Published in final edited form as:
Gut. 2014 April ; 63(4): 622–634. doi:10.1136/gutjnl-2012-304241.

Claudin-1 Regulates Intestinal Epithelial Homeostasis through the Modulation of Notch Signaling

Jillian L. Pope[2], **Ajaz. A. Bhat**[1], **Ashok Sharma**[1], **Rizwan Ahmad**[1], **Moorthy Krishnan**[1], **Mary K. Washington**[3], **Robert D. Beauchamp**[1,2,4], **Amar B. Singh**[1,5], and **Punita Dhawan**[1,2]

[1]Department of Surgery, Vanderbilt University Medical Center, Nashville, TN-37232

[2]Department of Cancer Biology, Vanderbilt University Medical Center, Nashville, TN-37232

[3]Department of Pathology, Vanderbilt University Medical Center, Nashville, TN-37232

[4]Department of Developmental Biology, Vanderbilt University Medical Center, Nashville, TN-37232

[5]Department of Medicine, Vanderbilt University Medical Center, Nashville, TN-37232

Abstract

Objective—Claudin-1 expression is increased and dysregulated in colorectal cancer and causally associates with the dedifferentiation of colonic epithelial cells, cancer progression and metastasis. Here, we have sought to determine the role claudin-1 plays in the regulation of intestinal epithelial homeostasis.

Design—We have used a novel Villin-claudin-1 transgenic (Cl-1Tg) mouse as model (with intestinal claudin-1 overexpression). Effect of claudin-1 expression upon colonic epithelial differentiation, lineage commitment, and Notch signaling were determined using immunohistochemical, immunoblot and real time PCR analysis. The frequently used mouse model of DSS-colitis was used to model inflammation, injury and repair.

Results—In Cl-1Tg mice, normal colonocyte differentiation program was disrupted and goblet cell number and muc-2 expressions were significantly downregulated while Notch- and ERK1/2-signaling were upregulated, compared to the wild type (WT)-littermates. Cl-1Tg mice were also susceptible to colonic inflammation and demonstrated impaired recovery and hyperproliferation following the DSS-colitis. Our data further show that claudin-1 regulates Notch-signaling through the regulation of MMP-9 and p-ERK signaling to regulate proliferation and differentiation.

Conclusion—Claudin-1 helps regulate intestinal epithelial homeostasis through the regulation of Notch-signaling. An upregulated claudin-1 expression induces MMP-9 and p-ERK signaling to activate Notch-signaling, which in turn inhibits the goblet cell differentiation. Decreased goblet cell number decreases muc-2 expression and thus enhances susceptibility to mucosal inflammation. Claudin-1 expression also induces colonic epithelial proliferation in a Notch-

To whom correspondence should be addressed: Punita Dhawan, Departments of Surgery and Cancer Biology, Vanderbilt University Medical Center, Nashville, TN-37232, USA, Tel: (615)-322-4755; Fax: (615)-322-6174; punita.dhawan@vanderbilt.edu.

Competing Interests: None

dependent manner. Our findings may help understand the role of claudin-1 in the regulation of IBD and CRC.

Keywords

Claudin-1; Notch; Differentiation; Mucin-2; Inflammation

INTRODUCTION

Inflammation of the underlying colonic mucosa is a key characteristic of inflammatory bowel diseases (IBD) including Crohn's disease (CD) and ulcerative colitis (UC). Although the etiology of IBD remains unknown, malfunctioning of the colonic epithelial barrier has emerged as a key characteristic of the IBD pathogenesis.[1] The general postulation is that dysregulated mucosal barrier facilitates the access of the luminal antigens across the epithelium and thus induces immune activation and thereby inflammation.[2]

The mucosal barrier consists primarily of two key constituents: extracellular mucus consisting primarily of the glycoprotein mucin-2 (muc-2) secreted by the goblet cells, and the single layer of epithelium.[3] Tight junctions, the most apical cell-cell adhesions, are the primary regulators of the epithelial barrier function.[4] Indeed, modulation of the muc-2 expression, goblet cell number and tight junction (TJ) integral proteins are known characteristics of IBD patients.[5] Furthermore, mice deficient in muc-2 protein demonstrate an elevated production of pro-inflammatory cytokines and develop colitis spontaneously[6]. In the colon, Notch activation modulates muc-2 expression, expression of tight junction proteins, and the balance between proliferation and differentiation in the enterocyte progenitor pool.[7–10]

The claudin family of proteins is an integral component of the structure and function of TJs. The 27 known claudin family members are expressed differentially among various tissues and their expression can be altered under pathological conditions including inflammatory disorders such as IBD and cancer.[4] Notably, expression of the claudin-1 protein is increased in the areas of active inflammation.[11] Of interest, a correlation between increased claudin-1 expression and neoplastic transformation was also noted in colitis-associated cancer.[12] Also, loss of claudin-1 leads to severe dehydration and post-natal death in mice.[13] We have reported a causal association of claudin-1 expression with sporadic colon cancer growth and progression.[14] However, the role of claudin-1 in the regulation of epithelial homeostasis, mucosal inflammation and IBD remains unknown.

In the current study, using Cl-1Tg mice as a model, we report a previously unknown role of claudin-1 in the regulation of Notch-signaling and colonic epithelial homeostasis. We demonstrate that colonic claudin-1 overexpression increases MMP-9 and p-ERK expression, Notch-signaling and the overall colonocyte population while decreasing the goblet cell number and muc-2 expression. We further demonstrate that Cl-1Tg mice are susceptible to colitis induced using dextran sodium sulfate (DSS) and demonstrate sustained inflammation and hyperplasia even when subjected to recovery following DSS-colitis.

Materials and Methods

Generation and characterization of Claudin-1 Transgenic Mice

The human claudin-1 cDNA was cloned into the pBS plasmid vector under the control of the intestine specific villin promoter, a generous gift from Dr. Sylvie Robine.[15] Two Villin-claudin-1 positive founder lines were obtained on a C57BL/6 genetic background. Mice were maintained following the Institutional Animal Care and Use Committee (IACUC) guidelines of Vanderbilt University. Cl-1Tg mice were maintained in the C57BL/6 background, and offspring genotyped using DNA isolated from the tail genomic DNA. Presence of the transgene was verified by PCR using two different PCR primer sets (Table S1).

Induction of Colitis by Dextran Sodium Sulfate (DSS)

Eight- to ten-week-old mice were used for all studies unless noted otherwise. Mice received standard chow diet during the experiment. Control mice received untreated water ad libitum and the colitis group received indicated amounts of DSS dissolved in water as described in details in the supplemental material.

Measurement of Colitis Severity and Histological Scoring

Colitis severity was estimated by measuring body weight loss, colon length, and colon weight. Paraffin-embedded sections were stained for H&E or with antibodies to indicated proteins (Table S2). Blinded to genotype and treatment, H&E stained sections were scored for parameters quantifying colitis, which include inflammation and crypt damage. Categories were given a score range from 0 to 4 as indicated.

Statistical Analysis

Graphpad Prism 5 was used for statistical analysis (San Diego, CA). Data were analyzed using a student t-test or Newman-Keuls post test following one-way analysis of variance. Data are expressed as means ± SEM. *P* values less than .05 were considered significant.

Results

Characterization of the Cl-1Tg mice

Claudin-1 expression is upregulated under multiple intestinal pathological conditions including IBD and colorectal cancer.[11,12,14] We further confirmed increased claudin-1 expression in Crohn's disease and Ulcerative colitis patient samples (S1). To further investigate the role of claudin-1 in intestinal homeostasis, we developed Cl-1Tg mice using a construct in which the human claudin-1 cDNA was placed under the control of the murine intestine-specific villin promoter (Figure 1A). As predicted, robust claudin-1 overexpression was observed in the colon, small intestine, and cecum of the transgenic mice (Figure 1B), but not in other organs (S2-A). Immunohistochemical analysis using anti-claudin-1 antibody further confirmed the increase in claudin-1 expression, and indicated that it was localized largely to the membrane and throughout the entire crypt in Cl-1Tg mice (Figure 1C).

The claudin family comprises 27 known members, which form homo and hetero-dimers.[16] Genetic manipulation of specific claudin family members alters expression of other claudin family members, possibly due to compensation. Therefore, we sought to determine whether claudin-1 overexpression alters expression of other claudins and/or E-cadherin and β-catenin. Both immunoblot and immunofluorescence analysis using colons from Cl-1Tg and WT mice demonstrated decrease in claudin-7 expression while expression of claudin-3,-4,15, Occludin, E-cadherin, and β-catenin remained largely unaltered (Figure 1E, 1F and S2-B).

Next, we examined the effects of claudin-1 overexpression upon TJ structure and function. Electron microscopic examination revealed no significant morphological changes in the TJ structure of the colonic epithelial cells between age- and sex-matched WT and Cl1-Tg mice. Similarly, epithelial permeability as determined by rectal administration or Ussing chamber analysis using FITC-dextran (4 kDa) was not altered between WT and Cl-1Tg mice. However, trans-epithelial resistance (TER) increased in Cl-1Tg mice *versus* WT-littermates (S3 and S6, $p<0.05$).

Claudin-1 overexpression altered epithelial cell differentiation

Although, Cl-1Tg mice did not differ from WT mice in their appearance and/or gross physiology, histological evaluation suggested potential alteration in the goblet cell number in the colon of Cl-1Tg mice *versus* WT mice (Figure 1D). To evaluate further, we performed Periodic Acidic Schiff (PAS) staining for mucins produced by goblet cells in small intestine (SI) and colon (Figure 2A,B). Indeed, a decrease in number of PAS-positive cells in the SI and colon of the Cl-1Tg mice compared to WT mice was observed (S4-A, $p<0.001$). To further confirm, Alcian blue staining was used to identify acidic proteins commonly found in mucus-containing cells (S4-B). Among the mucins that constitute the colonic mucus barrier, muc-2 is the most abundant[3,17] and is often used as a marker of goblet cell homeostasis. Therefore, we further performed immunohistochemical analysis to examine muc-2 expression in the colon of Cl-1Tg and WT mice. We documented a significant decrease ($p<0.0001$) in muc-2 positive cells in the colon of Cl-1Tg mice compared to WT mice (Figure 2B and S4-A).

The secretory goblet cells are one of the four cell types within the intestinal epithelium.[18] To determine whether other cell types of the absorptive or secretory lineages were also altered, we performed immunohistochemical analysis using Carbonic Anhydrase-I (marker of colonocytes) and Chromagranin-A (marker of enteroendocrine cells). We detected an increase in carbonic anhydrase staining in the colon of Cl-1Tg mice *versus* WT mice (Figure 2B). A slight increase in Chromagranin-A positive cells was also observed in the small intestine and colon of the Cl-1Tg mice and WT mice (Figure 2-A,B). Staining for lysozyme (marker for Paneth cells; small intestine) did not detect major changes in Cl-1Tg mice compared to WT mice (Figure 2A). Overall, intestinal overexpression of claudin-1 appeared to have altered the epithelial lineage commitment in the mouse colon and small intestinal epithelium.

The molecular/signaling mechanism/s that regulate colonic epithelial cell differentiation are also important in the regulation of colonic epithelial proliferation. Therefore, we determined

the potential effect of the colonic claudin-1 overexpression on proliferation. We observed a significant increase in proliferation in the colon of Cl-1Tg mice compared to the colon of WT mice (Figure 2C, $p<0.001$). Further determination of the pathways involved in cell proliferation/apoptosis demonstrated a marked increase in the phosphorylation of ERK-1/2 in the colon of Cl-1Tg *versus* WT mice (Figure 2D).

Cl-1Tg mice are susceptible to DSS-colitis and demonstrate impaired recovery

Loss of goblet cells characterizes IBD patient samples[19] and mice with genetic deletion of muc-2 develop spontaneous colitis.[6] Claudin-1 expression is upregulated in areas of active inflammation in IBD patients.[11] Therefore, in light of the dysregulated goblet cell differentiation and decreased muc-2 expression in Cl-1Tg mice, we further determined whether these mice are susceptible to mucosal inflammation/ regeneration/repair during colitis, using a commonly used DSS-mouse model of colitis. WT and Cl-1Tg mice were subjected to drinking water containing DSS (5% wt/vol) for a period of 7 days followed by regular drinking water for 5 days to recover. Mice were weighed daily and monitored for signs of distress (see Supplementary Methods). On day-4 of DSS-treatment, a significant body weight loss was observed in the DSS-treated Cl-1Tg mice compared to WT mice ($p<0.001$) and this continued until day-7 of DSS-administration (Figure 3A). Apart from body weight, we observed a significant increase in the colon weight/length ratio ($p<0.05$) in DSS-treated Cl-1Tg *versus* WT mice (Figure 3B). Histopathological analysis further supported the severity of inflammation in DSS-treated Cl-1Tg *versus* WT mice (S5). However these mice did not recover, lost more than 20% of body weight by day 9 and were therefore sacrificed.

In further studies, we decreased the dosage of DSS to 3.5% wt/vol, while keeping the duration of DSS-administration constant (7 days) followed by regular drinking water for 5 days to recover. The DSS-treated Cl-1Tg mice again showed a decrease in body weight as early as day-4 (*versus* WT mice) and the trend continued until day-7 (Figure 3C). The H&E staining showed epithelial damage and loss of the crypt structure in DSS-treated WT mice. The epithelial damage was enhanced in Cl-1Tg mice and showed severe loss of crypt structure. Most interestingly, during the recovery phase, the WT mice showed complete recovery of the DSS-colitis dependent body weight loss while Cl-1Tg mice demonstrated impaired recovery (Figure 3C). Furthermore, the recovering Cl-1Tg mice exhibited hyperplastic elongated crypts compared to the extensive normal regenerative crypts in WT mice (Figure 3D). Histopathological scoring for the inflammation, depth of inflammation, and crypt damage confirmed the significantly higher degree of the depth of inflammation and epithelial injury in DSS-treated Cl-1Tg mice *versus* WT mice (Figure 3E). Furthermore, during recovery, Cl-1Tg mice continued to display high scores for both inflammation and epithelial injury while the WT mice recovered almost completely.

The DSS-treated Cl-1Tg mice demonstrated persistently low muc-2 expression and an elevated and sustained immune response

The epithelial and mucosal barrier serve as the prime protective layers from luminal antigens [3]. Since, DSS-treated and recovering Cl-1Tg mice showed persistent inflammation, we determined the changes in epithelial permeability and status of muc-2 expression in these

mice. Ussing Chamber was utilized to determine the changes in TER and trans-mucosal permeability (S6). For the integrity of mucus layer, IHC was performed using anti-muc-2 antibody and number of positively stained, intact cells/crypt was quantified (Figure 4A). The TER decreased in both mice groups when subjected to DSS-colitis however decrease was more pronounced in Cl-1Tg mice ($p<0.0001$). Further, an increasing trend in TER in the recovering WT mice (*versus* DSS-colitis group) contrasted with the persistent decrease in Cl-1Tg mice (S6). The permeability for FITC-Dextran increased in both mice groups in response to DSS-treatment (*versus* respective controls). However, the trans-mucosal permeability demonstrated a reversing trend towards the control levels in the recovering WT mice compared to a persistently increased trans-mucosal permeability in the recovering Cl-1Tg mice though differences were statistically insignificant (S6). We observed decreased muc-2 expression in DSS-treated WT and Cl-1Tg mice compared to respective control mice. While muc-2 expression levels recovered to control levels ($p<0.05$) in recovering WT mice, it failed to recover to similar levels in the recovering Cl-1Tg mice ($p<0.001$). Furthermore, in Cl-1Tg mice the goblet cells in absence of optimal muc-2 synthesis lost their characteristic goblet like shape, a characteristic similar to that seen in muc2−/− mice earlier. [6]

During colitis there is an infiltration of immune cells accompanied by changes in cytokine gene expression that occurs in response to the ensuing damage.[20] One component of the immune infiltrate is CD3+ T-lymphocytes that are critical effectors of the mucosal immune activation. A significant increase in CD3+ cells was observed in DSS-treated Cl-1Tg compared to WT mice ($p<0.001$). Again as in muc-2 expression, the increase in CD3+ cells infiltration in recovering WT mice returned back to the levels in control (water) mice. However, recovering Cl-1Tg mice retained a significantly higher level of CD3+ cells ($p<0.01$; Figure 4B), suggesting sustained immune activation. To further define the changes in immune activation, we compared mRNA expression levels of the key inflammatory cytokines, TNFα, IFN-γ, IL-10 and chemokine KC/ CXCL1 using qRT-PCR. An increased expression of the inflammatory cytokines was observed in DSS-treated WT and Cl-1Tg mice. However, Cl-1Tg mice showed significantly increased and sustained cytokine production including TNF-α and IFN-γ even 5-days post-DSS treatment (the recovery phase) when the cytokine levels in DSS-treated WT mice had returned to control levels (Figure 4C). To further confirm these findings, we examined cytokine protein levels using total colon lysates from mice that underwent recovery following DSS-treatment (S7). Outcome was consistent with the data from qRT-PCR analysis and demonstrated significant increases in TNFα ($p<0.05$), IL-1β ($p<0.01$), IL-4 ($p<0.05$) and IP-10 ($p<0.01$). Thus, our results suggested that sustained loss of muc-2 expression and increased cytokine expression in the Cl-1Tg mice may underlie the sustained immune activation in these mice.

Proliferation and apoptosis were altered in Cl-1Tg mice following DSS treatment and recovery

In addition to the sustained immune activation, we observed impaired epithelial recovery in recovering Cl-1Tg mice while colonic crypts underwent hyperplasia. Under similar conditions, WT mice showed normal regenerating crypts (Figure 3D). Further determination of the proliferation and apoptosis showed sharply decreased cell proliferation while

caspase-3 positive cells increased in DSS-treated WT mice which was well in accordance with previous reports.[21] Interestingly, both the DSS-dependent decrease in proliferation and the increase in apoptosis were higher in DSS treated Cl-1Tg mice compared to control mice (Figure 5A,C&D, $p<0.001$). In contrast, during the recovery, we found no significant difference in the apoptosis between the WT and Cl-1Tg mice. At the same time, Cl-1Tg mice demonstrated increased p-ERK1/2 expression and hyperproliferation compared to WT mice (Figure 5B&C, $p<0.001$). Combined, the dynamic balance between the proliferation and apoptosis appeared to be dysregulated in Cl-1Tg mice, which combined with sustained inflammation and altered differentiation results in impaired recovery and hyperplasia.

Notch-signaling is upregulated in Cl-1Tg mice

The Notch-signaling pathway is the critical regulator of the intestinal epithelial cell fate determination.[22,23] Apart, Notch-signaling regulates Muc-2 expression [24],[18], and has a critical role in the regulation of mucosal inflammation and proliferation.[25] Therefore, we examined the status of Notch-signaling (using Hes-1 expression as marker) in DSS-treated and recovering WT and Cl-1Tg mice (Figure 6A). Hes-1 expression was higher in the control as well as DSS-treated Cl-1Tg mice (*versus* control or DSS-treated WT mice respectively). Interestingly, similar to the muc-2 expression, Hes-1 expression also reverted back to the control levels in the recovering WT mice. In contrast, Hes-1 expression remained increased in the recovering Cl-1Tg mice compared to the control and/or DSS-treated Cl-1Tg mice highlighting the inherent defect in the regulation of Notch-signaling in these mice.

Therefore, we further determined the changes underlying Notch-activation in Cl-1Tg mice. To activate Notch-signaling, a proteolytic cleavage releases the Notch intracellular domain (NICD), which is then transported to the nucleus to induce transcription of a number of genes including Hes-1.[8] Hes-1, inhibits expression of Math1 and thus muc-2, both of which are markers of secretory cell lineage.[7] Using immunoblot and real time qPCR analysis, increased NICD and Hes-1($p<0.01$, 2.5-fold) and decreased Math-1 ($p<0.001$, 3-fold) expressions were documented in the colon of Cl-1Tg *versus* WT mice (Figure 6B). We also observed increase in NICD and Hes-1 and a decrease in Math-1 expression in $SW480^{claudin\text{-}1}$ cells (stably overexpressing claudin-1)(Figure 6C). Similar increase in Notch signaling (NICD, Hes-1 and Math-1 expression) was observed in goblet cell-like Ls174T cells in response to stable claudin-1 overexpression (Figure 7A). Claudin-1 overexpression also inhibited the levels of PAS-immunostaining and differentiation-associated proteins TFF3 and KLF4 in these cells, similar to Cl-1Tg mice (Figure 7B). Inhibition of Notch-signaling using DAPT reverted the claudin-1-dependent effects upon differentiation and inhibited proliferation in these cells (Figure 7C–E).

An association of claudin-1 with matrix-metalloproteases and potential role in MMP-9 activation is reported. [26] Therefore, we determined the expression of active-MMP-9 in Cl-1Tg mice. Immunoblot analysis demonstrated increased expression of active MMP-9 in Cl-1Tg mice (*versus* WT mice, Figure 8A). Increased expression of active-MMP-9 was also observed in $SW480^{claudin\text{-}1}$ and $LS174T^{claudin\text{-}1}$ cells [14, and Figure 8B&C]. Similar to

Cl-1Tg mice, we also observed increased p-ERK1/2 expression in claudin-1 overexpressing cells (Figure 6C&8C).

We then examined functional importance of Notch, MMP-9 and p-ERK1/2 signaling in claudin-1-dependent effects. Inhibition of MMP-9 activity, using MMP specific inhibitor GM6001, inhibited NICD expression and induced differentiation in claudin-1 overexpressing Ls174T cells (Figure 8B&C,E,S8) without affecting the proliferation and p-ERK1/2 expression (Figure 8C&D). Of note, goblet cell number increases in MMP-9 knockdown mice. [28] Inhibition of Wnt/β-catenin signaling (yet another important pathway in intestinal differentiation/proliferation) using a specific inhibitor pyruvinium (100nM, 24hrs) did not affect the NICD or MMP-9 expression (data not shown).

We then determined the functional importance of ERK1/2 activation. Inhibition of p-ERK1/2, using U0126, inhibited NICD expression while inducing apoptosis (cleaved caspase-3 expression) and differentiation. However, inhibition of ERK activation did not affect active-MMP-9 expression or proliferation (Figure 9A–C).

Discussion

Claudin-1 is a key constituent of the tight junction complex, however, recent studies, including ours, have highlighted other potential functions of claudin-1.[29,30] Recent studies have demonstrated marked increase in claudin-1 expression in colon cancer [14] as well as the areas of active inflammation and its correlation with neoplastic transformation. [11,12] However, no study till date has determined the potential causal role of claudin-1 expression in the regulation of mucosal inflammation. In this study, using a novel transgenic mouse model with intestinal claudin-1 overexpression, we unravel a novel and previously unknown role of claudin-1 in the regulation of Notch-signaling, epithelial differentiation and mucosal inflammation.

Importantly, Notch-signaling is one of the master regulators of colonic epithelial differentiation and cell lineage determination of secretory cell lineage, especially goblet cells.[18,31] The principal secretory product of goblet cells is muc-2, a key constituent of the mucus layer that protects the mucosal epithelial layer.[32,33] Notably, Notch activation and muc-2/goblet cell depletion is a characteristic associated with mucosal inflammation and colon cancer.[5–7,34–36] Thus, it becomes important to investigate how Notch-signaling is regulated under physiological and pathological conditions. Our data suggest that claudin-1 expression may serve as one of the dynamic regulators of Notch-signaling. Our studies using qPCR analysis showed that the expression of Notch receptors and ligands known to be upregulated in colon cancer [37] is not altered in Cl-1Tg mice (data not shown). However, inhibition of MMP-9 inhibited NICD expression and differentiation. Therefore, we postulate that the increase in claudin-1 expression increases proteolytic cleavage of the Notch-receptor to release NICD, which in turn translocates to the cell nucleus and regulate the transcription of Notch-target genes to regulate the colonocyte differentiation and cell fate determination. Importantly, claudin-1 associates with various metalloproteinases including MT1MMP and MMP-2 and induces their activation.[14,38] While γ-secretase is the known regulator of Notch-cleavage, its proteolytic action to cleave Notch is facilitated by matrix

metalloproteinases (MMPs) including MMP-7 and MMP-9. In this regard, MMP-7 was shown to be necessary for γ-secretase mediated Notch1-cleavage in pancreatic cells and its downstream effects.[39] Furthermore, Notch-activity is modulated in the colon of mice genetically manipulated for MMP-9 expression/activity.[28] Notably, the phenotype of Cl-1Tg mice observed in our current study strongly resembles with the phenotype of MMP-9−/− mice under normal physiological condition or when subjected to DSS colitis. These findings support a role of claudin-1 in the regulation of Notch-signaling and colonic homeostasis. Future studies where Cl-1Tg mice are interbred with mice manipulated to inhibit Notch-signaling will further clarify such functional correlation and is part of our ongoing investigation.

Despite the obvious defects in Notch-signaling and altered differentiation, we did not observe any gross morphological/developmental defects in Cl-1Tg mice. However, when subjected to DSS-colitis and recovery, not only were these mice susceptible but also demonstrated sustained immune activation and inflammation even when the source underlying this immune-activation/inflammation (DSS) was removed. Moreover, epithelial regeneration and repair was compromised in Cl-1Tg mice when subjected to inflammatory insult. Our data shows muc-2 expression remains significantly decreased in DSS-treated Cl-1Tg mice. Furthermore, despite a baseline increase in TER, the DSS-dependent decrease in TER or increase in trans-mucosal permeability were relatively pronounced in Cl-1Tg mice. Also, during the recovery decreased muc-2 expression was accompanied with persistent decrease in TER and increased trans-mucosal permeability in Cl-2Tg mice. Thus, our data suggest that the impaired mucosal barrier function and epithelial differentiation may underlie the impaired epithelial recovery in DSS-treated Cl-1Tg mice, which in turn may underlie the sustained cytokine activation, immune activation and inflammation in these mice. Data from muc-2 deficient and MMP-9−/− mice support such a postulation as similar sustained immune-activation and hyperproliferation have been observed in these mice.[6,28]

When stimulated with proinflammatory cytokines (including IFN-γ and TNF-α), the intestinal epithelial cells may also secrete chemokines, directing migration and activation of leukocytes. CXCL10(IP-10) chemokine, which was significantly induced in recovering Cl-1Tg mice compared to the WT mice, chemoattracts activated T-cells as well as monocytes.[40] In accordance, we observed increased and sustained CD3+ cell infiltration in DSS-treated and recovering Cl-1Tg mice. Of interest, IP-10 is upregulated in pathogen-induced acute inflammation,[41] and IFN-γ is the major inducer of IP-10.[42] Furthermore, IL-1β has also been recently shown to induce IP-10, especially in combination with TNF-α and IFN-γ.[41] Notably, in recovering Cl-1Tg mice there was sustained increase in TNF-α, IL-1β and IFN-γ expression. Combined, we suggest increased cytokines and chemokines in recovering Cl-1Tg mice help sustain the inflammation by T-cell recruitment.

Another possibility that may underlie sustained inflammation in Cl-1Tg mice is the potential defect in antigen clearance. A relatively compromised mucus layer due to the decrease in muc-2 expression could enable enhanced passage of luminal antigens in DSS-treated Cl-1Tg mice *versus* WT mice. An impaired recovery in these mice could further help sustain access of the luminal antigens into the mucosa. (S-4&6). Apart, we have observed marked changes

in the expression of claudin-7 in the colon of Cl-1Tg mice. Of interest, genetic ablation of claudin-7 results in epithelial cell sloughing, significantly higher levels of cytokines, MMP-3 and MMP-7 and spontaneous colitis.[43] Also, claudin-1 is also a co-receptor for Hepatitis-C-virus.[44] However, further studies are required to ascertain such possibilities.

Sustained immune activation, dependent injury and subsequent regenerative response initiates a vicious cycle leading to chronic inflammation, which in presence of oncogenic gene mutation promotes epithelial transformation and neoplastic growth. Our findings that Notch and ERK 1/2 activation, and proliferation are significantly increased in the colon of Cl-1Tg mice (*versus* WT littermates) and colonic crypts in Cl-1Tg mice demonstrate hyperplasticity during the recovery from DSS-colitis suggest that inflammatory mechanisms may help promote the role of claudin-1 as colon cancer promoter. Similar claudin-1 mediated regulation of ERK activation in human liver cells has been demonstrated previously.[27] Co-operation between Notch and ERK1/2-signaling in the regulation of proliferation and differentiation has been reported.[45] Ras-activated breast cancer cells acquire tumorigenic properties when Notch signaling is activated.[46] Furthermore, dysregulated epithelial differentiation and proliferation constitutes the core of cancer progression and metastasis. Taken together, central outcome from our current study is that claudin-1 regulates colonic epithelial cell differentiation in a Notch-dependent manner. Dysregulation of claudin-1 expression modulates MMP-9 and p-ERK expression/activity to induce Notch-signaling and dysregulate colonic epithelial homeostasis favoring inflammatory conditions and hyperplasia. Our current findings are well in accordance with our earlier findings where genetic manipulation of claudin-1 in colon cancer cells had inverse effect upon epithelial differentiation.[14] Taken together, our data support a novel role of claudin-1 in the regulation of Notch-signaling and colonic homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: This work was supported by NIH grant CA124977 (P. Dhawan), DK088902 (A.B. Singh), the VICC P30CA68485 and the DDRC DK58404. We would like to thank Dr. Means for critically reviewing the manuscript.

References

1. Turner JR. Intestinal mucosal barrier function in health and disease. Nat Rev Immunol. 2009; 9:799–809. [PubMed: 19855405]
2. Chichlowski M, Hale LP. Bacterial-mucosal interactions in inflammatory bowel disease: an alliance gone bad. Am. J. Physiol. Gastrointest. Liver Physiol. 2008; 295:G1139–G1149. [PubMed: 18927210]
3. Kufe DW. Mucins in cancer: function, prognosis and therapy. Nat. Rev. Cancer. 2009; 9:874–885. [PubMed: 19935676]
4. Singh AB, Sharma A, Dhawan P. Claudin family of proteins and cancer: an overview. J Oncol. 2010; 2010:541957. [PubMed: 20671913]
5. Maloy KJ, Powrie F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. Nature. 2011; 474:298–306. [PubMed: 21677746]

6. Van der Sluis M, De Koning BAE, De Bruijn A, et al. Muc2-Deficient Mice Spontaneously Develop Colitis, Indicating That Muc2 Is Critical for Colonic Protection. Gastroenterology. 2006; 131:117–129. [PubMed: 16831596]
7. Qiao L, Wong BCY. Role of Notch signaling in colorectal cancer. Carcinogenesis. 2009; 30:1979–1986. [PubMed: 19793799]
8. Zheng X, Tsuchiya K, Okamoto R, et al. Suppression of hath1 gene expression directly regulated by hes1 via notch signaling is associated with goblet cell depletion in ulcerative colitis. Inflamm Bowel Dis. 2011; 17:2251–2260. [PubMed: 21987298]
9. Dahan S, Rabinowitz KM, Martin AP, et al. Notch-1 signaling regulates intestinal epithelial barrier function, through interaction with CD4+ T cells, in mice and humans. Gastroenterology. 2011; 140:550–559. [PubMed: 21056041]
10. Sikandar SS, Pate KT, Anderson S, et al. NOTCH Signaling Is Required for Formation and Self-Renewal of Tumor-Initiating Cells and for Repression of Secretory Cell Differentiation in Colon Cancer. Cancer Research. 2010; 70:1469–1478. [PubMed: 20145124]
11. Weber CR, Nalle SC, Tretiakova M, et al. Claudin-1 and claudin-2 expression is elevated in inflammatory bowel disease and may contribute to early neoplastic transformation. Lab Invest. 2008; 88:1110–1120. [PubMed: 18711353]
12. Kinugasa T, Akagi Y, Yoshida T, et al. Increased claudin-1 protein expression contributes to tumorigenesis in ulcerative colitis-associated colorectal cancer. Anticancer Res. 2010; 30:3181–3186. [PubMed: 20871038]
13. Furuse M, Hata M, Furuse K, et al. Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. Journal of Cell Biology. 2002; 156:1099–1111. [PubMed: 11889141]
14. Dhawan P, Singh AB, Deane NG, et al. Claudin-1 regulates cellular transformation and metastatic behavior in colon cancer. J. Clin. Invest. 2005; 115:1766–1776.
15. Marjou el F, Janssen K-P, Chang BH-J, et al. Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis. 2004; 39:186–193. [PubMed: 15282745]
16. Niessen CM. Tight Junctions/Adherens Junctions: Basic Structure and Function. J Investig Dermatol. 2007; 127:2525–2532. [PubMed: 17934504]
17. Coant N, Mkaddem SB, Pedruzzi E, et al. NADPH Oxidase 1 Modulates WNT and NOTCH1 Signaling To Control the Fate of Proliferative Progenitor Cells in the Colon. Molecular and Cell Biology. 2010; 30:2636–2650.
18. van der Flier LG, Clevers H. Stem Cells, Self-Renewal, and Differentiation in the Intestinal Epithelium. Annu. Rev. Physiol. 2009; 71:241–260. [PubMed: 18808327]
19. Tytgat KM, van der Wal JW, Einerhand AW, Büller HA, Dekker J. Quantitative analysis of MUC2 synthesis in ulcerative colitis. Biochemical and Biophysical Research Communications. 1996; 224:397–405. [PubMed: 8702401]
20. Műzes G, Molnár B, Tulassay Z, Sipos F. Changes of the cytokine profile in inflammatory bowel diseases. WJG. 2012; 18:5848–5861. [PubMed: 23139600]
21. Araki Y, Sugihara H, Hattori T. In vitro effects of dextran sulfate sodium on a Caco-2 cell line and plausible mechanisms for dextran sulfate sodium-induced colitis. Oncol. Rep. 2006; 16:1357–1362. [PubMed: 17089061]
22. Nakamura T, Tsuchiya K, Watanabe M. Crosstalk between Wnt and Notch signaling in intestinal epithelial cell fate decision. J Gastroenterol. 2007; 42:705–710. [PubMed: 17876539]
23. Fre S, Hannezo E, Sale S, et al. Notch lineages and activity in intestinal stem cells determined by a new set of knock-in mice. PLoS ONE. 2011; 6:e25785. [PubMed: 21991352]
24. Okamoto R, Tsuchiya K, Nemoto Y, et al. Requirement of Notch activation during regeneration of the intestinal epithelia. Am. J. Physiol. Gastrointest. Liver Physiol. 2009; 296:G23–G35. [PubMed: 19023031]
25. Okamoto R. Epithelial regeneration in inflammatory bowel diseases. Inflammation and Regeneration. 2011; 31:275–281.
26. Miyamori H, Takino T, Kobayashi Y, et al. Claudin Promotes Activation of Pro-matrix Metalloproteinase-2 Mediated by Membrane-type Matrix Metalloproteinases. Journal of Biological Chemistry. 2001; 276:28204–28211. [PubMed: 11382769]

27. Suh Y, Yoon C-H, Kim R-K, et al. Claudin-1 induces epithelial-mesenchymal transition through activation of the c-Abl-ERK signaling pathway in human liver cells. Oncogene. 2012 Nov 19. Online First:
28. Garg P, Ravi A, Patel NR, et al. Matrix metalloproteinase-9 regulates MUC-2 expression through its effect on goblet cell differentiation. Gastroenterology. 2007; 132:1877–1889. [PubMed: 17484881]
29. Singh AB, Sharma A, Smith JJ, et al. Claudin-1 up-regulates the repressor ZEB-1 to inhibit E-cadherin expression in colon cancer cells. Gastroenterology. 2011; 141:2140–2153. [PubMed: 21878201]
30. Singh AB, Sharma A, Dhawan P. Claudin-1 expression confers resistance to anoikis in colon cancer cells in a Src-dependent manner. Carcinogenesis. 2012; 33:2538–2547. [PubMed: 22941059]
31. VanDussen KL, Carulli AJ, Keeley TM, et al. Notch signaling modulates proliferation and differentiation of intestinal crypt base columnar stem cells. Development. 2012; 139:488–497. [PubMed: 22190634]
32. Bergstrom KSB, Kissoon-Singh V, Gibson DL, et al. Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa. PLoS Pathog. 2010; 6:e1000902. [PubMed: 20485566]
33. Johansson MEV, Phillipson M, Petersson J, et al. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc. Natl. Acad. Sci. 2008; 105:15064–15069. [PubMed: 18806221]
34. Garg P, Jeppsson S, Dalmasso G, et al. Notch1 regulates the effects of matrix metalloproteinase-9 on colitis-associated cancer in mice. Gastroenterology. 2011; 141:1381–1392. [PubMed: 21723221]
35. Yang W, Velcich A, Lozonschi I, et al. Inactivation of p21WAF1/cip1 enhances intestinal tumor formation in Muc2−/− mice. The American Journal of Pathology. 2005; 166:1239–1246. [PubMed: 15793302]
36. Yang K, Popova NV, Yang WC, et al. Interaction of Muc2 and Apc on Wnt Signaling and in Intestinal Tumorigenesis: Potential Role of Chronic Inflammation. Cancer Research. 2008; 68:7313–7322. [PubMed: 18794118]
37. Vooijs M, Liu Z, Kopan R. Notch: architect, landscaper, and guardian of the intestine. Gastroenterology. 2011; 141:448–459. [PubMed: 21689653]
38. Oku N, Sasabe E, Ueta E, et al. Tight junction protein claudin-1 enhances the invasive activity of oral squamous cell carcinoma cells by promoting cleavage of laminin-5 gamma2 chain via matrix metalloproteinase (MMP)-2 and membranetype MMP-1. Cancer Research. 2006; 66:5251–5257. [PubMed: 16707450]
39. Sawey ET, Johnson JA, Crawford HC. Matrix metalloproteinase 7 controls pancreatic acinar cell transdifferentiation by activating the Notch signaling pathway. Proc. Natl. Acad. Sci. 2007; 104:19327–19332. [PubMed: 18042722]
40. Fang YS, Zhu LM, Sun ZG, et al. Tumor necrosis factor-α pathway plays a critical role in regulating interferon-γ induced protein-10 production in initial allogeneic human monocyte-endothelial cell interactions. Transplant. Proc. 2012; 44:993–995. [PubMed: 22564606]
41. Yeruva S, Ramadori G, Raddatz D. NF-kappaB-dependent synergistic regulation of CXCL10 gene expression by IL-1beta and IFN-gamma in human intestinal epithelial cell lines. Int J Colorectal Dis. 2008; 23:305–317. [PubMed: 18046562]
42. Cassatella MA, Gasperini S, Calzetti F, et al. Regulated production of the interferon-gamma-inducible protein-10 (IP-10) chemokine by human neutrophils. Eur. J. Immunol. 1997; 27:111–115. [PubMed: 9022006]
43. Ding L, Lu Z, Foreman O, et al. Inflammation and disruption of the mucosal architecture in claudin-7-deficient mice. Gastroenterology. 2012; 142:305–315. [PubMed: 22044670]
44. Evans MJ, Hahn von T, Tscherne DM, et al. Claudin-1 is a hepatitis C virus coreceptor required for a late step in entry. Nature. 2007; 446:801–805. [PubMed: 17325668]
45. Sundaram MV. The love-hate relationship between Ras and Notch. Genes & Development. 2005; 19:1825–1839. [PubMed: 16103211]

46. Mittal S, Subramanyam D, Dey D, et al. Cooperation of Notch and Ras/MAPK signaling pathways in human breast carcinogenesis. Mol Cancer. 2009; 8:128. [PubMed: 20030805]

Significance of the study

What is already known about this subject?

- Claudin-1, a tight junction protein, is a key regulator of epithelial barrier function and genetic deletion of its expression in mice results in post-natal death.
- In colon cancer samples and cells lines, claudin-1 expression is highly increased and dysregulated.
- In colon derived cell lines, manipulation of claudin-1 expression affects differentiation, cellular transformation and tumorigenic as well as metastatic abilities.
- Claudin-1 expression is increased in colitis-associated cancer.

What are the new findings?

- Claudin-1 plays a key role in the regulation of colonic epithelial homeostasis. Modulation of its intestinal expression alters the normal intestinal epithelial cell differentiation in mice resulting in decreased goblet cells and muc-2 expression.
- Claudin-1 regulates Notch activation in mice and colon cancer cells through the modulation of MMP-9 and p-ERK signaling.
- Increased intestinal claudin-1 expression in mice renders susceptibility to colitis and causes impaired recovery following DSS-induced colitis. In accordance, claudin-1 expression is increased in patients suffering from ulcerative colitis and Crohn's disease.

How might it impact on clinical practice in the foreseeable future?

- The results from this study will provide insight into the understanding the contribution of claudin-1 to colonic epithelial homeostasis, differentiation, and epithelial injury and repair. These cellular aspects are directly related to the intestinal inflammation and neoplastic growth. Our current findings highlight the importance of claudin-1 as a potential novel therapeutic target for intervention in inflammatory bowel disease.

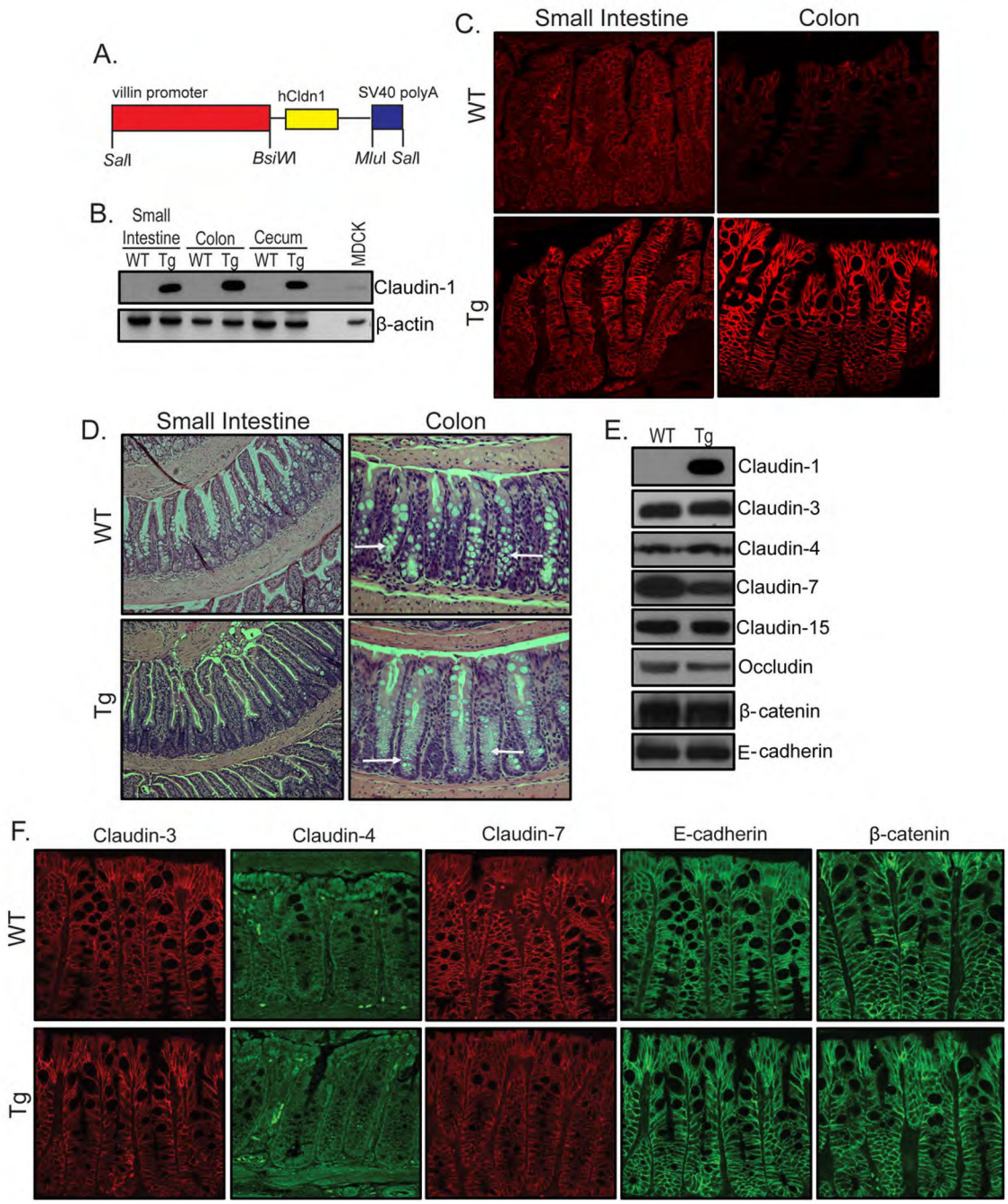

Figure 1. Generation of Claudin-1 Transgenic mice

(A) Schematic of Villin-claudin-1 expression vector. (B) Immunoblot analysis using tissue lysates from wild type (WT) and Cl-1Tg mice were utilized to determine the expression of Claudin-1 expression. β-actin was used as loading control. MDCK cell lysate served as positive control. (C) Immunostaining for claudin-1 in the small intestine and colon of WT and Cl-1Tg mice. (D) Representative H&E staining for the colon of WT and Cl-1Tg mice. (E) Immunoblot analysis; and (F) Immunostaining for tight junctions and adherens junctions proteins in WT and Cl-1Tg mice.

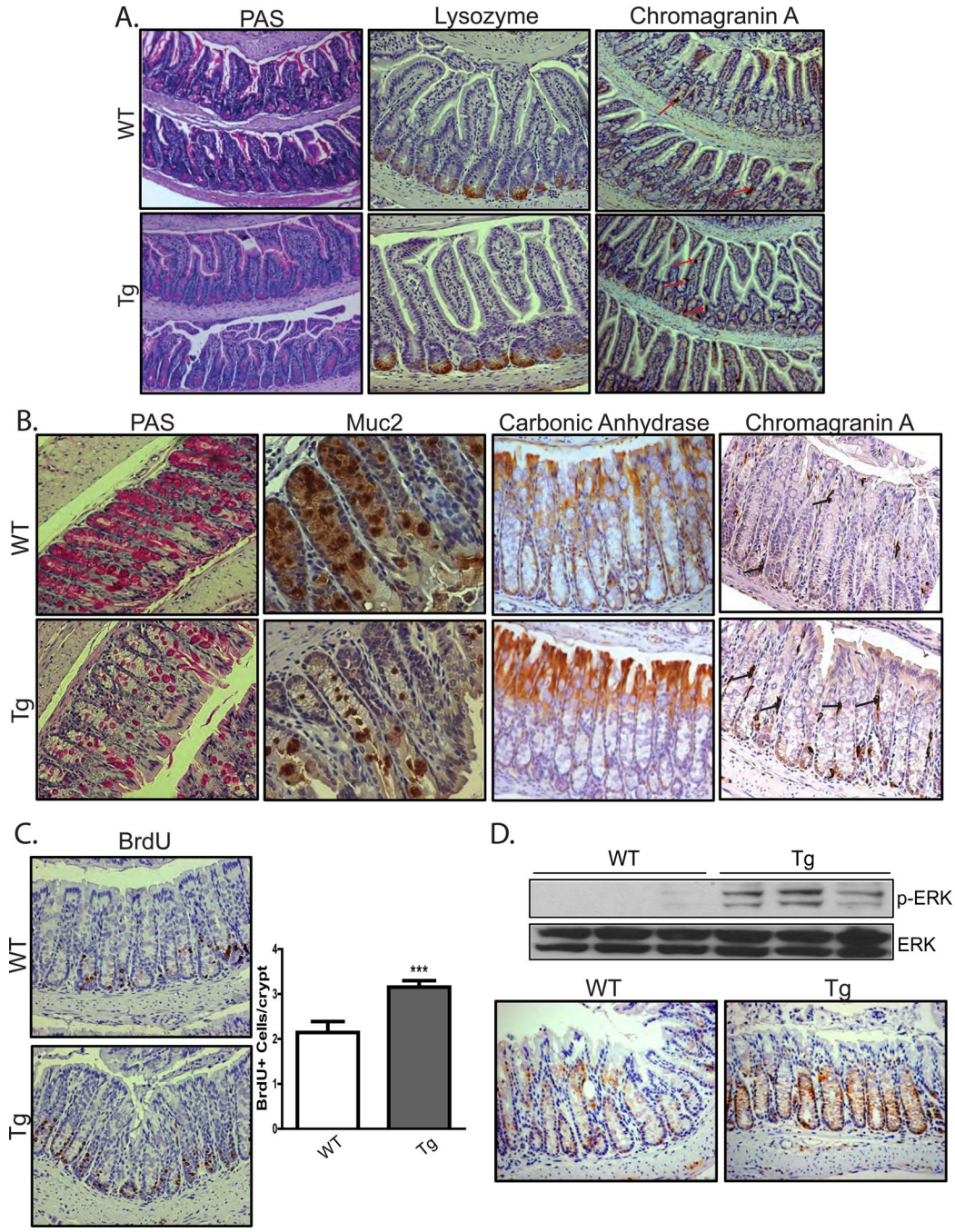

Figure 2. Claudin-1 overexpression alters intestinal epithelial cell lineage and increases proliferation

(A) Immunostaining for PAS, Lysozyme, and Chromagranin-A in small intestine; (B) Immunostaining for PAS, Muc-2, Carbonic Anhydrase-I, and Chromagranin-A in colon; (C) Immunostaining of colon for BrdU incorporation and quantification; and (D) Immunoblot and immunostaining to determine p-ERK expression in WT and Cl-1Tg colons. ***p<0.001

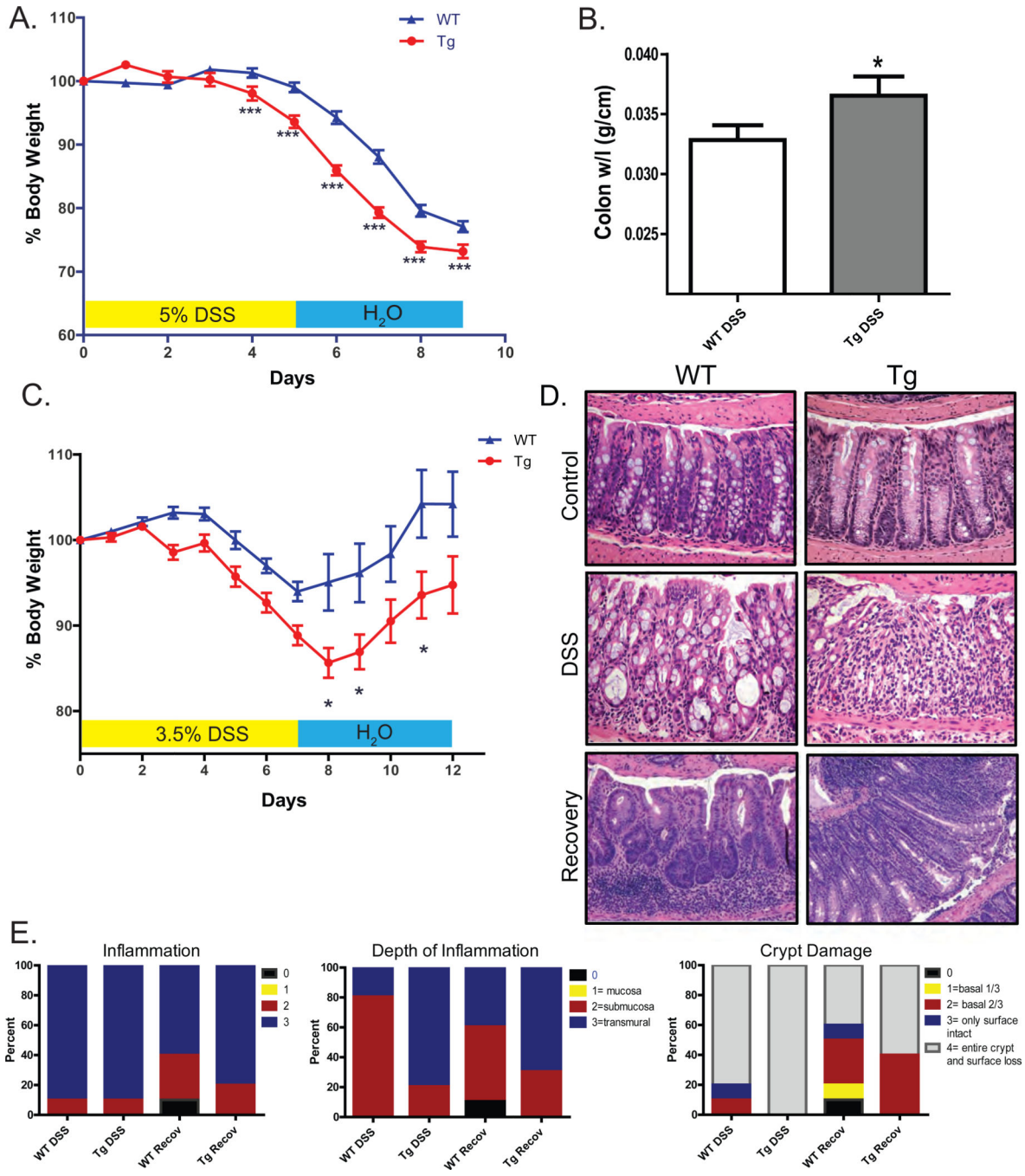

Figure 3. Claudin-1 Tg mice are susceptible to DSS-colitis

WT and Cl-1Tg mice were given DSS (5% w/v) for 7-days and (A) percent change in body weight and (B) Colon weight/length (g/cm) in control and DSS-treated animals were monitored. Further, WT and Cl-1Tg mice were exposed to DSS (3.5% w/v) for 7-days and then allowed to recover for 5-days. Following parameters were then determined: (C) Percent change in body weight; (D) Representative H&E staining; and (E) Histopathologic scoring of the inflammation, depth of inflammation, and crypt damage. Data are represented as the percentage of mice per group with the indicated score. *p<0.05, ***p<0.001

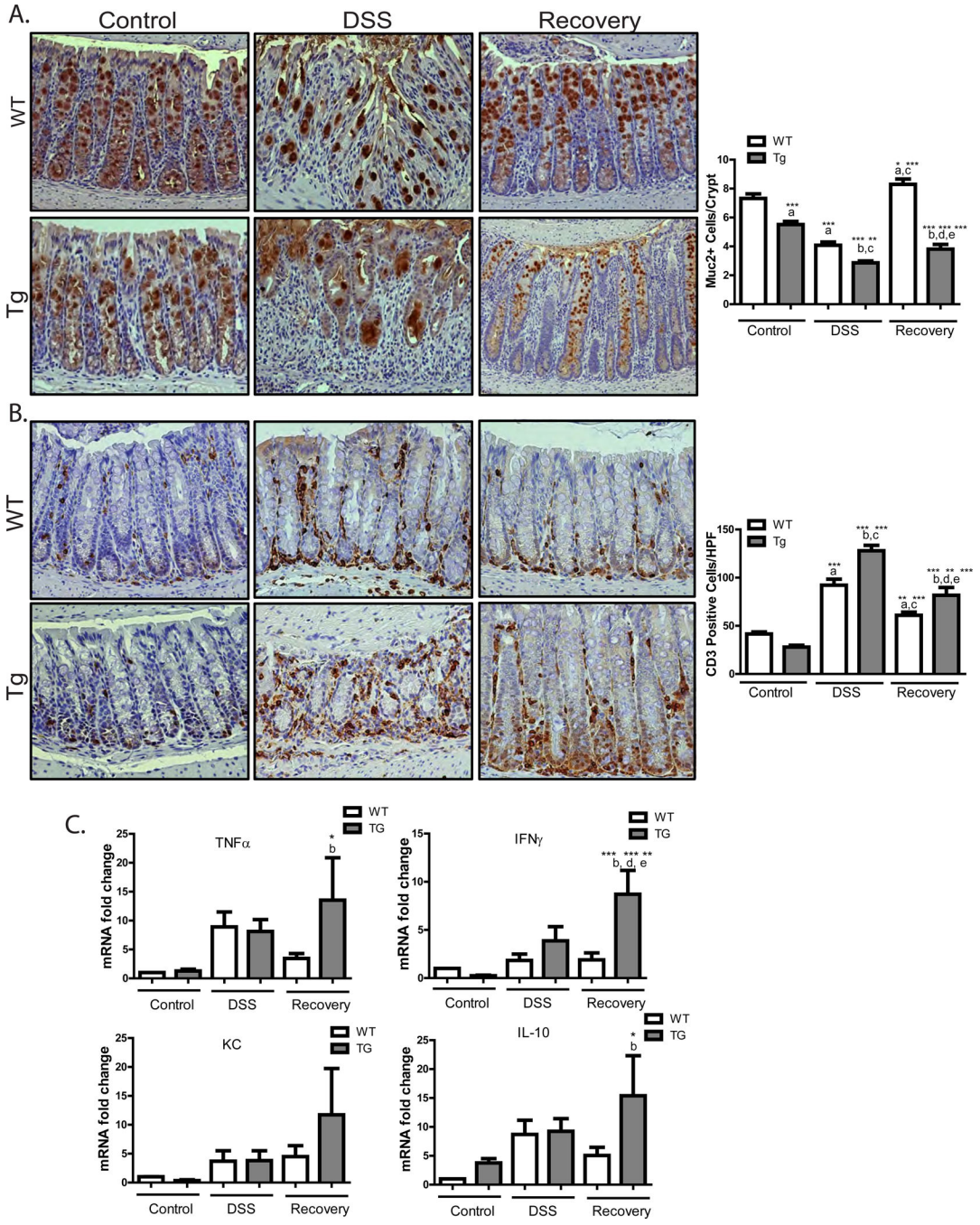

Figure 4. Cl-1Tg mice have sustained inflammation during recovery from DSS-colitis

WT and Cl-1Tg mice exposed to either water alone, DSS (7 days), or DSS and then switched to regular water (5 days; recovery). These mice were then (A) Immunostained and quantitated for muc-2 expression using anti-muc-2 antibody; and (B) Immunostained and quantitated for T-cell infiltration using CD3, a T-cell marker, in the colon from control (water), DSS (day-7) and recovery (day-12), WT and Cl-1Tg mice. [a] compared to WT-control, [b] compared to Cl-1Tg-control, [c] compared to WT-DSS, [d] compared to WT-recovery

(C) Quantitative RT-PCR analysis of TNFα, IFN-γ, K C a nd IL-10 expression. *p<0.05, **p<0.01, and ***p<0.001.

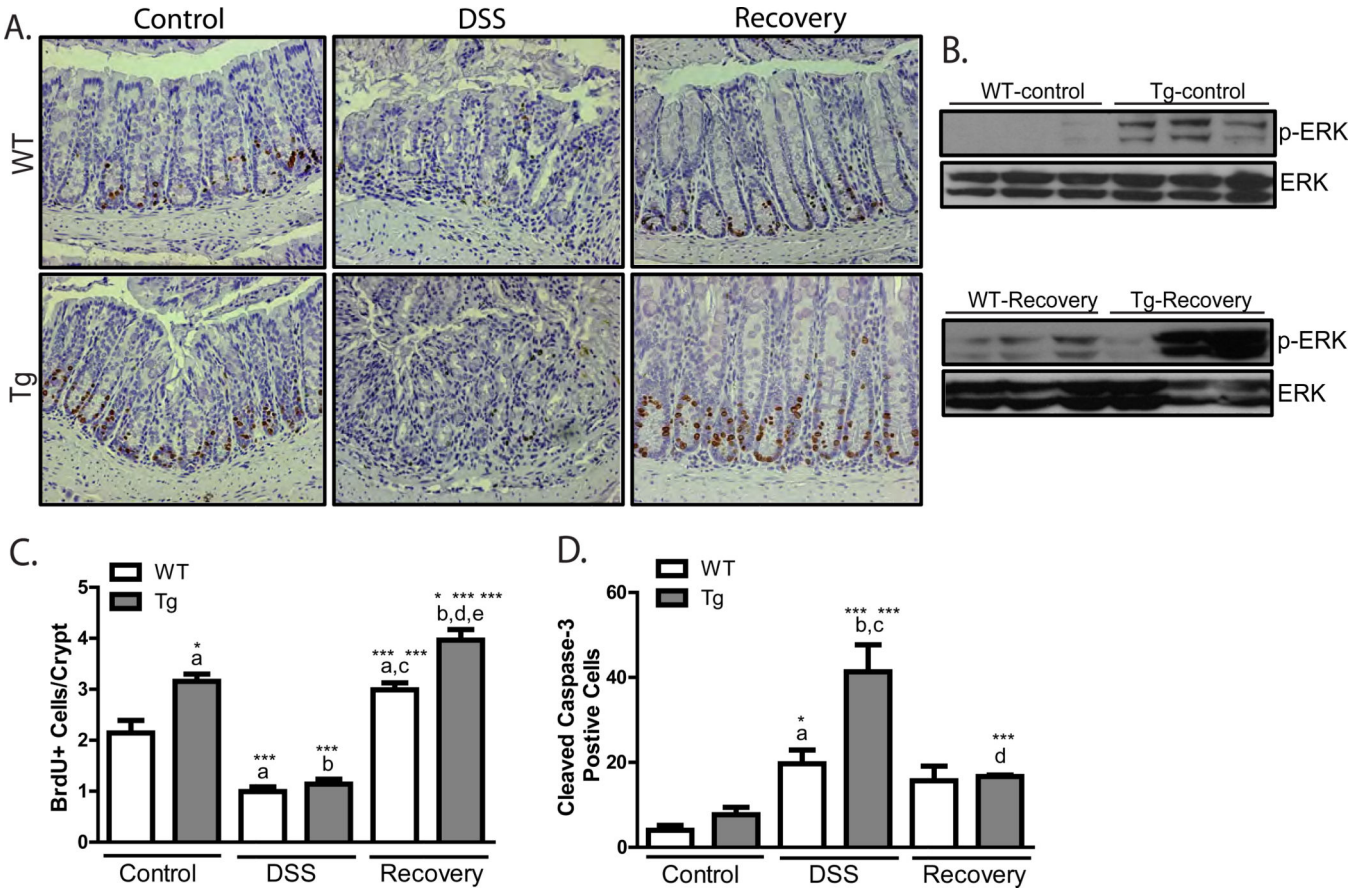

Figure 5. Proliferation and Apoptosis are altered in Cl-1Tg mice undergoing DSS treatment and recovery

(A) Proliferation was determined using immunohistochemical analysis to determine BrdU incorporation in the colon of WT and Cl-1Tg mice exposed to water, DSS and DSS-recovery; (B) Immunoblot analysis to determine the expression of pERK1/2 and total ERK1/2 in colon tissue samples. Quantification of (C) BrdU+ cells/crypt (50 crypts/mice, n#3), and (D) Cleaved caspase-3 positive cells in control, DSS and recovery samples. [a] compared to WT-control, [b] compared to Cl-1Tg control, [c] compared to WT-DSS, [d] compared to WT recovery. *p<0.05, **p<0.01, and ***p<0.001.

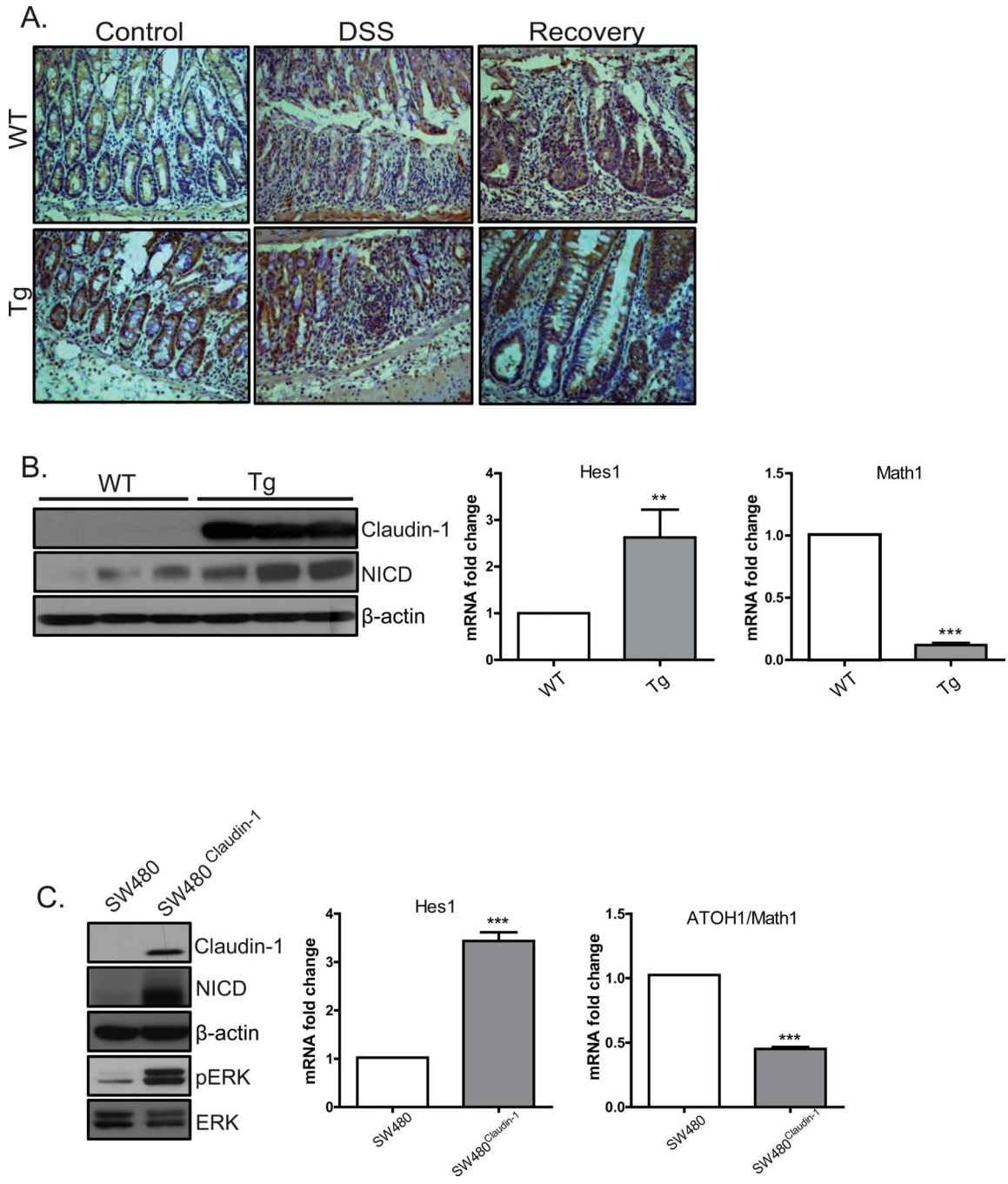

Figure 6. Increased Notch-signaling in Cl-1Tg mice and colon cancer cells during DSS injury and recovery

(A) Immunostaining for Hes1 expression to determine Notch activity during DSS-treatment and recovery. (B) Notch activity was assessed via immunoblotting for NICD expression and real-time PCR for Hes1 and Math1 in WT and Cl-1Tg colon tissue. **p<0.01, ***p<0.001. (C) Notch activity was assessed via immunoblotting for NICD and real-time PCR for Hes1 and Math1 expression in Claudin-1 overexpressing SW480 cells.

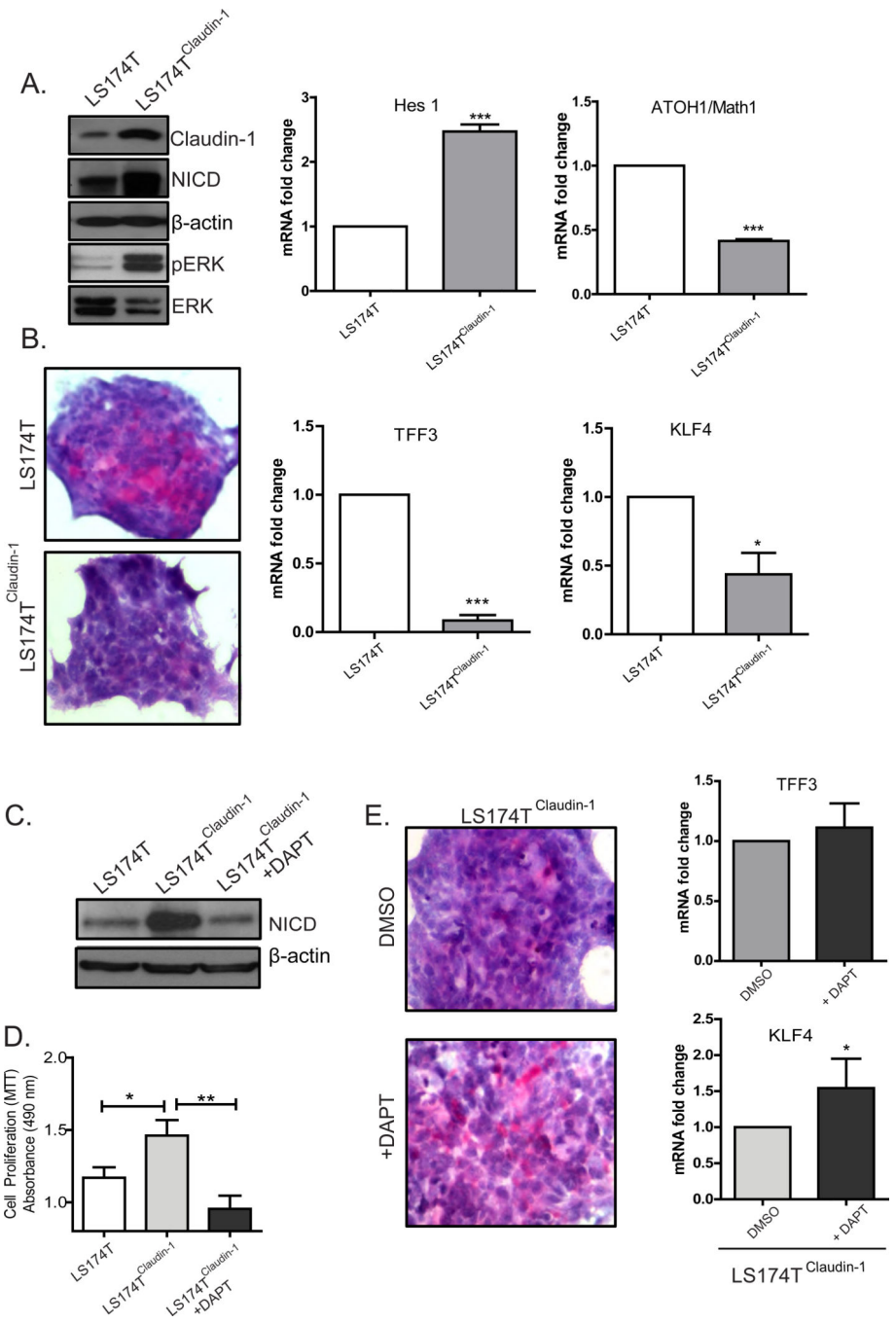

Figure 7. Claudin-1 Overexpression alters Notch signaling, proliferation and differentiation in colon cancer cells

(A) Notch activity was assessed *via* immunoblotting for NICD and real-time PCR for Hes1 and Math1 expression in goblet cell-like LS174T cells stably overexpressing claudin-1(LS174T$^{Claudin\text{-}1}$). (B) LS174T$^{Claudin\text{-}1}$ and control cells were assessed for changes in goblet cell differentiation by PAS immunostaining and qRT-PCR for TFF3 and KLF4. *p<0.05, ***p<0.001.(C) LS174T$^{Claudin\text{-}1}$ cells were treated with a γ-secretase inhibitor, DAPT (100μM, 48h); (C) inhibition of NOTCH activity; (D) Cell proliferation-MTT assay;

and differentiation was assessed *via* (E) PAS staining and qRT-PCR for differentiation markers TFF3 and KLF4.

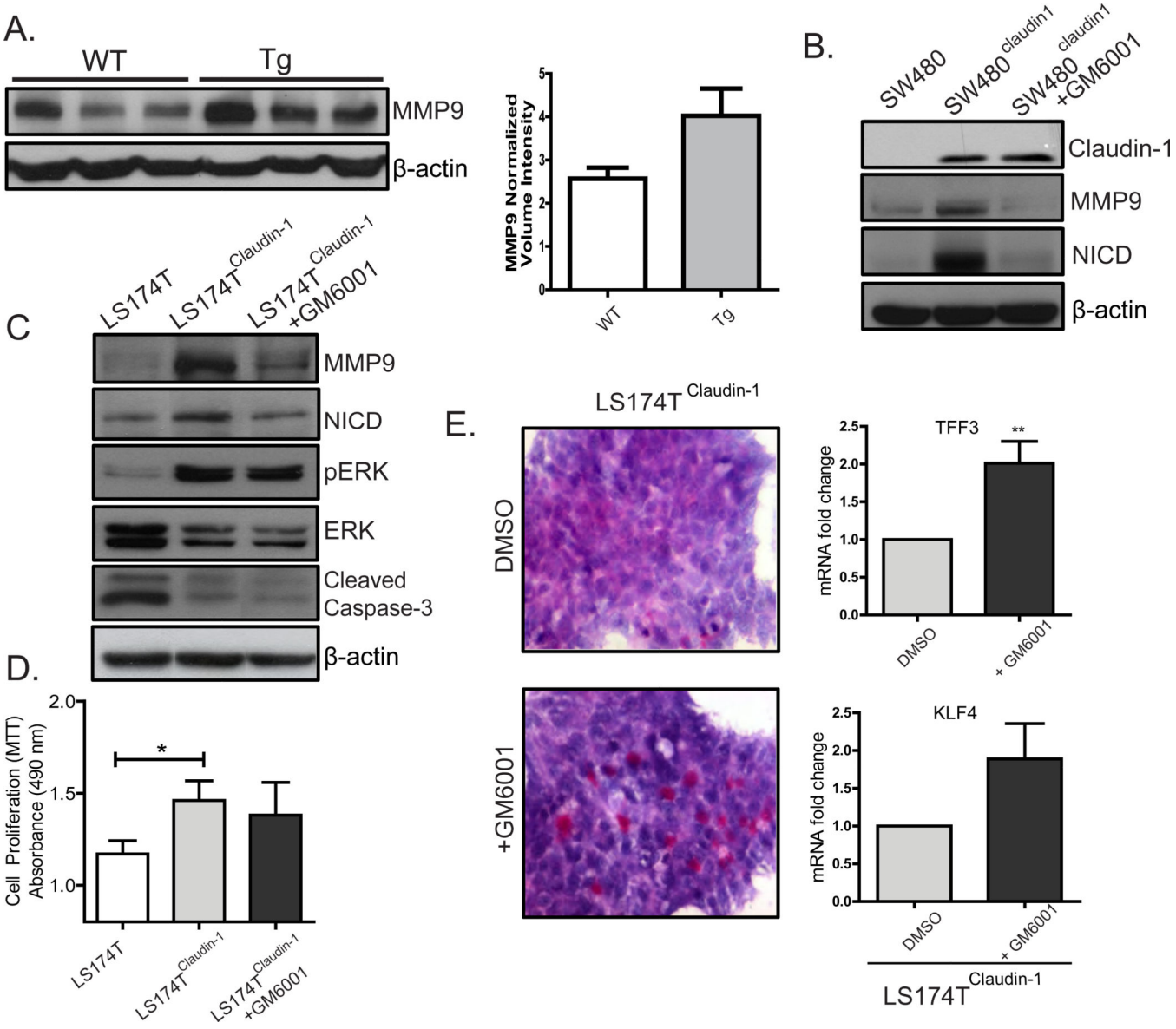

Figure 8. Claudin-1 induced Notch signaling is mediated through the regulation of MMP-9

(A) Immunoblot and densitometric analysis for MMP-9 expression in the colon of WT and Cl-1Tg mice. (B) LS174T$^{Claudin-1}$ cells were treated with a MMP inhibitor, GM6001 treatment (40μM, 48h) and effects on MMP-9 and NICD expression were determined in: (B) SW480 and SW480$^{claudin-1}$ cells; (C) LS174T and LS174T$^{Claudin-1}$ cells; (D) Cell proliferation-MTT assay; (E) differentiation (PAS staining and qRT-PCR for differentiation markers TFF3 and KLF4). *p<0.05, **p<0.01.

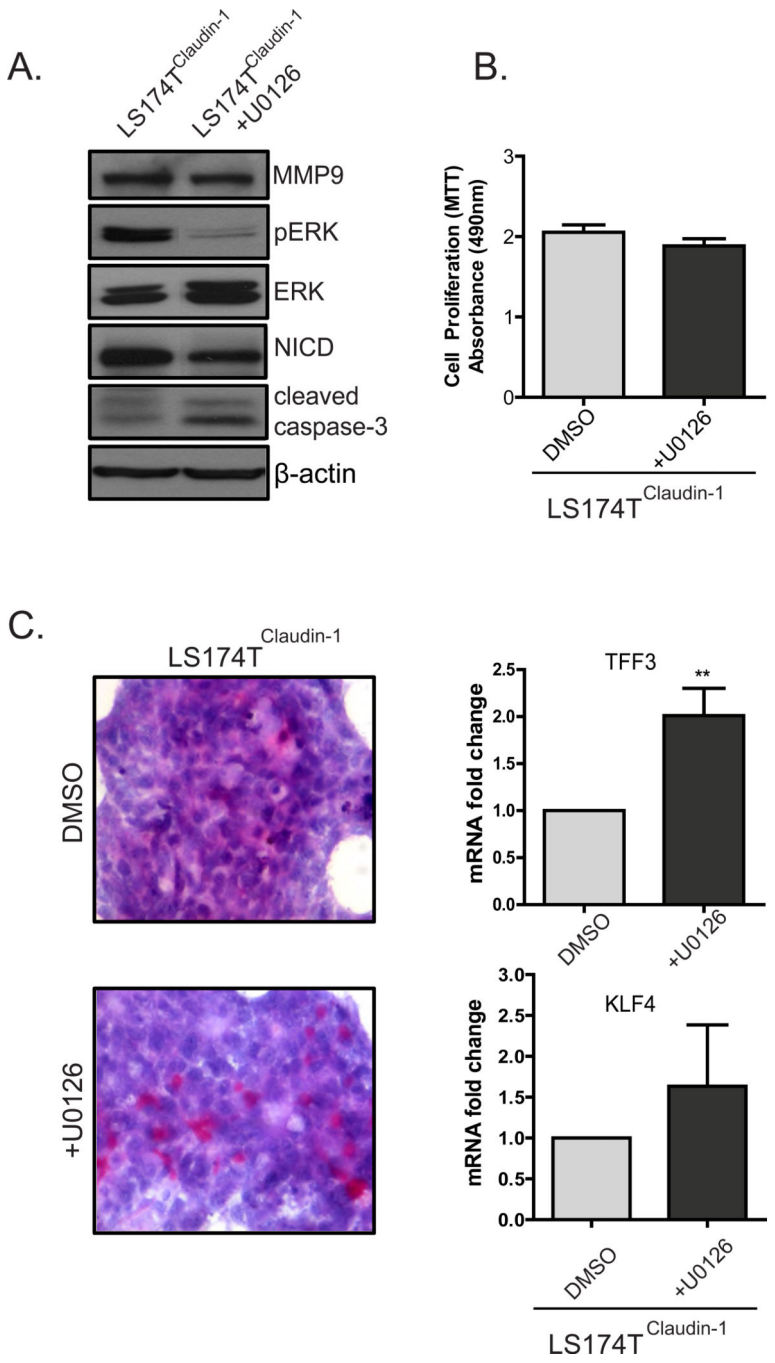

Figure 9. ERK signaling regulates Notch activation, apoptosis and differentiation in Claudin-1 overexpressing cells

Ls174T$^{claudin-1}$ cells were treated with U0126 (10μM for 24 hrs); (A) Immunoblot analysis of MMP9, p-ERK, NICD and cleaved caspase-3; (B) cell proliferation-MTT assay; (C) PAS staining and real-time PCR analysis of differentiation specific markers TFF3 and KLF4. **p<0.01