of vigorous proliferation of epithelial progenitors is thought to be an important innate defensive mechanism, which contributes to expel colonized pathogens, confines bacterial spreading, and localizes inflammation (27–30). Consistent with previous studies (31), Ki67+ proliferating cells were abundant at the bottom of virtually all the crypts in control mice, whereas Ki67+ proliferating cells were nearly absent in multiple crypts of RBP-JΔIEC mice (Fig. 7A, 7B). The lack of proliferating cells was remarkable in the crypt areas with prominent goblet cell hyper-
Hes1+ cells in the bottom of every crypt in control mice; these data also raise the possibility that RBP-JΔIEC mice are defective in epithelial cell turnover. We therefore performed an in vivo EdU incorporation assay. For the quantification of the rate of colonic epithelial turnover, we counted the number of EdU+ cells per crypt at early (4 h after i.p. injection of EdU) and late (96 and 144 h after i.p. injection of EdU) phases of colonic turnover. Consistent with Ki67-staining data, the number of EdU+ cells was significantly reduced in the colonic epithelium of RBP-JΔIEC mice at early phase (Fig. 8A). Subsequently, upward migration of EdU-labeled cells was slower in multiple crypts of RBP-JΔIEC mice compared with those in RBP-JIEC mice at late phases (Fig. 8B, 8C). Thus, ablation of epithelial Notch signaling results in impaired epithelial turnover, leading to the defects in the innate defense and the healing of intestinal epithelium.

**Discussion**

We have demonstrated that epithelial Notch signaling contributes to maintenance of the rapid epithelial cell turnover, whose breakdown leads to colonic inflammation caused by persistent bacterial colonization and impaired healing of inflamed mucosa. We observed that exposing RBP-JΔIEC mice to a CV environment predisposes them to more severe colitis mediated by Th17-dominant immune response. Antibiotic depletion of intestinal bacteria prevented the inflammatory response in RBP-JΔIEC mice. These observations imply that intestinal microbiota play a significant role in colitis development. The spontaneous colitis in RBP-JΔIEC mice resembles the disease observed in mice with IEC-specific deletion of protein O-fucosyltransferase 1 (Pofut1ΔIEC mice) (33). Because Pofut1 mediates O-fucosylation of Notch, which is required for ligand-binding activity (34), the ablation of Pofut1 results in defective Notch signaling, leading to goblet cell hyperplasia. Pofut1ΔIEC mice develop spontaneous colitis as late as 9 mo after birth. The colitis in Pofut1ΔIEC mice was at least partially explained by an alternation in the intestinal microbiota, particularly the accumulation of spiral-shaped bacteria in the colonic crypts. By contrast, we did not observe accumulation of the spiral-shaped bacteria in RBP-JΔIEC mice at any age; moreover, the composition of commensal bacteria was comparable between RBP-JΔIEC mice and control littermates. Thus, colitis development in RBP-JΔIEC mice is unlikely due to any major alternations in the intestinal microbiota. The chronic inflammatory response in these mice most likely arises from the aberrant bacterial translocation into the colonic mucosa caused by retarded epithelial turnover.

IEC barrier dysfunction has been implicated in increasing the risk for inflammatory disorders in the gut (22). Likewise, RBP-JΔIEC mice displayed abnormal bacterial translocation into the colonic mucosa. Under normal physiological conditions, secretory lineages of IECs express Notch ligands on their cell surface and activate Notch signaling in juxtaposing cells to inhibit their differentiation into secretory cell lineages. Because of this lateral inhibition, secretory cells such as goblet cells are not normally found adjacent to each other (35). Goblet cells are able to form functional TJs with absorptive enterocytes, although TJs formed between absorptive cells are different from those formed between goblet cells and absorptive enterocytes. In support of this notion, RBP-JΔIEC mice displayed increased permeability to rectally administrated FITC-labeled dextran, even though TEM analysis demonstrated that
expression of p27Kip1 is restricted at the villi and not the crypts in mice, with massive goblet cell hyperplasia. Scale bars, 200 μm. At each time point, three mice per group were analyzed, and representative staining data are shown. For quantification analysis, 20 crypts per mouse are randomly selected, and the number of EdU+ cells per crypts was counted. **p < 0.01 (Mann–Whitney U test).

Diverse environmental factors including physical stress, digestive enzymes, drugs, and infectious agents cause epithelial cell damage in the gut. To deal with the damage, epithelial progenitors vigorously proliferate to totally renew IECs within 3–4 d (K. Hase, unpublished observations). This rapid turnover of IECs enables prompt wound healing after removal of damaged cells. Mice with a heterozygous deletion of Klf5, a transcription factor responsible for proliferation of crypt cells, exhibited increased sensitivity to DSS because of a slower rate of proliferation and migration of epithelial cells in the region of injured mucosa (38). The rapid epithelial turnover is also essential to expel invasive intestinal pathogens such as Trichuris trichiura and Shigella flexneri (28, 29), indicating that epithelial cell turnover contributes to host defense functions. Therefore, if these mechanisms are impaired during the epithelial renewal, there is an increased risk for epithelial injury, microbial infection, and colonization (27). In RBP-JΔIEC mice, epithelial proliferation is nearly arrested around the lower crypt region (10, 39). Activation of Notch signaling results in upregulation of CDK inhibitors, which render epithelial progenitor cells cell cycle arrested. In addition, HES1 serves as a transcriptional repressor for MATH1, a basic helix-loop-helix transcription factor. Because expression of MATH1 in epithelial progenitor cells facilitates their differentiation into the secretory lineages and a reciprocal decrease in proliferating progenitors (35). This model is consistent with our observation that reduction of proliferating cells is frequently observed in areas of severe goblet cell hyperplasia in RBP-JΔIEC mice. Notably, bacterial translocation into the colonic lamina propria is also detected in the same areas. Based on these data, we propose that the defect in epithelial cell turnover affects the epithelial defense mechanisms and enhances bacterial translocation into the colonic mucosa. The increased bacterial burden leads to recruitment and activation of inflammatory cells, resulting in chronic inflammation of the colon in RBP-JΔIEC mice. Our data demonstrated that epithelium-intrinsic Notch signaling plays an important role in intestinal immune homeostasis by maintaining epithelial defense mechanisms.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Expression of cytokine mRNA in colonic tissue. Q-PCR analysis of colonic tissue from 14-18-week-old SPF RBP-J**IEC** mice with or without the sign of colitis and control RBP-J**F/F** littermates was performed using specific primers for the indicated genes. After histological examination, individuals with total histological score of 2 or more were considered to be positive for colitis. Data were normalized to expression of Actb (encoding b-actin). Values are mean ± SD of 8 (control), 4 (colitis negative) and 5 (colitis positive) mice. Differences among three groups were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett test. When variances were not homogeneous, the data were analyzed by the Kruskal Wallis test. *p < 0.05, **p < 0.01 (Dunnett or Kruskal Wallis test).
Supplemental Figure 2. Flow cytometric analysis of inflammatory cells in the colonic tissues of RBP-JΔIEC mice. (A) LPLs isolated from 14-18-week-old CV RBP-JΔIEC mice and control RBP-JF/F littermates were characterized by their surface expression of CD3ε, CD4, CD11b, CD11c and B220. Data are representative of three independent experiments. (B) The frequency of CD11b+ cells, B220+ cells and CD4+ T cells in colonic lamina propria cells are shown as mean ± SD (n = 3). ** p < 0.01 (Student’s t test). (C) CD4+ T cells from colonic lamina propria of 14-18-week-old mice were analyzed for cytokine-expression profiles by intracellular cytokine staining. Data are representative of three independent experiments. (D) The frequency of CD4+ T cells expressing the indicated cytokines is shown as mean ± SD (n = 3). *p < 0.05 (Student’s t test).
Supplemental Figure 3. The composition of commensal bacteria is comparable between RBP-J<sup>ΔIEC</sup> mice and control littermates. The composition of commensal bacteria in these mice was analyzed by Q-PCR using primer sets for specific groups of indicated bacteria. Data were shown as normalized value to the amount of total bacteria that was detected using primers for pan-eubacteria. Values are mean ± SD (n = 3).
Supplemental Figure 4

A

Gene / Actb

(x10^-3)  Def b1  (x10^-3)  Def b3  (x10^-3)  Def b4  (x10^-3)  Ccl6  (x10^-3)  Camp

B

Gene / Actb

(x10^-4)  Pla2g2a  (x10^-4)  Lysozyme 1  (x10^-4)  Defa (all cryptidins)

C

RBP-J^+/^-  RBP-J^dEC
Supplemental Figure 4. Expression of antimicrobial products in the intestinal epithelium of SPF RBP-J-IEC mice and control RBP-J-F/F littermates. (A) The expression of the indicated genes in colonic epithelial cells was analyzed by Q-PCR using specific primers. Data were normalized to the expression of Actb. Values are mean ± SD (n = 6). (B) mRNA expression of Paneth cell-specific antimicrobial genes in colonic epithelial cells was analyzed by Q-PCR. Data were normalized to the expression of Actb. Values are mean ± SD (n = 6). *p < 0.05, **p < 0.01 (Mann-Whitney U test). (C) Colonic tissue sections were stained with anti-lysozyme polyclonal antibodies to detect Paneth cell metaplasia (brown). Scale bars represent 200 μm. Data are representative of two independent experiments. (D) Expression of antimicrobial products in the small intestinal epithelium of SPF RBP-J-IEC mice and control RBP-J-F/F littermates. The expression of the indicated genes in small intestinal epithelial cells was analyzed by Q-PCR using specific primers. Data were normalized to the expression of Actb. Values are mean ± SEM (n = 8). *p < 0.05 (Student’s t test).