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Original Article

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Cooperation of Wnt/β-catenin and Dll1-mediated Notch pathway in Lgr5-positive intestinal stem cells regulates the mucosal injury and repair in DSS-induced colitis mice model

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Abstract

Background: Lgr5-positive cells located in the basal layer of crypts have self-regenerative and proliferative differentiation potentials of intestinal stem cells (ISCs), maintaining a balance of regeneration–repair in mucosal epithelium. However, the mechanisms of mucosal repair that are regulated by ISCs in ulcerative colitis (UC) remain unclear.

Method: Colon tissues from patients with UC were collected to test β-catenin and Notch1 expression by using Western blot and quantitative real-time polymerase chain reaction (PCR). *β-catenin*$^{fl/fl}$ mice, *β-catenin*^{Tg} mice, and *Dll1*$^{tm1\ Gos}$ mice were used to cross with *Lgr5-EGFP-IRES-creERT2* mice to generate mice of different genotypes, altering the activation of Wnt/β-catenin and Dll1-mediated Notch signaling in ISCs *in vivo*. Dextran sulfate sodium (DSS) was used to induce a colitis mice model. Intestinal organoids were isolated and cultured to observe the proliferation and differentiation levels of ISCs.

Result: β-catenin and Notch1 expression were significantly increased in the inflamed colon tissues from patients with UC. Wnt/β-catenin activation and Dll1-mediated Notch pathway inhibition in Lgr5-positive stem cells promoted the expressions of E-cadherin, CK20, and CHGA in colonic organoids and epithelium, implying the promotion of colonic epithelial integrity. Activation of Wnt/β-catenin and suppression of Dll1-mediated Notch pathway in Lgr5-positive ISCs alleviated the DSS-induced intestinal mucosal inflammation in mice.

Conclusions: Lgr5-positive ISCs are characterized by self-renewal and high dividend potential, which play an important role in the injury and repair of intestinal mucosa. More importantly, the Wnt/β-catenin signaling pathway cooperates with the Notch signaling pathway to maintain the function of the Lgr5-positive ISCs.

Keywords: intestinal stem cell; ulcerative colitis; Lgr5; β-catenin; Notch

Introduction

The characteristic pathologic changes of ulcerative colitis (UC) are absence of the mucus layer and reduction of goblet cells [1]. The order renewal, regeneration, and repair process of intestinal stem cells (ISCs) at the base of the colonic crypts are often disrupted in mucosal inflammatory injury [2]. Under physiological conditions, the intestinal epithelial cells (IECs) are replenished from ISCs every 3–4 days to maintain a continuous mucosal barrier [3] but damage to the epithelium by various factors can affect the repair process for restoring the barrier function [3, 4]. Clinically, mucosal healing is a prognostic indicator for long-term remission and reduced surgical risk for patients with UC [4, 5]. Although there are many studies on the mechanisms that maintain the dynamic balance between the inflammatory damage and regenerative repair of mucosa in inflammatory bowel disease (IBD), the exact mechanisms remain unclear.

Leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5) is an important ISCs marker and its positive cells are intestinal stem cells with multidirectional differentiation potential [6, 7]. Under the regulation of several signaling pathways, Lgr5-positive ISCs repair the damaged epithelium and maintain intestinal homeostasis upon self-renewal and differentiation [8]. It has been demonstrated that Wnt/β-catenin and Notch pathways are crucial for intestinal homeostasis. Lgr5 is the most important downstream target gene of the Wnt/β-catenin signaling pathway [6], whereas Lgr5 binds to R-spondin-1 and associates with the formation of the Wnt-Fz-LRP5/6 complex, which collectively initiates the Wnt/β-catenin signaling pathway and

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promotes the proliferation of ISCs [9–11]. Meanwhile, Notch signaling regulates the differentiation function of ISCs. The two ligands Dll1 and Dll4 mediate the activating and inhibiting effects of Notch signaling, respectively. Dll1 and Dll4 also maintain the balance of crypt regeneration and repair in the inflammation-injured colon together [12]. A previous study reported that Dll1-specific knockout mice had an increased number of goblet cells [12]. Notch and Jag/Dll ligands activate Notch signaling, leading to the release of secreted enzymes that cleave the intracellular segments of Notch into the nucleus to initiate transcription of the corresponding target genes [13]. The target gene of Notch signaling, Math1, induces the differentiation of ISCs towards a secretory cell lineage [14, 15], whereas the other target gene, Hes1, induces the ISCs that are differentiating towards an absorptive cell lineage [16]. Math1 cooperated with Hes1 to mediate the Notch signaling-regulated differentiation of Lgr5-positive ISCs, maintaining the intestinal homeostasis. Although Wnt/β-catenin and Notch signaling regulate the proliferation and differentiation of ISCs together, the balance between the Wnt/β-catenin and the Notch signaling may be disrupted in IBD, and the intestinal mucosal damage is not repaired properly. The mechanism of how these two signaling pathways interact to regulate the proliferation and differentiation of ISCs under the pathological state of intestinal inflammation remains unclear.

In this study, we used *β-catenin*$^{fl/fl}$ mice, *β-catenin*^{Tg} mice, and *Dll1*$^{tm1\ Gos}$ mice crossed with *Lgr5-EGFP-IRES-creERT2* mice, altering the activation of Wnt/β-catenin and Notch signaling in ISCs *in vivo*, and constructed the dextran sulfate sodium (DSS)-induced mouse colitis model. Then we isolated and cultured intestinal organoids to observe the proliferation and differentiation of organoids with indicated β-catenin and Notch signaling activation. We aimed to understand the regulation of Lgr5^{+} ISCs by the Wnt/β-catenin and Notch signaling under intestinal inflammatory conditions and its role in the repair of mucosal barrier damage.

Methods

Study design

In this study, we collected intestinal tissues from patients with UC and healthy volunteers, and detected the expression of β-catenin and Notch1 by using quantitative real-time PCR (qRT–PCR) and Western blot. We next observed the expression localization of β-catenin and Notch1 in the intestinal mucosa by using immunofluorescence staining.

In terms of animal studies, we referred to the previous animal experiment protocol and used DSS to induce mouse colitis [17]. First, we used *β-catenin*$^{fl/fl}$ mice, *β-catenin*^{Tg} mice, and *Dll1*$^{tm1\ Gos}$ mice crossed with *Lgr5-EGFP-IRES-creERT2* mice to generate mice with different activation of the β-catenin and Dll1-mediated Notch1 pathway in ISCs. Next, we added DSS in water to induce colitis and analysed the degree of inflammation by using the disease activity index (DAI) and histological hematoxylin and eosin (H&E) staining, and further assessed the integrity of the intestinal barrier by using immunofluorescence staining. Finally, intestinal crypts of these mice were extracted for 3D culture to assess the effects of ISCs with different activation of the β-catenin and Dll1-mediated Notch1 pathway on the epithelial barrier.

Human tissue samples

Non-inflamed and inflamed intestinal mucosa were collected from 31 patients with active UC who underwent surgery at the Department of Colorectal Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine (Shanghai, China) from February 2008 to March 2023. Normal intestinal tissues were obtained from 33 patients who underwent endoscopic biopsy due to colon polyps as healthy controls. Moreover, inflamed and paired distant non-inflamed colon tissues were obtained from eight patients with active UC. The diagnosis of UC was based on clinical symptoms, endoscopic and radiological examination, and histological changes [18, 19]. The study was approved by the Ethics Committee of Xinhua Hospital (No. XHEC-NSFC-2022–113). All subjects enrolled in this study had signed the broad consent.

Mice and DSS-induced murine colitis model

All mouse studies were approved and all animals were manipulated according to the protocols approved by the Animal Care and Use Committees of Xinhua Hospital, and animal care was conducted in accordance with institutional guidelines. *β-catenin*$^{flox/flox}$ mice, *β-catenin*^{Tg} mice, *Dll1*$^{tm1\ Gos}$ mice, and *Lgr5-EGFP-IRES-creERT2* mice were purchased from Beijing Vitalstar Biotechnology Co., Ltd and were crossed to generate *Lgr5-EGFP-IRES-creERT2; β-catenin*$^{flox/flox}$ mice, *Lgr5-EGFP-IRES-creERT2; β-catenin*^{Tg} mice, *Lgr5-EGFP-IRES-creERT2; Dll1*$^{tm1\ Gos}$ mice, and *Lgr5-EGFP-IRES-creERT2; β-catenin*^{Tg}*; Dll1*$^{tm1\ Gos}$ mice for the DSS-induced murine colitis model. To induce colitis, 8-week-old mice were fed with 3% of DSS (MP Biomedicals; Shanghai, China) in their drinking water and were monitored for their body weight and stool characteristics. All mice were euthanized after 7 days of induction.

DAI and histological pathological scoring

Body weight, stool character, and occult or rectal bleeding were monitored daily after DSS administration to assess the DAI score of each mouse on a scale of 0–4. The DAI score for each mouse was calculated by dividing the scores by three, as listed in Supplementary Table 1. The histological scores of the mice with inflammation were assessed on a scale of 0–3 based on neutrophils infiltration, crypts, cross-section involvement, and erosion or ulceration formation by two pathologists who were independent of this study (Supplementary Box 1).

Crypt isolation and 3D culture

Colons of mice were obtained and opened longitudinally, then washed with phosphate buffered saline (PBS). Tissues were incubated at 37°C in ethylene diamine tetraacetic acid (EDTA) buffer that included 2 mM of EDTA (Sangon Biotech Co., Ltd; Shanghai, China), 43.4 mM of sucrose (Sangon Biotech Co., Ltd), 54.9 mM of d-sorbitol (Sangon Biotech Co., Ltd), and 0.5 mM of DL-dithiothreitol (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min to dissociate the crypts. The isolated crypts were incubated by using a TrypLE Express (Thermo Fisher Scientific) for 1 h at 37°C to break them up into individual cells. Colon epithelial cells were suspended in 50% matrigel (Corning; Corning, NY, USA) with a 50% advanced DMEM/F12 medium (Thermo Fisher Scientific). After 20 min of incubation to polymerize the matrigel, WENR medium (containing Wnt-3a, EGF, Noggin and R-spondin-1) was added.

Immunohistochemistry and immunofluorescence

Paraffin-embedded tissues were deparaffinized in xylene and rehydrated with a descending concentration of ethanol before the antigen was retrieved by heating a citrate buffer. After being treated with 5% goat serum (Beyotime; Shanghai, China), the slides were incubated with specific primary antibodies at 4°C overnight. After being washed with PBS, the slides were incubated with appropriate AlexaFluor-labeled secondary antibodies (Beyotime).

Organoids in the plates were fixed by using 4% paraformaldehyde for 45 min at 4°C. The organoids were treated with PBS containing 10% goat serum, 10% dimethyl sulfoxide, and 2% Triton-X100 for 1 h before they were incubated with specific primary antibodies for 2 h at room temperature. After being washed with PBS, the organoids were incubated with appropriate Alexa Fluor-labeled secondary antibodies (Beyotime) for 2 h at room temperature. The slides were sealed in mounting media and then observed under a laser scanning confocal microscope (OLYMPUS, Japan).

RNA and protein extraction from patients

Patient tissue samples were washed with pre-cooled PBS and cut and homogenized to extract ribonucleic acid (RNA) using TRIzol reagent (Vazyme; Nanjing, Jiangsu, China) and proteins with 1% NP-40 lysis buffer (Sangon Biotech Co., Ltd) supplemented with a protease inhibitor cocktail, respectively.

Western blot

The total protein was extracted from the human tissues by using NP-40 as described above. Lysates that comprised protein of 20 μg/well were loaded and separated by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto the nitrocellulose membrane. The membrane was blocked with 5% non-fat milk (Sangon Biotech Co., Ltd) for ≥1 h at room temperature and was then incubated with a primary antibody at 4°C overnight. After the membranes were washed three times with tris-borate saline containing 0.1% Tween-20 (Sangon Biotech Co., Ltd), the specific protein was visualized by using horseradish peroxidase (HRP)-conjugated secondary antibody (Beyotime) and enhanced chemiluminescence (Millipore, Bedford, MA, USA).

qRT–PCR

The total RNA was extracted from the tissue by using TRIzol reagent as described above. Reverse transcription was performed on 500 ng of the total RNA by using a HiScript IV RT SuperMix for qPCR kit (Vazyme). qRT–PCR was carried out by using the Universal SYBR qPCR Master Mix (Vazyme) and an Applied Biosystems 7500 Fast real-time PCR system (Thermo Fisher Scientific). The relative expression of mRNA was evaluated by using the $2^{-\Delta\Delta Ct}$ method and was normalized to the expression of β-actin. The sequences of PCR primers that were used in this study are listed in Supplementary Table 2.

Statistical analysis

Graph Pad Prism software version 6.0 (San Diego, CA, USA) was used for statistical analyses. Quantitative data are presented as the mean ± standard deviation values. Unpaired Student's *t*-test was used to compare the difference between two single groups. One-way ANOVA with the Dunnett's multiple comparison test was used for the experiments with more than two independent groups. For DAI assessments, two-way ANOVA was performed to assess statistical significance. All statistical tests were two-sided and considered significant with a *P*-value of <0.05.

Results

β-catenin and Notch1 expression are increased in inflamed colon tissues

To explore the mechanism of β-catenin and Notch1 in Lgr5-positive cells, we analysed the expression of β-catenin and Notch1 between healthy controls and patients with active UC by using qRT–PCR. A total of 31 patients with active UC who underwent surgery were included for analysis. As shown in Supplementary Table 3, the median age at surgery was 46.0 years (interquartile range 35.0–56.0). Among them, none had a lesion in the rectum (E1), 2 patients (6.5%) had lesions in the left colon (E2), and 29 patients (93.5%) had lesions in the extensive colon (E3) according to the Montreal classification [20]. Thirty-one patients (100%) had received Mesalamine treatment. Three patients (9.7%) had received biologics treatment, 14 patients (45.2%) had received immunomodulators treatment, and 23 patients (74.2%) had received steroids treatment. All patients were treated with medication and eventually underwent surgery due to medical treatment failure or serious complications. The mRNA levels of β-catenin were significantly increased in inflamed colon tissues compared with the healthy controls (Figure 1A).

Consistently with the findings, the β-catenin mRNA levels were also markedly increased in inflamed and paired unaffected intestinal tissues from eight patients with UC (Figure 1B). Additionally, an obvious increase in Notch1 mRNA levels was evaluated in inflamed intestinal tissues from patients with UC compared with healthy controls and paired unaffected tissues (Figure 1C and D). By performing Western blot analysis of the inflamed and paired adjacent non-inflamed mucosa from six patients with UC, we found moderately to dramatically increased protein levels of β-catenin and Notch1 in inflamed tissues (Figure 1E). Immunofluorescence showed an increased immunofluorescence intensity of β-catenin in mucosal glands in inflamed colon tissues with co-staining of E-cadherin (Figure 1F). Increased expression of Notch1 and its ligand (Jag1) was also observed in mucosal glands in inflamed colon tissues by using immunofluorescence staining (Figure 1G). Altogether, these results indicated that β-catenin and Notch1 are highly expressed in inflamed intestines, which could be associated with the dysregulation of intestinal homeostasis in UC.

Besides, we also observed that *β-catenin* was expressed in Lgr5-positive cells in the small intestines and colons of adult *Lgr5-EGFP-IRES-CreERT2* mice by co-staining *β-catenin* with *Lgr5* (Supplementary Figure 1A). The expression of *Jag1*, the ligand of *Notch1*, was also observed in colon tissues (Supplementary Figure 1B), which indicated the activation of Notch signaling in the mucosal glands of colons from *Lgr5-EGFP-IRES-CreERT2* mice. These data provided a theoretical basis for our next step in constructing transgenic mice.

β-catenin knockout in Lgr5-positive cells aggravates DSS-induced colitis in mice

To explore the function of β-catenin in Lgr5-positive cells, we used $\beta\text{-catenin}^{flox/flox}$ mice and *Lgr5-EGFP-IRES-creERT2* mice to generate *Lgr5-EGFP-IRES-creERT2;* $\beta\text{-catenin}^{flox/flox}$ mice ($\beta\text{-catenin}^{fl/fl}$) and established the DSS-induced acute mice-colitis model. Compared with their *WT* (*Wildtype*) littermates, the $\beta\text{-catenin}^{fl/fl}$ mice developed more serious intestinal inflammation, manifested as a shorter colon length (Figure 2A), a higher DAI score (Figure 2B), more severe colonic tissue damage, and a higher pathological score (Figure 2C).

It has been shown that disruption of the intestinal barrier plays a critical role in the development of intestinal inflammation. Next, we examined the intestinal barrier integrity in $\beta\text{-catenin}^{fl/fl}$ mice and their *WT* littermates. Alcian blue/periodic acid-Schiff (ABPAS) staining of colon tissues showed that the number of goblet cells was further decreased in the DSS-induced $\beta\text{-catenin}^{fl/fl}$ mice (Figure 2D).

In the DSS-fed mice, especially in the DSS-fed $\beta\text{-catenin}^{fl/fl}$ mice, there was decreased E-cadherin, CK20, and CHGA expression, which revealed that mice with *β-catenin* knockout in Lgr5-positive cells had more intestinal damage with the destruction of

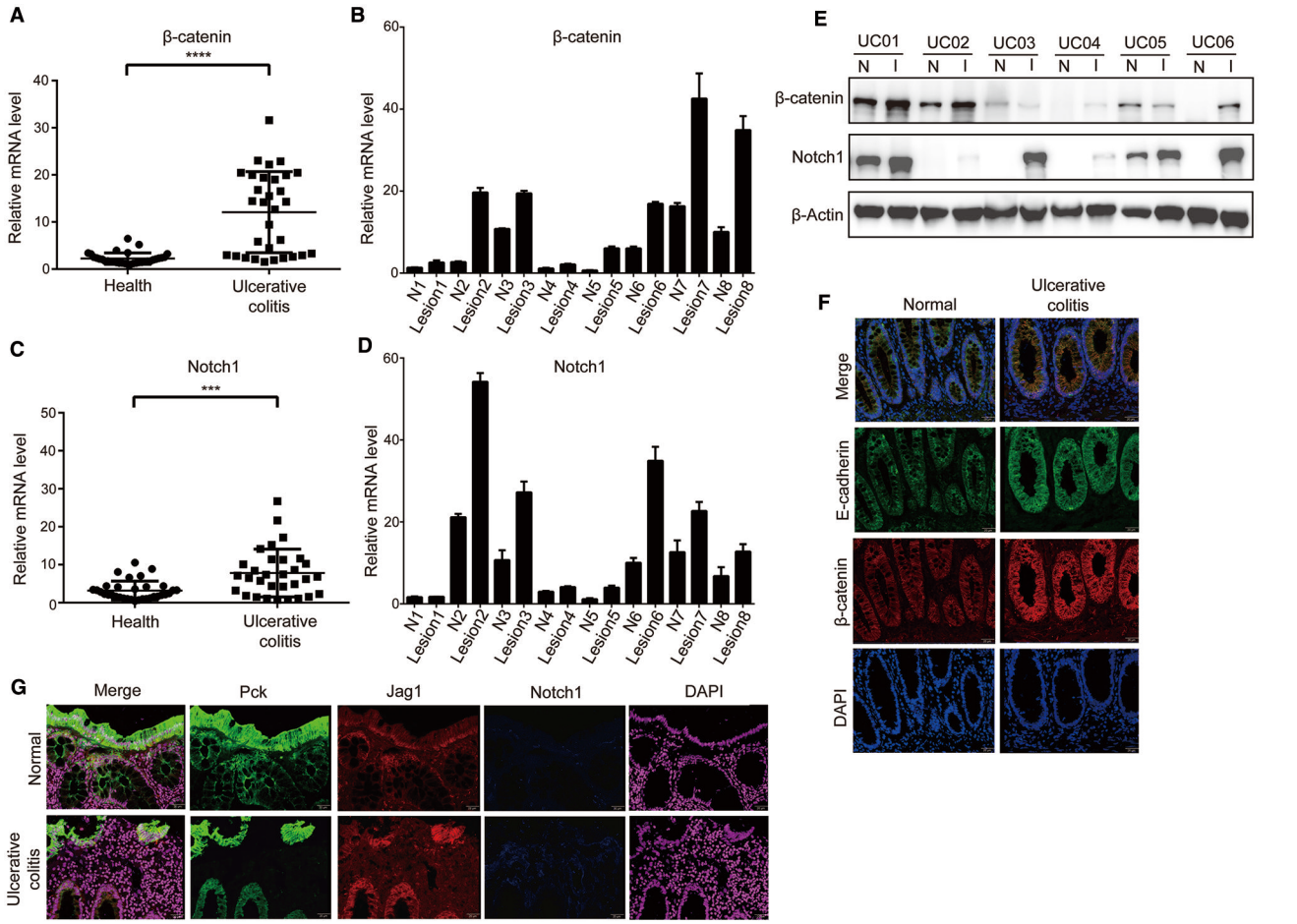

Figure 1. β-catenin and Notch1 expression are increased in inflamed colon tissues from IBD patients. (A) qRT–PCR analysis of β-catenin expression between healthy controls and patients with active UC. (B) qRT–PCR analysis of β-catenin expression in paired inflamed and unaffected intestinal tissues from patients with UC. (C) qRT–PCR analysis of Notch1 expression between healthy controls and patients with active UC. (D) qRT–PCR analysis of Notch1 expression in paired inflamed and unaffected tissues from patients with UC. (E) Western blot analysis of β-catenin and Notch1 expression in inflamed and paired unaffected intestinal tissues from six patients with UC. (F) Representative images of immunofluorescence co-staining with β-catenin and Notch1 in inflamed and unaffected tissues. Scale bar, 20 μm. (G) Representative images of immunofluorescence co-staining with Notch1, Pan-Cytokeratin (Pck), and Jag1 in inflamed and unaffected tissues from patients with UC. Scale bar, 20 μm. (A)–(D) Unpaired Student's *t*-test was performed to assess statistical significance. $*P < 0.001$; $**P < 0.0001$; N = normal, I = inflammatory, UC = ulcerative colitis.

the epithelial tight junction (Figure 2E–G). Collectively, these data demonstrated that *β-catenin* knockout in Lgr5-positive cells aggravated the DSS-induced intestinal mucosal inflammation in mice.

β-catenin activation in Lgr5-positive cells alleviates the DSS-induced intestinal mucosal inflammation in mice

We next used *β-catenin*^{Tg} mice and *Lgr5-EGFP-IRES-creERT2* mice to generate *Lgr5-EGFP-IRES-creERT2; β-catenin*^{Tg} mice (*β-catenin*^{Tg}) and established a DSS-induced colitis murine model as mentioned above. Consistently with the above findings, we observed that *β-catenin*^{Tg} mice exhibited alleviated colitis, manifested as a longer colon length (Figure 3A) and lower DAI scores (Figure 3B), as well as milder colonic tissue destruction and lower pathology scores (Figure 3C). *β-catenin*^{Tg} mice had more goblet cells compared with their *WT* littermates after DSS induction by ABPAS staining (Figure 3D). In the DSS-fed *β-catenin*^{Tg} mice, there was milder destruction in E-cadherin, CHGA, and CK20 compared with that in the *WT* mice (Figure 3E–G). In summary, these data revealed that β-catenin activation in Lgr5-positive cells alleviated the DSS-induced intestinal mucosal damage in mice.

Activation of *β*-catenin facilitates colon epithelial integrity in organoids

To further explore the role of β-catenin in the colonic epithelial barrier *in vitro*, we isolated colonic crypts and cultured mice organoids. As shown in Figure 4A, isolated crypts from the colons of mice were cultured and their characteristics were observed each day for ~10 days. Then, β-catenin, the intestinal barrier marker E-cadherin, CK20, intestinal enteroendocrine cells marker CHGA, and intestinal epithelial proliferation marker Ki67 were detected by using immunofluorescence staining. Compared with organoids from WT mice, the expressions of E-cadherin, CK20, CHGA, and Ki67 were increased in colonic organoids that were isolated from *β-catenin*^{Tg} mice but were decreased in colonic organoids from *β-catenin*$^{fl/fl}$ mice (Figure 4B–G). These data suggested that β-catenin could protect the intestinal barrier from damage and promote the proliferation of IECs and intestinal barrier repair.

Abrogation of Dll1-mediated Notch signaling activity in Lgr5-positive cells alleviates DSS-induced colitis in mice

Previous studies have reported that Notch signaling regulates the differentiation function of ISCs [14, 15] and Dll1—the important ligand of Notch signaling—promotes ISCs differentiation into

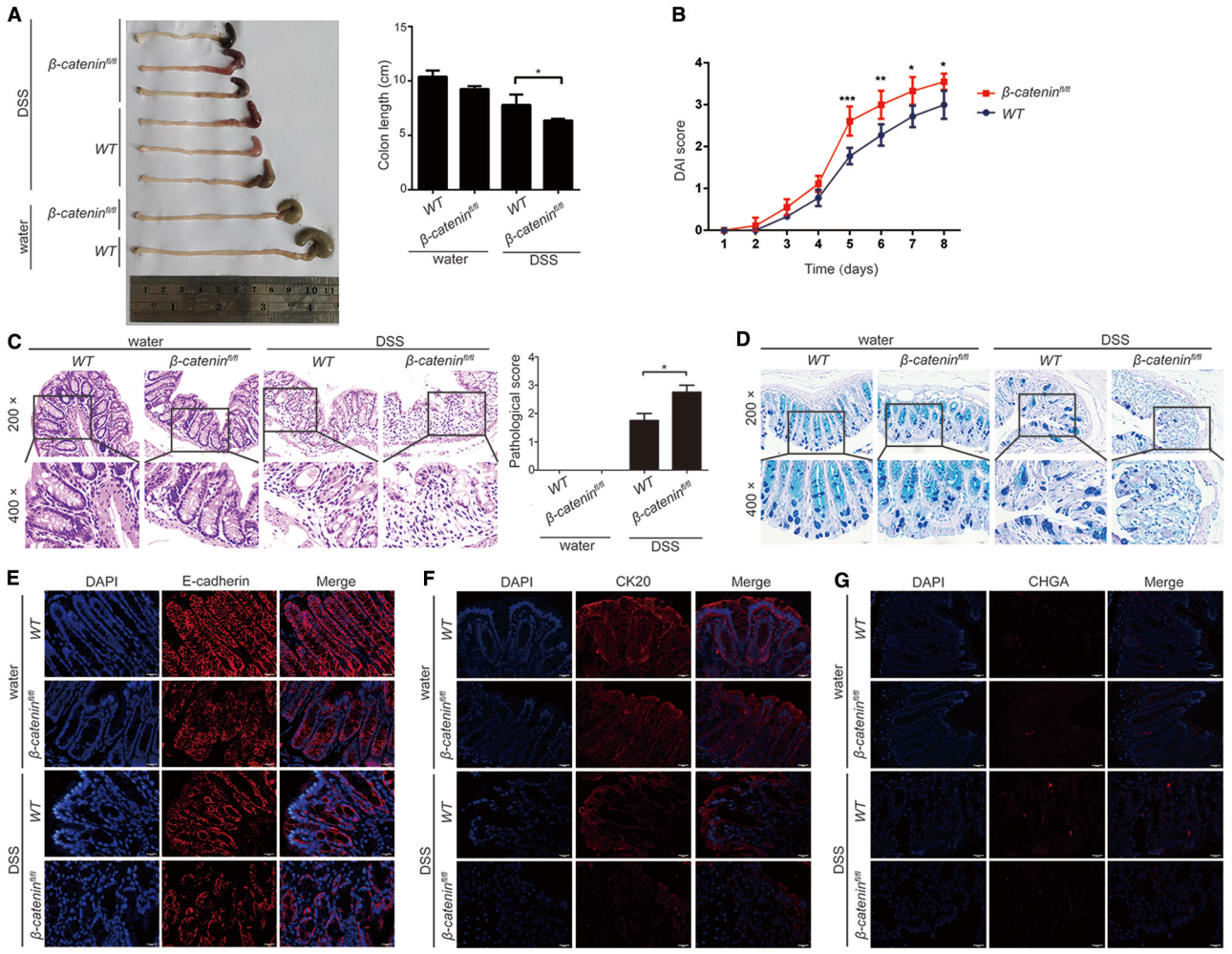

Figure 2. β-catenin knockout in Lgr5-positive cells promotes DSS-induced intestinal mucosal inflammation in mice. *β-catenin*$^{flox/flox}$ and *Lgr5-EGFP-IRES-CreERT2* mice were crossed to generate *Lgr5-EGFP-IRES-creERT2; β-catenin*$^{flox/flox}$ mice (*β-catenin*$^{fl/fl}$) that showed tamoxifen-inducible KO of β-catenin in LGR5-positive ISCs. (A) *β-catenin* knockout increased shortening of colon length and (B) DAI score of DSS-induced colitis mice. (C) Representative images of H&E staining and pathological scores of colon tissues from DSS-induced murine colitis model of *β-catenin*$^{fl/fl}$ and *WT* mice (magnification: 200×, upper panels; 400×, lower panels). (D) Representative images of Alcian blue/periodic acid-Schiff staining of colon tissues from *β-catenin*$^{fl/fl}$ and *WT* mice (magnification: 200×, upper panels; 400×, lower panels). (E)–(G) Representative immunofluorescence images for E-cadherin, CHGA, and CK20 in colonic tissues from different groups of *β-catenin*$^{fl/fl}$ and *WT* mice. Scale bar, 20 μm. (A) One-way ANOVA, (B) two-way ANOVA, and (C) the Mann–Whitney *U* test were performed to assess statistical significance. *$P < 0.05$. *WT* = *Wildtype*, DSS = dextran sodium sulfate.

goblet cells. Thus, we next explored the role of Notch signaling in Lgr5-positive cells in the DSS-induced colitis mice model. To this end, we used *Dll1*$^{tm1\ Gos}$ mice and *Lgr5-EGFP-IRES-creERT2* mice to generate *Lgr5-EGFP-IRES-creERT2; Dll1*$^{tm1\ Gos}$ mice (*Dll1*$^{tm1\ Gos}$) to abrogate Notch signaling activity in Lgr5-positive cells. All mice were randomly assigned into the indicated groups and sacrificed after 7 days of DSS-induced colitis. The *Dll1*$^{tm1\ Gos}$ mice developed slighter intestinal inflammation, which manifested as a longer colon length, a lower DAI score, less colonic tissue damage, and a lower pathological score (Figure 5A–C). Consistently with the above findings, the *Dll1*$^{tm1\ Gos}$ mice had a higher number of goblet cells compared with their *WT* littermates after DSS induction by ABPAS staining (Figure 5D), which is consistent with previously indicated results that Dll1-mediated Notch signaling regulates stem cell differentiation toward secretory cells [12]. Furthermore, the expressions of E-cadherin, CK20, and CHGA were higher in the DSS-fed *Dll1*$^{tm1\ Gos}$ mice compared with their *WT* littermates (Figure 5E–G). Taken together, Notch signaling inhibition in Lgr5-positive cells alleviated DSS-induced intestinal mucosal inflammation in mice via regulating the differentiation of stem cells and protecting epithelial barrier integrality.

β-catenin activation with Notch inhibition facilitates colon epithelial integrity in organoids

Next, we further explored the role of β-catenin activation and Dll1-mediated Notch signaling inhibition in colon epithelial integrity in colonic organoids. We isolated colonic crypts and cultured mice organoids from *Dll1*$^{tm1\ Gos}$ mice, *β-catenin*^{Tg} mice, and *β-catenin*^{Tg}*, Dll1*$^{tm1\ Gos}$ mice (generated by *β-catenin*^{Tg}*; Lgr5-EGFP* mice crossed with *Dll1*$^{tm1\ Gos}$*; Lgr5-EGFP* mice). As shown in Figure 6A–D, colonic organoids that were isolated from *β-catenin*^{Tg}*, Dll1*$^{tm1\ Gos}$ mice had increased expression of E-cadherin, CK20, and CHGA compared with the organoids from *Dll1*$^{tm1\ Gos}$ mice and *β-catenin*^{Tg} mice. These data suggested that β-catenin activation with Notch inhibition protected intestinal barrier integrality. We also found that the Ki67 expression in colonic organoids from *β-catenin*^{Tg}*, Dll1*$^{tm1\ Gos}$ mice was significantly increased (Figure 6E), which suggested that β-catenin activation with Dll1-mediated Notch signaling inhibition promoted the proliferation of IECs. Thus, these data indicated that β-catenin activation with Notch inhibition facilitated colon epithelial integrity in organoids.

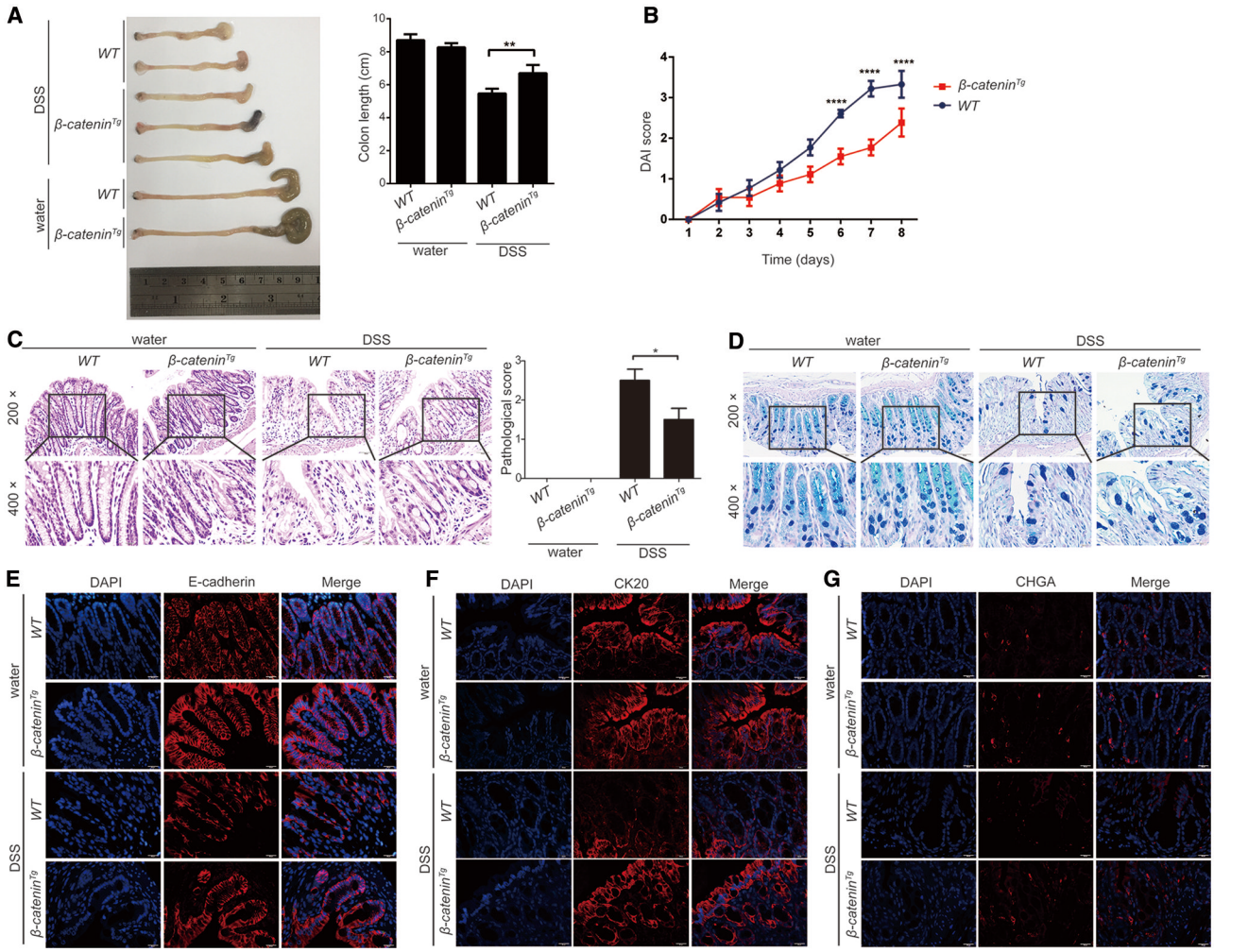

Figure 3. β-catenin activation in Lgr5-positive cells alleviates DSS-induced intestinal mucosal inflammation in mice. *β-catenin*^{Tg} and *Lgr5-EGFP-IRES-CreERT2* mice were crossed to generate *β-catenin*^{Tg}*; Lgr5-EGFP* mice (*β-catenin*^{Tg}) that showed tamoxifen-inducible overexpression of *β-catenin* in LGR5-positive ISCs. (A) *β-catenin* activation decreased shortening of colon length and (B) DAI score of DSS-induced colitis mice. (C) Representative images of H&E staining and pathological scores of colon tissues from DSS-induced murine colitis model of *β-catenin*^{Tg} and *WT* mice (magnification: 200×, upper panels; 400×, lower panels). (D) Representative images of Alcian blue/periodic acid-Schiff staining of colon tissues from *β-catenin*^{Tg} and *WT* mice (magnification: 200×, upper panels; 400×, lower panels). (E)–(G) Representative immunofluorescence images for E-cadherin, CHGA, and CK20 in colonic tissues from different groups of *β-catenin*^{Tg} and *WT* mice. Scale bar, 20 μm. All data are shown from three independent experiments. (A) One-way ANOVA, (B) two-way ANOVA, and (C) the Mann–Whitney *U* test were performed to assess statistical significance. *$P < 0.05$; **$P < 0.01$. *WT* = *Wildtype*, DSS = dextran sodium sulfate.

Discussion

In this study, we discovered that Wnt/β-catenin activation and Dll1-mediated Notch signaling inhibition in Lgr5-positive ISCs could alleviate mucosal inflammation in a DSS-induced mouse colitis model. We also found that Wnt/β-catenin activation and Dll1-mediated Notch signaling inhibition in Lgr5-positive cells facilitate E-cadherin, CK20, CHGA, and Ki67 expression in organoids. Thus, our data suggested that Wnt/β-catenin activation and Dll1-mediated Notch signaling inhibition in Lgr5-positive cells alleviated intestinal inflammation by regulating colon epithelial integrity and mucosal barrier repair.

The ISCs of the intestinal crypts regulate the self-renewal of the epithelium and the sustained regenerative repair to maintain intestinal epithelial homeostasis [21–23]. In this process, ISCs can differentiate into functional cells along the "crypt–villus axis," such as absorptive IECs, enteroendocrine cells, and goblet cells. It has been demonstrated that Lgr5 clearly marks ISCs [24]. Lgr5 is specifically expressed in the crypt-based columnar cells [6, 8] and Lgr5-positive cells can regenerate various types of IECs. Thus, Lgr5 could be considered a specific marker of colonic ISCs. It has been previously reported that transplantation of Lgr5-positive ISCs can treat intestinal mucosal damage in the DSS-induced mouse colitis model [25]. In addition, some studies have described the idea of using colon stem cells for *ex vivo* expansion and then autologous transplantation to the site of the colon injury to treat UC [26, 27]. These results and hypotheses may provide researchers with a new therapeutic strategy for the treatment of UC.

Wnt/β-catenin signaling exerts important regulatory effects on the proliferation of Lgr5-positive ISCs. Previous studies have reported that the Wnt pathway with the inhibited TGFβ/BMP pathway promotes regeneration of the human colonic epithelium to repair injured intestinal mucosal [28] whereas, in transgenic mice expressing the Wnt pathway inhibitor Dickkopf-1, it was found that intestinal crypt destruction and epithelial proliferation were significantly inhibited [29]. In contrast, the ISCs displayed a highly proliferative state when using the Wnt pathway agonist R-spondin [10]. We also found that β-catenin knockout in Lgr5-positive cells could inhibit Ki67 expression in colon

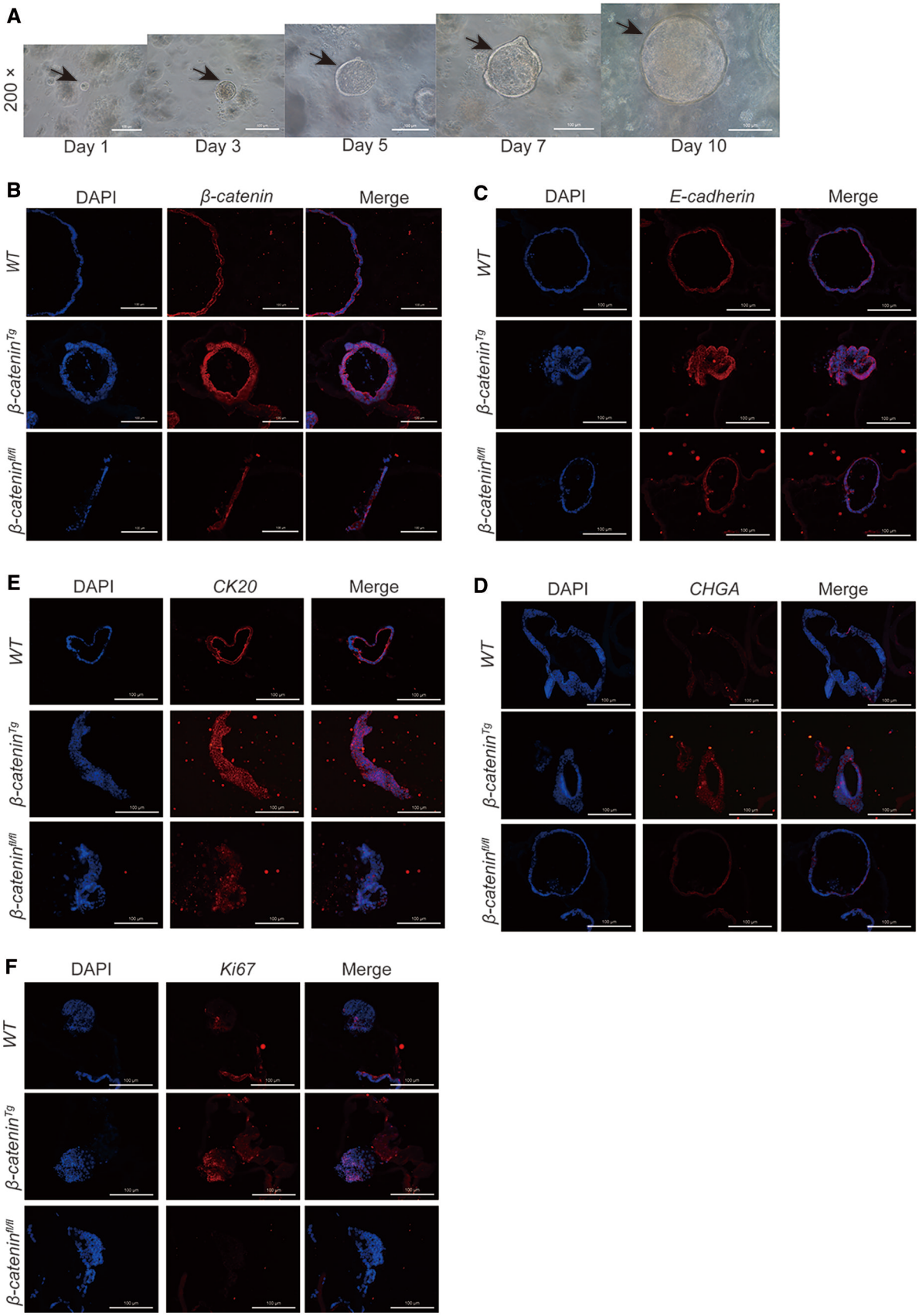

Figure 4. Activation of β-catenin facilitates colon epithelial integrity in organoids. (A) Representative images of colon organoids from WT mice. (B)–(F) Representative immunofluorescence images for β-catenin, E-cadherin, CK20, CHGA, and Ki67 in colon organoids isolated and cultured from *β-catenin*$^{fl/fl}$ mice, *β-catenin*^{Tg} mice, and WT mice, respectively. Scale bar, 100 μm.

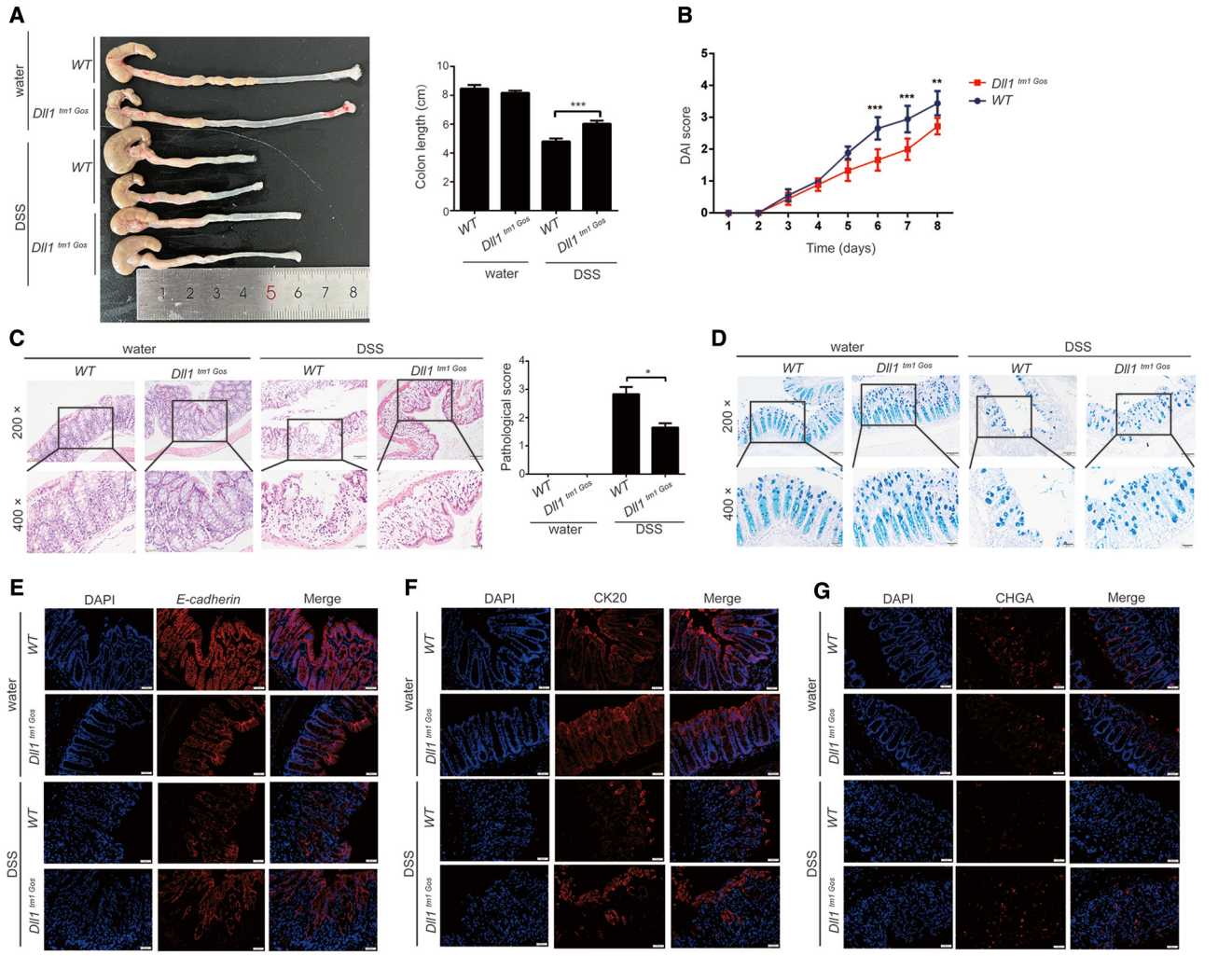

Figure 5. Abrogation of Dll1-mediated Notch signaling activity in Lgr5-positive cells alleviates DSS-induced colitis in mice. *Dll1*$^{tm1\ Gos}$ and *Lgr5-EGFP-IRES-CreERT2* mice were crossed to generate *Lgr5-EGFP-IRES-creERT2*; *Dll1*$^{tm1\ Gos}$ mice (*Dll1*$^{tm1\ Gos}$) that showed tamoxifen-inducible KO of *Dll1* in LGR5-positive ISCs. (A) Dll1 knockout increased shortening of colon length and (B) DAI score of DSS-induced colitis mice. (C) Representative images of H&E staining and pathological scores of colon tissues from DSS-induced murine colitis model of *Dll1*$^{tm1\ Gos}$ and *WT* mice. Upper scale bar, 50 μm; lower scale bar, 20 μm. (D) Representative images of Alcian blue/periodic acid-Schiff staining of colon tissues from *Dll1*$^{tm1\ Gos}$ and *WT* mice. Upper scale bar, 50 μm; lower scale bar, 20 μm. (E)–(G) Representative immunofluorescence images for E-cadherin, CK20, and CHGA in colonic tissues from different groups of *Dll1*$^{tm1\ Gos}$ and *WT* mice. Scale bar, 20 μm. (A) One-way ANOVA, (B) two-way ANOVA, and (C) Mann–Whitney *U* test were performed to assess statistical significance. $^{*}P < 0.05$; $^{***}P < 0.001$. *WT* = *Wildtype*, DSS = dextran sodium sulfate.

organoids that were isolated from *β-catenin*$^{fl/fl}$ mice while Ki67 expression was increased in Lgr5-positive cells with *β-catenin* activation. These results further indicated that Wnt/β-catenin is the crucial driving force in promoting the proliferation of ISCs.

Another important link between the Wnt pathway and intestinal mucosal homeostasis is the interaction between mucin expression and Wnt pathway activation. Through the association of the cytoplasmic tail region, MUC2 can interact with β-catenin, and this interaction is able to prompt β-catenin nuclear translocation, contributing to Wnt pathway activation [30]. However, β-catenin can also directly affect the transcription process of different mucin genes such as MUC2 [30]. In our study, β-catenin knockout in ISCs contributed to decreased goblet cells in DSS-induced colitis and impaired CHGA expression in intestinal organoids. This molecular mechanism implied to researchers that the decrease in MUC2 expression in IBD is not only associated with a decrease in goblet cells, but also abnormal regulation of the Wnt pathway.

Notch signaling regulates the differentiation of ISCs. Conditional knockout of RBP-j as well as tissue Notch intracellular domain (NICD) release using γ-secretase inhibitors would cause differentiation of ISCs into secretory cell lineage [31]. Similar results were obtained in juvenile mice that were deficient in the Notch pathway target gene Hes1 [16]. The Notch pathway can negatively regulate the differentiation of secretory cell lines after inhibition of the target gene Math1 [14]. Moreover, loss of Math1 can cause abnormal differentiation of mucosal goblet cells, resulting in the loss of the colonic mucus layer, thereby inducing UC [15]. The above results suggest that Math1 promotes the differentiation of ISCs into secretory cell lines, while Hes1 promotes the differentiation of ISCs into absorptive cell lines, and both maintain intestinal homeostasis. In our study, abrogation of Dll1-mediated Notch signaling activity in ISCs alleviated the decrease in goblet cells, which was consistent with the previous study [12], indicating that Notch signaling affected the repair of intestinal mucosal damage in IBD by regulating the differentiation of Lgr5-positive cells.

Although Wnt/β-catenin and Notch signaling have demonstrated important roles in intestinal homeostasis, how the signaling pathways interact to regulate ISCs activity and the

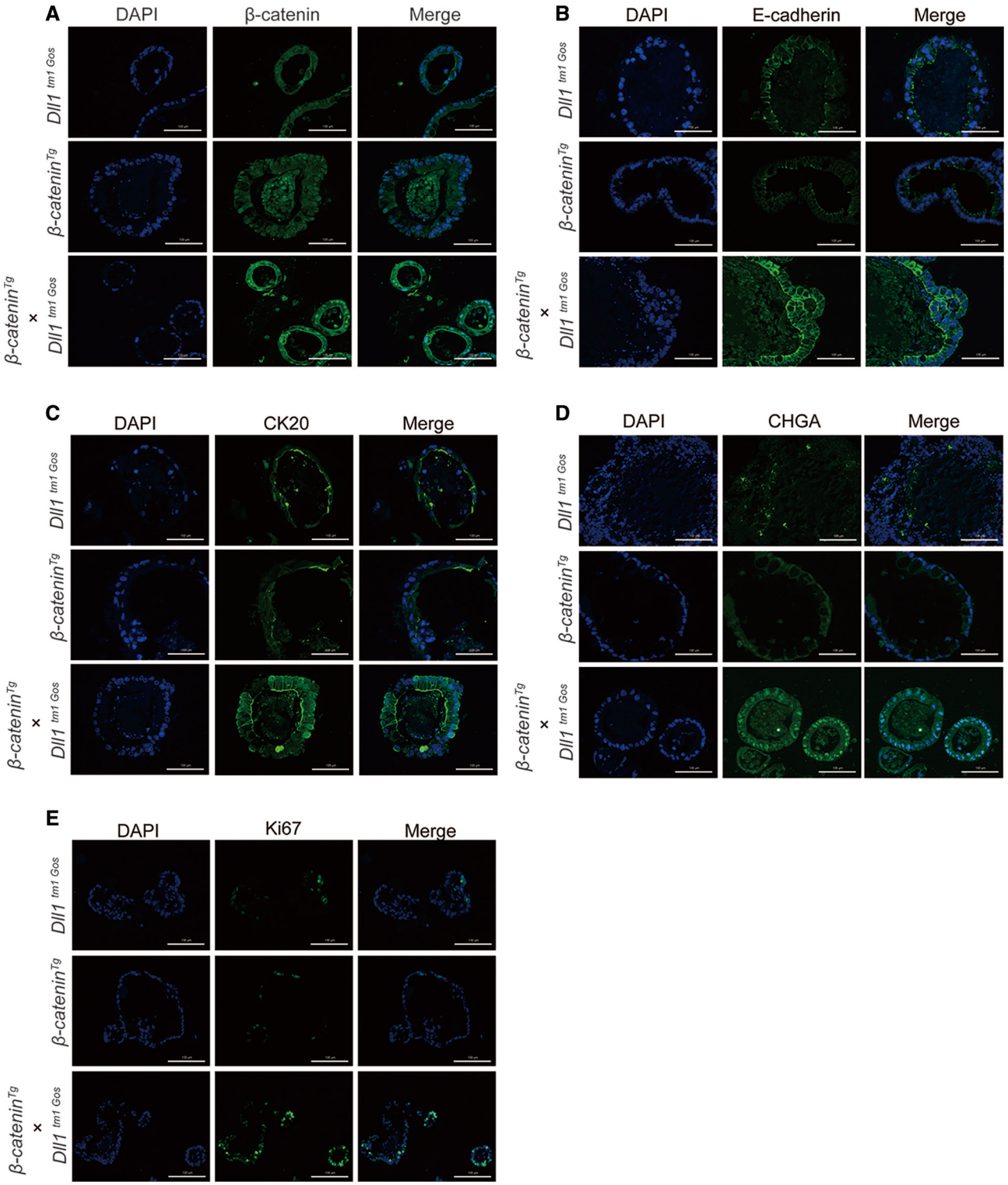

Figure 6. β-catenin activation with Notch inhibition facilitates colon epithelial integrity in organoids. *β-catenin*^{Tg}; *Lgr5-EGFP* mice and *Dll1*$^{tm1\ Gos}$; *Lgr5-EGFP* mice were crossed to generate *β-catenin*^{Tg}, *Dll1*$^{tm1\ Gos}$; *Lgr5-EGFP* mice (*β-catenin*^{Tg}, *Dll1*$^{tm1\ Gos}$). (A)–(E) Representative immunofluorescence images for β-catenin, E-cadherin, CK20, CHGA, and Ki67 in colon organoids isolated and cultured from *Dll1*$^{tm1\ Gos}$ mice, *β-catenin*^{Tg} mice, and *β-catenin*^{Tg}, *Dll1*$^{tm1\ Gos}$ mice. Scale bar, 100 μm.

differentiation direction remains unclear. In our study, by using β-catenin transgenic mice crossed with Dll1 conditional knockout mice and isolating cultured colon stem cells, we found that organoids were more proliferative and intestinal barrier indicators were upregulated. Previous studies have reported that increased ligand WNT3, decreased NICD in ISCs, and differentiation of ISCs into a secretory cell line were demonstrated after the administration of Notch pathway impedance antibodies [32]. Upon Wnt pathway disinhibition and upregulation shortly after Notch pathway blockade, Lgr5-positive ISCs would differentiate into secretory cell lines [32]. Our study is consistent with the above findings. Some studies have also reported that the expression of the Notch pathway and the activity of ISCs could be restored after reducing the output of the Wnt pathway by using

LPR6 antibody [32]. Therefore, Notch signaling is required to maintain the proliferation and differentiation of ISCs in parallel. The Notch pathway in the maintenance of ISCs activity and differentiation will inhibit the activity of Wnt signaling. There is an antagonistic relationship between the two pathways to maintain intestinal homeostasis. Under IBD pathological conditions, the balance of the Wnt and Notch signaling pathways may be broken, which will cause intestinal dysfunction, and the proliferation and differentiation of ISCs will be affected.

Given the prominent role of the Wnt/β-catenin and Notch1 pathways in regulating cell proliferation and tissue repair, it is reasonable to expect the effect of Wnt/β-catenin and Notch1 molecules to drive mucosal regeneration. It seems paradoxical that β-catenin and Notch1 fail to prevent the onset of intestinal mucosal barrier disruption despite their high expression in colitis. However, this seems to be related to the crosstalk of the Wnt/β-catenin and Notch1 pathways with other cellular signaling pathways that together regulate cell proliferation and tissue repair. Previous studies have revealed that many external cues control the life and death of epithelial cells during IBD [33]. These cues are transduced by various cellular signaling pathways, such as NF-κB, MAPK, STAT, and the Hippo/YAP signaling [33, 34]. Taking the interaction of β-catenin with NF-κB as an example, in colitis, most studies have shown that Wnt activation inhibits NF-κB signaling mainly through direct interaction of β-catenin with NF-κB [35]. However, it has also been demonstrated that TNFRSF19 is a target of β-catenin/TCF and may activate epithelial NF-κB signaling in a feedback loop, but was found in colorectal cancer [36]. Similarly to NF-κB, β-catenin and Notch1 molecules also interact with c-JUN/MAPK, STAT, and Hippo/YAP [34]. On the other hand, Wnt/β-catenin and Notch ligand expression is highly compartmentalized in the gut [37] and previous studies have identified multiple pericryptal stomal cell populations that constitute the basic ecological niche of intestinal epithelial stem cells [34, 38]. Relevant to the pathobiology of IBD, at least some of these cell populations dynamically adjust their abundance or ligand pool during colitis, thereby controlling neighboring stem cells without altering tissue gene expression [39, 40]. Previous studies have reported that immune cells are able to control epithelial stem cell dynamics either directly through inflammatory cytokines or through Wnt signaling and the Notch1 pathway [41, 42]. In other words, the effect of the Wnt/β-catenin and Notch1 pathway in IBD is a very researchable proposition and our experiments have only intuitively demonstrated the effect of Wnt/β-catenin with Notch1 on intestinal barrier repair in animal experiments, so multi-omics studies are needed to further reveal its effect in enteritis in the future.

In conclusion, intestinal mucosal injury is an important pathological change in IBD, and Lgr5-positive ISCs are characterized by self-renewal and high dividend potential, which play an important role in the repair of intestinal mucosa injury. Meanwhile, Lgr5-positive ISCs receive fine regulation from Wnt/β-catenin signaling and Notch signaling. The Wnt/β-catenin signaling pathway cooperates with the Notch signaling pathway to maintain the function of the Lgr5-positive ISCs. The combined treatment scheme of multi-target combined signaling pathways and the transplantation therapy of Lgr5-positive ISCs may provide new application prospects and therapeutic strategies for the treatment of IBD.

Supplementary Data

Supplementary data is available at *Gastroenterology Report* online.

Authors' Contributions

P.D. and W.X. conceived the study. W.O. analysed the data and wrote the manuscript. Y.W. and Z.H. were involved in mice reproduction and assisted in some analyses. W.D. and L.C. reviewed and revised the manuscript. All authors approved the final version.

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Conflicts of Interest

None declared.

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